ICES

Zooplankton Methodology Manual

Edited by

R.P. Harris, P.H. Wiebe, J. Lenz,
H.R. Skjoldal and M. Huntley




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PREFACE

Zooplankton are the diverse, delicate and often very beautiful, assemblage of animals that drift the waters of the world's oceans. These microscopic organisms play a key role in the pelagic food web by controlling phytoplankton production and shaping pelagic ecosystems. In addition, because of their critical role as a food source for larval and juvenile fish, the dynamics of zooplankton populations, their reproductive cycles, growth, reproduction and survival rates are all important factors influencing recruitment to fish stocks. It is this latter role which has made zooplankton ecology of particular interest to ICES.

The International Council for the Exploration of the Sea, ICES, is the oldest intergovernmental organization in the world concerned with marine and fisheries science. Since its establishment in Copenhagen in 1902, ICES has been a leading scientific forum for the exchange of information and ideas on the sea and its living resources, and for the promotion and coordination of marine research by scientists within its member countries. Each year, ICES holds more than 100 meetings of its various working groups, study groups, workshops and committees.

Membership has increased from the original eight countries in 1902 to the present 19 countries which come from both sides of the Atlantic and include all European Coastal states except the Mediterranean countries eastward of, and including, Italy. ICES established a Study Group on Zooplankton Production in 1992 chaired by Hein Rune Skjoldal, of the Institute of Marine Research, Bergen, Norway. The Study Group were given as terms of reference to:

(a) review existing methods for measuring biomass and production processes;
(b) make proposals for improvement and standardization of methods, and prepare a methodological manual;
(c) consider the need for laboratory and seagoing workshops to intercalibrate experimental methods and evaluate new technology.

The Study Group has met eight times, in March 1992 in Bergen; in March 1993 in Las Palmas; in March 1994 in Plymouth; in June 1995 in Woods Hole; in March 1996 in Bergen; in March 1997 in Kiel, May 1998 in Santander, and May 1999 in Reykjavik. In 1997 Roger Harris of the Plymouth Marine Laboratory, United Kingdom, assumed the chairmanship.

The Study Group decided at the first meeting to produce a Zooplankton Methodology Manual recognizing the need for improvements and standardization in methods for studying this important and challenging group of organisms. To assist in the review of methods and to provide input to the issue of standardization and improvement of methods, three special workshops were convened. The first was a seagoing workshop onboard RV *Johan Hjort* and RV *A.V. Humboldt* on zooplankton sampling methods (June 1993). The two others were laboratory workshops at the University of Bergen, on production methods using the copepods *Acartia tonsa* (October 1993) and *Calanus finmarchicus* (April 1994). A fourth workshop was arranged by US GLOBEC in Hawaii using marine copepods (April 1994). Results from these workshops have been incorporated by the Study Group in producing this Manual.

ICES changed the status of the Study Group to a Working Group on Zooplankton Ecology (WGZE) in 1994. The Working Group has taken over the task of completing work with the Manual.

The Scope of the Zooplankton Methodology Manual is to provide an *updated review* of basic methodology used in studies of zooplankton including recommendations on improvements, harmonization and *standardization* of methods. The chapters aim to maintain a balance between being introductory and comprehensive. They provide an overview of methods that are useful, for example to graduate students who are starting in a new field. They emphasize the sources of error and the strengths and weaknesses of methods for various purposes and tasks. It has not been possible, however, to go into great detail for all methods, and reference to recent reviews and detailed descriptions of methods is used where possible and appropriate.

Each chapter begins with a review of methods which in most cases is accompanied by recommendations regarding choice and conduct of methods. These reviews consider the background and history of the methodology, the basic principles, sources of variability, equipment and procedures, comparative evaluation of alternative methods, general recommendations, and extensive literature references. Where possible detailed descriptions of standard protocols are included. The aim is to give practical instructions on *how* to carry out particular measurements and procedures. Equipment, procedures, data analysis and interpretation are described, where possible. These protocols either define standard methods, or give examples of little-known methods. If many methods are used, or many instruments, guidance is given on the most highly recommended, or the most often used, or likely to be used. In some cases it proves difficult to propose an agreed standard protocol. It is however, possible to provide guidelines that reduce the variability in methods and contribute towards harmonization and standardization.

The various chapters of the Manual have been reviewed by the ICES WGZE, and in addition peer reviewers from outside this group have evaluated each chapter independently. Grateful thanks are due to these reviewers for their valuable contribution to the overall project.

Each chapter is authored by an expert, or group of experts, selected from both members of the WGZE and other international specialists. The writing has been organized and co-ordinated by the main author assisted by co-authors. Chapter 1 provides an introduction to zooplankton. Chapters 2, 3, 4 and 5 consider sampling and experimental design, collecting zooplankton, techniques for assessing biomass and abundance, and the specialized methodology required for protozooplankton enumeration and biomass estimation. Chapters 6 and 7 describe new and emerging optical and acoustic techniques for estimating zooplankton biomass and abundance. In chapters 8, 9 and 10, methods for measuring zooplankton rate processes are described; feeding, growth and reproduction, and metabolism. Chapter 11 gives a modern account of methods for population genetic analysis of zooplankton, and Chapter 12 a comprehensive treatment of modelling zooplankton dynamics.

While striving to be a comprehensive treatment of modern methods in zooplankton ecology, it is inevitable that some topics have not been covered. In particular it was the original intention to include chapters on methods for investigating zooplankton behavior, and for studying population dynamics. The former chapter was never commissioned, while the latter although originally written as part of the ICES Manual project, was ultimately published as a separate scientific article; Aksnes *et al.* 1997. Estimation techniques used in studies of copepod population dynamics – a review of underlying assumptions. *Sarsia*, **82**:279–298. This may still be referred to as being

complementary to the work. The original concept of the Zooplankton Methodology Manual included a related CD-ROM to include data, graphics and video images, particularly relating to sampling methods, and deriving from the seagoing workshop. This is not included with the Manual, however the WGZE are still considering the preparation and distribution of such a CD-ROM.

The ICES WGZE has been encouraging and co-ordinating zooplankton monitoring activities in the ICES area, and this Manual should contribute to these activities. Similarly, the development of major international initiatives with a particular focus on zooplankton, particularly the IGBP/SCOR/IOC co-sponsored Global Ocean Ecosystem Dynamics (GLOBEC) project, and the Living Marine Resources module of the Global Ocean Observing System (GOOS-LMR) make the publication of this Manual particularly timely. While not formally adopted by either program, the ICES Zooplankton Methodology Manual will contribute significantly to the standardization of methodology that both GLOBEC and GOOS-LMR strongly endorse.

The preparation of the Zooplankton Methodology Manual has by definition been a team effort. The members of the WGZE and the Editors have lead in this, over the years of development. It is a great pleasure to acknowledge the enthusiasm, dedication and patience of all the authors and co-authors during this process. I am particularly grateful to Dr Sarah Stafford, Clare Nehammer and Teresa Netzler of Academic Press who have all worked with me during the editing and production of the Manual.

Plymouth
December 1999

Roger Harris

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1 Introduction

J. Lenz

1.1 INTRODUCTION

Zooplankton occupies a key position in the pelagic food web as it transfers the organic energy produced by unicellular algae through photosynthesis to higher trophic levels such as pelagic fish stocks exploitable by man. The availability of zooplankton of the right size and at the right place and time during the first feeding period of fish larvae constitutes the famous match/mismatch hypothesis (Cushing 1990). Apart from predation, it is regarded as the most important environmental factor controlling the year class strength of a large number of commercial fish stocks known to be subject to strong fluctuations. Zooplankton grazing also largely determines the amount and composition of vertical particle flux. This not only fuels the benthos community but contributes to the removal of surplus anthropogenic CO_2 from the atmosphere through sedimentation and burial of organic and inorganic carbon compounds.

It is thus important to increase our comparatively sparse knowledge of all aspects of zooplankton ecology by a joint effort on the basis of intercomparable methods for understanding and predicting the impact of environmental changes on fish stocks. We also need to know more about the role of zooplankton in modeling the cycling of biogeochemical key elements such as carbon, nitrogen and phosphorus in the sea.

During the last decade, zooplankton research has gained a fresh impetus as mirrored by the very well attended International Symposium on Zooplankton Production, which took place in Plymouth in August 1994 under the auspices of the International Council for the Exploration of the Sea (ICES) (Harris 1995). In the keynote lecture, the 'pivotal role of zooplankton' in controlling phytoplankton production and shaping pelagic ecosystems was stressed by Banse (1995). The great significance of zooplankton research is also recognized in two current large international research programs. Within the Joint Global Ocean Flux Study (JGOFS) zooplankton plays an important role in regulating particle flux to the deep sea. The impact of climatic change on zooplankton population dynamics influencing the recruitment success of pelagic fish stocks forms the main focus of Global Ocean Ecosystem Dynamics (GLOBEC).

1.2 GENERAL DEFINITIONS

The term 'plankton' was coined by the German founder of quantitative plankton and fishery research Victor Hensen (1887). It is derived from the Greek word 'planao' meaning to wander and it has the same etymological root as 'planet'. It embraces all those organisms drifting in the water whose abilities of locomotion are insufficient to

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withstand currents, as opposed to nekton, the community of actively swimming organisms such as large crustaceans, cephalopods, fishes and aquatic birds and mammals. Although the adjective 'planktonic' has been established for a long time and is widely used, a plea is made in favor of the more correct term 'planktic' in which the syllable 'on' is deleted. The same problem is correctly solved in the corresponding term 'benthic' which is already in wider use than 'benthonic'.

Although exposed to the forces of turbulence and currents, almost all zooplankton species have developed some means to move, at least to change their vertical position within the water column. Protozoans use either flagella and cilia or change their specific weight by ion exchange or incorporation of oil droplets, for instance. Movement by cilia is also common in many invertebrate larvae, for example in polychaete trochophores, mollusks and echinoderm larvae. Medusae and salps move by peristaltic contractions, and lobate ctenophores and pteropods move by flapping their lobes and wings respectively. Chaetognaths as ambush predators, which catch their prey in one swift movement, contract their longitudinal muscles and use their fins. Pelagic polychaetes and crustaceans have developed a large variety of special swimming appendages like parapodia and swimming legs. Appendicularia use their tails as oar blades. Small cephalopods employ the same mode of propulsion as the adults and fish larvae already possess rudimentary fins.

Zooplankton may be distinguished from phytoplankton either on the basis of morphology or mode of nutrition, autotrophic or heterotrophic. From the latter viewpoint zooplankton may be defined as the community of all phagotrophic organisms. According to their food preferences they can be classified as herbivorous, detrivorous, omnivorous or carnivorous. Heterotrophic plankton also include the osmotrophic bacteria, termed bacterioplankton. Mixotrophy, the combination of auto- and heterotrophy, is quite commonly found in flagellates and other protozoans like foraminiferans, radiolarians and ciliates, and it occurs in some metazoan phyla, too, for instance in cnidarians and mollusks. A further distinction is made between obligatory and optional mixotrophy.

Species spending their whole life in the pelagic realm are termed holoplanktic as opposed to meroplankton, which drift in the sea only for part of their life cycle. Among the protozoans, the majority of flagellates and ciliates as well as all pelagic foraminiferans and radiolarians belong to the first group. Also classed in the same group are from among the metazoans the siphonophores, ctenophores, pelagic polychaetes, heteropods, pteropods, ostracods, copepods with few exceptions, hyperiids, euphausiids, chaetognaths, appendicularians and salps.

Concerning the meroplankton, we distinguish between species which switch over from plankton to nekton during their juvenile stage, for example cephalopods and fish, and those which change from a planktic to benthic life and vice versa. Here we find a large variety of life strategies. A special case is the life cycle of most hydrozoans and scyphozoa, which exhibit two alternating generations. A summer generation of pelagic medusae with sexual reproduction is followed by a winter generation of benthic polyps with vegetative propagation.

Two contrasting modes of behavior are found among the other meroplankton groups, depending on their main habitat. We have plankton organisms with benthic resting stages and benthos organisms with planktic larvae. Cysts are produced by protozoans, flagellates and ciliates, and resting eggs by rotifers, cladocerans and some copepod families (Madhupratap *et al.* 1996) for surviving unfavorable environmental conditions. Propagation through planktic larvae is found in many benthos groups, for example

polychaetes, mollusks, echinoderms, bryozoans, and barnacles and decapods among the crustaceans. Depending on the time period spent as drifting plankton and the availability of food, the larvae are either lecithotroph, living off their yolk reserves, or planktotroph, feeding on phyto- and zooplankton.

1.3 SIZE CLASSIFICATION

Marine zooplankton comprises a large variety of different organisms with some ten thousands of species if meroplankton is included. Their sizes range from tiny flagellates, a few μm large, up to giant jellyfish of 2 m diameter, and thus span six orders of magnitude. To cope with this enormous size range – volume and weight span eighteen orders of magnitude – a first attempt at size classification was already undertaken as early as 100 years ago in the first days of quantitative plankton research (Schütt 1892). He distinguished between micro-, meso- and macrozooplankton. This first classification has since been extended and modified several times. The latest revision (Sieburth *et al.* 1978) is now widely accepted (Figure 1.1).

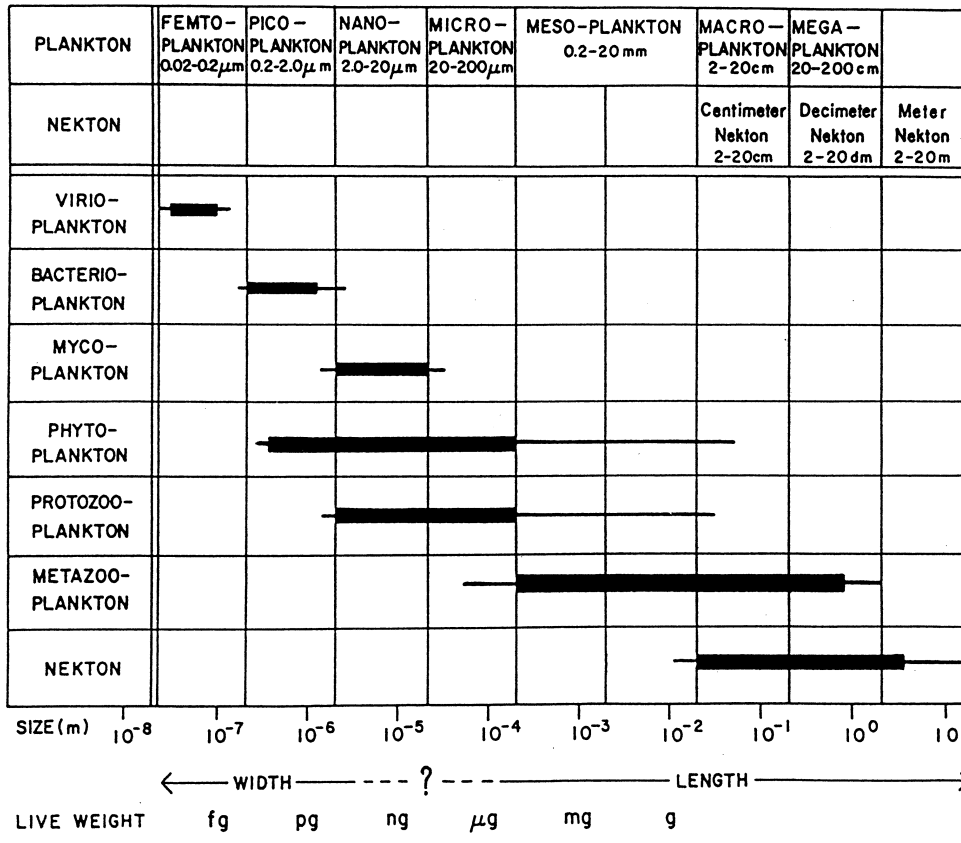

Fig. 1.1 Size spectrum of different taxonomic-trophic compartments of plankton including the size range of nekton modified after Sieburth *et al.* (1978) used with permission.

Zooplankton ranges over five size classes from nanoplankton to megaplankton. Since the finest mesh size of plankton gauze was, for a long time, 20 μm, this value defined the lower boundary of the so-called 'net plankton' which could be sampled by plankton nets, neglecting the smaller nano- and picoplankton. For this reason, the size class division (in Figure 1.1) is based on a factor of 2 instead of 1. Each size class covers one order of magnitude except mesozooplankton, which covers two orders. This exception is justified by the fact that the doubled size range corresponds to the size spectrum of traditional zooplankton samples taken by a mesh size of 200–330 μm. Such samples usually contain the bulk of crustacean plankton and, if present, of meroplanktic larvae as well. Moreover, the limits of this size class (0.2–20 mm) almost exactly match the size range of copepodites and adult copepods that generally constitute the dominant zooplankton group.

The main constituents of nanozooplankton (2–20 μm) are heterotrophic nanoflagellates feeding on bacteria. Most other protozoans, especially the ciliates, belong to the next size class, the microzooplankton (20–200 μm). This size class also covers the eggs and early development stages of crustacean plankton and meroplanktic larvae. Small hydromedusae, ctenophores, chaetognaths, appendicularians, doliolids, fish eggs and larvae together with the older stages of crustacean plankton and meroplanktic larvae comprise the mesozooplankton (0.2–20 mm) already mentioned.

In the next two size categories, species numbers diminish. Macrozooplankton (2–20 cm) are the larger specimens of hydromedusae, siphonophores, scyphomedusae, ctenophores, mysids, amphipods, euphausiids, salps and eel larvae, for instance. Only a few organisms reach the size of megazooplankton (20–200 cm). These are mainly large

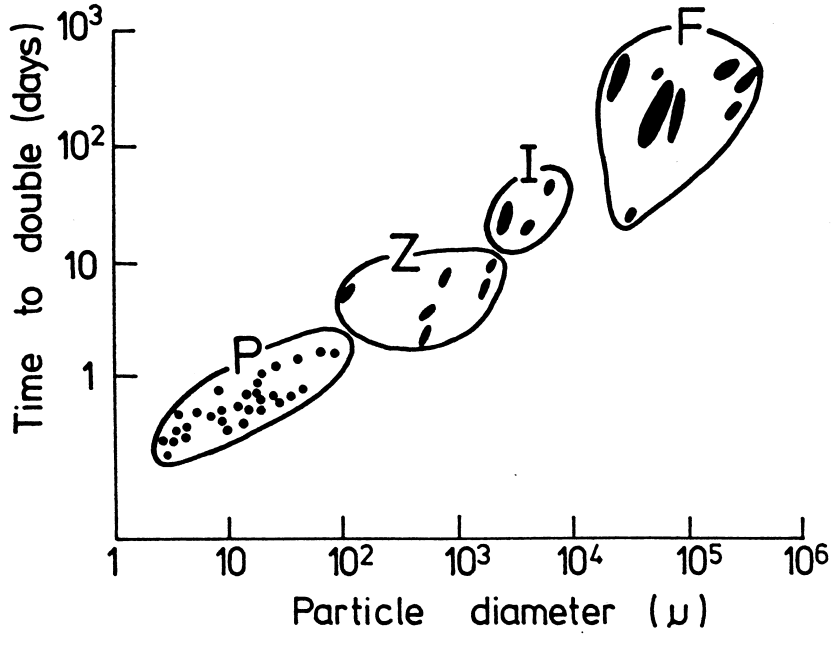

Fig. 1.2 The relationship between organism (particle) size expressed as equivalent spherical diameter and doubling time from data for phytoplankton (P), herbivores (Z), invertebrate carnivores or omnivores (I) and fish (F) from Sheldon *et al.* (1972) modified by Steele (1977) used with permission.

jellyfish, siphonophores and scyphozoa, and pelagic tunicates, pyrosomes and chain-forming salps.

Body size is a decisive factor in governing growth rate and doubling time of plankton organisms. Since within the pelagic food web most predators swallow their prey organisms undivided, body size also determines food-chain relationships. An example for its significance is the famous diagram by Sheldon *et al*. (1972) and modified by Steele (1977) as shown in Figure 1.2.

The methods described and discussed in this manual deal with net plankton, mainly with micro-, meso- and macrozooplankton which embrace most of the dominant species in meso- and eutrophic ecosystems.

1.4 MAIN SYSTEMATIC GROUPS

Another way of classifying planktic organisms is to consider their systematic position and biochemical composition. The first step is to distinguish between proto- and metazooplankton. Among the protozoans the ciliates, especially naked ciliates, form the ecologically most important group. Their division rate is so rapid as to enable them to almost keep pace with phytoplankton. Thus they react immediately to algal growth and take advantage of the food supply offered. In neritic ecosystems they are often the first grazers of the phytoplankton spring bloom (Smetacek 1981).

Characteristic of metazooplankton is a comparatively long life-span, ranging from several days in rotifers and few weeks in small crustaceans to several years in large euphausiids in polar regions. A long life-span generally goes hand in hand with a long development period. This is most pronounced in crustaceans where in some groups organisms have to pass through many larval stages before reaching sexual maturity.

Since crustaceans are represented in zooplankton mainly by eight orders, cladocerans, ostracods, copepods, cirripeds, mysids, amphipods, euphausiids and decapods, these organisms generally dominate the samples. They are collectively termed crustacean plankton as opposed to the so-called gelatinous plankton. In the latter group the organisms are characterized by a gelatinous body, the most prominent representatives being the common jellyfish (Scyphomedusae). Gelatinous zooplankton comprises various systematic groups, indicating that this particular mode of adaptation to planktic life in the sea was arrived at through independent evolutionary processes. We refer to cnidarians with hydromedusae, siphonophores and scyphomedusae, the ctenophores and the pelagic tunicates with pyrsomes, doliolids, salps and appendicularians. The latter are included not because of their body constitution but because they have gelatinous houses which are regularly abandoned and rebuilt. Occasionally, a single species in other groups has acquired a gelatinous body too, for example in heteropods and ostracods. The high water and also salt content of these organisms causes a substantial shift in the ratio between organic matter content and wet weight, dry weight and inorganic matter content as compared with crustacean plankton, for instance. It further enables gelatinous species to grow very fast manifested by the huge swarms frequently encountered.

Other ecologically important groups, not belonging to crustacean or gelatinous plankton, are rotifers in brackish waters, pteropods, chaetognaths, and especially fish larvae.

1.5 SPECIES DIVERSITY

Table 1.1 gives an overview of the main marine zooplankton groups with their approximate species numbers. The figures in brackets are very rough estimates since relevant data are lacking. This is especially true of the meroplanktic larvae, where estimates rely on the number of the parental benthos and nekton species. The figures quoted may therefore give only an idea of the vast species diversity in these groups. Summing up the figures listed in Table 1.1, one arrives at a total of around 36 000 species. Holoplankton with about 6000 protozoan (16%) and about 4000 metazoan species (11%) makes up roughly a quarter (27%). The bulk (73%) consists of the lesser known meroplankton with uncertain estimates, and the total should therefore be taken as only a tentative measure of the order of magnitude.

In most aspects of zooplankton research, accurate species identification is a necessary but often not easily achievable prerequisite. The help of experts in systematics might be needed. Even if only the biomass distribution is investigated during a survey by applying bulk methods like volume or weight measurements, at least a rough inspection of the main species composition would provide valuable information.

A first impression of the vast diversity of zooplankton organisms may be obtained from the excellent drawings by the Scottish planktologist James Fraser (1962), reproduced in Figures 1.4–1.16 at the end of this chapter. The following contains a short but in no way exhaustive list of introductory guides, plankton books, identification sheets and recent monographs on various zooplankton taxa.

There exist several introductory guides which are well written and contain a large number of informative drawings. Newell and Newell (1973) present boreal plankton, Wickstead (1965) tropical plankton and Tregouboff and Rose (1957) subtropical plankton in the Mediterranean Sea. Todd and Laverack (1991) provide a special introduction to neritic boreal zooplankton, this differs from other guide books in presenting photographs of preserved specimens as encountered by the normal investigator. Two very readable and well illustrated books which give a general account of the life of North Atlantic plankton are Fraser's (1962) *Nature adrift* and Hardy's (1970) *World of plankton*, whereas Raymont's (1983) *Zooplankton* is recognized as a comprehensive textbook.

The identification sheets, edited by ICES since 1947, are a collection of leaflets dealing with all zooplankton taxa from Foraminifera to fish larvae occurring in the northeast Atlantic and its adjacent seas. Most of them contain an identification key and table of geographical distribution. A table of contents of the sheets is found in Todd and Laverack (1991).

The monographs listed below also include accounts of the biology of various zooplankton groups. Dinoflagellates (Taylor 1987) play an important role in the systematically diverse group of heterotrophic flagellates. Ciliates are described by Corliss (1979), Foraminifera by Hemleben *et al.* (1989) and Radiolaria by Anderson (1983). The Hydro- and Scyphomedusae are presented by Russell (1953, 1970) in two volumes. Siphonophores are dealt with by Alvariño (1971) and Mackie *et al.* (1987) and ctenophores by Reeve and Walter (1978). An account of boreal meroplanktic larvae, especially of polychaetes and mollusks, is given by Thorson (1946). Heteropoda are described by Tesch (1949) and Pteropoda by Pruvot-Fol (1942) and Tesch (1946, 1948, 1950). Juvenile cephalopods are treated by Sweeney *et al.* (1992).

Details on the crustacean plankton are found in the following monographs: Cladocera (Egloff *et al.* 1997), Ostracoda (Angel 1993), Copepoda (Rose 1933; Marshall and Orr

Table 1.1 Systematic overview of the main zooplankton taxa with approximate species numbers (figures in brackets are very rough estimates), plankton status (holo- or meroplankton) and size class distribution (cf. Figure 1.1). Crosses indicate the distribution: rare +, common ++, dominant +++.

Systematic groups	Holo–Mero Plankton		Nano 2–20 μm	Micro 20–200 μm	Meso 0.2–20 mm	Macro 2–20 cm	Mega 20–200 cm
Protozooplankton							
Heterotrophic Flagellates (600)	+++		+++	+++	+		
Ciliata (1000)	+++		+	+++	+		
Foraminifera (Globigerina) 30	+++			++	++		
Radiolaria 4500	+++			+	+++	+	
Metazooplankton							
Cnidaria							
Hydromedusae (500)	+	+++			++	++	+
Siphonophora 150	+++				+	++	+
Scyphomedusae 250	+	+++			+	++	++
Ctenophora 80	+++				+	++	+
Rotatoria (100)		+++		++	++		
Polychaeta (100)	+++				++	++	+
Polychaeta Larvae (3000)		+++		+	++		
Mollusca							
Heteropoda 30	+++				++	++	
Pteropoda 100	+++				+++	+	
Cephalopoda 500	+	+++			++		
Veliger Larvae (10 000)		+++		+	+++		
Crustacea							
Cladocera 30	+	+++			+++		
Ostracoda 350	+++				+++	+	
Copepoda							
Calanoida 1200	+++	+		+	+++		
Cyclopoida 100	+++			+	+++		
Harpacticoida 100	+++			+	+++		
Cirripedia Larvae (800)		+++		+	+++		
Mysidacea 600	+++				++	++	+
Amphipoda (Hyperiidea) 300	+++				+++	+	
Euphausiacea 90	+++				++	++	
Decapoda 200	+++				+	+++	
Decapoda Larvae (6000)		+++			+++	+	
Echinodermata Larvae (2000)		+++			+++		
Chaetognatha 50	+++				++	++	
Tunicata							
Copelata 60	+++				+++	+	+
Thaliacea 45	+++				++	++	+
Fish Larvae (3000)		+++			+++	+	

1955; Mauchline 1998), Cirripedia (Southward 1987; Anderson 1994), Mysidacea (Mauchline 1980), Hyperiidea (Bowman and Gruner 1973; Vinogradov *et al*. 1996), Euphausiacea (Mauchline and Fisher 1969; Mauchline 1980), Decapoda (Omori 1974; Dall *et al*. 1990) and decapod larvae (Gurney 1939). Chaetognaths are described by Alvariño (1965) and Pierrots-Bults and Chidgey (1988). Pelagic tunicates are treated by Fraser (1981) and Bone (1997) and fish larvae by Fahey (1983) and Moser *et al*. (1984).

1.6 ECOLOGICAL POSITION

The ecological role of an organism is largely determined by its position and significance in the food web. Decisive characteristics are body size, food spectrum and feeding type. Filter-feeders like copepods, euphausiids and pelagic tunicates, feeding on different size spectra of phytoplankton, organic detritus as well as on nano- and microzooplankton, are primarily herbivores and omnivores. As secondary producers they thus occupy the second and to some extent third level in the food web. Various filter techniques are employed. Copepods and euphausiids use their highly structured mouthparts and feeding appendages, appendicularians a fine-meshed funnel net inside their house and thaliaceans a ciliary mucous net inside their barrel-shaped body. Ciliates, rotifers and many meroplankton larvae feed by means of ciliary currents. Thecosome pteropods employ large mucous nets for trapping their prey.

Raptional feeders are typically predators. Cnidarians and ctenophores catch their prey with stinging or sticky tentacles. Pelagic polychaetes, heteropods, gymnosome pteropods and cephalopods among the mollusks, hyperiids (amphipods) and fish larvae may be regarded as active hunters, whereas chaetognaths are described as ambush predators.

Cladocerans, ostracods and mysids occupy an intermediate position between the typical filter-feeding and the raptional zooplankton. It should, however, be stressed that the above classification has no strict demarcations, since there exist many exceptions in various groups. Ciliates, for instance, have carnivorous members feeding on their own relatives. Some scyphomedusae (Hansson 1997) and ctenophores (Greve 1981) prefer to prey on related species. Similarly in pteropods, gymnosomes feed on thecosomes. There are quite a number of carnivorous species among copepods and euphausiids too, and even a very typical filter-feeder like *Calanus finmarchicus* is able to switch to raptorial feeding, at least under laboratory conditions.

Because of their worldwide distribution, abundance and dominance, the following three groups may be regarded as the most significant secondary producers, the naked ciliates among the protozoa, and the copepods and euphausiids among the metazoa, both the latter specially in boreal and polar regions. Appendicularians and salps are also of importance in some areas, but their occurrence is of a more seasonal character and they are not as ubiquitous as the former groups. Ctenophores can have a high ecological impact as predators, as has been demonstrated in the Black Sea by the recently introduced species *Mnemiopsis leidyi* (Harbison 1994). Scyphomedusae like *Aurelia aurita* can function as top predators in inshore seas and structure the food web by top-down control (Behrends and Schneider 1995).

The functioning of food webs depends on the balance between bottom-up and top-down control. Bottom-up is the resource-driven control. In the pelagic realm, it is primarily exercised by the supply of nutrients determining the amount of primary production. Recently, the pivotal role of zooplankton grazing in controlling phyto-plankton growth has been stressed (Banse 1995) in explaining the apparent paradox of

the so-called 'high' nutrient 'low' chlorophyl regions. A high grazing pressure by herbivores prevents phytoplankton blooms.

The top-down control is particularly marked in the microbial food web, where ciliates are the top predators. They themselves are controlled by filter-feeding metazooplankton, thus connecting the microbial with the classical food web (Figure 1.3). Both food web types coexist in all areas of the ocean, but their relative significance changes with region and season. The classical food chain dominates in eutrophic cold-water and upwelling ecosystems. The microbial food web, however, is most significant in oligotrophic warm-

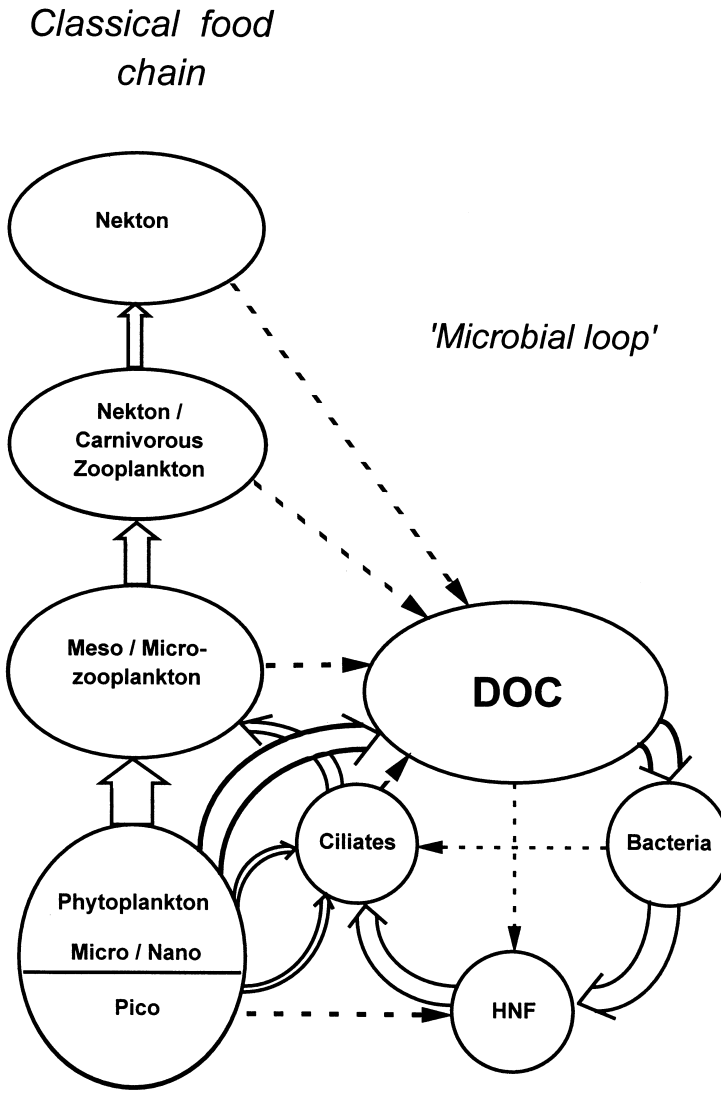

Fig. 1.3 Simplified food web structure with microbial loop, microbial food web and classical food chain. Microbial food web includes microbial loop and autotrophic picoplankton and nanoplankton $< 5\ \mu$m. (DOC = dissolved organic carbon and HNF = heterotrophic nanoflagellates.) Modified after Lenz (1992) used with permission.

water systems and during summer stratification in nutrient-depleted surface waters in higher latitudes (Lenz 1992).

1.7 DISTRIBUTION PATTERN

Zooplankton distribution is governed by water depth, trophic status of the area and temperature regime, to mention the most important factors. Water depth separates neritic from oceanic plankton. Neritic plankton inhabits inshore waters up to about 200 m at the shelf edge. Characteristic of neritic plankton is a high proportion of meroplankton larvae and species with benthic resting eggs. The proximity to the sea bottom favors an exchange between plankton and benthos communities.

Oceanic zooplankton on the other hand is characterized by a general absence of meroplankton and the presence of distinct vertical migrators. Among them are, for instance, larger copepod species and euphausiids. The daily migration, rising at dusk and descending at dawn, often extends over several hundred metres. Seasonal vertical migration as exhibited by many members of the copepod genus *Calanus* in higher latitudes even reaches down to depths of 500–1000 m. The epipelagic (0–200 m) and mesopelagic zones (200–1000 m) are the main domain of zooplankton. Below 1000 m depth in the bathypelagical, their concentration generally decreases logarithmically with depth (Vinogradov 1997).

Species diversity is generally governed by temperature regime and evolutionary age of ocean areas. The highest diversity is thus found in tropical and subtropical regions and the lowest in extreme environments such as polar zones and brackish water areas. Due to the steady water exchange through ocean currents many species enjoy a wide and often even worldwide distribution within their climatic boundaries. This is especially true for the warm-water sphere comprising all three oceans. Sporadic occurrences of neritic species observed since the turn of the century in increasing numbers are now generally explained by the high amount of ballast water transported and discharged all over the world by international ship traffic (Carlton and Geller 1993).

In studying the distribution and seasonal cycle of marine zooplankton, one is always faced with the problem that the sea is a highly dynamic environment with a steady motion and mixing of water masses. Whereas a limnologist, working in a small or medium-sized lake, can be almost certain of dealing with the same population during an investigation period, this is more the exception than the rule for a marine planktologist working in the sea. Because of prevailing currents and advection of new water masses, it is often virtually impossible to follow the same population of organisms (Huntley and Niiler 1995). Observations at a fixed station therefore generally represent a mixture of 'sequences' of different plankton populations passing by and 'seasonal changes' of the same populations. Every plankton sampling program should be accompanied by a detailed oceanographic documentation, especially salinity measurements and increasingly use of hydrodynamic and particle-tracking models, for identifying the impact of water mass changes (Chapter 2).

1.8 GROWTH AND METABOLISM

Growth and metabolism of zooplankton organisms are governed by the interaction of a number of forces which may be either internal or external. Internal factors are body

size and physiological properties, such as range of temperature tolerance, developmental stage and physiological state. Feeding activity, for instance, depends on the molting cycle in crustaceans. External factors are food supply and nutritional properties of food, as well as various environmental factors such as temperature, salinity and oxygen saturation. One must distinguish between potential growth rate under optimal conditions and actual growth rate which is often reduced by one or more of the factors mentioned above. An additional factor affecting population growth is top-down control through predation.

Metabolism as well as growth rate is an allometric function of body size. Small organisms have a comparatively higher metabolic rate and grow faster than large ones. This allometric relationship is described by the general equation

$$R = aW^b \tag{1.1}$$

R stands for respiration or any other metabolic rate including growth. W is body size, usually measured as dry weight, and a and b are constants. Whereas a depends on physiological properties of the organisms and environmental conditions, b is a uniform constant attaining the approximate value of 0.75 (Peters 1983). An increase in body size, expressed as volume or weight, by a factor of 1000, which corresponds to a ten-fold increase in body length, results in a reduction of metabolic rate by a factor of 5.6. This value gives an idea of the difference in growth rate between members of two neighboring size classes, for example microzooplankton and mesozooplankton.

Among the external factors governing growth, food supply and temperature are most important. The availability of food varies with season in higher latitudes. Thus the majority of organisms have adapted their life cycle in such a way that they encounter optimal conditions during their reproduction period.

There is also a relationship between food supply and energy conversion. The sparser the food the better the digestion. During phytoplankton blooms 'superfluous feeding' has been observed in herbivorous copepods. Their feces contained many undigested phytoplankton cells. In considering energy conversion it is advisable to distinguish between physiological efficiency in individual organisms and ecological efficiency expressing food chain efficiency. The first may be more than twice as high as the latter, where generally only a section of secondary producers, for example herbivorous copepods, are related to total primary production.

Water temperature in the open sea ranges from about $-2\,^{\circ}C$ to approximately $32\,^{\circ}C$. Zooplankton organisms inhabit all regions of the sea according to their physiological temperature adaption and tolerance limits. These may be narrow, stenotherm, as in polar and deep-sea plankton, or eurotherm, as in temperate and neritic waters of higher latitudes. Within the given tolerance limits of individual species, and within the whole range of sea temperature for the total plankton community, the intensity of all vital processes depends on the prevailing temperature conditions. This temperature dependency is based on the reaction speed of physicochemical processes in living organisms and is described by the van't Hoff law or the so-called Q_{10} rule. For a temperature rise of $10\,^{\circ}C$, an increase in metabolic rate of two to three times is generally observed (Schmidt-Nielsen 1979). Special adaptations to extreme temperatures in polar seas, for instance, are possible to some degree, but the general validity of this rule for all poikilotherm organisms is not suspended.

Knowing body size, developmental stage and physiological state of a plankton organism and the temperature conditions, it is thus possible to calculate the potential growth rate. In marine copepods, where dominant species often vary comparatively little

in body size, temperature has been demonstrated as the main factor governing their growth rate (Huntley and Lopez 1992).

Physical stress factors such as reduction in salinity and oxygen content limit species distribution and diminish growth and body size in those species which are able to tolerate these adverse environmental conditions. Thus brackish water organisms are usually smaller than their marine relatives because of the extra energy spent in physiological adaption.

Assimilated energy is converted both into somatic growth and egg production. Female copepods on reaching the adult stage after the last molting spend all their surplus energy on egg production. Thus egg production replaces somatic growth. Special methods have been developed to measure egg production in order to include it in estimates of zooplankton production.

1.9 REPRODUCTION AND DEVELOPMENT

Vegetative propagation through binary fission predominates in protozooplankton, whereas sexual reproduction is the common mode of propagation in metazooplankton. There are, however, quite a number of exceptions from this rule. The pelagic foraminifera, the Globigerina, multiply, for instance, by multiple fission, producing either vegetative cells or flagellated gametes. Vegetative propagation through budding occurs in hydromedusae and is a regular part of the life cycle of thaliaceans.

Parthenogenesis, the asexual propagation through unfertilized eggs, is a special achievement in rotifers and cladocerans. After a period of propagation through parthenogenesis a sexual generation appears in which the females produce fertilized eggs. These are protected by a thick shell and hibernate on the sea bottom until parthenogenic females hatch in spring.

Vegetative propagation and parthenogenesis have the great ecological advantage that a large population can be built up in a short period. These organisms are thus able to optimally utilize favorable feeding conditions.

Equally advantageous is a direct development from egg or first larval stage to adult, as we have with the ephyra in scyphomedusae. Such a development, which is found in ctenophores, ostracods and chaetognaths, for instance, is usually much faster than passing through a number of different larval stages, as is typical for copepods and euphausiids. Copepods have to pass six naupliar and five copepodite stages before molting into adults.

1.10 STANDING STOCK AND PRODUCTION

The relationship between standing stock and production of a population depends on food supply and climatic conditions and varies largely with season in most areas. The stronger the seasonal impact, generally the greater the variation. A very decisive factor is the temperature governing the growth rate. In cold-water ecosystems dominant species need to maintain a relatively high biomass to assert their predominance, since production rate is slow. In warm-water ecosystems it is possible to build up a large population from a low standing stock because of the high growth rate. The ratio between production and biomass of a species is an important index of its population dynamics and indicates the turnover rate of organic matter. Under optimal food conditions, the highest turnover

rate is thus observed in small organisms in the tropics and the lowest in large organisms in polar regions.

Many zooplankton species, especially those in cold-water and upwelling ecosystems, have developed various life strategies to adjust their growth and reproduction period to optimal environmental conditions. One mode of adaption to cope with unfavorable seasonal conditions is to produce resting eggs, as already mentioned. It is common in cladocerans and also occurs in a number of neritic copepod species (Madhupratap *et al.* 1996; Williams-Howze 1997). Another strategy is the evolution of a diapause stage as observed in members of the genus *Calanus* and its relatives, where copepodite stages IV and V, after having accumulated a large lipid reserve, migrate down to great depths and drift there motionless until the beginning of the next growth season.

1.11 CONCLUSION

When measuring zooplankton biomass and estimating its production, one usually tends to deal with the whole community. By employing different mesh sizes in nets and sieves it is possible to separate the main components from each other, for instance microzooplankton from mesozooplankton or small herbivores from larger carnivores by means of size fractionation. This approach may occasionally prove quite successful, depending on the size spectra and diversity of the species. Bulk parameters obtained in this way may provide useful data for modelers. It is, however, always necessary to realize that such data are no more than a methodological compromise, for reality is far more complex. The trophic role and ecological significance of zooplankton communities depends on the diversity, behavior and interaction of their species. These communities are often dominated by so-called key taxa, which play the main role in channeling energy up the food web and exercising top-down control through grazing or predation. Future research should put more emphasis on special environmental adaptations of these predominant species and their ecological significance in forming the food web (Verity and Smetacek 1996).

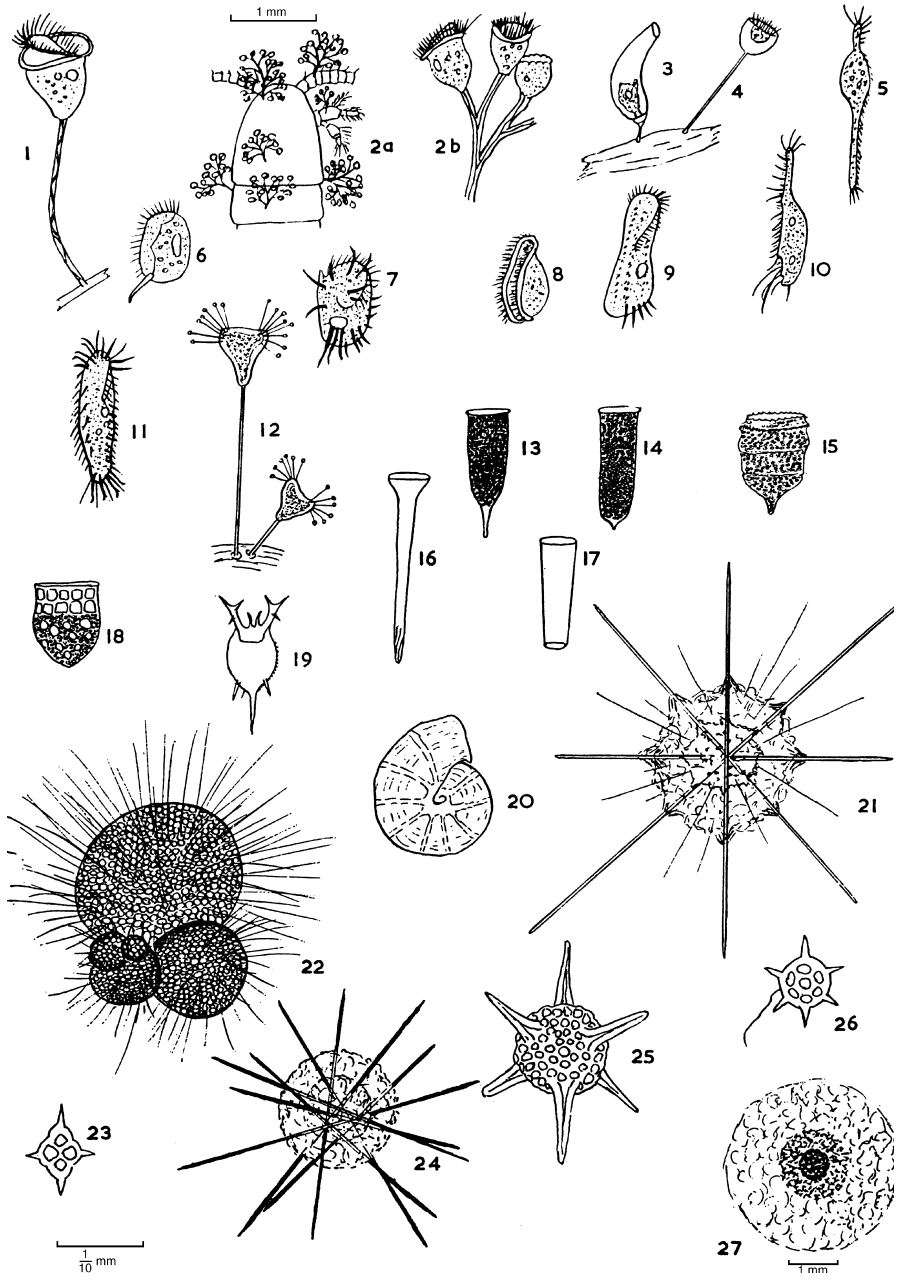

Fig. 1.4 Protozoa. See page 27 for key.

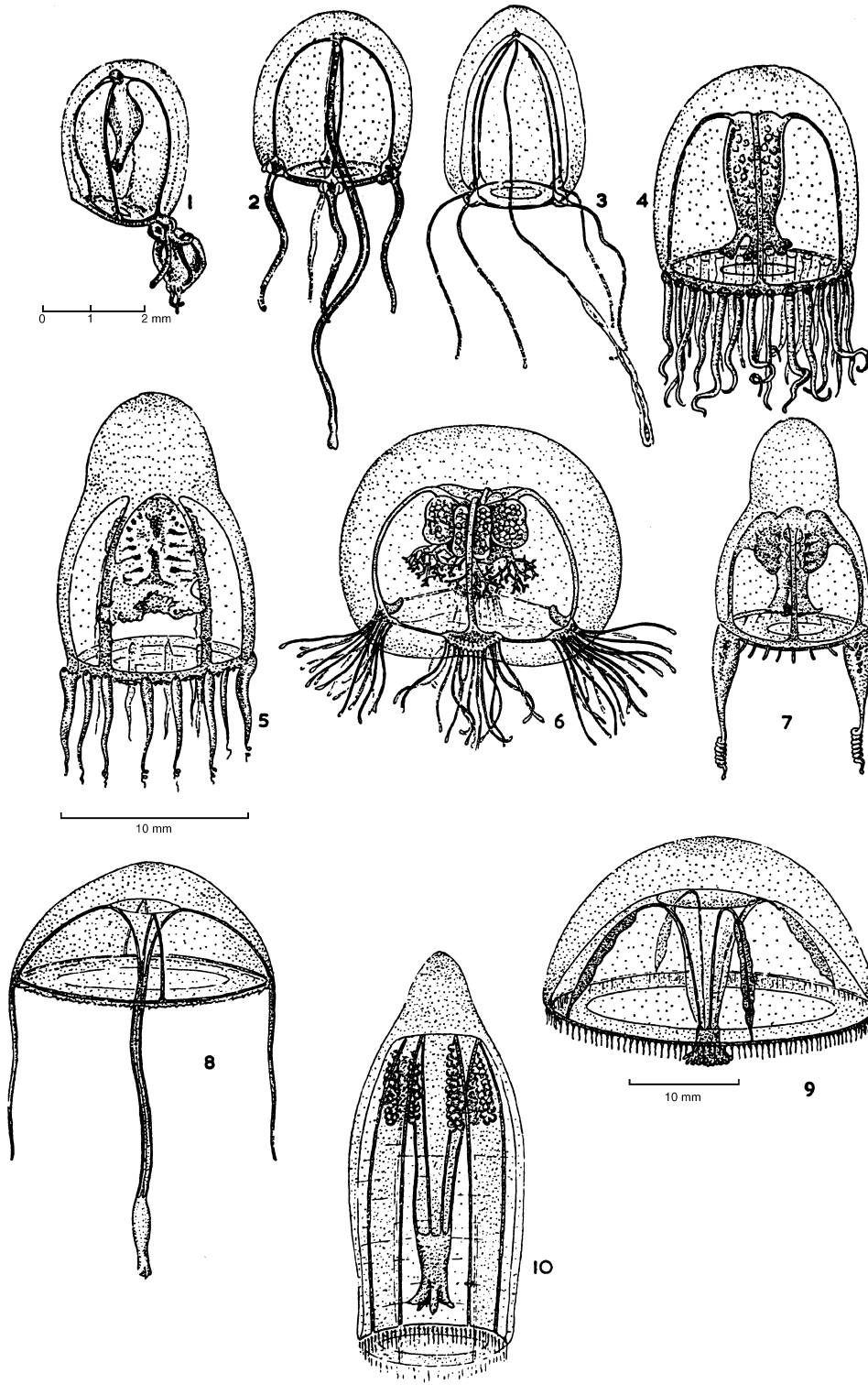

Fig. 1.5 Small jellyfish. See page 27 for key.

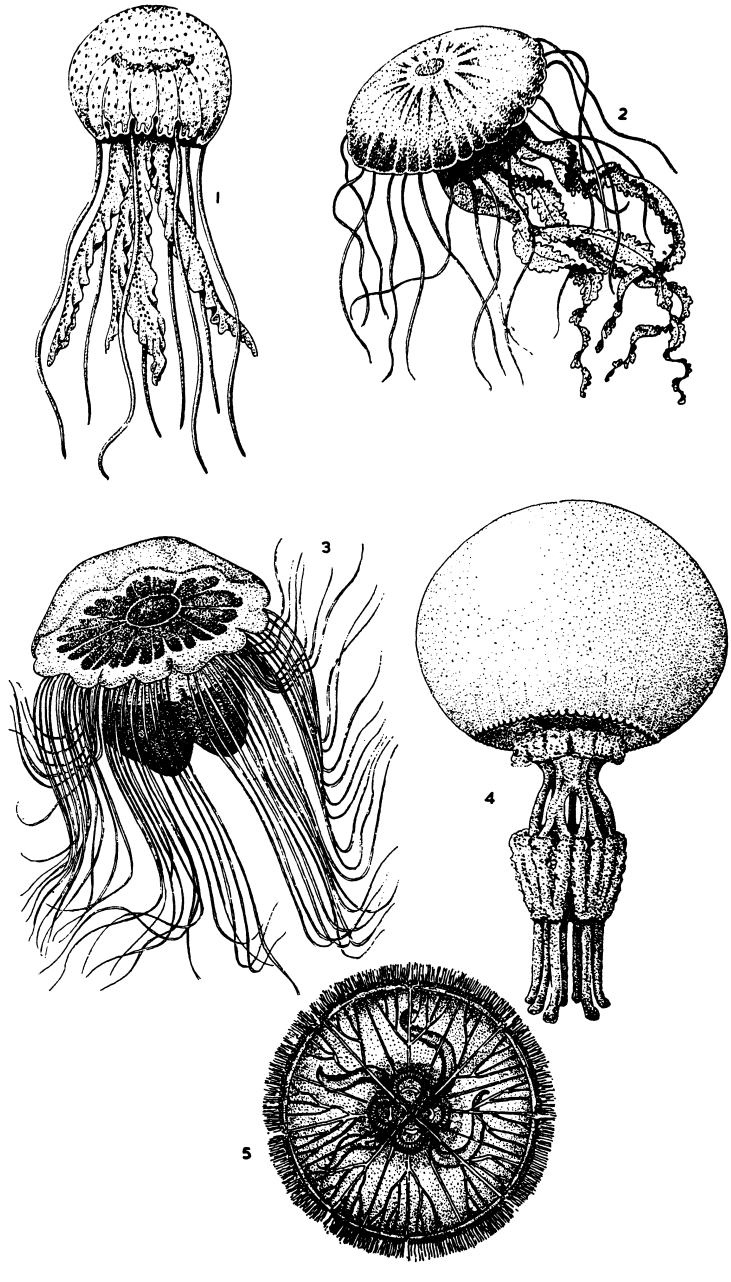

Fig. 1.6 Large jellyfish. See page 27 for key.

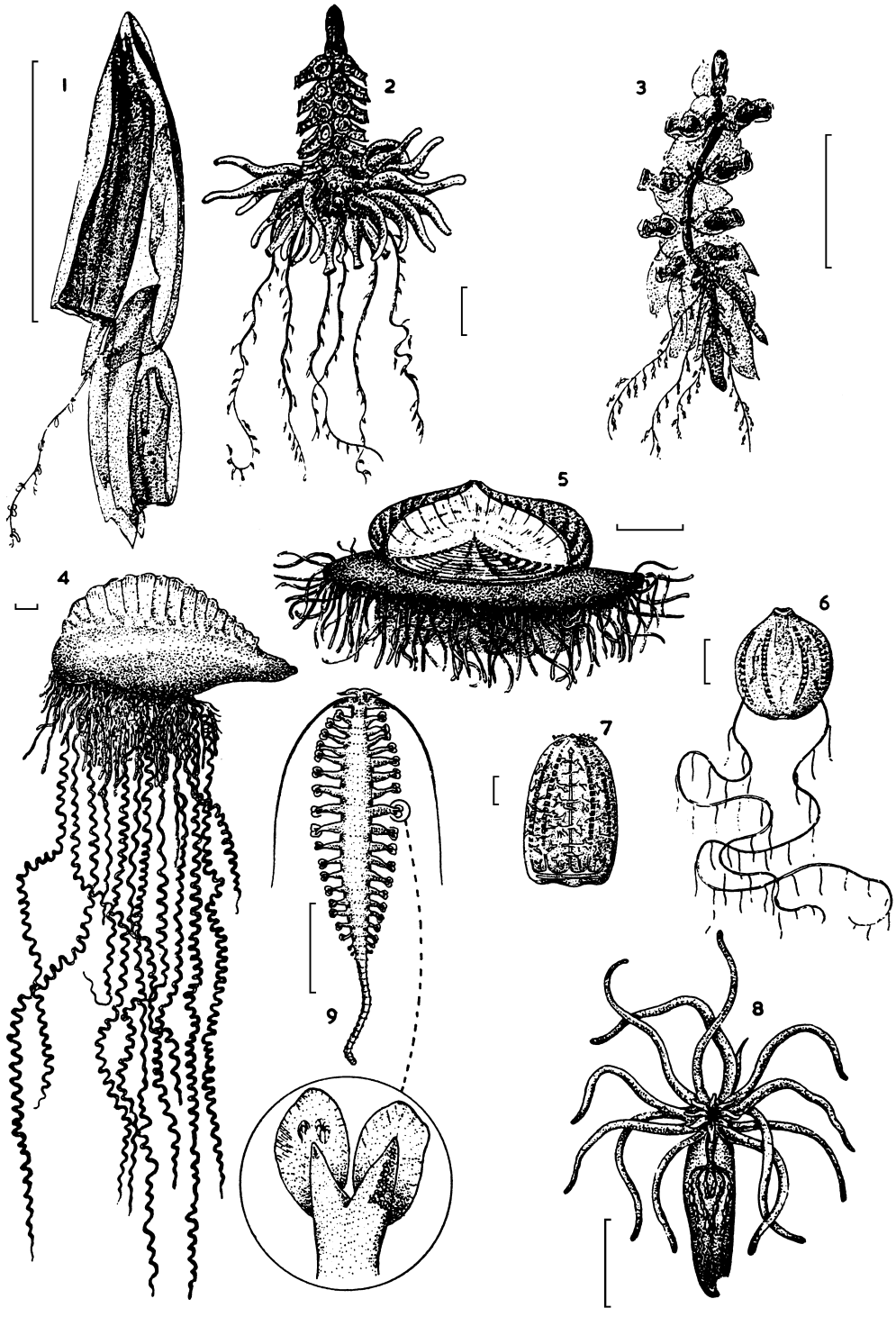

Fig. 1.7 Other Coelenterates etc. See page 27 for key. Scale lines all represent 1 cm.

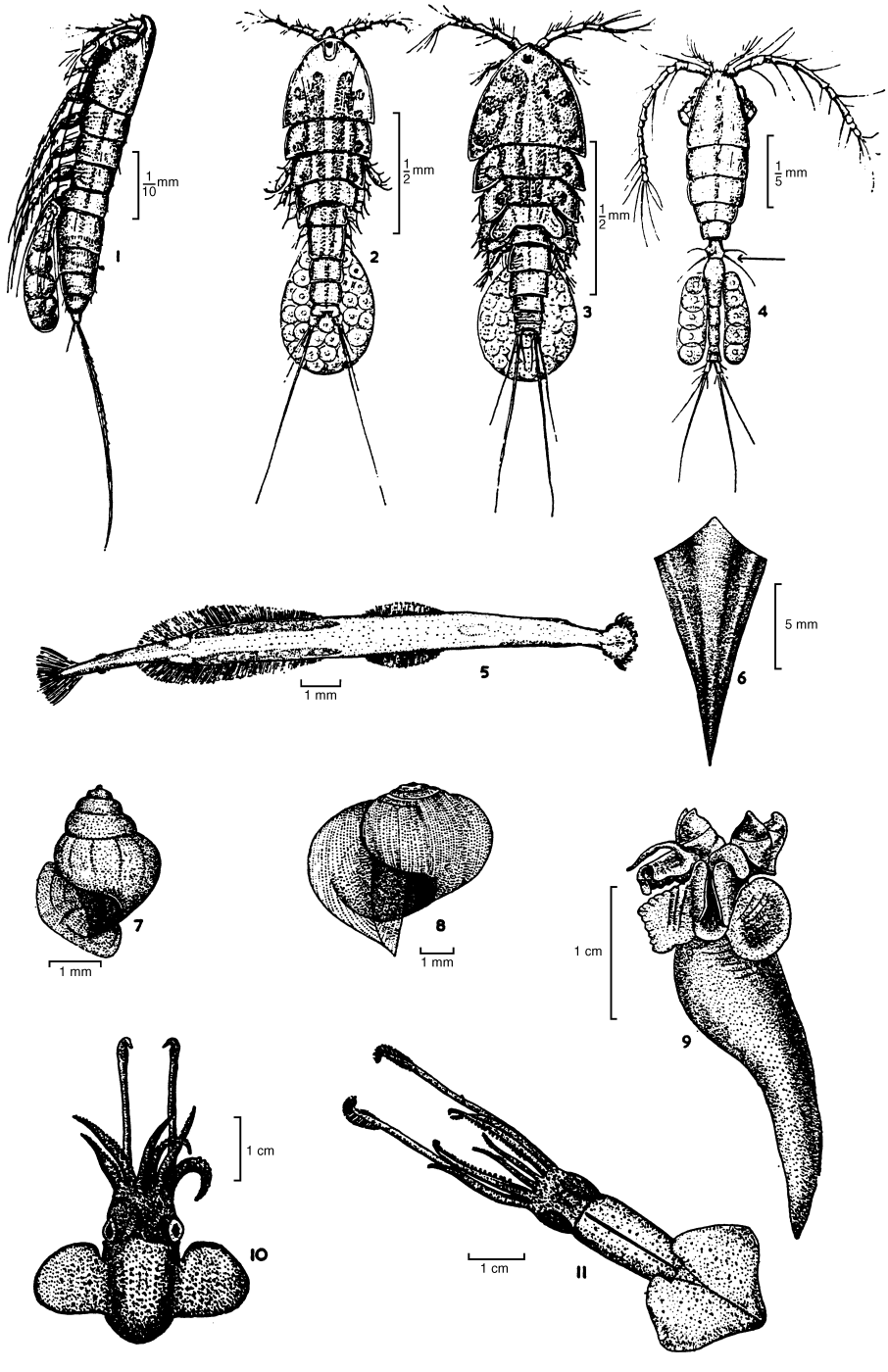

Fig. 1.8 Various kinds of zooplankton. See page 27 for key.

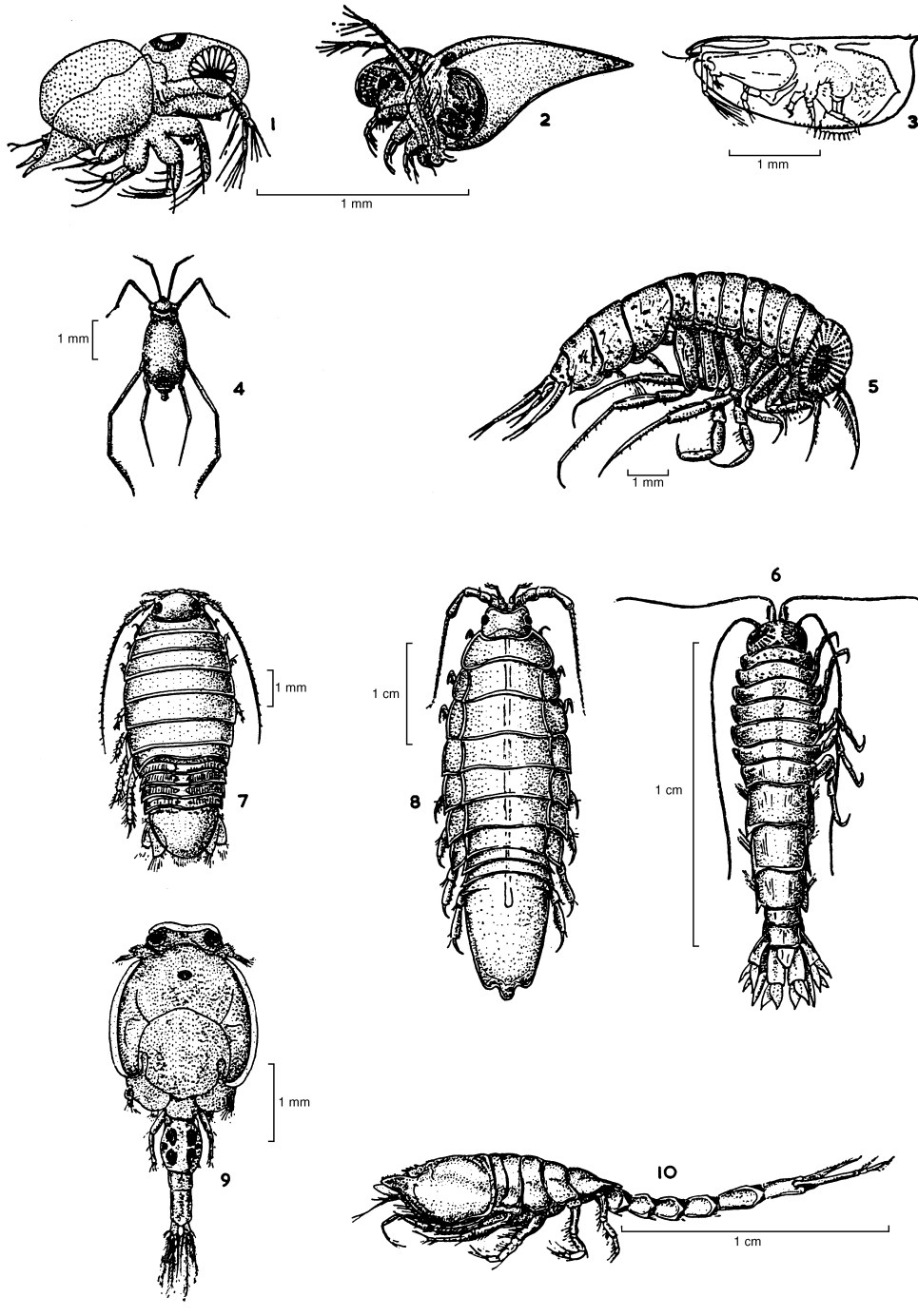

Fig. 1.9 Various planktonic Crustacea etc. See page 27 for key.

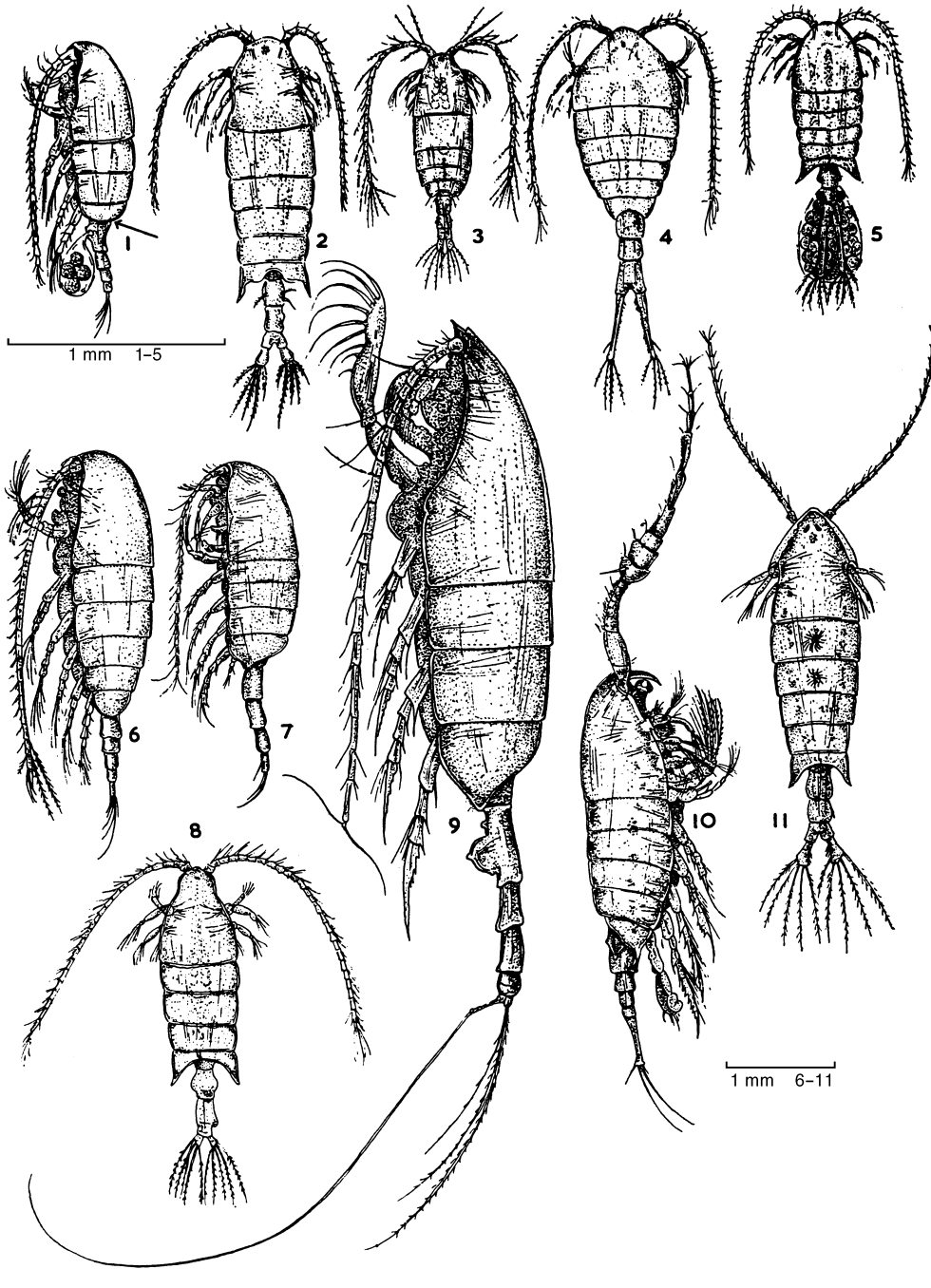

Fig. 1.10 Calanoid copepods. See page 28 for key.

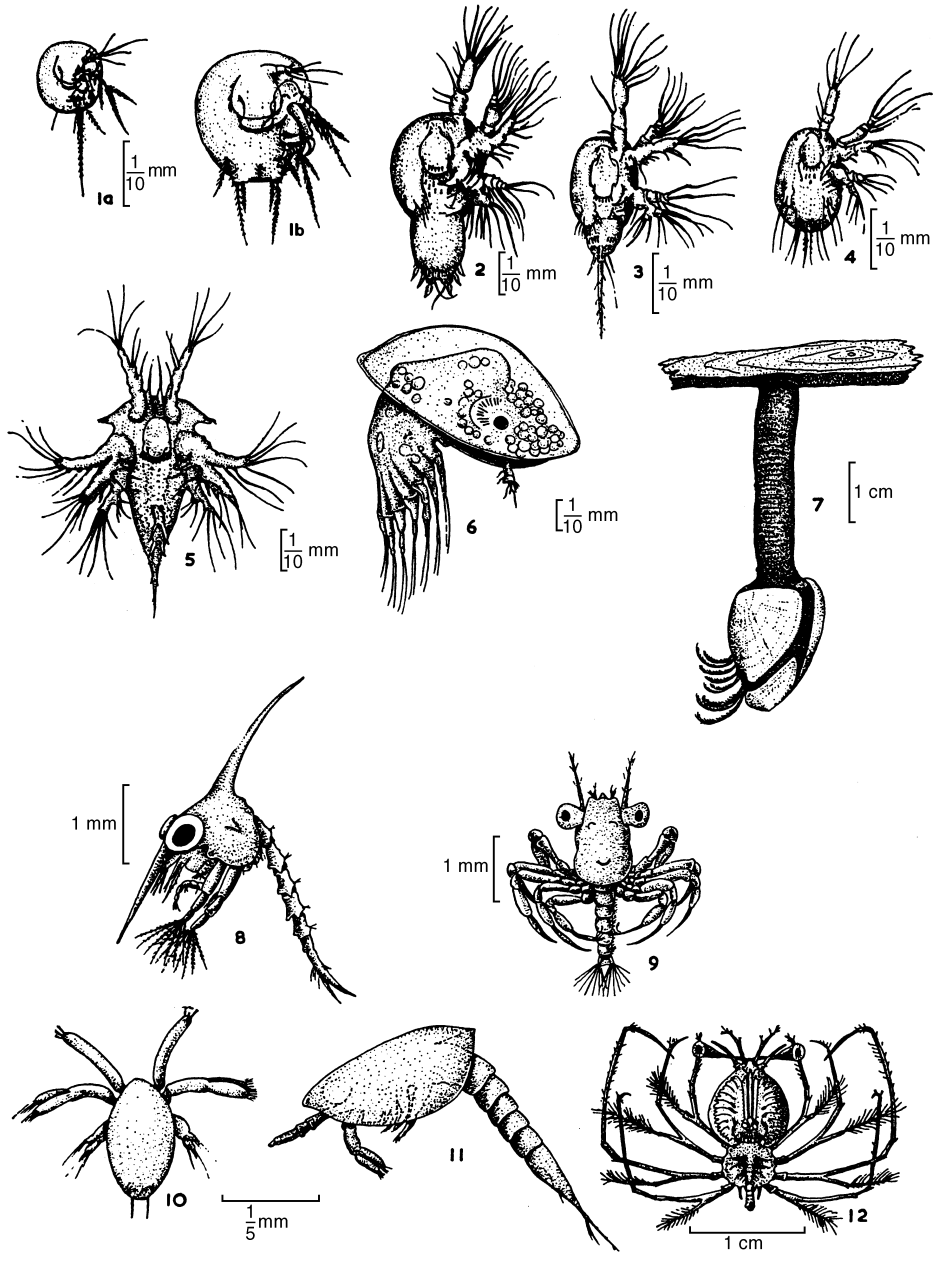

Fig. 1.11 Crustacean larvae etc. See page 28 for key.

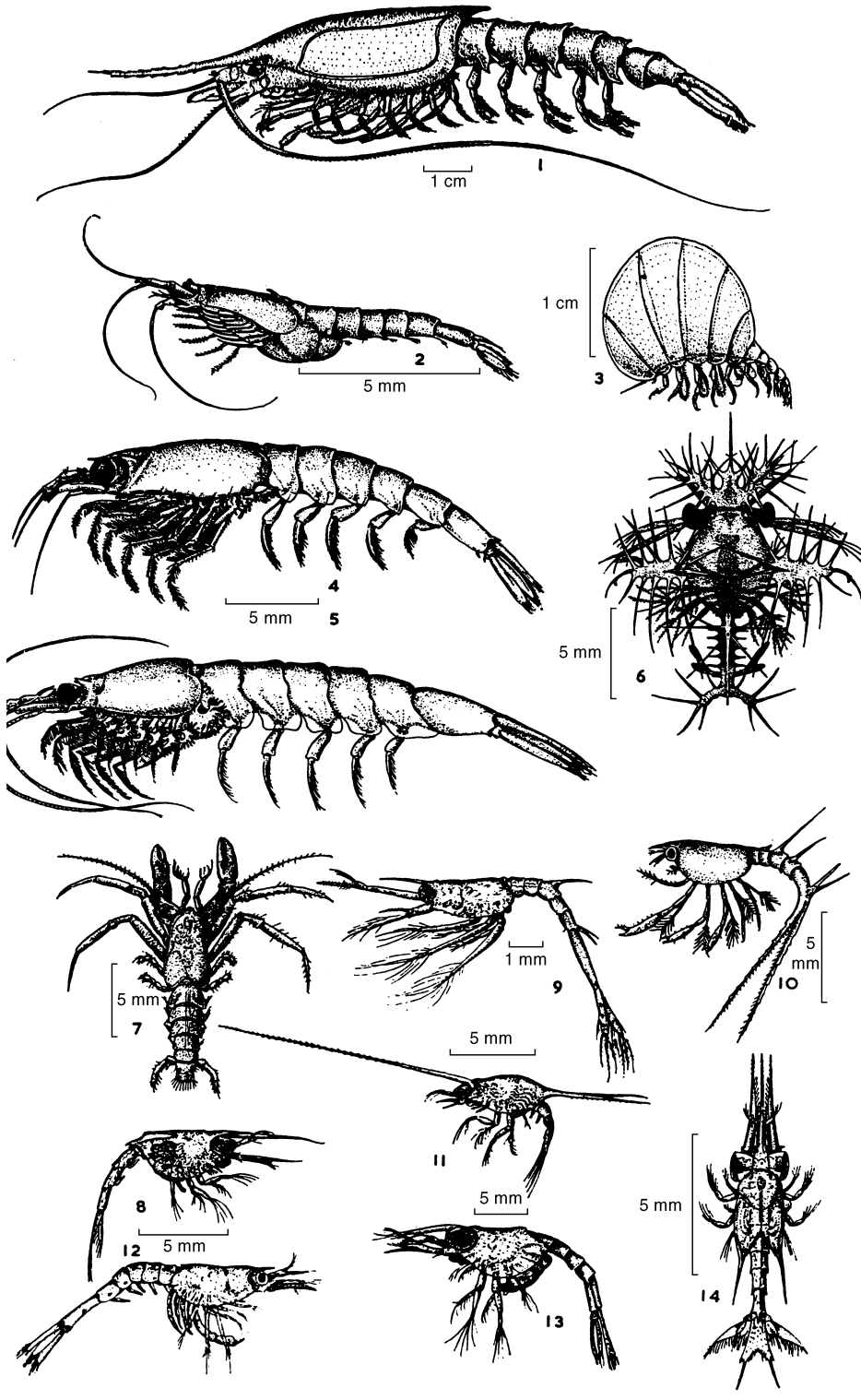

Fig. 1.12 Crustaceans. See page 28 for key.

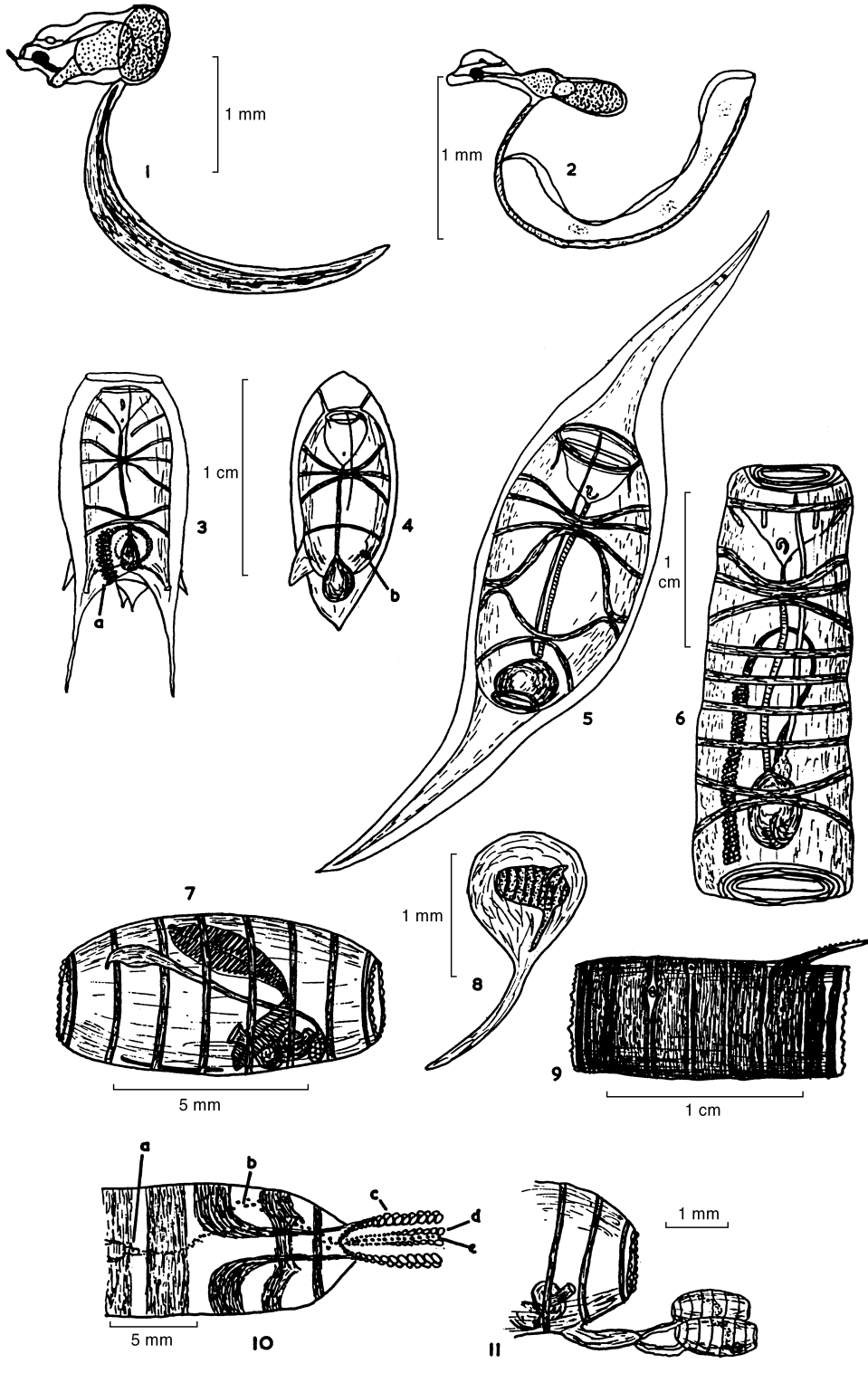

Fig. 1.13 Planktonic Tunicata. See page 28 for key.

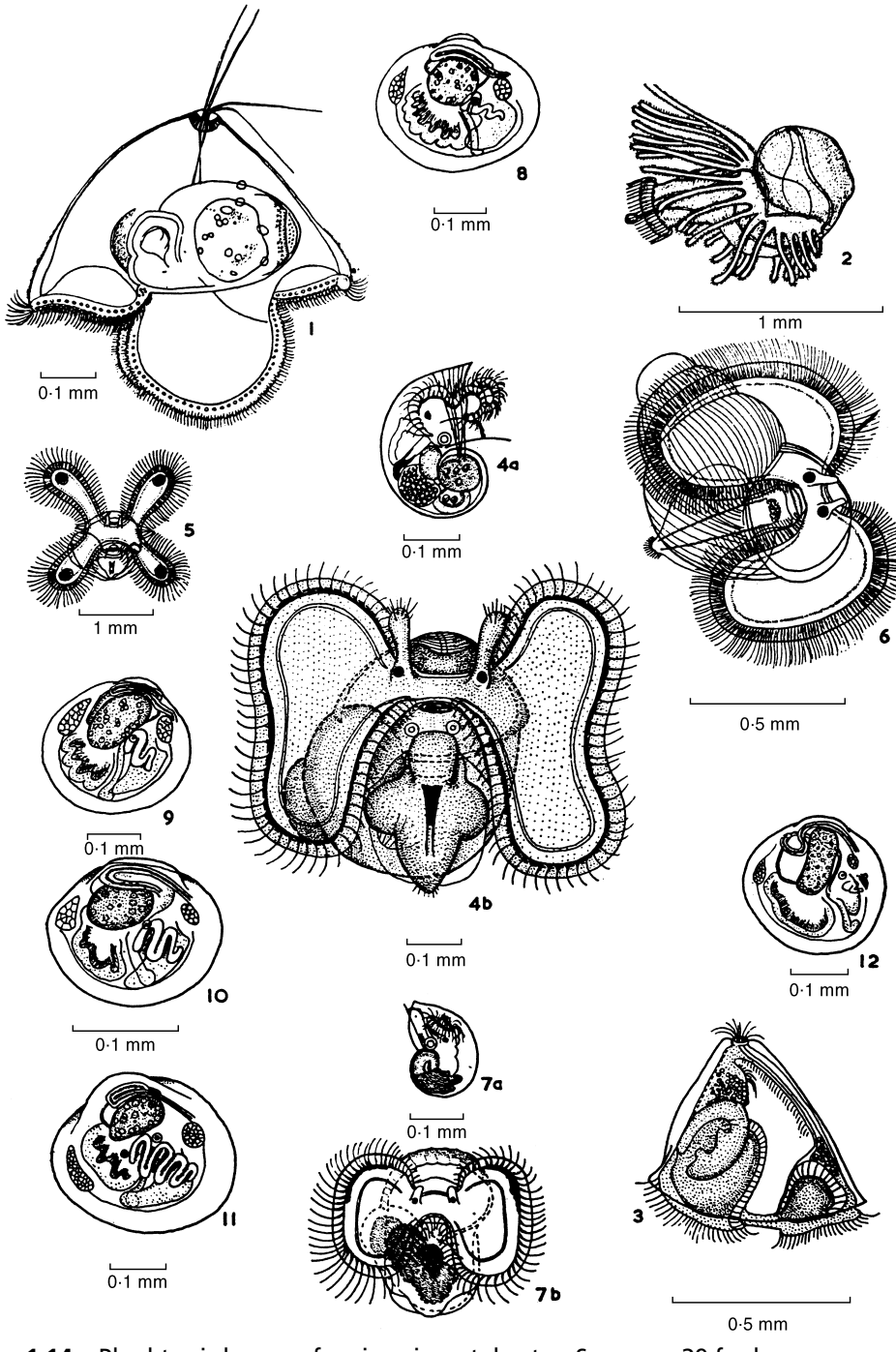

Fig. 1.14 Planktonic larvae of various invertebrates. See page 29 for key.

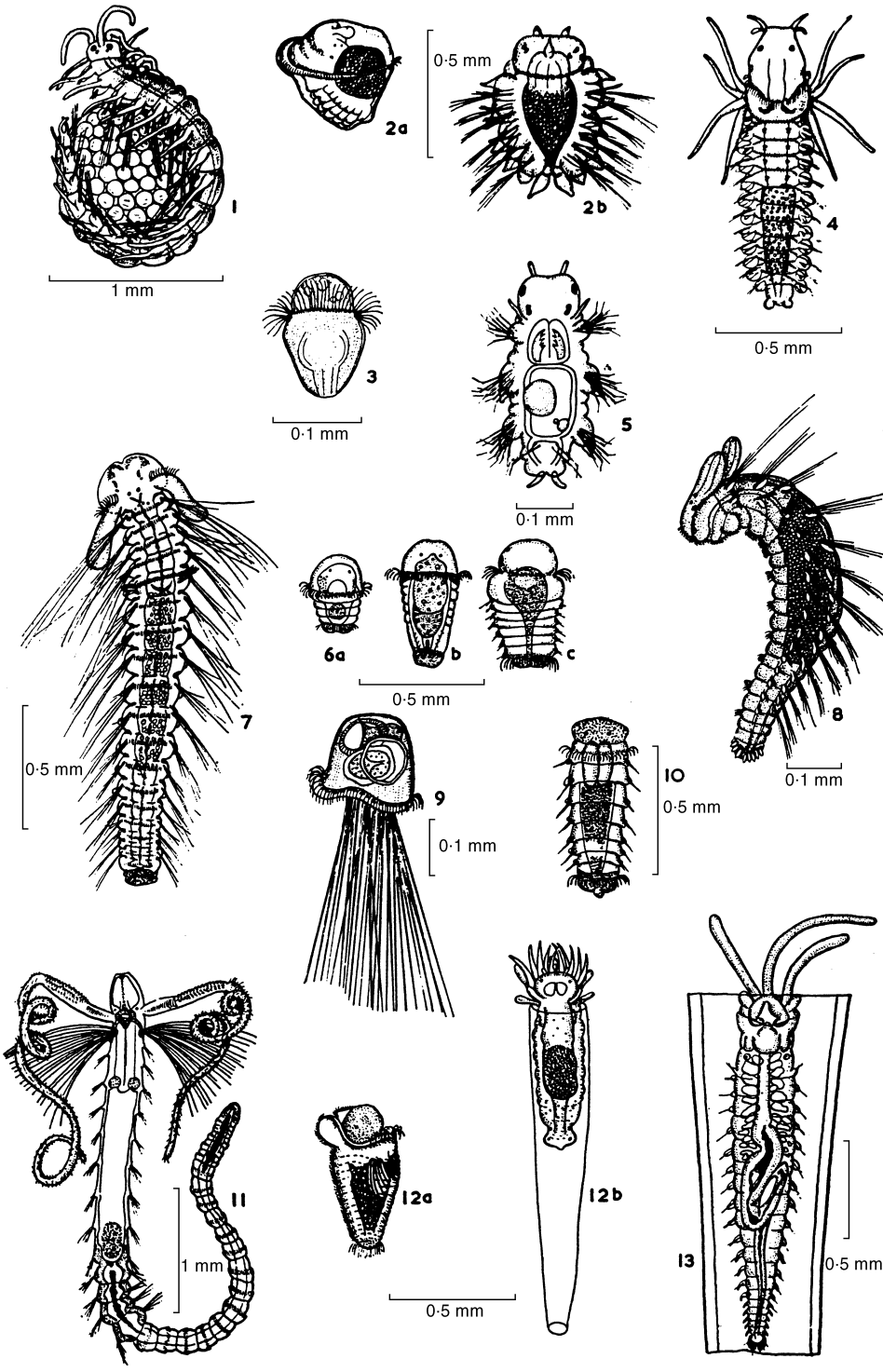

Fig. 1.15 Planktonic stages of polychaete worms. See page 29 for key.

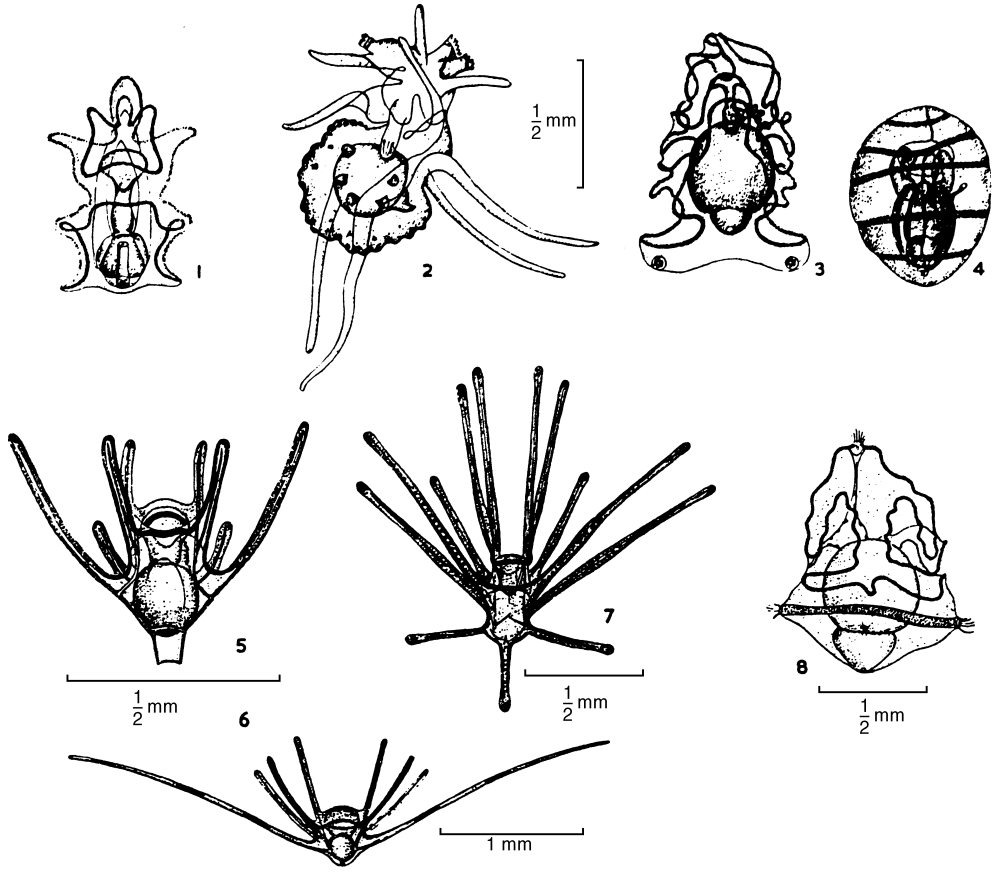

Fig. 1.16 Echinoderm larvae etc. See page 29 for key.

Captions for Figures 1.4–1.9.

Fig. 1.4 Protozoa
Ciliata: (1) *Vorticella marina*, (2a) *Zoothamnion marinum*, (2b) at a reduced scale showing colonies on the copepod *Eurytemora hirundoides*, (3) *Cothurnia gracilis*, (4) *Cothurnia havniensis*, (5) *Epiclintes retractilis*, (6) *Aegyria monostyla*, (7) *Euplotes harpa*, (8) *Aegyria oliva*, (9) *Amphisia pernix*, (10) *Stichochaeta pediculiformis*, (11) *Oxytricha pellionella*. Suctoria: (12) *Acineta tuberosa*. Tintinnoidea: (13) *Parafavella elegans*, (14) *Parafavella edentata*, (15) *Ptychocylis minor*, (16) *Salpingella ricta*, (17) *Tintinnus tubulosus*, (18) *Dictyocysta magna*. Radiolaria: (19) *Challengeron neptuni*, (21) *Acanthometron pellucidum*, (24) *Acanthochiasma fusiforme*, (25) *Hexalonche philosophica*. Foraminifera: (20) *Nonion pompilioides*, (22) *Globigerina bulloides*. Silicoflagellata: (23) *Dictyocha fibula*, (26) *Distephanus speculum*, (27) *Thalassicola nucleata*. All to the same scale except (2b) and (27) (Fraser 1962) used with permission.

Fig. 1.5 Small jellyfish
(1) *Hybocodon profiler*, (2) *Sarsia tubulosa*, (3) *Dipurena ophiogaster*, (4) *Podocoryne borealis*, (5) *Leuckartiara octona*, (6) *Bougainvillia principis*, (7) *Amphinema rugosum*, (8) *Eutima gracilis*, (9) *Eutonina indicans*, (10) *Aglantha digitale*. (2), (3), (4), (6) and (7) to the same scale as (1); (8)–(10) to the same scale as (5) (Fraser 1962) used with permission.

Fig. 1.6 Large jellyfish
(1) The pearl jelly, *Pelagia noctiluca*, (2) *Chrysaora hyoscella*, (3) the big stinger or lion's mane, *Cyanea*, (4) the cauliflower jelly *Rhizostoma octopus*, (5) the moon jelly *Aurelia aurita*. (1) Rarely exceeds 3 inches, (5) reaches about 1 foot, but the others may occasionally be up to 3 feet across (Fraser 1962) used with permission.

Fig. 1.7 Other Coelenterates etc.
Siphonophores or pseudo-siphonophores: (1) *Chelophyes appendiculata*, (2) *Physophora hydrostatica*, (3) *Agalma elegans*, (4) *Physalia physalis*, the Portuguese man o' war, (5) *Velella velella*, the by-the-wind sailor. Ctenophores (comb-jellies): (6) *Pleurobrachia pileus*, the sea gooseberry, (7) *Beroe cucumis*. (8) A larva of the sea-anemone *Cerianthus*, called *Arachnactis* larva. (9) A pelagic polychaete worm, *Tomopteris helgolandica*. The scale lines all represent 1 cm (Fraser 1962) used with permission.

Fig. 1.8 Various kinds of zooplankton
Harpacticoid and cylopoid copepods: (1) *Microsetella norvegica*, (2) *Tigriopus fulvus*, (3) *Idya furcata*, (4) *Oithona similis*. Chaetognatha: (5) *Sagitta elegans*. Pteropoda (shells): (6) *Clio pyramidata*, (7) *Spiratella* (= *Limacina*) *retroversa*, (8) *Spiratella helicina*, (9) *Clione limacina*. Small Cephalopoda: (10) *Sepiola*, (11) *Brachiotheuthis*. The arrow in (4) indicates the joint between the cephalosome and abdomen (Fraser 1962) used with permission.

Fig. 1.9 Various planktonic Crustacea etc.
Cladocera: (1) *Podon leuckartii*, (2) *Evadne nordmanni*. Ostracoda: (3) *Conchoecia elegans*. Insecta: (4) *Halobates micans*. Amphipoda: (5) *Themisto abyssorum*, (6) *Hyperia galba* male (the female is much broader). Isopoda: (7) *Eurydice pulchra*, (8) *Idothea balthica*. Copepoda: (9) *Caligus rapax*, the sea louse, a semi-parasitic copepod. Cumacea: (10) *Dyastylis rathkei* (Fraser 1962) used with permission.

Captions for Figures 1.10–1.13.

Fig. 1.10 Calanoid copepods
(1) *Pseudocalanus elongatus*, (2) *Centropages typicus*, (3) *Acartia longiremis*, (4) *Temora longicornis*, (5) *Eurytemora hirundoides*, (6) *Calanus finmarchicus*, (7) *Metridia lucens*, (8) *Candacia armata*, (9) *Pareuchaeta norvegica*, (10) *Anomalocera patersoni* male, (11) female. The arrow in (1) indicates the joint between the cephalosome and abdomen (Fraser 1962) used with permission.

Fig. 1.11 Crustacean larvae etc.
Copepoda: (1a) and (1b) 1st and 3rd stages of the nauplius of *Tigriopus*, (2) 4th nauplius of *Calanus*, (3) 5th nauplius of *Centropages*, (4) 5th nauplius of *Oithona*. Cirripedia: (5) 2nd nauplius of acorn barnacle, *Balanus*, (6) cypris stage of same, (7) adult goose barnacle, *Lepas*. Decapoda: (8) 3rd zoea of crab, *Portunus puber*, (9) megalopa of same, (12) phyllosome of spiny lobster, *Palinurus*. Euphausiacea: (10) 2nd nauplius of *Thysanoessa inermis*, (11) 2nd calyptopis of *Thysanoessa longicaudata* (Fraser 1962) used with permission.

Fig. 1.12 Crustaceans
Mysidacea: (1) *Gnathophausia zoea*, (2) *Gasterosaccus sanctus*. Amphipoda: (3) *Mimonectes loverni*, an exotic species. Euphausiacea: (4) *Thysanoessa inermis*, (5) *Meganyctiphanes norvegica*. Larvae of various Decapoda: (6) *Sergestes*, an oceanic prawn, (7) and (8) *Eupagurus*, hermit-crab, (9) *Pontophilus*, (10) *Nephrops*, Norway lobster, (11) *Porcellana*, pea-crab, (12) *Crangon*, shrimp, (13) *Galathea*, (14) *Munida*, squat lobster (Fraser 1962), used with permission.

Fig. 1.13 Planktonic Tunicata
Appendiculata: (1) *Oikopleura dioica*, (2) *Fritillaria borealis*. Thaliacea: (3) *Thalia democratica*, solitary stage showing the budding stolon (a) which gives rise to a chain of aggregate stages, (4) *Thalia democratica*, aggregate stage with a single embryo (b) which will eventually become a free solitary stage, (5) *Salpa fusiformis*, aggregate stage, (6) *Salpa fusiformis*, solitary stage, with stolon, (7) *Dolioletta gegenbauri*, gonozoid, complete with sexual organs. The eggs from this stage hatch into 'tadpoles'. (8) A later stage of the 'tadpole' showing the remains of the outer capsule and the oozoid developing inside, (9) the late oozoid or 'old nurse' now devoid of almost all its internal organs, but with broad muscle bands and balancing organ (statocyst), a nerve center and the remains of the dorsal process, (10) a view from above of the oozoid at its functional stage with a prominent dorsal process, (a) the buds developing on the stolon, (b) the buds migrating to the dorsal process, (c) double rows of lateral zooids, the trophozoids which serve only to catch food for the whole, (d) median rows of phorozoids, and (e) the youngest migrating buds which will become new gonozoids, (11) a later stage of a phorozoid, now broken free from the dorsal process of the oozoid and acting as a bearer for two developing gonozoids. These will eventually become the free living sexual form figured in (7) (Fraser 1962) used with permission.

Captions for Figures 1.14–1.16

Fig. 1.14 Planktonic larvae of various invertebrates
(1) Pilidium larvae of nemertine worm, (2) Actinotrocha larva of Phoronis, (3) Cyphonautes larva of a sea moss, *Membranipora*. Gasteropoda (univalve mollusks): (4) *Nassarius reticulata*, (a) larva just after hatching, (b) old larva with shell forming, (5) *Nassarius incrassata*, old larva, (6) *Actis minor*, old larva with shell forming, (7) *Littorina littorea* (periwinkle), (a) young larva, (b) later larva. Lamellibranchiata (bivalve mollusks): (8) *Tellina* sp., (9) *Macoma baltica*, (10) *Cardium edule* (cockle), (11) *Mytilus edulis* (mussel), (12) *Pecten opercularis* (queen, or small scallop). (1)–(7) from Thorson, (8)–(12) from Jørgensen (Fraser 1962) used with permission.

Fig. 1.15 Planktonic stages of polychaete worms
(1) *Autolytus prolifer*, with eggs, (2) *Harmothoe imbricata*, (a) metatrochophore, (b) nectochaete stage, (3) *Phyllodoce groenlandica*, old trochophore, (4) *Phyllodoce maculata*, old larva, (5) *Nereis pelagica*, nectochaete stage, (6) *Nephthys ciliata*, (a) young trochophore, (b) and (c) metatrochophores, (7) *Polydora coeca*, old larva, (8) *Pygospio elegans*, old larva, (9) *Myriochele danielsseni*, young larva, (10) *Nephthys coeca*, nectochaete stage, (11) *Megalona papillicornis*, old larva, (12) *Pectinaria auricoma*, (a) young larva, (b) old larva in a gelatinous tube, (13) *Lanice conchilega*, old larva in a gelatinous tube. All from Thorson (Fraser 1962) used with permission.

Fig. 1.16 Echinoderm larvae etc.
(1) Bipinnaria larva of a starfish, *Asterias glacialis*, (2) Brachiolaria larva of *Asterias rubens*, (3) Auricularia larva of a sea cucumber, *Synapta digitata*, (4) Doliolaria larva, or pupa, of *Synapta*, (5) Pluteus larva of a brittle star, *Ophiura texturata*, (6) Pluteus larva of a brittle star, *Ophiothrix fragilis*, (7) Pluteus larva of a sea urchin, *Echinocardium cordatum*, (8) Tornaria larva of the acorn-worm, *Balanoglossus* (Hemichordata). (1)–(4) are to same scale (Fraser 1962) used with permission.

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2 Sampling and experimental design

H.-R. Skjoldal, P.H. Wiebe and K.G. Foote

2.1 INTRODUCTION

Biological and oceanographic phenomena associated with zooplankton occur over enormous time and space scales, so that it is not possible to know the actual distribution of zooplankton. What is directly known is derived by sampling, or measurement at so-called sampling stations. Additional information comes from modeling studies and from experimentation in the laboratory. Integration of the different approaches in ecological studies characterizes current efforts to understand the structure and dynamics of zooplankton populations in the world's oceans.

The design of a scientific study of zooplankton depends on the purpose of the study. The first step in designing a study is therefore to define the purpose. This involves identification and delimitation of the scientific questions, hypotheses, issues to be addressed, and objectives for a particular study. Then, the dominant processes affecting the zooplankton that are the objects of study need to be identified. The processes may be physical, chemical, or biological. A key design issue concerns the temporal or spatial scale or scales at which the dominant processes are operating. The scales may range from long-term (decadal or longer) and large-scale climatic processes to biochemical and physiological processes in cells and individuals. The scales of the dominant processes determine the appropriate scale of an investigation, although it is important to recognize that interactions with processes acting on other scales may bias or in some way alter the direct effects being studied (Haury *et al*. 1978). Numerical definitions of scales of spatial patterns are given in the same work.

Typically in marine ecology and oceanography, there is a continuum of scales from the very fine to the very large, where various processes are interlinked (Haury *et al*. 1978; Dickey 1990). For example, changes in climatic patterns on a large scale may cause an alteration in wind events, changing levels and patterns of turbulence on the smallest scales. This may affect the growth conditions of phytoplankton species and the feeding conditions for zooplankton and fish larvae. The effects of these fine-scale processes may in turn be compounded to alter the population dynamics and distribution of species of plankton and fish at the scale of regional seas and ocean basins. The interlinked nature of processes and scales is an important feature and a great challenge when designing studies of zooplankton.

Three lines of approach may be taken to address a given issue in zooplankton ecology; these are theoretical, experimental and observational. As appropriate, the three approaches may be used singly or in combination. A theoretical approach should be

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used to the extent possible and may assist in consideration of scales and in the further design of supplementary experimental and field studies. For example, mathematical models are being used increasingly to simulate ocean physics and plankton dynamics. Experimental studies in the laboratory or with enclosed mesocosms in the field may be used for more careful observational scrutiny and manipulations to reveal underlying mechanisms and processes. Both theoretical and experimental studies can aid in planning a field study by helping to determine which parameters and variables to measure and with what spatial and temporal resolution. A research strategy based on the combined use of the different approaches is advantageous and can provide a synergistic benefit to the research effort. Full integration should be the aim, where theoretical considerations may lead to better focused field observations and experiments, which again may form the basis for improved theoretical or modeling efforts.

Once the approach has been decided the specific methods for sampling and analysis must be chosen. The investigator's expertise in one or several methods often dictates the research topics to be addressed. Ideally, however, methods should be developed, standardized, and described for wider use in the scientific community. The choice of methods will involve compromising between the expertise required and the associated cost, and the need to address a given scientific issue. This can be illustrated, for instance, by the various methods used for sampling and describing the abundance and distribution of zooplankton. Sophisticated methods dependent on acoustical and optical technology may be the ideal choice. However, high costs and technical and operational requirements may prevent the use of such technologies. Instead, more traditional and simpler methods based on vertical or oblique net hauls may be the realistic alternative in some cases.

The final step in the design of a study is to prepare a detailed work plan. This involves considering the sampling location and frequency, sampling size, and whether pooling of samples or sub-sampling should be used for particular analyses. It also involves determining which variables should be measured and the specific methods to be used for the measurements. Statistical considerations should be a central element in the design of a study. Prior knowledge of the accuracy and precision of specific analyses and natural variability at different scales should be used to guide the design of a sampling and measurement program.

There are two foci in this chapter: principal conceptual issues that individuals or groups of researchers face when preparing to conduct extensive and intensive studies of plankton populations, and design criteria and strategies for sampling the oceans for zooplankton.

2.2 CONCEPTUAL ISSUES

2.2.1 Increased emphasis on species dynamics

Zooplankton constitute a central component of marine ecosystems, providing the link between the phytoplankton primary production and higher trophic levels, such as commercially exploited fish stocks. Because of this, zooplankton may be important from an economic perspective as well as from an ecological perspective.

In ecology, there has been much emphasis on the flow of energy and cycling of matter in ecosystems. The concept of trophic levels (Lindeman 1942) has allowed simplified quantitative descriptions of complex ecological systems. This has formed the basis for addressing such issues as ecological transfer efficiencies and trophic structure. Classical

box models of the type constructed by Odum (1972) and Platt *et al*. (1981) have been used to summarize and illustrate trophic structures and the associated flow patterns and budgets of energy and matter. The broad International Biological Programme (IBP) initiative in the 1970s was based on these concepts of trophic levels, energy flow, and production. The Joint Global Ocean Flux Study (JGOFS) of the International Geosphere–Biosphere Programme (IGBP) can be seen as a continuation of this research tradition, with emphasis on carbon flow and budgets in the ocean basins.

There is at present, however, movement toward greater emphasis on individual zooplankton species and their population dynamics in marine ecology. This is at least partly based on the recognition that physical processes have great influence on plankton, and that this influence must be addressed at the individual species level. This shift in emphasis from bulk energy flow to species dynamics is clearly reflected in the current IGBP Global Ocean Ecosystem Dynamics (GLOBEC) program (US GLOBEC 1991a; IGBP 1997, 1999). GLOBEC addresses the influence of climatic and physical oceanographic forcing on marine ecosystems with particular focus on zooplankton population dynamics. The vertical distribution of zooplankton determines the advective regime which they are in, and therefore influences their horizontal distribution. This again influences the overlap in distribution with predator populations. The vertical distribution of a species also determines the likelihood of being seen and eaten by visual predators such as planktivorous fish. Thus, behavior is important for both distribution and trophic interactions and ultimately for the population dynamics.

2.2.2 Integration of disciplines: zooplankton, between physics and fish

A central issue in marine ecology is the degree to which marine ecosystems are controlled by bottom-up versus top-down processes (Verity and Smetacek 1996). In broad terms, climatic and physical oceanographic forces affect the level and timing of phytoplankton primary production, with consequences for zooplankton, fish and other predators up the food chain. Physical forces can also affect populations of zooplankton and fish directly, particularly through their effect on fish recruitment. Physically driven variability sets the stage for strong biological interactions, cascading downward as well as upward in the trophic structure. Thus, bottom-up and top-down control are linked, although operating on different time scales and through different mechanisms.

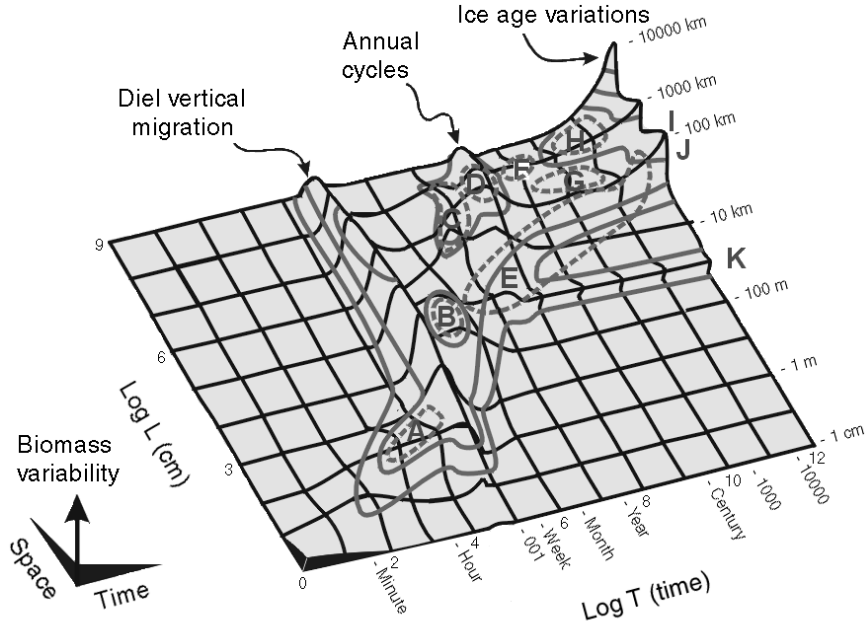
To reveal the roles of zooplankton in controlling marine ecosystem dynamics, studies of zooplankton should, to the extent possible, be carried out by integrating physical, biological, and fisheries scientific disciplines. Such an ecological approach generally requires a collaborative effort by a team of scientists.

In situations where a broad ecological approach is not possible and a more focused research issue on zooplankton is pursued, there is still a need for descriptive supporting information on the environment of the zooplankton. Such information may include hydrography, currents, light conditions, phytoplankton fluorescence, biomass, production, and distribution and numerical density of fish. When designing a study, careful consideration should be given to the practicability of obtaining such information. Modern research vessels are often equipped with conductivity-temperature-depth (CTD) sonde, acoustic Doppler current profiler (ADCP), *in situ* fluorometer, and echo sounders which can give descriptive information on the physical environment, phytoplankton, and fish. Portable equipment can be used when carrying out field research with smaller vessels. Zooplankton sampling gear may need to be equipped

with environmental sensors to record detailed environmental conditions at the time and place of collection.

2.2.3 Integration of scales: from climatic to turbulent

The choice of appropriate temporal and spatial scales and the key processes involved when addressing a given zooplankton issue are important parts of the design considerations. Physical, chemical, and biological processes affecting zooplankton occur at different temporal and spatial scales. Knowledge of these scales forms the basis for deciding what the appropriate scales should be for a measurement program as part of the design consideration for a given zooplankton study. The temporal and spatial scales are linked, as illustrated by the so-called Stommel-diagram (Figure 2.1; Haury *et al.* 1978). Phytoplankton with approximately one-day generation times are affected by small-scale physical processes such as vertical mixing and stratification of the upper ocean layer. Zooplankton with generation times of weeks to months are more strongly affected by larger-scale processes, such as advection with ocean currents and ocean-shelf exchanges. Fish with typical generation times of several years may exhibit large-scale migratory



A Micro patches
B Swarms
C Upwelling
D Eddies and rings
E Island effects
F El Nino type events
G Small ocean basins
H Biogeographic provinces
I Currents and ocean fronts – length
J Currents – width
K Oceanic fronts – width

Fig. 2.1 A conceptual model of the time–space scales of zooplankton biomass variability in ocean ecosystems (redrawn from Haury *et al.* 1978).

patterns and are affected by long-term and large-scale patterns of climatic and physical variability particularly impacting their reproduction.

It is important to note that the different scales are interlinked and nested in a hierarchical manner. This can be illustrated from physical and biological perspectives. Climatic variability or change at the global scale is associated with variability and changes in regional weather patterns (e.g. changes in storm frequencies and intensities). This may lead to changes in currents and water mass distributions and in dynamics of the upper mixed layer of the ocean, cascading downward to affect the patterns of small-scale turbulence. The biological effects of changes in small-scale turbulence may propagate upward in the hierarchy of scales. Thus, small-scale turbulence may influence the species composition and growth of phytoplankton as well as the encounter rates between plankton predators and prey, and thereby the food production and feeding success of zooplankton and fish larvae. This may in turn alter the growth rate and population dynamics of zooplankton and fish.

The nesting of scales can also be illustrated by an example from fish ecology. As visual predators, pelagic planktivorous fishes depend on light to detect and capture their zooplankton prey. Their feeding and feeding success depends therefore on small-scale processes involving visual range, visibility, distribution and behavior of zooplankton prey. The small-scale feeding processes are linked to the day–night cycle of light, vertical migration and behavior of zooplankton prey, as well as the vertical migration, schooling, and feeding behavior of the fish. The day–night cycle is in turn related to seasonal patterns in the plankton production and in the migration, growth, and maturation of the fish. The seasonal pattern is again related to large-scale shifts in distribution patterns within the multi-annual life cycle of the fish. An example of the role of multiple scales in predation is illustrated by observing juvenile herring predation on copepods (Kils 1992), which is also discussed from a sampling perspective in section 7.2.5.

2.2.4 Integration of approaches: from theory to field

As noted in the introduction to this chapter, there are three different approaches available for studying a zooplankton issue: theoretical, experimental, and observational. Theoretical considerations should direct experimental work and field observational programs. For instance, the use of hydrodynamical and population dynamical models may be of help in deciding the spatial and temporal scales and resolution for a measurement program. In recent years, progress has been made in theoretical work on zooplankton. Based on life-history theory, the behavior, distribution patterns, and life cycles of zooplankton species have been explained and predicted (Aksnes and Giske 1990; Giske *et al*. 1994, 1997; Fiksen and Giske 1995). Models for growth and population dynamics of zooplankton (see also Chapter 12) have been developed and refined (Carlotti and Nival 1992; Carlotti and Radach 1996; Carlotti and Hirche 1997; Zhou and Huntley 1997). There have been a number of model studies of the dynamics and distribution of zooplankton in relation to ocean currents and hydrodynamic flow fields using coupled 2- and 3-dimensional physical models (Hannah *et al*. 1998; Lynch *et al*. 1998; McGillicuddy *et al*. 1998). Progress has also been made in the modeling of acoustical properties of zooplankton as a basis for quantification of their distribution and abundance by echo sounders (Stanton *et al*. 1996, 1998; Wiebe *et al*. 1996a, 1997; Benfield *et al*. 1998; Martin Traykovski *et al*. 1998; Francis *et al*. 1999a,b).

Experiments would normally be conducted to examine a hypothesis of how one or several factors influence a given feature or zooplankton process. Experiments may also

be carried out to determine rates or coefficients used in modeling or budget calculations. The strength of a good experiment is the ability to examine the role of one or several factors under conditions where other factors are kept as constant or controlled as possible. An inherent challenge with experiments is the difficulty found in simulating natural conditions; with the associated difficulty of extrapolating results obtained in the laboratory to nature (Reeve 1977).

Experiments with mesocosms may represent a compromise between laboratory experiments and field observations (Grice and Reeve 1982). Mesocosms may achieve quasi-natural conditions with respect to physical conditions and small plankton, while at the same time allowing some degree of control and experimental manipulation. Mesocosms have been used to study, for instance, effects of nutrient enrichments and variable densities of zooplankton and small predators such as fish larvae or juvenile fish on plankton composition and production (Madin *et al.* 1996). Small mesocosm-like experimental chambers have been used to demonstrate the effects that fish predators have on the diel feeding behavior of copepods (Bollens and Sterns 1992). Mesocosms are not identical and replication may therefore be a problem in an experimental design even if similar conditions are sought (Pilson *et al.* 1979; Grice and Reeve 1982; Skjoldal *et al.* 1983). The limited size of mesocosms may also make them unsuitable environments for large organisms such as fish. This may limit the applicability of mesocosms and the extrapolation of results obtained in mesocosms to natural conditions.

Field observational programs are carried out for many different purposes. Field investigations may be carried out to verify predictions from theoretical or experimental studies. An experimental approach may, in turn, be used for field investigations. Natural events may cause large changes in physical and biological conditions, which may be taken as the basis for a diagnostic analysis of the dynamics of the changes. Comparison of contrasting situations in different regions may also be utilized to reveal underlying mechanisms and driving forces for variability in marine ecosystems.

While we strive through our studies to reveal and gain understanding of the basic mechanisms that govern processes in nature, our preconceptions based on the current paradigm may be erroneous. Nature contains the 'truth' which is there to be seen through unbiased observations. Thus, there is a need to keep a balance by staying focused and guided by theory, and remaining unbiased and open-minded for observations of new phenomena. Unbiased observations can form the basis for further development of theory and for planning new experiments. Thus, the three approaches, described above in Section 2.1, should be fully integrated. Theory should be tested by experimentation and field observations. Experiments may help clarify and explain field observations. Field observations in turn may suggest new experiments and further development of the theoretical foundation.

2.2.5 Integration of pattern and process

Pattern (distribution and abundance) and process (biological and physical rates) are both important in ecology. Determination of the population dynamics of zooplankton requires knowledge of the number of individuals in the population and their time rate of change. The rate of change in the number of individuals depends on recruitment and mortality, and rates of change in the properties of individuals through feeding, metabolism, and growth. The production of zooplankton is in principle the product of biomass and rate of production.

Information on abundance or numerical density and on physiological rates is required

for studies involving trophodynamics. The abundance data provide information on prey and predators and form the basis for calculations of feeding of predators and mortality of prey. Information on changes in abundance may also form the basis for extracting information on underlying rates of recruitment and mortality by population dynamical calculations. There may be limitations, however, to the spatial and temporal resolution in descriptions of abundances, limiting the ability to extract information on the underlying dynamics. Measurements of rates may provide valuable help in this respect, facilitating interpretation of observed patterns in abundance and budget calculations.

The variability in abundance may differ fundamentally from that of rates. The spatial and temporal variability in zooplankton biomass tends to be much larger than the variability in physiological rates, and most effort needs to be spent on determining biomass distribution for the purpose of estimating zooplankton production (Zhou and Huntley 1997). There is, however, an inherent difficulty in comparing abundances and rates. The number of organisms (N) changes with time t according to an exponential relationship

$$N = N_o \exp(-mt) \tag{2.1}$$

where m is the mortality rate of an exponential coefficient.

Individual growth (W) also satisfies an exponential-type relationship with growth rate (g) as an exponential coefficient

$$W = W_o \exp(gt) \tag{2.2}$$

For comparisons of variability, therefore, it is most appropriate to compare the variance in log-transformed abundances with the variance of rates.

Different processes may affect abundances and rates in various manners and scales. Thus, the abundance of zooplankton in a given area may be determined by the hydrodynamical flow field and the advective regime, the intrinsic rates of recruitment and growth, and the rate of predation by, for instance, schools of planktivorous fishes. Advection and predation may be determined by large-scale features related to meteorology, ocean currents and fish migrations. Rates of feeding, growth, and egg production by adult zooplankton females may, however, depend more on small-scale and local features such as the degree of mixing in the upper ocean layer and turbulence. In field investigations, integrating observations of zooplankton abundance and distribution with information on rates determined in shipboard experiments and incubations is often essential. This may be necessary to determine rates of feeding, metabolism, and growth under standardized or simulated *in situ* conditions.

Depending on the purpose of study, careful consideration should be given to the allocation of effort given to measurements of abundances and rates respectively. Generally, a combination should be sought where measurements of abundances and rates can lend mutual support to each other.

2.2.6 Integration of technologies and methods

The various methods of sampling and analyzing zooplankton have different strengths and weaknesses. There can, therefore, be a benefit in combining the different methods to obtain broader and more representative results.

A given zooplankton net may sample representatively within a limited size range due to the impact of mesh size and net avoidance. These issues are discussed in Chapter 3. The combined use of different nets may be required to obtain samples over a wider size range. This approach may be used to cover all life stages, from eggs to adults, of a given

species or to cover the size ranges of prey and predators involving zooplankton. Where planktivorous fish are important predators, it may also be useful to include sampling with fish trawls to supplement sampling with zooplankton nets.

Acoustics is a powerful method for collecting information about zooplankton distributions and densities. There is a limitation, however, in the ability to discriminate among organisms of different size, taxonomic affinity, and material properties, such as sound speed contrast and density contrast between the organism and sea water. Acoustical recordings should be verified by net sampling to identify the sound scatterers and to apply algorithms to calculate the numerical densities of different species, groups, or size classes of zooplankton (e.g. Wiebe *et al*. 1997). Acoustics may provide quantitative information on the numerical density and distribution of fish which often prey on zooplankton.

Optical methods based on *in situ* video recordings or photography may be used to quantify the numerical density of zooplankton and to study their distribution and behavior *in situ* (see Chapter 7 on optical methods for additional detail). Optical methods may be used in combination with acoustics to identify the organisms producing echoes (e.g. Benfield *et al*. 1998). They may also be used in combination with net sampling to indicate or quantify avoidance by organisms of nets and to provide information about delicate zooplankton organisms which are destroyed by the nets (Benfield *et al*. 1996).

Ideally, zooplankton nets and *in situ* sampling systems involving acoustics and/or optics should be equipped with environmental sensors recording physical parameters such as temperature and salinity, water transparency, and phytoplankton chlorophyl fluorescence. This would allow more detailed descriptions of the environmental conditions in relation to zooplankton distribution and abundance.

2.3 DESIGN OF OCEANOGRAPHIC CRUISES AND SURVEYS

2.3.1 Survey design considerations

A single sampling station is a time interval and region of space that should be small compared to the respective time and space scales of variation of the phenomenon being studied. Sampling stations may span intervals of time and occupy regions of space that are comparable to or larger than the characteristic or significant scale sizes of variation. At the start of this section, however, sampling stations are assumed to be sufficiently restricted so that observations in time and space can be linked without ambiguity.

The spatial arrangement and performance in time of a set of sampling stations define a cruise or survey design. These depend on many different considerations (Foote 1995), the first of which must be the objective of the sampling. The entire set of possible survey objectives need not be described in order to illustrate the importance of an objective to the design. For example, mapping a distribution pattern may be the main objective of a survey. In this case, the design will aim to cover the entire region of occurrence or at least the main area of concentration. It will aim to do this with sufficient sampling density so that the distribution where not sampled can be estimated by interpolation, as opposed to extrapolation.

Determination of the abundance of a resource or size of an animal stock, as by echo integration (section 6.2.5), may involve a survey design similar to that used in mapping a distribution pattern, although the aims are quite different. In particular, abundance

estimation requires comprehensive or synoptic knowledge for purposes of integration, while mapping requires the same information for essentially point-wise estimation. Estimation of production may also require comprehensive surveying on the large scale, first to define areas and degrees of production, then to average or integrate these.

Process studies are generally performed on the small scale to define or elucidate the nature of a process. Sampling over a rather limited region of space, as along a line transect crossing a hydrographic front, may be sufficient.

The design of a survey also depends on the sea area to be covered. Land masses, skerries, currents, possible presence of ice, and likely weather patterns may play major roles in designing a survey. The presence of gradients, as in bottom topography or in the concentration of nutrients, may define a geometrical framework for designing the pattern of sampling stations. Navigational hazards connected with the particular sea area, including breadth of passages between land masses, often temper or completely determine designs that otherwise aim to collect samples along gradients.

Sampling in the arbitrary sea area generally requires a suitable platform, such as a research vessel. What is suitable for one task and sea area, however, may be inadequate for another. The time that the vessel is available may be equally decisive, for without a guaranteed minimum time period, the coverage may necessarily only be partial. If the vessel is not available at the proper season, then movements of the resource to a new sea area or to greater or lesser depths, or changing conditions associated with season, may hinder or even prevent observation. Examples are abundant, as illustrated by seasonal changes in the depth of *Calanus finmarchicus* in the Norwegian Sea (Østvedt 1955; Hirche 1991) and effects of ice on the availability of euphausiids to measurement in Antarctic waters (US GLOBEC 1991b).

Prior knowledge is generally advantageous in designing a survey. The presence of hydrographic structures, for example, layers or flow fields, may be inseparably linked to the occurrence or absence of zooplankton. Advance observations of animal distribution may also be valuable in design considerations. For example, krill distribution may be marked by the presence of feeding baleen whales. Prior knowledge is also valuable in concentrating sampling effort on the target resource. Its use may, however, be perilous, in so far as the prior knowledge may be partial, resulting in erroneous exclusion of important areas from the survey region.

Models, like prior knowledge, may also be exploited in designing a survey. One aim may be concentration of sampling effort on the survey object; another may be the need to associate different phenomena as defined by their respective biological or oceanographic fields. In this case a model may be useful for assigning the degree of sampling in the presence of specific features.

2.3.2 Survey design types

Some general survey design types relative to zooplankton sampling are described here. These and others that are more specifically designed for fisheries surveying are presented in greater detail in Simmonds *et al.* (1992).

SYSTEMATIC DESIGN

In the absence of prior information about a resource to be mapped or other phenomena to be studied, most information is gained, for a given effort, by so-called systematic sampling. Accordingly, information is collected at stations positioned on a square grid or along parallel, equally spaced transects or along zigzag transects with a constant

vertex angle. By sampling systematically, maximal information is gathered for defining the underlying spatial structure. This may be quantified through the covariance function or variogram, and further exploited in geostatistical methods of analysis (Isaaks and Srivastava 1989; Cressie 1991). Examples include minimum-variance interpolation schemes for mapping and computation of the estimation variance (Foote and Stefansson 1993).

RANDOM DESIGN

In a random design, an attempt is made to perform sampling as though the underlying phenomenon lacks structure or for which possible structure is unimportant. The scheme may involve placement of stations at positions that are determined by a random selection procedure or placement of transects with random spacing if parallel or with random orientation otherwise. In a true random design, there is nothing preventing stations or transects from being located at arbitrarily close distances. If structure can be neglected, as indeed it is in random designs, then the full apparatus of conventional statistical sampling theory may be invoked for purposes of inference. Derivation of an unbiased measure of variance is possible, although this may not be particularly realistic, depending on the degree of underlying structure. In fact, the variance estimate is realistic only when the spatial process has the characteristics of noise. While data may be collected according to a random design, they may still be analyzed with a view towards structure. The covariance function or variogram may be computed as before. It will be degraded at least somewhat because of the non-uniformity of sampling, but it can still reveal structure which may then be used in interpolation and variance estimation.

STRATIFIED RANDOM DESIGN

The stratified random design places stations or transects randomly within predefined strata. It thus attempts to avoid a conspicuous source of error with the pure random design, namely that due to the placement of stations or transects in close proximity. Analysis of data may proceed in the neglect of structure by treating the data as though they were derived from a genuinely random design. This requires the neglect of auto-correlation. The data may also be analyzed by means of geostatistics. Structure may be defined through the covariance function or variogram, followed by modeling and inference.

PREFERENTIAL DESIGN

If prior knowledge is available, it may be desirable to sample preferentially, hence non-systematically, on the large scale. In particular, more effort may be placed in some areas than in others. At some scale size, where there is no reason to sample preferentially, the sampling should be done systematically, to achieve maximal information for the allocated effort.

OTHER DESIGN TYPES

In addition to the four design types described above, with the preferential being a general category, there are other useful types. In narrow passages, for example, navigational considerations may strictly limit the placement of stations. An *ad hoc* design may represent the only possible solution. Limitation in time, as due to foul weather, may also require an *ad hoc* design to salvage what remains of a truncated cruise or survey. Under certain circumstances, for example, surveys performed over quite limited geographical regions, a multi-stage survey may be performed. Such an adaptive design type may be

composed of an initially coarse systematic survey design followed by finer systematic designs applied over rather small areas identified as having the highest concentrations of target animals. Other survey types, especially combinations, may be defined. In general, however, the aim remains the same, to derive maximal information with the available surveying resources given the constraints of sailing conditions including weather and navigational hazards.

2.3.3 Sampling in flow fields

As indicated in section 2.3.2, a number of techniques are now available for mapping a static field and determining the level of confidence that can be placed in the features that are contoured. However, while the stations may be fixed in space, the water is not, and in surveys of a region which take a number of days, the effects of a rapidly evolving property field as a result of the current field must be considered. The question is: how to remove the effects of the flow field before attempting to objectively map a property field?

One approach is to use the mean transport field in the sampled region to apply an 'advective correction' by adjusting the originally sampled locations to their 'synoptic' position at a time central to the sampling of the field as a whole (McGillicuddy *et al*. in press). The flows used in the correction can either be climatological mean flows or mean flows based on current data collected during the course of the sampling using, for example, an acoustic Doppler current profiler (ADCP).

Using Observational System Simulation Experiments (OSSEs) of the GLOBEC broad-scale sampling on Georges Bank (US GLOBEC 1991c), McGillicuddy *et al*. (in press) examined the errors resulting from incomplete spatial sampling of the Bank (under-sampling) and from space–time smearing due to the relatively long period (10 days) required to sample the field compared to the mean flow around the area. Although simple mapping error accounted for most of the error in the simulations, the reduction in error by correcting for the displacement of observation locations due to the mean flow was significant. In regions of strong flows and relatively long survey periods, the need to apply this or a similar approach should be considered.

In the course of sampling at a station, the ship often moves or drifts away from the position at the start of the station work. A practical issue is determining when to steam back to station before continuing a sampling program. As described above, many programs employ a sampling strategy with a fixed number of stations distributed over the area of interest. Each station nominally represents an area (volume) defined by a polygon created by drawing a line halfway to the adjacent stations and splitting the distance for any gaps that might be left by the process (Figure 2.2). When biological data are ultimately used to make contour plots or to compute statistical properties of an area involving more than one station, one view is that the observation made at that station location is representative of the entire area (volume) defined by the station polygon. It follows that any single sample taken elsewhere in the polygon is as representative of that area (volume) as any other sample taken somewhere else in the polygon. From the sampling point of view, therefore, the need is to ensure that all of the observations made with the various sampling devices (CTD, pump, Multiple Opening/Closing Net and Environmental Sensing System, MOCNESS, Bongo, *inter alia*) fall within the polygon. Deployment of these sampling systems may take several hours during which time the ship's position in the polygon will change.

From the biological perspective, there may not be a need to steam back to a single set of station coordinates, for example, the station center point, unless the ship is beyond the

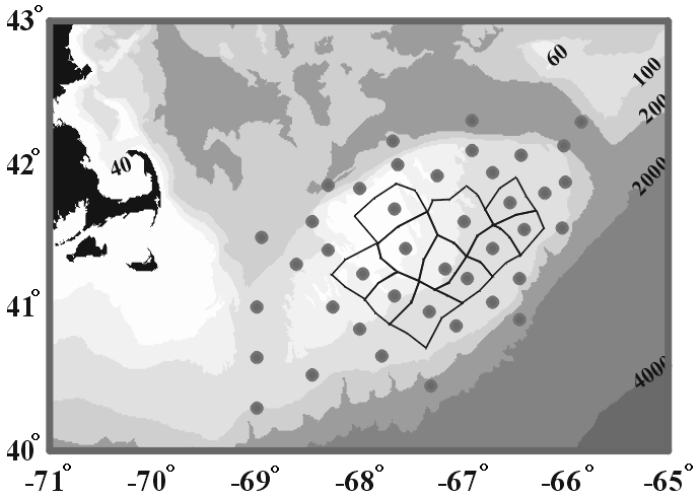


Fig. 2.2 The US GLOBEC Georges Bank broad-scale station positions and examples of the 'polygon' area they represent.

polygon boundary or, in the course of sampling, the ship has left the polygon. On the other hand, for hydrographic data, the assumption cannot be made that any observation within the polygon is as representative as any other. The fields of physical water properties are often fairly regular and smoothly varying, and suitable for interpolation between observations. One value of having standard station locations is that the properties at a location can be compared through time. Choosing a radius of some distance (e.g. 3–4 nautical miles) could relieve the vessel of the necessity of returning to station in most cases, while assuring a reasonable ability to create time series of water properties at stations. A simple rule that would assist in making a decision about whether to steam back toward the central station location on a given survey grid is: Steam back if the ship is more than halfway to the next station. Otherwise, make the observations where the ship ends up after the last instrument has been brought on board.

It might be argued that there is a need to have all observations from the different instruments made "at the same place". Intercomparisons between biological and physical data are internally consistent because most biological instrument systems carry CTDs. The CTD profile does not need to be at exactly the same location in the station area as net tow or pump collections, only within the sampling polygon. While the argument is valid that up to halfway to the next station is no longer on station, the fact is that the water being sampled has also moved during the sampling period. So, while the exact location of the station can be pinpointed, and occupied, the water mass is no longer the same. This water mass change is influenced by the sample-water depth, time of year, weather conditions and other physical conditions. Indeed, this effect is one area of study. Whether or not to return to the exact station location would ultimately depend on the particular goals of an experiment.

2.3.4 Examples of field programs

There are numerous examples of actual ocean sampling programs that employ one or more of the sampling designs described in section 2.3.2. As emphasized previously in this chapter, the particular sampling design chosen must be matched to the objectives of the research and the particular aspects of the area of study.

Ocean-basin-scale sampling has rarely been done extensively. The early oceano-

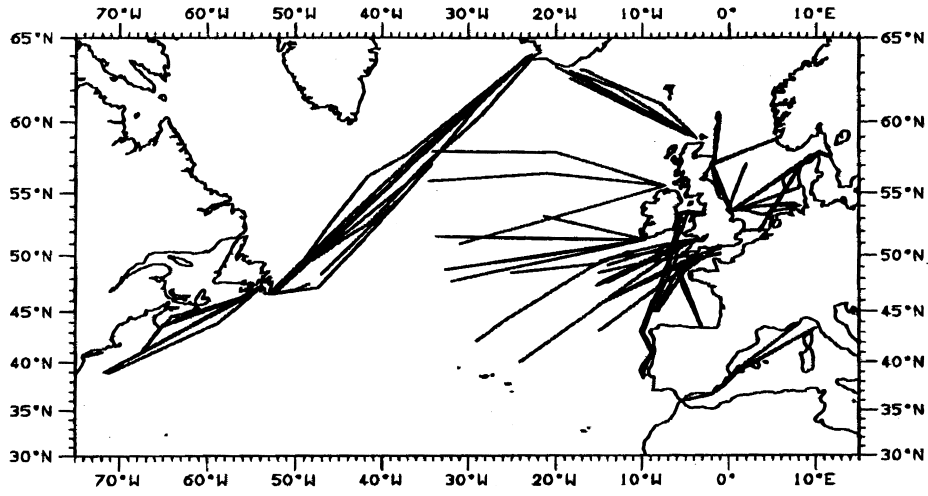


Fig. 2.3 Standard Continuous Plankton Recorder tow routes sampled during 1997. Reproduced from the 1997 annual report of the Sir Alister Hardy Foundation for Ocean Science.

graphic expeditions were often global in scope, but only sparsely sampled the world's oceans in time and space. The only extensive ocean basin scale sampling that has taken place over time and space was started in the North Atlantic in the late 1940s and continues to this day. The Continuous Plankton Recorder (CPR) surveys are carried out with the sampling instrument towed behind commercial vessels operating fixed transects across shelf seas and ocean basins (Figure 2.3). The results of the survey are often aggregated and presented as temporal trends representative for given subdivisions of a sea area such as the North Sea (Taylor 1995).

Long-term macro-scale monitoring programs employ a systematic sampling scheme for a fixed geographic region, i.e., a fixed set of sampling stations along line transects or a grid. Examples of a fixed grid of sampling stations for zooplankton monitoring including ichthyoplankton, which have been at the heart of long-running time series, are the US Marine Resources, Monitoring, Assessment, and Prediction (MARMAP) sampling program (Figure 2.4; Sherman *et al.* 1996) on the eastern seaboard of the United States and the California Cooperative Oceanic Fisheries Investigation (CalCOFI) grid (Chelton *et al.* 1982) off the west coast of the United States. Stations are positioned along transects running more or less perpendicular to the shelf and continental slope isobaths or coastline. In the case of CalCOFI, during the early years of the program, a large number of the station locations were sampled and the sampling occurred frequently within a year (as often as monthly). Increased knowledge about the distribution of regional properties and reduced resources to support the program have resulted in sampling at fewer stations and less frequently in time (quarterly or less often).

The Norwegian MARE COGNITUM program (Skjoldal *et al.* 1993) has investigated the ecology of the Nordic Seas with focus on interactions between zooplankton and pelagic fishes, notably herring and blue whiting. In the first part of the program, macroscale surveys were conducted to map and quantify the distributions of meso- and macrozooplankton and micronekton in relation to the distribution of water masses and fish. Sampling was carried out with a combination of vertical net hauls, MOCNESS,

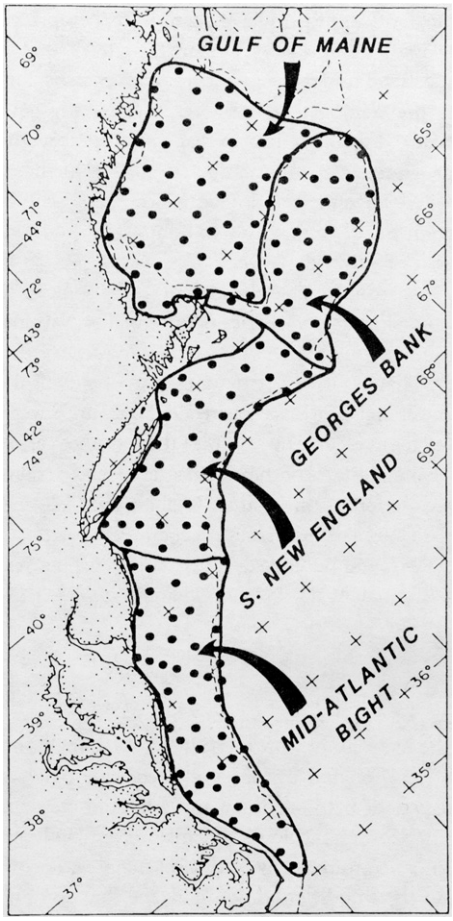


Fig. 2.4 Stations of the Marine Resources, Monitoring, Assessment, and Prediction (MARMAP) Program, conducted between six and eight times per year from 1977 to 1987 (Sherman *et al*. 1996). Sampling at these stations has continued since then, but at a lower level of effort.

pelagic fish trawls, and acoustical recordings. The target organisms were distributed over a wide vertical range, from the surface to 600–800 m depth. This required extensive sampling, with about 12 h spent on sampling stations and about 12 h on cruising during each 24 h survey period. To cover the large area of about 2 million km^2 quasi-synoptically, three research vessels were used over a period of 7 weeks.

Norwegian investigations of the horizontal and vertical distributions of zooplankton in the Barents Sea have been carried out since the beginning of the 1980s. Aims have included collection of primary data for multi-fish-species research, determination of predator–prey interactions, and understanding of long-term fish growth rates. Since 1986, regular surveys have been conducted in the autumn when the ice cover is at its annual minimum. Data on zooplankton, nutrients, and chlorophyl have been collected systematically over an area in excess of 1 million km^2. In 1995, a dual survey was conducted by R/V *G. O. Sars* from 16 August to 1 October and by R/V *Johan Hjort* from 25 August to 9 October. The composite sampling patterns for zooplankton by MOCNESS and WP2 net, nutrients, chlorophyl, salinity, temperature, and water are illustrated in Figure 2.5.

Meso-scale sampling design is strongly dependent upon the frame of reference for the sampling. If the feature is fixed geographically, then station locations are also usually

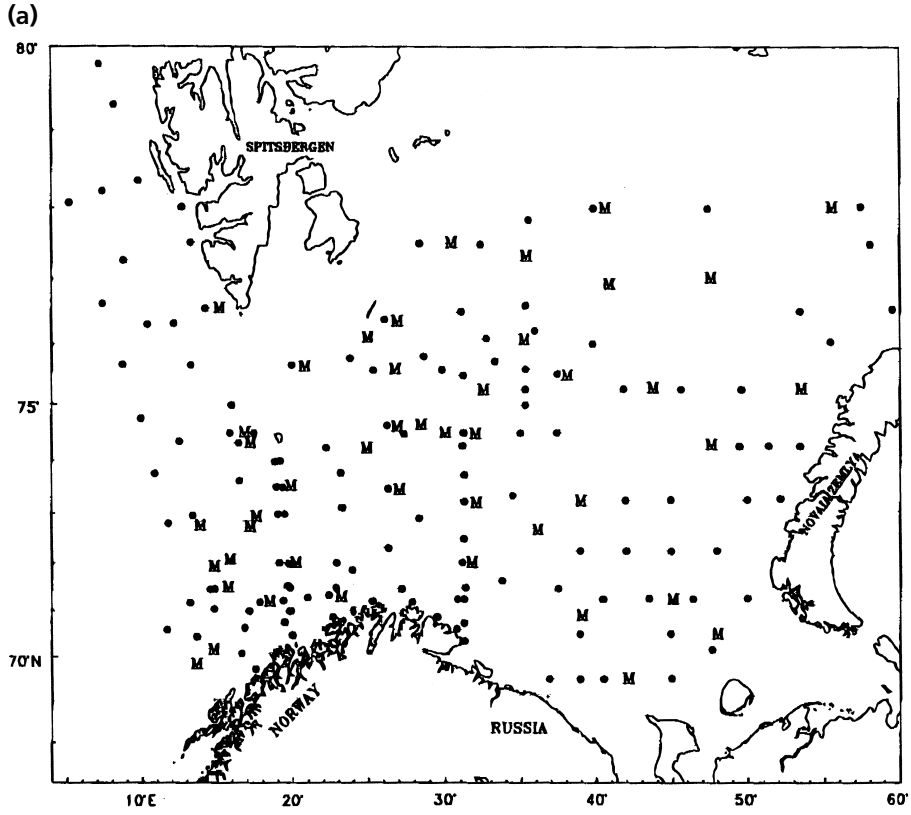


Fig. 2.5 Locations of sampling stations in the Barents Sea during the autumn survey in 1995 with R/V *G. O. Sars* and R/V *Johan Hjort*. (a) Locations of stations for zooplankton by MOCNESS (M) and WP2 net, nutrients, and chlorophyl.

Continued overleaf

fixed for repeated sampling of the area. This is exemplified by the Georges Bank sampling program. The US GLOBEC Northwest Atlantic Georges Bank Study (US GLOBEC 1992) adopted a strategy that involved a coordinated sequence of broad-scale surveys and process-oriented cruises. In the broad-scale surveys which took place during the first 6 months of the year for a 5-year period beginning in 1995, Georges Bank was covered by sampling at 38 to 41 standard stations positioned in an irregular grid (Figure 2.6). The stations in the grid were placed by taking into account the bottom topography and the dominant water flow-field in the area. Additional stations were occupied depending on weather conditions and available time at locations intermediate between the standard stations in order to better define patches of fish larvae encountered at the primary stations. The broad-scale surveys provided a background of updated information against which to plan and conduct the process-oriented cruises where selected processes were studied in more detail. These process cruises took place in alternate years beginning in 1995 (Wiebe *et al.* 1996b).

In contrast, substantial sampling has been done of meso-scale features embedded in a larger flow field or moving relative to a fixed reference frame. The sampling of meso-scale eddies such as Gulf Stream rings (the Ring Group 1981; Joyce and Wiebe 1983)

(b)

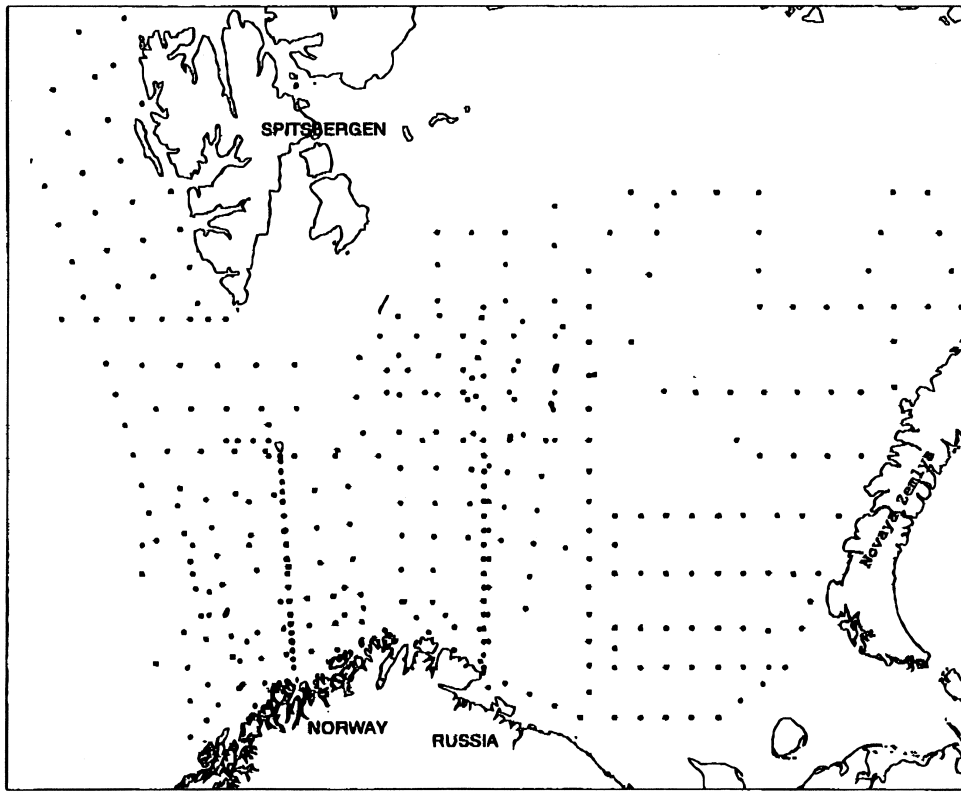


Fig. 2.5 continued (b) Locations of stations for hydrography by CTD sonde and water bottles.

and mode eddies in the western Atlantic Ocean (Robinson 1983) has required the use of star patterns and line transects to characterize the physical and biological structure of the eddy. These sampling patterns had to be adjusted both within a cruise and between cruises to the same eddy feature to account for their movement over the ocean floor. In sampling the eddies, sampling was most intense in the gradient regions between the inside core structure of the eddy and the outside medium, and less intense where the gradients were weaker. A star-shaped pattern was often used for rapid 'synoptic' surveys of the physical structure of the eddies. High-resolution towyo sections were also done in gradient regions to define the physical and biological structure (Wiebe *et al.* 1992).

Coarse-scale sampling designs are usually intended to examine in detail the biological spatial heterogeneity within a study area and its relationship to the physical properties and dynamics of the area. Often the effort consists of point source sampling, continuous sampling, or both along the trackline of a square or rectangular grid 2 to 10 km on a side. At this scale, the flow of water during the course of the sampling cannot, in most cases, be ignored, and the sampling is often carried out relative to a drifter/drogue (Niiler *et al.* 1995; Davis *et al.* 1996; Limburner and Beardsley 1996) that is designed to follow the movements of the water mass being sampled. (Examples of grids relative to drifter position are the 1966 zooplankton patch study (Wiebe 1970), a series of experiments on

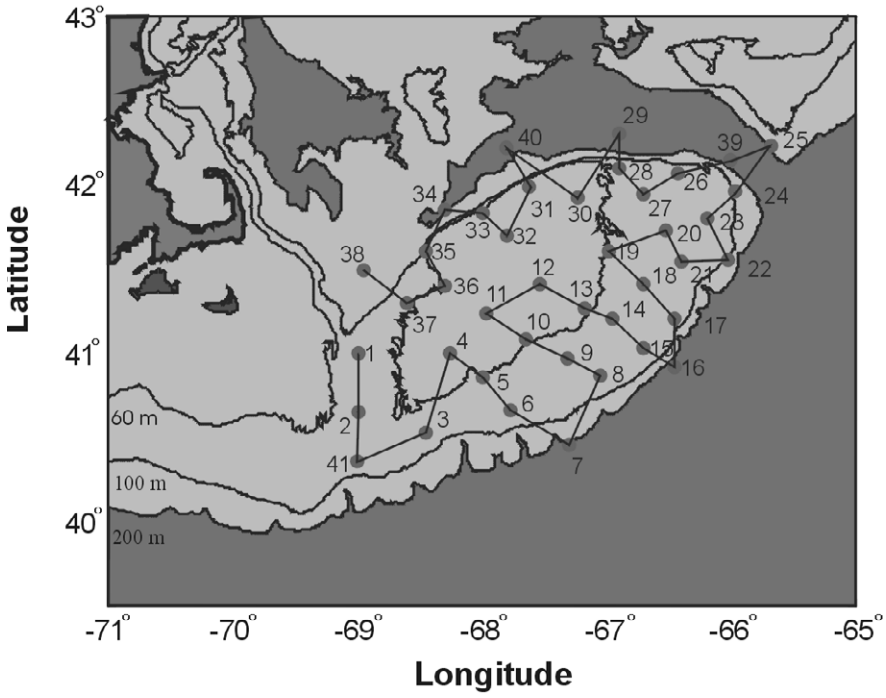


Fig. 2.6 The US GLOBEC Georges Bank Program broad-scale sampling station plan for 1998. The first broad-scale sampling was conducted in June 1994 with a sampling design (with 48 stations) that was subsequently modified into a pattern used in 1995 with 38 stations. Station 39 was added during the 1996 field season, Station 40 was added in 1997, and Station 41 was added in 1998.

Georges Bank (Davis *et al*. 1996; Benfield *et al*. 1998; Pershing and Wiebe in press), and an Antarctic krill experiment (Greene *et al*. 1994).)

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3 Collecting zooplankton

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3.1 INTRODUCTION

From the beginning of modern biological oceanography more than 100 years ago, remotely operated instruments have been fundamental to observing and collecting organisms. For most of the twentieth century, biological sampling of deep ocean has depended upon winches and steel cables to deploy a variety of instruments. The samplers developed over the years generally fall into three classes (Table 3.1):

- Water-bottle samplers that take discrete samples of relatively small volumes of water (a few liters)
- Pumping systems that sample intermediate volumes of water (tens of liters to tens of cubic meters)
- Nets of many different shapes and sizes that are towed vertically, horizontally, or obliquely and sample much larger volumes of water (tens to thousands of cubic meters).

Traps to collect animals in midwater or rising off the seafloor have been used less often to collect marine zooplankton.

Early depth-specific collecting nets opened or closed mechanically, either with weighted 'messengers' traveling down the towing cable by gravity to trigger a trip mechanism, or by a pressure- or flow-meter activated release. In the 1950s and 1960s, conducting cables and transistorized electronics were adapted for oceanographic use, and more sophisticated net systems began to do more than collect animals at specific depth intervals. Multiple net systems now routinely carry sensors to measure water properties such as temperature, pressure/depth, conductivity/salinity, phytoplankton fluorescence/biomass, and beam attenuation/total particulate matter. They also measure net properties such as volume of water filtered, net speed, and altitude from the bottom, as well as net function such as an alarm to tell when a net closes. In spite of their advanced features, all instruments deployed from cables to collect organisms are limited in their temporal and spacial coverage. This is not only because of the large amount of time it takes to collect a sample (tens of minutes to an hour or more, and many hours to complete an entire multiple net haul), but also because of the time required (hours to a day or more) to identify and count individuals by species under a microscope.

This chapter deals with improvements in mesozooplankton and macrozooplankton (in a size range $>200\,\mu$m to ~ 3 cm) net sampling as well as other sampling techniques for describing the vertical and geographic distribution and biomass estimation of these

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Table 3.1 Summary of zooplankton sampling gear types.

Sampling gear	Kind of sampling	Size fraction	Resolving scale		Typical operating range	
			Vertical	Horizontal	Vertical	Horizontal
A. CONVENTIONAL METHODS						
Water bottes	Discrete samples	Micro/Meso	0.1–1 m	–	4000 m	–
Small nets	Vertically integrating	Micro/Meso	5–100 m	–	500 m	–
Large nets	Vertical, obliquely, horizontally integrating	Meso/Macro	5–1000 m	50–5000 m	1000 m	10 km
High-speed samplers	Obliquely, horizontally integrating	Meso/Macro	5–200 m	500–5000 m	200 m	10 km
Pumps	Discrete samples	Micro/Meso	0.1–100 m	–	200 m	–
B. MULTIPLE NET SYSTEMS						
Continuous Plankton Recorder	Horizontally integrating	Meso	10–100 m	10–100 m	100 m	1000 km
Longhurst–Hardy Plankton Recorder	Obliquely, horizontally integrating	Meso	5–20 m	15–100 m	1000 m	10 km
MOCNESS	Obliquely, horizontally integrating	Meso/Macro	1–200 m	100–2000 m	5000 m	20 km
BIONESS	Obliquely, horizontally integrating	Meso/Macro	1–200 m	100–2000 m	5000 m	20 km
RMT	Obliquely, horizontally integrating	Meso/Macro	1–200 m	100–2000 m	5000 m	20 km
HYDRO-BIOs Multinet	Vertically, obliquely, horizontally	Micro/Meso	2–1000 m	100–2000 m	5000 m	5 km
C. ELECTRONIC OPTICAL OR ACOUSTICAL SYSTEMS						
Electronic Plankton Counter	High resolution in the horizontal/vertical plane	Meso	0.5–1 m	5–1000 m	300 m	100s km
In situ Silhouette Camera Net system	High resolution in the horizontal/vertical plane	Meso	0.5–1 m	5–1000 m	1000 m	10 km
Optical Plankton Counter	High resolution in the horizontal/vertical plane	Meso	0.5–1 m	5–1000 m	300 m	100s km
Video Plankton Recorder	High resolution in the horizontal/vertical plane	Meso	0.01–1 m	5–1000 m	200 m	100s km
Ichthyoplankton Recorder	High resolution in the horizontal/vertical plane	Meso	0.1–1 m	5–1000 m	200 m	10 km
Multifrequency Acoustic Profiler System	High resolution in the horizontal/vertical plane	Meso/Macro	0.5–1 m	5–1000 m	100 m	10 km
Dual-Beam Acoustic Profiler	High resolution in the horizontal/vertical plane	Meso/Macro	0.5–1 m	1–1000 m	800 m	100s km
Split-Beam Acoustic Profiler	High resolution in the horizontal/vertical plane	Meso/Macro	0.5–1 m	1–1000 m	1000 m	100 km
ADCP	High resolution in the horizontal/vertical plane	Meso/Macro	10 m	5–500 m	500 m	100s km

Notes: Most vertical nets are hauled at a speed of 0.5–1 m s^{-1}. Normal speeds for horizontal tows are $\sim$2 kn (1 m s^{-1}) and for high speed samplers $\sim$5 kn (2.6 m s^{-1}). For further categorization of pumping systems which are used by a number of investigators see the review by Miller and Judkins (1981)

zooplankton. Substantial improvements have been achieved since the appearance of the UNESCO Manual (Tranter and Smith 1968).

Research requirements and the species of zooplankton of interest will dictate the sampling method used. In most cases some form of capture sampling will be needed to acquire specimens for taxonomic and/or experimental purposes. Nets are the most common method of capture, but the use of pumps and large water bottles has increased as a means of collecting zooplankton for biomass estimation and for collection of larval stages of zooplankton that nets do not sample effectively. Plankton traps are much less commonly used. Since 1968, there have been no radical advances in net sampling, but only a series of incremental improvements in the way nets are configured and integrated with instruments for the measurement of environmental parameters. Communication with instruments on net samplers through electrical, fiber optic conductor cable or by acoustic link are now commonly used to transmit data to and from the sampling device. These instrument packages allow accurate depth directed net sampling of zooplankton, while at the same time measuring other important physical and biological parameters when zooplankton are collected. This combination of instruments and nets has resulted in more efficient use of ship and sampling time as well as increased understanding of zooplankton ecology.

3.2 A SURVEY OF SAMPLING DEVICES

3.2.1 Pumps and traps

Pumps in various configurations have been used to sample plankton since at least the work of Hensen (1887). They offer advantages over towed nets, particularly in habitats with very high animal density. Volume filtered can be measured much more reliably, and interference from mesh clogging can be monitored when filtration is on deck. Depth of sampling is readily controlled with no contamination from surrounding levels and the desired parameter (T, chlorophyl, ammonia) can be measured in the same water inhabited by the animals collected plus serial sampling is simplified. However, volumes filtered with pumps of reasonable scale are small relative to towed nets. Limits to capacity are set by the power required to move large volumes and by hose friction, and depths that are commonly sampled with pumps are within 200 m of the surface. An exception is the MULVPS pumping system (Bishop *et al.* 1992) which has been used to sample to 1000 m and more.

Several pump configurations give satisfactory service, including centrifugal (Gibbons and Fraser 1937), diaphragm (Mullin and Brooks 1976) and vacuum, a large chamber on deck is evacuated, then opened to a hose with submerged intake (Lenz 1972). For systems delivering water to a ship's deck by hose, the main factors limiting transfer capacity are static lift from sea to deck and hose friction. If the pump is on deck, the actual motive force for pumped water is atmospheric pressure, which is limited to 10 m of static head. In most pumps, the available suction head is less, since some is required to prevent cavitation in the pump. If the pump is at the sampling depth, the available static head can be greater, but hose friction still limits transfer. Small bore hose has greater friction loss than large bore, so that large hoses improve flow more than expected from their larger transfer cross section. All joints, bends and elbows add friction, which must be accounted for in design. A set of design considerations and component selection guidelines for pump samplers involving hose with filtration on deck is given by Miller

and Judkins (1981). Recent systems include a high capacity ($2.8\,m^3\,min^{-1}$) seagoing design by Harris *et al.* (1986), and a handy system for coastal boats from Omori and Jo (1989). A similar design, known as the Pacer Pump has been used by Durbin *et al.* (1987) in the US GLOBEC Georges Bank Program to sample naupliar stages of copepods as part of a population dynamics study on the Bank.

Another sampler configuration packages pump and filter together to be lowered by power cable (Mohlenberg 1987) eliminating the overside handling of hose and hose friction. The advantage of monitoring filtration on deck is lost, but can be partly replaced by telemetering flow meters in the pump stream. Clogging is indicated by reduced flow. Mohlenberg showed that his $0.42\,m^3\,min^{-1}$ net-pump was about equally as efficient as a WP-2 net, the zooplankton net described in the report of the Working Party 2 (Tranter 1968), except for capture of adult female copepods. Female *Calanus* and *Pseudocalanus* avoided the pump more effectively than the net when the pump was held stationary in the vertical. Another possibility not yet realized is to modify the Mohlenberg apparatus for multiple sampling by adding a filter carousel. The overall package would be about the size and shape of the CTD-sampling rosette.

Studies of the capture efficiency of pumps relative to nets are reviewed by Taggart and Leggett (1984). On the whole the comparisons are favorable enough to support use of pumps when their advantages are needed. Singarajah (1969) has studied the response of individual zooplankters to narrow suction intakes, they do leap clear when subjected to strong flow gradients. All but the smallest gelatinous zooplankton are badly damaged by impeller pumps, so they are mainly satisfactory for hard-bodied forms, particularly crustaceans. There is evidence that the smallest nauplii stages of *Calanus finmarchius* are damaged and lost in sampling with a centrifugal pump, but not with a diaphragm pump (Durbin, unpublished data).

3.2.2 Nets and serial samplers

SIMPLE NET SAMPLERS

A detailed description of simple plankton nets and their use was given by Tranter (1968), and these recommendations are still valid today. The recommended plankton net is still the WP-2 net with an open-mesh-filtering area to mouth area ratio of at least 6:1. A flow meter mounted in the mouth should be used whenever possible. The flow meter should not be located in the center of the net mouth opening, but in a position about halfway from the net mouth center to the net rim (Smith, Counts and Clutter 1968). The center position generally gives an over estimate of the flow into the net. The working group in 1968 recommended use of the TSK flowmeter (Tokyo Seimitsu Co. Ltd), but since then there have been a number of excellent new flow meters developed and manufactured, therefore we do not recommend any single brand of meter.

The main advantages of ring nets over multiple net samplers is their ease of use and low cost, they can be used with simple hydrographic cables, and can easily be towed from any type and size of vessel.

MULTIPLE SAMPLE INSTRUMENTS

There are a number of net samplers that collect multiple zooplankton samples and these fall into three main types. The first type is based on the principle of collecting animals on a continuous ribbon of netting and includes the Continuous Plankton Recorder (Hardy 1939), the Longhurst Hardy Continuous Plankton Recorder (Longhurst *et al.* 1966), the Autosampling and Recording Instrumented Environmental Sampling System (Dunn *et*

al. 1993a, 1993b) and the highspeed Gulf-III OCEAN sampler (Nellen and Hempel 1969).

The Continuous Hardy Plankton Recorder (CPR)

The Continuous Hardy Plankton Recorder (CPR) is a high speed zooplankton sampler designed to be towed in near surface waters over long distances from ships of opportunity (see Fig. 2.3). The original CPR was designed by Alister Hardy to be used to study patchiness of plankton in the Antarctic on the Discovery Expedition, 1925–1927 (Hardy 1926). During the 1930s, the CPR was deployed in the North Sea to monitor seasonal and annual changes in the plankton (Hardy 1935). Since that time, except for a break during the Second World War, the CPR has continued to be deployed on a monthly schedule in the North Sea and North Atlantic (Warner and Hays 1994). In addition to its plankton-sampling role, the CPR has the capability to carry environmental sensor packages under its box-section tail. Electromagnetic flow meters can be fitted to the exit apertures of the CPR to measure the volume of water sampled (which has a theoretical maximum of 3 m^3 per 10 nautical miles of towing).

CPR operation The CPR is designed to be towed at speeds up to 25 knots in the surface mixed layer (Hays and Warner 1993) by the non-scientific crews of commercial ships going about their regular business. The recorder is deployed from the ships mooring deck, off a davit or A-frame on a 10 mm steel-wire rope using the ship's winches. The CPR towing depth of approximately 10 m is maintained by an in-built diving plane and by regulation of the length of the towing wire. Water enters the CPR through an aperture of 1.27 cm^2, travels down a tunnel 5 cm × 10 cm in cross section where it passes through a graduated silk filter of mesh size 270 mm, and finally exits the machine via a tunnel and aperture (10 cm × 3 cm cross section) to the rear. As the CPR passes through the water, an impeller drives a take-up spool which moves the filter silk across the filtering aperture where it is covered by a second layer of silk and wound into a storage tank containing formaldehyde. The silk is wound across the aperture at a rate of approximately 10 cm per 10 nautical miles of tow. This rate can be controlled by adjusting the angle of the impeller blades. At the end of the tow, the CPR is retrieved, and the crew fill out a form detailing the times and locations of CPR deployment and retrieval, and any alterations in ship course. Upon docking, the CPR is unloaded and returned to the laboratory where the silks are processed using a standard procedure (Colebrook 1960).

Treatment of samples In the laboratory, the silk is removed from the storage tank and unwound. Using the tow data provided by the ship's crew, and assuming a constant tow speed, the silk is marked out and labeled in sections corresponding to 10 miles of towing. A visual estimate of the greenness of the silk is then made with reference to a standard color chart. The silk is then cut into the 10 mile sections and distributed for plankton analysis. Full details of analysis procedures are given by Rae (1952) and Colebrook (1960). The plankton is identified on the silk in three stages. The first examination, for phytoplankton, is of 20 fields of view along a traverse (a sub-sample of about one thousandth of the filter silk) under 450 × magnification. The species in each field are identified and the number of fields of view (out of 20) in which that species was present is recorded. The second examination is of both the filter and covering silk for selected zooplankton species at 48 × magnification (a sub-sample of about 1/40 of the silk). All the species identified are counted and recorded. Finally the whole sample is

examined for large (generally >2 mm) organisms that are counted and recorded. The samples are then sprayed with borax-buffered 4% formalin, labeled, packaged and placed in an archive.

Longhurst Hardy Plankton Sampler (LHPR)

The most commonly used multiple cod-end sampler is the Longhurst Hardy Plankton Sampler (LHPR). The sampler is effective in collecting large numbers of samples and performs best in waters where net clogging due to phytoplankton or jellyfish is not a problem, as an accurate measure of the volume of water filtered is essential for accurate biomass or animal concentrations to be made. The LHPR performs best when samples are taken in a horizontal or upward oblique direction. The LHPR can be towed at speeds up to 6 knots and can take a series of samples on a single haul for studies of vertical or horizontal distribution (Coombs *et al.* 1985, 1992; Conway and Williams 1986; Haury and Wiebe 1982; Wiebe 1970; Williams and Conway 1988). The original single net system (e.g. 280 μm mesh aperture) described by Longhurst *et al.* (1966) has been superseded by an improved twin net system (e.g. 53 μm and 200 μm mesh) described by Williams (1983). More recent improvements incorporated in the LHPR system used by the Plymouth Marine Laboratory include real-time data display and deck control of sample acquisition.

Essentially the system consists of a modified high speed net frame (2.5 m in length and 76 cm diameter) in which is fitted a 200 μm mesh aperture conical net terminating in a cod-end unit. In the cod-end unit two rolls of filtering gauze are advanced by an electric motor to give a sequential series of samples. Volume filtered for each sample is recorded from a flowmeter mounted in a conical nose-cone on the front of the sampler. The nose-cone inlet aperture can vary between 20 cm to 40 cm diameter, depending on plankton concentration, typically 35 cm diameter giving samples from about 20 m^3 of water filtered during a 2 min advance interval.

A similar, but smaller, finer net system (35 to 100 μm mesh aperture, typically 53 μm) with a separate cod-end unit can be mounted on top of the main sampler frame to give a concurrent series of fine mesh samples for studies of smaller organisms (e.g. copepod nauplii and copepodite stages). Flow into the fine mesh system is via a small conical nose cone (inlet diameter 3 to 7 cm, depending on plankton concentration) with flow rate monitored by a flowmeter in an inlet tube assembly between the inlet cone and short tubular filtration net (typically giving samples each from about 400 l of water filtered on a 2 min sample advance).

Advantages of the use of the LHPR system are the large number of samples that can be taken (up to 100 for each of the fine and coarse mesh systems), the wide size range of organisms that can be sampled by the twin net arrangement and the extensive flow validation data available for similarly designed high-speed samples with conical nose-cones. Disadvantages of the single net/multiple cod-end configuration include net hang-up/residence time and inadequate discrimination between consecutive samples (Haury 1973; Fasham *et al.* 1974; Haury *et al.* 1976). Improvements in the more recent systems, including optimum net design, correct choice of inlet cone aperture and sampling interval have minimized the problems of filtration efficiency; similarly the inclusion of blank gauze between consecutive samples has removed any confusion between adjacent samples. However, a degree of operator experience is still required for optimum sampling integrity. Checks on sampling validity are available both from double oblique sampling, where samples on the ascent and descent profiles are compared (Pipe *et al.*

1981) and by analysis of the filtering net residue after a haul in comparison with the catches of the cod-end samples.

Recent developments of the LHPR system include the addition of chlorophyl fluorescence and conductivity (salinity) sensor for real time display with the existing temperature, depth, flow and gauze advance signals. Transmission is via a single-cored cable enabling its use on a wide range of research vessels.

Autosampling and Recording Instrumented Environmental Sampling System (ARIES)

The Autosampling and Recording Instrumented Environmental Sampling System (ARIES) is a highly modified LHPR developed for concurrent physical, biological and chemical sampling at sea and this is done by collecting serial plankton and water samples (Dunn *et al*. 1993).

Mechanics of ARIES The main frame of the sampler is constructed from sea-water resistant aluminum scaffolding tube. The principal sampling components are all inside the main frame and protected from any accidental damage. A major advantage of this frame is that any damage can be easily repaired in the field. The open design of the sampler means that a large number of sensor packages can be carried and they are not isolated from the flow of water.

Cable telemetry is not used, allowing deployment from a wide variety of vessels. It is normally deployed on 11–14 mm wire rope at a towing speed of 4–5 knots (2–2.5 m s^{-1}). Real-time depth data is required for control and safe operation of the sampler, therefore a commercially available acoustic telemetry system is used to transmit these data to the towing vessel.

Plankton sampler In the ARIES system, the ribbon of mesh layers of the LHPR is replaced with a single belt carrying a series of individual cod-end bags, each bag being placed over the tail of the conical collecting net for a selected sampling period. The belt, feed spool and take-up spool are housed in a simple rectangular cassette, bolted to a fixed base plate. The cassette is removable as a complete unit to allow a fast turn around of the sampling system. The take-up spool is driven directly by a DC motor and gearbox mounted in an underwater housing located below the baseplate; 110 cod-end bags, of 250 μm mesh, are spaced along the length of the belt and held in place by Velcro strips. Indexing holes, positioned directly above each bag, ensure exact alignment of each cod-end with the tail of the collecting net. The holes are detected by an optical sensor attached to the front of the cassette, which signals the control unit to start or stop and drive the motor.

Water sampler The design of the water sampler is similar to that of a conventional rosette sampler. Sixty free-flooding tubes with hinged sealing lids are placed in a double ring around a cylindrical carousel. The lids of each tube are connected together internally by a length of silicone elastic. Prior to sampling the lids are held open, against the tension of the elastics, by integral pins slotted into pivoting levers. At the end of each sampling period, the levers at each end of the next tube in the carousel are simultaneously turned by a rotating arm, thus releasing the lids and closing the tube. The rotating arm is driven by a DC motor and high-reduction gearbox, mounted in an underwater housing at one end of the carousel. The 6° angle of rotation required to close each tube is controlled accurately by the main control unit and a shaft encoded, with 1° resolution, mounted on the rotating arm.

Electronics The control unit consists of a commercially available single-board microcontroller and a custom interface card. On-board application software was also developed in-house. In addition to controlling both samplers, the control unit digitizes data from an integral pressure sensor and flowmeter, mounted in the mouth of the plankton net. All data are stored in solid-state memory, together with the date and any time information and status flags, indicating sampler operations. The control unit is programed by a host computer subsequent to deployment. User-selectable plankton and water sampling rates between 1 and 60 min are available, whilst depth and flow data can be logged at intervals ranging from 1 s to 60 min.

Gulf III OCEAN sampler

This type of sampler uses a carousel containing a number of nets that rotate and take sequential samples. The Gulf III high speed sampler has been used since the 1950s with various modifications for the purpose of primarily sampling fish larvae in European waters. The most recent modification of this type of sampler is the OCEAN sampler (Dunn *et al.* 1993). The body of the sampler is based on the Dutch version of the Gulf III plankton sampler. It is made of HE30 aluminum with a reinforced tow-point and depressor attachment point. It incorporates three tail fins to provide good stability and the fins are fixed by shear bolts and secured with lanyards. Instead of single sampling net and cod-end, the internal frame incorporates four 250 μm nets each with a mouth diameter of 150 mm and a length of 150 cm, each with a detachable 68 μm cod-end. The nets are arranged in a circle and attached to a fixed circular disk. The nets are tensioned at the rear of the frame using shock-cord loops. Attached to the disk is an underwater housing, containing a DC electric motor and reduction gearbox positioned immediately in front of the stationary disk. The rotating disk has eight, rather than four, sequential indent positions, allowing the sampler to be closed completely whilst maneuvering to the next sampling depth. The mating faces of both disks are covered with bondable PTFE material to provide a low friction water-tight seal.

In order to maintain a flow-rate into the four small nets, comparable to the flow in a standard Gulf III, the sampler is fitted with a reducing nose-cone, which also houses the flowmeter. This nose-cone is coupled to the sampling hole in the rotating disk by a flexible hose to ensure an adequate, non-turbulent flow of water into each sampling net.

Electronics and telemetry The electronic control unit is mounted on top of the main sampler body. This is a commercially available acoustic telemetry system, modified for use with the OCEAN sampler. Data such as date, time, ship's position and sea-bed depth are processed by a personal computer and displayed graphically.

Gulf V plankton sampler

The Gulf V sampler, which is a modified Gulf III sampler (Gehringer 1952) is an effective sampler for ichthyoplankton and macrozooplankton (Nellen and Hempel 1969). It can be towed at high speed (5 knots) and it was shown to filter more water and catch more plankton than the Gulf III. The increased performance of the Gulf V was due to the removal of the case of the Gulf III. Added advantages of this sampler were a lower weight, ease of handling and a lower cost.

MULTIPLE NET SAMPLERS

The second group of samplers, the multiple net samplers, is based on the principle of opening and closing a series of individual plankton nets in succession.

There are a number of commonly used multiple net samplers, the Multiple Plankton Sampler (MPS) (Bé 1962), the HYDRO-BIOS Multinet (Weikert and John 1981), the RMT 1 + 8 (Baker *et al.* 1973), MOCNESS (Wiebe *et al.* 1985), and the BIONESS (Sameoto *et al.* 1980). All use square mouth-opening nets and come in a variety of sizes. The BIONESS, MOCNESS, and RMT 1 + 8M are towed horizontally or obliquely while the MPS can be towed horizontally, vertically or obliquely. All of the samplers are similarly effective in collecting mesozooplankton. When the same mesh was used in the BIONESS and MOCNESS nets there was little difference in the biomass of mesozooplankton collected by the two different samplers. The BIONESS is generally towed at a speed of 3–4 knots and the MOCNESS at 1.5–2 kn. There was some indication that the higher speed of the BIONESS may increase the pressure inside the nets and result in more extrusion of zooplankton through the mesh of the nets than occurs in the slower MOCNESS. The BIONESS was more efficient in capturing the larger forms of zooplankton such as shrimp, krill, and juvenile fish, and this is attributed to the higher towing speed.

It is recommended that when using multi-net samplers a calibrated flowmeter be used, preferably in the mouth of the frame of the sampler. If this is not possible a flowmeter on the frame outside the net should be used. When no flowmeter is available the volume of water filtered can be estimated using the ship's speed and the accurate time of opening and closing each of the nets. If the nets are not clogged it can be assumed that approximately 85%–90% of the water in front of the net will be filtered. These multiple net samplers generally perform best when towed obliquely in an upward direction.

BIONESS

The BIONESS is a multiple net (10 nets) opening and closing zooplankton sampler that is made in two sizes, with either 1 m^2 or 0.25 m^2 mouth-opening nets. The 10 nets of the BIONESS are stacked horizontally one behind the other and open sequentially with one net opening as one is closed. The horizontal stacking reduces the frontal area of the frame to a minimum thereby reducing sampler visibility and the frontal pressure wave of the sampler. Each of the 10 horizontal dropping bars is lead filled and weighs approximately 25 kg. The entire sampler has a weight in air of approximately 800 kg. The nets and frame of the BIONESS are dyed and painted a dark gray color to reduce visibility. The BIONESS can be towed from a conductor cable, allowing continuous communication with the sampler, or from a non-conducting cable with the data stored in the BIONESS computer and retrieved when the sampler is on the deck of the ship. A cable with a minimum breaking strength of 6000 kg and a winch with a pulling load of at least 3000 kg is recommended.

The filtration area to mouth area ratio of the nets is normally 10:1 resulting in a filtration efficiency of close to 90% for a clean net towed at 1.5 $m\,s^{-1}$. The normal towing speed is 1.5 $m\,s^{-1}$ but the BIONESS can be towed safely up to speeds of 3.0 $m\,s^{-1}$. However, there will be a drop in filtration efficiency at the higher speeds unless the R ratio of the nets is increased. The filtration efficiency is measured by comparing the difference in the flow between a flowmeter in the mouth of the net and the flow of a meter mounted on the outside of the BIONESS frame. The mouth angle of the BIONESS when towed at 1.5 $m\,s^{-1}$ is near 0° from the vertical but it increases to about 15° from the vertical at speeds near 2.5 $m\,s^{-1}$.

The BIONESS may be towed at speeds as low as 0.5 $m\,s^{-1}$, but this may result in the zooplankton being captured only in the front part of the nets, making it difficult to wash the animals out of the net. A minimum speed of 1 $m\,s^{-1}$ is recommended with the

optimum speed being 1.5 m s^{-1}. Extrusion of zooplankton through the mesh of the nets increases with increasing speed, therefore the advantages of high speed (i.e. >1.5 m s^{-1}) may be outweighed by the loss of the smaller zooplankton through the mesh.

Information obtained during a tow includes temperature, salinity, depth, speed through the water, pitch angle of the sampler, flow through the net, filtration efficiency, and net count indicating which of the 10 nets is open. An Optical Plankton Counter (OPC) fluorometer, and video camera can easily be added to the BIONESS. When the BIONESS is brought on to the ship's deck after a tow, the zooplankton are located near the cod-end of the net and require only a small amount of washing to collect the zooplankton in the cod-end bucket. However, if the BIONESS is held in the wash of the ship's propeller for any length of time during retrieval, the collected animals will be distributed throughout the net and this will require more washing of the nets to recollect the sample in the cod-end buckets. The buckets should all be numbered in sequence, as should the nets, to prevent confusion as to which net is associated with which bucket. The contents of each of the buckets are poured into numbered pails and processed during the next tow.

The handling methods of the BIONESS during launch and retrieval from the water will vary according to the capability of the ship's equipment. It is important that the ship be moving forward during both of these operations. The BIONESS is most easily handled with the use of an A-frame or large crane from the stern, but side towing is possible with a large crane. Because of the compact design of the BIONESS it can safely be handled in rough seas and moderately high winds.

LOCHNESS sampler

The LOCHNESS (Dunn *et al.* 1993b) is basically a large BIONESS sampler designed to capture fish larvae. The frame is 3 m high, 3 m wide and 2 m deep and houses five nets of 2 mm mesh each with a mouth opening of 2.3 m^2. The sampler is designed to be towed straight level at 4 kn (2 m s^{-1}). The control unit, motor housing, flowmeters and topside data processing system are identical to that used by the OCEAN sampler.

Multiple Opening/Closing Net and Environmental Sensing System (MOCNESS)

The Multiple Opening/Closing Net and Environmental Sensing System (MOCNESS) is a family of net systems based on the Tucker Trawl principle. There are currently eight different versions of MOCNESS designed for capture of different size ranges of zooplankton and micronekton (Table 3.2; Wiebe *et al.* 1985). Each is designated according to the size of the net mouth opening and in two cases the number of nets it carries. The original (Wiebe *et al.* 1976) was a redesigned and improved version of a system described by Frost and McCrone (1974).

The MOCNESS-1/4 and the Double MOCNESS-1/4D carry nine and eighteen 1/4 m^2 nets respectively usually of 64 μm mesh and have been used to sample the larger micro-zooplankton. The MOCNESS-1 (Wiebe *et. al.* 1976) and the Double MOCNESS-1D carry nine and twenty 1 m^2 nets respectively usually of 335 μm mesh and are intended for macrozooplankton sampling. There are four mid-water systems: the MOCNESS-2 (with 2 m^2 nets), the MOCNESS-4 (with 4 m^2 nets), the MOCNESS-10 (with 10 m^2 nets) and the MOCNESS-20 (with 20 m^2 nets). These systems typically carry five or six nets of 3.0 mm circular mesh; however, the MOCNESS-2 was equipped with 505 μm mesh nets, and the MOCNESS-10 has been used with ten nets. All nets are dyed dark blue or black to reduce contrast with the background.

All MOCNESS systems use the same underwater and shipboard electronics. The nets are opened and closed sequentially by commands through a single conductor armored cable from the surface. The electronics has 16-bits of resolution and the basic data

Table 3.2 MOCNESS system dimensions and weights (Wiebe *et al.* 1985).

System	No. of nets	Width of frame	Height of frame	Net width	Mouth area @ 45° towing angle	Length of net	Approx. weight in air	Recommended wire diameter
MOCNESS-1/4	9	0.838 m	1.430 m	0.50 m	0.5 m^2	6.00 m	70 kg	6.4 mm
MOCNESS-1/4-Double	18/20	1.430 m	1.430 m	0.50 m	0.5 m^2	6.00 m	155 kg	7.4 mm
MOCNESS-1	9	1.240 m	2.870 m	1.00 m	1.0 m^2	6.00 m	150 kg	7.4 mm
MOCNESS-1-Double	18/20	2.560 m	2.870 m	1.00 m	1.0 m^2	6.00 m	320 kg	12.1 mm
MOCNESS-2	9	1.650 m	3.150 m	1.41 m	2.0 m^2	6.00 m	210 kg	11.8 mm
MOCNESS-4	6	2.140 m	4.080 m	2.00 m	4.0 m^2	8.44 m	460 kg	11.8 mm
MOCNESS-10	6	3.410 m	4.690 m	3.17 m	10.0 m^2	18.25 m	640 kg	11.8 mm
MOCNESS-20	6	5.500 m	7.300 m	4.47 m	20.0 m^2	14.50 m	940 kg	17.3 mm

stream consists of temperature, depth, conductivity, frame angle, flow counts, net number, and net response. An acquisition/controller computer retrieves data from the underwater unit at a rate of up to 2 times per second. Temperature (to approximately 0.01 °C) and conductivity are measured with SEABIRD sensors. A modified TSK-flowmeter (Tsurumi-Sikie-Kosakusho Co. Ltd; see Longhurst *et al.* 1966 for a description of the flowmeter modification) is normally used to measure flow past the net. A model 2031 General Oceanics flowmeter has been used less frequently. Both the temperature and salinity sensors and the flowmeter are attached to brackets which are mounted on the top portion of the frame so that they face directly into the flow when the frame is at a towing angle of 45 °. An electronic pendulum angle transducer (Humphrey) measures the angle of the towed net through the water. A GPS unit providing latitude and longitude can be integrated into the data stream. The electronics and mechanical frame can be modified to accommodate optional sensors, for example transmissometer, fluorometer, submarine photometer, and bottom finding transducer (altimeter). Furthermore, acoustical and video (Davis and Gallager 1993) systems have been adapted for use on MOCNESS-1.

The MOCNESS flowmeter should be calibrated before and after each cruise. This can be done in a flow-tank or in the field by mounting the flowmeter(s) on a frame that can be towed over a measured distance. For field calibrations, paired runs over the measured distance should be made in opposite directions and averaged to eliminate errors introduced by naturally occurring water movements.

A microcomputer (together with disk drive and printer) are the deck unit and permit shipboard real-time data acquisition and processing as well as net control. Salinity (to approximately 0.01%), net oblique velocity and vertical velocity, and volume filtered by each net is calculated after each string of data has been received by the computer. Raw and processed data are stored on disk (in separate files) and processed data can be printed out. Plots of net depth versus time, temperature and salinity versus depth, temperature versus salinity, and latitude versus longitude are made during a tow and displayed on the computer screen.

A motor/toggle release assembly is mounted on the top portion of the frame and stainless-steel cables with swaged fittings are used to attach the net bars to the toggle release. A stepping motor in a pressure-compensated case filled with oil turns the escapement crankshaft of the toggle release that sequentially releases the nets to an open then closed position on command from the surface.

All MOCNESS systems incorporate the same basic design, with the nets, the underwater electronics package, the environmental and net monitoring sensors, and electro/mechanical net release mechanism mounted on a rigid frame, and many of the components are interchangeable.

The MOCNESS systems are designed to be towed at a 45° angle which is usually obtained with a ship speed of approximately 2 kn when difference in vertical current shears are minimal. Higher angles indicate higher net speed and vice versa. An algorithm for the calculation of net speed is given in Wiebe *et al.* (1985). Current practice is to 'fly' the system so that it is moving through the water at 2 ± 0.5 kn. If net speed drops below 1.5 kn, ship speed should be increased by 1/2 to 2 kn. Although not precisely equivalent, the net can also be flown by maintaining the net angle between 55° and 35°. Both speed and angle should be monitored closely because most of the complications to tows have occurred during excessively low or high speeds.

Any single conducting armored cable (where the conductor is used to transmit the signal and the armor is used as the ground return) will serve for the sending of data

between the underwater and deck units. The wire must be strong enough to withstand any realistically conceivable tension which might be experienced under tow. With the MOCNESS-1, wire tension on 0 to 1000 m tows of approximately 3000 lb (1360 kg) have been seen. Normal practice is to insist on a safety factor of at least 2 and preferably 3 in the breaking strength of the wire (e.g. 6000–9000 lb or 2730–4090 kg). For the larger MOCNESS-1D, -2, -10, and -20 trawls, a heavier conducting cable is required. A 0.68 inch (17.3 mm) diameter cable with a breaking strength more than 40 000 lb (18 200 kg) for these systems has been typically used in the USA. However, wire breaking strength is not the only factor that should be considered in choosing a wire diameter.

Multinet sampler

The Bé Multiple Plankton Sampler (Bé *et al.* 1959) and its improved HYDRO-BIOS multinet (Weikert and John 1981) are square-mouth samplers. The HYDRO-BIOS sampler contains five nets (0.25 m^2) that are closed on command from the deck via a conductor cable or by pressure release mechanisms that are preset to activate at predetermined depths. The sampler has three tow bridles in the mouth of the sampler which results in some degree of avoidance of the sampler by the larger mesozooplankton and macrozooplankton. This is a useful sampler for taking stratified depth samples of mesozooplankton during a vertical tow. Samplers such as the BIONESS, MOCNESS and RMT 1 + 8 are designed for horizontal or oblique towing.

RMT 1+8

The RMT 1 + 8 was the first opening and closing rectangular trawl to be widely used (Clarke 1969 and Baker *et al.* 1973). The RMT 1 + 8 was modified to have three 1 m^2 nets with a mesh size of 0.32 mm and three 8 m^2 mouth area nets with a mesh of 4.5 mm so that stratified sequential samples could be taken (Roe and Shale 1979). The different sized mesh nets allowed the capture of mesozooplankton, macrozooplankton and micronekton during the same tow resulting in considerable saving in ship's time.

Signals are sent to the sampler by acoustic communication to open and close the nets as desired. The RMT 1 + 8 and the multiple RMT mouth angles are sensitive to the towing speed, the greater the speed the greater the angle. At a speed of 2 kn the angle of the RMT 1 + 8 was 61° and 42° for the multiple RMT. The multiple RMT has successfully sampled to depths of 4500 m, deeper than most conductor cable systems. These depths were attained primarily because of the use of wire rope rather than expensive conductor cables, and because of the acoustic control.

3.3 FACTORS INFLUENCING MESOZOOPLANKTON SAMPLES

The variety of kinds of sampling devices for zooplankton is in part a reflection of the problems inherent with any particular sampler design. Factors which have been the subject of many studies include avoidance of the sampler by the organisms, clogging of the net meshes, and extrusion of animals through the mesh (escapement). These subjects and related topics are reviewed in this section.

3.3.1 Extrusion of zooplankton from nets

Animals which are captured by a net during the course of hauling it through the water can 'escape' the net by passing through the open area of netting mesh. In some cases, escapement is simply a case of individuals being smaller than the diameter of mesh

opening. However, it is possible that water pressure associated with flow through the mesh will force or extrude an individual larger than the mesh opening through, thus enhancing escapement. The speed of the net directly influences the water pressure within the net and therefore the amount of animal extrusion through the mesh. Generally it is recommended that vertically towed nets be towed at speeds of $1\,m\,s^{-1}$ or slower when sampling for mesozooplankton.

A fundamental parameter governing the performance of a net is R, the ratio of open area of net mesh to the area of the mouth opening of the net. The open area of a net mesh is determined by the total area of net mesh forming the net, $\boldsymbol{\alpha}$, and its porosity, $\boldsymbol{\beta}$, which is defined as the open area fraction of the mesh size. Thus:

$$\boldsymbol{R} = \frac{\boldsymbol{\alpha} \times \boldsymbol{\beta}}{\boldsymbol{A}} \tag{3.1}$$

Porosity values may be obtained from gauze manufacturers. Table 3.3 provides an example of the porosity of some selected mesh sizes taken from the Tetko Inc. General Catalogue No. 2000 for Nitex Swiss Nylon Monofilament. For a given net mesh opening, there can be more than one thread diameter. Generally as thread diameter increases, the strength of the netting increases, but the mesh porosity is reduced and more total mesh is required to achieve the same open mesh area to mouth opening ratio (R).

The 1968 joint working group (Tranter 1968) recommended that the WP-2 ring net has a filtration ratio of mesh area to mouth area of 6:1. This ratio is sufficient for nets of $>200\,\mu m$ mesh vertically towed in waters that are not rich in phytoplankton. Sampling in phytoplankton bloom conditions will make it necessary to increase the R ratio or shorten the duration of the tow to overcome the clogging problem (additional information is provided in section 3.3.2). The R ratio for oblique and horizontally towed nets at speeds of about $1.5\,m\,s^{-1}$ should be increased to at least 10:1 to compensate for the greater flow rate at these speeds and to reduce internal water pressure in the net.

The problem of zooplankton extrusion through the mesh of the net was examined by Nichols and Thompson (1991). They towed a series of high speed ($9–10\,m\,s^{-1}$) nets of 61, 90, 124, 190, and 270 μm mesh sizes and an open net area to the mouth area of the nose cone ratio of between 23 and 40:1. They described a mathematical model that showed a mesh size of 75% of the copepod carapace width would capture about 95% of the animals present. They reported significant loss of *Calanus* copepodite stages 1 and 2 through mesh size 190 μm, with only about half of the stage 1 copepodites captured by this mesh. A 124 μm mesh was needed to capture all of the stage 1 copepodites of *Calanus* spp. The equation relating the number of copepods per m^{-3} retained by the net

Table 3.3 Porosity of Nitex mesh.

Product Number	Mesh opening (μm)	Open area as % of total area
HC 3-500	500	49
HC 3-400	400	47
HC 3-300	300	50
HC 3-202	202	47
HC 3-150	150	51

(N_{caught}) as a function of the total number per m^{-3} available to capture (N_{total}) to copepod width/mesh size ratio (R) was

$$\frac{N_{caught(i)}}{N_{total(j)}} = \frac{1}{1 + \exp[-8.9(R - 1.0)]} \tag{3.2}$$

or

$$R = 1 + \frac{\ln\left(\frac{N_{caught(i)}}{N_{total(j)}} - 1\right)}{-8.9} \tag{3.3}$$

To adequately sample early copepodite stages of *Pseudocalanus* and *Paracalanus* species required mesh sizes of 61 and 35 μm respectively. This study applied only to high speed nets that generate high internal water pressures in the nets increasing the amount of copepod extrusion. Conventional vertically towed nets at speeds of 1 m s^{-1} may or may not produce the same degree of copepod extrusion through a similar size mesh. It is recommended that the above mesh sizes be used as a guide for sampling at the lower speeds commonly used for vertical and oblique sampling.

Any obstruction in front of the net mouth may lead to avoidance of the sampler by the larger forms of zooplankton and may also create problems in recording an accurate water flow through the net (Tranter 1968). It is recommended that nets without frontal obstructions be used when sampling larger forms of mesozooplankton (> 5 mm). There are no published data dealing with the influence of net-bridles on catch rates of mesozooplankton, however, whenever possible it is best to use samplers without bridles.

Inaccurate measurement of water flow through the net is an important source of error in estimating concentrations of zooplankton, therefore it is important that flowmeters mounted in the mouth of the net be used if the sampler design will allow. The calibration of the flowmeter is a critical component in an accurate measure of flow. Many commercial meters are supplied with calibration tables or curves, however, through usage the calibration of a meter will change due to wear and therefore meters should be calibrated before each sampling program. Calibration after a sampling program is a risky procedure because of the possibility of net loss during the program, leaving the researcher with volume values that cannot be verified by calibration.

Calibration of the meters is best done in a tow tank through which the meters are towed at calibrated speeds and times to measure distance. If a tow tank is not available meters can be calibrated by towing them vertically on an open frame, without nets, over a known depth and speed. Towing the meters horizontally over a measured distance also can be used to calibrate meters at various speeds. Care must be taken to measure the speed of the vessel through the water and not over the bottom.

There is no convincing evidence that increasing the size of the mouth area of zooplankton nets increases the efficiency of the sampler (Pearcy 1983). The concentrations of animals per volume of water have not been shown to increase with larger mouth areas for the same type of net and mesh size. The larger mouth area only provides a larger volume of filtered water increasing the likelihood that rare animals will be caught. A 0.75 m diameter net is adequate for the common species of mesozooplankton in northern and temperate coastal zones. In oligotrophic waters the size of the net mouth area may have to be increased to filter larger volumes of water to collect a reasonable sample size of animals.

3.3.2 Clogging of net mesh

'Four places of decimals in a computed coefficient can hardly offer compensation for an error so fundamental as the variation in the straining capacity of the net' (Kofoid 1897). The design of a plankton net is a critical element in effective quantitative sampling. The amount of water that can be efficiently filtered depends upon net shape, mesh size, mesh area, netting porosity, filtering area, and the mesh area to mouth opening ratio. Smith, Counts, and Clutter (1968) conducted an extensive series of instrumented net tows using nets of different shapes (cone, cylinder-cone, and cylinder) and mesh sizes in what they defined as coastal or green water and oceanic or blue water. They measured percent efficiency of each net by using a flowmeter mounted in the net mouth and one mounted outside. They considered a net to begin to have significant clogging problems if the filtration efficiency during a tow fell below 85%. Smith, Counts and Clutter (1968) found that the clogging rate of a net was affected by four factors:

1) The composition and density of suspended material in the water. Coastal waters with generally higher particle loading than oceanic waters will cause clogging to occur more rapidly.
2) The mesh size – the smaller the mesh size, the faster the net clogged.
3) The ratio of filtering area to mouth area – the smaller the ratio the faster the clogging.
4) The form of the net – a cylinder cone resisted clogging the best, closely followed by the cylinder net.

There are important sampling implications which result from progressive net clogging since the water column will not be sampled uniformly. As the pressure difference between the inside and outside of the net increases, more organisms will be extruded through the mesh. Water will be pushed out of the way of the net and the disturbance (a bow wave) will provide a cue that could trigger an avoidance response by the animals in front of the net.

The results of this study provided a basis for two equations that are particularly useful in the design of nets.

$$\mathbf{Log_{10}}(R) = \mathbf{0.38}^{*}\mathbf{Log_{10}}(V/A) - \mathbf{0.17} \qquad \textbf{Green Water} \tag{3.4}$$

$$\mathbf{Log_{10}}(R) = \mathbf{0.37}^{*}\mathbf{Log_{10}}(V/A) - \mathbf{0.49} \qquad \textbf{Blue Water} \tag{3.5}$$

where R = filtering area/mouth area, A = mouth area (m^2), and V = volume of water to be filtered (m^3). These equations enable an investigator to develop a net design to meet the conditions that are likely to be met during the course of a study. Given a volume of water that needs to be filtered to catch sufficient individuals to provide a statistically valid sample and the mouth size of the net system, the mesh area required to prevent clogging can be computed.

3.3.3 Avoidance

Avoidance, the active swimming of zooplankton out of the capture path of a net, is the most serious bias affecting the catch of the larger meso- and macrozooplankton. While there have been numerous field, laboratory, and theoretical studies concerning avoidance effects, few solutions that effectively eliminate the problem exist.

The effects of avoidance are net-size dependent. McGowan and Fraundorf (1966) studied the relationship between the size of net used and estimates of zooplankton diversity. They took a series of net tows in an area off Baja, California using nets of 20, 40, 50, 80, 100, and 140 cm diameter. The tows were taken obliquely and the length of tow was regulated so that each net tow filtered the same amount of water. Two night series and one day series of tows were taken and the nets were used in random order to minimize the effects of patchiness. A total of 140 species of mollusks, euphausiids, larval fish, and larval squid were counted. The ability to catch species was $140 > 100 = 80 = 40 > 60 > 20$ cm diameter net. One conclusion was that the various species can avoid little nets better than large ones. When ordering the nets according to their ability to estimate abundances, the result was $140 > 100 > 80 > 60 > 40 > 20$. On a per unit volume, the larger the net, the larger the catch. By using the larger net, the avoidance error in this study was reduced. However, although the 140 cm net did the best job, it only took an average of 54.8 species/tow and cumulatively only captured 99 of the species out of the 140 caught by all of the tows. Thus, even the best net in this experiment seriously underestimated the number of species present in the area.

Fleminger and Clutter (1965) did an elaborate tank experiment to examine the effects of net size and lighting conditions on the avoidance of towed nets by a mysid and six copepod species. Net sizes used were 43, 32, and 22 cm diameter and they were towed about 30 cm s^{-1} through the tank in full light, reduced light, and darkness. The smaller nets caught significantly fewer mysids, changes in light resulted in significantly more caught in darkness, and avoidance tended to be less when population numbers were higher. For the copepods, smaller nets caught fewer individuals, but the level of avoidance differed among the species; changes in light had no effect on the abundance estimates; and avoidance tended to be less in denser populations. The differences in avoidance observed between these two taxa were ascribed to differences in visual acuity, mysids could 'see' in the lit medium, and tended to shun foreign objects and to aggregate more.

The theoretical aspects of zooplankton avoidance have been examined by Barkley (1964, 1972). In order to avoid a net, the required individual escape velocity increases proportionately with an increase in either towing speed or net radius and decreases in proportion to increases in the reaction distance or the initial offset of the individual from the center of the net mouth. Because minimum escape velocities decrease rapidly as the reaction distance decreases below optimum values, it is quite inefficient to reduce the net opening to low values. Thus, in the model runs and assumptions specified by Barkley, a net with a 300 cm radius was several times more effective than a 50 cm net. The basic problem that still exists for most zooplankton is that both the reaction distance to an approaching net and the escape velocities of individuals are poorly known. Wiebe *et. al.* (1982) applied the Barkley theory to a series of collections made with a 1 m^2 and a 10 m^2 MOCNESS from which an euphausiid, *Nematoscelis megalops* was counted and sized. There was significant differential day/night avoidance of both net systems, but there was no difference in estimates of catch rate between the nets. The results indicated that increasing the mouth area by a factor of 10 did not effectively reduce the avoidance of the net because the individuals apparently began their avoidance reaction further in front of the larger net. One conclusion of this study was that since vision was the likely sensory system used by *N. megalops* to detect the net approach, active measures to reduce net detection were needed to reduce the avoidance effect as described below in Effect of ambient light, and Mesh and frame color.

EFFECT OF AMBIENT LIGHT

The level of ambient light influences the degree of net avoidance by the larger forms of macrozooplankton (Sameoto *et al.* 1980; Wiebe *et al.* 1982; Sameoto 1983) and fish larvae (Heath and Dunn 1990) as evidenced by larger numbers of these animals captured per unit volume of water at night. There is no evidence that avoidance of samplers by copepods is affected by ambient light. When possible it is best to sample mesozooplankton at night, because this sampling will also provide a better estimate of macrozooplankton as well as an equally valid estimate of mesozooplankton in the same sample. It is important that the ship's deck lights are turned off when sampling at night. The bright lights from the ship will make the net visible to the macrozooplankton and at the same time may attract some species and repel others from the vicinity of the ship which will result in biased estimates of these species.

A study of the effect of artificial light on reducing net avoidance by euphausiids during daylight demonstrated an increase in catch of euphausiids of between 10–20 times that obtained when no light was used. It is believed that the light has a blinding effect on the euphausiids making it less likely that the animals will see the net. This effect was reduced at night to an increase of about 2–3 times as many caught with the light on, compared to no light (Sameoto *et al.* 1993). The light used in the experiments was a 125 W video light pointing straight ahead of the sampler. Recent studies using a flashing strobe light in the same manner as the above experiment showed a strobe light flashing at 10 s intervals had the same effect in reducing avoidance as the continuously shining video light (Sameoto, unpublished data).

MESH AND FRAME COLOR

The recommended mesh material for mesozooplankton nets is Nylon Nytal 7 P, with a mesh aperture width of 200 μm. Especially for sampling larger organisms (macrozooplankton), it is recommended to avoid bright colored nets. The color of the mesh should be one that makes the sampler less visible in the water such as a dull green, blue or gray, white nets should be avoided. The frame of the sampler should also be a dull dark color similar to the net with the bright metal rings or frames of the sampler painted to reduce light reflection. These color recommendations will help reduce macrozooplankton avoidance of the net.

3.4 HANDLING TOWED SAMPLERS

Precision towing of multiple net systems requires reasonably fine control of winch speed, especially in the range of 1–30 m min^{-1}. The net system is usually paid out at 30–40 m min^{-1}; occasionally when angle and speed are optimized and the system is well below the surface, a rate of 50 m min^{-1} may be used. Hauling speeds are generally between 10–20 m min^{-1}, although on shallow tows with finely spaced strata, a rate as low as 1 m min^{-1} may be required to evenly sample a stratum and at the same time filter adequate amounts of water. Under windy conditions the ship should steam into the wind during a haul. When winds exceed 10 kn, there is sufficient wind set so that the towing course should be chosen to put the wind and swell on the side of the bow which corresponds to the side of the ship where the net is to be launched and recovered. This should keep the wire out from under the ship. It will also give the bridge some advantage in keeping the ship moving ahead at a slow speed if under calm conditions the ship has trouble reducing its speed to that optimal for towing.

Under calm sea surface conditions, there is no preferred towing point, from the side or stern are equally good. In high winds or heavy swell and a ship's course into the wind and sea, towing from the side has the advantage of minimizing the effect of ship pitch on the wire and net. Severe pitch can seriously affect the quality of the catch (jerking of the nets up and down can damage the organisms) as well as stress the nets and frame (causing net blowouts) and damage the cod-end buckets. Towing from the ship's pitch pivot point will minimize this effect.

The handling of heavy samplers in a rough sea is dangerous and there is a high risk of losing the gear due to tow cable breakage, but there are ways of reducing this. The best means of reducing the sudden high peak load on the tow cable due to waves and swells causing the upward acceleration of the ship is to use a constant tension winch. Mitchell and Dessureault (1992) described a control unit with a pressure relief valve inserted in the hydraulic circuit of a winch that maintained a constant tension on a towing cable. This unit showed significant reduction in the cable tension peaks and marked improvement in the towed behavior of the BIONESS. A reduction in the total cable tension by a factor of 4 to 5 occurred when the pressure relief valve was used. Without the tension compensation there was significant relationship between the movement of the ship and the motion of the sampler, but no relationship existed when the winch was compensating for the ship's vertical motion. This not only makes the operation safer but it also provided better depth accuracy and control when sampling specific depth strata. The use of the controller made the towing, launching and retrieval of the BIONESS and other heavy gear much safer and easier in rough seas.

A less expensive but less effective method of keeping a load on the towing cable when the ship is pitching is to use a bungy cord attached to a roller block on the cable. The bungy cord applies a load to the cable at 90° from the cable. When the cable slackens in a swell the slack is taken up by the stretched bungy cord thereby reducing the snap in the cable when the tension returns to the cable and thereby reducing the likelihood of the cable parting. This method of loading the cable is not as effective as the constant tension winch in protecting against cable breakage.

The relationship between wire diameter and meters of wire out to get the net system to a given depth is also an important consideration. At any given tow speed, a given diameter cable will have an inherent angle which depends on its weight per unit length and its drag (a function of surface area). As a general rule, the larger the diameter cable, the larger the ratio of weight per unit length to drag and therefore the steeper the inherent angle at a given speed. Larger cable usually permits less wire to be paid out to get to a given depth and therefore cuts the time to shoot a net to depth. Larger cable also seems to tow straighter (with less catenary) thus reducing stalling of the net when hauling in.

When is it too rough to tow? The decision not to tow will depend on the stability of the ship in rough seas, the flexibility in adjusting the towing point between the side of the ship and the stern, and the ease of launch and recovery from the deck. MOCNESS systems have been towed in winds up to 40 kn and seas of 8–15 ft (2.4–4.6 m), but the collections under these conditions have not always been of the best quality. Perhaps the best advice is to be conservative until enough experience has been gained to judge the feasibility of towing under marginal conditions. This decision should be made with the safety of personnel during launch and recovery foremost in mind. While handling experience and ship's capability play a large part in the decision, personnel safety should always be the dominant factor.

3.5 CARE OF TOWING CABLES

It is important the all-metal towing cables whether conductor or wire rope type be properly cleaned and lubricated. The frequency of this maintenance will depend on the frequency and the conditions of use. Operations in tropical waters require much more frequent cable maintenance than those in colder regions due to the higher rate of corrosion at warmer temperatures.

3.6 HANDLING SAMPLES AND SAMPLE PRESERVATION

Quantitative work starts with careful rinsing of the plankton net, using an appropriate flow of sea water from the outside part of the gear, washing plankton quantitatively from the net material and concentrating it in the cod-end. The water jet should remove the organisms from net material but not damage them. Windows of the cod-end should be equipped with gauze of the same aperture as the net. Balachandran (1974) proposed closed plankton buckets, without side or bottom windows, especially to keep fragile organisms of tropical waters in good conditions. Closing nets like WP-2 net (UNESCO, 1968) should be rinsed only at the lower part after stratified sampling. Otherwise organisms from the abundant near surface plankton could stick in the part above the closing rope and contaminate the sample. This has to be taken into account especially for vertical stratified taxonomic studies. Finally the cod-end will be screwed off to pass the specimen on into a jar or in a bucket for further treatment. The use of filtered sea water for concentrating and passing the material on is essential to avoid any contamination. Take care that sampling containers or buckets are properly marked to avoid mistakes, especially if several catches will be performed at the same location.

Nets should be kept wet or damp between stations to avoid successive clogging of meshes by dried organic matter. The net material should always be checked to be sure not to use damaged gauze. Nets should be washed with a dilute alkali solution (detergent) after cruises, then rinsed with fresh water, dried and stored in bags.

It is necessary to know the amount of filtered sea water for calculation of abundance or of biomass concentration. The use of a calibrated flow-meter is best for that purpose. Calibration must be done at a speed which is in the range of the towing velocity of the gear, for example at 45 m min^{-1}. The variability of the mechanical TSK (Tsurumi-Seiki-Kosakusho Co., Ltd, Yokohama) flowmeter was about 4% during calibration conditions (Postel 1990). Be sure that the materials of the flowmeter do not react to temperature. Teflon-made instruments alter their revolution properties at low temperatures. The flowmeter should be mounted in that part of the net entrance where flow properties are optimal, for example in the center or in a quarter of the diameter. The latter version is needed because bridles in the center of the net opening area interfere with the flow (Tranter 1968). The revolutions correspond to a length of the water column (in meters) which has passed the net. The calibration factor determines the ratio of revolutions per meter. The amount of filtered water (m^3) is finally the product of flowmeter revolutions, calibration factor and net opening area (m^2). If the construction of the gear allows attachment of a second flowmeter outside the net, the ratio between both indicates the filtration efficiency. Samples from tows of less than 70% efficiency should be neglected (UNESCO, 1968). Electronic instruments permit on-line registrations through conductivity cable and allow stopping the tow in the worst conditions, or off-line readings by the memory probe for later consideration.

The estimation of the filtered water column without a flowmeter is generally not recommended. The use of the length of the released wire, multiplied by the net opening area, is restricted to vertical hauls. The calculation 'time × speed × net opening area' allows estimations independent of the towing direction (vertical, horizontal, oblique). The proper functioning of the flowmeter could be tested in calm weather, either by measuring the released wire versus revolution and given towing velocity, or by measuring the horizontal distance and ship speed if the ship is equipped with a precise navigation system, i.e. GPS (Geo-Positioning System). The reduction of the amount of filtered water by clogging effects in eutrophic waters on one hand or the influence of drifting ships during vertical towing on the other, cannot be considered without flowmeter. Particularly when sampling deeper levels, a drifting ship continues the filtration process, even when the winch is stopped for the time a messenger takes to reach a mechanical releasing mechanism. The ship drift depends on the size of ship and is proportional to the wind velocity. The effect became dominant in comparison to clogging (in shelf areas), at wind velocities larger than $6\,m\,s^{-1}$ (wind force 4). It was assessed on R/V *A.V. Humboldt*, 1200 gross tons. The influence increased drastically at wind velocities of $12\,m\,s^{-1}$ (wind force 6), especially if the closing depth of a vertical operating net (WP-2) was deeper than 50 m. The relationship

$$y = 0.64x_1 + 0.05x_2 - 3.9 \tag{3.5}$$

was statistically significant for x_1 and allowed an approximate correction of the underestimated amount of filtered water y (m^3) from wind velocity x_1 ($m\,s^{-1}$) and closing depth of the net x_2 (m).

Wire angles are another problem of drifting boats. The gear does not reach the intended depth for vertical hauls if no depth recorder is operating on-line, i.e. the depth is measured by the meter wheel only. In that case the wire angle correction must be performed by trigonometric rules. Then the final wire length z_f is the intended depth z divided by the cosine of the wire angle a, which is assessed by a clinometer, for example, to reach 200 m depth at a wire angle of 15°,

$$z_f = 200/\cos 15$$

$$z_f = 207\ \text{m} \tag{3.6}$$

Important sources of error in the collection of zooplankton samples can occur after the net is retrieved from the water and taken on deck. It is important to wash the net properly so that all the contents are moved into the cod-end. When washing the net it is best to raise the net vertically and hold it in this position while washing with seawater from the outside of the net. Care should be taken not to get the washing water in the mouth of the net since this can introduce organisms from the washing hose and contaminate the sample. Special care should be given to the cod-end after the plankton bucket is removed to ensure that no animals remain in the seams of the net near the cod-end.

The type of preservative used to fix the zooplankton will depend on the purpose for which the samples were taken. An in-depth discussion of the various techniques for zooplankton fixation and preservation is given in *Zooplankton fixation and preservation* edited by Steedman (1976a). In general, a seawater formalin solution containing about 4% formaldehyde buffered with sodium borate and strontium chloride is recommended when animals are collected for taxonomic purposes. For long term preservation of calcareous shelled zooplankton, it is very important that the pH of the preserving fluid

be maintained at about 8.2. With too little buffering, the calcareous shells will dissolve and with too much buffering the shell-binding protein may soften (Steedman, 1976b). Frequent monitoring and buffering of the pH of the preserving fluid should be done during the first days after the initial preservation of a sample and every few weeks thereafter for a period of 3 to 6 months (Turner 1976). It should be remembered that formaldehyde is a carcinogen and should be handled accordingly.

3.7 COLLECTION OF LIVE ZOOPLANKTON FOR EXPERIMENTAL STUDIES

Zooplankton for laboratory experiments must be collected with great care. Sampling procedures are designed to minimize physiological stress and physical damage to the organism during capture and during treatment immediately after the animals are brought on deck. In general, sampling protocols call for the capture and transfer of organisms to be performed quickly and gently. Direct sunlight or bright deck lights are avoided during transfer operations. Special care is taken to ensure that the seawater for reception of the animals is at ambient temperature and salinity and is free of contaminants that may be encountered on ships. One net system that has been widely used to collect animals for 'live work' was described by Reeve (1981). A net similar in design has a 1 m diameter ring net equipped with 333 μm mesh and a large 32 cm diameter by 46 cm tall cod-end bucket.

3.7.1 Copepods

To capture live copepods ring nets are typically towed either vertically or obliquely at low speed ($<0.5\,\mathrm{m\,s^{-1}}$ forward and 0.1–$0.5\,\mathrm{m\,s^{-1}}$ upward, depending on depth). A general rule of thumb is that the angle of the towing wire with the sea surface should be $>45^{\circ}$. Some investigators pull in the net by hand or allow it to drift with the ship. This method is not feasible for larger taxa which may be residing deep in the water column, requiring tows of longer duration, 20–30 min or more depending on the depth of tow. To reduce damage to antennae and setae, the nets are preferably fine-mesh relative to the size of the animal (e.g. 150 μm mesh for female *Calanus finmarchicus*) although larger mesh sizes may be used, particularly during phytoplankton blooms or other conditions where net clogging or cod-end overcrowding due to the abundance of small organisms is a problem. The plankton is usually collected in a large-volume (5–20 l) cod-end. While some investigators use cod-ends with no drainage at all, others prefer that several screened holes are drilled near the top of the cod-end, in order to allow flow of water into the quiescent bottom part during towing.

Unless the cod-end volume is very large, the catch should be diluted immediately upon arrival on the ship's deck. Plastic, 4 l jars make excellent reception containers. They are filled with approximately 3 l of clean, ambient seawater and placed into seawater maintained at an appropriate temperature in thermally insulated coolers. Because there may be layering within the cod-end, it is prudent to pour a small portion of the catch into each jar to start, then return and add more according to a visual assessment of plankton density. When correctly diluted, copepods will resume normal swimming behaviors. With a large-bore pipette, copepods can be transferred directly from the jars to petri dishes for sorting. The reception containers should be scrubbed with hot water and rinsed with sea water between uses in order to reduce bacterial contamination.

3.8 OTHER ZOOPLANKTON INSTRUMENTS USED IN CONJUNCTION WITH NETS

To sample zooplankton efficiently the researcher should know as much as possible about the vertical distribution of the various components of the zooplankton community before the net samples are taken. There are relatively inexpensive instruments and methods that can provide this information. The OPC (discussed in detail in Section 7.3) provides *in situ* information on the concentration and size of zooplankton with depth when it is combined in a CTD profiling package. The size frequency data from the OPC can also be used to make an estimate of the wet biomass of the mesozooplankton (Heath 1995; Stockwell and Sprules 1995). The OPC data is presented during the vertical profile allowing the researcher to decide immediately which depth intervals are of interest to him for sampling with nets.

A commercial video camera in a pressure case can provide qualitative and quasi-quantitative data of zooplankton as it is lowered through the water column. The advantage the video camera has over the OPC is that larger organisms can be identified. There are a number of special video instruments that have been developed to identify and quantify zooplankton if only a few dominant species or developmental stages are present.

The use of acoustic sounders with multiple or single frequency will tell the researcher in which areas there are changes in patterns of organism distribution and this information will suggest regions of interest for other methods of sampling such as net sampling. In a zooplankton sampling program all types of information should be combined to create as much insight as possible into the distribution of the zooplankton before and during a net sampling program.

3.8.1 Optical plankton counter

This commercially available instrument provides *in situ* counts of zooplankton in the size range of 0.2–20 mm spherical diameter. The OPC is a non-video optical instrument for studying zooplankton distribution and abundance. Its initial design has been described by Herman (1988). The submersible version comprises a sampling tunnel with a 22 * 2 * 0.4 cm collimated light beam from a stack of 640 nm light emitting diodes (LEDs) to a photodiode receiver. The light attenuance of the water is monitored simultaneously to counting and sizing particles from 0.250 mm to approximately 20 mm equivalent spherical diameter (ESD). Data including time marks of 0.5 s intervals are reported to a deck unit via a single conductor cable. This time interval corresponds to a volume of water of 6.4 l and a spatial resolution of 1.3 m at a towing speed of 5 kn (2.6 m s^{-1}). A laboratory version having a shorter light beam can detect live and preserved plankton down to a lower size limit of about 0.1 mm. The maximum count rate (i.e. the product of flow velocity towing speed), and particle concentration, is 200 counts s^{-1} for both versions due to the response time of the sensor (see Herman 1992 for further technical details). The data are logged on to disk in a simple format accessible to subsequent processing, for example with standard spreadsheet software. Size discrimination of the particles allows the identification of species and stages of species.

The OPC can be towed horizontally at high speed (up to 12 kn), dropped vertically in a profiling mode and used as a bench sampler through which the samples are passed to get counts and sizes of zooplankton. The vertical profiling OPC can be used as a reconnaissance tool combined with a CTD to locate the depths at which concentrations

of various sizes of zooplankton are found. The concentration profiles are used to guide the net sampling to particular depths of interest thus making the most efficient use of the net sampling and shiptime. By targeting certain depth strata, maximum information about the vertical distribution of zooplankton can be gained from the nets with the least amount of shiptime and sample analysis cost. A towed OPC has been used to carry out high speed surveys for zooplankton over large geographic areas (Herman *et al*. 1991; Huntley *et al*. 1995).

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4 Biomass and abundance

L. Postel, H. Fock and W. Hagen

4.1 INTRODUCTION

Some decades after Johannes Müller noticed floating organisms in the sea, which he called 'Auftrieb', Victor Hensen first claimed in 1887 that the sea could be studied *quantitatively* (Mills 1989). This achievement was linked with the beginning of fishery research, especially the question of food web relationships. Hensen (1887) tried to compare the productivity of plankton with that of agriculture. Brandt (1898) intensified this effort determining the nutritive value of plankton by analyzing its water, ash, protein, lipid and carbohydrate content. Finally he compared the productivity of fishermen and farmers on a monetary basis (Brandt 1894), which was the first attempt at a socio-economic study. The holistic view of ecology was promoted by Odum and Odum (1959) in the middle of the twentieth century. Organic carbon and energy were introduced as integrating units during that time.

Knowledge of plankton community structure and how it functions depends on answering *how much*, *which*, *where* and when plankton occurs. Patterns of zooplankton concentration in terms of numbers or biomass reflect pattern forming processes in a wide range of space and timescales (Haury *et al.* 1978).

The required *accuracy* to reflect a typical distribution pattern depends on an appropriate sampling design (see Chapter 2) and the *precision* of the methods being employed.

Depending on mesh size and the area of investigation, net samples contain a variable mixture called seston. It consists mainly of living, but sometimes also of dead, organic and inorganic particles (Table 4.1). Hensen (1887) named all floating organisms 'plankton' whether living or dead. Nowadays plankton stands for the living part of seston, detritus for dead organic and inorganic particles. The organic part is known as tripton, and the inorganic component of detritus and of plankton organisms can be

Table 4.1 Potential fractions of net samples and their terms.

	Living organisms	Dead organic matter	Inorganic matter
Seston	×	×	×
Plankton	×		
Detritus		×	×
Tripton		×	

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determined as ash content. The methods commonly used to analyze mixed samples do not distinguish between living and dead organisms, except for the determination of adenosine triphosphate (ATP).

Fractionation of samples by different mesh dimensions enables plankton to be classified according to size. Floating particles larger than 0.2 mm are mostly zooplankton, termed mesozooplankton (0.2–2 mm) and macrozooplankton (>2 mm) (cf. Chapter 1). The fractions smaller than 0.2 mm, the microzooplankton, are often contaminated by chain-forming phytoplankton.

The methods described in this chapter refer to the size range larger than 50 μm. Copepods and their developmental stages are the most important part of this plankton fraction, which also includes cladocerans, larger crustaceans, chaetognaths, tunicates, cnidarians, etc.

These taxonomic groups differ in water and ash content as well as in biochemical components, which affects their nutritive value for predators and their importance within nutrient cycles.

In principle there are two main types of quantitative procedure, biomass determinations and counting methods. A combined method involves both counting the organisms and calculating their biomass, using individual biomass factors, determined directly or by individual morphometric characteristics (Figure 4.1).

The methods finally used rely on the purpose of the study, the cost effectiveness, the technical conditions of laboratories and the requirements of precision.

The concentration of biomass or biovolume is generally expressed as average mass per volume of water, for example mg m^{-3}, or related to the sea surface as mg m^{-2}.

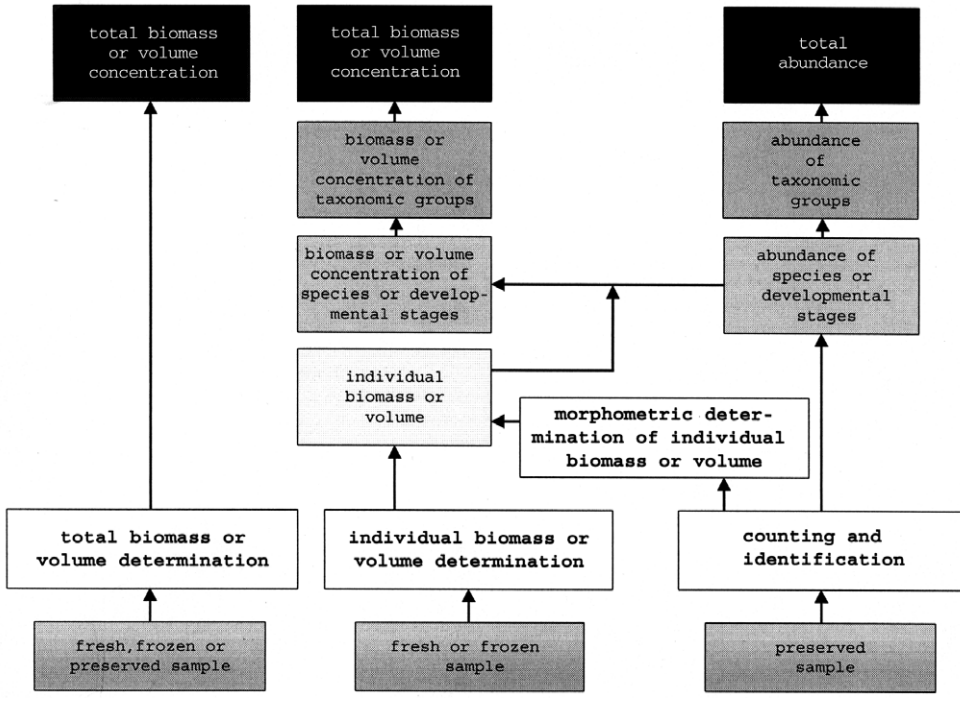


Fig. 4.1 Pathways of quantitative zooplankton work.

Information on the integrated water column is recommended. In the current literature 'weight' is frequently used instead of 'mass' (dry weight, for example). This is incorrect according to the international agreement on units (SI, Système International d'Unités). Strictly speaking weight is an expression of power, and is mass multiplied by gravitational force.

The concentration of individuals is called 'abundance' and is expressed as number m^{-3} and number m^{-2} respectively.

There are numerous published data, produced by different methods. They are comparable to some degree using conversion factors (4.3.4).

4.2 SHIPBOARD SAMPLE TREATMENT

A net retains a fraction of the total amount of plankton, which depends on both escapement and avoidance (see Chapter 3). For example, the WP-2 net (mesh size 200 μm) collects the fraction between 0.2 and 10 mm (Tranter and Smith 1968). Organisms outside this range are occasionally taken and should be excluded by sieving. Consequently, the authors recommend the use of different gears, appropriately designed for each size class, and the combination of sampling with a *sieving technique* to determine the concentration of plankton within the size fraction concerned. Summation would allow the determination of the total amount of plankton.

Furthermore, the *separation of size classes* by a filter column is a helpful tool to roughly distinguish taxonomic groups and/or developmental stages. The ecological implication of body size (Peters 1983) requires such a procedure for the application of size dependent formulae for the calculation of metabolic rates (see Chapters 8 to 10). Seda and Dostálková (1996) validated live sieving statistically by comparing the sieved fraction with microscopic sizing of individuals. In the case of cladocerans they achieved efficacy of separation of 90%, using a mesh size of three quarters of the body length. The procedure is subject to substantial individual error. Therefore, the procedure should preferably be done by the same personnel during a cruise, using stepwise gentle rinsing manipulations and checking the effectiveness of separation microscopically from time to time. A reverse filtration approach may guarantee a gentle procedure. A stepwise filtration of sub-samples improves the quantitative separation.

Taking samples for simultaneous measurements of different properties in zooplankton is often necessary. This requires either multiple hauls on the same station or dividing the same sample. The first method is time consuming if no multiple gear is available and the results may be affected by patchiness. The second increases the amount of errors due to the *splitting procedure*. The variability between splits was quantified in land-based laboratories (Kott 1953; van Guelpen *et al*. 1982; Sell and Evans 1982). The coefficients of variation of different sub-sampling devices varied between about 5% and 30%, depending on the construction and the species assemblage. Gravity dependent splitting techniques become unreliable on moving ships. The problem might be avoided using a kind of beaker approach. Modifying a description by van Guelpen *et al*. (1982), the known amount of sample is poured from one measuring cylinder to another. The procedure is repeated back and forth until the sample is homogeneously dispersed and split in halves. Remaining organisms should be rinsed with filtered sea water equally to the sub-samples. This method produced a coefficient of variation of 9% to 14%.

Further treatment and storage on the ship depend on the purpose of sampling. Samples for biomass determinations are ordinarily rinsed with distilled water to

eliminate the salt of adherent sea water which would enlarge the mesozooplankton dry mass by 2% to 7% (Hopkins 1982) or by 3% on average (Omori 1978). The *question of rinsing* has caused some controversy about its negative effect on the organic content by bursting of cell membranes, if hypotonic fluids are used. For example, Platt *et al.* (1969) found no loss on adding 1 l distilled water either for 30 s or for 1 h. However, Omori (1978) observed a remarkable decrease in biomass within the first 15 s of the procedure, applying distilled water instead of isotonic (3.35%) ammonium formate or sea water. The loss is logarithmically related to the amount of rinsing fluid (Figure 4.2). The relationship based on Omori's (1978) Table 2 using the difference in biomass between samples rinsed with sea water and distilled water respectively, reduced by an average amount of 3% adherent salt. The percentage was similarly observed for dry mass, ash-free dry mass, carbon and nitrogen content (of chaetognaths and calanoid copepods). The equation in Figure 4.2 could be used for corrections. Considering Omori's (1978) results, removal of the interstitial sea water would correct the biomass positively by about 3%, accepting a loss up to 30% at the same time. However, the inorganic component of the organisms itself exceeds the adherent salt by up to one order of magnitude (cf. Table 4.2). Therefore, it is better to determine the total amount of ash directly than to remove the minor part with distilled water.

There are different methods of *preservation and storage*. Samples for direct biomass determination such as dry mass (Figures 4.1 and 4.3) will be rapidly frozen (after removing excess water by filtration) in a freezer at −18 °C or in dry ice. For longer storage, samples should be kept in airtight containers to prevent dehydration (Omori and Ikeda 1984). The material could be also dried immediately (see page 91) and stored in an airtight desiccator at −18 °C (Omori 1978) for weighing at a land-based laboratory (see page 91).

Samples for species enumeration and identification are kept in 4% formaldehyde (high-grade) solution, in jars of about 250 ml, made of brown polyethylene or glass. Concentrated formalin (37–40%), buffered by borax (sodium tetraborate, $Na_2B_4O_7{\cdot}10H_2O$), is added in a sample to fixative ratio of 9:1, using a graduated cylinder or a dosing feeder (i.e. 90 ml sample, 10 ml formaldehyde) as soon as possible,

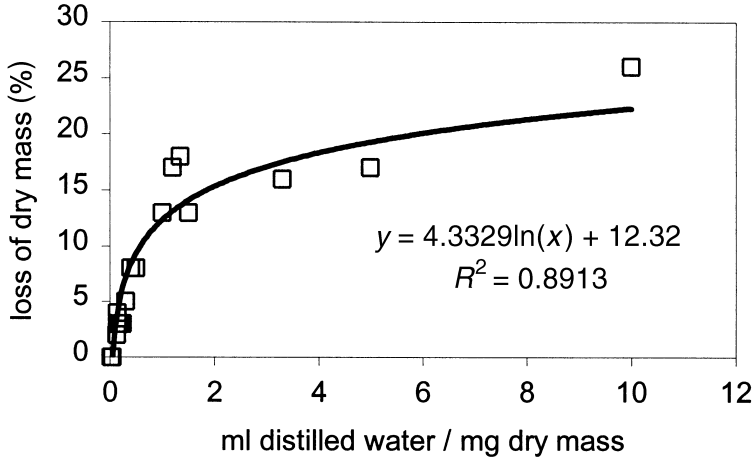


Fig. 4.2 Loss of dry mass depending on rinsing with distilled water, compiled using data of Omori (1978); see text for details.

at best within 5 min (Steedman 1976). The jar must be gently rotated to mix the contents, repeated several times within 1 h. Containers should be filled completely to avoid sloshing of organisms. Buffering of fixative should be done before a cruise, adding 2 g of borax to 98 ml of 40% formaldehyde. This results in a pH of about 8 to 8.2, which is suitable for mixed plankton and general taxonomic work. Without buffering, oxidation of organic matter during storage bears the risk of decreasing the pH, leading to a dissolution of calcareous structures of the animals. A pH above 8.2 may damage crustacean tissues and gelatinous organisms, on the other hand. Hexamine (hexamethylene-tetramine) as a buffering agent has the disadvantage of crystalizing around organisms, if sample fluid evaporates during later examination in an open dish. It is also highly carcinogenic. Miscellaneous preservation techniques for special taxonomic objectives are recommended by Steedman (1976).

The sample jar must be labeled (not on the lid, to prevent mistakes). An additional label (of water-resistant material, e.g. greaseproof paper) should be placed in the jar. Labeling should be done by pencil or marker. Ensure that lids are tight and sealed. Samples should be stored at low constant temperature and in the dark. The level of preservative, its pH and the tightness of lids must be checked from time to time, at least just after the cruise. If the pH has altered, remove fixative, using a net with mesh smaller than the dimensions of the plankton, and add a new formaldehyde solution of 4%.

Caution is needed if working with concentrated formaldehyde. Splashes in the eyes or on skin are removed by washing with cold running water for about 10 min (Steedman 1976). Spills must be removed immediately by copious amounts of water, and good ventilation of the laboratory is recommended.

Field logs should be prepared so that researchers who did not attend the cruise are able to obtain all the information needed to calculate the final results, abundance and biomass concentration, and to interpret extraordinary occurrences. The logs should include the project, cruise number, name of the ship, the collector's name, net type (mesh size), type of haul (vertical, horizontal, oblique), station number and/or geographical coordinates, date, time (start and end of the haul), depth level (m), wire angle (°), wire length out (m), towing speed ($m\,s^{-1}$), towing time (if necessary for determination of filtered water), flow meter serial no., flow meter readings (or amount of filtered water), comments (for example removal of macroplankton such as jelly fish, contamination with phytoplankton, and other points).

4.3 BIOVOLUME AND BIOMASS DETERMINATIONS (By W. Hagen)

The determination of the total plankton biomass or biovolume is rapid compared with enumeration and identification techniques. Many samples can be processed in parallel. These measurements are suitable for mixed samples, as well as for samples of selected individual species.

There is a wide variety of biovolume and biomass measurements. The effort involved in sample treatment increases within the conventional methods from settling volume, to ash-free dry mass (organic mass) and is generally large in biochemical procedures.

The methodological bias decreases stepwise: first, when the amount of excess and interstitial water is reduced by measuring displacement volume or wet mass instead of settled volume; secondly, when body fluids are totally removed during dry mass determination; and finally, when the inorganic substances are subtracted. The flow diagram in Figure 4.3 illustrates these stepwise procedures.

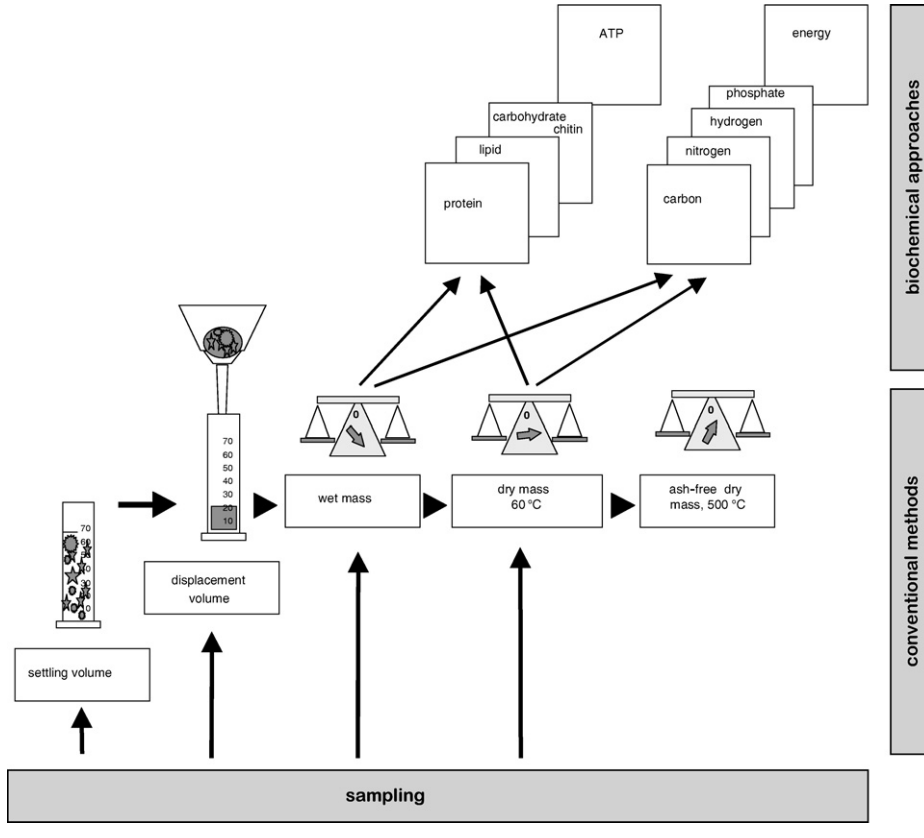


Fig. 4.3 Pathways of biomass determination.

4.3.1 Volumetric methods

Volumetric methods are the only choice if samples are also to be used for species identification. They are also recommended as a quick-look procedure, i.e. on board a ship, and in cases where no micro-balance or other sophisticated equipment is available. There are two principles: the direct volume estimation of settled plankton, or the measurement of the amount of water displaced by the plankton. The first method is more gentle but less precise.

SETTLING VOLUME

Preserved samples are poured into graduated cylinders of 50–100 ml or 500 ml (Lillelund and Kinzer 1966) and carefully mixed. The last parts of the sample should be rinsed with filtered sea water into the cylinder using a wash bottle. The precision of cylinders depends on their size. It is stated by the manufacturer, for example 50 or 100 ml cylinder ± 0.5 ml, 250 ml $\pm$ 1 ml and 500 ml $\pm$ 2.5 ml. The precision of conical sedimentation chambers, which may also be used, changes similarly with the amount of sediment and graduation: 0–2 ml $\pm$ 0.1 ml, 2–10 ml $\pm$ 0.5 ml, 10–40 ml $\pm$ 1 ml, 40–100 ml $\pm$ 2 ml, 100–1000 ml $\pm$ 10 ml.

Particles settle gently by gravity. Vibrations should be avoided. Settling time must always be the same, commonly 24 h (Lohmann 1908, p. 194). The readings of settled

volume are satisfactory if shapes of plankton species do not differ drastically, for instance in pure copepod samples. Long appendages, gelatinous organisms and specimens with significant buoyancy make the method less precise (Hensen 1887; Beers 1976a). Larger jelly fish should be rinsed, removed and handled separately in any case.

For a rough overview it is sufficient to estimate the amount of settled plankton in storage containers, for example at the end of the cruise. This quick-look method is easy to realize by comparing the settled content in sample bottles with an empty bottle, which has been calibrated.

The influence of the different shapes of organisms on settling volume may become evident, unlike displacement volume where this effect is minimized. The ratio between both methods varied by a factor of 4 to 51 in the Baltic Sea (Lohmann 1908), with an annual average of 17 ($\pm$18). Such a range was frequently reported from different marine and fresh water regions (Balvay 1987; Beers 1976a). The measurements themselves are reproducible (Lillelund and Kinzer 1966). The effect of different forms can be reduced both by splitting samples in quantities smaller than 20 ml and summating the results of sub-samples afterwards (ibid.). A precondition is that the diameter of the graduated cylinders is not smaller than the length of the largest organisms (Sheard 1947).

Most authors use the 24 h settling time mentioned above; some wait for 48 h. Beers (1976a) quoted Hardy and Gunther (1935), who found a decrease in settling volume of 35%, mostly by 80% to 95% after 1 year compared to 24 h.

These findings are more an effect of the volume loss of preserved samples, described by Ahlstrom and Thrailkill (1962, 1963), than of settling duration. The shrinkage of organisms starts within the first 10 to 15 min after fixation with formaldehyde. The most remarkable change in biovolume occurs within the first day. The process slows down significantly 3 to 7 days later. Stability is reached between 1 month and up to 1 or 2 years after preservation. Samples of copepods are much less affected than gelatinous plankton (Omori 1978). Ahlstrom and Thrailkill (1963) recommended starting volume (biomass) determination no earlier than 3 days after preservation.

DISPLACEMENT VOLUME

The disadvantages of the settling volume method can be reduced by estimation of displacement volume to a certain degree. It is less time consuming and the influence of body shape and appendices on volume are reduced. However, fragile organisms may be damaged when interstitial fluid is removed, especially by the preferred use of vacuum instead of gravity.

Displacement volume can be determined in two ways. The plankton, from which interstitial water has been removed, is added to a known volume of sample fluid in a graduated cylinder. The increase in volume indicates the plankton volume. More convenient is the determination of the difference between the known sample volume and the amount of fluid which is separated by filtration.

There is a wide variety of equipment for both procedures, discussed by Frolander (1957) and Beers (1976a). Taking all critical aspects into account the following treatment is recommended. The plankton sample should be concentrated using a sieve with a smaller mesh size, preferably half the size of that of the sampling net. Its volume is read in a burette with 0.1 ml measuring interval and a precision of $\pm$0.05 ml. Next the excess and interstitial water is removed using a filter apparatus connected to a suction bottle (e.g. 250 ml) which collects the filtrate. If organisms remain in the burette, they can be rinsed down by a known volume of sea water, using a pipette. The sample is drained gently by vacuum. Omori and Ikeda (1984, p. 88) recommend a vacuum of 250 mmHg

(33 kPa = 0.33 atm). Finally the filtrate is measured in the burette that was used for the total sample at the beginning. The use of a burette has the advantage of precise volume measurements. On the other hand, the measurable sampling volume is restricted to 50 ml only and the outlet of < 10 mm is too narrow for the larger zooplankton. A graduated cylinder of known precision (see page 88) is recommended in that case.

Sucking plankton out of water takes about 3 to 5 min for displacement volumes less than 10 ml. The amount of interstitial fluid removed by vacuum is about 1.5 times greater than by gravity draining; the duration is about six times shorter (Frolander 1957). The use of a water-jet pump (Lillelund and Kinzer 1966) needs approximately the same time as gravity draining.

Methodological errors were in the range of 7% to 9% whichever draining method was used, whether the vacuum pump (Frohlander 1957), the water-jet pump (Lillelund and Kinzer 1966), alcohol (Tranter 1959) or air pressure (Yentsch and Herbard 1956). The error increased if the displacement volume was in the range of the reading interval of the burette or smaller (Tranter 1959). The difference in displacement volume determinations by two observers, the individual error, was 1% (ibid.). The varying water content of different species influenced the results most significantly. The coefficients of variation were about 35% when seasonal ratios between displacement volume and dry mass were compared.

One millilitre displacement volume corresponded to 128 mg dry mass for copepods, 160 mg for euphausiids, 74 mg for mixed plankton and 24 to 49 mg for salps (Tranter 1959). These variations due to the species composition are one order of magnitude smaller than those of settling volume. The latter amounted to about 900% (Bigelow and Sears 1939; quoted by Beers 1976a).

4.3.2 Gravimetric methods

With regard to the large bias of settling volume technique Hensen (1887) tried first to weigh samples after squeezing (wet mass) and again after drying (dry mass). However, Lohmann (1908) considered the methods unsuitable to estimate the pure plankton biomass because of the remaining sea water in the first case and the adhering salt in the latter. Moreover dry mass may include inorganic body substances and re-suspended sediment due to tidal currents etc.

WET MASS, FRESH MASS AND LIVE MASS

The live mass of organisms may differ from fresh mass because the loss of organic material causes dying organisms to shrink. Fresh mass is exclusively from samples which are not preserved, for example by formaldehyde or by deep-freezing. The wet mass of samples preserved by formaldehyde suffers an increasing loss of biomass, depending on the time of preservation (see page 88). Live and fresh mass are seldom measured in marine planktology. Since balances cannot be used aboard ship, these values could be determined only in the laboratories of shore stations.

Wet mass is closely related to displacement volume (Frolander 1957). Frolander quoted Riley *et al.* (1949), who weighed wet mass 20 times before and after rolling the sample on filter paper. The difference of both conditions was comparable with that of displacement volume determined by gravity and by vacuum respectively. The methods differ depending on how the remaining plankton was measured, whether volumetrically or by weighing. The elimination of excess and interstitial water in a sample should be generally done by vacuum filtration as described on page 89.

The net wet mass comprises the organic and inorganic content of organisms, including the body fluids when the previously determined mass of a filter is subtracted. If paper or glass fiber filters are used, the determination of their mass might be simplified by using an average obtained by weighing 10 filters. This procedure is acceptable due to the relatively low precision of the entire method. However, it is important to moisten the filters before weighing. Gauze filters with a mesh size smaller than those of the sampling gear could also be applied. They can be cleaned and used again for the next set of samples. Their rims should be fixed by heating to prevent the loss of fibers, which would alter their tared mass. The best way is to use a soldering iron and a template of heat resistant material, for example Teflon, for cutting out the gauze filters and fixing their rims.

A large sample should be either processed stepwise using several filters to minimize clogging or by processing a sub-sample only (splitting techniques, see section 4.2). This is also recommended for the following gravimetric procedures.

The variability of the results of mixed samples originates mainly from the significantly varying quantity of body fluid of different taxonomic groups which amounts to 69% of pteropods, 84% of copepods and 87% of chaetognaths and decapods according to recalculations following Omori (1969). Gelatinous zooplankton, like cnidarians, ctenophores and tunicates, contains up to 99% water (see Chapter 1).

DRY MASS

The removal of body fluid leads to dry mass, a method widely used since the sixties. The method starts with the *preparation of glass fiber*, GF/C filters, before a cruise. The filters should be washed with distilled water to remove any particles and incinerated at 500 °C to burn off any possibly organic substances. Temperatures above this recommendation may alter the filtering characteristics. This careful procedure is essential when expecting very small amounts of drying material. The filters must be kept dry (eventually dried again) before pre-weighing. This is done in a vacuum desiccator using calcium chloride ($CaCl_2$) or silica gel as the drying agent.

The filters are weighed on a micro-balance with a reading accuracy of 0.1 mg, after adaptation to the weighing-room temperature. Since the glass-fiber filters cannot be numbered, every tared filter should be stored in a labeled plastic bag or other suitable container.

Vacuum filtration of samples is performed on board ship as described on page 89, avoiding the rinsing problem (see section 4.2). Only sub-samples should be used (see section 4.2) or several filters in case of large amounts of plankton.

The *samples* on the filters *are stored* at −18 °C in an aluminum dish, each covered by its numbered plastic bag and transferred to a land-based laboratory for dry mass determination. Samples which were dried on the ship are transported also in their bags in airtight desiccators at −18 °C (see section 4.2.4) and dried again up to mass constancy before weighing.

The storage of sample plus filter in an aluminum crucible has two advantages. It prevents losses, (a) during the numerous manipulations (putting sample plus filter into the plastic bag; transfer in drying crucibles; transfer into a weighing crucible, etc.) and (b) by possible leakage of organic matter after dying and shrinking of organisms before freezing and finally after thawing the samples in the land-based laboratory. All solid substances remain in the aluminum crucible. It would be a mistake to decant the melted fluids, losses up to 50% would be incurred (Williams and Robins 1982; Postel 1990).

Drying at 60 °C in a drying oven for about 24 h is recommended (Lovegrove 1962, 1966). A higher temperature should be avoided preventing the loss of any volatile organic material, for example up to 8% of the lipids at 105 °C (ibid.).

Organisms with hydrated skeletons contain bound water which is only removed at temperatures above 140 °C and is still present in samples dried at 60 °C. The problem is known for some phytoplankton groups, larger crustaceans such as brachyura, and gelatinous plankton (Madin *et al.* 1981; Larson 1986; cited according to Clarke *et al.* 1992). Båmstedt (1974) successfully used 70 °C according to Raymont *et al.* (1969, 1971a) to dry mysids, decapods, and copepods for biochemical analyses.

The results in terms of mass and chemical composition are comparable to the results obtained using the evaporation procedure in a vacuum desiccator, which would take several weeks. The drying time depends on the amount of matter and the size of organisms, ranging from a few hours to a day. Mass constancy was reached after 18–22 h in a test with samples of 1.6 g to 2.7 g dry mass (Postel 1990).

Freeze-drying (lyophilization) is more gentle than oven drying. It results in a comparatively higher dry mass of 2% (organic mass, carbon, and nitrogen respectively) (Omori 1978). Fudge (1968) also found 3% more lipids, and 1% more protein, chitin and ash in freeze-dried material.

Samples for the lyophilization are initially deep-frozen. Water is removed through sublimation under vacuum, which has to be chosen according to the sublimation pressure curve (see user manual). The vacuum controls the drying procedure. To start the process, heating energy is applied to the frozen material by means of temperature-controlled shelves. Their capacity should not exceed 60 °C (see above). The water vapor is trapped at the very cold condenser. Approximately 99.5% of the water is removed during the main drying process. The end point is reached when the temperature difference between the product and the shelf is smaller than 5 °C. (The water of crystallization could be sublimated at a very low vacuum, within a final drying phase.)

Freeze-drying in a single chamber system rules out drops of pressure and guarantees the quality of the product during the entire drying process, unlike a double chamber system.

The dried samples on filters in aluminum crucibles are adapted to temperature conditions of the weighing room in a vacuum desiccator with a drying agent for 1 h and weighed with the same precision as used for the empty filters.

Dried plankton is often hygroscopic, consequently mass may increase during the *weighing procedure* due to uptake of humidity. Lovegrove (1966) observed an increase of 2% of dry mass during 5 min. This increase could be reduced to 0.05% in a weighing room with low humidity, a weighing duration of 30 s and an average dry mass of $\leqslant$300 mg (Postel 1990). Båmstedt (1974) exposed dried material for 20 h at 17 °C uncovered and recorded an increase in mass of about 3%. Therefore, balances with lockable weighing chambers are recommended to reduce the possible influence of drafts and varying humidity. A small container of desiccant could be placed in each chamber, and more desiccant placed inside the housing of the balance to improve the stability of readings (Downing, cited in McCauly 1984).

The first stable result of the micro-balance should be accepted.

The aluminum crucibles must be re-weighed after washing with a detergent, rinsing with distilled water, drying and cooling in a desiccator. If the determination of ash content follows, the crucibles are re-weighed as the last step of the entire procedure. (Do not forget to mark the crucibles with the filter number.)

The concentration of dry mass is finally calculated according to

$$\text{Dry mass (mg m}^{-3}\text{)} = \text{dried sample with filter in aluminum dish (mg)} - \text{filter (mg)} - \text{aluminum dish (mg)} / \text{amount of filtered water (m}^{-3}\text{)} \quad (4.1)$$

Possibly sub-sampling steps have to be taken into account.

The *variability of results* depends in the case of mixed samples on the plankton composition, the amount of salt in the interstitial water and on the precision of the method. The largest contribution to variability comes from the inter-specific differences in ash content (Table 4.2). The concentration of salt in interstitial water ranges between 2% and 7% (Hopkins 1982). The precision of the method is about 6% (Postel 1990). This results from 10 pairs of samples, collected with a combination of two WP-2 UNESCO standard nets. To minimize the influence of patchiness, the average of the amounts of dry mass differences between the parallel net samples was related to the

Table 4.2 Ash content of different taxonomic groups and size classes of zooplankton (N = number of samples).

Plankton group	Ash (% of dry mass)	Region	Author
Small mesoplankton	27.0 ± 19.5	Kiel Bight (Baltic Sea), seasonal variability (N = 11)	Brandt (1898)
		North Pacific	Omori (1969)
Pteropods	28.5–46.6	(N = 4)	
Amphipods	10.0–37.7	(N = 3)	
Decapods	11.9	(N = 1)	
Mysids	10.2	(N = 1)	
Euphausiids	8.0–8.5	(N = 3)	
Copepods	1.9–6.4	(N = 18)	
Chaetognaths	4.2–4.8	(N = 2)	
		Continental shelf south of New York	Curl (1962)
Tunicates	77.1–69.0	(N = 2)	
Ctenophores	75.0–70.9	(N = 2)	
Cnidarians	69.0–49.1	(N = 3)	
Pteropods	64.2–24.3	(N = 3)	
Calanoid copepods	22.8–17.6	(N = 2)	
Euphausiids	22.4–18.6	(N = 2)	
Chaetognaths	21.6	(N = 1)	
Size classes:		Off Angola/Namibia (April, 1997)	Postel (unpublished)
55–100 μm	35.5 ± 21.1	(N = 38)	
100–200 μm	28.2 ± 18.8	(N = 38)	
200–500 μm	22.8 ± 21.6	(N = 43)	
500–1000 μm	12.4 ± 8.5	(N = 43)	
> 1000 μm	28.7 ± 15.6	(N = 43)	

average of the 20 observations. The precision concerns all steps of the procedure such as sample treatment, storage, drying, and weighing.

ASH-FREE DRY MASS

Ash-free dry mass is also called dry organic mass (Omori and Ikeda 1984). Ash, i.e. all inorganic substance, varies depending on the species composition (see Table 4.2). Sampling in shallow areas involves the risk of collecting a significant amount of mineral particles due to wind or tidal mixing during events.

The determination of *ash* follows the dry mass analysis (cf. Figure 4.3). The already dried and weighed samples on glass-fiber filters in their aluminum dishes are used. They are combusted in a muffle furnace at 500 °C for at least 12 h (Salonen *et al*. 1976) because the authors found traces of organic material after 3 h combustion.

An exact temperature is also important for the optimal oxidation process. A lower temperature does not guarantee complete combustion while some inorganic salts might become volatile at a higher temperature (Paine 1964). This is important to consider if using older muffle furnaces where temperature variations up to 70 °C are possible.

Båmstedt (1974) analyzed the effect of different starting conditions for the incineration of copepod plankton. Starting at room temperature and reaching 500 °C within half an hour was the method which produced the smallest confidence intervals between parallel samples and was most convenient, when compared to either starting at room temperature and increasing the temperature by 50 °C every half an hour until 500 °C was reached, or starting at the maximum temperature. In the latter case there is the risk that inorganic components form an inclusion of organic matter which is difficult to remove (Brandt 1898).

Finally, aluminum dishes with the remaining ash are cooled to room temperature in a desiccator and re-weighed. The calculation of ash-free dry mass concentration is:

$$\begin{aligned}\textbf{Ash-free dry mass (mg m}^{-3}\textbf{)} = {} & \textbf{dried sample with filter in} \\ & \textbf{aluminum dish (mg)} - \textbf{ash on} \\ & \textbf{filter in aluminum dish (mg)/} \\ & \textbf{amount of filtered water (m}^{-3}\textbf{)}\end{aligned} \qquad (4.2)$$

Possibly sub-sampling steps have to be taken into account also here.

The determination of ash content should be made as described. Data obtained in parallel during CHN (carbon, hydrogen, nitrogen) analysis or energy determinations by microbomb calorimeter are too low, because calcareous substances decompose during high-temperature combustion (>900 °C).

4.3.3 Biochemical methods

Other methods to quantify the organic mass of zooplankton use the organic carbon and energy content. Total protein represents only one of the three main organic components, the others being lipids and carbohydrates. It is preferably used as a scaling factor for enzymatic activity measurements (see Chapter 10).

Adenosine triphosphate (ATP) is involved in energy-requiring reactions of living cells and therefore a reliable index of living organic material. It is more-or-less related to organic carbon (Holm-Hansen 1973; Traganza and Graham 1977).

Nucleic acid (DNA) content as a measure of biomass is of doubtful value since it also occurs in detritus.

Studies on the flux of matter and energy require information on the amount of all major compounds and elements and the nutritive value of zooplankton. The latter depends on the quality of the organic matter, which is determined by the proportion of proteins, carbohydrates, and lipids.

The stoichiometry of carbon, nitrogen and phosphorus in zooplankton has become more and more important in the context of the nutrient flux between autotrophs and heterotrophs in both the marine and the freshwater environment (for example Corner and Davis 1971; Elser and George 1993; Elser and Hasset 1994; Anderson and Hessen 1995).

The organic components, the elemental composition, and the energy content are correctly determined by *direct analyses*, which involve mostly an oxidation. However, there are also some *coarse calculations* commonly used (see Figure 4.11).

1) The energy content may be derived from the proximate composition of main organic components. The energy equivalents of lipids (39.4 kJ g^{-1}), proteins (23.7 kJ g^{-1}) and carbohydrates (17.2 kJ g^{-1}) allow its rough calculation, if the proportion of the major organic components are known (Brody 1945). The equivalents assume total oxidation. Considering the incomplete oxidation by organisms, the factors are somewhat lower (see Figure 4.11).
2) An approximate energy content can be calculated from organic carbon according to Salonen *et al.* (1976) by converting 1 g C into 46 kJ or using a related equation (see Figure 4.11).
3) The energy content and the approximate biochemical composition were calculated by Gnaiger and Bitterlich (1984) using a stoichiometric approach. They studied the organs of silver carp to determine the composition of carbon, hydrogen, nitrogen and phosphorus, and the ash content. Vollenweider (1985) examined a similar attempt by Spoehr and Milner (1949) to apply the technique in both phyto- and zooplankton research, and found some discrepancies. These arose mainly from varying environmental conditions, which influenced the availability of elements and consequently the inter- and intra-specific biochemical composition of organisms (Hessen and Lyche 1991; Elser and Hasset 1994; and Table 4.3). Omori and Ikeda (1984) were reserved in recommending the technique for the same reasons. Also, elements occur in sources other than the three main organic components, for example nitrogen originates also from non-protein sources such as chitin and various kinds of amino acids and peptides. This drawback is also relevant to the following procedure.
4) Protein content may be calculated from the organic nitrogen content, using the 'Kjeldahl' factor of 6.25 (= 100/16), traditionally used to convert organic nitrogen to total protein (Brandt 1898; Brody 1945; Winberg 1971), based on the empirical protein formula $(C_{24}H_{38}N_6O_8)_n$ of Playfair (Wolff 1876). The mass of nitrogen (84 g) is about 16% of the total molecular mass (538 g). The use of this factor assumes first, that there is no variability within protein composition and second, that nitrogen is exclusively a component of protein. Factors found in the literature range from 5.8 (including zooplankton, algae and bacteria) by Gnaiger and Bitterlich (1984) to 7.7 (for *Neomysis integer*) by Raymont *et al.* (1966). Equivalent values for animals vary between 5.0 and 8.3 (Frutont and Simmonds 1958, cited by Raymont *et al.* 1966). These factors are frequently used in commercially available 'protein analyzers'. The difference between calculated and directly determined

protein was used by Gnaiger and Bitterlich (1984) to estimate the non-protein nitrogen.

5) Holm-Hansen (1973) converted ATP to cellular organic carbon of living organisms using a factor of 250, based on over 30 species ranging from bacteria to macrozooplankton. Omori and Ikeda (1984) cited the papers of Balch (1972) and Traganza and Graham (1977), which included the controversial statements regarding the stability of the C:ATP ratios. Finally, Karl (1993) recommended ratios from 50 to 150 as suitable for 'micrometazoans' and those of 200 to 350 for unicellular microorganisms (see page 131).
6) The difference approach is commonly practiced; this determines two of the major organic components by biochemical analysis, for example total proteins and lipids (of ash-free dry mass) and calculates the missing one (e.g. carbohydrates) by completion to the total ash-free dry mass (Orr 1934; Nakai 1955; Matondkar *et al.* 1995). Brandt (1898) directly measured the elements C, H and N, and one of the main organic compounds, the fat content, and calculated the protein and carbohydrate content of dried plankton. The approach failed. Apart from analytical problems (too low lipid content and too high ash content), the inter- and intraspecific variations of the stoichiometric properties were neglected, always using constant average formulae of organic compounds (see Table 4.4).

Figure 4.4 reviews the relationships between biochemical determinations of biomass in terms of carbon, proteins, energy and ATP (marked by white ellipses and circle), and of elements (N, P, H) and organic compounds (lipids and carbohydrates/chitin) related to them.

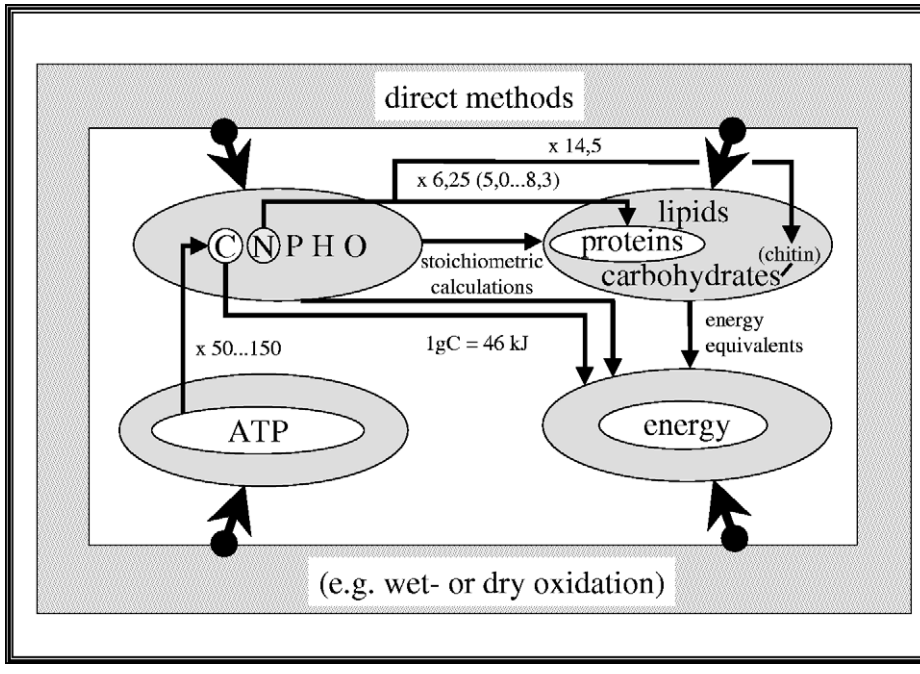


Fig. 4.4 Links between different biochemical approaches to determine zooplankton biomass.

SAMPLE PREPARATION

Fresh, fresh frozen and fresh dried samples are used. Giese (1967) suggested keeping fresh material frozen until treatment on shore, for example on dry ice (−78.5 °C) to stop enzymatic action. Such samples can be stored for several days; for longer storage, material should be dried (see page 91). Lovegrove (1966) also recommended freezing at −78 °C. Biochemical properties are kept constant, using fast fixation and storage in liquid nitrogen (−196 °C). However, temperatures of −20 °C and −30 °C are frequently reported. Raymont *et al.* (1971a) found no remarkable and systematic difference between the biochemical composition of euphausiids, whether they were treated fresh or after storage at −20 °C. Le Borgne (1982) noticed no influence of storage duration on CHN content when samples were kept at −30 °C, whether for 2 months or for 6 months. The samples should be kept in airtight containers to avoid any dehydration.

Freeze-drying (see page 91) is gentle and the most useful method for biochemical purposes. Dried material must be kept in a desiccator, otherwise it absorbs moisture.

Formalin-preserved samples are unsuitable for biochemical studies. Rinsing the samples with isotonic ammonium formate (like Omori 1978) should be avoided in all cases.

Sub-samples (replicates) require well-homogenized material to standardize their composition. Extraction could be incomplete using larger particles. Larger particles of non-macromolecule material bias the results even in small samples.

Freeze-dried material meets the best conditions for clean homogenization. An agate mortar and pestle are useful for grinding, for larger quantities an agate mill is recommended.

Frozen material is thawed at room temperature and kept in a water–ice bath at 4 °C. Samples are ground using a Potter–Elvehjem homogenizer with Teflon pestles. Samples are cooled by ice during the procedure.

ELEMENTAL ANALYSIS

Elemental analysis was primarily used to construct gross formulae of organic compounds. Protein, lipids, and carbohydrates consist mostly of carbon, nitrogen, hydrogen, oxygen and traces of phosphorus and sulfur (Table 4.3).

While all organic compounds include carbon in similar proportions, the occurrence of nitrogen is mainly restricted to protein, and that of phosphorus to lipids (Table 4.3). Carbon is therefore a more universal stock parameter. The other elements play no role as biomass proxies. Zooplankton dry mass contains about 40% organic carbon, the content of organic nitrogen and hydrogen is in the range of 10%, while that of organic phosphorus is mostly less than 1% (Table 4.4). However, phosphorus is of central importance in energy metabolism and in the synthesis of proteins, storage components, and skeleton material. It is an elemental part of nucleic acids, enzymes, and compounds of different energy level (adenosine tri-, di- and monophosphate). The relative amounts of the latter components characterize the metabolic activity of organisms (Atkinson 1971). Schneider (1990) pointed out the lack of knowledge of phosphorus levels in marine zooplankton, relative to the comprehensive studies of carbon and nitrogen.

Sulfur plays a role in increasing buoyancy of plankton by reducing the relative amount of the denser sulfate ions.

Larson (1986) distinguishes marine zooplankton according to the elemental content into three groups: the gelatinous plankton (cnidarians, ctenophores, salps) with very low and variable nitrogen and carbon percentages; the non-gelatinous group (crustaceans,

Table 4.3 Average elemental composition of the main organic compounds and an example of ratios of elements (according to Rogers 1927, assembled by Omori and Ikeda 1984; Wolff 1876 (without a sulfur content of protein of 0.5–1.5%, noticed by Brandt 1898); Gnaiger 1983).

Rogers (1927)	**Protein**	**Lipid**	**Carbohydrate**
C	51.3	69.0	44.4
H	6.9	10.0	6.2
N	17.8	0.6	–
P	0.7	2.1	–
Ratio of elements			
C:N	2.9	113.2	–
H:N	0.4	16.4	–
N:P	25.4	0.3	–

Wolff (1876)	**Protein** $(C_{24}H_{38}N_6O_8)_n$	**Chitin** $C_6H_{15}NO_6$	**Lipid (oil acid)** $C_{18}H_{34}O_2$	**Carbohydrate (sugar)** $C_6H_{12}O_6$	**Carbohydrate (glycogen)** $(C_6H_{10}O_5)_n$
C	53.53	46.35	76.60	40.00	44.44
H	7.06	6.44	12.10	6.67	6.17
N	15.61	6.01	–	–	–
O	23.80	41.20	11.40	53.33	49.38

Gnaiger (1983)	**Protein** $(C_{4.83}H_{7.58}N_{1.35}O_{1.5}S_{0.025})_n$	**Lipid** $(C_{18.8}H_{33}O_2)_3$
C	53.06	77.63
H	6.94	11.36
N	17.30	–
O	21.97	11.01
S	0.73	–

larvaceans) with high elemental content of low variability; and the semi-gelatinous plankton (mollusks, chaetognaths) which falls between the two other groups.

Data on carbon, nitrogen and phosphorus content of various taxonomic groups of the marine zooplankton, published between 1939 and 1989, were pooled by Schneider (1989, 1990). Based on this compilation, the author calculated averages of atomic ratios, which are presented in Table 4.5. The magnitudes of N:P ratios of copepods determined and collected by Ikeda (1977), based on measurements of respiration and excretion of ammonia-nitrogen and inorganic phosphate-phosphorus, agree with those in Table 4.5. They vary between 10.8 and 19.0. Ikeda (1977) added O:N and O:P ratios of copepods, other crustaceans and chaetognaths, ranging from 1.61 to 15.6 and from 110 to 89 respectively. Those of mixed zooplankton increased up to about 200 in both cases.

Despite some uncertainties regarding the correlation of the elemental composition and the biochemical make-up of organisms, the ratio between elements gives some hints on their physiological condition. For example, the C:N ratio indicates shifts in the occurrence of the main organic components, especially proteins and non-proteins (Vollenweider 1985). Because carbohydrates are of no great importance in animal tissue, it characterizes especially the ratio between proteins and lipids. The existence of lipids is indicated by a C:N ratio of more than 2.9 (see Table 4.3). The variation within

Table 4.4 Variation of elemental composition in some taxonomic groups (% of dry mass), *N* = number of samples.

Plankton group					Region (number of samples)	Author
	C	**N**	**H**			
Small mesoplankton	14.8–42.8	1.8–5.6	2.9–6.4		Kiel Bight (Baltic Sea), seasonal variability (*N* = 11)	Brandt (1898)
Copepods	–	9.2	–		Kiel Bight	
Copepods	45.2	9.4	7.1		Fresh water	
Pteropods	17.0–29.0	1.5–6.0	1.1–3.8		North Pacific (*N* = 4)	Omori (1969)
Amphipods	25.9–48.4	6.1–8.2	4.4–7.5		(*N* = 3)	
Decapods	41.1	9.3	6.7		(*N* = 1)	
Mysids	42.4	11.0	6.7		(*N* = 1)	
Euphausiids	38.7–47.2	10.0–10.7	6.7–7.6		(*N* = 3)	
Copepods	39.0–66.6	5.1–13.1	6.7–10.3		(*N* = 18)	
Chaetognaths	43.5–47.7	10.7–11.1	7.2–7.6		(*N* = 2)	
	C (total)	**N (Kjeldahl)**		**P (total)**		
Tunicates (salps, Pyrosoma)	7.2–10.4	0.3–1.5		0.14–0.28	Continental shelf south of New York (*N* = 2)	Curl (1961)
Ctenophores	6.4	0.2–1.1		0.12–0.16	(*N* = 2)	
Cnidarians	8.2–22.5	0.4–1.4		0.02–0.16	(*N* = 3)	
Mollusks (pteropods)	26.3–28.3	2.2–5.0		0.26–0.58	(*N* = 3)	
Calanoids	32.5–41.7	4.7–7.1		0.39–0.84	(*N* = 2)	
Euphausiids	33.4–37.0	5.2–7.1		0.94–1.16	(*N* = 2)	
Chaetognaths	–	7.8		0.20–0.57	(*N* = 1)	
	C	**N**		**P**		
Tunicates	4.2–62.3	0.4–13.9		0.05–0.20	(*N* = 20, 18, 8)	Assembled by
Salps	4.2–11.6	0.4–2.8		–	(*N* = 17,15, –)	Schneider
Larvaceans	50.4–62.3	11.3–13.9		–	(*N* = 3, 3, –)	(1989, 1990)
Cnidarians, ctenophores	0.9–32.4	0.02–11.0		0.02–0.44	(*N* = 82, 75, 32)	
Copepods	32.2–67.5	5.1–13.1		0.53–1.96	(*N* = 197, 197, 36)	
Euphausiids, mysids	31.1–55.4	6.2–12.1		0.75–2.60	(*N* = 76, 76, 38)	
Decapods	23.4–59.6	3.8–11.7		–	(*N* = 27, 26, –)	
Other crustaceans	22.5–48.4	4.0–10.8		0.64–2.26	(*N* = 50, 50, 21)	
Mollusks	17.0–52.0	1.5–10.4		0.23–0.60	(*N* = 36, 31, 12)	
Chaetognaths	19.3–52.0	5.4–15.0		0.37–0.95	(*N* = 34, 35, 20)	

Table 4.5 Atomic C:N:P ratios of phytoplankton (Redfield *et al.* 1963) and of zooplankton groups (Schneider 1990). Values in the left column are related to 100 parts carbon, those in the right column according to phosphorus = 1.

	C:N:P	C:N:P
Phytoplankton	100:15.1:0.94	106:16:1
Non-gelatinous zooplankton		
Copepods	100:19.0:0.77	129:25:1
Euphausiids, mysids	100:21.1:1.19	84:18:1
Other crustaceans	100:17.6:1.40	71:13:1
Semi-gelatinous zooplankton		
Mollusks	100:16.1:0.51	195:31:1
Chaetognaths	100:22.9:0.73	137:31:1
Gelatinous zooplankton		
Cnidarians, ctenophores	100:22.1:0.60	166:37:1
Salps	100:15.8:0.67	149:24:1

the taxonomic groups of the North Pacific Ocean (Table 4.4) ranges between 3.5 and 13.2. C:N varied between 6.1 and 13.4 (Figure 4.5) for mixed mesoplankton in Kiel Bight (Baltic Sea), with high values in late fall and winter (October to February) and the lowest ratio in late spring (May) according to Brandt (1898).

The traditional technique of mineralization of organic compounds is the high-temperature combustion method, developed by Liebig and Dumas in the middle of the nineteenth century and improved by Pregl at the turn of the century. There are also wet chemical analyses, such as the Kjeldahl procedure to determine nitrogen. Organic phosphorus is only measurable after wet combustion and hydrolysis.

In contrast with ash-free dry mass determination (see page 93), which is based on weighing the inorganic remains after combustion, the determination of elements is based on quantifying the liberated product, such as carbon dioxide. The detection methods range from the gravimetric procedure (used in the past), gas volume determination, and titrimetric analysis, to physicochemical techniques with increasing sensitivity. Automatic and semi-automatic elemental analyzers using different detecting procedures are

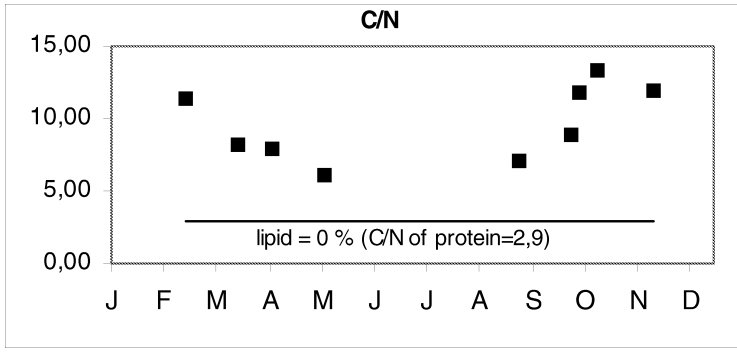


Fig. 4.5 Seasonal variability of the C:N ratio of mixed mesoplankton in Kiel Bight (Baltic Sea), according to Brandt (1898).

often applied. The highest resolution is obtained by the mass spectrometer and other advanced and expensive equipment.

ORGANIC CARBON (AND HYDROGEN)

Liebig (1840) combusted organic material by the addition of oxygen in the presence of cupric oxide. Emitted carbon dioxide and steam are absorbed by sodium carbonate and calcium chloride respectively, in U-glass tubes. Carbon and hydrogen are analyzed simultaneously. The mass differences of the tubes with absorbents are equivalent to the emitted elements. One mg CO_2 corresponds to 0.2729 mg carbon, 1 mg H_2O is oxidized to 0.1119 mg hydrogen, according to their atomic masses. This gravimetric method was used by Brandt (1898) and co-workers first in planktology. The results are roughly comparable with those of today (Table 4.4).

The *wet oxidation* method removes carbon dioxide by strong oxidizing compounds, for example with a mixture of potassium dichromate and concentrated sulfuric acid (e.g. Strickland and Parsons 1968) or potassium persulfate (Menzel and Vacarro 1964).

Carbon dioxide is quantified by the back titration of the unused oxidizer (Kay 1954, cited by Szekielda and Krey 1965) and by gas chromatography (Dal Pont and Newell 1963). Strickland and Parsons (1968), Parsons *et al.* (1984) and Wetzel and Likens (1991) recommended the same method, invented by Johnson (1949; cited in these papers) and adapted it for spectrophotometry. These procedures allow processing of samples without any sophisticated equipment. However, they are time consuming (less than one to two samples per hour), and they may also lead to an overestimation of 10% to 20% due to the oxidation of highly reduced substances like lipids. Curl (1962) reported the same finding concerning inorganic substances such as chloride. However, he warned 'Attempts to remove chloride by washing with distilled water result in appreciable losses of ... carbon-containing compounds'. On the other hand, Salonen (1979) quoted Sharp (1973), arguing that the wet oxidation is probably incomplete.

Compared with the uncertainties of the laborious wet oxidation, the high temperature combustion (dry oxidation) is faster and more precise (Parsons *et al.* 1984). Dried organic substances are combusted at 950 °C in the presence of catalysts. The carbon dioxide is not gravimetrically determined any more. It is measured by a gas burette (Curl 1962), infrared detection (Menzel and Vacarro 1964; Latja and Salonen 1978; Salonen 1979), thermal conductivity (Gordon 1969; Kerambrun and Szekielda 1969; Sharp 1974), electrical conductivity (Szekielda and Krey 1965) and gas chromatography as well (Omori and Ikeda 1984). The indirect detection methods use standard substances for calibration, for instance oxalic acid and glucose.

The *micro-method* of Salonen (1979) 'makes the full use of the potential rapidity and sensitivity of the infrared gas analyser'. It detects quantities preferably from 0.01 μg C to 2 mg and more. Consequently, this allows determination of the carbon content of the individual biomass of small organisms such as copepod nauplii or rotifers (Latja and Salonen 1978). For example, one copepod nauplius with a dry mass of about 0.2 μg, is one order of magnitude larger than the required material (carbon = dry mass × 0.6 according to Cushing *et al.* 1958). The dried organisms are introduced by forceps into the combustion tube. It is possible to make 60 to 180 measurements per hour, depending on oxygen flow rate. The precision is better than 1% at the 1 μg carbon level.

A number of special conditions (oxidation temperature, moisture level and electrostatic charge of samples, the flow rate of oxygen, salt content of samples, the use of different substances for calibration and preservation techniques, the ratio between the

organic and inorganic fraction of total carbon in aquatic invertebrates, sample size) have been studied by Salonen (1979), and Salonen and Sarvala (1978, 1980). Some of *the problems* are briefly reviewed here.

High temperatures of 900 °C to 1000 °C are used for complete oxidation. This temperature range poses a problem for discriminating between organic and inorganic carbon. Inorganic carbon, like bicarbonates and magnesium carbonate, starts decomposing at 510 °C (Paine 1964), and not all organic carbon is oxidized at 750 °C. The oxidation of both organic and inorganic carbon takes place at 950 °C (Salonen and Sarvala 1978; Salonen 1979). Fortunately, there are few specimens with significant amounts of inorganic carbon (carbonate) in the mesozooplankton. There are some shell-containing mollusks (pteropods), in which about a tenth of total carbon is inorganic carbon (Curl 1961). Curl (1962) determined the inorganic carbon in these cases, measuring the evolved CO_2 by acidification of the sample with 6N hydrochloric acid and heating to 100 °C, as well as determining total carbon. Others measured it by analyzing carbon during the high temperature ignition of ash (Hirota and Szyper 1975; Salonen *et al*. 1976; Salonen and Sarvala 1978; Gnaiger and Bitterlich 1984). Baudouin and Ravera (1972) measured the calcium content of the sample, assuming all inorganic carbon is present as calcium carbonate. In all cases, organic carbon was determined by the difference between the inorganic part and the total carbon content.

A high flow rate of oxygen, at least up to 1300 ml min^{-1}, and the temperature of 950 °C guarantee a complete oxidation of organic matter.

Dried samples tend to be influenced by their electrostatic charge, which causes a loss of weighed material. The samples might be moistened by a small amount of distilled water. The moisture level of samples does not influence carbon oxidation.

Salonen and Sarvala (1980) tested the influence of preservation techniques on carbon content determination, concluding that storage and preservation should be avoided. However, the carbon content of organisms kept alive until analysis was reduced due to starvation. Despite this phenomenon, this recommendation is not practicable in marine research. Heat- or freeze-dried material is acceptable if leaked substances are included in the carbon determination (see the discussion on losses of organic material by rinsing of fresh material, section 4.2) and by thawing of frozen samples (see page 97). No losses were observed using frozen samples which were treated with formaldehyde. Salonen and Sarvala (1980, 1985) concluded that losses by leakage were probably substituted by formaldehyde carbon. However, this qualitative result is not suitable in practice even if it has already been used (Kankaala and Johansson 1986).

Dry oxidation of carbon is mostly performed by *elemental analyzers*, which may optionally determine other elements, such as nitrogen, hydrogen, sulfur, and oxygen, either simultaneously or successively. The sensitivity of analyzers has improved recently and allows traces of carbon to be measured. This will hopefully increase the amount and the quality of biomass factors for zooplankton individuals. The unsatisfactory situation in this respect, described by Latja and Salonen (1978), has not been adequately rectified up to now. The lack of reliable data is still a 'bottleneck' in plankton research (see section 4.4).

The analyzers work in the following way: rinsing of the system and sample injection, combustion, reduction (nitrogen analysis), homogenization of product gases, the separation of gases, detection and calculation of results, taking calibration and sample weight into account.

The dried samples are weighed in tin, aluminum, silver, or platinum capsules (the two latter metals are used in the case of hydrochloric acid treatment to remove inorganic

carbon before the analysis). They are injected into the combustion chamber by an autosampler. There is generally an automatic weight entry.

The combustion is the most critical step of the whole process and ultimately affects the accuracy and precision of the final result. It is performed in the combustion chamber in helium as carrier gas with oxygen pulses. The heater temperature is adjustable. Briefly, there are temperatures of about 1800 °C by the combustion of capsules, if tin caps are used which have a low melting point of 232 °C. For capsules of higher melting points combustion is more difficult. Platinum (m.p. 1773 °C) is able to withstand combustion totally. The direct oxygen injection guarantees a quantitative combustion. Products of the oxidation are separated in the helium carrier gas flow, using a multiple elemental analyzer (e.g. after reduction of nitrogen oxides to elemental nitrogen at 650 °C). Helium carrier gas is commonly used and allows more precise measurements than argon.

Carbon is measured by infrared detection, by thermal conductivity or gas chromatography. When multiple analyzers are used, hydrogen and nitrogen are also measured by thermal conductivity, hydrogen sometimes by an infrared detector. Gas chromatography techniques depend on pressure and flow rate changes. The solid state pressure transducer guarantees constant pressure, unaffected by flow rate, barometric or altitude changes. During multiple analysis elements are detected successively, when one element is measured, the others are bound in adsorption traps. After detection the following element is thermally desorped and quantified.

The simultaneous analysis of several elements lasts about 3 to 9 min, depending on the equipment.

The sample size and detection ranges differ. There are 'wide band' analyzers with sample sizes of 0.02–800 mg dry mass and detection ranges of 4 μg to 30 mg carbon. Those with higher sensitivity require a sample size of 1 μg to 200 mg (nominal 2 mg). The manufacturer gives a theoretical detection range of 0.02 μg to 2.5 μg carbon. The lower limit is 5 to 6 times larger in practice. The sensitivity for hydrogen and nitrogen is generally lesser by one order of magnitude. Their detection ranges are narrower.

The precision of the micro-method mentioned is 0.3% of relative standard deviation at the level of 2 mg test substance.

The cost of such equipment is approximately 50 to 70 thousand US dollars. Up to $1 per sample is needed for consumables, such as gases, capsules and reagents used as catalysts and for calibration.

ORGANIC NITROGEN

Nitrogen is, like carbon, measurable by both dry and wet oxidation methods. Automated versions are available for both procedures.

The traditional *method of Dumas* determines nitrogen by oxidizing dried organic substances at 900 °C using oxygen. The combustion gases formed are carried by carbon dioxide across the copper oxide (CuO) catalyst to complete the oxidation. Formed nitrogen oxides are reduced by copper. Elemental nitrogen is trapped in a graduated tube over potassium alkaline solution (nitrometer). The volume, multiplied by the atmospheric pressure and divided by the gas constant and the absolute temperature is the amount of nitrogen mass.

Thermal conductivity is the usual detection method of nitrogen analyzers. Their calibrations are stable over longer periods. Daily variations, depending on the laboratory temperature and the air pressure, are normally compensated for automatically.

Strickland and Parsons (1968) recommended the Dumas method. It is precise and environmentally friendly. In contrast to most variants of Kjeldahl wet digestion, it does

not need any toxic catalysts. There is a close and highly significant correlation of the results of both procedures, which guarantees the comparability of data.

The *wet oxidation method of Kjeldahl* consists of three steps: mineralization, distillation and detection (Bradstreet 1965; Giese 1967; Kleber *et al*. 1987). First, the total organic nitrogen compounds must be transferred into a form ready for detection, for example by titration. It starts with heating the dried material with concentrated sulfuric acid (free of nitrogen), using boiling chips or lens beads and adding suitable catalysts, i.e. a tip of a selenium reaction mixture (SeO_2; KSO_4; $CuSO_4$). Heating the flasks might be performed on a Kjeldahl rack. The products are carbon dioxide, water and ammonia. The latter reacts with sulfuric acid, forming ammonium sulfate $(NH_4)_2SO_4$. The mineralization is complete when the solution becomes clear and SO_2 stops vaporizing. After 1 h of additional boiling the samples are cooled down and quantitatively transferred into a graduated flask, adding some distilled water and two drops of the phenolphthalein indicator. After pouring this mixture in a chamber of Parnas and Wagner still, the addition of 'Kjeldahl' alkaline solution (33% NaOH) liberates ammonia, which is transferred by steam and trapped in the receiver. It includes 2% boric acid and two drops of Tashiro indicator (0.2% methyl red, 0.1% methyl blue, both in ethanol, 2:1). The formed ammonium borate alters the pH >5 and the color from red to green in about 10 min. The distilled amount of the alkali solution is acidimetrically measured, using 0.01N H_2SO_4. The endpoint is determined by the change, back to its original color. One ml of 0.01N H_2SO_4 is proportional to 0.14 mg nitrogen. A calibration is needed, distilling 1, 2, 4, 8, and 12 ml NH_4Cl (1 ml = 0.1 mg N) with 5 ml of 33% NaOH. Then the straight line is the ratio between the introduced amount of nitrogen (mg) and the used sulfuric acid (ml). Finally, the averages of the results are corrected by a mean blank and related to dry mass. Ammonia obtained by Kjeldahl distillation may also be determined colorimetrically, using the Nessler's reagent (Strickland and Parsons 1968; Golterman and Clymo 1969; Salonen *et al*. 1976) or by complexing with ninhydrin and hydrindantin (Strickland and Parsons 1968). According to Holm-Hansen (1968) the latter method is quicker, simpler and more sensitive than the first. Giese (1967) and Golterman and Clymo (1969) mentioned some other methods of wet digestion and detection.

About 22% of nitrogen might originate from non-protein (NPN) sources in marine zooplankton, according to measurements by Raymont *et al*. (1971a) in *Meganyctiphanes norvegica*. Mayzaud and Martin (1975) compiled their own data and additional data from the literature and noted 16% of NPN for phytoplankton, 19% for *Calanus finmarchicus* (stage V), 14% for adults, 12% for *Meganyctiphanes norvegica*, 20% for *Neomysis integer*, and 37% and 42% for two chaetognath species, *Sagitta hispida* and *S. elegans*, respectively.

The above mentioned *calculation of protein* from organic nitrogen by the conversion factor of 6.25 or similar, might be improved by determining the protein specific part of nitrogen only, separated from the soluble, non-protein fraction shown in Figure 4.6.

The Kjeldahl procedure is the convenient method to analyze the different nitrogen fractions, starting with the total sample. Then the non-protein organic nitrogen (NPN) is measured after dispersing the dried material in a test tube by trichloric acid (TCA), heated for 10 min in a boiling water bath and cooled down, then stored in a refrigerator at 4 °C overnight. Afterwards it is homogenized and centrifuged. The protein nitrogen might be determined by calculating the difference between total and NPN or directly. In the latter case, the sediments are washed with distilled water and quantitatively transferred to Kjeldahl flasks.

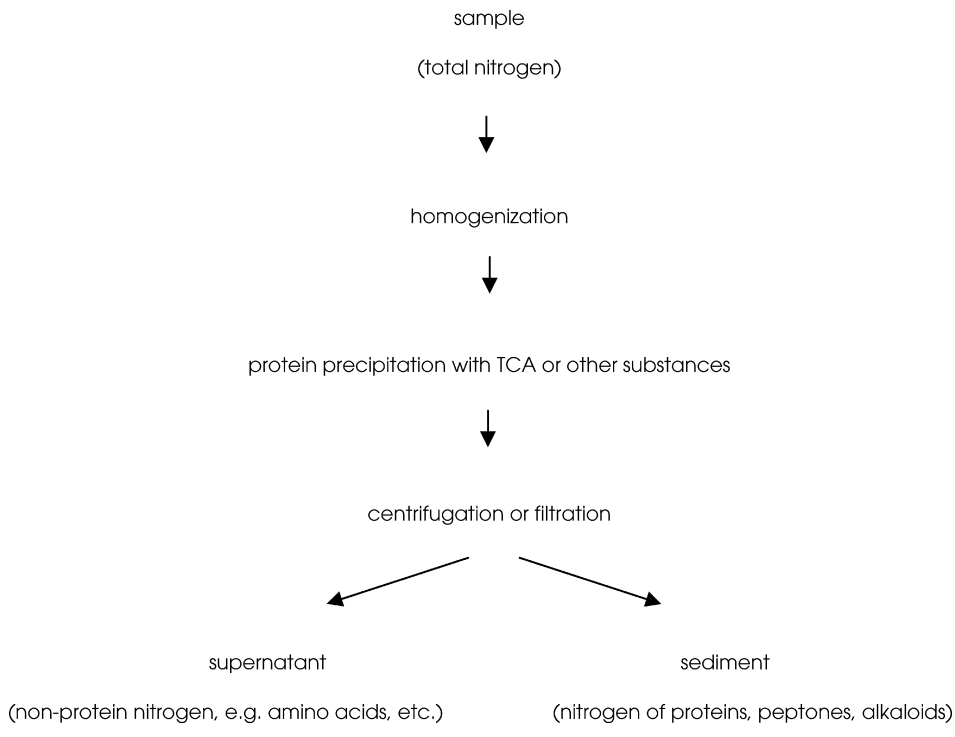


Fig. 4.6 Differentiation of protein and non-protein nitrogen components of organisms (Kleber *et al.* 1987).

Aliquots of Kjeldahl digest can also be used for total phosphorus determination. The oxidation by persulfate, used for determination of organic phosphorus, might also be applied for the measurements of nitrogen compounds (Grasshoff *et al.* 1983).

ORGANIC PHOSPHORUS

Because phosphorus determinations of zooplankton are rather rare, the procedures are outlined in more detail than the other elemental approaches.

It is important to distinguish between the inorganic and organic phosphorus compounds. In principle, the inorganic part is determined in one sub-sample. The organic phosphorus fraction is converted into the inorganic one in another sub-sample and determined as total phosphorus. The difference between the results is equal to the organic fraction.

The *inorganic phosphorus* reacts with ammonium molybdate reagent, forming a yellow complex (heteropoly-molybdenum-phosphorous acid). It is subsequently reduced to the highly colored blue complex by ascorbic acid and stimulated by antimony (Murphy and Riley 1958, 1962; Strickland and Parsons 1968). The intensity of the blue color is proportional to the phosphate concentration and colorimetrically determined at an approximate wavelength of 885 nm in comparison to distilled water or air. Blanks and turbidity have to be taken into account, even the calibration by KH_2PO_4 solution. The orthophosphate determination might become more sensitive by extraction procedures, whether of the yellow or the blue complex (Golterman and Clymo 1969).

The *organic phosphorus*, however, does not react with molybdate reagent. It must be

first digested or *hydrolyzed* by a strong acid at a temperature of 140 °C. Strickland and Parsons (1968) and Golterman and Clymo (1969) recommended oxidizing the organic matter *by* heating the homogenate in the presence of *PCA (perchloric acid, $HClO_4$)*. The method was originally described by Robinson (1941) and Hansen and Robinson (1953). Golterman and Clymo (1969) suggested an additional method of Redfield *et al.* (1937), the digestion with sulfuric acid, which allows the determination of organic nitrogen as well. The description includes the use of hydrogen peroxide, depending on the amount of organic matter. Another recommendation concerning sulfuric acid includes the hydrolysis of organic phosphorus compounds by heating in a pressurized autoclave for 5 to 8 h at 140 °C (Harvey 1948). Menzel and Corwin (1965) found the Harvey method acceptable, but of limited use, because an autoclave is needed. Omori and Ikeda (1984) recommended this procedure according to Szyper *et al.* (1976), using a water bath (90–100 °C) for 1 h.

Menzel and Corwin (1965) generally criticized PCA and sulfuric acid digestion methods. They are less precise (because of high and varying blanks), time consuming, and the boiling of PCA is potentially dangerous, especially in contact with organic matter. Therefore the authors examined the *wet digestion* of organic phosphorus compounds with *potassium persulfate* ($K_2S_2O_8$) as an oxidizing agent, which was already recommended for the complete oxidation of dissolved organic carbon by Menzel and Vacarro (1964). Full recovery ($\pm 1\%$) was obtained by persulfate digestion of zooplankton samples, in comparison with the sulfuric acid digestion of Redfield *et al.* (1937). The oxidation was performed in an acid solution of 5% potassium persulfate, prepared using 15 ml of 9.0N H_2SO_4, diluted by double distilled water up to 100 ml in the cold and adding 5 g $K_2S_2O_8$ (Koroleff in Grasshoff *et al.* 1983). Menzel and Corwin (1965) used dried zooplankton material, which was ground and suspended in distilled water. The suspension was filtered and the filtrate digested. Eight ml of freshly prepared oxidation solution were added to 50 ml of sample in 125 ml borosilicate flasks. They were either autoclaved for half an hour at 120 °C and a pressure of approximately 10^5 Pa or placed in a boiling water bath for 1 h. Afterwards the sample is made up to a final volume of 60 ml with distilled water and cooled down by tap water. Finally, the developed orthophosphate is determined.

The potassium persulfate method has been continually used in zooplankton studies, for example by Le Borgne (1982), Schneider (1988), Anderson and Hessen (1991) and Gismervik (1997a, 1997b). The two latter studies used whole animals for digestion, either frozen or fresh (cf. conditions to prevent losses of organic material in section 4.2). Anderson and Hessen (1991) performed phosphorus analyses of 3 to 30 copepods or cladocerans, with an average dry mass of about 160 mg in 7 ml scintillation minivials. The organisms were sorted into pre-weighed polycarbonate capsules. All vials and capsules with caps were cleaned of any phosphorus by soaking in antimony-molybdate solution and rinsing twice in double-distilled water. The samples were dried first overnight at 60 °C and weighed as described on page 91. Then they 'were oxidized overnight with 200 μl of H_2O_2 at room temperature to make the exoskeletons more hydrophilic and to avoid incomplete digestion of animals floating on the surface'. Digestion was performed by adding 2 ml of 1% $K_2S_2O_8$ solution (10 g per 1000 ml bi-distilled water) and autoclaving for 1 h at 120 °C. This concentration is sufficient for the digestion of organic matter including up to 444 μg carbon, because 2 mol of $K_2S_2O_8$ are needed to oxidize an amount of organic carbon equivalent to 1 mol CO_2. Finally, the standard molybdate-blue analysis of orthophosphate is followed. Anderson and Hessen (1991) described the preparation of standards carefully. They added 0–50 μl of an

orthophosphate solution ($100\,mg\,l^{-1}$ P) to minivials containing the empty polycarbonate capsules used as blanks in the weighing procedure. The added water evaporated overnight. The following digestion procedure was carried out according to the sample treatment. The antimony-molybdate complexing reagent and the ascorbic acid reductant were prepared to yield correct final concentrations after 200 μl were added to each solution to give a nominal final sample volume of 2.6 ml. The absorbance at 880 nm in 1 cm cuvettes was linear with added orthophosphate over the whole range of 0–5 μg P per vial.

The phosphorous components of organisms, which are of central importance in metabolism, are esters of phosphoric acid in the chemical sense. It might be important to know the single components instead of the total, for example in case of the determination of phospholipids and of nucleic acids (Dagg and Littlepage 1972). The compounds can be separated by differences in solubility and hydrolyzibility, as shown in Figure 4.7 (Kleber *et al*. 1987).

ORGANIC COMPOUNDS

An overview of the relative biochemical composition of dried zooplankton taxa is presented in Figure 4.8. It is based on minima, maxima and partly on averages of data sets, compiled by Raymont *et al*. (1964), Omori and Ikeda (1984) and Raymont (1983). The original data were globally collected from the end of the nineteenth century to the late 1970s.

The proteins form more than 50% of organic matter in all taxonomic groups, followed by the lipids. Carbohydrates are generally less than 15%; chitin reaches up to 10% in crustacean plankton only. There is a high degree of variability within the group of copepods in all the components, due to the differences of food quality of filter and raptorial feeders, the different habitat preferences, and to some extent the seasonal cycles. A dominant lipid storage in deeper waters or polar regions causes a relative low protein content and *vice versa*. This also explains the huge range of the chaetognaths. Some outliers and extreme values might be also caused by methodological differences.

The data in Figure 4.8 are generally supported by papers published after the compilations mentioned above, for example by Goswami *et al*. (1981), Percy and Fife (1981, 1983), Morris and Hopkins (1983) and Båmstedt (1986). Stephen *et al*. (1979) noticed no significant differences between neritic and oceanic euphausiids and mysids. Protein is the dominant metabolic reserve of zooplankton in regions with constant food supply, for example in tropical, estuarine areas (Madhupratap *et al*. 1979) or of neuston in the Mediterranean Sea (Champalbert and Kerambrum 1978). This was also underlined by measurements of protein catabolism (Quetin *et al*. 1980). Falk-Petersen (1981) reported contrary results from subarctic regions, where the lipid fraction was of high importance and strong seasonal variability occurs.

A single biochemical component is insufficient to describe fully the nutritive value of organisms. This conclusion was drawn by Mayzaud and Taguchi (1979) from the composition of organic particles in neritic habitats.

PROTEINS

Proteins are the major component in protoplasm. They are responsible for the structure of cells and organisms and for a large variety of physiological functions. The macromolecules are formed by up to 20 proteinogenic amino acids, linked by peptide bonds. The reaction between the NH_2- and COOH-groups liberates water, which is

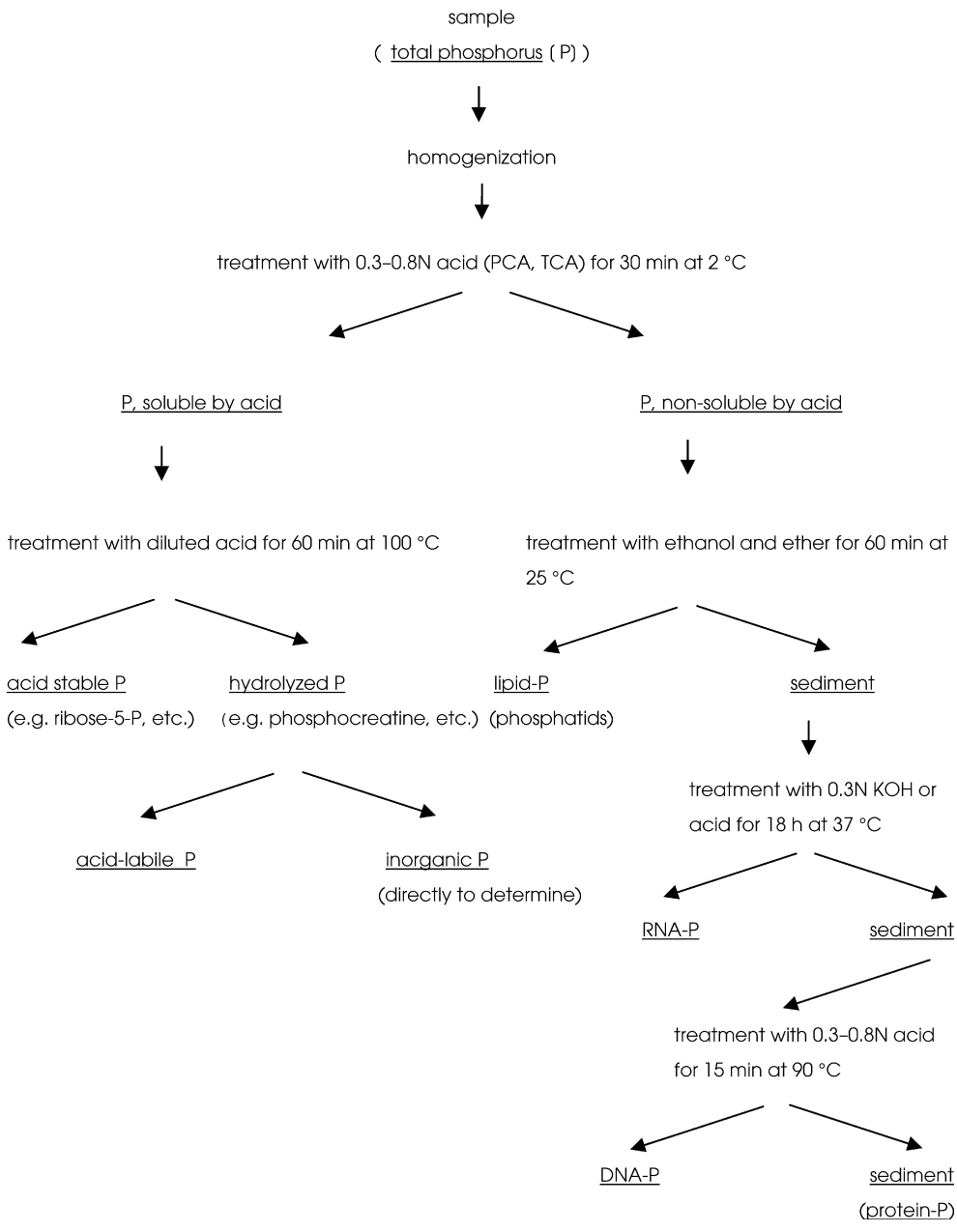


Fig. 4.7 Separation of the different phosphorus components of organisms (Kleber *et al.* 1987).

needed in the opposite case during hydrolysis. The sequence of the more than 100 amino acids is unique for every protein. The pure amino-acid chain is called the primary structure. There are secondary, tertiary and quaternary structures, formed by specific bonds, which might be irreversibly destroyed (denatured) at high temperature, by ultraviolet radiation, etc. Besides protein, there are similar compounds, like the shorter

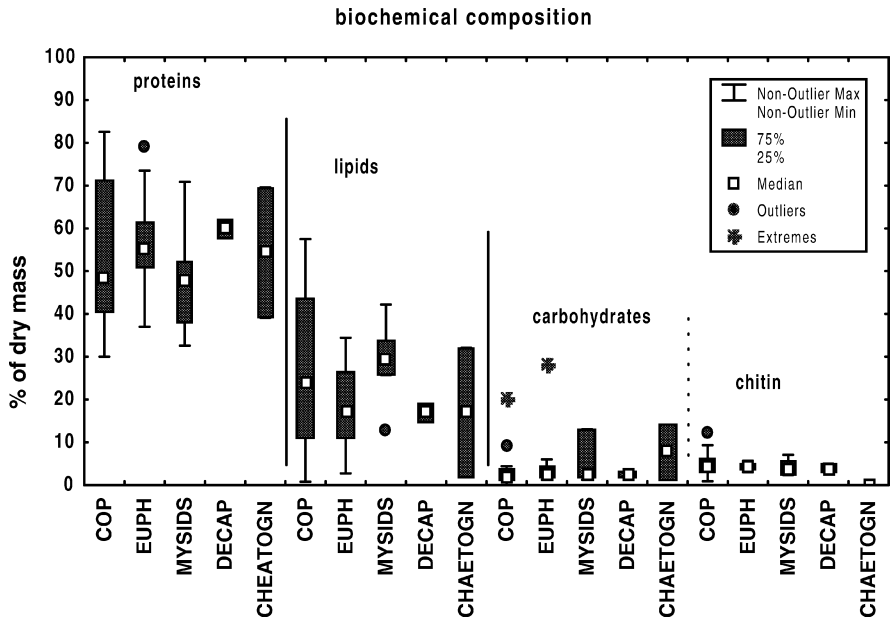


Fig. 4.8 Relative biochemical composition of dried copepods, euphausiids, mysids, decapods, and chaetognaths, showing minima, maxima and averages of data sets, with 27, 16, 6, 2, and 2 inputs for the respective taxa, compiled by Raymont *et al.* (1964), Omori and Ikeda (1984) and Raymont (1983).

amino-acid chains, the poly- or oligopeptides, and the composed proteins, the proteids. They consist of a protein part and prosthetic groups, such as metals, phosphoric compounds, dyes, lipids, carbohydrates, and nucleic acids.

All these specific characteristics imply analytical possibilities on one hand, the diversity of compounds underline the likely interference between them on the other.

The protein determination is not frequently used as an expression of zooplankton biomass. Krey (1951, 1952, 1958) and Krey *et al.* (1957) tried to introduce the determination of albumin equivalents by biuret reaction as such. However, less than 5% of papers, referred to in *Aquatic Science and Fisheries Abstracts* (ASFA) since the middle of the 1970s according to the key words 'zooplankton' and 'protein', are focused on biomass determination, like Berdalet *et al.* (1997). The introduction of more sensitive carbon analyzers at the same time and some analytical uncertainties of the protein methods (e.g. Hopkins *et al.* 1984) might have caused this development. In 45% of publications protein was mentioned in the context of biochemical composition of zooplankton. A quarter of authors used it as a scaling factor for metabolic activity measurements, for example Skjoldal (1981), Bidigare *et al.* (1982), Blazka *et al.* (1982), Folkvord *et al.* (1996) Hernández-León *et al.* (1995) and others.

Another application of protein studies is the detection of specific 'stress proteins', which indicate the physiological stage of organisms (for a review see Hoffmann and Parsons 1994).

The determination of amino acids, the constituents of proteins, is an interesting aspect of food web studies, considering the need of essential amino acids by animals. For example, Claustre *et al.* (1990, 1992) compared the amino acid composition of

phytoplankton and fecal pellets of copepods, and concluded which phytoplankton taxa were utilized by the mini-crustaceans. Martin-Jezequel *et al.* (1989) found the preferred food of fish larvae with this approach. Cowey and Corner (1962, 1963a, 1963b) examined the food chain phytoplankton–zooplankton–fish by means of certain amino acid compositions. Their attempt to determine the protein content by summing up the amount of amino acids demands their complete estimation, which is quite a laborious undertaking.

There are several *principles of direct protein determinations*. They are more accurate than the calculation from organic nitrogen (Conover and Corner 1968; Beers 1976a). The most frequently applied methods are based on the property of proteins to form chemical derivatives and to bind dyes. Both principles are used by *colorimetric methods*, like the biuret reaction, the Lowry and the Smith assay on one hand and the Bradford procedure on the other. The rapid Warburg–Christian determination is a *spectrophotometric method* using the proteins' ability to absorb ultraviolet light at 280 nm due to aromatic amino acid radicals. A *fluorometric approach* is based on the reaction of primary amines with fluoresamine and *o*-phthalaldehyde (OPA) forming fluorescent derivatives (Castell *et al.* 1979). It is considered as a very sensitive method (1 to 5 μg, using OPA). However, the possibly accompanied amines and the varying fluorescence signals of different proteins prevent a general application. *Gravimetry* is one quantitative determination of protein which is known to be exact, but which is laborious and therefore not recommended in the relevant literature.

Colorimetric and spectrophotometric methods are mainly used for routine measurements in biochemistry, and also in the field of zooplankton studies.

These assays are mostly performed using bovine serum albumin (BSA) as the reference protein. A standard curve is generated (except in the Warburg–Christian method), plotting the absorbance versus the amount of protein. The absorbance (optical density) is a linear function of concentration which holds within the limits of the Beer–Lambert Law (cf. Alexander and Griffiths 1993). Practically, the unknown protein amount must be diluted and measured at concentrations that fall into the limits of the sensitivity of the method. The protein is added first, then the water to the same final volume. The tubes are well mixed after each addition. The color-producing reagent is always added last, and the reaction may need to be accurately timed.

The range of concentrations giving a linear curve, the substances that interfere, and the sensitivity are important factors in the evaluation of a photometric or colorimetric method.

The *biuret reaction* was one of the first colorimetric determinations of proteins and is known to be rapid, but less precise (Gornall *et al.* 1949). It is specific for substances of two or more peptide bonds, i.e. molecules larger than dipeptides. The biuret positive substances react with copper salts (e.g. $CuSO_4$) in a strong alkaline solution. They form a red to blue purple color complex, which is determined at the extinction maximum of 540 nm. The intensity is proportional to the amount of peptide bonds. Ammonium ions and lipids are disturbing substances. However, the formation of precipitation by lipids mostly takes longer than the color reaction. Performing the measurements after 5 to 15 min instead of the normal 25 min may avoid the problem (Suelter 1990). The calibration curve is linear between 1 mg and 10 mg.

Raymont *et al.* (1964, 1969, 1971a) measured the protein content of homogenized fresh crustaceans within this range. The homogenate was passed through a GF filter (cellulose interferes with the reagent) to remove chitinous debris and the clear filtrate was used to determine the optical density at 540 nm.

Alexander and Griffiths (1993) described a method with a concentration range between 0.2 and 2 mg protein. Krey *et al.* (1957) worked within a similar range. When Krey (1951, 1958) adapted the biuret method for plankton research he used dried material hydrolyzed by NaOH. The error of the method was $\pm 10\ \mu g$ within an albumin range of 0 to 3 mg. Båmstedt (1974) also used dried plankton and avoided the variable results after modifying the method of Raymont *et al.* (1964). He included a NaOH hydrolysis as did Krey *et al.* (1957) and 10 min heating in a water bath. After centrifugation and shaking with diethyl ether to eliminate any turbidity caused by lipids the protein was determined in the aqueous phase of the supernatant at 550 nm. The standard curve was constructed between 0 to 5 mg, similar to Krey *et al.* (1957).

Hopkins *et al.* (1984) recommended a sample treatment for the parallel performance of the biuret method and the gravimetric determination of the total lipid (see page 103) from the same material. The procedures diverged after an extraction step by a chloroform-methanol mixture and centrifugation. The supernatant included the lipids. The sediment was dried prior to a protein determination. The dried material was treated by a procedure like that used by Båmstedt (1974); however, the material was not mixed with NaOH and biuret reagent at the same time. Biuret was added after the 15 min hydrolysis and before the mixture was boiled for 10 min. The calibration curve between the absorbance at 550 nm and BSA was linear in a range between 2 and 20 mg BSA. Furthermore, the paper includes some hints concerning the accuracy of the method.

The sensitivity of the biuret method increases 100 times in the *Lowry protein assay* (Lowry *et al.* 1951; McDonald and Chen 1965; Hartree 1972; Peterson 1977; Dortch *et al.* 1985) by adding the Folin–Ciocalteau reagent. First the copper–protein complex is formed in the alkaline solution. Next the cupric ions produced enhance the effectiveness of the reaction within 1 s. Certain amino-acid residues, mainly tyrosine and tryptophan, are involved. The complex reduces the phosphomolybdate-phosphotungstate, components of the Folin–Ciocalteau–phenol reagent, forming an intensive blue color (molybdenum blue reaction). The color may finally vary a little due to the specific occurrence of the involved amino acids.

Folin's reagent is only stable in acid conditions. Added to the alkaline copper–protein solution, the compounds must be mixed immediately to finish the reduction by the Cu–protein complex before the reagent is degraded. The extinction is generally measured at 720 nm in a 1-cm cuvette after 30 min and within 1 h. The calibration curve is linear between 5 and 100 μg protein (Kleber *et al.* 1987). Alexander and Griffiths (1993) note a range of 20 to 200 μg, measured at 700 nm. Measurements at different wavelengths demand specific extinction coefficients and lead to linear standard curves in three distinct concentration ranges, at 500 nm from 25 to 100 μg, at 660 nm between 2 and 30 μg and at 750 μm from 1 to 20 μg (Markwell *et al.* 1981). There are more wavelengths cited in the literature than those mentioned here and the different recommendations are linked with many slight modifications in the procedure itself.

The reaction proceeds by the same substances as the biuret reaction, but more intensively. Lipids and salts also cause undesirable precipitation. An addition of SDS (sodium dodecyl sulfate) eliminates the interference by lipids (Markwell *et al.* 1981).

The method is frequently used because of its sensitivity although it is time consuming. The dependence of the color reaction on the varying occurrence of tyrosine and tryptophan in proteins is a clear restriction. For example, Cowey and Corner (1963a, 1963b) found that the tryptophan was totally lacking during the amino-acid screening in *Calanus helgolandicus* and *C. finmarchicus*.

A modification of the Lowry assay, using bicinchoninic acid (BCA) in place of Folin–Ciocalteau reagent, has been developed by Smith *et al.* (1985) and Walker (1994). The *BCA method* (*Smith assay*) is comparable in sensitivity and easier to perform. The BCA and cupric ions which are produced in peptide bonds under alkaline conditions form a violet color complex. This 'one step method' avoids the dependence on the amino-acid composition and is not influenced by detergents such as Triton-X-100 and SDS. The favorable range of the method is 0.2 to 50 μg, measured at 562 nm.

The *Bradford method* is a protein dye-binding assay (Bradford 1976; Krüger 1994) and generally popular. The color of 'Coomassie-Brilliant Blue G-250' or 'SERVA Blue G' in diluted acid solution changes proportionally as the dye binds to protein, shifting the absorption maxima from 465 to 595 nm. Depending on the amount of the color complex, the absorption maximum may vary between 595 and 620 nm, which is a certain disadvantage of the method. On the other hand, the procedure is rapid (5 min incubation only) and simple. Its sensitivity is similar to the Lowry method. The linear part of the calibration curve is situated between 5 and 50 μg protein (Kleber *et al.* 1987). Alexander and Griffiths (1993) recommend a micro-method with a concentration range of 0 to 20 μg. Substances which interfere with the reaction are mainly detergents. There are special treatments to diminish such influences (Suelter 1990). The color intensity depends on radicals of alkaline and aromatic amino acids (Pingoud and Urbanke 1997).

Berges *et al.* (1993) compared the Lowry, the BCA (Smith) and the Bradford protein method using homogenates of marine diatoms. They found varying results due to standards being used and to the application of hydrolysis. Algal protein was determined best, using bovine serum albumin (BSA) in the Bradford technique while bovine gamma-globulin (BGG) is preferable for the Lowry method. For the BCA procedure no dependence was observed on either standard. Precipitation of protein by trichloric acid (TCA) and re-dissolving in 1N NaOH led to 60% smaller quantities in the Lowry and BCA methods, but not in case of the Bradford method. The accuracy of the methods was not compared. However, the Bradford method was recommended because of its comfortable treatment and the comparably smaller disturbance by non-protein compounds found in the marine phytoplankton.

The spectrophotometric *Warburg–Christian* (1941) method is a direct absorption measurement within the UV range (cf. Manchester 1996). Proteins absorb most light at 280 nm, primarily due to tyrosine and tryptophan residues. Therefore, the results vary with the content of these amino acids. The measurement is fast and not as specific as the techniques described above, but it is non-destructive and the sample can be recovered. This allows a rapid reading to obtain an approximate protein concentration followed by a colorimetric method with a sample dilution suitable for the linear range of the certain calibration curve. Sometimes this may be the only possibility for getting a protein concentration, if only a small sample is available for additional purposes. The sensitivity of the method is in the range of 0.02 to 3 mg protein. Calibration is not needed. Nucleic acids are the commonest interfering substances, which absorb light about 10 times stronger than proteins at 280 nm. Since proteins absorb more weakly than nucleic acids at 250 nm, a correction mode is recommended according to Layne (1957):

$$\text{protein (mg ml}^{-1}) = 1.55A_{280\text{ nm}} - 0.76A_{260\text{ nm}} \tag{4.3}$$

The dependence of the method on the amino-acid composition, especially the occurrence of tryptophan makes the method less suitable in zooplankton research like the Lowry method (see above). The recommendations of Whitaker and Granum (1980) may help in

this respect. According to them, the difference between the extinction (A) at 280 nm and 235 nm is mainly a result of peptide bonds. Consequently, the concentration of

$$\text{protein (mg ml}^{-1}) = (A_{235\text{ nm}} - A_{280\text{ nm}})/2.51 \quad (4.4)$$

does not depend on the composition of amino acids or on contamination by nucleic acids. Homogenates, including insoluble proteins, may become clear by adding SDS just before measuring the extinction; 0.1% of the final concentration is suitable (cf. Welch and Scopes 1981).

The advantages and disadvantages of the different approaches used are summarized in Table 4.6. If the protein content of the sample is unknown, the spectrophotometric method is convenient for obtaining a first clue as to the concentration range. The modification of Whitaker and Granum (1980) might be especially suitable for zooplankton, which sometimes contain no tryptophan. The material remains available for other determinations with colorimetric methods, which are more sensitive, except the classical biuret reaction. The method of Bradford and the modified Lowry (BCA) method according to Smith *et al*. (1985) are both sensitive. In contrast to the original Lowry method, the BCA assay does not depend on the occurrence of tyrosine and tryptophan. Berges *et al*. (1993) recommend the Bradford method because it is rapid, sensitive, and easy to perform. There are assay kits available for both the BCA assay (e.g. Sigma Chemical Co.) and the Bradford micro-procedure (e.g. Bio-Rad Laboratories).

The portion of insoluble protein in comparison to the soluble fraction is probably smaller than the differences originating from the various techniques and miscellaneous standards. Walker *et al*. (1987) identified only 5% (of deep sea holothurians) protein as insoluble.

There is no recent information on the accuracy of the different methods and no standardization in plankton research has taken place. The determination of proteins is reviewed from the biochemical perspective by Stoschek (1990), Copeland (1994), Coligan *et al*. (1995) and Price (1996).

LIPIDS (by W. Hagen)

Lipids usually have a low polarity and dissolve well in organic solvents such as chloroform or methanol. In contrast to other organic molecules, lipids are therefore commonly defined in textbooks according to their physical characteristics, a major criterion being solubility. There is however no universally accepted definition for the term 'lipids' (Christie 1987). Responsible for the 'fattiness' are the long hydrocarbon chains, which form the unipolar 'backbone' of the various lipid types. Lipids comprise an extremely heterogeneous group of organic compounds from very simple to highly complex structures. The group includes typical lipid classes such as hydrocarbons and fatty acids as well as wax esters, triacylglycerols, phosphoglycerides, sphingolipids and sterols. Due to their diversity and specific physico-chemical properties, lipids serve a multiplicity of functions in all biological systems (Hadley 1985).

Lipids are of special importance in the marine environment. In contrast to proteins and carbohydrates, lipids provide buoyancy due to their lower density as compared to sea water. Hence, many plankton and nekton species incorporate large amounts of lipid to remain suspended in the water and thus conserve energy, which would otherwise be wasted in the struggle against sinking. The limited compressibility and negligible thermal expansion of lipids is of importance for strong vertical migrants such as copepods or euphausiids. For instance, lipid-rich copepods ascending from great depths do not suffer

Table 4.6 Protein methodology, measuring principles, suitable detection ranges, advantages, disadvantages and some examples of references according to Suelter (1990), Alexander and Griffiths (1993), Pingoud and Urbanke (1997).

Method	Principle involved	Useful range (mg)	Advantages	Disadvantages	Reference
Colorimetric detection of derivates					
Biuret	Cu_2^+ reaction with peptide bonds; formation of a colored complex (biuret reaction)	0.2 . . . 1 to 10 . . . 20	Easy, fast	Generally less sensitive; interferences by NH_4^+, Tris and Good's buffer, lipids	Gornall *et al.* (1949); Hopkins *et al.* (1984); Alexander and Griffiths (1993)
Lowry	1) Cu_2^+ reaction with peptide bonds 2) Reduction of phospho-molybdate and phospho-tungstate mainly by tyrosine (tyr) and tryptophan (tryp)	0.025 to 0.1 (500 nm)* 0.002 to 0.03 (660 nm)* 0.001 to 0.02 (750 nm)*	Sensitive	Tedious; exact timing required; many interfering substances, cf. biuret reaction, dependence on tyr, tryp	Lowry *et al.* (1951); Peterson (1977); Dortch *et al.* (1985) *Markwell *et al.* (1981)
BCA	Cu_2^+ reaction with peptide bonds and with bicinchoninic acid	0.0002 to 0.05	Sensitive like Lowry, no tyr, tryp dependence	Interfering substances: NH_4^+, EDTA	Smith *et al.* (1985); Walker (1994)

Colorimetric detection of bound dyes					
Bradford	Coomassie-Brilliant-Blue bonds with basic and aromatic amino-acid radicals	0 to 0.02	Fast, sensitive, easy, few interfering substances	Expensive, requires greater precision; interfering substances, Triton	Bradford (1976); Alexander and Griffiths (1993); Krüger (1994)
Spectro-photo-metric detection					
Warburg–Christian	Absorption of aromatic amino-acid radicals (mainly of tyrosine, tryptophan) at 280 nm	0.02 to 3	Fast; non-destructive, sample can be recovered, no calibration needed	Interference by nucleic acids, correction possible; tyr, tryp dependence to be corrected	Warburg and Christian (1980); Manchester (1996)
Fluoro-metric detection	Primary amines form fluorescent derivatives with fluoresamine and *o*-phthalaldehyde (OPA)	0.001 to 0.005 (using OPA)	High sensitivity	Varying fluorescence signals of different proteins	Castell *et al.* (1979)

from a pronounced increase in volume (Sargent 1976). Osmolarity problems also do not occur, since lipids are hydrophobic compounds.

One of the most important general characteristics of lipids is their capability to store energy in a very efficient way. On average, carbohydrates contain only about 17 kJ g^{-1} and proteins 24 kJ g^{-1}, whereas the average energy content of lipids is about 39 kJ g^{-1} in aquatic animals (Brody 1945; Winberg 1971). Hence, compared to the other organic compounds about twice as much energy per unit mass can be accumulated as lipid. In contrast to proteins, for example, the entire lipid molecule can be oxidized to generate energy (Båmstedt 1986). Only minimal amounts of water are deposited with the hydrophobic lipids, which allows a very compact and economic energy storage. Triacylglycerols are the usual energy reserves of all terrestrial animals and these compounds play an important role as storage lipid also in many marine organisms. The other major storage lipids, wax esters, are largely restricted to the marine environment where they clearly predominate in some taxa, particularly in calanoid copepods from higher latitudes and greater depths (Lee and Hirota 1973; Sargent and Henderson 1986; Hagen *et al.* 1993). Due to their rapid biosynthesis and specific composition, these lipids allow a very efficient energy storage, which is of vital importance especially in ecosystems with a periodically limited food supply such as the deep sea or the polar oceans.

In addition, life-history traits of pelagic organisms can be derived from the nature of the lipids accumulated. According to findings by Sargent *et al.* (1981) different lipid classes may indicate different feeding strategies. Wax esters are stored as long-term energy reserves and particularly marine herbivorous copepods are characterized by wax ester storage (Hagen *et al.* 1993). These calanoid copepods cease feeding in autumn, go into a resting stage ('diapause'), and overwinter at depth until the next spring, when they reascend to surface layers to reproduce and to feed (Hagen and Schnack-Schiel 1996). In contrast, species which are deficient in wax esters but store triacylglycerols instead, such as the Antarctic krill *Euphausia superba* (Hagen *et al.* 1996), do not stop feeding during the dark season but seek alternative food sources. In fact, winter feeding of krill had been suggested by Sargent *et al.* (1981) based on the fact that *E. superba* deposits triacylglycerols, long before this behavior was actually observed (e.g. Marschall 1988; Daly 1990).

More detailed analyses of lipid composition may help reveal dietary preferences of the species investigated. The concept of lipids as trophic markers makes use of the fact that specific fatty acids are characteristic of specific groups of phytoplankton, such as 16:1 (n − 7) for diatoms or 18:4 (n − 3) for dinoflagellates (Harrington *et al.* 1970; Lee *et al.* 1971; Falk-Petersen *et al.* 1990). These marker fatty acids are incorporated largely unaltered by phytophageous species revealing their dominant diet (Graeve *et al.* 1994a, 1994b). Lipid markers may provide trophic level resolution, however, they usually cannot indicate species-specific relationships. Their major advantage over the much more tedious gut content analyses is their integration of trophic information over a period of weeks and months yielding long-term mean feeding preferences (Graeve *et al.* 1994b). Apart from this information on nutritional ecology, detailed fatty acid and fatty alcohol analyses may also reveal adaptive differences in the lipid economy of species with a different evolutionary or biogeographical background. For instance, typical polar copepod species which are most affected by the seasonality of food supply seem to have developed the highest efficiency to store their lipid reserves (Kattner *et al.* 1994; Kattner and Hagen 1995; Albers *et al.* 1996).

The unique properties of lipids in marine organisms have for a long time created a

strong commercial and scientific interest in these compounds. By the end of the nineteenth century marine scientists such as Hensen and Brandt in Kiel became interested in the chemical composition of plankton (Hensen 1887). Brandt and his co-workers modified analytical procedures developed by Liebig for agricultural purposes and applied these techniques to their pioneering planktological investigations which also included lipid determinations (Brandt 1898; Brandt and Raben 1919/1920). Although lipids of marine organisms were not analyzed in great detail in these early studies, the lipid data provided useful information on the life history of the zooplankton species investigated.

It is noteworthy that already at the turn of the century Rosenfeld (1904) investigated the relationships between storage lipids of carnivores and their dietary lipids. For these studies he used marine organisms which he found best-suited for such objectives. Rosenfeld (1904) determined the content, iodine value and saponification value of the lipids extracted from various marine carnivores and from their prey (crustaceans, fish, etc.). These data, although rather crude, were used to characterize the different lipids and to allow conclusions about the transfer of lipids from one trophic level to the next. Similar investigations which attempt to elucidate dietary links via trophic 'marker lipids', are still frequently carried out in marine lipid studies (e.g. Lee *et al.* 1971; Falk-Petersen *et al.* 1990; Graeve *et al.* 1994a, 1994b). These much more sophisticated investigations were possible with the development of modern analytical techniques. Methods such as thin-layer chromatography (TLC) and especially gas-liquid chromatography (GLC) revolutionized the analysis of (marine) lipid compounds (e.g. Christie 1982; Kates 1986).

The *total lipid content* is the fraction of lipid that can be extracted from entire specimens or tissues, expressed as the percentage of wet mass or dry mass. The extraction of total lipids is usually carried out with a chloroform and methanol solution in the ratio of 2:1 (by volume), with 0.01% butylhydroxytoluene added as antioxidant (chloroform has recently been substituted by the less toxic dichloromethane). The procedure is based on the rapid and efficient methods of Folch *et al.* (1957) and Bligh and Dyer (1959), which have been modified in various details depending on the respective requirements (Figure 4.9). The total lipid content can be obtained by photometric methods such as the sulphophosphovanillin method (Zöllner and Kirsch 1962; Barnes and Blackstock 1973) and the dichromate method (Pande *et al.* 1963) or by gravimetric determinations. As an advantage over gravimetric analyses, the photometric methods require only very small amounts of lipid. However, careful calibration with known lipid quantities is needed, since the same amount of lipid may produce different photometric responses depending on the degree of unsaturation (Zöllner and Kirsch 1962). According to Christie (1982) the method of Folch *et al.* (1957) extracts 95–99% of the lipids from tissues, as compared to 99% for the sulphophosphovanillin method. Gangliosides and glycolipids may occasionally be lost with the aqueous phase. Hopkins *et al.* (1984), in connection with their protein determinations, extensively studied potential sources of error when lipids are extracted gravimetrically. These authors give valuable hints for avoiding artefacts.

The total lipid extracts can be separated into their various *lipid classes* by adsorption chromatography (column chromatography, thin-layer chromatography). Column chromatography may separate larger quantities, but thin-layer chromatography (TLC) is more rapid and sensitive (Christie 1982) and usually the preferred method. During TLC the samples are applied to glass plates coated with a thin layer of silica gel (stationary phase) using an appropriate solvent mixture as mobile phase (e.g. Stahl 1969). To quantify the separate lipid classes the TLC plate may be sprayed with, for example, a

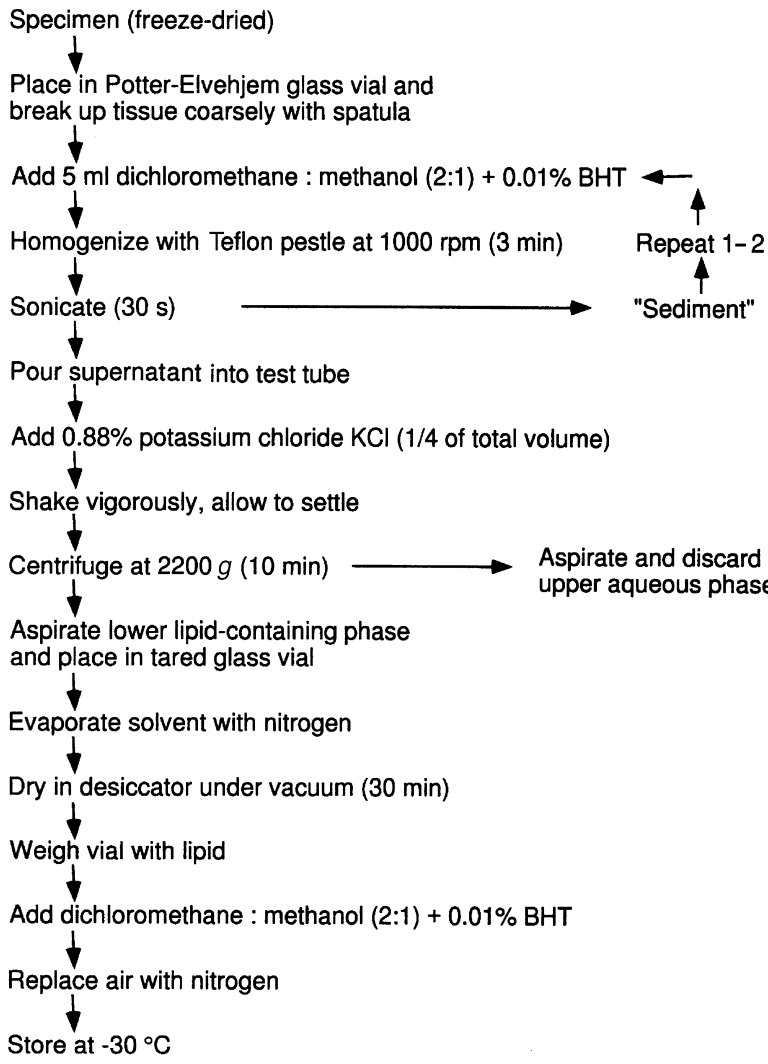


Fig. 4.9 Extraction procedure for gravimetric determination of the total lipid content of zooplankton specimens.

chromic acid solution, then heated and the amount of charred material determined by a scanning photo-densitometer (Christie 1982; Olsen and Henderson 1989).

High-performance liquid chromatography (HPLC) is an alternative technique to quantify lipid classes. Lipid analyses by HPLC used to be limited by detector resolution, but there has been much progress lately and light scattering detectors now achieve satisfactory lipid separations (Christie 1987, 1989). A rapid technique frequently applied to quantify lipid classes, the Iatroscan, combines the principle of TLC with a flame-ionization detector (FID). Instead of TLC plates lipid samples are separated on quartz rods (150 mm long with a diameter of 1 mm) coated with a thin layer of sintered silica in a TLC chamber with the appropriate solvent mixture (Ackman 1981). Subsequently, the rods are advanced through the FID flame and the separate lipid compounds are ionized. The intensity of the electrical signal is proportional to the amount of organic substance

entering the flame. This allows the quantification of lipid classes after careful calibration with known standards (see e.g. Fraser *et al.* 1985). With respect to copepod wax esters, Miller *et al.* (1998) pointed out that the use of commercially available standards leads to a significant underestimation of wax esters. They suggest using copepod wax esters from bulk-extracted material for calibration.

Using preparative TLC the separate lipid compounds can be recovered from the plates and analyzed in greater detail by gas-liquid chromatography (GLC), which determines the quantitative *fatty acid and fatty alcohol* compositions of these lipids. GLC is a type of partition chromatography with gas as the mobile phase and a liquid as the stationary phase. GLC analyses used to be time-consuming procedures. In the beginning of the GLC era the analyses were carried out on short (ca. 2 m) stainless steel columns or fragile glass columns packed with celite and coated with paraffin as the liquid phase, which required rather large lipid samples. In modern equipment, flexible wall-coated open tubular capillary columns of fused silica (50 m length) are used for the separation of single components and provide an excellent resolution. A minute lipid sample ($< 1\ \mu g$) is injected via an injection port into the gas phase where it is volatized and passed onto the liquid phase in the column, usually a polar polyester. The lipid components are carried through the column by hydrogen or helium (carrier gas) using specific temperature programs. The various fatty acids and alcohols have deviating affinities (retention) for the stationary phase due to their different chemical and physical properties and hence separate while they travel down the column. Emerging from the column in narrow peaks the constituents are quantified by, for example, a flame-ionization detector. The results are converted to electrical signals, amplified and recorded by an integrator. The peaks of the individual components can be identified from their retention times relative to known standards. Prior to the GLC analysis the fatty acids are usually derivatized to fatty acid methyl esters (and the alcohols sometimes to acetates or other less polar derivatives) for better detection. The whole procedure, of which only a very basic description has been given above, takes less than 1 h to obtain quantitative results (for details see, e.g., Kates 1986; Christie 1989). GLC analyses, especially of wax esters, have been further accelerated after the development of a method, which allows the simultaneous determination of fatty acid methyl esters and free fatty alcohols (Kattner and Fricke 1986). A GLC may also be connected to a mass spectrometer (GLC-MS), a powerful tool for the identification and verification of specific lipid components based on their ionization and fragmentation.

CARBOHYDRATES

Carbohydrates are a diverse group of compounds. The empirical formula $C_x(H_2O)_y$ expresses the chemical meaning as hydrates of carbon. They are the predominant constituents of dried plant material (60%). They form 20% of the content of bacteria and 12% of phytoplankton. Only 2% of the dry zooplankton consists of carbohydrates (Baretta-Bekker *et al.* 1992) with ribose of nucleic acids as the main component, followed by the percentage of glycogen and traces of free sugars (Raymont *et al.* 1968; Mayzaud and Martin 1975).

Carbohydrates are grouped according to their complexity. The simplest units are the sugars, which form larger units, the oligo- and polysaccharides, by condensation of water. For example, glycogen finally consists of about 10^5 to 10^7 glucose molecules. The opposite way, the decomposition, is based on hydrolysis.

Besides the complex sugars, there are derived products of carbohydrates, deoxy-sugars and amino-sugars. Chitin, the material of the exoskeletons of crustaceans,

belongs to mucopolysaccharides consisting of amino-sugars. Chitin is a polymer of about 2000 *N*-acetyl-D-glucosamine monomers. It is degraded very slowly and is of no nutritive value, although it is known as the most common bio-polymer in the sea. However, chitin is a valuable proxy for the past trophic stages of aquatic environments, especially if recorded in laminated sediments. Furthermore, Roff *et al.* (1994) recommended a radiochemical method for determining productivity of arthropods based on the chitin synthesis rate.

Brandt (1898) calculated the amount of non-chitin *carbohydrates* by the difference between the entire dry mass and the analyzed percentage of protein, chitin, lipids and ash.

Direct measurements follow the principle to hydrolyze the polysaccharides and to determine the evolved soluble monosaccharides colorimetrically using a calibration with glucose. There are two methods generally recommended in the aquatic science literature, the determination using the phenolic anthrone reagent and the more precise MBTH (3-methyl-2-benzothiazolinone hydrazone hydrochloride) method. Two additional methods are used especially in the marine zooplankton research, the procedure according to Mendel *et al.* (1954) and the more frequently used Dubois *et al.* (1956) method. Finally, the amount of certain carbohydrate fractions can be determined according to Clarke *et al.* (1991) using HPLC systems equipped with a pulsed electrochemical detector (PED).

In the following the two generally recommended methods and the Dubois *et al.* (1956) procedure are briefly reviewed.

The *anthrone method* is described by Strickland and Parsons (1968), Golterman and Clymo (1969), and Parsons *et al.* (1984) for sea water samples and suspensions of organisms. The method makes use of the hydrolyzing and dehydrating effect of concentrated sulfuric acid and the formation of the blue-green chromophores of furfural derivatives. Those are degradation products of the carbohydrates when combined with polyaromatic compounds, such as anthrone, phenol and others (Parsons *et al.* 1984). The duration of hydrolysis performed at 100 °C depends on the complexity of the carbohydrate involved and is finished after 15 min. At this time the extinction coefficients of the various sugars differ minimally. Then the measurements of the blank, standards, and samples against distilled water should be done within 3 h at 620 to 630 nm.

Sensitive and more accurate than the anthrone method is the so-called *MBTH method.* It is also carefully described in Parsons *et al.* (1984). In principle, the particulate carbohydrates are hydrolyzed in a sealed glass ampule with 10 ml 0.1N HCl for 20 h at 100 °C in a constant temperature bath. The cooled sample is then filtered through an ignited glass-fiber filter. The filtrate is neutralized with NaOH solution and the monosaccharides become reduced to alditol with potassium borhydride during 4 h at room temperature. Alditol is oxidized with periodate forming two moles of formaldehyde per one mole of monosaccharides, which is determined spectrophotometrically with MBTH at 635 nm against a water/acetone mixture. The concentration of carbohydrates is calculated using the extinction, reduced by the blank (distilled water instead of a sample), multiplied by a calibration factor and related to the sample volume. The calibration is performed applying glucose solution.

From the two methods especially used in zooplankton research, Raymont and Krishnaswamy (1960) and Raymont and Conover (1961) applied the method of Mendel *et al.* (1954) and Kemp and Heijningen (1954) respectively on one hand, Marshall and Orr (1962) introduced the procedure of Dubois *et al.* (1956) on the other. Finally, Raymont *et al.* (1964) compared both and found the *Dubois method* more practical than the Mendel method. It requires only 10 to 20 mg wet mass instead of 100 mg while the yield is two times larger (0.45% vs. 0.21% carbohydrate of wet mass)

and the scatter of about 10 replicates is significantly smaller (0.43–0.48% vs. 0.12–0.29%) probably because of the usage of phenol reagent. The Dubois method is additionally more rapid and simple. Following this procedure, Raymont *et al.* (1964) placed a single *Neomysis integer* of about 20 mg wet mass in a tube with 1 ml distilled water, 1 ml 5% phenol solution and 5 ml concentrated sulfuric acid. After 20 min the optical density of the developed brown color was read at 490 μm and compared with the standard curve for glucose. The readings were corrected by the results of the blank (1 ml distilled water instead of the sample). Eighteen replicates provided an average percentage of carbohydrate of 2.4 ± 0.4 at the 95% confidence level, related to dry mass. The results had been obtained for wet mass first and then converted to dry mass conditions considering an actual water content of 78%. The preparation of this sample had been done without any rinsing with distilled water, deviating from earlier studies (Raymont and Krishnaswamy 1960; Raymont and Conover 1961) and without homogenizing. Later, Raymont *et al.* (1969) applied a Potter–Elvehjem homogenizer. Another modification concerned the use of deep-frozen material (Raymont *et al.* 1971b). This might be linked to a loss of organic matter, if specimens are drained from 'excess' water, when thawed.

Båmstedt (1974) found the Dubois procedure to be also suitable for dried samples and recommended the use of 10 mg pulverized material for one determination. He added about 1 ml distilled water, waited some minutes until the sample was thoroughly wet and followed the next steps of the method. The spectrophotometer readings were done against a control prepared in the same way as the samples but without plankton. The standard curve has been arranged between 0 and 125 μg glucose. The error was several times smaller than those of the above mentioned procedure of Raymont *et al.* (1964). Five replicates of five concentrations between 1 and 10 mg dry mass which is approximately equivalent to a range between 0.013 and 0.13 mg carbohydrate led to coefficients of variations from 7.03 to 2.45%, naturally decreasing with enlarging the sample amount.

Chitin is measured separately from the other carbohydrates rather as a solid substance by weighing (Raymont *et al.* 1964, 1969, 1971b; Båmstedt 1974; Omori and Ikeda 1984) than colorimetrically by the determination of the single glucosamine units (Strickland and Parsons 1968; Mayzaud and Martin 1975). An application of the HPLC technique is a special case in the frame of secondary production measurement suggested by Roff *et al.* (1994). A fourth method, the already mentioned calculation of chitin first requires the measurement of the nitrogen content of the exoskeletons by wet oxidation using the Kjeldahl method (cf. protein determination). The results are multiplied by 14.5 (see Figure 4.4).

The *gravimetric procedure* is performed in five steps: the elimination of proteins, the test of their successful removal, a purification of the remaining exoskeletons, followed by drying and ashing, both linked with weighing steps.

Raymont *et al.* (1964, 1969, 1971b) used homogenized fresh material of about 250 mg per sample and boiled it in a glass test tube with a 50% NaOH solution for 2 h and left it overnight for digestion. The remaining chitinous exoskeletons were then washed with distilled water and tested for full removal of proteins by biuret reaction (cf. protein determination). Subsequently, the chitin debris was washed with dilute hydrochloric acid, then with distilled water, dried in tared crucibles at 60 °C for 24 h and re-weighed. Finally, the loss of mass during ashing at 500 °C for a couple of hours was taken to be that of chitin. Since 1969 the authors skipped the washing with HCl and used potassium hydroxide solution instead of sodium hydroxide. The average percentage of four

determinations of *Neomysis integer* chitin amounted to $7.1 \pm 0.65\%$ at the 95% confidence level.

The method suitable for fresh material did not work in case of dried material, because the strong alkaline solution was found to be too viscous for complete sedimentation when samples are centrifuged. Therefore, Båmstedt (1974) standardized the method of Orr (1934) using powdered material. It was placed in tared centrifuge tubes, weighed again and treated successively with fluids (1 ml $10\,mg^{-1}$) to remove all organic components apart from chitin:

1) for 3 min in 1N HCl in a bath of boiling water,
2) for 20 min with 4N NaOH again in the water bath, and
3) washing the residuals with distilled water, with 96% ethanol and finally with diethyl ether.

Between all steps, the sample was centrifuged and the supernatant was decanted. The centrifugation was generally done for 10 min at 3000 rpm, except after the last step when a hand centrifuge was used to avoid explosions.

At the end the residues of the sample were rinsed into a tared crucible using distilled water and dried at 60 °C for 24 h (Omori and Ikeda 1984). The pure chitin content was calculated as the mass difference between the dry mass and the remains after incineration at 500 °C for some hours (cf. dry mass and ash-free dry mass determination).

As in the case of total carbohydrate estimations, Båmstedt (1974) determined the error of the method using the coefficient of variation. Five replicates of dried samples were recorded at five different sample amounts between 10 and 50 mg which was equivalent to a chitin content between 0.8 and 4.0. The average coefficients of variation ranged between 5.95 and 2.50 showing again the higher the amount of material, the lower the variations between the replicates. Mayzaud and Martin (1975) *hydrolyzed the chitin* by HCl at 100 °C for 4 to 8 h, neutralized the hydrolyzate with sodium hydroxide solution until the phenolphthalein indicator turned pink, acetylated the evolved chitin monomers glucosanine with acetylacetone and condensed the product with *p*-dimethylaminobenzaldehyde forming a red solution whose extinction was measured at 530 nm. This is the method described in detail by Strickland and Parsons (1968). The conversion of the extinction readings to chitin requires a calibration with D(+)glucosamine hydrochloride as a standard at a final concentration of 150 $\mu g\,ml^{-1}$ of distilled water. The interference due to other carbohydrates and proteins could be avoided by removing them by applying an ion exchange procedure, or by determining their concentration and subtracting them from the total, considering factors for the carbohydrate concentration of 0.014 and for protein of 0.0025.

ENERGY CONTENT

Energy units enable processes such as consumption, respiration, growth, and reproduction to be compared on a common basis. The metabolic activity as a result of different physiological processes is directly measurable by heat-flow calorimeters, such as the BioActivity Monitor of Suurkuusk and Wadsö (1982). This equipment uses an open flow chamber, works on the heat-conduction principle (Calvet and Prat 1963; Wadsö 1974), ensures isothermal conditions and allows an unlimited experimental period (Gnaiger 1983). Before such dynamic techniques were available, biomass-specific caloric equivalents were determined and used as input variables in energy flow studies. In marine research, we remember the pioneering work on invertebrates by Rinke (1938). Lindeman's synthesizing influence on trophodynamic theory (Lindeman 1942) enhanced this

development, which culminated in the integration of ecosystem ecology and ecological energetics (e.g. Phillipson 1966). This was noted by Cummins and Wuycheck (1971), who tabulated the existing energy equivalents of a wide range of several trophic levels, habitats and taxonomic categories. The calorie (cal) has been used as the energy unit. One calorie is the amount of heat needed to increase the temperature of 1 g of water from 14.5 °C to 15.5 °C at an atmospheric pressure of 101 325 Pa (1 atm). Therefore, the unit gram calorie (g cal) has been synonymously applied (e.g. Golley 1961). Since 1975 the joule (J) has become the accepted SI unit: 1 cal is converted to joules by a factor of 4.1868.

Theoretically, the total energy content of organisms amounts to 22.6 J per milligram organic mass. It ranges from the energetic equivalent of lipid (39.8 J mg^{-1}) to that of carbohydrate (17.2 J mg^{-1}). These limits become narrower in nature. The tissues of the majority of aquatic species range between 21.8 J mg^{-1} and 25.1 J mg^{-1} ash-free dry mass (AFDM) following the compilation of Prus (1970). This range supports Cummins and Wuycheck (1971), who expected the gross average to be near the energy content of protein. Figure 4.10a shows the gross mean energy content (related to AFDM) of detritus and six trophic categories in terrestrial and aquatic environments (after Cummins and Wuycheck 1971), in relation to the averages of lipids, proteins and carbohydrates (Brody 1945). Figure 4.10b illustrates the scatter in different plankton groups which vary in total between 18.7 J mg^{-1} and 31.1 J mg^{-1} AFDM (Prus 1970). Most of the values lie within the range of proteins in all three categories. The remaining variability is probably caused by differences in methodology as well as by variations in sexual state and physiological conditions of the species. For example, the seasonal variation within a calanoid copepod population (*Euchaeta norvegica*) amounted to 5 J mg^{-1} in dried female adults and only 1 J mg^{-1} in dried eggs (Båmstedt 1986). Assuming 10% variance in ecological field programs, variations of about 2–4 J mg^{-1} AFDM have been considered as significant (Cummins and Wuycheck 1971).

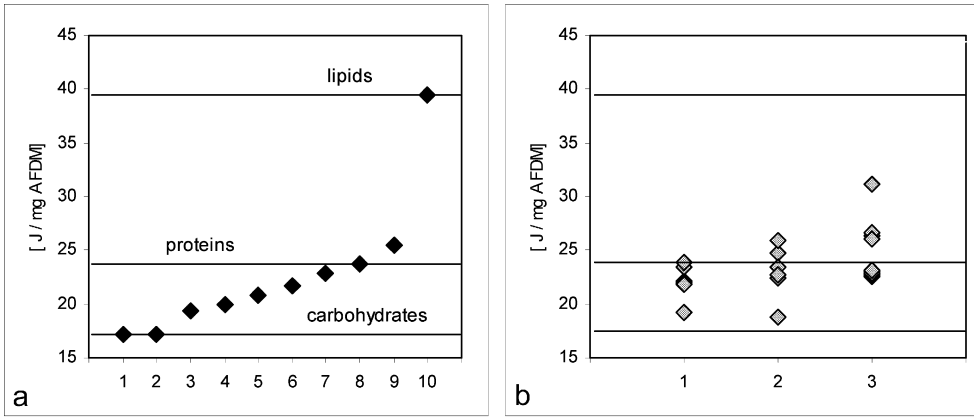


Fig. 4.10 a) Average energy content of the ash-free dry mass (AFDM) of carbohydrates (1, line), terrestrial detritus (2), aquatic primary producers (3), terrestrial producers (4), aquatic microconsumers (5), aquatic detritus (6), aquatic macroconsumers (7), protein (8, line), terrestrial macroconsumers (9) and lipids (10, line), according to Cummins and Wuycheck (1971); b) Average energy content of AFDM of crustaceans: euphausiids, amphipods, isopods, and cirripedes (group 1), cladocerans (2), and copepods (3), according to Prus (1970).

Platt *et al.* (1969) drew attention to the relatively low seasonal changes of plankton concentration per volume of water, expressed in energy units instead of as dry mass or even individual numbers. These authors found the 100-fold variability in abundance reduced to a 14-fold difference in dry mass concentration and to a 7 to 8-fold variation expressed in energy units. The authors quoted Odum and Smalley (1959) who observed the same in terrestrial populations and attributed the phenomenon to some form of compensatory or homeostatic mechanism controlling population energy flow.

The energy content of organic matter is *indirectly determined by calculation* either from the percentage of the organic constituents or from carbon (and other elements) using their average energy content, the so called 'caloric equivalents' (see Figure 4.4). Calculation examples for the different approaches are presented in Figure 4.11.

Using the average equivalents for proteins, lipids, and carbohydrates requires a precise component analysis (see page 107) as well as the choice of proper conversion factors.

One weak point of such an analysis might be the incomplete drying procedure and consequently the overestimation of organic mass. Another is the determination of protein from nitrogen using a fixed conversion ratio (6.25) in spite of an obviously varying organic N content (see section 4.3.3.). Thirdly, the amount of lipid depends on the extraction method (cf. page 113). The more effective solvents extract low energy lipids in a higher proportion, reducing the average energy content (Beukema 1997). These are serious problems, when determining two components and calculating the percentage of the third by difference.

When using caloric equivalents of organic constituents one should be aware of the following facts. First, these values represent the 'physical' heat content, produced by complete combustion in a pure oxygen atmosphere, a point which is also problematic in bomb calorimetry (see below), while the metabolic oxidation is incomplete. In other words, the maximum energy available from protein catabolism amounts to 20.1 J mg^{-1} only, not 23.7 J mg^{-1}. The difference is 'lost' by the chemically bound energy of the excretion products (see below). Secondly, the composition of organic compounds itself may vary, even if the average energy equivalents remain constant. For example, the occurrence of free glucose within the carbohydrate fraction reduces the accepted factor by its low energy content of 15.4 J mg^{-1}. The seasonal variation of lipids alone ranged between 32.7 J mg^{-1} and 39.4 J mg^{-1} in the studies of Héral and Deslous-Paoli (1983) owing to the variable content of low-energy monoglycerides (22.2 J mg^{-1}) and high-energy free sterols (43.1 J mg^{-1}).

Beukema (1997) considered some of these aspects and suggested the use of values of 17.2 J mg^{-1} for carbohydrates instead of the sometimes recommended 17.6 J mg^{-1}, of 21.4 J mg^{-1} for protein instead of 23.7 J mg^{-1}, and of 35.6 J mg^{-1} for lipids instead of 39.4 J mg^{-1} (see Figure 4.11, 5a, 5b).

Stanczykowska and Lawacz (1976) employed a C, H, N, O elemental analyzer and calculated the energy content from the percentage of elemental fractions. The formula (Figure 4.11, 2) originates from the coal industry (Krevelen and Schuyer 1959) and illustrates the attempt to find suitable conversion factors. The limitations of such an elemental approach have been considered already in the discussion of the stoichiometric approaches by Spoehr and Milner (1949), Gnaiger and Bitterlich (1984) and Vollenweider (1985) in the introductory part of section 4.3.3.

Platt *et al.* (1969) predicted the energy content of dried zooplankton from its carbon and ash percentage (Figure 4.11, 3). The standard error is given as 1.059 J g^{-1} dry mass. Salonen *et al.* (1976) criticized the results as being slightly too high because they are

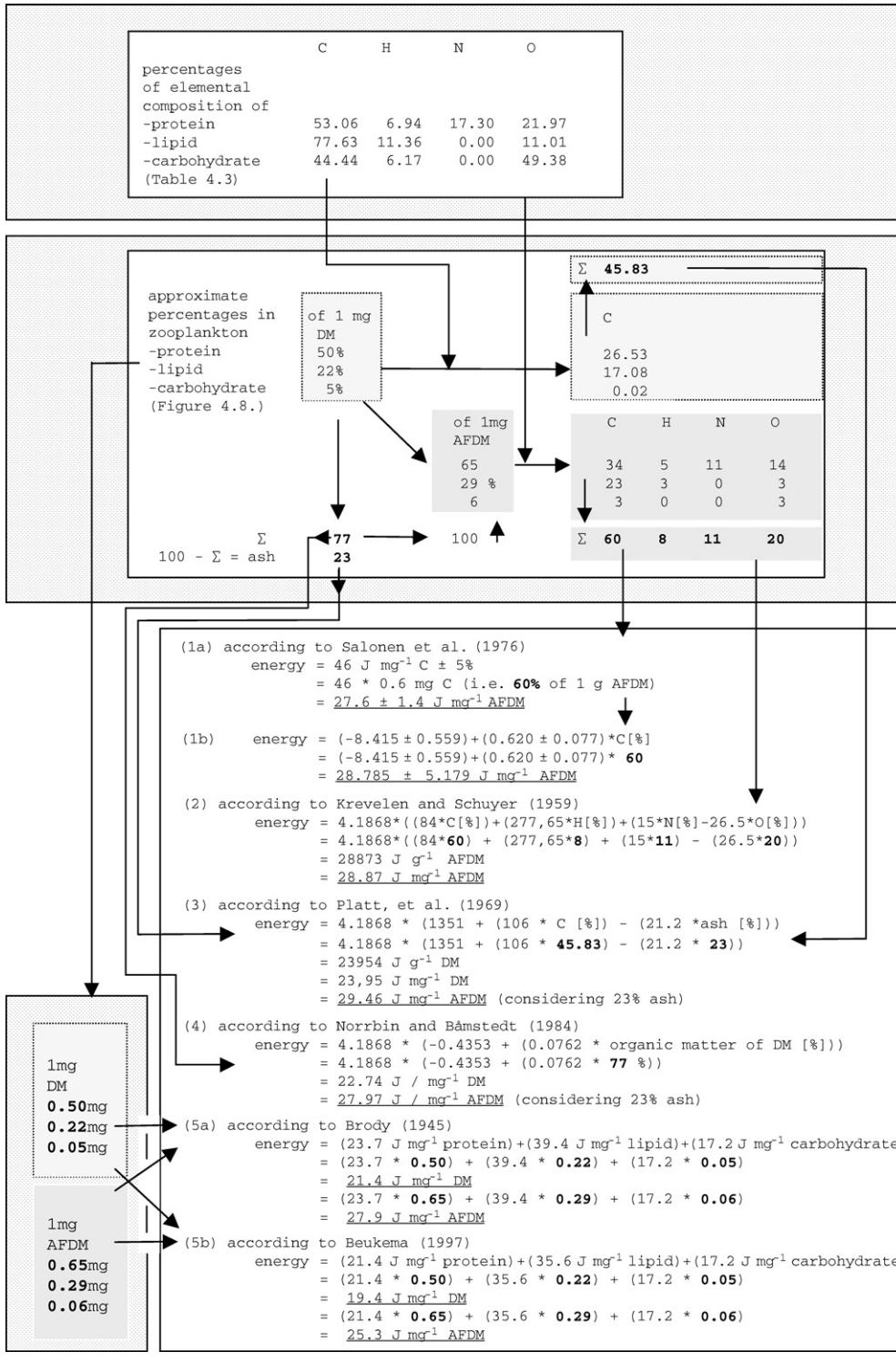
based on wet combustion which underestimates carbon (see page 101). They recommended the equation of Norrbin and Båmstedt (1984) which enables the energy content of dried zooplankton to be calculated in terms of dry mass and organic mass, considering the ash content (Figure 4.11, 4).

Salonen *et al.* (1976) found a significant reduction in interspecific scatter in the energy content, if it was related to carbon (CV = 3.6%) instead of to dry mass or to AFDM (CV = 10.9 and 16.1, respectively). Besides, a micro-carbon determination has been developed (Salonen 1979) which needs 10^4 times less material and 20 times less time than a direct determination by 'micro-bomb calorimetry' using the Phillipson device. These advantages make a reliable conversion factor with satisfactory accuracy quite possible. Winberg (1971) recommended using a value of 41.9 kJ g^{-1} carbon as an approximation. Salonen *et al.* (1976) proposed the use of 46 kJ g^{-1} carbon (see Figure 4.11, 1a) with a precision of $\pm 5\%$ ($p < 0.95$). However, Beukema (1997) noted that this precision is restricted to the determination and does not reflect the potential variability of the biochemical composition of the material. Salonen *et al.* (1976) considered the problem and proposed an equation that takes the percentage of carbon in ash-free dry mass into account (see Figure 4.11, 1b). Nevertheless, uncertainty remains because the same amount of carbon corresponds to an energy content about 1.4 times higher, if it originates from lipids and not from carbohydrates.

The calculations given in Figure 4.11 yield similar results. Those of formulae 5a and 5b differ by the yield of total and physiological oxidation. This often-discussed difference is about half of that originating from the inter-specific variations considered in formula 1b.

The so-called *direct methods* are based on an oxidation process and utilize one of two measurement principles, either the measurement of the *heat content* or *the amount of oxygen consumed*. In the latter case, the utilized oxygen is converted to energy. Strictly speaking, this method could also be considered as an indirect one.

The *wet oxidation* with dichromate and the conversion of the consumed oxygen by an 'oxycaloric coefficient' of 14.2351 kJ g^{-1} oxygen is based on reactions restricted to carbon-containing material (Ivlev 1934; Maciolek 1962). The method is rather seldom used (Beers 1976), because of the incomplete oxidation, especially of nitrogen-containing material (e.g. Ostapenya and Sergeev 1963). Henken *et al.* (1986) employed a special equipment for the determination of chemical oxygen demand (COD), including a thermoreactor, a commercially available kit of reagents, and a spectrophotometer. This method allows the parallel treatment of 12 samples for 2 h at 148 °C. Only 1.5 mg of dried material per sample are needed. Each sample is suspended in 2 ml distilled water. Next, 2 ml of the reaction mixture (6.129 g $K_2Cr_2O_7$ in 60 ml distilled water made up to 500 ml with concentrated H_2SO_4 containing 10 g l^{-1} $AgSO_4$) are added. The oxidation is carried out with an excess of $K_2Cr_2O_7$. The H_2SO_4 masks the chloride and $AgSO_4$ is used as a catalyst for the oxidation. The COD is estimated by a spectrophotometric determination of the Cr^{+3} formed, measuring the difference between the samples and the reagent blank at 623 nm (Golterman and Clymo 1969). After converting the oxygen demand into energy units, the latter are corrected for the incomplete protein oxidation, using a value of 3.9775 kJ g^{-1} protein (Elliott and Davidson 1975). Besides the disadvantage that the correction requires the determination of protein, the corrected results remained lower by 8% than those derived from parallel bomb calorimeter measurements and from calculations based on biochemical composition (Henken *et al.* 1986). The authors explained the difference as being due to the lack of consideration of nitrogen compounds not bound to proteins. They suggested a total N correction by a factor of 1.18 to 1.23 based on the deviation between the results of wet combustion


C H N O
percentages of elemental composition of
-protein 53.06 6.94 17.30 21.97
-lipid 77.63 11.36 0.00 11.01
-carbohydrate 44.44 6.17 0.00 49.38
(Table 4.3)
approximate percentages in zooplankton
-protein
-lipid
-carbohydrate
(Figure 4.8.)
of 1 mg DM
50%
22%
5%
of 1mg AFDM
65
29 %
6
Σ 45.83
C
26.53
17.08
0.02
C H N O
34 5 11 14
23 3 0 3
3 0 0 3
Σ 60 8 11 20
Σ 77
100 - Σ = ash 23
100
(1a) according to Salonen et al. (1976)
energy = 46 J mg-1 C ± 5%
= 46 * 0.6 mg C (i.e. 60% of 1 g AFDM)
= 27.6 ± 1.4 J mg-1 AFDM
(1b) energy = (-8.415 ± 0.559)+(0.620 ± 0.077)*C[%]
= (-8.415 ± 0.559)+(0.620 ± 0.077)* 60
= 28.785 ± 5.179 J mg-1 AFDM
(2) according to Krevelen and Schuyer (1959)
energy = 4.1868*((84*C[%])+(277,65*H[%])+(15*N[%]-26.5*O[%]))
= 4.1868*((84*60) + (277,65*8) + (15*11) - (26.5*20))
= 28873 J g-1 AFDM
= 28.87 J mg-1 AFDM
(3) according to Platt, et al. (1969)
energy = 4.1868 * (1351 + (106 * C [%]) - (21.2 *ash [%]))
= 4.1868 * (1351 + (106 * 45.83) - (21.2 * 23))
= 23954 J g-1 DM
= 23,95 J mg-1 DM
= 29.46 J mg-1 AFDM (considering 23% ash)
(4) according to Norrbin and Båmstedt (1984)
energy = 4.1868 * (-0.4353 + (0.0762 * organic matter of DM [%]))
= 4.1868 * (-0.4353 + (0.0762 * 77 %))
= 22.74 J / mg-1 DM
= 27.97 J / mg-1 AFDM (considering 23% ash)
1mg DM
0.50mg
0.22mg
0.05mg
(5a) according to Brody (1945)
energy = (23.7 J mg-1 protein)+(39.4 J mg-1 lipid)+(17.2 J mg-1 carbohydrate)
= (23.7 * 0.50) + (39.4 * 0.22) + (17.2 * 0.05)
= 21.4 J mg-1 DM
= (23.7 * 0.65) + (39.4 * 0.29) + (17.2 * 0.06)
= 27.9 J mg-1 AFDM
1mg AFDM
0.65mg
0.29mg
0.06mg
(5b) according to Beukema (1997)
energy = (21.4 J mg-1 protein)+(35.6 J mg-1 lipid)+(17.2 J mg-1 carbohydrate)
= (21.4 * 0.50) + (35.6 * 0.22) + (17.2 * 0.05)
= 19.4 J mg-1 DM
= (21.4 * 0.65) + (35.6 * 0.29) + (17.2 * 0.06)
= 25.3 J mg-1 AFDM

(without N correction) and of bomb calorimetry. This proposal is based on results from dried material with protein contents of about 50% and 70,% which is appropriate for zooplankton (see Figure 4.8). This general uncertainty restricts the advantages of the method to the fact that it is fast and requires a small sample amount. Finally, Henken *et al*. (1986) investigated the variation by comparing replicates of the energy determination using three methods: component analysis, bomb calorimetry, and a COD approach. The coefficients of variation (CV) were similar and smaller than 2% in all cases.

Oxygen bomb calorimetry measures the quantity of *heat produced by a complete dry combustion* of organic constituents in a pure oxygen atmosphere. There are two types, the isothermic or adiabatic and the diathermic or non-adiabatic. In the first case the temperature change is compensated and the energy required is measured (e.g. McEwan and Anderson 1955). The other type detects a temperature difference proportional to the heat change produced. It is used in ecological studies in different versions which are distinguished according to their sensitivity. The '*Parr type*' (Parr Instruments), a semi-microcalorimeter, was commonly used in ecological research up to the middle of the 1960s, and is also called a water jacket calorimeter. The operational range is from 10 mg up to 150 mg dry mass. Quantities of 40–70 mg are preferably used and 70 mg was found to be optimal in a test series of samples between 6 mg and 95 mg (Comita and Schindler 1963). The operation is time consuming and allows the treatment of two samples per hour. Time is needed for filling the water bucket and weighing it, for equalizing temperatures between the bomb and the surrounding water, and for the combustion itself. The use of a chart recorder instead of a thermometer and of two bomb heads can save some time (Paine 1971). These disadvantages were reduced by the *micro-calorimeter* of *Phillipson* (1964). The procedure takes 8–10 min from closing the bomb to completion of the data output, which is generally done on a chart recorder. Richman (1971) recommended this equipment for plankton studies because it operates over a suitable range between 1 mg and 50 mg dry mass, preferably between 10 mg and 15 mg. There is no water jacket and the temperature difference is measured between the heated and the constant block by thermocouples. The precision depends on the number of thermocouples employed. An insulating layer covering the bomb reduces heat exchange with the surroundings. Phillipson (1964) reported a precision of CV below 2.5%, similar to the results of Henken *et al*. (1986).

Scott and Marlow (1982) introduced a *micro-calorimeter* with a range of 0.4–4.0 J. The

Fig. 4.11 Examples of calculations of the energy content of zooplankton using (1a) the regression between the carbon percentage of organic matter (ash-free dry mass, AFDM) and the energy content, (1b) an average energy equivalent of organic carbon, (2) the regression between the percentages of carbon, hydrogen, nitrogen and oxygen and the energy content of AFDM, (3) the multiple relationship between organic carbon and ash percentages to the energy content of dry mass (DM), (4) a relationship between dry mass and the energy content, (5) the percentages of proteins, lipids and carbohydrates in both dried and organic material (with and without ash), and the conversion factors for them, assuming the total (a) and 'physiological' oxidation (b). The calculations are based on the same conditions (dotted boxes), the percentage of elements in the organic compounds according to Table 4.3 and on the approximate zooplankton composition of proteins, lipids, and carbohydrates according to Figure 4.8. The steps of the calculation are indicated by arrows. The values used are printed in bold type.

greater sensitivity was achieved by a hundred-fold amplification of the measured voltage difference. The lower detection limit of 0.4 J corresponds to 20 μg benzoic acid, which means a fifty-fold higher sensitivity than is obtained with the Phillipson bomb calorimeter. For example, 100 rotifers are sufficient for an accurate measurement. However, the micro-technique of carbon determination (Salonen 1979) only requires organic material in the range of one-tenth of a single rotifer (see page 101) which means a thousand-fold higher sensitivity. The calorimetric measurement certainly considers the varying percentage of organic components, in contrast to the already mentioned unified conversion from carbon to energy units. Another advantage of the micro-calorimeter is the use of a five times smaller oxygen pressure than the Phillipson apparatus. A lower pressure might positively influence the efficiency of the combustion and temperature recording owing to a reduction of temperature and turbulence following ignition (Paine 1971). The precision of the method is reported with CV of 4%.

Generally, the *procedural steps* are similar for the non-adiabatic calorimeters. The freeze-dried zooplankton samples are pulverized and homogenized in an agate mortar, and pressed into pellets to avoid the loss of material during filling the bomb with oxygen at 30–35 $\times$ 10^5 Pa. A previously dried and weighed pellet (see page 91) is placed in a tared platinum pan and combusted using platinum fuse wire in a pure oxygen atmosphere. The liberated heat is potentiometrically measured and recorded. The graphs are analyzed and the voltage converted to energy using a calibration by substances of known energy content. Benzoic acid 'for calorimetry' is mainly used in the expected measuring range. Its energy content is declared by the manufacturer, for example 26 433 $\pm$ 4 J g^{-1} (Phillipson 1964). Heavy combustible material, for example gelatinous plankton with a high salt content, might be mixed with a known amount of benzoic acid. Percy and Fife (1981) added 10–30% to samples of ctenophores and hydromedusae. Sometimes nitrocellulose filters were combusted together with the material. For example, the energy content of Millipore filter (5 μm pore size) amounts to 12 998 $\pm$ 154 J mg^{-1} (Comita and Schindler 1963).

A step-by-step description of the Phillipson calorimeter technique, including a detailed list of equipment needed, calculation sheet, calibration procedure, and graphic analyzing method, is given by Prus (1975) and Fraschetti *et al.* (1994).

Figure 4.12 shows the main procedures of dry and wet combustion and some steps for corrections, modified after Cummins and Wuycheck (1971).

There are some *corrections* recommended for potential acid formation, fuse wire and endothermic processes by carbonates as well as for some errors associated with the material combusted.

Acid production (Golley 1961) is a minor source of error. Nitric and sulfuric acid formation takes place under pressure during the combustion of organic compounds and requires a reduction of the result by 1–2% (Paine 1971).

Fuse wire glows during ignition, producing about 8–12 J per combustion depending on the amount of wire. A correction by -0.4 J mg^{-1} for platinum wire and -1.4 J mg^{-1} for nickel-chromium is recommended by Mott and Parker (1959), cited by Paine (1971). Additionally, fuse wire contaminates the sample residuals. Therefore ash determination should be separately performed in any case.

Chemical reactions within the bomb are complex. The decomposition of carbonates is a serious problem and has to be taken into account, if more than 25% of dry mass is carbonate. This might be the case in zooplankton groups with a high amount of ash (see Table 4.2). The decomposition of $CaCO_3$ at 898 °C into CaO and CO_2 is an endothermic process, which theoretically consumes 1.8 J mg^{-1} $CaCO_3$. In practice, Paine (1966)

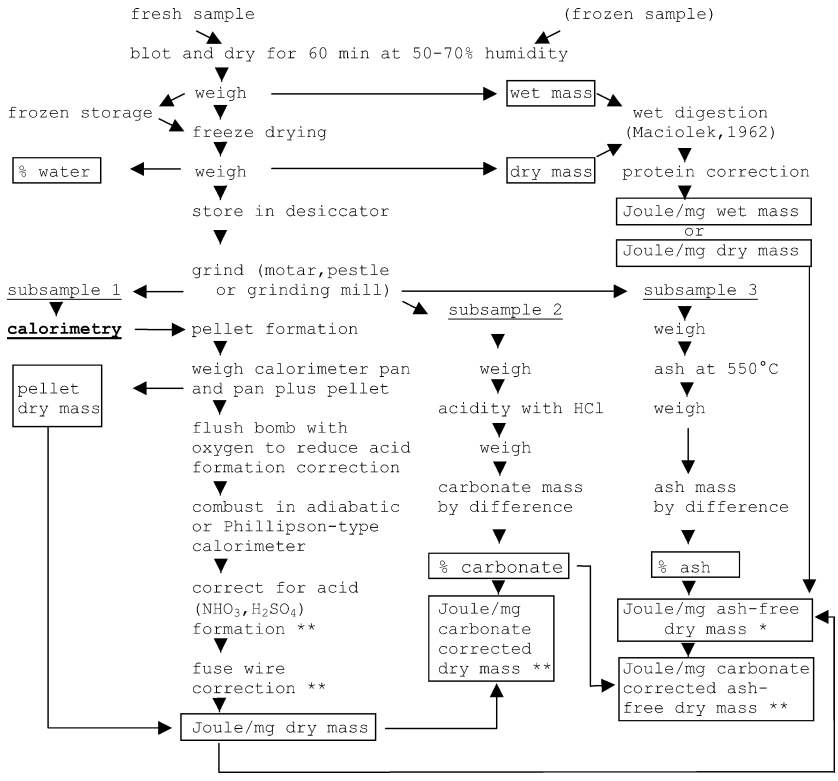


Fig. 4.12 Wet and dry combustion procedure of energy measurements modified according to Cummins and Wuycheck (1971). *, about 10% correction for the lesser physiological energy content (Kersting 1972; Salonen *et al.* 1976; Beukema 1997; see text); ** corrections that are often negligible.

empirically determined a value of $-0.586\ \mathrm{J\ mg^{-1}}$. The difference between the two values is probably due to compensatory effects of the complex chemical reactions. The correction requires a carbonate determination as indicated in Figure 4.12 or by weighing a sub-sample before and after the combustion at 925 °C (Cummins and Wuycheck 1971). The decomposition and sublimation of carbonates are further reasons to determine the ash content separately in a muffle furnace at 500 °C (see page 93).

Norrbin and Båmstedt (1984) analyzed the correlation between the percentage of 35 'unburned residues from combustion in the bomb calorimeter (x), and percentage ash in the material (y)' mainly of copepods and found $y = 6.6105 + 1.0581x$ ($r = 0.964$). This equation allows some correction of samples lacking ash determination.

Hydrated skeletal material produces an error if the bound water is not removed during the dry mass determination process. It affects the ash-free dry mass values owing to the subtraction of the ash mass (see page 93). This effect is minimized by using a two-step freeze-drying procedure which includes a final drying phase (see page 91). In any case it is important to prevent the uptake of humidity before and during weighing of the material pellets.

It has been already noted that there is no complete oxidation of nitrogen-containing products to N_2 in nature but this does occur in bomb calorimetry. Kersting (1972) proposed a correction for the physiologically irrelevant part of energy. Assuming

ammonia as the main excretion product, the nitrogen correction amounts to $-24.7\ \mathrm{J\ mg^{-1}}$ organic nitrogen (urea excretion is of minor importance in zooplankton, but it would require a correction of $-30.1\ \mathrm{J\ mg^{-1}}$).

A correction would imply the determination of the organic nitrogen content. However, Salonen *et al.* (1976) published energy values of 19 species of fresh water invertebrates (copepods, cladocerans, mysids, isopods, amphipods, etc.) in two versions, with and without the nitrogen correction of Kersting (1972). The differences between the averages of both data sets are significant at $p < 0.05$, independent of whether the energy content is related to ash-free dry mass (AFDM) or organic carbon (org.C). The regressions presented here are significant at $p < 0.05$ and allow the determination of the 'physiological' energy values from data obtained by bomb calorimetry:

$$[\mathrm{J.mg\ AFDM^{-1}}]_{\mathrm{N\ corrected}} = -2.978 + 1.032 \times [\mathrm{J.mg\ AFDM^{-1}}]_{\mathrm{N\ uncorrected}} \quad (4.5)$$

or

$$[\mathrm{J.mg\ org.C^{-1}}]_{\mathrm{N\ corrected}} = -4.059 + 0.994 \times [\mathrm{J.mg\ org.C^{-1}}]_{\mathrm{N\ uncorrected}} \quad (4.6)$$

In general, the correction amounts to about 10%. It corresponds to the difference between the results obtained according to formula 4a in Figure 4.11, using the energy equivalents from total combustion (Brody 1945), and 5b, taking the physiologically relevant factors (Beukema 1997) into account.

However, Salonen *et al.* (1976) suggested avoiding such considerations in energy budget calculations at the ecosystem level, because excretion products become further degraded by micro-organisms.

Relating the energy content to ash-free dry mass or organic carbon is useful as it produces comparable energy equivalents. Relating it to dry mass might be more useful from the trophodynamic point of view because it provides a measure of the relative energy value to the consumer of a given mass of food (Percy and Fife 1981).

Weighing up the *pros and cons*, direct calorimetry (i.e. measuring the amount of heat production of the combustion) is the most suitable way to obtain accurate data. This method accounts for all variations of the material, between species, seasons, etc., the internal composition of the main groups of organic compounds, and the varying energy value for carbon depending on the specific composition of these compounds. The major disadvantages are the laborious processing and the necessarily large sample amount. Even for the micro-version of the Phillipson device of Scott and Marlow (1982) a 1000-fold higher sample amount is needed as compared to the micro–carbon method of Salonen *et al.* (1976). Wet oxidation as a means of determining the chemical oxygen demand is faster than bomb calorimetry because of parallel sample processing and it needs much less material. However, the oxidation of protein nitrogen is incomplete and demands a correction by 18–23%. When using the recommended $3.98\ \mathrm{J\ mg^{-1}}$ protein value as a correction factor, the results are not directly comparable to those obtained by high-temperature combustion. They are still smaller by about 8% since the non-protein nitrogen is not considered. Consequently, these results would be in the same range as the incomplete, i.e. physiological oxidation, neglecting the excretion products. This amounts to about 10% and is taken into account by the so-called nitrogen correction of Kersting (1972), by the application of the reduced energy equivalents for the main organic compounds (Beukema 1997) or by the equations proposed in the previous text (see Figure 4.11). Calculations of energy content from the organic or elemental composition,

especially the carbon content, provide suitable results. Salonen *et al*. (1976), for instance, reported a CV of $\pm 5\%$ (Figure 4.11, 1a) using the energy equivalent of carbon, and $\pm 18\%$ (Figure 4.11, 1b) considering samples of multispecies composition. The general disadvantage of conversion factors or equations is that they are invariant in relation to the internal composition of the main components or the origin of carbon.

The precision of direct determination of energy content ranges between 2% and 4%. Principally, the use of freeze-dried material is recommended for the reliable removal of bound water. Complete homogenization during pulverization of the samples minimizes the variability between sub-samples. The recommended corrections (see Figure 4.12) are less important except for the endothermic degradation of carbonates in bomb calorimetry, when the carbon content is larger than 25%.

Specific energy data should be related to ash-free dry mass, because a large number of comparable equivalents exists for a wide range of organisms. However, reference to carbon results in lower variability than that for AFDM and much lower than for dry mass. The dry mass related energy content is suggested for trophodynamic studies.

ADENOSINE TRIPHOSPHATE (ATP)

All the previously mentioned methods for determining biomass or energy do not distinguish between live and dead organisms in mixed samples. Even desoxyribonucleic acid (DNA), the carrier of the genetic information in living cells, is stable in dead organisms until complete degradation and would not be a suitable proxy for living material. Non-viable pelagic organic matter is called tripton (see Table 4.1) and consists of molting products, remnants of 'sloppy feeding', excrements, and dead organisms. Its occurrence is a function of both formation and relative increase due to selective feeding on living organisms.

Carbon values using ATP estimated from microplankton studies seem to be reliable and give values consistent with other estimates. For example, less than half of the organic carbon content belongs to living organisms within this size fraction, for example in depths below 100 m off San Diego, California (Figure 4.13a) and near the ice edge or in the open waters of the Bransfield Strait (Antarctica) where grazing pressure is highest on live microplankton at station 11 (Figure 4.13b). Even plankton larger than 333 μm may be non-viable, between 10%, 30% and 60% (depending on the C/ATP conversion factor used – whether 227, 178 or 90) being reported in the upper 200 m off Monterey, California (Traganza and Graham 1977).

Budget calculations on matter transfer or energy flow especially suffer from the uncertainties stemming from the non-viable organic material. They rely on *in vitro* activity or productivity measurements which are extrapolated to *in situ* conditions considering the *bio*mass in a certain volume of water.

In routine programs it is difficult to *separate plankton from tripton*, especially in smaller size classes. For example, the use of light traps taking advantage of positive phototactic behavior is not applicable to all zooplankton groups (Behrendt and Krocker 1990). Microscopic analysis in connection with biomass calculations is very laborious, and better methods were sought. The search for an indicator of exclusively living material began in the 1960s with special reference to marine bacteria, phytoplankton, zooplankton and sediments (e.g. Holm-Hansen and Booth 1966; Hamilton and Holm-Hansen 1967; Holm-Hansen 1970). Such an indicator should occur in all living cells and be restricted to them. Its cellular concentration should be unaffected by environmental influences. Methods of detection should be sensitive, accurate, precise, and easy to employ.

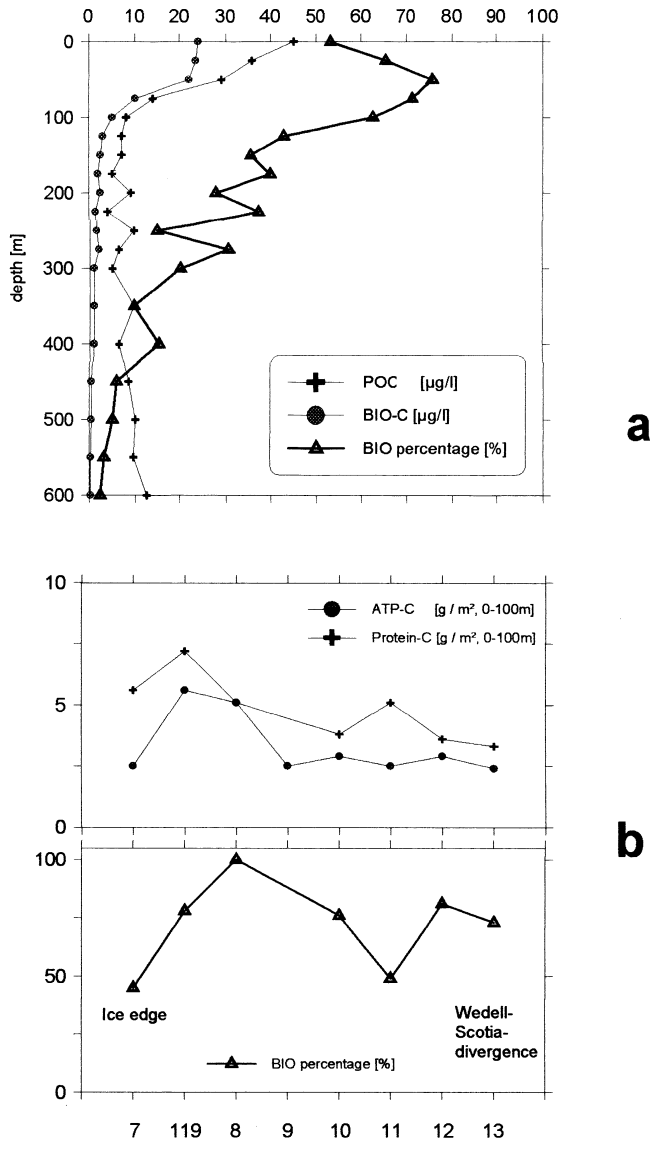


Fig. 4.13 a) Vertical distribution of total particulate organic carbon (POC) in comparison with viable organic carbon (BIO-C) derived from ATP measurements and converted by C : ATP = 250 for microplankton (0.4 μm to 150 μm) expressed as (BIO) percentage of total POC at 32°41′N, 117°35′W, January 1969, modified according to Holm-Hansen (1973); b) Transect of stations showing values for the upper 100 m, Bransfield Strait, Antarctica, January 1994. Protein carbon (derived from protein which was multiplied by 0.513; cf. Table 4.3) compared with ATP carbon (ATP $\times$ 250) of microplankton ($>$0.45 μm). Bottom panel: living material (BIO) expressed as percentage of total organic carbon. Numbers on the abscissa indicate the stations. Modified from Berdalet *et al.* (1997).

Adenosine triphosphate (*ATP*) satisfies these requirements. Ninety-five per cent is produced in the mitochondria by the activity of the respiratory chain in animal cells. Its amount is restricted and it is regenerated quickly. For example, human ATP molecules are produced and utilized about 2400 times a day. The average time between their production and utilization is less than 1 min (Libbert 1986). A short half-life following cell death and autolysis is a consequence. Holm-Hansen and Booth (1966) found only minimal quantities of ATP in killed bacteria and algae, approximately a thousand times less than in living material. This degradation takes place within 1 h at least (Conklin and McGregor 1972). The current methodological guidelines, however, consider a decay within seconds.

Karl (1993) reported a fairly constant level of the intracellular ATP pool in all living cells. Balch (1972) described a stable carbon to ATP ratio over a 23-day starvation period in *Calanus finmarchicus*, during which the individual amounts of ATP, carbon, and nitrogen decreased proportionally. Hamilton and Holm-Hansen (1967) observed a close correlation between the concentration of viable cells and of ATP in phytoplankton cultures outside the exponential growth phase. However, during the limited time of extremely active growth, the ATP content of cells increased by a factor of 4 to 5. Similar findings (a factor of 8) were reported for dry mass specific ATP concentrations by Skjoldal and Båmstedt (1977) during the reproduction period of various zooplankton species. They interpreted the ATP content as a reliable indicator of reproduction activity. Generally, Hamilton and Holm-Hansen (1967) detected an average content of ATP relative to carbon of 0.4% for bacteria (total range 0.3% to 1.1%), 0.35% for algae and 0.5% for *Calanus helgolandicus*. The percentage of 0.4 justified the often recommended factor of 250 to convert ATP to carbon content. The actual measured values, however, required factors ranging from 91 to 333 with a mean of 228. This result is similar to that found by Tranganza and Graham (1977) for mixed zooplankton. Ernst (1958) proposed a C:ATP ratio of 50 for nematodes, which was confirmed by Balch (1972) for *Calanus finmarchicus*, taking non-lipid carbon as a basis, and by Skjoldal (1981) for some tropical zooplankton. Karl (1993) observed C:ATP ratios from about 200 to 350 (by mass) in unicellular micro-organisms and in micrometazoans, ratios from 50 to 150. These variations are considered a serious problem for the general application of ATP as an appropriate indicator for living biomass (Omori and Ikeda 1984).

However, an evaluation and standardization of the methods, especially the extraction procedures and the preparation of samples for carbon determination, on one hand, and considering the basis for appropriate correction factors on the other, would give some hope of minimizing these discrepancies.

Amounts as small as 10^{-15} mol of ATP are detectable, and therefore it was initially used to determine the biomass of bacteria in sea water samples. The frequent application in micro- and meiobenthic studies is explained by the high detritus content in sediments which is very difficult to separate from the amount of viable organisms using other methods. Studies of zooplankton are rare and were mainly published in the second half of the 1970s. Some of them focused on methodological aspects (Balch 1972; Skjoldal and Båmstedt 1977; Traganza and Graham 1977; Karl *et al.* 1978; Skjoldal 1981). Some authors applied the method to test the influence of temperature in the vicinity of a power plant on the mortality of zooplankton (Anraku and Kozasa 1978), or employed it in a transatlantic study (Traganza *et al.* 1979), or for the comparison of the percentage of live specimens in various plankton groups (Mayzaud and Taguchi 1979; Gordienko 1980; Boswell *et al.* 1980). Maranda and Lacroix (1983) observed a

significant change in the ratio between zooplankton ATP and dry mass in a tidal estuary.

There are several analytical *methods of ATP quantification*, for example high-performance liquid chromatography, either for discrete samples or for continuous flow analyses (Karl 1993). However, the preferred procedure is the use of the *bioluminescence reaction* of the American firefly *Photilus pyralis* (De Luca and McElroy, 1974, 1978). This goes back to an observation of McElroy (1947), who noticed that a firefly extract in which the bioluminescent reaction had stopped emitted light again after addition of energy-donating ATP. The duration of the reaction was proportional to the amount of ATP added (cited by Graf 1977).

Generally, during enzymatic reactions an enzyme–substrate complex (ES) is formed first, and secondly a product (P) is derived by regeneration of the enzyme (E). In the special case, D(−)-luciferin is the acting substrate (S), and luciferase the enzyme. Luciferin is oxidized to oxyluciferin, the product (P) which might inhibit the reaction finally. Magnesium ions catalyze the process. ATP is the energy donator for the light reaction and converted to energetically lower APM (adenosine monophosphate). One quantum of light ($h\nu$) is emitted for one hydrolyzed ATP molecule at the pH optimum of 7.75 and in an emission maximum of 562 nm (Seliger and McElroy 1960). The reactions follow in principle:

$$\mathbf{E + S + ATP} \overset{\mathbf{Mg^{++}}}{\rightleftharpoons} \mathbf{(E\text{-}S\text{-}AMP) + diphosphate} \tag{4.7}$$

$$\mathbf{(E^*S^*AMP) + O_2} \longrightarrow \mathbf{E + P + CO_2 + AMP} + h\nu \tag{4.8}$$

The light emission depends only on ATP concentration, if the other components of the reaction are present at saturated concentrations. Accordingly the kinetics of the luminescence reaction, using the extremely purified firefly lantern extract (FLE), consist of a short period of light emission 0.3 s after starting the reaction followed by a phase of declining emission of about 20 min (Figure 4.14). The long duration might be a result of the already regenerated enzyme (DeLuca and McElroy 1974, cited by Graf 1977).

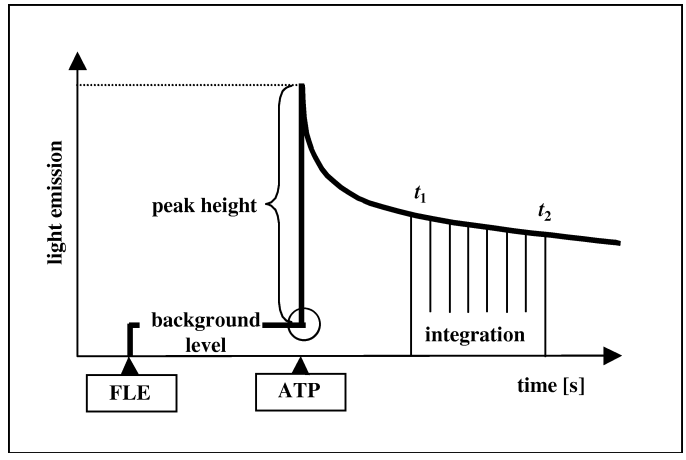


Fig. 4.14 Principal luminescence curve of an ATP measurement after the successive introduction of the enzymatic solution, prepared from firefly lantern extract (FLE), and the ATP-containing sample (ATP) with the integration period between t_1 and t_2. Modified from Graf (1977).

The total procedure includes *several steps*, the preparation of gently caught samples, the ATP extraction, its determination, the calibration of the relative luminescence units (RLU) or the counts per minute (CPM), and the extrapolation of ATP to biomass concentrations.

Balch (1972) *prepared samples* by dropping live individuals into buffer for extraction and breaking them up using forceps. Tranganza and Graham (1977) gently transferred single or mixed species catches onto a screen (see page 89 for applying vacuum or pressure of about 0.3 atm) and immersed this filter into the extraction solution in a glass tissue grinder. This must be done quickly because desiccated organisms lose their viability within seconds. The extraction was started immediately. The filter was removed before grinding. Homogenization of the individuals was found to be the best preparation for effective ATP extraction (Karl *et al.* 1978).

Following extraction, the samples are stored frozen at $-20\,^{\circ}C$ until assayed, if not directly processed. At this stage they are extremely stable. ATP losses are less than 1% per year in properly buffered solutions (Karl 1993). Some manufacturers' standard protocols recommend centrifugation at 10 000 *g* for 1 min and transfer of the supernatant to a fresh test tube before storage until the ATP readings.

Skjoldal and Båmstedt (1977) used a different procedure. They rinsed the animals with distilled water (see section 4.2), fixed the ATP status with liquid nitrogen ($-196\,^{\circ}C$) for 2 min and longer (up to 2 h) and stored the samples afterwards at $-26\,^{\circ}C$ until freeze-drying (see page 91). The dried animals were stored in a desiccated state at –26 °C before weighing and extraction of adenosine phosphates. The different treatment has been tested. The authors reported no disagreement in ATP content whether the animals were killed in the extraction buffer or in liquid nitrogen with a successive lyophilization and extraction. However, storage at $-26\,^{\circ}C$, without fixation by liquid nitrogen, resulted in a 50% less ATP content.

The *ATP* can be rapidly *extracted* and stabilized in solution using boiling buffers, cold acids, or a variety of organic solvents (Lundin and Thore 1975). Boiling with TRIS buffer (0.02–0.05M, pH 7.4–7.7) at 95–100 °C for 3–8 min is the generally accepted procedure (Holm-Hansen and Booth 1966; Skjoldal and Båmstedt 1977). The latter authors used 25–50 ml buffer for samples of 1 mg to 1 g dried zooplankton. It is strongly recommended that the extraction buffer is at its boiling point at the time of sample addition otherwise the procedure remains incomplete. Karl *et al.* (1978) found this especially relevant for multicellular organisms which were not homogenized. In such cases, thermal gradients occur within the particular sample material and hinder a complete extraction. They recommended a cold acid procedure (combined with homogenization of the specimen) in 0.6N H_2SO_4 and ethylenediamine tetra-acetic acid (EDTA) for 5 min. This treatment yields twice as much ATP as the boiling procedure. Skjoldal (1981) similarly applied a trichloric acid (TCA, 0.25M) with EDTA (9.3 mM) extraction of 20–30 min, including a homogenization lasting 1.5 min.

The *ATP detection by bioluminescence* is outlined by Parsons *et al.* (1984) and by Omori and Ikeda (1984), mainly following Holm-Hansen and Booth (1966). It requires first the preparation of reagents.

Firefly lantern extract is commercially available in both crude and purified versions and is stored at $-20\,^{\circ}C$. The content of a 50 mg vial is hydrated for activation in 5 ml of distilled water. The enzymatic activity stabilizes after 4–6 h. This period must be considered before starting the analysis. Parsons *et al.* (1984) recommend keeping the solution in the dark at about 10 °C. Karl (1993) allows room temperature. A crude FLE

solution also contains endogenous ATP and enzymes producing ATP from other extracted nucleotides. The endogenous ATP is consumed during the aging process. However, slowly reacting adenylate kinases might influence the light emission decay curve during the reaction.

The aged 50 mg FLE is combined with 10 ml each of 0.04M $MgSO_4$ (catalyst) and arsenate buffer ($KHAsO_4$, 0.1M, pH 7.4, arsenate to avoid bacterial contamination) followed by 1 h incubation at room temperature (Karl 1993). Non-optimal concentrations of both, the catalyst and the buffer, negatively influence the luminescence strength (Chapelle and Levin 1968; Kimmich *et al.* 1975, cited according to Graf 1977).

The concentrations are finally adjusted to the detection ranges by buffer. Karl (1993) noted that if a single vial of 50 mg desiccated FLE is diluted to a working volume of more than 50 ml, the bioluminescence reaction would become limiting for luciferin (which must be available in a saturated concentration, see above).

The aged enzyme-substrate solution must be used within 24 h. The measurements take place at room temperature. Any one of a range of commercially available instruments, such as fluorometers, spectrophotometers and liquid scintillation counters, are suitable for measuring the light emission. In order to obtain efficient and reliable data with maximum sensitivity a specially designed luminometer (e.g. Berthold Co., Wildbach, Germany) is required. The ATP extract, i.e. the sample extract, and the prepared FLE are poured in a cuvette at room temperature and the light emitted is recorded as relative luminescent units (RLU) or counts per minute (CPM). Using a calibration, these units are converted to ATP concentrations.

The light emission is measured in two ways, either by the *peak height* or the *integrating mode*. Figure 4.14 illustrates the course of the curve during an ATP measurement. It starts with a potential background level (blank) of light emission due to equipment-specific noise as well as interferences by the endogenous ATP content of the enzymatic solution, bacterial contamination, etc. This blank is not considered by the integrating mode but by initial peak height measurement, which is consequently accepted as the more reliable measure of the luminescence yield (Rasmussen and Nielsen 1968). However, its accuracy relies on a rapid and proper mixing of all reactants which is best achieved using an automatic injection system (Stanley 1992). A slow injection lowers the maximum light emission. The peak height response is not detectable by all of the instruments mentioned.

The other mode integrates the signal within a predetermined period (10–20 s) during the decay phase. The time is automatically set by the first light emission in an ATP photometer. The higher sensitivity and reliable mixing conditions counteract the unfavorable measuring moment within the reaction process. The detection is performed when energy for light emission has already significantly decreased, the nucleotide turnover has started, and accompanying enzymes of a less purified FLE system are mostly active (see above). Manufacturers' instructions give information on ways of minimizing the problem in the latest generation of instruments.

The *precision* of the ATP method according to Holm-Hansen and Booth (1966) amounts to 10% at the 95% confidence level. Hansen (1993) reported a precision of the peak high assay procedure of $\pm$1–2% of the mean using the same standard for eight measurements. The accuracy is determined using standards.

The measured ATP value in RLU or CPM must be converted using a *calibration* curve. This is created by a dilution set of the ATP sodium salt in the expected concentration range using buffer and its assay with the luciferin-luciferase reagent. Tranganza and Graham (1977) choose 1 ng to 1000 ng of ATP per ml of buffer. The

standard is prepared by dissolving 12.3 mg disodium triphosphate in 1 l of distilled water (stored frozen in 1 ml aliquots, it is stable over months). Further dilution of 1 ml in 100 ml buffer leads to a corresponding concentration of 0.2 ml equal to 20 ng ATP. In commercially available ATP bioluminescence assay kits, the exact ATP content of the standard is determined individually for each lot and indicated. The treatment of the calibration solution must be identical with that of the organisms, including the extraction procedure. This means that plankton extracted with H_2SO_4 is not comparable with a calibration curve treated with TRIS (Karl and Larock 1975, cited by Graf 1977). The light intensity emitted is directly proportional to the concentration of ATP in the sample. The linearity holds over 4 to 5 orders of magnitude of ATP concentration. Therefore, the use of a calibration factor based on three averaged determinations of standards is justified instead of a curve (Parsons *et al.* 1984). A repeated check of the calibration factor between the measurements is recommended (e.g. before, after, and in the middle of a set or after every fifth sample). Changing enzymatic activity of the prepared FLE solution requires new calibrations for a set of determinations.

The presence of ions in the sample extract, enzymatic inhibitors or activators, etc. (Karl 1993) interferes with the assay, especially when the measurements are done during the declining luminescence phase (integration mode). The application of an *internal standardization*, the 'standard addition' technique, allows correction of these influences. For this purpose a known amount of ATP (disodium triphosphate solution) is added to the sample of unknown ATP content, and the added amount still detectable despite the interferences is measured. The quantity of ATP addition depends on the expected ATP level of the samples. Readings of the internal standard which are 2 to 5 times higher than those of the samples guarantee best accuracy in the calibration (Karl 1993).

The internal standardization is applied in two ways. One is the determination of a recovery factor (R) and correction of the sample's ATP content, which has already been calculated using the blank and a calibration factor (curve). The recovery factor (R) is calculated according to Bancrofft *et al.* (1974, cited by Graf 1977), as:

$$\mathbf{R = (ATP_{sample + standard} - ATP_{sample})/ATP_{standard}} \tag{4.9}$$

The recovery factor should be larger than 0.75 (Parsons *et al.* 1984). The estimated ATP content is calculated by multiplying the blank-corrected luminescence units of the sample with the calibration factor and dividing the result by the recovery factor R.

Another approach, which includes the calibration and the correction in one step, is to calculate an internal calibration factor k basing on three averaged replicates of each component, the internal standard (sample + standard), the blank, and the sample, as follows:

$$\boldsymbol{k = \frac{ATP_{standard}}{(RLU_{sample + standard} - RLU_{blank}) - (RLU_{sample} - RLU_{blank})}} \tag{4.10}$$

where k is the amount of ATP per RLU (or CPM respectively). The internal calibration factor k is finally used to calculate the ATP content of the sample set from the relative luminescence units (RLU) or counts per minute (CPM) respectively, according to:

$$\mathbf{ATP_{sample} = \boldsymbol{k} \times RLU_{sample}} \tag{4.11}$$

The calculation of the ATP concentration in the marine environment must consider possible dilution factors of the homogenate and sub-sampling procedures as well as the volume of water filtered by the plankton net.

The *conversion of ATP content to biomass*, for example carbon, depends on a reliable ratio between both. While the ATP content of organisms may vary with their physiological condition, the body carbon content is influenced by both morphology and storage properties. Some parts or compounds that consist of organic carbon do not include any ATP, for example exoskeletons or lipids. Consequently, interspecific variations and different life phases might influence the C:ATP ratio. These problems are satisfactorily soluble only by actual calibrations. Representative sub-samples of exclusively living plankton must be selected and treated in the same manner for ATP and carbon as those that include partially non-living organic matter. Time for such work could be saved during cruises if the samples for ATP analysis are fixed by liquid nitrogen and the selection of specimens is made with help of the light trap mentioned above (Behrendt and Krocker 1990).

Total carbon determination (see page 101) requires an aliquot of the sample homogenate. It must be taken prior to the extraction procedure (cf. Tranganza and Graham 1977).

Consequently, ATP determination is a sensitive tool for the identification of living mass, i.e. biomass. It is quite accepted for microbiological and micro- and meiobenthic investigations for different reasons. In the latter case, the difficulty in discriminating the large amount of detritus in such samples necessitates the application. Such conditions are also met for mesozooplankton in eutrophic waters, density discontinuities, etc. Distinguishing the non-living material from biomass is ultimately needed, for example for the extrapolation of *in vivo* matter and energy flux to *in situ* conditions. In cases of high sample densities, the determination of ATP might be a real alternative to the laborious work of species identification and subsequent conversion to biomass using morphometric measures or individual factors. However, some problems must be solved before.

The first precondition is the standardization of the different steps of the method (ATP fixation by liquid nitrogen, homogenization, total carbon samples from aliquots taken before ATP extraction, cold acid extraction, bioluminescence reading by peak high determination, internal standardization). An adaptation of commercially available standard protocols, delivered with ATP bioluminescence assay kits, for zooplankton purposes might be helpful in these respects.

The determination of actual C:ATP ratios by the analysis of a limited amount of selected live samples is the second task and should be a part of a sampling program.

A third problem, the avoidance of sampling stress on the organisms and its consequences on the ATP level (Skjoldal and Båmstedt 1977) requires an extremely gentle sampling technique. At least, its potential influence must be checked by an actual test of energy charge.

Caution is also needed concerning the ATP content of the 'Aufwuchs', formed by micro-organisms settled on detrital material.

New approaches for determining the mass of living material, for example the application of biochemical cell death detection methods or video techniques, linked with an automatic analysis of living (motile) and non-living particles, should be adapted or developed.

4.3.4 Conversion factors and equations

The use of the methods discussed here depends on the purpose, the equipment available, the sampling procedure, and whether the same material is required for another analysis or not. Sometimes it is not possible to achieve the optimal technique. A conversion from one biomass proxy to another may eventually be the only compromise to compare the outcomes with values from the literature.

The ratios between the results of different methods depend on their analytical suitability as well as their precision and accuracy. They are also affected by variances originating from natural sources, such as morphology and body composition of different species, their life stages and in some cases their metabolic states. All these factors have to be taken into account when using conversion procedures.

Constantly related biomass values are the result of standard relations between them. Their conversion is simply the application of percentages listed in Tables 4.2 (ash content of dry mass), 4.3 (elemental composition of organic compounds), 4.4 (elemental composition of various dried taxonomic groups) and 4.5 (atomic ratios in zooplankton groups), briefly reviewed in section 4.3.3 and demonstrated in Figure 4.8 (biochemical composition of different dried taxonomic groups). Energy equivalents are discussed on page 122 and the conversion between ATP and organic carbon on page 131. While these percentages mainly concern biochemical aspects, Table 4.7 and the last column of Table 4.8 also include ratios of the conventional biomass parameters. The last compilations mentioned are specified for total zooplankton and mesozooplankton (some taxonomic groups), and cover different geographical regions, i.e. environmental conditions. Authors and other specifications are listed in Table 4.9.

However, these (constant) percentages (ratios or equivalents) are restricted to coarse estimates. The widely used compilation produced by the ICES Committee on Terms and Equivalents (Cushing *et al.* 1958) is included in Table 4.7 as well as conversion factors for settled volume (SV), displacement volume (DV), wet mass (WM), dry mass (DM), ash-free dry mass (AFDM), organic carbon (C), nitrogen (N), phosphorus (P), and proteins.

Uncritical use of conversion factors may lead to incorrect interpretations. Natural variability or methodological errors which are not related to the amount of biomass may produce a certain degree of scatter in the data and may influence the slope of a relationship. This is reflected in Table 4.7 by the ratios of author 3 (Bode *et al.* 1998) which vary with the amount of biomass. The underlying relationship is clearly non-linear. In most cases biomass relations are not a case of simple proportionality (Wiebe *et al.* 1975). The authors described this with the help of the power function $y = ax^{\beta}$. The influence of the exponent β is demonstrated in Figure 4.15, on both normal and logarithmic scales. The simple ratio (percentage) is characterized by $\beta = 1$. This corresponds to the linear regression model $y = a + x$. Non-linear relationships are expressed by $\beta \neq 1$.

The use of the power function is appropriate if the $\log_{10}$ (usually written as lg) of the two biomass parameters is linearly related in a plot, i.e. if β is constant. Then $\lg y = \lg a + \beta \lg x$. The constant a and $\lg a$ are known as the axis intercept of the y axes, describing the deviation from 0 or $\lg 0 = 1$ respectively (not shown in Figure 4.15).

Most of the relationships compiled in Table 4.8 follow this type. The part of the equations which corresponds to the simple ratio or percentage, the antilog of the intercept a, is listed in the last column. From a mathematical point of view, it has the same meaning as the ratios in Table 4.7.

Table 4.7 Conversion factors of zooplankton biomass: displacement volume (DV), wet mass (WM) and dry mass (DM) standardized to settling volume (SV); WM, DM, ash-free dry mass (AFDM), protein, organic carbon (C), nitrogen (N), and phosphorus (P) related to displacement volume; AFDM, protein, C, N, and P related to dry mass; protein and C normalized to AFDM, and phosphorus standardized to nitrogen, according to Bode *et al.* 1998 (3), Cushing *et al.* 1958 (4), Hernández-León *et al.*, personal communication (5), Larson 1986 (6), Postel 1990 (9), and Wiebe 1988 (10).

Parameter and units	Conversion factor	Author (cf. Table 4.9)	Remarks
cm^3 DV SV cm^{-3}	0.35	10 and 1	Recalculated from 0.013 cm^3 DV mg^{-1} DM (10) and 0.383 cm^3 SV mg^{-1} DM (1) from Table 4.8; without gelatinous plankton
cm^3 DV SV cm^{-3}	0.06	10 and 9	Recalculated from 0.24 cm^3 DV mg^{-1} DM (9) and 0.383 cm^3 SV mg^{-1} DM (1) from Table 4.8; including gelatinous plankton
mg WM SV cm^{-3}	195	4	ICES Committee on Terms and Equivalents
mg DM SV cm^{-3}	26		
mg WM cm^{-3} DV	800	4	ICES Committee on Terms and Equivalents
mg DM cm^{-3} DV	160		
mg DM cm^{-3} DV	*42*		*0.1 cm^3* of DV, coastal upwelling, NW Spain, 90% copepods
	71	3	1.0 cm^3
	111		*10.0 cm^3*
	200		*100.0 cm^3*
mg DM cm^{-3} DV	132 $\pm$ 26		Oligotrophic, subtropical waters, mixed
mg AFDM cm^{-3} DV	91 $\pm$ 16	5	mesozooplankton
mg protein cm^{-3} DV	25 $\pm$ 5		
mg C cm^{-3} DV	96	4	ICES Committee on Terms and Equivalents
mg C cm^{-3} DV	*10*	3	*0.1 cm^3* of DV, coastal upwelling, NW Spain, 90% copepods
	21		1.0 cm^3
	41		*10.0 cm^3*
	81		*100.0 cm^3*
mg N cm^{-3} DV	16	4	ICES Committee on Terms and Equivalents
mg N cm^{-3} DV	*3*	3	*0.1 cm^3* of DV, coastal upwelling, NW Spain, 90% copepods
	4		1.0 cm^3
	6		*10.0 cm^3*
	9		*100.0 cm^3*
mg P cm^{-3} DV	1.3	4	ICES Committee on Terms and Equivalents

continued

Table 4.7 *continued*

Parameter and units	Conversion factor	Author (cf. Table 4.9)	Remarks
mg DM mg^{-1} WM	0.20	4	ICES Committee on Terms and Equivalents
mg DM mg^{-1} WM	0.035–0.05		gelatinous zooplankton: hydromedusae, siphonophores, scyphomedusae, ctenophores, thaliaceans
		6	semi-gelatinous zooplankton:
	?0.05–0.15		chaetognaths
	0.035–0.30		mollusks
	0.10–0.30		non-gelatinous zooplankton: crustaceans
mg C mg^{-1} WM	0.12	4	ICES Committee on Terms and Equivalents
mg N mg^{-1} WM	0.020		
mg P mg^{-1} WM	0.0016		
mg AFDM mg^{-1} DM	0.696 $\pm$ 0.048	5	Oligotrophic, subtropical waters, mixed mesozooplankton
mg AFDM mg^{-1} DM	*0.80*		*0.1 mg* of DM, coastal upwelling, NW Spain, 90% copepods
	0.81	3	1.0 mg
	0.80		*10.0 mg*
	0.78		*100.0 mg*
mg AFDM mg^{-1} DM	0.20–0.50	6	gelatinous zooplankton: hydromedusae, siphonophores, scyphomedusae,
	0.20–0.35		ctenophores, thaliaceans
	0.40–0.75		semi-gelatinous zooplankton: mollusks
	0.65–0.85		non-gelatinous zooplankton: crustaceans
mg protein mg^{-1} DM	0.192 $\pm$ 0.025	5	oligotrophic, subtropical waters, mixed mesozooplankton
mg C mg^{-1} DM	0.60	4	ICES Committee on Terms and Equivalents
	0.03–0.15		gelatinous zooplankton: hydromedusae, siphonophores, scyphomedusae,
	$<$0.01–0.05		ctenophores thaliaceans
mg C mg^{-1} DM	0.04–0.10	6	semi-gelatinous zooplankton:
	0.15–0.40		chaetognaths
	0.20–0.30		mollusks
	0.35		polychaetes
	0.30–0.60		non-gelatinous zooplankton: crustaceans
	0.20		*0.1 mg* of DM, coastal upwelling, NW Spain, 90% copepods
mg C mg^{-1} DM	0.22	3	1.0 mg
	0.29		*10.0 mg*
	0.38		*100.0 mg*
mg N mg^{-1} DM	0.10	4	ICES Committee on Terms and Equivalents

continued

Table 4.7 *continued*

Parameter and units	Conversion factor	Author (cf. Table 4.9)	Remarks
			gelatinous zooplankton:
	<0.01–0.05		hydromedusae, siphonophores, scyphomedusae,
	<0.01–0.01		ctenophores
	0.01–0.03		thaliaceans
mg N mg^{-1} DM		6	semi-gelatinous zooplankton:
	0.05–0.10		chaetognaths
	0.02–0.04		mollusks
	0.09		polychaetes
	<0.01–0.05		non-gelatinous zooplankton: crustaceans
mg N mg^{-1} DM	*0.040*	3	*0.1 mg* of DM, coastal upwelling, NW Spain, 90% copepods
	0.046		1.0 mg
	0.051		*10.0 mg*
	0.57		*100.0 mg*
mg P mg^{-1} DM	0.008	4	ICES Committee on Terms and Equivalents
mg protein mg^{-1} AFDM	0.279 ± 0.050	5	oligotrophic, subtropical waters, mixed mesozooplankton
mg C mg^{-1} AFDM	*0.25*	3	*0.1 mg* of AFDM, coastal upwelling, NW Spain, 90% copepods
	0.27		1.0 mg
	0.37		*10.0 mg*
	0.48		*100.0 mg*
mg N mg^{-1} C	0.17	4	ICES Committee on Terms and Equivalents
mg P mg^{-1} C	0.013		
mg P mg^{-1} N	0.078	4	ICES Committee on Terms and Equivalents

Only the two last relationships in this compilation satisfy the requirements of a linear regression. The first eleven equations provided by Wiebe *et al.* (1975), corrected by Wiebe (1988) and added to by Postel (1990) include additionally routines to calculate confidence limits for the estimated constants and slopes. They are applied as the following example demonstrates.

How many grams of ash-free dry mass (AFDM) correspond to 1 g of dry mass (DM)? The equation $\lg \text{AFDM} = -0.077 + 1.006 \lg \text{DM}$ (Table 4.8) was derived from data in a transition of coastal upwelling and oceanic waters of Northwest Africa within the range 0.3–7.8 g DM and 0.2–7.5 g AFDM (Postel 1990), suitable for the magnitude of biomass in question. It leads to $\lg \text{AFDM} = -0.077 + 1.006 \times 0 = -0.077$. The confidence limits of the expected estimate are calculated according to $\lg \text{AFDM} \pm 1.98 \times [0.001830\{1.006 + ((\lg \text{DM} + 0.0897)^2 \times (27.4717)^{-1})\}]^{1/2}$ which is $-0.077 \pm 1.98 \times [0.001830\{1.006 + ((0 + 0.0897)^2 \times (27.4717)^{-1})\}]^{1/2}$.

Consequently, the lg AFDM is in the range -0.077 ± 0.085. Using the antilog, 1 g DM corresponds to 0.838 g AFDM, according to the simple ratio (Table 4.8, last column), and may actually occur between 0.689 g and 1.019 g with a probability of 95%.

Table 4.8 Regression formulae of different models to convert zooplankton biomass according to Wiebe *et al.* 1975, Wiebe 1988 (10) and Postel 1990 (9), including formulae to calculate the confidence limits of the estimated variables, according to Bode *et al.* 1998 (3), Balvay 1987 (1), Ohman and Smith 1995 (8), as well as Oosterhuis and Baars 1985 (7). For details see text; for specifications concerning sample description, geographical region, etc., see Table 4.9.

y	*x*	Intercept *a*	Slope *b*	*r*	*N*	Range and units	For confidence limits see:	Author, remarks	$x = 1$, i.e. antilog *a* e.g. cm^3 DV mg^{-1} C
lg DV	lg C mass	−1.434	0.820	0.980	87	C: 0.2–130 mg DV: 0.01–2.5 cm^3	a	(10) Wiebe *et al.* (1975),	0.037
lg WM	lg C mass	−1.537	0.852	0.959	70	C: 0.2–30 mg WM: 0.008–0.65 g	b	Wiebe (1988)	0.029
lg DM	lg C mass	0.499	0.991	0.985	195	C: 0.05–120 mg DM: 0.15–340 mg	c		3.155
lg DV	lg DM	−1.842	0.865	0.980	163	DM: 0.8–340 mg DV: 0.008–2.5 cm^3	d	Geometric mean functional	0.014
lg WM	lg DM	−2.002	0.950	0.970	95	DM: 0.8–75 mg WM: 0.008–0.65 g	e	regression model (Ricker 1973):	0.010
lg DV	lg WM	0.139	1.003	0.949	77	WM: 0.008–0.65 g DV: 0.01–0.8 cm^3	f	$\lg y = a + b \lg x$ ($\lg = \log_{10}$)	1.377
lg WM	lg DM	−1.947	1.050	0.794	421	DM: 0.1–100 mg WM: 0.0003–1 g	Bé1		0.011
lg DV	lg DM	−1.887	1.007	0.748	404	DM: 0.1–100 mg DV: 0.001–1 cm^3	Bé2		0.013
lg DV	lg WM	0.005	0.981	0.866	403	WM: 0.0002–1 g DV: 0.001–1 cm^3	Bé3		1.012
lg DM	lg SV	−2.292	1.150	0.845	166	SV: 12–720 cm^3 DM: 0.3–7.8 g	g	(9) Postel	0.005
lg AFDM	lg DM	−0.077	1.006	0.995	166	DM: 0.3–7.8 g AFDM: 0.2–7.5 g	h		0.838

continued

Table 4.8 *continued*

Formulae to calculate confidence limits of the estimates using the regressions a) to h) listed on previous page and the Student distribution at $p < 0.05$ according to Ricker (1973):

a)	DV	$\pm 1.98 \times [0.022095 \times \{1.011 + ((\lg C - 0.8310)^2 \times 69.3796^{-1})\}]^{1/2}$
	C	$\pm 1.98 \times [0.032483 \times \{1.011 + ((\lg DV + 0.7573)^2 \times 47.1915^{-1})\}]^{1/2}$
b)	WM	$\pm 2.00 \times [0.018282 \times \{1.014 + ((\lg C - 0.2076)^2 \times 22.0394^{-1})\}]^{1/2}$
	C	$\pm 2.00 \times [0.024992 \times \{1.014 + ((\lg C + 1.3663)^2 \times 16.1224^{-1})\}]^{1/2}$
c)	DM	$\pm 1.96 \times [0.016987 \times \{1.005 + ((\lg C - 0.6456)^2 \times 117.7726^{-1})\}]^{1/2}$
	C	$\pm 1.96 \times [0.017311 \times \{1.005 + ((\lg C - 1.1383)^2 \times 115.5700^{-1})\}]^{1/2}$
d)	DV	$\pm 1.96 \times [0.019299 \times \{1.006 + ((\lg DM - 0.8369)^2 \times 106.4068^{-1})\}]^{1/2}$
	DM	$\pm 1.96 \times [0.025782 \times \{1.006 + ((\lg DV + 1.1181)^2 \times 79.6486^{-1})\}]^{1/2}$
e)	WM	$\pm 1.98 \times [0.011558 \times \{1.011 + ((\lg DM - 0.6473)^2 \times 19.9736^{-1})\}]^{1/2}$
	DM	$\pm 1.98 \times [0.012808 \times \{1.011 + ((\lg WM + 1.3868)^2 \times 18.0252^{-1})\}]^{1/2}$
f)	DV	$\pm 1.99 \times [0.023822 \times \{1.013 + ((\lg WM + 1.3706)^2 \times 17.3509^{-1})\}]^{1/2}$
	WM	$\pm 1.99 \times [0.023701 \times \{1.013 + ((\lg DV + 1.2347)^2 \times 17.4390^{-1})\}]^{1/2}$
Bé1)	WM	$\pm 1.96 \times [0.085121 \times \{1.002 + ((\lg DM - 0.2343)^2 \times 87.7505^{-1})\}]^{1/2}$
	DM	$\pm 1.96 \times [0.077210 \times \{1.002 + ((\lg WM + 1.7010)^2 \times 73.2612^{-1})\}]^{1/2}$
Bé2)	DV	$\pm 1.96 \times [0.093843 \times \{1.002 + ((\lg DM - 0.2408)^2 \times 85.9032^{-1})\}]^{1/2}$
	DM	$\pm 1.96 \times [0.092518 \times \{1.002 + ((\lg DV + 1.6446)^2 \times 63.8768^{-1})\}]^{1/2}$
Bé3)	DV	$\pm 1.96 \times [0.054240 \times \{1.002 + ((\lg WM + 1.6813)^2 \times 89.1699^{-1})\}]^{1/2}$
	WM	$\pm 1.96 \times [0.056355 \times \{1.002 + ((\lg DV + 1.6439)^2 \times 85.8228^{-1})\}]^{1/2}$
g)	DM	$\pm 1.98 \times [0.047762 \times \{1.006 + ((\lg SV - 1.9148)^2 \times 20.7583^{-1})\}]^{1/2}$
	SV	$\pm 1.98 \times [0.036101 \times \{1.006 + ((\lg DM + 0.0897)^2 \times 27.4717^{-1})\}]^{1/2}$
h)	AFDM	$\pm 1.98 \times [0.001830 \times \{1.006 + ((\lg DM + 0.0897)^2 \times 27.4717^{-1})\}]^{1/2}$
	DM	$\pm 1.98 \times [0.001810 \times \{1.006 + ((\lg AFDM + 0.1667)^2 \times 27.7735^{-1})\}]^{1/2}$

continued

Table 4.8 *continued*

y	*x*	Intercept *a*	Slope *b*	*r*	*N*	Range and units	Author, remarks	$x = 1$, i.e. antilog *a*, e.g. mg DM mg^{-1} AFDM
lg DM	lg AFDM	0.090 ± 0.020	1.009 ± 0.017	0.988	90			1.23
lg DM	lg C	0.582 ± 0.036	0.897 ± 0.042	0.928	65			3.82
lg DM	lg N	1.276 ± 0.022	0.955 ± 0.051	0.933	48	DV: 0–1.6 cm^3	(3) Bode *et al.* (1998)	18.88
lg DV	lg DM	-1.692 ± 0.070	0.824 ± 0.054	0.857	64	DM: 2–160 mg	geometric regression	0.02
lg DV	lg C	-1.242 ± 0.062	0.771 ± 0.079	0.817	87	AFDM: 1–160 mg	model (Sokal and Rohlf	0.06
lg DV	lg N	-0.672 ± 0.030	0.865 ± 0.078	0.817	87	C: 0.5–55 mg	1981)	4.70
lg AFDM	lg C	0.410 ± 0.041	0.963 ± 0.047	0.958	97	N: 0.2–10 mg	lg $y = a + b \lg x$	2.57
lg AFDM	lg N	1.157 ± 0.013	0.961 ± 0.027	0.956	97			14.35
lg DM	lg protein	0.668	0.763	0.945	about 50	DM: 7–900 μg protein: 0.8–100 μg	(2) Berges *et al.* (1990, 1993) geometric mean functional regression model (Ricker 1973): lg $y = a + b \lg x$	4.656
lg WM	lg SV	2.290	0.948	0.92	127	SV: 0.5–75 cm^3	(1) Balvay (1987)	195
lg DM	lg SV	1.417	0.843	0.92	128	WM: 19–16 200 mg	geometric mean	26.122
lg DM	lg WM	−0.691	0.893	0.96	549	DM: 6–1510 mg	functional regression	0.204
lg ash mass	lg DM	−0.782	0.928	0.91	549	ash: 1–150 mg	model (Ricker 1973):	0.165
lg AFDM	lg DM	−0.098	1.018	0.998	549		lg $y = a + b \lg x$	0.798
lg AFDM	lg DV	−1.482	1.157	0.958	160	DV: 3–650 cm^3 AFDM: 0.15–80 g	(8) Ohman and Smith (1995) model II or functional regression of $\log_{10}$ transformed values	0.033
N mass	C mass	−0.0247	0.2324	0.995	119	C: 0.2– 100 mg N: 0.04–25 mg	(10) Wiebe (1988) $y = a + bx$	0.208
Protein	AFDM	0.029	0.24	0.950	18	AFDM: 8–30 μg protein: 1–8 μg	(7) Oosterhuis and Baars (1985), $y = a + bx$	1.069

Table 4.9 Specifications of the zooplankton material used for developing conversion routines compiled in Tables 4.7 and 4.8.

Authors		Zooplankton material
1	Balvay (1987)	Mesozooplankton >200 μm, Lake Geneva, seasonal study
2	Berges *et al.* (1990) Berges *et al.* (1993)	*Artemia franciscana* culture
3	Bode *et al.* (1998)	90% copepods, >250 μm, Galician shelf waters, NW Spain, seasonal study except times with meroplankton and gelatinous plankton >10%; data standardized to volume of water (m^3)
4	Cushing *et al.* (1958)	Zooplankton
5	Hernández-León *et al.* (personal communication)	Mesozooplankton >200 μm, oligotrophic, subtropical waters, Canary Islands
6	Larson (1986)	Zooplankton of different taxonomic groups, with special attention to gelatinous plankton, compiled according to Beers (1966), Curl (1962), Ikeda (1974), Omori (1969)
7	Oosterhuis and Baars (1985)	Copepods (*Temora longicornis*) culture, grown at different food levels and temperatures
8	Ohman and Smith (1995)	Macrozooplankton (>505 μm) , California Current, different seasons
9	Postel (1990)	Mesozooplankton, >200 μm, coastal upwelling and oceanic regions off Northwest Africa, August to November, fixation by 4% formalin
10	Wiebe *et al.* (1975) Wiebe (1988)	Zooplankton 0.35–100 mm size (mesh sizes 240, 333 μm; data of Bé, obtained by 202 μm), North Atlantic (western part of the Central Gyre, New York Bay, Gulf of Mexico), South Atlantic, Peru upwelling, different seasons

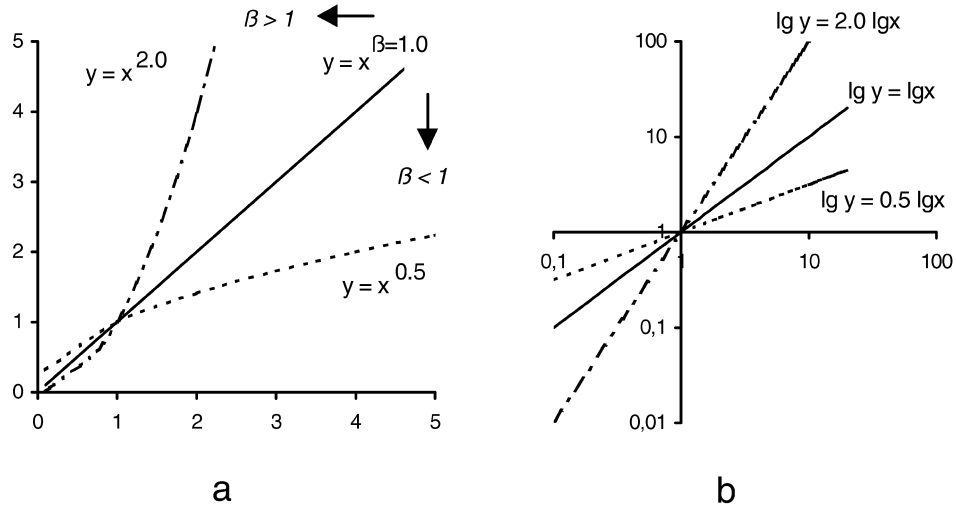


Fig. 4.15 Power functions $y = ax^{\beta}$ with $\beta = 1$ and $\beta \neq 1$ in normal and in logarithmic presentation. For details see text.

The borders of the confidence limits are equally different from the biomass converted by a factor of 1.22, because (0.838/0.689) = (1.019/0.838).

Details of the procedures to construct such relationships are described by Ricker (1973) and to a certain degree by Wiebe *et al*. (1975). The method is based on the general concept of Ricker (1973) determining the best fit by the sum of the least products instead of the least squares of Gauss (of the vertical and horizontal deviations of every data point from the regression line). Consequently, the sums of the deviation's products are identical, which guarantees matching equations for y on x and x on y respectively. In case of the Gauss procedure two separate equations, one for each direction, would be necessary. (Practically, the parameters of the Ricker formula are the averages of those of the two Gauss equations.)

The approximate conversion factors of zooplankton biomass in Table 4.7 are grouped into seven categories. The first is standardized to settling volume, and the others to displacement volume, wet mass, dry mass, ash-free dry mass, carbon, and nitrogen. The regression equations in Table 4.8 are categorized according to authors, because of particular data processing and application of mathematical procedures. Table 4.9 gives information on the origin of plankton material and other specifications.

Generally, conversions should be performed in the original range of the available ratio or relationship. Mostly they are constructed from data obtained at natural concentrations (see Table 4.8, column 7). An equation with estimated confidence limits is recommended rather than a simple ratio. The potential influence of the formula applied should be considered in terms of the taxonomic level, as well as the plankton size class, the geographical region, the season, and the 'slight' modifications of the methods of biomass determination. Coarse conversion factors cannot replace measurements, if they are focused on detailed outcomes. Especially in interdisciplinary studies, we are often in the unsatisfactory situation of measuring with highest precision and making the results comparable by the use of less appropriate factors. As far as possible, the organic matter should be measured directly in such cases on a common basis. These aspects are discussed in greater detail by Wiebe *et al*. (1975), Balvay (1987), and Bode *et al*. (1998).

4.4 ABUNDANCE AND SPECIES IDENTIFICATION

Abundance is defined as the number of individuals per unit volume of water. Its determination was the beginning of quantitative plankton research (Hensen,1887; Lohmann 1908). Lenz (1968), Edmondson (1971), Boltovskoy (1981), Omori and Ikeda (1984) and McCauley (1984) have reviewed methods for microscopic analysis, particularly including dissection, staining and mounting procedures.

Abundance and species identification allows the parallel quantification and identification of specimens and is the basis of any community analysis (cf. section 4.5). Unfortunately, it is time-consuming and also requires a considerable amount of experience. This restricts the number of samples that can be analyzed with an acceptable effort. Attempts to overcome these problems by automatic acoustical or optical methods (cf. Chapters 6 and 7) may overcome the problem of under-sampling. However, they are limited to uniform samples (e.g. cultures in laboratories), to size class specific analyses or to a coarse separation of taxonomic groups with significant differences in shape. A coupling of such procedures with computerized image analysis is still limited because of the necessary sophisticated technical equipment and software.

In contrast to biomass determination, numbers of organisms do not take account of their size, which is a significant limitation of the method. The calculation of biomass is a way of overcoming this problem if suitable individual biomass factors or proper morphometric approaches are applied (cf. Table 4.12). Such biomass determination is zooplankton specific and neglects any potential contamination of samples by phytoplankton and detritus.

Routinely, identification is performed for taxonomic groups and developmental stages. These may be classified according to characteristics of nutrition, biology, behavior, and their affinity with particular water masses, with the aim of characterizing ecological situations. Taxonomic skill is required for the identification of single species. This is evident, if, for example, one considers the world-wide total of about 11 500 known copepod species (Humes 1994).

Besides experience, identification literature (Table 4.10) is needed as well as species inventories (check lists) for bio-geographical provinces (e.g. Razouls and Durand 1991). Reference lists for species based on current taxonomic descriptions are convenient. A reference collection of the sample specimens should be stored. The species names should be according to the *International Code of Zoological Nomenclature* (Stoll *et al.* 1964, cited after Omori and Ikeda 1984). The holo- or the syntype specimens are stored in Museums of Natural History.

Computer aided data storage requires species codes, suitable for the international

Table 4.10 Examples of frequently used references for the identification of zooplankton. In addition, there are various monographs on plankton groups, noted in Chapter 1. Additional CD-ROM based information is available from the Expert Centre for Taxonomic Identification (http://wwweti.eti.bio.uva.nl).

Author, editor	Remarks
Introductory references (student level)	
Newell and Newell (1973)	General
Smith (1977)	Coastal marine plankton and invertebrate larvae
Todd and Laverack (1991)	Coastal marine plankton
Wickstead (1965)	Tropical plankton
References specific for various taxonomic groups	
Boltovskoy (1981)	South-west Atlantic
Brandt and Apstein (1901–1938)	Nordic Seas
ICES (1939)	ICES area, North Atlantic
Riedl (1983)	Mediterranean Sea
Trégouboff and Rose (1957)	Mediterranean Sea
Yamaji (1971)	Japanese coastal waters
References specific for one taxonomic group	
Guglielmo and Ianora (1995)	Copepods, Magellan Strait
Rose (1933)	Copepods, French coast
Sars (1903)	Norwegian waters
Schram (1986)	Crustaceans, general
Seymour Sewell (1929)	Copepods, Indian Ocean

data exchange, provided for example by the Nordic Code Centre of the Swedish Museum of Natural History (RUBIN Code) or from the United States NODC (National Oceanographic Data Centre) of NOAA (National Oceanic and Atmospheric Administration).

Training of the staff in different laboratories storing their results in a joint database is essential for harmonizing the methods and improving the skills necessary to identify the species or taxonomic groups. These are essentials for the quality assurance within international monitoring programs.

How is microscopic analysis performed? Sample preparation on board a ship and recommendations concerning storage are described in section 4.2. The laboratory procedure starts with the filtration of the sample through meshes smaller than the mesh size of the sampling gear. The filtrate is used again after the analysis for any further storage. The organisms are suspended in filtered tap water for analysis. This is necessary for health and safety considerations, as formalin is a carcinogen. The procedure should be carried out under a fume-hood. The dilution of the total sample will be chosen according to experience to reach an appropriate concentration for the analysis. Mostly, 200 to 300 ml are found to be a suitable total sample volume. Sometimes 100 or 500 ml has to be chosen.

The volume of material in the sample is often such that it demands sub-sampling into aliquots. The volume of the total sample, measured in a graduated cylinder, is noted as the reference amount. The sample is then poured into a beaker to allow a thorough mixing until the organisms are distributed randomly before taking an aliquot. Repeated sub-sampling by the Stempel pipette (Hensen 1887; Figure 4.16) produces a coefficient of variation of 7–9%, applying a bulb pipette of 14–15%, and a Folsom splitter of 5–18%. The variability between total counts amounts to 0.3–2.5% (van Guelpen *et al.* 1982). The respective ranges are a result of the various sizes and shapes of the organisms. Treatment using pipettes is 5 to 8 times faster than the splitter technique. It's limitation lies in the size of plankton, if it is larger than the pipette's diameter. The Kott splitter (Kott 1953) is more convenient in comparison to the Folsom splitter (Sell and Evans 1982; Griffiths 1984). The Kott produces eight sub-samples at the same time, while the Folsom splits samples into halves and increases the error from step to step (Behrends and Korshenko, personal communication).

Routine sorting requires a dissecting stereo-microscope (Figure 4.16). Unfortunately, the range of magnification is only suitable for larger zooplankton. Smaller mesozooplankton, such as copepods and their developmental stages, should be analyzed using an inverted microscope, which allows manipulation of the specimen as in case of stereomicroscope and combines opportunities for a general survey with a 50 × magnification and for the analysis of details with a factor of 80 × and larger. An inverted microscope needs a counting chamber with high transparency.

There are two types of *counting chambers*, closed trays with defined volumes, like the Sedgwick–Rafter cell (Whipple *et al.* 1927) which holds 1 ml, and open chambers, such as the Bogorov tray containing 10 ml (Arndt 1985) or more. Closed types are preferably used in microzooplankton studies. The trays may be provided with a grid or with sections to allow a better orientation and to avoid a repeated counting of the same organism. The size of chambers and sections depends on the magnification applied. One counting strip should be fully covered with the 50 × magnification. The Mini-Bogorov-chamber in Figure 4.16 is easy to produce in a workshop and has the following dimensions: the length, width, and height are 70, 40, and 8 mm respectively. The counting path is 6 mm deep, its width amounts to 3 mm, the section walls are 1 mm

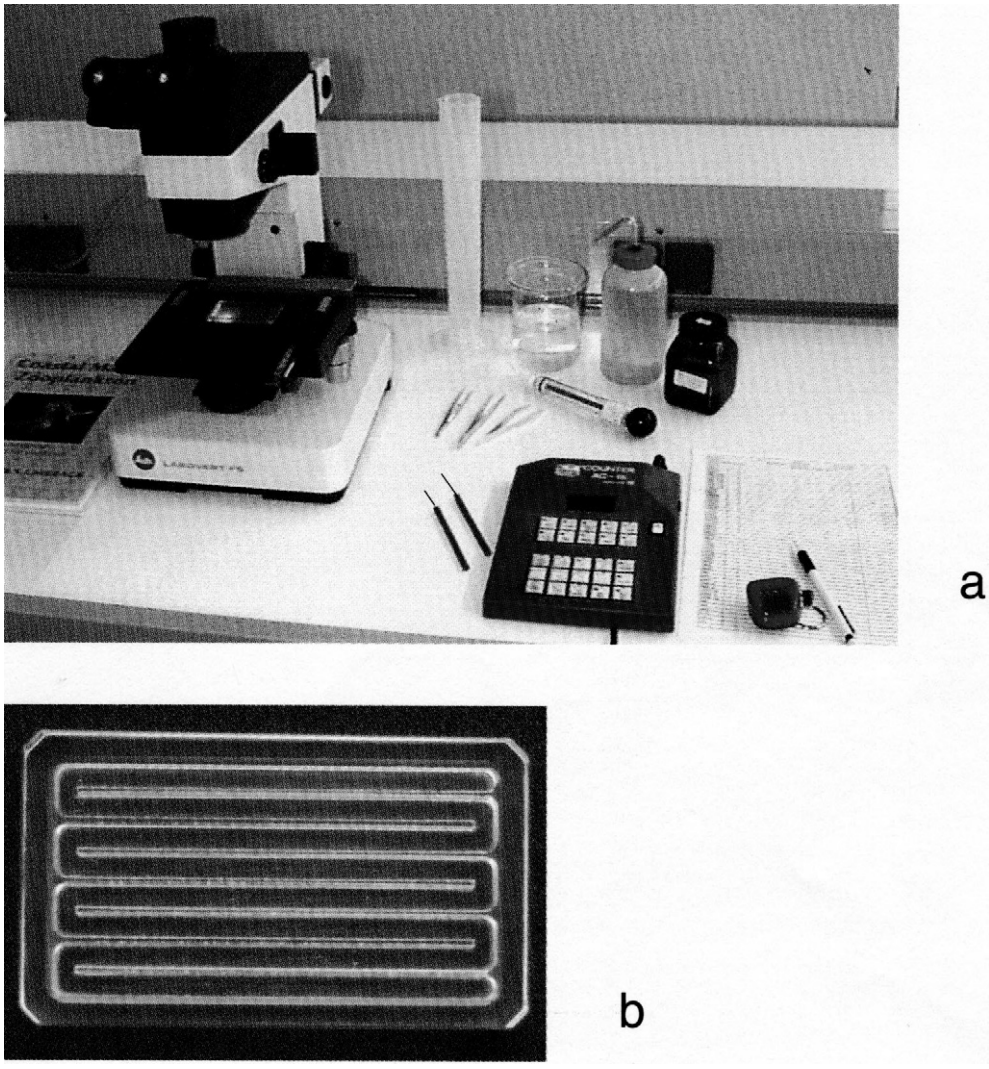


Fig. 4.16 (a) Working place for zooplankton counting and species identification showing an inverted microscope (30 to 250 ×) with a mini-Bogorov chamber containing a sub-sample, needles, forceps, Stempel pipette, the total sample in a beaker (the precision of its graduation might be determined with a graduated cylinder), a wash bottle, the sample container and two examples of counting devices (near the protocol). (b) Mini-Bogorov chamber (modified according to Arndt 1985), approximately original size (see text).

wide, and their height is 4.5 mm. The sides and walls are tapered sloping at top. The tray is made of clear plastic and finally polished. The table of the microscope is adapted to carry the tray (Figure 4.16).

The Sedgwick–Rafter cell has a constant volume, thus the chamber itself measures the volume of the sub-sample. The (Mini-)Bogorov tray is filled with a known aliquot (e.g. 0.5 or 1 ml – which has to be considered for calculation of abundance) and finally made

up to the top (10 ml) with filtered tap water. The surface must be level to avoid any reflections. Therefore, the outer walls are 1.5 mm higher, than those of the counting paths.

Some organisms, for example, cladocerans, tend to float in the surface film. An addition of detergents, or the palmityl alcohol [$CH_3(CH_2)_{14}CH_2OH$] (Desmarias (1997) reduces their surface tension and promotes sinking to bottom. This makes it easier to focus on all animals in the same way.

Other *sorting media* are glycerol and propylene glycol or lactic acid used for clearing tissues of small crustaceans (Omori and Ikeda 1984).

Contamination by large quantities of phytoplankton makes analysis more difficult. In this case staining of animals by adding Eosin Y is a helpful tool. A few drops are enough for a 100 ml sample volume. Several hours should be allowed for staining (Edmondson 1971).

Lund *et al.* (1958), Cassie (1971), Frontier (1981) and others have considered the statistical aspects of *counting errors*, which allow the necessary amount of organisms for counting to be established. The accuracy of results required depends on the purpose of the work. To detect differences between total abundance in space or time of 100%, an accuracy of 50% is adequate 'and any time spent in making more accurate estimates is largely wasted' (Lund *et al.* 1958). Generally, an error of $\pm 20\%$ is felt acceptable.

If all organisms are randomly distributed, following the Poisson distribution, the accuracy of a sample and the precision of a single count depends only on the number of specimens counted (Cassie 1971). This can be read from Table 4.11 (a test for departure from randomness is the chi-square test; e.g. Sokal and Rohlf 1995). The table precision in percentage terms which might be reached if the corresponding number of specimens is counted, together with the respective range of organisms within their actual number is expected to fall. Accordingly, a doubling of precision requires a fourfold effort in counting. For example a precision of $\pm 20\%$ requires the analysis of 100 specimen while those of $\pm 10\%$ needs 400 counts (Lund *et al.* 1958). The 0.95 confidence limits (CL_{95}) are also calculated from the number of counts (n):

$$\mathbf{CL_{95}\,[\%] = \pm 2\,(100/\sqrt{n})}$$

Table 4.11 Precision obtained at the 95% confidence level for differing numbers counted (after Lund *et al.* 1958 and Lenz 1968)

Organisms counted	Precision in % of count	Range
4	± 100	0–8
11	± 60	4–18
16	± 50	8–24
25	± 40	15–35
44	± 30	31–57
64	± 25	48–80
100	± 20	80–120
178	± 15	151–205
400	± 10	360–440
1600	± 5	1520–1680

Table 4.12 References on the determination of individual biomass (biovolume) factors suitable for calculation of biomass from abundance (cf. Figure 4.1).

Author	Principle	Remarks
General aspects		
Beers (1976)	General remarks on biomass estimates based on size measurements	Size measurements time consuming, many approximations because of irregularities in shape
Lohmann (1908) (cited according to Beers 1976)	Body shape equivalent to geometric configuration(s); conversion of volume to mass by multiplication with specific density of organisms	Currently used for micro-zooplankton because of the difficulty of direct biomass determination; density of sea water ca. 1.026
Miller (1966) (M) Robertson (1968) (R) Krylov (1968) (K) (cited according to Beers 1976)	Relatively constant relationships between some size measures, (generally length) and individual biomass or biovolume; method requires ability to make taxonomic identifications	M: length vs. wet mass for Chaetognatha, Ctenophora, Euphausiacea, etc. – California Current; R: length vs. dry mass calanoid copepods – North Sea; K: copepods East China Sea + compilation from Russian literature
Chislenko (1968)	3D-dimensional forms vs. biomass of marine plankton without the need to determine the species	Series of nomograms, assuming specific density = 1
McCauly (1984)	Error discussion: influence of the variability of individual biomass estimates and the number of specimens counted on the total error	Assumption of Poisson distribution; total variability is about 15% if coefficient of variation (c.v.) of dry mass estimation is 1–5% and 50 individuals are counted; (if dry mass variability = 10% then 100, and if 15% then 600 individuals must be counted to keep the total c.v. of 15%)
Sprules *et al.* (1981) (cited according to McCauly, 1984)	Computer-based caliper system to speed up the measurements of individuals	400 to 700 individuals in 15 to 20 min
McCauly (1984)	Statistics of papers on individual dry mass determination (1972–1982, fresh water crustacean plankton)	23% direct determinations, 77% by length-dry mass relationships (45% of them – original studies, 55% used relationships from literature)
Examples of length-biomass (volume) relationships		
Svetlichny (1983)	Planktonic copepod body volume calculated from length and width of cephalo-thorax	List of necessary coefficients of 68 marine species (females and males)
Chojnacki (1983)	Length-volume or dry mass relationships of copepods	Four species, sex and single copepodites stages considered, Baltic Sea

Table 4.12 *continued*

Examples of length-biomass (volume) relationships (continued)		
Mauchline (1998)	Relationships of prosome length or total length and body volume, wet mass, dry mass, and ash-free dry mass of calanoid copepods	Extensive compilation
Ruttner-Kolisko (1977) (cited according to McCauly 1984)	Formulae for calculating the volume of rotifers according to their body length, width, and height	Fresh- and brackish water species
Dumont *et al.* (1975)	Body length-dry mass relationships of cladocerans, copepods, and rotifers	Fresh- and brackish water species
Persson and Ekbohm (1980)	Methods to determine individual dry mass and construct relationships of body length and dry mass and estimate average population biomass	Fresh water crustaceans
Kulka and Corey (1982)	Length and dry mass relationships of euphausiids	*Meganyctiphanes norvegica*, *Thysanoessa inermis*, Bay of Fundy
Schneider (1988)	Relationship between the diameter (d) and the wet mass (wm) of *Aurelia aurita*	Jelly fish, Baltic Sea wm (g) = $0.088 \times d^{2.75}$ $N = 59$ (range of d = 1–28 cm)
Madin *et al.* (1981)	Length to carbon relationships of *Thaliacea (Tunicata)*	Salps; supplemented by literature data
Kankaala and Johansson (1986)	Individual variations and the length-biomass (organic carbon) relationship	Cladoceran *Bosmina maritima*, copepod *Eurytemora affinis* Baltic Sea
Bottrell and Robins (1984)	Seasonal variations of length and dry mass, carbon, nitrogen relationships	Copepod *Calanus helgolandicus* Celtic Sea
Durbin and Durbin (1978)	Seasonal variations of length and dry mass relationship	Copepod *Acartia clausii*, Narragansett Bay
Chisholm and Roff (1990)	Length or width – biomass relationships; seasonal variability	Tropical neritic copepods, Jamaica
Mizdalski (1988)	Length, wet-, dry-, and ash-free dry mass data of various zooplankton taxa	Length/biomass relationships for some euphausiids and calanoids, Antarctic
Examples of direct determination of individual biomass		
Cummins and Wuycheck (1971)	Energy content of invertebrates	Extensive compilation
Petipa and Borichenko (1985)	Carbon content of copepods	Indian Ocean
Snow (1972)	Seasonal variability of animal size and the effect on its energy content	Fresh water cladoceran *Daphnia pulicaria*
Tanskanen (1994)	Seasonal variability of individual carbon content	Copepod *Acartia bifilosa*, northern Baltic Sea
Durbin and Durbin (1992)	Short-term variability (weeks) of biomass in terms of carbon and nitrogen	Copepod *Acartia hudsonica*, Narragansett Bay

Ricker (1937) cited according to Lund *et al.* (1958) provided formulae to estimate the error ranges on probability levels of 95% and 99%, respectively:

$$\text{Lower } CL_{95} = n + 1.42 - 1.960\sqrt{(n + 0.5)}$$
$$\text{Upper } CL_{95} = n + 2.42 + 1.960\sqrt{(n + 1.5)}$$
$$\text{Lower } CL_{99} = n + 2.82 - 2.576\sqrt{(n + 1.2)}$$
$$\text{Upper } CL_{99} = n + 3.82 + 2.576\sqrt{(n + 2.2)}$$

In practice, one or more counting chambers with the same concentration should be analyzed until 100 specimens of the most abundant taxonomic groups are reached in a sample. The estimations of the remaining, less common, groups are less precise (Table 4.11). If the counting procedure is continued until 100 specimens of the other groups are reached, neglecting the more abundant groups, the different sub-sample sizes must be considered in the successive calculations. This procedure has some merits, but may lead to additional errors. Finally, the remaining part of the total sample can be surveyed for rare species which may be qualitatively noted as present. The estimation of the accurate amount of species to be expected is treated in section 4.5.

The *calculation of abundance* (individuals/m^3) needs to consider the number of counts (n), the part of the sample counted (k), i.e. the proportion of total volume to sub-sample volume(s), and the amount of water filtered by the sampling net (m^3):

$$\text{ind.}/m^3 = (n \times k)/\, m^3$$

Finally, the need for inter-calibration between joint observation programs of different laboratories should be emphasized. For example, eight laboratories round the Baltic Sea analyzed parts of the same sample (Leppänen *et al.*1990). From 15 taxonomic groups, 10 were analyzed with any differences being expected by subtracting the counting error according to Table 4.11 and an error due to the splitting technique when partitioning the total sample. Reasons for the remaining deviations were an insufficient number of organisms counted (expected counting error of $\pm 32\%$), the non-random distribution of gelatinous individuals in the sample, and taxonomic uncertainties regarding the identification of nauplii.

4.5 ANALYSIS OF COMMUNITY STRUCTURE (By H. Fock)

Communities are defined as associations of different populations co-existing in space and time (Begon *et al.* 1990). These associations have specific properties, for example composition, diversity, ratio of rare to common species, certain indicator species or biomass production. The main objectives of community analysis are to investigate these patterns and the processes that generate them (Wilbur and Travis 1984). The study of patterns has been primarily descriptive, either in a way of describing species richness, diversity measures and its temporal development and spatial distribution and comparing it to other patterns, or in relating given associations to environmental gradients.

If such comparisons are a major issue of an investigation, the sampling design necessary for sufficient statistical analysis has to be considered in the planning phase (Green 1979, cited by Andrew and Mapstone 1987; see also Chapter 2). Andrew and Mapstone (1987) recommended applying statistical power analysis in planning an investigation, possibly based on pilot studies. Statistical power is defined as the probability of correctly rejecting a false null hypothesis (Zar 1996). If the difference

between two samples is expected to be small, statistical power can be increased by increasing the number of replicate measurements, i.e. sample size. For larger expected differences the sample size can be smaller. An elaborate discussion of the application of power analysis prior to starting an investigation is given by Andrew and Mapstone (1987).

The scope of this chapter is to focus on basic assumptions in the analysis of community structure, i.e. choice of indices and methodological approaches, rather than to give a detailed guide to multivariate procedures, for which space is too limited and comprehensive textbooks are available (Digby and Kempton 1987; Bakus 1990; Burd *et al.* 1990). Table 4.13 provides a short digest of software packages; much more information is available on the Internet.

In the analysis of community patterns these steps are distinguished:

- estimation of species numbers
- diversity and similarity indices
- detection of groups (i.e. classification)
- analysis of spatial formations.

In the analysis of processes knowledge can be gained either from experimental work or from network analysis. This chapter will only refer briefly to the latter. Hypothetical questions will present examples for the possible applications of methods and indices.

Finally, it has to be recognized that the properties of a community (diversity, processes and spatial formation) have to be regarded as dynamic properties, i.e. they change over time as community development proceeds.

4.5.1 Estimation of species numbers

Estimating the total number of species in a community is a crucial task. This section focuses on aspects concerning the comparison of estimates of different samples, for example subregions in a given area.

The first approach aims at species richness, i.e. the real number of species in an ecosystem including those that were not sampled. If replicates were taken, the species richness within a sample of n replicates can be estimated by extrapolation using either jack-knifing or bootstrapping techniques. Both methods resample the observed species and relate the estimated species richness to an upper sample size. According to Heltshe and Forrester (1983) and Palmer (1990) the first-order jack-knife estimate $JACK1$ of species richness can be calculated as:

$$JACK1 = SO + r_1(n-1)/n \tag{4.10}$$

where SO is the total number of observed species, r_1 is the number of species observed only once in the replicates and n is the number of replicates. The same authors present a bootstrap estimation:

$$BOOT = SO + \sum_{j=1}^{SO}(1-p_j)^n \tag{4.11}$$

where p_j is the proportion of replicates in which species j is present. In bootstrapping the parenthesis term has to be calculated for each observed species, while in jack-knifing only one calculation has to be carried out. However, results of Palmer (1990) indicate that the bootstrap estimate is more precise.

Table 4.13 Specialized software packages for ecological community analysis. This table can only provide a very short overview of available software. More information can be obtained from the Internet, for example 'The Ordination Homepage' at http://www.okstate.edu/artsci/botany/ordinate/

Name	Selected features	Distributor(s)
Ecological Analysis package	Operates only in connection with SAS software, includes detrended correspondence analysis, principal coordinates analysis, polar ordination, transformation operations, cluster analysis	EcoAnalysis Inc., 221 E. Matilija Street, Ojai, CA 93023, USA
PRIMER	Includes multivariate analysis, cluster analysis, analysis of similarity	Plymouth Marine Laboratory, Prospect Place, Plymouth PL1 3DH, UK
PC-ORD	Including Mantel tests, TWINSPAN and 'multiway indicator species analysis'	MjM Software Design, PO Box 129, Glenneden Beach, OR 97388, USA
TWINSPAN	Twinspan is part of a program package consisting of DECORANA (multivariate ordination, correspondence analysis), TWINSPAN (Two-way classification of samples and species) and TABLCORN (Table operations to convert files into the required Cornell Condensed Format)	Mark Hill, ITE Monks Wood, Abbots Ripton, Huntingdon, Cambs PE17 2LS, UK
ECOPATH	Ecopath with EcoSim is an approach and a free Windows software for construction and analysis of mass-balance models and feeding interactions or nutrient flow in ecosystems including the calculation of network diversity indices	Vılly Christensen International Center for Living Aquatic Resources Management Headquarters: Bloomingdale Bldg, 205 Salcedo St, Legaspi Vıllage, 1229 Makati City, Philippines Mailing address: MCPO Box 2631, 0718 Makati City, Philippines
Biodiversity Pro	Free software to conduct multivariate analysis of communities, to calculate alpha- and beta-diversity measures and to analyze similarity of samples	PJD Lambshead and GLJ Paterson, The Natural History Museum in London and JD Gage, Scottish Association for Marine Science, Oban, Scotland

If species number–area relationships are known, these can be applied to calculate regional estimates. Haedrich (1985) presented an approach to assess regional species richness for benthic fauna and mesopelagic fishes depending on species–area–productivity relationships.

Example 1: How many species occur in a study area of given size?
To solve this task bootstrapping and jack-knifing can be applied. As a result estimates for a larger area are obtained, provided that samples were taken with the same methodology.

If the samples are of different size (numbers of specimens collected), comparisons between samples can be achieved by relating species numbers to the smallest sample size by rarefaction (Magurran 1988; Sanders 1968). Sanders (1968) developed a spreadsheet technique, in which the species in a sample of, for example, 250 individuals are ranked according to their abundance and given percentage values of their proportion within the community. If the reduced sample size for comparison is chosen to be 100 individuals, each specimen within this reduced sample then represents 1% and all the species representing 1% or more from the original sample are included in the reduced rarefied sample, whereas species representing less than 1% are omitted. Hurlbert (1971) applied the rarefaction method to a formula in which rarefied values are calculated for every species and summed:

$$RF(n) = \sum_{i=1}^{SO}\left\{1 - \left[\binom{N - N_i}{n} \Big/ \binom{N}{n}\right]\right\} \tag{4.12}$$

where $RF(n)$ is the number of species in the standardized (rarefied) sample, n is the size of the standardized sample, N is the total number of individuals in the original sample, and N_i is the number of individuals of species i. Though the formula seems complicated, the idea behind it is easy to understand. For each species the probability of not being in the sample after it is reduced to size n is subtracted from the probability of being in the original sample (which is 1). The difference represents the probability of being in the sub-sample. The probability P of not being in the sub-sample follows a hypergeometrical distribution of random draws without replacement (Zar 1996):

$$P = \binom{N_i}{z}\binom{N - N_i}{n - z} \Big/ \binom{N}{n} \tag{4.13}$$

with $z = 0$ (expected number in the sub-sample). Since $(N_i/0) = 1$, P becomes

$$P = \binom{N - N_i}{n} \Big/ \binom{N}{n} \tag{4.14}$$

Rarefaction leads to a loss of information for the larger sample. Problems arise when a number of sub-samples with different species dominance patterns are pooled and the species numbers for the sub-samples are recalculated from the pooled total. Rarefaction then can overestimate the rarefied species number for the sub-samples considerably (Gray 1997). In turn, rarefaction can be used to calculate a sufficient sample size (Omori and Ikeda 1984).

Example 2: Which device samples species diversity more efficiently?
If one intends to use a new type of sampling gear which filters a different volume (i.e. unequal sample size) of water than previously used equipment, the sampling efficiency

in terms of estimating species numbers can be investigated by comparing the different devices by means of rarefaction and subsequent analysis of similarity with the NESS index (see section 4.5.2).

4.5.2 Diversity and similarity indices

Diversity measures are obtained in order to describe the quality of a community, as either 'complex' and 'rich' or 'simple'. Similarity measures are obtained to describe whether observed communities differ in composition or not, often as a result of environmental changes. Diversity measures can be obtained for a single or for several samples, whereas similarity measures always require a comparison of several samples.

Species diversity measures (except species richness, see section 4.5.1) depend on species numbers and the relative abundances of species. Graphical presentation of abundances and transformation of data to different scales has led to development of four main species rank-abundance distribution models: logarithmic series, log normal series, geometric series and MacArthur's broken stick model (for calculation see Giller 1984; Magurran 1988; Krebs 1999). The slopes of the distribution graphs and the species-axis intercept describe the diversity by means of species richness and evenness of abundance distributions within the given community. However, the distribution model approach requires empirical fitting of the data to one or another model and problems arise if the various communities studied fit to different models and cannot be compared directly (Magurran 1988). This is exemplified in Figure 4.17, where species rank is plotted against abundance data from Table 4.14 (a 'Whittaker plot', after Krebs 1999). Up to rank 5 the log normal as well as the broken stick distribution fit well to the data, however, in total the geometric series distribution deserves the best fit.

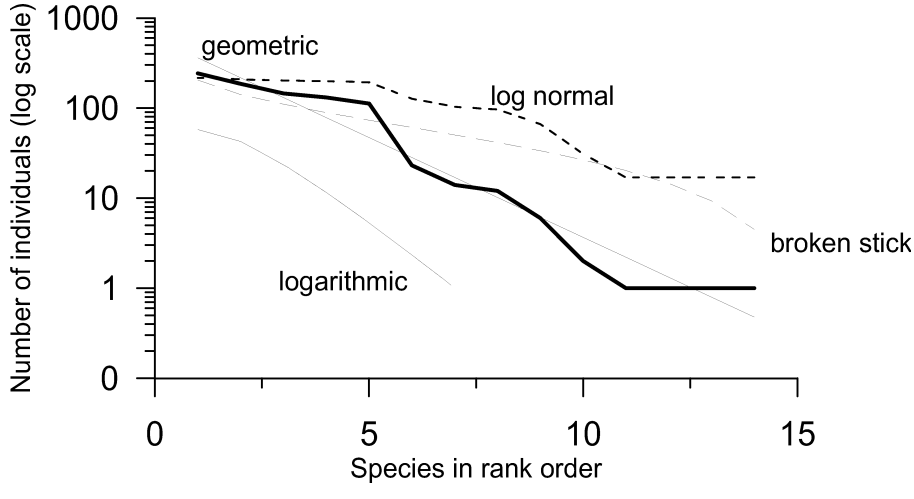


Fig. 4.17 Whittaker plot (log abundance per species versus species rank) of the sample data set (Table 4.11) for different distribution models and original data (bold line). Up to rank 5 log-normal and broken stick distributions fit well, but in total the geometric series fits best. Therefore, the application of distribution models can only serve as an aid to understand species distributions (see also Krebs 1999).

Table 4.14 Sample data set for copepods in the German Wadden Sea, 1996.

		March	April	May	June	July	August	Sept	Oct	Nov
Temora longicornis	Calanoida	3	33	38	48	16	6	8	19	15
Pseudo/Paracalanus	Calanoida	14	81	8	10	8	3	7	6	8
Calanus helgolandicus	Calanoida	0	0	0	0	0	0	0	1	1
Acartia clausi	Calanoida	10	96	55	39	3	3	11	13	12
Acartia longiremis	Calanoida	0	0	0	0	0	0	0	5	1
Acartia discaudata	Calanoida	0	0	0	0	0	0	0	1	0
Centropages hamatus	Calanoida	0	5	1	9	3	2	2	1	0
Eurytemora affinis	Calanoida	0	1	0	0	0	0	0	0	0
Indet. CII	Calanoida	7	26	22	30	5	3	6	4	9
Oithona similis	Cyclopoida	1	2	2	1	4	1	1	1	1
Corycaeus anglicus	Cyclopoida	0	0	1	0	0	0	0	0	0
Oncea sp.	Cyclopoida	0	0	0	0	1	0	0	0	0
Microsetella sp.	Harpacticoida	1	4	5	0	0	1	0	1	0
Euterpina acutifrons	Harpacticoida	1	5	45	32	1	2	2	40	3

Table 4.15 Common diversity indices. OS = observed number of species, p_i = proportional abundance of species i, N = total number of individuals per sample (after Magurran 1988; Omori and Ikeda, 1984).

Index name	Diversity formula
Shannon–Wiener diversity	$H' = -\sum_{i=1}^{OS} p_i \ln p_i$
Pielou's index of evennness (also evenness component diversity)	$J' = \frac{H'}{H_{max}} = \frac{H'}{\ln OS}$
Simpson's diversity	$D = \sum_{i=1}^{OS} p_i$
Margalef's diversity	$D_{MARG} = (OS - 1)/\ln N$

Therefore, indices based on proportional abundances (Shannon index, Simpson's index, Table 4.15) or total abundance and species number (Margalef's index) are widely used in plankton literature. According to Krebs (1999) the Shannon measure of diversity is more sensitive to rare species, whereas Simpson's index puts weight on the common species.

The measures of diversity strongly depend on sample size and the number of species involved. In order to analyze differences in diversity between two samples with different numbers of species, the relationship between measured diversity and maximum possible diversity is important to know. This relationship is named evenness or 'equitability' (Omori and Ikeda 1984). Thus, samples of different size with different diversity values might have the same evenness, if dominance patterns within the samples are comparable. The problem of dominance patterns is related to the similar issue in calculating rarefied species numbers (see section 4.5.1). Pielou's index of evenness (see Table 4.12) has proved to be best suited for general use and is recommended (Omori and Ikeda 1984).

For comparisons of samples with the same species number the significance of the difference of two diversity measures H'_1, H'_2 can be tested. In the case of two samples of sizes $N1$ and $N2$ without replicate measurements the difference $\Delta H'_{1-2}$ is compared with a distribution of randomized differences (Solow 1993). The randomization is achieved by combining both data sets $N1 + N2$ and randomly redistributing the species onto two new data sets of same size $N1$ and $N2$ and repeatedly calculating the difference $\Delta H'_{r1-r2}$. The p value of significance is represented by the percentile of the random distribution, for which $\Delta H'_{1-2} \geqslant \Delta H'_{r1-r2}$ holds. While the forementioned randomization procedure is applicable to any index, Zar (1996) after Hutcheson (1970) presents a direct t-test applicable only on the difference of two Shannon–Wiener diversity values. The latter test has the advantage that it can be applied even when the number of species and total numbers of specimens are different between the two samples.

If m combined replicates were used to calculate the diversity of a sample, statistical properties of the diversity estimate (mean and standard deviation) can be derived from jack-knifing. Magurran (1988) points out that this is a very robust method to improve the diversity estimate and should be widely applied. It is applicable to any index A'. The new diversity estimate is calculated in two steps (Magurran 1988). First, while omitting each replicate once, m jack-knife estimates JV_i out of $m - 1$ combined

samples are calculated. Second, these values JV_i are converted into pseudovalues PV_i by

$$\boldsymbol{PV_i = mA' - (m - 1) \times JV_i} \tag{4.15}$$

The mean value of all PV_is is the best estimate of sample diversity A' and the difference between A'_1 and A'_2 of two samples can be tested.

Hurlbert (1971) also Bakus (1990) argue that diversity measures have little ecological meaning, since species extinctions and replacements due to severe disturbances can be disguised by little or no change in diversity values.

Example 3: Comparing the dominance structure of two communities

If the structure of two communities, i.e. the dominance structure, has to be compared, the calculation of Shannon's H' is recommended, since a good index of evenness is available for H'. If the evenness measure J' is the same for both samples, the difference in H' must be attributed to different sample sizes. If $J'_1 \neq J'_2$, the difference between the diversity measures can be analyzed in detail. If replicates are involved, jack-knifing can be used for testing the difference. Otherwise Zar's test on H' can be applied.

For example, the diversity values in April and May of the sample data set (Table 4.11) are $H'_{\text{april}} = 1.51$ and $H'_{\text{may}} = 1.65$, respectively, with corresponding evenness values of $J'_{\text{april}} = 0.69$ and $J'_{\text{may}} = 0.75$. Thus, the difference between the two diversity measures is not due to sample size. Applying the t-test (Zar 1996) yields a value $t = 11.2$, which is bigger than the tabulated $t_{0.05(2)} = 1.968$ with 327 degrees of freedom. This indicates a significant difference between the values for April and May.

Similarity and dissimilarity measures (S) reflect changes in species composition and, in the case of quantitative indices, of abundance as well. If no explicit formulation of dissimilarity exists, as for the Bray–Curtis index (see Table 4.17), similarity measures S can be converted to dissimilarity or distance measures by calculating $1 - S$. Similarity and dissimilarity measures are often used as input for multi-dimensional scaling operations, which can be operated as metric or non-metric MDS (see page 165). Therefore it is important to know, whether the similarity measure has metric or non-metric properties (see also Jackson *et al.* 1989). Metric measures do not change the distances between the samples (species), whereas non-metric measures change the ordering of the data by multiplying or weighting some parts of the numerator. For example, the Sörensen index weights the number of co-occurrences by a factor of 2 and by this over-emphasizes them relative to the non co-occurring objects. Therefore, multi-dimensional scaling of a Sörensen matrix should be carried out in the non-metric mode.

Prior to the calculation of similarities, data are usually standardized to reduce the values to a common scale of comparison (Bakus 1990). Standardization does not change the relative distance between data (except for double standardizations). Data are transformed, either if the effect of very abundant species is to be diminished or the importance of rare species is to be increased. The first point is especially the case in data sets with a large amount of zero entries (Burd *et al.* 1990). Furthermore, transformations are recommended, when multivariate procedures require data following a normal distribution. The proportion of variance explained by the first ordination axis is then increased (Burd *et al.* 1990). But as they point out, many parametric tests are robust against skewed data. To avoid transformation of zero values, either 1 (for logarithmic transformation) or 0.5 (for square root transformation) is added to the

Table 4.16 Common standardizations and transformations.

Operation	Algorithm
Standardization of series to mean zero and unit variance	$x_{i,new} = \frac{x_i - \bar{x}}{s^2}$ with $\bar{x}$ = mean, s^2 = variance
Standardization by column (species) totals	$x_{new} = x_i / \sum x_i$
Subsequent standardization by column (*i* species) and row (*y* sites) totals (double standardization)	$x'_{new} = x_i / \sum x_i,\ x''_{new} = x'_{new} / \sum x'_{y,new}$
Logarithmic transformation	$x_{new} = \log(x_i + 1)$
Square-root transformation	$x_{new} = \sqrt{x_i + 0.5}$
Reciprocal transformation	$x_{new} = \frac{1}{x_i}$

numerical value of abundance (Table 4.16). Transformation changes the relative distance between data.

Similarity measures are based either on presence–absence data (often referred to as qualitative indices) or on meristic data, i.e. counts of individuals (referred to as quantitative). Indices based on presence–absence data can be used either for site–site (sample–sample) or for species–species comparisons. In the case of species–species comparisons, they indicate co-occurrences of species rather than associations of species. Association implies a functional relationship, which can be negative, positive or none, whereas co-occurrence is 'yes' or 'no' (binary, Pielou 1977). Hence, similarity indices range from 0 to 1, whereas association indices range from -1 to 1, i.e. the numerator in association indices contains a difference of frequencies, for instance $ad - bc$, where a, b, c, and d are frequency values in a 2×2 contingency table (Jackson *et al.* 1989).

A wide variety of indices exists, of which a small portion is displayed in Table 4.17. It should be noted for (dis-)similarity measures whether conjoint absences are included.

A very general qualitative similarity measure is the NESS index (normalized expected species shared; see Grassle and Smith 1976; Trueblood *et al.* 1994). NESS takes into account the effect of different sample sizes, already discussed for the rarefaction assessment of species numbers in a sample of size n, and is defined as:

$$NESS_{12|n} = \frac{2ESS_{12|n}}{(ESS_{11|n} + ESS_{22|n})} \tag{4.16}$$

ESS can be understood as the sum of products of rarefied species numbers of two samples, either taken from two different samples of size n ($ESS_{12|n}$) or two sub-samples from the same sample ($ESS_{11|n}$):

$$ESS_{12|n} = \sum_{i=1}^{SO} \left\{ 1 - \left[\binom{N_1 - N_{i1}}{n} \Big/ \binom{N_1}{n} \right] \right\} \left\{ 1 - \left[\binom{N_2 - N_{i2}}{n} \Big/ \binom{N_2}{n} \right] \right\} \tag{4.17}$$

Table 4.17 Common similarity and dissimilarity indices. Using Jaccard and Sörensen index for species–species comparison changes parameter *a* to no. of samples with co-occurrence of species *i* and parameter *b* and *c* to no. of sole occurrences.

Index and type	Algorithm
Simple Absolute and Euclidean dissimilarity***	$D_{Absolute} = b + c - 2a$ $D_{Euclidean} = \sqrt{(b + c - 2a)}$ *a* = no. of co-occurring species, *b*, *c* = number of species only present either at *b* or *c* respectively
Jaccard index (presence–absence type)*	$S_J = \frac{a}{a + b + c} = \frac{a}{OS_{tot}}$ *a*, *b*, *c* = see above, OS_{tot} = total number of species in both samples
Sörensen index (presence–absence type, also known as Czekanowski index)*	$S_s = \frac{2a}{a + b + c}$ *a*, *b*, *c* = see above
Bray–Curtis similarity (meristic type, also known as Czekanowski's quantitative index)**	$S_{BC} = \frac{2\sum_i \min(x_{i1}, x_{i,2})}{\sum_i (x_{i1} + x_{i2})}$ where min = minimum value, x_{i1}, x_{i2} = densities of species *i* at sites (samples) 1 and 2
Morisita's similarity index (meristic type, modified by Horn 1966, also known as Morisita–Horn)**	$S_M = \frac{2\sum_i x_{i1} x_{i2}}{\sum_i x_{i1}^2 + \sum_i x_{i2}^2}$
Canberra metric dissimilarity (meristic type)*,**	$D_C = \frac{1}{OS_{tot}} \sum_i \frac{\lvert x_{i1} - x_{i2} \rvert}{(x_{i1} + x_{i2})}$
Bray–Curtis dissimilarity (meristic type)*	$D_{BC} = \frac{\sum_i \lvert x_{i1} - x_{i2} \rvert}{\sum_i (x_{i1} + x_{i2})}$

*After Bakus (1990); **after Bloom (1981); ***after Lamont and Grant (1979)

and for $ESS_{11|n}$ and $ESS_{22|n}$ respectively, where N_1 and N_2 are the numbers of specimens in samples 1 and 2, respectively.

NESS converges to the Sörensen presence–absence index for large n, and for $n = 1$, NESS behaves as the Morisita–Horn index (Trueblood *et al.* 1994). For small n, NESS is dominated by the most abundant species. It becomes increasingly sensitive to rare species as n increases.

While not taking the effect of different sample sizes into account, qualitative measures are susceptible to the influence of frequent species. Comparing six presence–absence (qualitative) indices of species–species similarities (not including NESS), Jackson *et al.* (1989) showed that both the Jaccard and the Sörensen index are influenced by the number of occurrences of the most frequent species rather than by joint co-occurrences. Clustering based on these indices produces species rankings similar to rankings obtained

from the frequency of occurrence alone. Thus, only a little additional information in terms of 'ecological similarity' and associations of species is provided by this analysis.

Under the condition of different sample sizes, Lamont and Grant (1979) concluded in an extended analysis of 21 out of 60 qualitative indices that the simple Absolute and the qualitative Euclidean dissimilarity can be highly recommended for reasons of versatility and environmental sensitivity.

In a comparison of meristic indices, Bloom (1981) found that the Bray–Curtis index reflects true similarity most accurately and is recommended.

Example 4: How similar are communities?

If the samples taken to compare two different communities were subject to patchiness, i.e. high variability in abundance within each group, a qualitative index should be used, for example the simple Euclidean index or NESS (see example 2). If it can be assumed that samples were taken from one type of community and the variability in abundance is due to an underlying physical gradient, for example the distance from a pollution source, the quantitative Bray–Curtis index should be applied.

4.5.3 Classification and ordination: the detection of groups

Classification and ordination can be carried out for two purposes: either for defining groups of species (Q-mode analysis) or groups of sampling units (R-mode analysis, Pielou 1984) in order to reduce data complexity to an interpretable level, or for defining characteristic assemblages of species, i.e. indicator species or recurrent groups. Whereas the first aim can be reached with the common suite of multivariate procedures (clustering, principal components analysis, factor analysis, multidimensional scaling), the latter requires iterative techniques such as repeated correspondence analysis on sub-groups or recurrent group analysis (see below).

Common to the multivariate procedures of the first group is the dependence on the initial input, which always is a symmetrical matrix containing similarity, dissimilarity or association measures of species or sites. Evidently, measures of similarity with a range between 0 (no relationship) and 1 (same relationship) are less effective than measures of association with a range from -1 (opposite relationship) to 1 in separating groups of variables. Common measures of association are listed in Table 4.18. Association measures always take conjoint absences into account.

The Pearson correlation coefficient as a measure of association delivers peculiar results if the data matrix contains more than 50% zero entries (Bakus 1990). Results from Jackson *et al.* (1989) show a clear and meaningful separation of species groups based on association measures compared to similarity measures.

MULTIVARIATE CLASSIFICATION TECHNIQUES

For a detailed guide to multivariate procedures see textbooks such as Bakus (1990), Burd *et al.* (1990) and Digby and Kempton (1987). Very common are clustering, principal components analysis and multidimensional scaling. Since these procedures do not allow simultaneous ordination of species and samples, they are performed either in the R- or the Q-mode analysis.

The results of clustering operations depend on the fusion rules to aggregate groups of objects (species, sites). Compared to a known cluster structure, average linkage (group average method, unweighted group mean method) and Ward's minimum variance method are best capable of reproducing the original structure (SAS 1990). From these

Table 4.18 Common measures of association.

Index name	Algorithm
Pearson correlation (can be converted to covariance measure when only numerator is taken)	$r = \frac{\sum_{OS}(x_{1i} - \bar{x}_1)(x_{2i} - \bar{x}_2)}{\sqrt{Var_1 Var_2}}$ with Var_1, Var_2 = variance of sample 1 or 2 respectively; x_{1i} = abundance of species i in sample 1, $\bar{x}_{1,2}$ = mean abundance of sample 1 and 2 respectively. $autocovariance = \sum_{os}(x_i - \bar{x})^2$
Phi coefficient (based on the 4 cells a, b, c and d of a 2 × 2-contingency table, derived from the chi-square statistic)*	$\varphi = \frac{ad - bc}{(a+b)(a+c)(b+d)(c+d)}$
Yule coefficient*	$Y = \frac{ad - bc}{ad + bc}$ for a, b, c, d see above

**After Jackson* et al. *(1989)*

Ward's method tends to find clusters of equal size, while average linkage tends to find clusters of equal variance. Single linkage clustering often produces the poorest structure.

Principal components analysis (PCA) is a very common procedure to derive a small number of linear combinations (principal components) of a set of variables (sites, samples) that retain as much of the information in the original variables as possible (SAS 1990). That means that the first principal component describes the axis to which most of the data set's total variance is attributed. The forthcoming principal components are perpendicular on the first, so that the axes are statistically independent. PCA calculates as many components as variables in the data set. PCA can be carried out on a correlation matrix, weighting all variables equally, or on a covariance matrix, so that the analysis is influenced by the highest values.

Multidimensional scaling (MDS) is a class of methods for estimating the coordinates of a set of variables (samples, sites) in a space of specified dimensionality from data measuring the distances between pairs of objects (SAS 1996). In metric MDS, the distances are reflected accurately, whereas non-metrical MDS is based on the rank order of distances.

Usually results of multivariate procedures are presented graphically. For cluster dendrograms a decision on the similarity/distance level has to be made, below which the clusters are accepted to represent groups. For PCA and MDS ordination graphs, groups are distinguished by framing variables lying close together. Cornelius and Reynolds (1991) discuss a variety of methods to locate borders between groups of data (discontinuities) within ordered ecological data so that framing in MDS is less arbitrary. Their approach applies a split moving window technique, in which a subset (window) of adjacent data points is split into two equal parts and differences or similarities between the two subsets are calculated. After calculating the difference measure which is assigned to the window midpoint, the window is shifted forward by one unit (time, space) and the calculation is repeated. The highest difference values mark the edge between two ecologically different groups and the groups can then be identified in an MDS plot.

Example 5: How to describe associations?

If data sets with many taxa and/or samples have to be analyzed, the application of multivariate methods with the Pearson correlation matrix as input is recommended. If for reasons described in the previous examples, other matrices have been computed (similarity or dissimilarity), these can also be used as input for MDS. For describing the major underlying pattern, either for temporal trends or spatial arrangements, these methods give good results. However, on the species level these methods may sometimes provide results which cannot be interpreted. For example, when species associations are to be defined and a great number of species belong to neither group, it remains unclear how these species should be classified.

Analyzing the sample data set (see Table 4.14) with different methods shows the effects of different association and distance measures as well as the effects of transformations. A look at the data shows that during August on average a very low abundance was recorded. Only the ordinations based on the Pearson correlation coefficient (Figure 4.18, B, D, F) reproduce this feature sufficiently, whereas the application of the Euclidean distance measure leads to results suggesting nearly no difference between the March, July, August, September, and November samples (Figure 4.18, A, C, E). Transformation reduces the effect of high values, so that April and May move to the center in Figure 4.18 F compared to Figure 4.18 D. The general configuration for the Pearson-based ordinations remains the same under transformation, whereas in Figure 4.18 E October moves to a marginal position compared to Figure 4.18 C. In contrast, in the ordinations based on the Pearson *r* (Figure 4.18 B, D, F) October always marked a marginal position. To conclude, for the sample data set presented here the ordinations based on the Pearson *r* seemingly represent community structure more appropriately than ordinations based on the Euclidean distance. In any case, a comparison with the original data set is recommended to interpret ordinations.

RECURRENT GROUP ANALYSIS

The recurrent group analysis (RGA) (Fager 1957) is used to identify characteristic species groups. Although based on a similarity measure, the affinity index *I*, its iterative procedure allows a meaningful separation of species often applied to ichthyoplankton studies (e.g. Doyle *et al.* 1995). It copes with the effect of dominance influence described above for the qualitative indices by subtracting a weight for the dominant species:

$$I = \frac{a}{\sqrt{(A)(B)}} - \frac{1}{2\sqrt{(B)}} \tag{4.18}$$

(after Doyle *et al.* 1995; with a = number of joint occurrences, A = total number of occurrences of the less frequent species A, B = total number of occurrences of the more frequent species B, I = Affinity Index).

As for cluster analysis it is necessary to define a minimum level of the affinity index, below which no relationship between species is anticipated. Doyle *et al.* (1995) use minimum values of *I* between 0.3 and 0.4. After calculating the affinity index, the species are tabulated in an affinity matrix in descending order with the species exhibiting highest affinities at top. Based on four rules, within the group of highest ranked species a group is searched in which all species are interrelated with each other. This group is outlined as the first recurrent group and removed from the table. From the remaining species, those interrelated only with one of the members of the first recurrent group are selected and

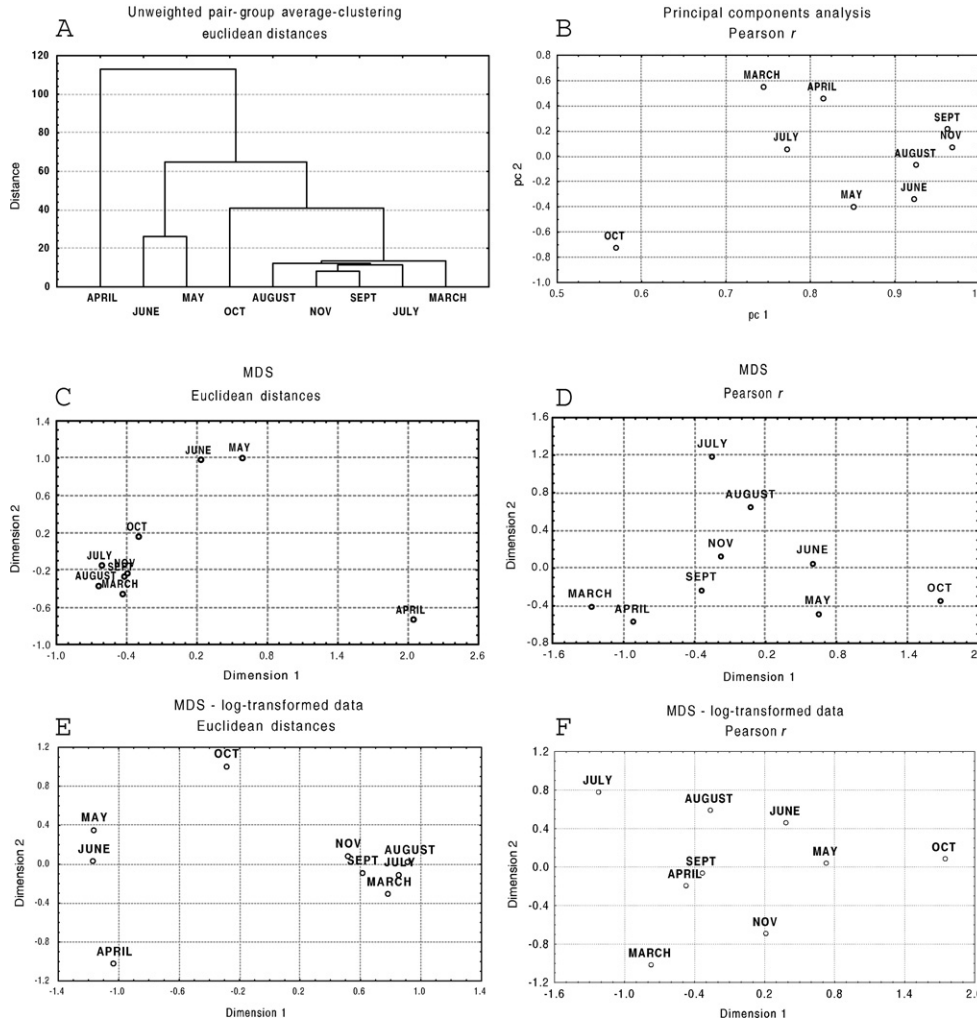


Fig. 4.18 Application of different ordination techniques and similarity/dissimilarity measures to the sample data set (Table 4.11). A, C, E: ordinations and classification based on Euclidean distance; B, D, F: ordinations based on Pearson moment correlations. MDS, multidimensional scaling. For explanation see text.

considered to be 'associated species' to this group. After removing the first group and its associated species, the remainder is analyzed in a similar way and the second recurrent group is outlined and so forth. Fager (1957) tested this approach and claimed that defining recurrent groups in such a way is in accordance with the known autecological features of the species considered.

Example 6: Analyzing relationships in calanoid copepods

If the number of taxa involved is rather small and a taxonomically homogeneous group is considered, for which the autecological features are well known, iterative sorting such as the recurrent group analysis can be applied. The advantage of this approach is to include a large amount of biological information.

MATCHING SPECIES AND SAMPLES: INDICATOR SPECIES ANALYSIS

At present four main approaches are commonly in use to define species–sample (i.e. site or season) relationships: two-way joining, correspondence analysis (including detrended CA), TWINSPAN, and the indicator value method of Dufrene and Legendre (1997).

The *two-way joining* is a combination of cluster classifications of both R- and Q-mode type. As a result, a two-way coincidence table is created in which each species has a density symbol for each site. Usually the table is reordered in such a way that similarities from the cluster structure are depicted and group members are placed in adjacent positions. Omori and Ikeda (1984) present an example, in which a Q-mode (species only) analysis table is reordered in such a way that associated species become adjacent to each other.

Correspondence analysis (CA) allows simultaneous ordination of rows and columns, separate R- and Q-mode analyses do not have to be made. Hence, CA has become a very popular method in ecology (Digby and Kempton 1987). In contrast to the above-mentioned multivariate methods, CA does not require an input matrix of similarity or distance measures. Based on meristic data (countings) each cell of the original data matrix is double standardized to obtain site and species scores.

Species and sample/site scores are defined as (after Digby and Kempton 1987)

$$a_i = f \sum_j x_{ij} b_j / r_i \tag{4.19}$$

$$b_j = f \sum_i x_{ij} a_i / c_j \tag{4.20}$$

with f = scaling factor, x_{ij} = abundance of species i at site j, a_i = the species score, b_j = the site score, r_i = total number for species i, and c_j = total number of individuals at site j.

Starting with an arbitrary set of scores for b_j, these are inserted into the equation for a_i and new values for a_i are calculated. These are used as input for the calculation of b'_j values, which are in turn used for the calculation of new a'_i, and so forth. The process proceeds until results converge and no further development is observed.

CA has two disadvantages which are removed in detrended CA (DCA, for a detailed discussion on the CA/DCA methodology see Palmer 1993): the horseshoe-effect of creating a curvilinear figure related to the first ordination axis, and a compression of data points at the marginal ends of the first axis.

The *TWINSPAN analysis* is widely applied in terrestrial ecology and also in marine studies (for the latter see Pearcy *et al.* 1996). Dufrene and Legendre (1997) give a brief description of the method. It basically consists of a repeated CA analysis on data subsets. The subsets are chosen in such a way that a subset of sites from the first CA is selected bearing a positive sign on the first CA axis. This subset is reanalyzed by CA and the portion of sites bearing a positive sign on the new first axis is again selected, until only two sites are left, either negatively or positively associated to the first axis of the last CA step. The species are not split into subsets. According to its abundance each species is separated into abundance classes, so-called 'pseudospecies', and in each step the 'pseudospecies' receive an attribute value indicating a positive or negative relationship to the selected subset of sites and samples respectively. TWINSPAN can only work efficiently when strong gradients underlying the data are present. Furthermore, the borderline to separate into one of the groups according to the sign the sites have on the

first axis is quite arbitrary for those sites, for which absolute scores are close to zero, since a slightly different ordination could make them switch onto the other side of zero.

The *indicator value method* of Dufrene and Legendre (1997), also known as 'multiway indicator species analysis', starts with a Q-mode analysis of sites. For each of the clusters of sites an indicator value is calculated for each species *i* by comparing the abundance within the site group to the abundance at all other sites and by weighting this measure with the ratio of number of sites within the group to the number of sites outside the group. The species indicator value can be calculated at any level of hierarchy within the cluster diagram, so that more general species can be adequately placed at higher levels than rather site-specific species, which are placed on the lower levels. Since the indicator value is strongly dependent on abundances, it is possibly biased by patchy distributions, as these are common in plankton.

Example 7: Analyzing relationships between sites or seasons and species

All methods mentioned in this section require a stable relationship between species and samples. This can be the case in time series, when species show a stable pattern of seasonal occurrence, or in spatial samplings. If a larger area has been sampled and patchiness is supposed to cause no severe bias (i.e. variability in subgroups is smaller than overall variability), analysis should start with CA/DCA. In the case of promising results, i.e. clear site-species clusters in relation to environmental factors, TWINSPAN can be applied subsequently. However, up to the present TWINSPAN mostly has been applied to sedentary species (benthos, vegetation). If a smaller area is considered and patchiness of plankton causes considerable variability, only qualitative statements should be made (see example 4). Possible limitations of TWINSPAN and the indicator value method are discussed above. As far as the author knows, the indicator value method has not been applied to plankton studies yet.

4.5.4 Analysis of spatial and temporal formations

The investigation of ecosystems always warrants the integration of processes at very different spatio–temporal scales (Postel 1983). Hence, understanding the interference between scale and pattern and the processes which form these patterns is the central problem in ecology (Levin 1992). The development of such a functional analysis in plankton ecology started when Colebrook (1969) pleaded for a shift from the analyses of frequency distributions to what he named a 'systematic variation approach'. Before then, investigations focused on fitting empirical data to theoretical distribution functions (for example Poisson, normal or negative binomial) and to calculate dispersion indices (see Elliot 1983, his chapter 5.6). However, this approach has three major disadvantages for the analysis of plankton (Fasham 1978):

1) distribution parameters will vary with sample size (tow length, sampled volume)
2) a frequency distribution is unable to depict the spatial arrangement of data
3) often more than one distribution function can be fitted to the data and, therefore, no conclusions on underlying processes can be drawn.

Three approaches to analysis of spatial (and of temporal) formations will be considered here, of which all take the spatial arrangement of samples into account:

1) comparison of different matrices
2) analysis based on semi-variograms
3) spectral analysis.

Although closely related, the latter two are different according to the data structure required. For linear transects with high frequency sampling or permanent sampling, spectral analysis can be applied. If spectral analysis is applied to coarsely sampled data, the analysis is subject to the 'aliasing' effect, i.e. high frequency processes with wavelengths smaller than the sampling interval are detected as low frequency processes with wavelengths longer than the sampling interval. This causes erroneous results (Postel 1983; Schlittgen and Streitberg 1995). For data sets with regional coverage and irregular spacing between sampling points, methods based on the analysis of semi-variograms are preferred.

Furthermore, it is possible to use PCA for detection of characteristic patterns in spatio–temporal data sets (Williams *et al.* 1993). Recently non-linear techniques, the so-called near-neighbor algorithm, have been applied to spatial analysis of salinity and chlorophyl data (see Strutton *et al.* 1997 for details).

The introduction given here concerns the integrative type of sample normally obtained for zooplankton. The individuals are not directly counted and positioned in their micro-scale environment, but integrated values for the total volume of sea water filtered for the sample are calculated and thus, only the spatial arrangement of the samples but not that of the organisms themselves can be analyzed. However, if the development and application of optical methods continues (Chapter 7), direct counts and positioning on the micro-scale for each individual will be available (see Currie *et al.* 1998). In this context indices based on the measurement of real spherical distances between individuals will be of importance, as they have already been applied in benthos and vegetation ecology (Andrew and Mapstone 1987; Currie *et al.* 1998).

The *comparison of two distance matrices* can be conducted in several ways.

One is to ask whether biological differences can be attributed to distance between sampling points. In this case the Mantel test for two matrices can be applied, of which one matrix D contains measures of ecological dissimilarity $d_{i,j}$ and the other matrix G geographic distances $g_{i,j}$ (detailed guide in Sokal and Rohlf 1995; examples in Mackas 1984, Planque and Ibanez 1997). This test is based on a correlation of the i,j-th element in either matrix, where i marks the position in the columns and j marks the position in the rows:

$$r_{Mantel} = \frac{1}{n-1} \sum \frac{(g_{i,j} - \bar{g})}{\sqrt{Var(g)}} \times \frac{(d_{i,j} - \bar{d})}{\sqrt{Var(d)}} \tag{4.21}$$

In the above case, the values belonging to the two different matrices are treated as two variables g and d. A high r_{Mantel} value indicates that biological dissimilarities increase with geographical distance. As a test, the r_{Mantel} value can be compared to correlation values after elements i,j have been randomly redistributed in both matrices, which then indicates the correlation coefficient caused by chance (permutation test). A significant relationship can be assumed if $r_{Mantel} > r_{random}$.

Furthermore the Mantel statistic can be calculated for different classes of geographic distances in order to depict the decrease of the r_{Mantel} values with increasing distance (Mantel correlogram). For calculation of the Mantel-correlogram the distance values are coded 1 for being in the distance class and zero otherwise. For each class of distances a separate r_{Mantel} value is calculated (example in Planque and Ibanez 1997).

The second way to apply matrix comparisons is to ask whether biological differences belong to environmental differences rather than to geographical distance. In this case the distance matrix G is replaced by a dissimilarity matrix E containing dissimilarities of the

samples based on more than one environmental factor. The correlation coefficient between elements $d_{i,j}$ and $e_{i,j}$ indicates which combination of environmental factors serves as 'best explanatory variables' to explain biological variability (example in Clarke and Ainsworth 1993).

In the *analysis of semi-variograms* the semi-variance $\gamma(h)$ (note: factor 0.5) is a measure to describe the difference between all points y_j and y_{j+h} in a data set, separated by the distance value h, by means of variance:

$$\gamma(\boldsymbol{h}) = \mathbf{0.5}\frac{\mathbf{1}}{\boldsymbol{n}}\sum(\boldsymbol{y_j} - \boldsymbol{y_{j+h}})^2 \tag{4.22}$$

Following Mackas (1984), it can be deduced from the spatial autocovariance structure of the regional data set, which is the autocovariance structure for y at distance lag h (for formula of covariance see Table 4.18), by subtracting the latter from the variance σ^2 with n degrees of freedom (E denotes a summing function for all pairs of stations in the data set and is omitted in equations 4.28–4.30):

$$\gamma(\boldsymbol{h}) = \boldsymbol{E}[\sigma^2 - \mathbf{autocovariance}_{\boldsymbol{h}}] \tag{4.23}$$

$$\gamma(\boldsymbol{h}) = \boldsymbol{E}[(\boldsymbol{y_j} - \mu)^2 - (\boldsymbol{y_j} - \mu)(\boldsymbol{y_{j+h}} - \mu)] \tag{4.24}$$

$$\boldsymbol{y_j^2} - \mathbf{2}\boldsymbol{y_j y_{j+h}} + \boldsymbol{j_{j+h}^2} = \mathbf{2}(\boldsymbol{y_j^2} - \mathbf{2}\mu\boldsymbol{y_j} + \mu^2 - \boldsymbol{y_j y_{j+h}} + \boldsymbol{y_j}\mu + \boldsymbol{y_{j+h}}\mu - \mu^2) \tag{4.25}$$

$$\mathbf{0.5}(\boldsymbol{y_j^2} - \mathbf{2}\boldsymbol{y_j y_{j+h}} + \boldsymbol{y_{j+h}^2}) = \boldsymbol{y_j^2} - \mathbf{2}\mu\boldsymbol{y_j} + \mu^2 - \boldsymbol{y_j y_{j+h}} + \boldsymbol{y_j}\mu + \boldsymbol{y_{j+h}}\mu - \mu^2 \tag{4.26}$$

Since $\Sigma y_j^2 = \Sigma y_{j+h}^2$ and the sample mean $\mu = \Sigma y_j/j = \Sigma y_{j+h}/j$, it follows that

$$\boldsymbol{y^2} - \boldsymbol{y_j y_{j+h}} = \boldsymbol{y^2} - \boldsymbol{y_j y_{j+h}} \tag{4.27}$$

In a semi-variogram the increase of semi-variance $\gamma(h)$ is described in relation to increasing distance h between two points. This relationship is essential for interpolating irregularly spaced data to grid points, for example by kriging (for a detailed introduction to kriging, see Davis 1984) in order to draw isopleth maps. It is also important for the analysis of sample variance, the autocorrelation structure and spectral analysis, since the power spectrum is the Fourier transform of the spatial autocovariance function at distance h (Mackas 1984).

Variogram analysis has been applied to both fishery (Barange and Hampton 1997) and zooplankton studies (Mackas 1984; Solow and Steele 1995) in order to define scales of patchiness (i.e. spatial autocorrelation). Solow and Steele (1995) present an elegant way of calculating confidence bands for the semi-variogram by randomly redistributing the data to spatial locations and recalculating a series of random semi-variograms. By this they can assess whether a variable is significantly distributed in patches as well as the corresponding patch size (original semi-variogram not within the confidence limits at certain distance values) or not distributed in patches (original semi-variogram within the boundaries of the random confidence limits).

Spectral analysis requires detrended data, either sampled continuously or at very short intervals. Since zooplankton is tedious to collect in such a way, continuous measurements of chlorophyl, salinity, and temperature are mostly considered in spectral analysis. However, using a shipboard high capacity pump, simple zooplankton parameters (either biomass or particles >0.375 mm spherical diameter) can also be obtained at acceptably high sampling frequencies (Mackas and Boyd 1979, Piontkovski *et al.* 1997).

The idea of spectral analysis is to decompose a continuous sequence of values (time

series, spatial transect) into a sum of sine and cosine waves of different frequencies and amplitude (Schlittgen and Streitberg 1995). The method to accomplish this process is Fast Fourier Transformation. In spectral analysis not the original data series, but its spatial or temporal autocovariance function is dissected into portions of variance per frequency, and the size of the portion indicates which frequency contributes most to the overall variance of the data series. In this way the strength of processes at very different scales can be compared in only one analysis.

This characteristic has made spectral analysis subject to wider interpretation of results than is the case for results of semi-variograms, since different processes can be assigned to variance maxima at different frequencies (Horne and Platt 1984; Steele and Henderson 1979; and also Walsh *et al.* 1977, for details on time series analysis). Furthermore, based on calculations of average drift speeds along the investigated transects, for example, implications on temporal duration of spatially resolved data as well as spatial extensions of temporal data and associated processes at different wavelengths and frequencies respectively can be drawn (see Horne and Platt 1984, their Figure 1). Low-frequency processes with long wavelengths were attributed to rotation (eddy formation) and buoyancy, whereas high-frequency processes with short wavelengths were attributed to turbulence and, in the case of phytoplankton, to patch formation by growth.

Two aspects of spectral density curves can be extracted for interpretation: (1) peaks indicate spatial heterogeneity at that particular wavelength, and (2) if distinct peaks are missing, the slope of the power spectrum – i.e variance versus frequency (cycles per unit scale) – is considered, both on a logarithmic scale. Usually this relationship is expressed for wave number instead of frequency (wave number = frequency $\times 2\pi$).

Model results and investigations in the sea have shown that the slopes of the log-variance versus log-wave number curves range between $-5/3$ and -3 (Fasham 1978). This indicates that low-frequency processes prevail in the sea. If isotropic turbulence, i.e. turbulence moving particles in all directions equally, is considered to be the force creating spatial variability, the slope narrows to $-5/3$. If turbulence is two-dimensional or if, for instance, eddy formation or thermocline processes overlay the pure isotropic turbulence, slopes up to -3 are obtained. Spectra driven by internal waves, i.e. periodic vertical movements of the pycnocline, in practice have a slope of approximately -2. Theoretically, such spectra driven by internal waves should show no considerable variance for frequencies greater than the Brunt–Väisällä frequency, which determines the size of eddies and is related to the density gradient between surface and pycnocline. However, as for turbulent diffusion, additional dissipation processes at the pycnocline cause transition of energy to higher frequencies and smaller spatial scales (Fasham 1978), so that a continous spectrum is obtained. Phytoplankton spectra taken from a model of Denman and Platt (1976, in Fasham 1978), consisting of growth and diffusion terms, show a relationship between eddy size d and growth rate α of phytoplankton. They assume that at size d a time constant τ can be described in which energy is transferred to an eddy of size $d/2$. If $\tau \ll \alpha^{-1}$, i.e. if growth is low compared to the time constant, slopes of the spectrum will be in the range of $-5/3$ to -3 as for turbulence spectra. However, if $\tau \gg \alpha^{-1}$, i.e. if growth rates are high, slopes will become less steep than the pure turbulence spectrum. In conclusion, less steep slopes indicate more intense processes at shorter wavelengths (higher frequencies or wave number, respectively) and thus increased patchiness not related to physical gradients. Steele and Henderson (1977) used this approach and calculated ratios

between spectra of chlorophyl and temperature. These ratios increased with wave number, indicating increased biological patchiness.

Example 8: Analysis of spatial formations

Depending on the type of input data, methods have to be chosen as described above. It is recommended to have one physical data set with which to compare the biological results.

4.5.5 Examination of processes within communities by network analysis

The reason for including this topic is that the network analysis approach provides an alternative view to diversity in community ecology. Instead of analyzing only the numerical properties of components of a system, these components are understood to be linked to each other by flows of matter, and that flows and components together determine the structure of the system. The direction of the flows is determined by the trophic status of each component. In the frame of this approach, a series of diversity indices of ecosystem organization has been developed (Kay *et al*. 1989). In contrast to this holistic perspective, traditional species diversity measures (see section 4.5.2) only describe the structure within the components of a system. Thus, organization indices provide a better refined measure of the realized and ultimate status of a system than species diversity indices (Table 4.19). It is evident that the 'development capacity' has no counterpart in terms of species diversity.

Ways of calculating the flows of matter are given by steady state models and inverse models. Both model types feature a system of linear equations, in which biomass data

Table 4.19 Comparison of network and species diversity measures and the levels of systems performance which they address. Since the network measures are based on information theory, the Shannon–Wiener index is compared to them. For comparison, all indices have the logarithm based on 10. Maximum values are calculated for T_i and p being equal for all components. Network indices after Ulanowicz and Goldman (1988) and Kay *et al.* (1989).

Level of performance	Network diversity	Species diversity
Realized in a system	$A = T\sum_{i}^{n}\sum_{j}^{n+2}\frac{T_{ij}}{T}\log\frac{T_{ij}T}{T_iT_j}$ A = ascendency, T_i = flows leaving i, T_j = flows reaching j, T_{ij} = flows leaving i and reaching j, T = total flows	$H_{SW} = -\sum_{OS} p\log p$ OS = number of observed species
Ultimate value	$C = -T\sum\frac{T_i}{T}\log\frac{T_j}{T}$ C = development capacity	
Maximum value	$C_{max} = -T\log\frac{T_i}{T} = T\log OC$ OC = number of observed components	$H_{max} = -\log p = -\log\frac{1}{OS}$ $= -\log 1 + \log OS = \log OS$

and specific flow rates are used to calculate flows between components. While the steady state models use *a priori* information on flows to calculate the indices, the inverse approach estimates flow parameters from existing biomass data by means of matrix algebra with inverse matrices (therefore 'inverse' approach) (for details see Ducklow *et al*. 1989; Venzina 1989). Recent literature on network analysis considering the ECOPATH software presents a combination of both approaches in such a way, that already known rates are presented to the model and remaining blanks are calculated by the inverse method (Pauly and Christensen 1993; Monaco and Ulanowicz 1997; Niquil *et al*. 1998).

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5 Sampling, preservation, enumeration and biomass of marine protozooplankton

D.J. Gifford and D.A. Caron

5.1 INTRODUCTION

Nano- and microzooplankton are defined operationally on the basis of size as heterotrophic plankton 2–20 μm and 20–200 μm respectively (Sieburth *et al*. 1978). They are ubiquitous and abundant in the euphotic zones of marine and freshwaters. Both categories contain a diversity of heterotrophic protistan taxa including flagellates, dinoflagellates, ciliates, and sarcodines. Heterotrophic flagellates are typically the most abundant nanozooplankton organisms, while ciliates and heterotrophic dinoflagellates usually dominate the microzooplankton. The sarcodines span size ranges from nanoplankton to mesoplankton (2–2000 μm) or greater. They are treated separately in this chapter because of their specific requirements for collection and enumeration. Here we use the term 'protozooplankton' to refer to nano- and microzooplankton inclusively, and also to those protozoa that exceed these size designations. Typical abundances of the various protozoan taxa are given in Table 5.1. The microzooplankton category also includes micrometazoa such as metazoan nauplii, larval stages of meroplanktonic forms, and rotifers. Micrometazoa are not covered here.

There are many reasons to sample the protozooplankton. These organisms perform a number of functions in pelagic ecosystems. They are major grazers of bacteria and phytoplankton (e.g. Banse 1992) and important nutrient recyclers (e.g. Berman 1991; Caron 1991). Forms such as plastidic ciliates, which contain functional chloroplasts or whole algal cells, are primary producers (e.g. Stoecker 1991). As prey for higher order consumers, nano- and microzooplankton constitute a trophic link between the microbial loop and the classical metazoan food web (e.g. Stoecker and Capuzzo 1990; Gifford 1991). Quantitative evaluation of any of these functions requires accurate estimates of nano- and microzooplankton numerical abundance and biomass. To estimate abundance and biomass it is necessary to collect, usually preserve, and enumerate the target organisms. With the exception of the sarcodines, collection methods are similar for the

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Table 5.1 Typical numerical abundances of nano- and microzooplankton taxa in estuarine, neritic and oceanic ecosystems.

Taxon	Location	Numerical abundance	Author
Heterotrophic flagellates	Sargasso Sea	100–300 ml^{-1}	Davis and Sieburth (1982)
	Gulf Stream	600–2800 ml^{-1}	Davis and Sieburth (1982)
	Marine Snow	1000–182 000 ml^{-1}	Caron *et al.* (1986)
	Georgia Coast, USA	300–3200 ml^{-1}	Sherr *et al.* (1984)
	Parker Estuary, USA	300–21 900 ml^{-1}	Wright and Lebo (1987)
	Red Sea	600–1200 ml^{-1}	Weisse (1989)
	Limfjord, Denmark	100–4200 ml^{-1}	Fenchel (1982b)
	Long Island estuaries, USA	800–85 000 ml^{-1}	Caron *et al.* (1989)
	North Atlantic	2000–4600 ml^{-1}	Verity *et al.* (1993)
Ciliates			
Aloricate ciliates	Halifax Harbour, Canada	2680–11 360 l^{-1}	Gifford (1988)
	North Atlantic	3303–7145 l^{-1}	Gifford *et al.* (1995)
	North Atlantic	1900–17 200 l^{-1}	Verity *et al.* (1993)
	North Pacific	500–28 000 l^{-1}	Strom *et al.* (1993)
	California Current	2400–18 000 l^{-1}	Beers and Stewart (1970)
	Coastal Peru	1100–5300 l^{-1}	Beers *et al.* (1971)
	Georges Bank	580–13 000 l^{-1}	Stoecker *et al.* (1989)
	Narragansett Bay, USA	5–2803 l^{-1}	Verity (1986)
	Baltic Sea	7300–230 000 l^{-1}	Kuuppo-Leinikki (1990)
	Mediterranean Sea	200–20 000 l^{-1}	Sherr *et al.* (1989)
	Oslofjord, Norway	2000–10 500 l^{-1}	Paasche and Kristiansen (1982)
	Grand Banks	2700–3870 l^{-1}	Paranjape (1990)
	Celtic Sea	2488–4000 l^{-1}	Burkill *et al.* (1987)
	Carmathen Bay, Wales	12 000 l^{-1}	Burkill *et al.* (1987)
Tintinnid ciliates	Halifax Harbour, Canada	0–1440 l^{-1}	Gifford (1988)
	North Atlantic	60–220 l^{-1}	Gifford *et al.* (1995)
	Narragansett Bay, USA	20–8181 l^{-1}	Verity (1986)
	Long Island Sound, USA	1000–9600 l^{-1}	Capriulo and Carpenter (1980)
	Oslofjord, Norway	320–4220 l^{-1}	Paasche and Kristiansen (1982)
	Grand Banks	170–990 l^{-1}	Paranjape (1990)

Heterotrophic dinoflagellates			
Total	North Atlantic	2–49 ml^{-1}	Verity *et al.* (1993)
	Baltic Sea	90–4800 ml^{-1}	Kuuppo-Leinikki (1990)
Athecate	Gulf of Maine	29–145 ml^{-1}	Shapiro *et al.* (1989)
	North Atlantic slope water	97–230 ml^{-1}	Shapiro *et al.* (1989)
	North Atlantic oceanic water	6–123 ml^{-1}	Shapiro *et al.* (1989)
	Gulf of Alaska	13–217 ml^{-1}	Shapiro *et al.* (1989)
Sarcodines			
Amebae	North Atlantic, neuston	10–100 l^{-1}	Davis *et al.* (1978)
	Carribbean Sea	1–10 l^{-1}	Davis *et al.* (1978)
	Narragansett Bay, USA	1–10 l^{-1}	Davis *et al.* (1978)
Foraminifera	North Atlantic	0.1–2 m^{-3} (adults)	Bé (1960)
	Red Sea	1–100 m^{-3} (adults)	Almogi-Labin (1984)
	Panama Basin	up to 10 m^{-3} (adults)	Bé *et al.* (1985)
	Panama Basin	up to 100 l^{-1} (juveniles)	Be *et al.* (1985)
	Arabian Sea	1–5 m^{-3} (adults)	Rao (1973)
Radiolaria (Polycystines and Phaeodaria)	Eastern Tropical Pacific	16–82 l^{-1}	Beers and Stewart (1971)
	Eastern Equatorial Atlantic	10–70 m^{-3}	Dworetsky and Morley (1987)
	Antarctic waters	10–100 m^{-3}	Morley and Stepien (1985)
Acantharia	Eastern Tropical Pacific	1–6 l^{-1}	Beers and Stewart (1971)
	North Atlantic	1–397 m^{-3}	Massera-Bottazzi *et al.* (1971)
	Eastern Equatorial Pacific	5–25 l^{-1}	Michaels (1988)

majority of nano- and microzooplankton taxa. Techniques of preservation and enumeration may differ for the nano- and microzooplankton, and in some cases are taxon-specific, depending on the objectives of the individual study. Alternatively, live counting may be done for some protozooplankton taxa. Here we review and summarize methods in common use in marine studies, citing alternative methods where appropriate. Although the review is directed primarily toward marine protozooplankton assemblages, many of the methods cited may also be applied to freshwater organisms, with appropriate modifications such as the use of buffers.

5.2 COLLECTION METHODS

5.2.1 Nano- and microzooplankton

Nano- and microzooplankton, unlike most other zooplankton groups, are sufficiently abundant in most waters to collect in small volumes of seawater without first being concentrated in some manner. This has the advantage of minimizing losses due to cell breakage during sample collection, although losses caused by preservation can occur. Samples of a few ml to a liter collected by any convenient method are preserved directly (see section 5.3), and abundances sufficient to allow enumeration are obtained by filtering or settling preserved subsamples onto membrane filters or glass slides. Because of their flexibility and fragility, many protozooplankters pass through or are destroyed by plankton meshes and are disrupted by turbulence (e.g. Gifford 1985). Thus, the use of nets and pumps to collect or manipulate protozooplankton is not recommended except for some size categories of sarcodines (discussed in section 5.2.2) and for tintinnid ciliates, which are encased in a protective lorica.

Go-Flo® or Niskin® bottles are the most commonly employed collection bottles for oceanographic work. These bottles have considerable appeal for collecting nano- and microplankton because they can be deployed in multiple arrays (rosettes) which collect water samples from discrete depths. Because rosettes typically are equipped for collecting physical/chemical data (salinity, temperature, pressure, fluorescence, light, light transmission, oxygen), they also provide detailed ancillary information against which to compare protozooplankton vertical abundances. Niskin bottles are available in a number of configurations and may have interior tubing, o-rings and/or springs composed of a diversity of materials. The tubing typical of early generations of Niskin bottles is toxic to a number of planktonic protists (Price *et al.* 1986). When water is collected and used for incubation, Niskin bottles outfitted with exterior springs, or non-toxic teflon-coated interior springs and silicone o-rings, are essential. Niskin bottles are deployed open and are closed at the depth of sample collection. Therefore they transit the surface microlayer, an area characterized by elevated abundances of microorganisms and concentrations of material including metals and other potentially toxic contaminants. If this is a concern, Go-Flo bottles can be used to collect bulk seawater. Go-Flo bottles are deployed closed, opened below the surface, and closed at the depth of water collection. They may be teflon-lined and equipped with o-rings of various composition. Both the silicone and vitan o-rings available for Go-Flo bottles are non-toxic to marine micro-organisms (Gifford, unpublished data). For rate measurements of oceanic protozooplankton, which are believed to be particularly susceptible to contamination, use of metal-free clean collection and handling techniques (Fitzwater *et al.* 1982) may be appropriate.

Densities of nano- and microplankton-sized protozoa in pelagic microenvironments such as the neuston (Sieburth *et al*. 1976), marine snow particles (e.g. Caron *et al*. 1986; Alldredge and Silver 1988; Davoll and Youngbluth 1990; Lochte 1991; Turley and Mackie 1995), the oxic/anoxic interface (e.g. Fenchel *et al*. 1990), sea-ice (e.g Garrison 1991; Garrison and Buck 1986), the pycnocline (e.g. Tiselius *et al*. 1994) and thin biological layers (Johnson *et al*. 1995) can be several orders of magnitude more abundant than abundances in the water adjacent to or surrounding these microenvironments (Table 5.1). Collection of samples from such microhabitats requires more specialized methods than are used for assessing protozooplankton dispersed in the water. We do not describe these methods in detail here, but note that neuston samples are collected using methods that employ screens, glass plates or rotating drums to sample the surface microlayer (e.g. Sieburth 1979). Macroscopic detrital aggregates (marine snow particles) are hand-collected in jars, syringes or tubes from surface waters of pelagic environments by divers, while macroaggregates in deeper waters are collected using submersibles or remotely operated vehicles (e.g. Alldredge and Silver 1988). Microorganisms from thin layers are collected using profiling/siphon systems specialized for sampling at fine (cm) scales (Donaghay *et al*. 1992; Johnson *et al*. 1995).

5.2.2 Planktonic sarcodines

The planktonic sarcodines are a polyphyletic group of protozoa possessing some type of pseudopod as a conspicuous feature of their body form. The term 'sarcodine' usually refers to the gymnamebae (the 'naked' amebae), the foraminifera and the actinopods (heliozoa, acantharia, polycystines and phaeodaria). The term 'radiolaria' is commonly employed for the polycystines and phaeodaria. Sarcodines span an enormous size range ($<10\,\mu$m to >1 m). Some amebae have maximal sizes smaller than $10\,\mu$m (Rogerson 1993), while other sarcodine species (planktonic foraminifera, some actinopods) begin ontogeny as swarmers that are several μm in size but eventually grow to macroscopic size. Although the terminology is counterintuitive for single-celled organisms, these larger sarcodines are commonly termed 'juveniles' and 'adults' based on their size and morphology. The capsules or tests of individual acantharia, radiolaria and foraminifera can be >1 mm in size. In addition, many species possess spines that radiate from the central skeleton and increase the overall diameter of the organisms considerably. Some polycystine radiolaria form gelatinous colonies of disks or spheres up to several centimeters in diameter, or thin cylinders more than 1 m in length.

A variety of methods have been used to collect planktonic sarcodines because of the tremendous size and structural range of this group, their taxonomic diversity, the different criteria used for their identification and enumeration, and the different goals for collecting these specimens. Most studies have employed standard plankton nets or high-volume pump systems (see Chapter 3), which rely on the durability of the organisms' calcium carbonate, silica or strontium sulfate skeletons to withstand the rigors of collection. Most of the information on the geographical and depth distributions of skeleton-bearing sarcodines has been obtained in this manner (e.g. Cifelli and Sachs 1966; Beers and Stewart 1971; Massera Bottazzi *et al*. 1971; Bé 1977; Michaels *et al*. 1995).

The use of plankton nets to collect planktonic sarcodines is strongly biased. Individuals within a taxon may range in size over many orders of magnitude and net collections are typically biased towards adult and large juvenile specimens (Caron and Swanberg 1990). Adult sarcodines are usually rare ($\leqslant 1\,\mathrm{m}^{-3}$) relative to juvenile

Table 5.2 Fixatives used to preserve nano- and microzooplankton. **Freshwater studies.

Taxon	Fixative	Concentration	Comments	Author
Heterotrophic flagellates	Formaldehyde	1%–5%	Can be used in conjunction with fluorescent stains; shrinks cells; no cell losses with unbuffered fixative	Bloem and Bar Gilissen (1988)**; Choi and Stoecker (1989); Sherr and Sherr (1993)
	Glutaraldehyde	0.3%–3%	Short shelf life; can be used in conjunction with fluorescent stains; shrinks cells; no cell losses with unbuffered fixative	Bloem and Bar Gilissen (1988)**; Choi and Stoecker (1989); Sherr and Sherr (1993)
	Modified Van der Veer's	2%	Shelf life unknown; less cell shrinkage than other aldehydes	Choi and Stoecker (1989)
Ciliates	Formaldehyde	2%–20%	Can be used in conjunction with fluorescent stains; preserves chlorophyl fluorescence in mixotrophic forms; significant losses of cell numbers; shrinks cells	Choi and Stoecker (1989); Stoecker *et al.* (1989, 1994); Stoecker and Silver (1987)
	Glutaraldehyde	0.5%–2%	Short shelf life; can be used in conjunction with fluorescent stains; preserves chlorophyl fluorescence in mixotrophic forms; significant losses of cell numbers; shrinks cells	Laval-Peuto and Rassoulzadegan (1988); Choi and Stoecker (1989)
	Basic Lugol's	0.5%–20%	Preserves more cells than aldehydes; more cell losses than acid Lugol's; masks chlorophyl fluorescence; shrinks cells	Gifford (unpublished)

	Acid Lugol's	0.6%–20%	Preserves more cells than aldehydes; masks chlorophyl fluorescence; shrinks cells	Choi and Stoecker (1989); Sime-Ngando *et al.* (1990); Stoecker *et al.* (1994); Putt and Stoecker (1989); Ohman and Snyder (1991); Wiakowski *et al.* (1994)**
	Bouin's	5%	Preserves similar cell numbers to acid Lugol's; shrinks cells	Jerome *et al.* (1993); Stoecker *et al.* (1994)
	Mercuric chloride	2.5%	Extremely toxic; preserves similar cell numbers to acid Lugol's; not widely used for marine work	Sime-Ngando *et al.* (1990)**; Pace and Orcutt (1981)**; Wiakowski *et al.* (1994)**
	Van der Veer's	4%	Acrolein component extremely toxic	Van der Veer (1982)
Heterotrophic dinoflagellates	Formaldehyde		Can be used in conjunction with fluorescent stains; preserves chlorophyl fluorescence in mixotrophic forms; losses of cell numbers and cell shrinkage not evaluated	Verity *et al.* (1993)
	Glutaraldehyde	0.5%–1%	Short shelf life; can be used in conjunction with fluorescent stains; preserves chlorophyl fluorescence in mixotrophic forms; losses of cell numbers and cell shrinkage not fully evaluated	Lessard and Swift (1986); Shapiro *et al.* (1989); Lessard (unpublished)
Sarcodines	Formaldehyde	5%	Strontium must be added to preserve acantharians; plankton net samples are usually buffered to prevent decalcification of foraminifera when large amounts of zooplankton biomass are present	Beers and Stewart (1970); Michaels (1988); Bé and Anderson (1976)

specimens which can be several orders of magnitude more abundant ($>10\,l^{-1}$). Thus, sampling with plankton nets is often a compromise between using relatively large mesh size ($>300\,\mu m$) to avoid clogging of the net when filtering large volumes of water to collect adult specimens (Bé 1959), and reducing mesh size (increasing the chance of clogging) to collect smaller species and juvenile specimens (Dworetzky and Morley 1987). In addition, some sarcodine species do not have mineralized skeletal structures. The abundances of these latter specimens have undoubtedly been underestimated using net/pump collection methods because of damage in the net or extrusion through the mesh. Significant losses of specimens occur even for species which possess skeletons (Michaels 1988). For these reasons, collecting methods using plankton nets probably underestimate total abundances of planktonic sarcodines to some degree, and severely underestimate the abundances of certain species and most juvenile sarcodines. The best possible estimates are obtained by a combination of sampling methods: large, durable sarcodines are collected by plankton nets, while the smallest and most abundant sarcodines can be collected in 1–30 l volumes using standard collecting bottles. This method is effective for juvenile foraminifera and small actinopods (mostly acantharia) which fall into the 'nano' or 'micro' size classes and have abundances in the range of 1–$100\,l^{-1}$ (Davis *et al.* 1978; Michaels *et al.* 1995). The entire contents of the bottle are concentrated with fine mesh netting ($5\,\mu m$) to avoid losses due to rapid settling in the bottle. The samples are then filtered onto polycarbonate filters, dried at 60 °C and examined with a dissecting microscope (Michaels *et al.* 1995). Concentrates can also be preserved appropriately (see section 5.3.3) and settled onto glass slides. Counts of small planktonic sarcodines obtained from bottle collections can be combined with counts of large specimens collected using plankton nets or pumps. The combined use of bottles and net/pump collections is currently the best available method for assessing abundances of planktonic sarcodines.

Gymnamebae ('naked' amebae) are particularly difficult to collect from plankton environments because they are small (usually $\leqslant 50\,\mu m$), occur at relatively low abundance in the plankton ($\leqslant 1\,l^{-1}$), and do not possess skeletal structures that are recognizable in preserved samples. Studies examining the abundance of amebae in the plankton have relied on their growth in Most Probable Number cultures to establish their abundances, with or without prior concentration of the amebae by gentle filtration (Davis *et al.* 1978; Rogerson and Laybourn-Parry 1992). Most studies have not attempted quantification. Consequently, little information is available regarding the distributions of amebae in the oceans. However, in most instances amebae appear to constitute a minor component of protozoan abundances in planktonic ecosystems, and our inability to estimate these populations probably affects our overall assessment of protozooplankton biomass only for some shallow marine and freshwater ecosystems (Arndt 1993; Murzov and Caron 1996).

Individual specimens or colonies of large planktonic sarcodines can also be collected in jars by divers. Because many species have large skeletons, spines and other projections as well as pseudopodial networks supported by these structures, many sarcodines are conspicuous in the oceanic plankton. These species are collected easily by divers in surface waters of tropical and subtropical oceans. Accumulations of sarcodine species at the surface of the ocean on calm days and aggregation in the convergence zones of Langmuir cells make large planktonic sarcodines relatively easy to collect by experienced divers. This collection method does not lend itself to quantification of sarcodine abundances, but it does provide specimens in excellent physiological condition: adult specimens of acantharia, radiolaria and foraminifera have been collected

routinely in this manner for laboratory studies (Bé *et al*. 1977; Swanberg 1983; Caron *et al*. 1995b).

The abundance of planktonic sarcodines can also be estimated using an underwater video plankton recorder (VPR) (Davis *et al*. 1992a, 1992b). This method (see Chapter 7) collects images of the organisms on video tape, together with ancillary chemical/physical data at the depth and location of sampling. Although a 'hard specimen' is unavailable using the VPR, the approach has great potential because it permits the collection of large amounts of data rapidly and without bias or losses due to disruptive sampling methods. Initial results using an instrument modified for use with planktonic sarcodines in the Sargasso Sea near Bermuda and in the eastern North Pacific indicate that this method may provide an alternative means of assessing the abundances and distributions of these delicate protozoa (Dennett, Michaels and Caron, unpublished data). Advances in video image processing will undoubtedly increase the speed, accuracy and applicability of this sampling method for assessing sarcodine abundances.

5.3 PRESERVATION AND ENUMERATION

Preservation and enumeration protocols vary considerably among protozooplankton and no single preservative or fixation method is appropriate for all taxa. Some protozoan taxa may possess skeletal materials and/or morphological and ultrastructural features that are susceptible to damage, dissolution or deterioration in preserved samples. Particular criteria used to count and identify different groups of protozoa require specific preservatives that maintain the integrity of these features.

5.3.1 Nanozooplankton

PRESERVATION

The choice of a preservative for nanoplankton must consider the method of enumeration that will be employed. Nanoplankton are typically enumerated by epifluorescence microscopy (see section on Nanozooplankton Enumeration), and preservatives must be used that will not interfere with the fluorescence characteristics of the cells. Mercuric chloride and Lugol's solution are two common preservatives that can interfere with autofluoresence. Regardless of the preservative employed, it is imperative to store the preserved samples cold ($\leqslant 5\,^{\circ}C$) and in the dark until they are processed. Nanoplankton are commonly preserved using formalin at 1%–5% final concentration or glutaraldehyde at 0.5%–1% final concentration (Table 5.2). These preservatives are compatible with most procedures for epifluorescence microscopy. A concentration of 1% of these solutions is most practical because the solutions usually do not need additional buffering if they are prepared with natural seawater (although buffering is often recommended). However, preservatives made with natural seawater must be filtered to remove microorganisms and precipitate that might form in the seawater during preparation. A common protocol is given in section 5.5 below.

Preservation/fixation methods to prepare nanoplankton for electron microscopy are more complex than methods used to prepare cells for epifluorescence microscopy. These methods are not discussed here. For a recent review of these procedures see Leadbeater (1993). Electron microscopy provides considerably more taxonomic information on nanoplankton assemblages than is achieved by epifluorescence microscopy, but quanti-

fication is difficult because of cell losses during preparation, the equipment required, and the labor-intensive nature of the methods.

NANOZOOPLANKTON ENUMERATION

Three basic approaches are commonly used to enumerate heterotrophic nanoplankton (HNAN): transmitted light microscopy, electron microscopy and epifluorescence microscopy. Techniques employing epifluorescence microscopy are most widely used. Current epifluorescence methods for enumerating phototrophic nanoplankton (PNAN) and HNAN employ fluorochrome stains to visualize the cells, and take advantage of the natural fluorescence of chlorophyl *a* to distinguish plastidic from aplastidic cells. The cells containing chlorophyl are assumed to be autotrophic. However, a number of studies indicate that up to 50% of PNAN cells may ingest particles (e.g. Porter 1988; Caron *et al*. 1990; Sanders *et al*. 1990; Berninger *et al*. 1992; Jones *et al*. 1993; Arenovski *et al*. 1995), rendering the distinction between PNAN and HNAN somewhat ambiguous.

In epifluorescence microscopy, a series of filters selects a specific band of light to excite the stained material, then selectively passes the excited light to the microscope's ocular lenses. Most standard research microscopes can be equipped for epifluorescence with filter sets designed for specific fluorochromes (reviewed by Sherr *et al*. 1993). The reasons for the popularity of epifluorescence microscopical techniques include ease of preparation, minimal losses of cells during preparation, relatively short preparation times, the ability to distinguish between PNAN and HNAN, and the relatively moderate cost of the equipment and supplies required to perform the methods. Many of the epifluorescent stains in common use are teratogens and/or mutagens, and must be used with due attention to safety. Detailed procedures and a discussion of the advantages and disadvantages of a variety of fluorochrome-staining procedures are reviewed by Sherr *et al*. (1993). Fluorochromes in common use include 4′6′-diamidino-2-phenylindole (DAPI), direct yellow 59 (primulin), fluorosceinisothiocyanate (FITC), 5-(4,6-dichlorotriazine-2-yl) aminofluoroscein (DTAF), and proflavine hemisulfate. These stains target specific cellular components such as cytoplasm, nuclear material, or lipids. For a compendium of fluorescent stains see Haugland (1996). A number of protocols have been developed for the various stains and combinations of stains. The choice of which stain to use is determined by the target taxa and objectives of the individual study. A 'generic' procedure is described in section 5.5, but the reader should refer to Sherr *et al*. (1993) for related methods and for specific precautions for each method. Image analysis techniques employing epifluorescence (Inoue and Spring 1997) offer a means of partially automating the enumeration of nanozooplankton (e.g. Sieracki and Viles 1990; Sieracki and Webb 1991; Viles and Sieracki 1992), but the cost of the necessary optical and photographic equipment, computers and software can be considerable.

Although epifluorescence microscopy is the most common method for determining numerical abundances of nanozooplankton assemblages, it does not resolve the ultrastructural features upon which many species descriptions are based, and hence does not permit identification of nanoplanktonic cells to the species level. Such taxonomic identification requires the use of electron microscopy and/or live examination. These are usually not feasible for most field studies. While these methods often provide more taxonomic resolution than the limited differentiations provided by epifluorescence microscopy, they are often 'semiquantitative' due to loss of cells during preparation. Transmitted light microscopy has been performed with samples settled in glass-bottomed chambers (Utermöhl 1958), but the quantitativeness of this method for small nanoplankton is questionable and there are often difficulties distinguishing small

autotrophs from protozoa. Transmission and scanning electron microscopy are also useful approaches for nanoplankton assemblages because more taxonomic information is usually obtained than is possible with epifluorescence microscopy. However, losses due to the concentration procedures necessary for electron microscopy and the cost- and labor-intensive nature of these methods make them difficult for routine counts of nanoplankton. Advantages, disadvantages and general protocols for these approaches are reviewed by Booth (1993) and Leadbeater (1993). Major taxonomic references for heterotrophic flagellates include Lee *et al.* (1985), Margulis *et al.* (1989) and Patterson and Larsen (1991).

One new approach for viewing nanoplankton that combines the ease of epifluorescence microscopical methods with the ability to distinguish nanoplankton taxa is based on the use of ribosomal RNA-targeted oligonucleotide probes (Lim *et al.* 1996). Oligonucleotides (short sequences of DNA bases) are labeled with reporter molecules that allow them (and the organisms to which they bind) to be visualized by epifluorescence microscopical protocols. The design and testing of species-specific oligonucleotide probes for nanoplanktonic protists, and the development of a quantitative protocol for applying them to natural samples (Lim 1996), provide a tool to study the population dynamics of small algae and protozoa in natural ecosystems using epifluorescence microscopy (Lim *et al.* 1999). This and similar approaches should greatly facilitate biogeographical studies of small nanoplankton.

5.3.2 Microzooplankton

Techniques for the preservation and enumeration of microzooplankton are often taxon-specific and the choice of methods is determined by the objectives of the individual study and consideration of the target taxa. To examine the entire suite of microzooplankton taxa, several sets of samples must be preserved and enumerated separately. Depending on the needs of the study and the preservatives employed, some taxa of microphytoplankton can also be enumerated in the same samples.

MICROZOOPLANKTON PRESERVATION

A number of fixatives are used to preserve microzooplankton, including formaldehyde, glutaraldehyde, Bouin's solution (a mixture of formalin, picric acid, and glacial acetic acid), and various formulations of Lugol's iodine solution. Aldehyde-based fixatives are used in both buffered and unbuffered form. Common buffers include sodium borate, calcium carbonate and hexamethylenetetramine. A generic fixation protocol is described in section 5.5.

Aplastidic ciliates

Fixation efficiencies and effects have been most thoroughly studied in planktonic ciliates (Table 5.2), where two general problems emerge: cell losses due to fixation and changes in cell volume due to fixation. The former affects determination of microzooplankton numerical abundances; the latter impacts calculation of microzooplankton biomass. In general, lowest losses occur with relatively high ($\sim$10% v/v) concentrations of acid Lugol's solution (Throndsen 1978) or with Bouin's solution (Stoecker *et al.* 1994). However, acid Lugol's and Bouin's solutions cause the greatest shrinkage of ciliate cells relative to other aldehyde fixatives (Jerome *et al.* 1993; Leakey *et al.* 1994; Stoecker *et al.* 1994), and significant shrinkage can occur immediately following fixation (Ohman and Snyder 1991). In their favor, iodine-based fixatives are not as toxic as aldehyde-based

formulations. Iodine is a heavy metal and has the additional advantage of adding mass to the preserved protozooplankters, thereby enhancing settling. The shelf life of microzooplankton samples under various preservation regimes has not been well studied (Sime-Ngando and Grolière 1991). Glutaraldehyde has a shelf life of the order of months and iodine-based fixatives deteriorate in light. Other formulations for Lugol's solutions are given in Throndsen (1978), although it should be noted that neutral and basic versions do not preserve cilia and flagella as well as the acid formulation. Quantitative Protargol Staining (QPS) employs Bouin's fixative in combination with protargol silver impregnation (Montagnes and Lynn 1987; Jerome *et al.* 1993; Montagnes and Lynn 1993; Skibbe 1994) and is an alternative method for preserving planktonic ciliates. The method has the advantages of being quantitative and providing taxonomic information, but it is extremely labor-intensive for routine sampling.

Plastidic ciliates and heterotrophic dinoflagellates

Cold buffered formalin or glutaraldehyde is generally used to preserve heterotrophic dinoflagellates (Lessard and Swift 1986; Verity *et al.* 1993) and plastidic ciliates (Stoecker and Silver 1987; Laval-Peuto and Rassoulzadegan 1988) because it does not interfere with chlorophyl fluorescence, enabling heterotrophic cells to be distinguished from autotrophic cells when enumerated using epifluorescence microscopy (see section on Microzooplankton Enumeration). If an epifluorescence microscope is not available, some ciliate and dinoflagellate taxa can be identified as autotrophs or mixotrophs on the basis of their known taxonomy. For example the ciliate genera *Laboea* and *Mesodinium*, which have distinctive morphologies, are obligate mixotrophs. Similarly, some dinoflagellate genera (e.g. *Protoperidinium*) are obligate heterotrophs. It should be noted that many plastidic microplankton cells, including ciliates, dinoflagellates, and a number of phytoplankton taxa, are mixotrophic (e.g. Dolan 1992; Stoecker 1992; Bockstahler and Coats 1993; Jacobson and Andersen 1994; Jones 1994; Skovgaard 1996; Stoecker *et al.* 1997) so that any division of microplanktonic protists into autotrophs and heterotrophs is approximate at best.

MICROZOOPLANKTON ENUMERATION

Inverted microscopy is the most common method used to enumerate microzooplankton in preserved samples. Microzooplankton from seawater samples are first concentrated by settling, then enumerated using an inverted microscope. The inverted microscope's light source and condenser illuminate the sample from above and the objective lenses view the sample from below through a glass plate in the bottom of the counting chamber. One advantage of the inverted microscope is use of the same chamber for settling and counting, thereby avoiding transfer and possible loss of material (Hasle 1978a). Another is that whole (as opposed to sectioned) microorganisms can be viewed without distortion. The inverted microscope method is typically used for organisms larger than 10 μm (primarily ciliates and heterotrophic dinoflagellates). If plastidic forms are enumerated separately, the microscope must be equipped to detect chlorophyl fluorescence (see section on Plastidic ciliates and heterotrophic dinoflagellates).

Taxonomic resolution of cilates is generally limited to the level of genus with most of the common preservation methods. Lugol's iodine preservatives have the disadvantage that material stains darkly, obscuring morphological detail. This can be overcome by clearing samples with sodium thiosulfate (Sherr and Sherr 1993), laundry bleach (Gifford, unpublished data) or bright light (Gifford, unpublished data) and post-fixing with 1%–5% buffered or unbuffered formalin. Ciliates stained using protargol (silver

impregnation) methods can be identified to species on the basis of ultrastructural characteristics (e.g. Lynn and Montagnes 1988; Lynn *et al.* 1988, 1991; Montagnes *et al.* 1988, 1990; Montagnes and Lynn 1993). However, most planktonic ciliate species have not yet been described on this basis, so that many specimens are likely to be unidentifiable. Major taxonomic references for planktonic ciliates include Fauré-Fremiet (1924); Kofoid and Campbell (1929, 1939), Kahl (1932), Lee *et al.* (1985), Marshall (1969), Maeda and Carey (1985), Maeda (1986), Montagnes and Lynn (1991) and Foissner (1991, 1994). Taxonomic identification of heterotrophic dinoflagellates on the basis of morphological characteristics is more feasible than for ciliates, at least at the level of genus. Identification of thecate forms to species level requires determination of the 'plate pattern' of the cells' cellulose plates, which is not practical when working with diverse microplankton assemblages. Athecate forms are identified on the basis of gross morphology. Taxonomic references for dinoflagellates include Ferguson-Wood (1968), Steidiger and Williams (1970), and Taylor (1976).

Live counting

Live counting is an alternative method for microzooplankton taxa which are sufficiently large to view with a dissecting microscope, have distinctive morphology so that they can be identified at relatively low magnification, and are sufficiently abundant to count in a relatively small sample volume (Dale and Burkill 1982; Sime-Ngando *et al.* 1990; Arndt and Mathes 1991). The primary literature should be consulted for details about the design of counting chambers for this purpose.

5.3.3 Planktonic sarcodines

LARGER PLANKTONIC SARCODINE PRESERVATION

Preservation techniques for the skeleton-bearing planktonic sarcodines (acantharia, foraminifera, and some radiolaria) for establishing abundances and biomasses are relatively straightforward. Formalin is the most commonly used preservative because it is also used to preserve other zooplankton. Most formulae consider preservation of the skeletal structures upon which much of sarcodine taxonomy is based. These structures, particularly the strontium sulfate skeletons of the acantharia and to a lesser degree the calcium carbonate skeletons of foraminifera, are susceptible to dissolution in unamended formaldehyde solutions, while the siliceous skeletons of radiolaria tend to be durable in unamended formaldehyde. These selective dissolution effects can result in strong biases in the enumeration of sarcodines in preserved samples. Calcareous skeletons of foraminifera can be preserved in formaldehyde (2% final concentration is common) buffered with either sodium tetraborate (borax) or hexamethylenetetramine (hexamine). Bé and Anderson (1976) recommended adding 5 ml of a buffered formaldehyde solution (30 g borax l^{-1}), 4.5 ml of propylene glycol, and 0.1 ml of propylene phenoxetol to 90 ml of plankton net collection in seawater to obtain a slightly alkaline solution that will maintain foraminiferan tests indefinitely. The pH should be checked periodically during long-term storage. Buffering formaldehyde with hexamine (200 g of hexamine l^{-1}) is also common practice. The strontium sulfate skeletons of the acantharia are highly susceptible to dissolution in common preservatives. This sensitivity is due to undersaturation of dissolved strontium in seawater, and can be corrected by the addition of strontium to preserved samples. For most species, the addition of strontium chloride at a final concentration of 80–160 mg l^{-1} works well (Beers and

Stewart 1970; Beers 1976; Michaels 1988). In all cases, samples of preserved planktonic sarcodines should be kept cool (5–15 °C).

LARGER PLANKTONIC SARCODINE ENUMERATION

Enumeration of planktonic sarcodines other than amebae is done with wet samples using a dissecting and/or compound microscope, or on filters for small foraminifera and actinopods (see section on Determination of Biomass: Conversion Factors). If concentrated samples of small sarcodines collected with bottles have been preserved in liquid, subsamples can be transferred to settling chambers and examined by inverted microscopy. Examination of sarcodines in seawater hinders the identification of some actinopods, but methods for removing the cytoplasm from these specimens to reveal diagnostic skeletal structures (e.g. low temperature ashing or peroxide treatments) may preclude making the measurements necessary to estimate volume, and therefore biomass (see below). Major taxonomic references include Schwiakoff (1926), Bé (1967), Massera Bottazzi and Nencini (1969), Strelkov and Reshetnyak (1971), and Nigrini and Moore (1979).

5.4 DETERMINATION OF BIOMASS: CONVERSION FACTORS

Determination of the biomass of nano- and microzooplankton in natural assemblages is based on estimation of cell volume and calculation of biomass (usually as carbon) from cell volume measurements and carbon : volume conversion factors. Biovolume estimates are based on microscopical measurements of individual organisms and conversion to biovolume using geometric shapes that best approximate the shape of the particular organism. Although many investigators make these measurements by eye, image analysis systems are beginning to provide a more rigorous and accurate approach for both nano- (Sieracki and Webb 1991; Viles and Sieracki 1992; Verity and Sieracki 1993) and microzooplankton (Roff and Hopcroft 1986). Conversion of protozoan biovolume to carbon units is a major consideration in the accurate determination of the biomass of protozooplankton in seawater samples. An estimate of 80 fg C μm^{-3}, derived largely from work with phytoplankton species, was commonly applied to protozoa until approximately a decade ago. This value was based on the assumptions that specific gravity was equal to 1.0, a value of 0.2 for the ratio of dry weight to wet weight, and a carbon content equal to 0.4 × dry weight (Beers and Stewart 1971; Beers *et al.* 1975).

5.4.1 Nanozooplankton

The factors used to convert nanozooplankton biovolume to carbon have varied by more than 4-fold in recent years, and there is still no clear consensus on the most appropiate value(s) (Table 5.3). A significant fraction of the variability associated with these conversion factors appears to be real, and can be related to preservation, growth conditions (e.g. temperature and light) and cell size. Preservation has a significant effect on nanozooplankton cell volume (Choi and Stoecker 1989). Preservation typically results in cell shrinkage, and the magnitude of this effect can be preservative- and taxon-specific. As discussed in the Nanozooplankton Enumeration section, taxonomic information is largely lacking for the nanoplankton when enumerated by epifluorescence microscopy, so taxon-specific information on shrinkage is not particularly helpful for natural samples. However, cell size-dependent conversion factors do exist and are commonly used for converting volume to carbon (Verity *et al.* 1992; Montagnes *et al.*

Table 5.3 Common volume : biomass conversion factors for nanoplanktonic protists.

Volume : carbon factor or regression	Comments	Author
$(\log C) = -0.314 + 0.712 \times (\log V)$ (range = 44–320 fg C μm^{-3}	Based on measurements of phytoplankton; C is carbon in pg; V is volume in μm^{-3}; range is based on cells 2–20 μm	Strathmann (1967)
80 fg C μm^{-3}	Assumes specific gravity = 1; dry weight = 20% wet weight; carbon = 40% dry weight	Beers *et al.* (1975)
180 fg C μm^{-3} 300 fg C μm^{-3}	Calculated from measurements of cell volume and carbon $cell^{-1}$ for *Ochromonas* sp. and *Pleuromonas* sp.	Fenchel (1982a)
220 fg C μm^{-3}	Derived from direct measurements of the heterotrophic nanoflagellate *Monas* sp.	Børsheim and Bratbak (1987)
160, 240 or 360 fg C μm^{-3}	Based on analysis of marine photosynthetic nanoplankton. Size dependent factors for cells of 10^3, 10^2 or 10^1 μm respectively	Verity *et al.* (1992)
183 fg C μm^{-3}	Derived from literature values, particulate carbon and nitrogen, and carbon : chlorophyl ratios	Caron *et al.* (1995a)
130–310 fg C μm^{-3}	Based on analysis of marine photosynthetic nanoplankton	Montagnes *et al.* (1994)

1994). The most accurate approximations of biomass that can be obtained at this time are probably provided by non-linear, size-dependent regressions (Verity *et al.* 1992; Montagnes *et al.* 1994) because they produce conversion factors that account for the higher relative carbon content of smaller nanoplanktonic cells (Table 5.3). However, there are still significant discrepancies between the conversion factors provided by these regressions and it should be noted that these factors were developed for nanophytoplankton. More work will be required to constrain nanozooplankton conversion factors.

5.4.2 Microzooplankton

Biovolume estimates for microzooplankton also are based on measurements made of individual organisms, in this case typically with an inverted microscope, and then converted to biovolume. If measurements are performed by eye, they are converted to volume using geometric shapes (e.g. sphere, cone, spheroid) that best approximate the shape of the particular organism. Image analysis techniques (see section on Nanozooplankton Enumeration) may also be used. Direct measurements of cell volume : carbon factors are available for aloricate ciliates and heterotrophic dinoflagellates (Table 5.4). Direct estimates are generally higher by a factor of about 2 than the indirect estimate

Table 5.4 Direct measurements of volume : biomass conversion factors for planktonic ciliates and heterotrophic dinoflagellates. B is bacterivorous. H is herbivorous.

Taxon	Volume : Carbon factor (pg C μm^{-3})	Author
Ciliates		
Uronema marinum (B)	0.86 $\pm$ 0.09 (10 °C) 0.76 $\pm$ 0.06 (20 °C)	Burkill (1978)
Athecate oligotrichs (H)	0.19 $\pm$ 0.01	Putt and Stoecker (1989)
Strombidium sp. (H)	0.126 (stationary phase) 0.148 (exponential phase)	Ohman and Snyder (1991)
Uronema marinum (B)	0.32 (stationary phase)	Ohman and Snyder (1991)
Heterotrophic dinoflagellates		
Lightly thecate forms (*Oblea* sp.)	0.140	Lessard (1991)
Heavily thecate forms (*Protoperidinium* sp.)	0.205–0.300	Lessard (unpublished data)
Athecate forms (*Gymnodinium* sp. and *Gyrodinium* sp.)	0.138–0.190	Lessard (unpublished data)
Heterotrophic and autotrophic dinoflagellates	$0.760 \times vol^{0.819}$	Menden-Deuer and Lessard (personal communication)

cited in section 5.4. These factors appear to vary with preservative, taxon, cell size, and possibly trophy. For aloricate ciliates (which are generally not primarily bacterivorous), the value of 190 fg C μm^{-3} measured by Putt and Stoecker (1989) for cells preserved with 2% acid Lugol's solution is commonly used. However, when other preservatives or concentrations of preservative are used (with concomitant changes in the degree of cell shrinkage), the factor must be adjusted accordingly (Jerome *et al.* 1993; Stoecker *et al.* 1994). Other conditions can also affect volume : carbon conversion factors. Some low values that have been published may be due to diet (Ohman and Snyder 1991). Conversion factors for heterotrophic dinoflagellates appear to vary with size, and the degree to which cells are armored (Lessard 1991, unpublished data) (Table 5.4). Direct measurements of carbon : nitrogen ratio are available for several taxa of marine planktonic ciliates (Table 5.5). The values are highly variable, ranging from about 3 to 8, and appear to vary with taxon and trophic habit.

5.4.3 Planktonic sarcodines

There are few direct measurements of sarcodine biomass, a result of the difficulties of collecting undamaged specimens for making the measurements, and because of the difficulties of making volume estimates of these species. Sarcodines form complex pseudopodial networks that occupy large volumes but contain large amounts of interstitial water between their cytoplasmic projections. Moreover, these networks are typically destroyed by common collection methods such as pumps and plankton nets. Michaels *et al.* (1995) addressed this problem by relating direct measurements of cellular carbon and nitrogen, performed on specimens collected individually by divers, to features of these protozoa that can be measured in preserved samples. The resulting

Table 5.5 Direct measurements of C : N ratios of marine planktonic ciliates. B is bacterivorous. H is herbivorous. P is plastidic. S is stationary phase. E is exponential phase.

Taxon	C:N	Author
Laboea strobila (P)	6–13 4–8	Putt and Stoecker (1989)
Strombidium capitatum (P)	3–8	Putt and Stoecker (1989)
Strombidium spiralis (H)	3–4	Putt and Stoecker (1989)
Uronema marinum (B)	3.7 (S)	Ohman and Snyder (1991)
Strombidium sp. (B)	7.1 (S) 4.9 (E)	Ohman and Snyder (1991)

conversion factors are variable and somewhat group- or species-specific but at present they provide the only straightforward method for obtaining the biomass of large, net-collected sarcodines (Table 5.6).

There are no published carbon : volume conversion factors for small ($< 50\ \mu$m) planktonic foraminifera and actinopods. Cell volume can be estimated for many of these specimens from the dimensions of skeletal structures in these juvenile specimens, and these values can be converted to carbon estimates using factors published for nanoplanktonic protozoa (Table 5.3). Amebae present a particularly difficult problem for assessing biomass because of their amorphous or irregular shape, and the lack of skeletal structures that can be measured in live or preserved specimens. Recently, Rogerson *et al.* (1994) have demonstrated that nuclear diameter of several uninucleate species is related to the cell volume of the amebae. This approach may provide a method for estimating the biomass of these species in preserved natural samples, but first it is necessary to devise a means of accurately enumerating the amebae themselves in preserved samples.

Table 5.6 Estimation of volume and volume : carbon factors of larger planktonic sarcodines collected near Bermuda. Values are based primarily on measurements made by Michaels *et al.* (1995) using specimens collected individually by divers. Some acantharia were collected by gentle drift tows. *Carbon values are related directly to the number of central capsules for colonial radiolaria rather than to colony volume because colonies are usually disrupted in net tows.

Taxon	Carbon : Volume factor	Feature used for volume measurement
Acantharia	2.6 fg C μm^{-3}	Spheroid encompassing the extreme of the radiating spicules
Foraminifera	89 fg C μm^{-3} (excluding adult *Orbulina universa*)	Longest linear dimension of the test, usually from the edge of the final chamber to the edge of the penultimate chamber
Solitary radiolaria	9–280 fg C μm^{-3} (highly species dependent)	Central capsule diameter
Colonial radiolaria	133 ng C $capsule^{-1}$	*Estimate of the number of central capsules in colony

5.5 STANDARD PROTOCOLS

5.5.1 Collection of nano- and microzooplankton

Because many protozooplankton taxa are fragile, water collected in Niskin or Go-Flo bottles must be handled gently to minimize losses (Gifford 1985). Drain water from the bottles immediately after collection. Water is drained slowly through acid-cleaned, wide-bore silicone tubing directly into sample bottles or storage vessels. Submerge the end of the drain tubing to minimize turbulent mixing that may damage the target organisms (Gifford 1985). If the objectives of the study require separation of mesozooplankton from the protozooplankton assemblage, pass the collected water through as coarse a mesh as possible, having as large a surface area as possible to reduce flow rates through the mesh. Submerge the mesh, again to minimize damage to the organisms (Gifford 1993).

5.5.2 Preservation of nanozooplankton

Use natural seawater of similar salinity to the samples to be preserved. Add sufficient formalin (37% formaldehyde) or 50% glutaraldehyde slowly and with stirring to a sufficient volume of natural seawater to yield a 10% solution (e.g. for 100 ml of glutaraldehyde fixative, add 20 ml of 50% glutaraldehyde to 80 ml of seawater). Filter the resulting 10% solution through a 0.45 μm filter (Millipore® HA membrane filters work well). Add the preservative at a ratio of 1:9 with the sample to obtain a final concentration of 1%. There are a number of acceptable modifications on this protocol, and several investigators have found slight variations in their efficacies for preserving nanoplankton from different pelagic ecosystems (see Sherr and Sherr 1993). A notable variation is the combined use of paraformaldehyde and glutaraldehyde to preserve delicate nanoplankton (Tsuji and Yanagita 1981).

5.5.3 Staining and enumeration of nanozooplankton

The staining procedure using the fluorochrome DAPI described here has the advantage that there is little overlap in the emission spectra of the fluorochrome and photosynthetic pigments used to distinguish autotrophic nanoplankton. This method has been used for samples from estuarine, coastal and oceanic locations. Verity *et al.* (1993) used a similar epifluorescent staining method for heterotrophic dinoflagellates smaller than 20 μm in size.

Prepare a 25 mm filtration apparatus by placing a wet 0.45 μm pore size Millipore® filter (as a backing filter to aid dispersion of cells on the filter) onto the filter base. Place a wet 0.2 or 0.8 μm blackened polycarbonate filter over the backing filter. Do not trap air bubbles. Clamp the filter funnel to the base. Add to the filter funnel a 5–50 ml subsample (depending on nanoplankton abundance) of water preserved in formalin, paraformaldehyde or glutaraldehyde. Reduce the volume to approximately 1–2 ml by gentle filtration ($\leqslant$5 in. Hg). Add DAPI working solution (100 μg ml^{-1}) to obtain a final stain concentration of 20–50 μg ml^{-1}. Stain for 5–7 min. DAPI stock solutions of 1 mg ml^{-1} in distilled water can be prepared and stored frozen for weeks. Working solutions should be prepared in distilled water and filtered (0.2 μm) for each staining session. Draw the stained sample down onto the filter. Without removing the vacuum, add approximately 2 ml of distilled rinse water. Remove the filter and mount immediately onto a glass slide.

Filters adhere well if a drop of immersion oil is first spread on the slide, or if the slides are thoroughly 'fogged' with one's breath. The latter procedure must be performed quickly to avoid air drying, but it produces very flat preparations. Immediately place a drop of immersion oil on the filter and cover with a coverslip.

All wetting and rinsing solutions are 0.2 μm filtered distilled water. Low-fluorescence immersion oil (e.g. Cargille® type A) should be used to reduce background fluorescence. DAPI is light sensitive and the stained samples, stock solution and working solution must be protected from direct light. Storage of preserved samples in liquid longer than 24 h is not recommended. Preserved samples should be stained within 24 h and stored frozen ($\leqslant -20\,^{\circ}C$) in the dark until examined. Sealing the edge of the coverslips to the slides with paraffin or fingernail polish is recommended for long-term storage.

Once prepared, samples can be viewed immediately (or after thawing, if frozen) at high magnification (400–1000 ×) using a compound microscope equipped for epifluorescence. Fluorochromes enable the visualization of all nanoplankton in the sub-sample while autofluorescence of the photosynthetic pigments allows PNAN to be distinguished from HNAN. DAPI-stained nanoplankton are visualized with UV excitation (e.g. Zeiss filter set no. 487702 or equivalent), and PNAN are differentiated by determining the presence of chloroplasts by using a filter set with appropriate excitation and emission characteristics (e.g. Zeiss filter set no. 487709 or equivalent). PNAN and HNAN are counted on the filters from known areas (usually fields of view or an area demarcated by an ocular grid) and expressed as individuals per unit area (e.g. cells per field of view). HNAN abundance is then calculated using the general equation in Sherr *et al*. (1993):

$$\mathbf{HNAN\,ml^{-1}} = \frac{\mathbf{(HNAN\ per\ field\ of\ view)} \times \mathbf{(area\ of\ filter\ covered\ by\ sample)}}{\mathbf{(area\ of\ field\ of\ view)} \times \mathbf{(DF)} \times \mathbf{(ml\ of\ sample\ filtered)}}$$

where DF is the dilution factor due to preservation (usually 0.9). Optimally, a total of about 500 cells are counted.

It is important that all regions of the filters be examined in conducting the microscopical counts because the distribution of microorganisms on the filters is not uniform. Also, it is advisable to obtain nanoplankton counts from multiple sub-samples (i.e. multiple filters) from a given sample because filter-to-filter variability can occur. The limits on the degree of thoroughness in this regard are often compromised by the magnitude of the study. Care should be taken that sample variability, which is often greater than counting error, is characterized adequately. Accurate determination of sample variability is particularly important when comparing nanoplankton abundances in experiments composed of several parallel treatments.

5.5.4 Preservation of microzooplankton

Microzooplankton may be preserved with formaldehyde, glutaraldehyde, Bouin's fixative, or various formulations of Lugol's iodine solution. All of these fixatives have advantages and disadvantages associated with their use (Table 5.2), and the aldehyde-based fixatives are toxic and should be used with appropriate safety precautions. Specific formulations are given in the references cited in Table 5.2 and are not repeated here. Regardless of the fixative employed, the sample should always be added to the preservative (not the converse) so that all material preserved experiences at least the minimum target concentration of fixative at all times. Ciliate and dinoflagellate cells tend to be fragile immediately following preservation, and samples should be allowed to sit undisturbed for at least 24 h before processing. When aldehyde-preserved samples are

analyzed by epifluorescence microscopy, the samples must be stored in the cold and dark until processed. In terms of preservation of chlorophyl autofluorescence, the shelf life of such samples is in the order of a few months.

5.5.4 Enumeration of microzooplankton

In the inverted microscope method, a well-mixed, preserved water sample is concentrated by settling in a plastic chamber consisting of a cylinder with a detachable bottom plate. The cylinder is removed following settling, leaving organisms on the bottom plate, which is an optical coverglass. A separate coverglass is placed over the top of the bottom plate. The volume settled depends on the abundance of the target protozooplankton taxa. Settling tubes ranging from 5–100 ml are commercially available. A variety of settling times have been recommended. One general rule of thumb is that the settling time in hours is equal to 0.3 cm h^{-1} (Margalef 1969). Lund *et al*. (1958) and Hasle (1978a, 1978b) give protocols for counting plankton samples with an inverted microscope. Depending on the abundance of the target taxa, organisms located in areas, transects, or the entire bottom plate may be counted. Magnification factor is tailored to the target taxa, but a magnification factor of a least 200 × is typically used to ensure resolution of small microzooplankton. If target organisms are to be measured as well as counted, a calibrated ocular micrometer is used. Alternative measurement techniques utilize digitization (e.g. Roff and Hopcroft 1986) and/or image analysis techniques (e.g. Sieracki and Webb 1991; Viles and Sieracki 1992; Verity and Sieracki 1993). Venrick (1978a, 1978b) and Lund *et al*. (1958) review the statistical considerations of subsampling and counting.

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6 Acoustical methods

K.G. Foote and T.K. Stanton

6.1 INTRODUCTION

Acoustics has a significant but still rather limited history of application in zooplankton studies. Simple reasons are the relative newness and complexity of the sampling tool. It is the overall goal of this chapter to establish a background sufficient for understanding current applications and contemplating new ones.

To achieve this goal, the chapter is divided into two major parts. In the first (section 6.2), principles, sources of variability, instruments, methods of data processing and analysis, and comparative approaches are presented. In the second part (section 6.3), measurement protocols, model computations, and examples are presented.

By necessity, since the present chapter is but one in a much larger work, some topics are neglected. These include current issues such as developments in broadband systems, measurement of morphology and physical properties of animals, and integration and interpretation of acoustical measurements.

Another neglected topic is integration of acoustical and optical systems. In a sense, this is somewhat extraneous to the present goal of establishing a firm foundation for appreciating acoustical methods. Clearly, the gains to be had from integrating methodologies, while paying careful attention to the engineering side of technology, are enormous.

6.2 GENERAL DISCUSSION OF PRINCIPLES, INSTRUMENTS, TECHNIQUES, AND COMPARATIVE APPROACHES

6.2.1 Review

BACKGROUND

The methodology involved in using acoustics to study zooplankton has its roots in fisheries acoustics. As in applications in fisheries research, acoustics can be used to provide rapid synoptic information on zooplankton distributions, for example through the echo sounder (Figure 6.1) and a standard display of echo data, the echogram (Plate 1), and be used in concert with direct sampling devices such as nets and pumps. Because of broad interest in fish, including the commercial application, acoustical methods for studying fish have long been established. Initial efforts in quantitative acoustic investigations of zooplankton used fish-related methods as a basis, and these have since evolved. A major difference between studies involving fish and zooplankton is the fact that fish, as acoustic scatterers, are relatively similar, neglecting differences due to the presence or absence of a swimbladder, varying principally with size, whereas aggregations of zooplankton typically contain a number of species with vastly different acoustic proper-

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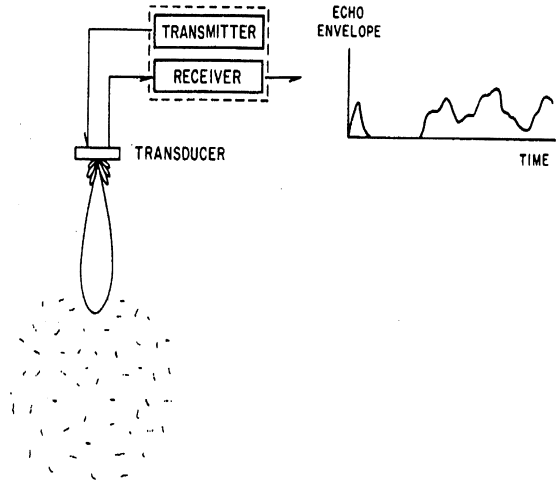


Fig. 6.1 Echo sounder and plot of echo voltage as a function of time.

ties that vary with both species and size. This difference has driven the evolution of zooplankton acoustics methodology. Central to this evolution has been the development of (1) echo sounders with many frequencies covering a wide range of frequencies, (2) mathematical inversion methods to infer meaningful biological parameters from the multi-frequency data, and (3) advanced acoustic scattering models that incorporate the major anatomical features of the zooplankton.

HISTORY

About two decades after the first development and application of acoustical methods in fisheries research, Holliday, Pieper, and others defined an approach appropriate for zooplankton. The first major paper on this subject was published by Holliday in 1977 and a review of many of the related advances in the field since then, along with references, is given in Holliday and Pieper (1995). The approach involves use of a multi-frequency acoustics system (up to 21 frequencies in their studies), physics-based acoustic scattering models, and a mathematical inversion technique which converts acoustic data into a size distribution of the biological sound scatterers. Other studies have involved measurement of material properties of zooplankton and development of acoustic scattering models, such as an early major paper on the subject by Greenlaw (1977). An important early systematic study is that of Greenlaw and Johnson (1982).

Although there have been significant departures in zooplankton acoustics methodology from fish acoustics methodology, there remains necessarily significant overlap between the two areas. For example, elements such as the sonar equation, echo integration, calibration, and instrument platforms are common to both. As a result, much of the material below applies to both fish and zooplankton acoustics methods.

6.2.2 Basic principles

ACTIVE SONAR EQUATION

The general phenomenon of echo registration and quantification is well described by the active sonar equation (Urick 1983; Medwin and Clay 1998). Briefly, this is an engineering solution to the wave equation for a noisy, vertically bounded fluid medium,

containing an acoustic source, targets, and a receiver. The equation is eminently useful for estimating echo levels expected from particular targets and for evaluating the performance of sonars apropos of target detection and maximum operating range. The equation is generally expressed in logarithmic form, hence its terms are additive. Given all of the uncertainties surrounding the incipient field of plankton acoustics, it is regarded as a most useful tool. A short explication is given here and some worked examples in section 6.3.4.

Source level

A flat transducer is assumed to be excited uniformly at a level under the cavitation threshold. The fluid medium thus remains intact, and the sonar performance is not limited by rupture of the medium or other non-linear phenomena that occur at high intensity. The source level of the combined transducer–sonar system is given by the equation

$$SL = 10 \log P + DI_T + 170.8 \tag{6.1}$$

where P is the transmitted acoustic power in watts, DI_T is the transmitting directivity index, and the numerical factor renders the units of SL to be decibels with respect to 1 μ Pa at 1 m distance. The acoustic power P is related to the electrical power P_{el} by the equation

$$P = \eta P_{el} \tag{6.2}$$

where η is the efficiency of the transducer. A typical number for ceramic piezoelectric transducers is 0.6. The transmitting directivity index is

$$DI_T = 10 \log(4\pi / \int b_T d\Omega) \tag{6.3}$$

where the transmit beam pattern b_T, expressed in relative intensity units, is integrated over the entire spherical range.

Transmission loss

In propagating over the distance r, a spherical wave attenuates because of spherical spreading and absorption according to the equation

$$TL = 20 \log r + \alpha r \tag{6.4}$$

where r is given in meters, and the absorption coefficient α is given in decibels per meter.

Scattering strengths

A single target is characterized by its backscattering cross section σ or target strength TS,

$$TS = 10 \log[\sigma/(4\pi r_o^2)] = 10 \log(\sigma_{bs}/r_o^2) \tag{6.5}$$

where σ is expressed in units of square meters, and r_o is a reference distance, typically but not always 1 m. In the second form of the equation, σ_{bs} denotes the differential backscattering cross section, which differs from σ by the factor of 4π. For an aggregation of unresolved targets, the characteristic measure of scattering strength is the total scattering strength, namely $S_V + 10 \log V$, where the mean volume backscattering strength is

$$S_V = 10 \log[N\sigma/(4\pi V)] = 10 \log(N\sigma_{bs}/V) \tag{6.6}$$

where σ is the average backscattering cross section of N scatterers in the sampling volume V,

$$V = (c\tau/2) r^2 \psi \tag{6.7}$$

where c is the speed of sound in the medium, τ is the transmit pulse duration, hence $c\tau/2$ is the minimum range separation for resolving two targets, and ψ is the equivalent beam angle,

$$\psi = \int b^2 d\Omega \tag{6.8}$$

where b^2 is the product of transmit and receive beam patterns, each expressed in the customary relative intensity units.

Echo level

The echo level is gauged in the fluid medium at the receiving transducer. For a single target on the acoustic axis,

$$EL = SL - 2TL + TS \tag{6.9}$$

For an aggregation of targets

$$EL = SL - 2TL + S_V + 10\log V \tag{6.10}$$

Noise level

There are two quite general noise states to be considered, those in which ambient noise or reverberation is the limiting factor. For present purposes, the first of these is the more interesting, hence only ambient noise is considered in the following. For simplicity this is assumed to be isotropic. It is generally associated with three distinct sources.

1) Wave action: the noise spectral level SPL due to wave action is described by the Knudsen equation (Bartberger 1965),

$$SBL_{amb} = 46 + 30\log(n_{SS} + 1) - 17\log(v/1000) \tag{6.11}$$

   where v is the transmit frequency in hertz, and n_{SS} is the sea state number.
2) Thermal noise: the spectral level is

$$SPL_{th} = -15 + 20\log(v/1000) \tag{6.12}$$

3) Receiver noise: internal electronic noise usually exceeds thermal noise, but for a well-designed, and expensive, receiver, this may approach SPL_{th}. In this case

$$SPL = SPL_{amb} + 2SPL_{th} \tag{6.13}$$

Ambient-noise-limited active sonar equation

Terms are collected. The basic equation is

$$EL - (NL - DI_R) = SNR \tag{6.14}$$

where DI_R is the receiver directivity index, and SNR is the signal-to-noise ratio. With increasing transducer directivity, less ambient noise enters the receiver, thus increasing the SNR and possibility of detecting targets. If SNR is replaced by the detection threshold DT, then solution of the same equation yields the maximum range of detection for the particular target.

TARGET STRENGTH MODELS

Associated with each animal is a measure of efficiency in sound scattering (Figure 6.2). The scattering is a complex function of size, shape, internal structure, material properties, and orientation, as well as acoustic wavelength. The scattering can be determined empirically through measurements, mathematically through physics-based predictions,

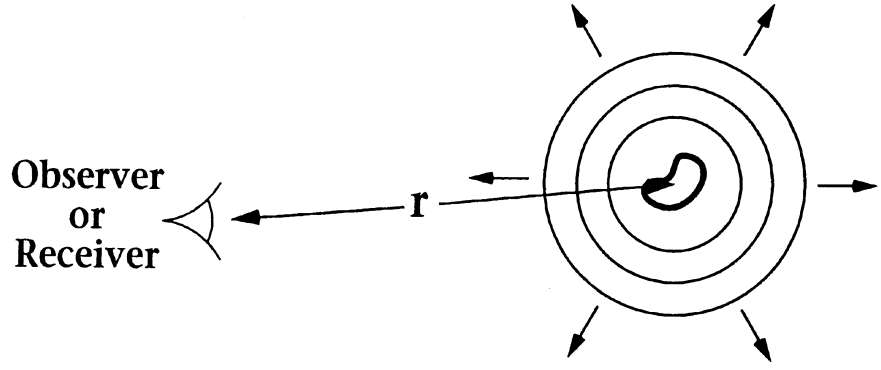


Fig. 6.2 Acoustic scattering by a target.

or in some combination in which certain parameters of the mathematical model are experimentally determined. Regardless of the approach, scattering can be described by the following equation:

$$p_{scat} = P_0 \frac{e^{ikr}}{r} f \tag{6.15}$$

where p_{scat} is the scattered pressure, P_0 is the amplitude of the incident acoustic wave, i is the imaginary number ($i = \sqrt{-1}$), k is the acoustic wave number ($k = 2\pi/\lambda$, where λ is the acoustic wavelength) and f is the acoustic scattering amplitude. Acoustic scattering descriptions focus on the scattering amplitude which, when given in logarithmic terms and evaluated in the backscatter direction, is the target strength:

$$TS = 10 \log |f_{bs}|^2 \tag{6.16}$$

where the reference distance r_o is implicitly assumed to be 1 m. This expression is similar in form to the one given earlier where the target strength is expressed in terms of the backscattering cross section. The backscattering amplitude is simply related to the backscattering cross section by the formula

$$|f_{bs}|^2 = \sigma/4\pi = \sigma_{bs} \tag{6.17}$$

where two forms of the backscattering cross section are given, the backscattering cross section and the differential backscattering cross section, respectively.

In the following sections, general approaches to physics-based mathematical models are summarized.

Categories of mathematical models

Because of the diversity of animal morphology and applications, there is a wide range of approaches to the mathematical description of acoustic scattering by zooplankton. The approaches range from the approximate through the exact, and from describing a single echo from an individual animal to an average of echoes from an aggregation of swimming animals. Furthermore, the animals come in many sizes, shapes, and material properties.

Governing factors in the description of acoustic scattering by any body are the boundary conditions. That is, the type of scattering model depends intimately upon the material properties of the object. For example, the scattering and associated formulations vary dramatically, depending upon whether the object is fluid-like (i.e., does not

support a shear wave), contains elastic material (i.e., supports a shear wave), or contains a gas inclusion. Because of this strong dependence upon type of material property, the scattering models for zooplankton can be categorized according to their class of material property. With this scheme, animals of similar material property, or gross anatomical group, can be described with the same type of scattering model.

Classification of zooplankton organisms according to material properties

Because of the fact that there are thousands of species of zooplankton, it is a practical issue that they be categorized in some way so that only a small number of acoustic scattering models need to be used to describe the scattering. A large number of zooplankton fall into each of the following categories: fluid-like, elastic-shelled, and gas-bearing. Because of the large numbers per category, acoustic scattering models have been developed for each category (Figure 6.3) (Stanton *et al.* 1998a). Experimental

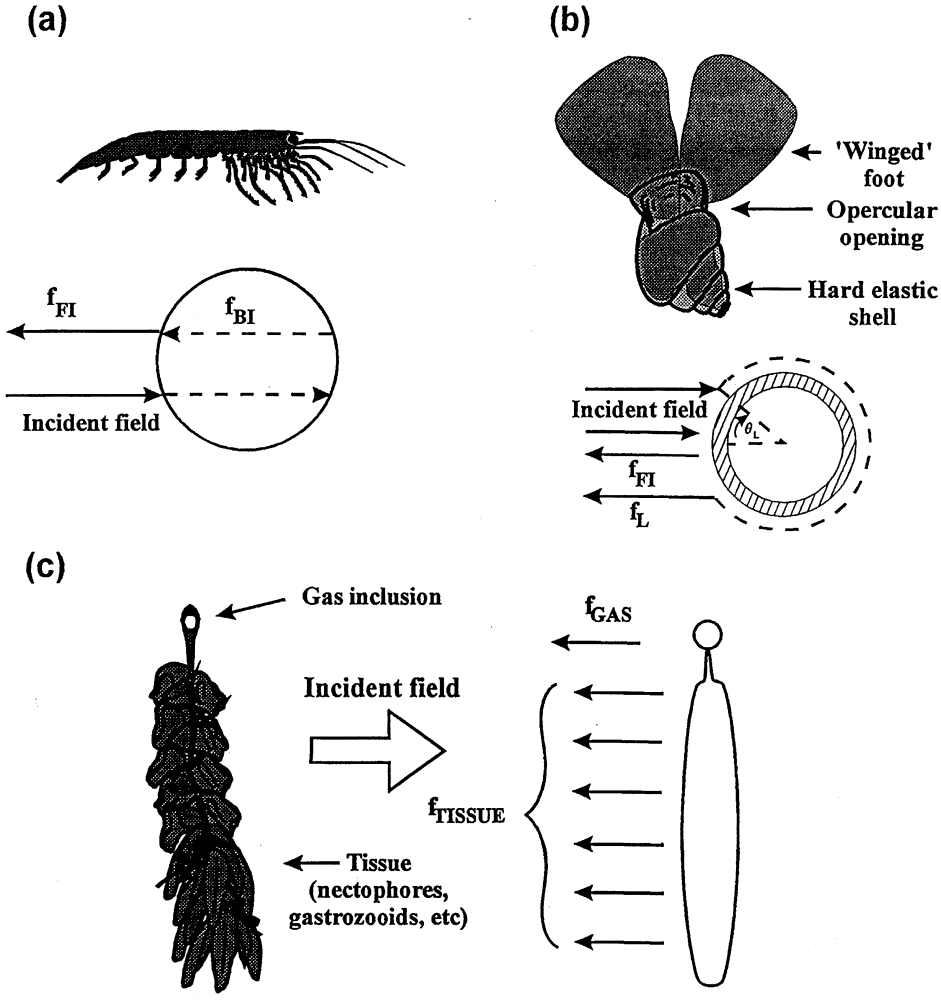


Fig. 6.3 Zooplankton from three gross anatomical groups (a) fluid-like, (b) elastic-shelled, and (c) gas-bearing. Associated scattering mechanisms are illustrated (From Stanton *et al.* 1998a).

Table 6.1 Relative echo energy normalized by biomass measured for animals with different material properties. The data show a variation of up to 19 000 : 1 when comparing the elastic-shelled gastropods with the fluid-like salps. Acoustic frequency: 200 kHz. The results are similar at other higher frequencies. (From Stanton *et al.* 1994).

	Relative (echo energy)/ (organism mass)		Physical information	
	Linear scale	Logarithmic scale (dB)	Average length (mm)	Average wet weight (mg)
Gastropod (elastic-shelled)	19 000	43	1.9	3.6
Siphonophore (gas-inclusion)	260	24	37	1400
Decapod shrimp (fluid-like)	270	24	16	38
Yellowtail fish (swimbladder-bearing)	140	21	450	1 700 000
Salp (fluid-like)	1	0	26	620

studies show that the average scattering levels, when normalized by biomass of the animal, can vary by orders of magnitude between animals with different material properties (Table 6.1).

Fluid-like organisms This category is chosen for any animal that does not contain gas or elastic material substantial enough to support a shear wave with significant energy. Copepods, euphausiids, and jelly fish are fluid-like.

Elastic-shelled organisms These are animals that have an elastic shell thick enough for a shear wave of significant energy to travel in it. Gastropods are in the elastic-shelled category.

Gas-inclusion organisms These are animals that contain enough gas to produce a substantial echo as a result of the gas. Siphonophores contain gas inclusions which, although much smaller than the surrounding tissue, can produce substantial echoes.

Classification according to shape

Within each category of material property, the shape of the animal must be taken into account. There is a wide range of shapes, varying from nearly spherical to very elongated, and nearly smooth to very rough. The shape can have a profound effect on the scattering properties of the animal (Stanton 1989, 1990; Stanton *et al.* 1998b). Also, the shape chosen to model the animal can have a dramatic effect on the scattering (Figure 6.4).

Emerging models

Numerous models have been developed to describe scattering by various groups of zooplankton. The models are of varying accuracy, complexity, and generality.

Fluid-like The most general methods involve an analytical approach based upon the Distorted Wave Born Approximation (DWBA) or a numerical approach based upon the Finite Element and Boundary Element (FEBE) methods. Each approach can accommodate an arbitrarily shaped animal. The DWBA approach is limited to animals whose

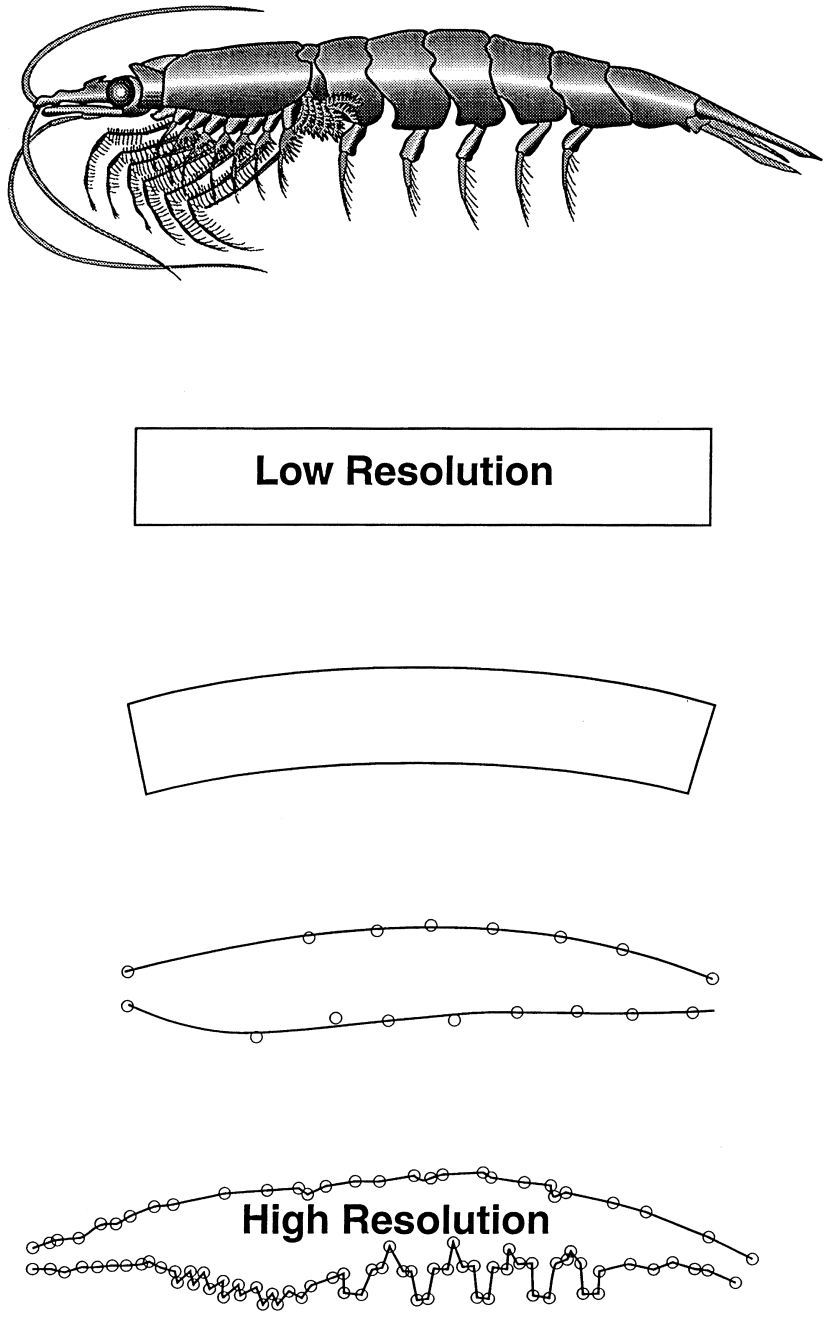


Fig. 6.4 Various shapes that may be considered to model the scattering by an animal. There are trade-offs between ease of use and range of applicability (From Stanton and Chu, in press).

material properties are similar to that of the surrounding water. This restriction is generally not a problem since most fluid-like zooplankton do have properties similar to water.

Details of the DWBA and FEBE models can be found in Stanton *et al.* (1998a) and Francis (1993), respectively. Each approach has its distinct advantages. The DWBA approach can sometimes be reduced to simple analytical forms for convenient calculations, while the numerical approach of the FEBE method is general and can be used to describe a wide range of material properties.

In an early application of the FEBE method to zooplankton, the copepod *Calanus finmarchicus* was modeled as a composite scattering body, with fluid-like oil sac embedded in a fluid-like prosome (Francis *et al.* 1999a). The forms of the two structures were determined from videomicroscopic images of the living animal, encased in a droplet of sea water, in both dorsal and lateral aspects. Each structure was represented by two material properties: the mass density and longitudinal-wave sound speed. The respective property differs between oil sac and prosome. In another early application, the euphausiid *Meganyctiphanes norvegica* was modeled by a fluid-like body having the actual shape of the head and thorax as determined by videophotography with macro lens of the anesthetized animal in both dorsal and lateral aspects (Francis *et al.* 1999b). Values of mass density and longitudinal-wave sound speed were derived by laboratory measurements on ensembles of specimens.

A simple approximate approach, which is convenient for computations, is the ray-based method. This predicts scattering based on only two ray-components of the scattered field (Stanton *et al.* 1993). In fact, this approach yields the average scattering due to an aggregation of animals with a distribution of orientation angles.

Elastic-shelled organisms This area is still in the early stages of development. In Stanton *et al.* (1994), a simple approximate model is used for all frequencies; in Stanton *et al.* (1998a), a two-ray model is used in the high frequency region; and in Stanton *et al.* (in press), a modal-series-based model is used to estimate the average scattering due to an aggregation of animals (all frequencies).

Gas-bearing This approach requires use of a model describing fluid-like animals in combination with a model describing the scattering by gas. This is described in detail in Stanton *et al.* (1998a) where both a general formula is given as well as an example for siphonophores.

Although analytical approaches are only referred to in cases of elastic-shelled and gas-bearing bodies, the FEBE model that is currently under development for zooplankton at the time of writing can, in general, be used to describe the scattering.

6.2.3 Sources of variability

Variability may arise from a number of sources. These are divided somewhat arbitrarily into two categories, associated with the act of measurement and with modeling.

MEASUREMENT

Instrument effects

The general assumption is made that instruments perform stably over time and in accordance with their calibration. In fact, performance may vary and, in general, will

vary with time, if only because of changing conditions of operation, power supply and thermal effects.

Medium influence

Acoustic measurements are affected by the state of the medium. Effects due to absorption and scattering may be especially troublesome at times. Air bubbles, for instance, may attenuate both transmitted signal and echoes. The presence of suspended particulate matter may cause reverberation that masks the echoes from sought targets. The signal-to-noise ratio may even be reduced below the detection threshold.

Behavioral effects

Animals may react while being observed, as in response to associated noise or light stimuli. Both changes in orientation and movements may change the effective sampling volume, hence perception of numerical density. Aggregation of animals may prevent single individuals from being observed, requiring other kinds of measurements, as, for example, to determine the backscattering cross section.

MODELING

Accuracy of mathematical models

Models are just that. As admitted in section 6.2.2, many acoustic scattering models are approximations. Their accuracy is thus fundamentally limited, such as to a particular size-to-wavelength regime.

Accuracy of model parameters

Exercise of the models in section 6.2.2 for a single organism requires knowledge of the morphometry of the animal and its physical properties. Determination of these characteristics also involves models, which are themselves subject to errors and at the least may involve rather complicated experimental procedures (Foote 1998). Application of the mathematical scattering models to animals *in situ* generally requires knowledge of their orientation. If this is unknown, which is frequently the case, the resultant uncertainty in model computations must constitute yet another source of variability.

6.2.4 Generic instruments

ECHO SOUNDER

An echo sounder may be viewed in its most primitive form as a box of electronics that controls the excitation of a transducer and reception of forthcoming echoes. In general, an operator chooses from among a set of parameters, allowing specification of the transmit power level, transmit pulse duration, and receiver bandwidth. When the echo sounder admits of calibration, it is called a scientific echo sounder.

The nominal box of electronics is often expanded with additional electronics and units for signal processing and display of the echo signal. In this configuration it is an echo sounder system. Throughout the present chapter the term echo sounder means scientific echo sounder system.

Three variants of echo sounding systems are described.

Single-beam echo sounder

Here the echo sounder is connected to a transducer with a single beam. This is the simplest and most traditional form (Figure 6.1).

Dual-beam echo sounder

The transducer is composed of an array of elements that is wired differently for transmission and reception. In transmission, all of the elements are excited at the same time. In reception, each of two beams is formed, one based on the entire array of elements and one based on a smaller central core of elements. The corresponding beams are respectively narrow and wide. As explained below in section 6.2.5, simultaneous reception with the two beams allows the beam pattern in a single-target direction to be determined.

Split-beam echo sounder

The transducer is divided into four quadrants. These are used in unison during transmission. In reception, each of four quadrant beams is formed, allowing formation of half-beams, whose phase differences can be found and used to determine single-target positions.

SONAR

A sonar resembles an echo sounder in that they both transmit sound and receive echoes. However, a sonar controls a transducer beam that can be moved, as by mechanical turning or electronic steering. The degree of complexity of a sonar is roughly an order of magnitude greater than that of an echo sounder. Similarly, the amount of information that is typically generated by a sonar which forms many receiver beams simultaneously is often more than an order of magnitude greater than that of an echo sounder.

ACOUSTIC DOPPLER CURRENT PROFILER (ADCP)

The acoustic Doppler current profiler (ADCP) is an echo sounder with signal processing that measures the Doppler shift in echoes received throughout the water column. The echoes typically take the form of volume or bottom or surface reverberation. The processing gauges the Doppler shift in the direction of the transducer, encouraging its use at an oblique angle with respect to the vertical in order to determine current velocity as a function of depth. In fact, the ADCP transducer typically forms four non-colinear beams, whose independent measures of velocity are combined to form a more robust estimate, also reducing the effects of strong single scatterers observed in only one of the beams.

ECHO INTEGRATOR

At an early time in its development, the echo integrator was a box of electronics that performed the mathematical operation of integration by analog circuitry. It is now performed by digital circuitry or by digital computer. The function is often incorporated with echo sounding systems.

POST-PROCESSING SYSTEM

In addition to echo integration, there are many other operations requiring powerful, essentially real-time processing, but with operator control or participation. For this reason, it is often useful to perform the post-processing through a dedicated system. An example is provided by the Bergen Echo Integrator (BEI) (Foote *et al.* 1991). This is a workstation-based system that allows the echo sounder data to be selected, displayed, and manipulated on a screen. This is particularly useful for (1) allocating the echo record to specific classes of scatterers, described further in section 6.2.5, (2) correcting erroneous

bottom detections and sample locations, and (3) storing the results of new echo integration computations in a data base.

6.2.5 Methods of data processing and analysis

ECHOGRAM

The most basic form of presentation of echo sounder data is the echogram. This is a two-dimensional representation of a succession of echo signals. Each signal is represented by a single vertically oriented line, with information on the intensity of backscattering typically encoded by the degree of darkening or by color.

In so far as the echo signal has been received, it is subjected to processing, if only in the form of amplification. Generally time-varied gain is applied to compensate for simple range dependent effects. If the transducer is moving, the echogram will, in a manner of speaking, image scatterers in a slice or wedge-shaped volume of water. If the transducer is stationary, the echogram will record the temporal variability of the vertical distribution of scatterers over the selected range interval.

Two examples of echograms are shown in Plates 2 and 3. In the first, a diverse aggregation of zooplankton is imaged in Storfjorden, Norway. The upper 250 m of the water column is shown, but echogram noise dominates at depths below about 175 m. The total sailed distance is about 3 nautical miles. The echogram was produced by the EK500/200-kHz echo sounder system. In the second figure, both blue whiting (*Micromesistius poutassou*) and zooplankton are imaged over the slope of the Continental Shelf west of the British Isles. The blue whiting is delimited by the pair of irregular lines in the mid-water region, and the zooplankton are observed between the lower edge of the dense concentration and the bottom. The total imaged depth range is 150–650 m. In addition, in the lower frame, the bottom channel is imaged from 10 m over to 5 m under the detected bottom. The total sailed distance is 5 nautical miles. The echogram was produced with the Bergen Echo Integrator, described briefly in section 6.2.4. The color scale to the right indicates the absolute value of volume backscattering strength.

ECHO INTEGRATION

The heart of echo integration is a simple mathematical operation, namely integration of the received signal intensity following application of the time-varied-gain function $20 \log r + 2\alpha r$. In absolute terms, the received signal intensity is expressed through the volume backscattering coefficient s_V. Its integral over the range interval $[r_1, r_2]$ is called the area backscattering coefficient s_a,

$$s_a = \int_{r_1}^{r_2} s_V(r) dr \tag{6.18}$$

This applies to a single signal. Often s_a is averaged over a number of pings or over some interval of sailed distance. In the case of n pings,

$$s_a = (1/n) \sum_{i=1}^{n} s_{a,i} \tag{6.19}$$

The units of s_a are square meters of backscattering cross section per square meter of surveyed area. The numbers are typically quite small for fish, say of the order of 10^{-8} to 10^{-2}, and often orders of magnitude smaller in the case of zooplankton. In the case of

fish, a scaling factor is generally used to allow the use of integers only (Knudsen 1990). Thus the quantity s_A is defined:

$$s_A = 4\pi 1852^2 s_a \tag{6.20}$$

with units of square meters of backscattering cross section per square nautical mile of survey area. In the case of zooplankton, s_A may still require use of numbers less than unity, hence floating-point registration, but the reference to one square nautical mile of survey area is convenient for large-scale applications.

Use of s_A is also convenient for deriving measures of biological density through the fundamental equation of echo integration, namely

$$s_A = \rho_A \sigma \tag{6.21}$$

Here σ is the characteristic backscattering cross section of the observed scatterers, and ρ_A is their numerical density in units of numbers of animals per square nautical mile.

A useful variant of the last equation is that relating the volume backscattering coefficient s_V to the numerical density of scatterers with respect to volume, namely

$$s_V = \rho_V \sigma \tag{6.22}$$

In this case, ρ_V is expressed in terms of the number of animals per cubic meter.

TARGET STRENGTH DETERMINATION

A rather large number of methods for determining target strength have been developed and applied mainly to fish. They are classified and summarized in Foote (1991a).

Since the methods are generic, they could under similar conditions also be applied to zooplankton. Some likely candidates for application are described very briefly in the following sections. Reference is made to the cited literature for more details.

Indirect *in situ* methods

In the case that the transducer directivity pattern is unknown for single targets, the principal measurement is that of echo energy or intensity, namely

$$\epsilon = g b^2 \sigma \tag{6.23}$$

where g is a general scaling factor consisting of gain factors due to receiver amplification and geometric factors depending on the target range and possible application of time-varied gain, b^2 is the product of transmit and receive beam patterns in the direction of the target, and σ is the target backscattering cross section.

Since ε can be measured and g is otherwise known from the detected target range and type of time-varied gain, the product $b^2\sigma$ can be determined. For the particular consideration of a single-beam transducer, σ can only be described by a statistical analysis of many determinations of the product term. This may be approached in several ways. In the intensity domain, the equation may be rewritten as $\varepsilon = \alpha\beta$, where $\alpha = g\sigma$ and $\beta = b^2$. Equating corresponding probability elements,

$$f_E(\epsilon) = \int_0^1 f_A(\epsilon/\beta) f_B(\beta) d\beta/\beta \tag{6.24}$$

where f denotes the probability density function (pdf) of the indicated variable, the integration is performed over the range of b^2, namely 0 to 1. In the amplitude domain, a

similar equation can be derived in terms of the quantity $\varepsilon^{1/2}$. In the logarithmic domain, the equation can be written as

$$z = x + y \tag{6.25}$$

where $z = \log \varepsilon$, $x = \log(g\sigma)$, $y = \log(b^2)$. If corresponding probability elements are equated, then

$$f_z(z) = \int_{-\infty}^{0} f_x(z - y) f_y(y) dy \tag{6.26}$$

where the range of integration is that of $\log(b^2)$, where b is bounded by 0 and 1.

There are a number of solutions to the resultant integral equations. In the intensity domain, Ehrenberg (1972) and Robinson (1982) approximated the pdf $f(\sigma)$ by polynomials. In the amplitude domain, Clay (1983) transformed variables, expressing the pdf of $\varepsilon^{1/2}$ as a convolution, allowing deconvolution by the Laplace Z-transform. In the logarithmic domain, Degnbol *et al.* (1985) have shown how to attach a non-negativity constraint to the linearized set of equations resulting from discretization of the integral equation, rendering the early solution by Craig and Forbes (1969) numerically stable and physically realizable.

Parametric approaches have also been pursued. Rayleigh statistics have been assumed by Petersen *et al.* (1976), Ehrenberg *et al.* (1981), and Ehrenberg (1983). The more general Rice statistics have been applied by Clay and Heist (1984).

Direct *in situ* measurements

Two specific cases are cited. In each, the beam pattern term in the equation for the echo energy or intensity due to a resolved single target can be determined from the manner of measurement.

Dual-beam method In this, two concentric transducer arrays are used in a cooperative manner (Ehrenberg 1974). The entire array of elements is used to transmit a rather narrow beam. Echoes are received simultaneously by the central core of elements, with wide beam, and by the entire array of elements, with narrow beam. Each of two equations can be written for the echo energy or intensity. For the equation with wide-beam reception, the narrow-beam pattern can be approximated, allowing its use in the equation for narrow-beam reception. Solution for σ is immediate.

Split-beam method The transducer array is divided into four quadrants. They act in concert under transmission, but separately in reception, allowing phase differences in each of the two planes to be determined. From these, the target direction can be determined, allowing specification of the beam pattern value and immediate extraction of σ from the equation for the echo energy. The method of split beams is judged superior to that of dual beams (Ehrenberg 1979). The first application to fish is described by Foote *et al.* (1984), with documentation of early measurements by Foote *et al.* (1986).

Other methods

A number of other methods are summarized in Foote (1991a). Apropos of zooplankton, two techniques in particular deserve mention. These are those based on morphometry, as by model computation, which is discussed in section 6.2.2, and cage measurement of an aggregation. An illustration of the second is that in which the target strength of Antarctic krill (*Euphausia superba*) was determined using measurements of volume scattering strength and *a priori* knowledge of numerical density of the animals in the cage (Foote *et al.* 1990).

POST-PROCESSING AND DATA ANALYSIS

Echo sounder data, like many other kinds of acoustical scattering data, are essentially underdimensioned in the sense of containing insufficient information for target identification. Further processing of echo data is thus generally necessary. In addition, use of the data to describe geographical distribution, abundance, and associated variance requires analysis of the post-processed data. Some details are given here.

Echo interpretation

Because of the underdimensional, or non-unique, character of echo sounder data, an important post-processing operation is allocation of the echo record to specific scatterer classes. In applications to fish, a number of pelagic and demersal species may be represented, and zooplankton such as euphausiids may also be considered.

A means to echo allocation is provided by the Bergen Echo Integrator (BEI), mentioned in section 6.2.4. This allows the operator to associate arbitrary regions of the echogram displayed on the screen with specific scatterer classes. When satisfied with this so-called interpretation process, the area backscattering coefficient is computed internally and entered in a data base according to selected degrees of horizontal and vertical resolution. A fine degree of resolution could be 0.1 nautical mile in sailed distance and 10 m or less in the vertical direction. Respective coarse degrees of resolution would be 5 nautical miles and 100 m or more.

Numerical density estimation

Single frequency The numerical density is determined from the echo integration equation (equation 6.21). Interpreted echoes are quantified by the area backscattering coefficient. When this is divided by the mean backscattering cross section characteristic of the scatterers, the result is a biological measure of numerical density.

Two frequencies The numerical density can be estimated by taking the difference between volume scattering strengths as measured at two frequencies. By assuming a scattering model and material properties, mean size and numerical density are estimated. Results are generally much more reliable when one of the frequencies is in the Rayleigh region and the other is above this (Greenlaw 1979). This method was developed for non-gaseous objects, but could be extended to gaseous objects.

Multiple frequencies The numerical density can be estimated by means of a mathematical inversion in combination with assumed acoustic backscattering models of the animals present (Holliday 1977; review in Holliday and Pieper 1995). With this approach, the volume scattering coefficient for the ith frequency can be written as

$$(s_v)_i = \sum_{j=1}^{N} \sigma_{ij} n_j \tag{6.27}$$

where n_j is the numerical density of the jth size class and σ_{ij} is the backscattering cross section of the jth size class at the ith frequency. Given measurements of the volume scattering strength at each frequency and model calculations of all backscattering cross sections, then the numerical densities of the various size classes can be estimated through the method of non-negative least squares (NNLS).

A wide range of frequencies needs to be chosen because both the Rayleigh and geometric scattering regions need to be covered for each desired size class. It is the transition point between these two regions that is exploited in the inversion (for gas-

bearing bodies, it is the resonance frequency of the gas inclusion or pocket that needs to be within the range of acoustic frequencies that are used). Typically one size class per frequency can be estimated.

Integration with other variables

Oceanographic variables Planktivorous fish such as herring depend on zooplankton, which in turn depend on or are influenced by ocean currents, upwelling zones, etc. While such connections are generally appreciated (Haury *et al.* 1978; Verheye and Richardson 1998), they can also be exploited quantitatively in the analysis of density data, as in stratification.

Bathymetric features In so far as bathymetry is influential in determining plankton-bearing currents, it may be coupled directly to density measurements of zooplankton. Strata may be defined solely in terms of bathymetric features, which may be linked to persistent water masses, as illustrated by Loeng (1991), and their flow-defined boundaries.

Other forms of echo visualization

With recent advances in computers and three-dimensional graphics, more advanced forms of presentation of echograms are possible. One interesting form is the so-called ribbon plot which, in essence, draws the echogram along the actual trackline of the ship. The result is a three-dimensional view of the acoustic scatter pattern (resembling the shape of a ribbon) (Plate 4). When measurements are made along a number of closely spaced parallel tracks, patches across tracklines can be inferred (Greene *et al.* 1996; Wiebe *et al.* 1997; Benfield *et al.* 1998). In fact, samples distributed over an area, spaced nearby or far apart, collected along parallel transects or according to other designs, give a basis for interpolation, as by kriging (Journel and Huijbregts 1978; Cressie 1991). This, however, exceeds the scope of the present work and is not considered further.

Another derivative of the two-dimensional echogram is the noise-processed echogram (Korneliussen 1998). An example is shown in Plate 5.

A different noise-removing algorithm has been applied to echo data by Watkins and Brierley (1996). Exemplary three-dimensional plots of integrated values in time and depth, both with and without noise correction, are shown in the cited work.

Multiple echograms formed concurrently but at different frequencies have been combined, as through so-called difference-frequency echograms in which respective values of volume backscattering strength measured simultaneously at different frequencies are subtracted and the resulting difference value is displayed. This is illustrated in Socha *et al.* (1996). In more recent work, R.J. Korneliussen has formed weighted-difference noise-processed echograms, showing magnitudes as degrees of shading of red or blue as the pixel-based differences are positive or negative, respectively.

6.2.6 Comparisons

A number of otherwise separate topics are treated in this section. In each case, considerations or alternatives are presented that may involve or require choosing – to be done on the basis of comparisons.

IDENTIFICATION

The customary forms of identification are physical capture and optical registration. The size, robustness, swimming speed, sensitivity, numerical density, and, in general, availability of the target organism to sampling are major factors in choosing gear for identifying acoustically registered organisms.

While there may be few general rules for sampling, guidelines may certainly be given. In the case of rather large, relatively sparsely distributed and robust organisms such as fish, catching by gill net, seine, pelagic or bottom trawl may be the only feasible means of identification. At the opposite end of the size and density spectrum, the very small and numerous and fragile may best be observed *in situ* as with the underwater video plankton recorder (Davis *et al*. 1992a, 1992b). Euphausiids, with intermediate properties, may be caught by pelagic trawl or plankton net, or may be observed optically, depending on the particular conditions present, including lighting and numerical density.

CHOOSING AN ACOUSTIC INSTRUMENT

In order to resolve two targets at ranges differing by Δr, the acoustic pulse duration τ must not exceed $2\Delta r/c$. Alternatively, use of the pulse duration τ allows resolution of two targets differing in range by

$$\Delta r = c\tau/2 \tag{6.28}$$

Resolution of individual targets in an aggregation may require use of a more directional beam, for targets that are well separated in distance may not differ significantly in range. As the beamwidth decreases, the sampling volume also decreases, decreasing the cumulative number of possible target echoes. In fact, the number of echoes does not change strictly with beamwidth, but the level of individual echoes may essentially vanish owing to the powerful effect of beam pattern loss on off-axis echoes. Reference is made here to the active sonar equation (equation 6.9) presented in section 6.2.2.

If the echo signal-to-noise ratio (SNR) is high, the sampling volume corresponding to a transmitted pulse of duration τ is approximately

$$V_s = \pi(r\Delta\theta/2)^2 c\tau/2 \tag{6.29}$$

where $\Delta\theta$ is the full beamwidth at the -3-dB level in radians. In general, however, the sampling volume also depends on the target echo level relative to the receiver detection threshold, hence it involves statistical considerations. The theory with formulae for numerical evaluation is given in Foote (1991b).

In marginal circumstances, when the SNR may be low, reference to the general theory (Foote 1991b) may be valuable. This indicates how the sampling volume may increase with increasing source level or decreasing detection threshold, system characteristics which may be chosen at the outset of a study or which, if given, define the performance bounds of the system.

SINGLE-ANIMAL METHODS

Resolving echoes from individual animals depends principally on the numerical density of the animals in relation to the sampling volume including pulse duration. For a given transducer size and geometry, the capacity to resolve individual echoes is essentially fixed. The performance of single-, dual-, and split-beam transducers of the same overall size and shape is essentially identical. Thus other factors need to be considered in choosing among the three types of transducers.

If there is no need to measure the target strength of individual scatterers, the principle of simplicity would dictate selection of a single-beam transducer. If there is a need to measure the target strength of individual scatterers, this can be accomplished with either of the three transducer types, as considered in section 6.2.5. However, there may be operational advantages in doing this directly with a dual- or split-beam transducer.

As mentioned earlier, there is a general advantage to choosing the split-beam

transducer over the dual-beam transducer (Ehrenberg 1979). However, should it be necessary to track an individual target, the split-beam transducer has a unique capacity, for this is the only one that resolves target position in three dimensions.

MULTIPLE-ANIMAL METHODS

In contrast to single-animal measurements, individual animals are not resolved in so-called multiple-animal measurements. A number of acoustic instruments can be used to observe and measure such aggregations. Choosing between these depends on the purpose of the measurement.

If the animals are distributed in a layer or shoal, they might be observed by a vertical echo sounder. The use of an echo sounder allows both visualization and quantification with a high degree of resolution.

If the measurements are similarly distributed but are sensitive to the passage of the transducer platform, avoidance reactions may preclude ordinary registration. A sonar, with its movable beam or beams, can image fish schools (Misund *et al.* 1995; Fernandes *et al.* 1998), enabling them to be counted (Hewitt *et al.* 1976). Patches of zooplankton of sufficient size and density can also be imaged. Because of the widespread use of automatic gain control (AGC) in sonar circuitry, quantitative information on backscattering is not readily available. If the AGC signal itself can be monitored, and the sonar calibrated, the volume backscattering coefficient can be measured.

An alternative to ordinary echo sounder measurement is measurement by ADCP (section 6.2.4). Its sum beam or beams are equivalent to echo sounder beams, although, like sonar beams, these are generally uncalibrated. In addition, they may suffer from limited dynamic range and coarse digitization, but the potential is admitted (Flagg and Smith 1989; Heywood *et al.* 1991; Roe *et al.* 1996, 1998).

PARAMETER RANGES FOR SCIENTIFIC ECHO SOUNDERS

Some parameter ranges are given in Table 6.2. For reference, some medium properties are given. These may be put in context by reference to some elementary relations and properties of the water medium. First, the relationship between frequency v and wavelength λ is

$$v\lambda = c \tag{6.30}$$

where c is the speed of sound in the medium. For the range of temperature 0–30 °C, the

Table 6.2 Nominal parameter ranges of scientific echo sounders.

Parameter	Range of values
Resonant frequency v	20–200 kHz
Wavelength λ	0.7–7 cm
Diameter	2–50 cm
Beamwidth	1–20°
Transmit power	10–1000 W
Pulse duration τ	0.1–10 ms
Pulse length relative to λ	10–50
Receiver bandwidth relative to v	1–10%

sound speed in sea water is roughly 1450–1550 m s^{-1}, and in fresh water is roughly 1400–1500 m s^{-1}. The frequency may also be expressed through the wavenumber

$$k = 2\pi/\lambda = 2\pi v/c \tag{6.31}$$

The product of the wavenumber and transducer radius a defines the beamwidth θ for a circular transducer, namely

$$\theta = 2\pi \arcsin(1.57/k) \tag{6.32}$$

For ordinary operations, the transmit waveform is usually a pulsed sinusoid with center frequency v and pulse length $c\tau$ that is long compared to λ. Signal filtering in the receiver may be characterized by a bandwidth that is generally small compared to v, say 1%–10%.

6.3 MEASUREMENT PROTOCOLS, MODEL COMPUTATIONS, AND EXAMPLES

6.3.1 Calibration

An essential part of any scientific use of acoustic instruments is calibration. This is necessary to ensure stability in performance over time for relative measurements. It is also necessary to determine the scaling factor for absolute measurements.

Three kinds of calibration measurements are described in this section. These may be performed separately or in conjunction with one another. Their frequency of performance need not be constant, especially since the overall performance of the system can be controlled by the standard-target method. Should a deviation be observed, the other measurements may help disclose the problem.

No matter what the actual or likely findings of a calibration exercise are, the exercise should be viewed as an essential operation. Generally it should be performed in conjunction with any valuable series of measurements and otherwise at regular intervals to monitor the system performance. Calibration is thus also part of the basic maintenance of the instrument.

TEST MEASUREMENTS

A number of so-called laboratory measurements can be performed without the need for transducer immersion. These measurements may include the impedance, or its reciprocal admittance, of various parts of the system including its frequency dependence. The impedance seen from the transducer terminals is especially interesting for monitoring purposes. The measurement may also include the time-varied-gain (TVG) functions or, more generally, the range compensation functions realized in the echo sounder. This measurement may be facilitated by use of a time-amplitude-frequency device (Knudsen 1985), by which a signal of known time delay, amplitude, and frequency can be introduced into the system, as at the transducer connection points. Comparison of the output signal with the expected function enables the operation of the TVG function to be verified.

STANDARD-TARGET METHOD

A standard target is suspended in a known part of the beam of the immersed transducer as normally mounted for use. The response of the system can be related to the

backscattering cross section of the standard target, and the constant of proportionality determined. Measurements of other objects can then be expressed in absolute physical units.

To illustrate the calibration protocol, the example of a scientific echo sounder with coupled echo integrator and hull-mounted transducer is assumed. The vessel platform is secured in sheltered and relatively deep water with little or no current. Fishing reels mounted on outriggers are fixed to the railings at predetermined positions, two on one side and one on the other. Before anchoring the vessel, a line is passed over the bow for passing the target suspension lines beneath the hull. When these are joined, the target, a standard sphere, is attached, wetted in a solution of household detergent and fresh water, and immersed. The sphere is centered in the beam by drawing and slackening the three lines, held clear of the hull by the outriggers, in coordinated fashion. This process is guided by observing the magnitude of the echo, and in the case of the split-beam echo sounder by the apparent position of the target in the beam. When the target is on the axis, so that its echo strength is a maximum, the sphere echo is integrated for a set period of time, with simulation of a constant vessel speed. The result of the echo integration M is compared to the expected theoretical result for the area backscattering coefficient s_A, namely

$$\mathbf{CM} = \mathbf{1852}^2\boldsymbol{\sigma}/(\boldsymbol{r}^2\boldsymbol{\psi}) \tag{6.33}$$

where σ is the backscattering cross section of the standard sphere for the particular sound speed at depth, determined by CTD sonde, r is the sphere range, and ψ is the equivalent beam angle of the transducer (Urick 1983). The constant of proportionality C is thus determined. When measurements are made on other targets, these can be expressed in absolute physical units of s_A by multiplying by the constant C.

The calibration exercise with standard target concludes with confirmation of the completeness of information and data entered on a standard form. The results of the calibration are later summarized in an instrumentation report for dissemination among interested parties and for archiving for possible future reference.

The standard-target calibration method is an ICES-recommended procedure. It is elaborated in Foote *et al.* (1987). Further details on the calibration of echo integrators are given by Foote and Knudsen (1994).

BEAM PATTERN MEASUREMENT

Another form of calibration involves measurement of the transducer directivity function in space. While measurement of the beam pattern is straightforward for a transducer detached from its mounting, it is a great challenge, for example to measure the beam pattern for hull-mounted transducers. A sophisticated method has been developed and realized by Reynission (1998) for this case. Accordingly, a standard sphere is moved to known positions in the beam and the echo strength recorded. Performance of the measurement over a sufficient cross section of the beam enables the beam pattern function to be defined. Usually, since this may be known from previous measurement or the geometric configuration itself, parameters may be confirmed or adjusted to reflect possible changes in the beam pattern. Again, significant discrepancies require detailed investigation, usually beginning with the transducer itself, but including both mechanical parts and electrical connections.

6.3.2 Elements of echo abundance surveying

The most general method of acoustic abundance surveying is that of echo integration. This has already been defined in a narrow sense in section 6.2.5 as a mathematical operation. However, it also has a much broader connotation, which is that of echo abundance surveying.

The elements of this method are well known (Foote 1989) and are displayed in Figure 6.5 (Foote and Stefansson 1993). Their order is somewhat arbitrary, but their presence is not. Each element represents an essential part of the surveying process.

EQUIPMENT

A certain minimum of equipment is required. This includes an echo sounder with directional transducer on a movable platform, physical capture gear or optical

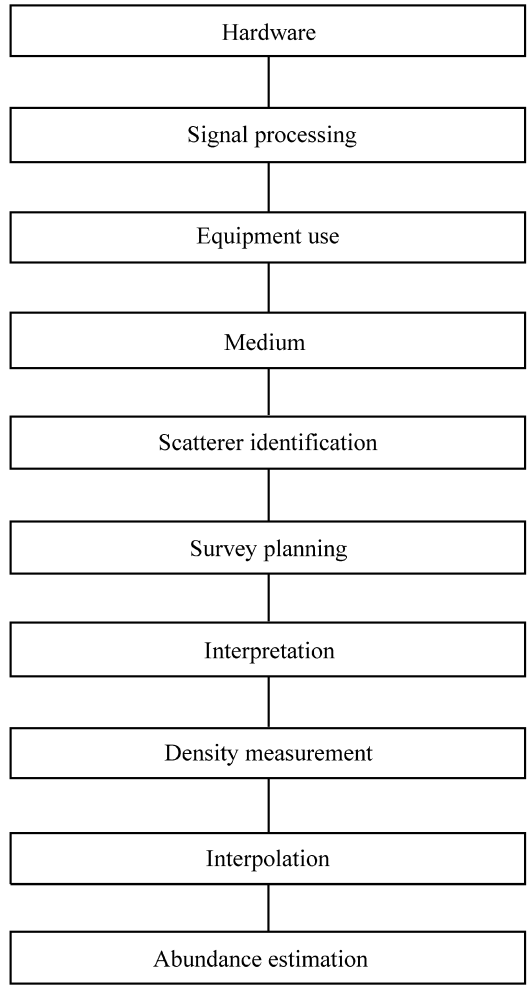


Fig. 6.5 Elements of echo abundance surveying, arranged in a chain analogy. Used with permission (Foote and Stefansson 1993).

registration device, a means of displaying the data in real time during their collection, an echo integrator, and a means of storing the data for further processing and analysis.

SIGNAL PROCESSING

A basic capacity for signal processing includes range compensation of the time-varied-gain (TVG) type. The common TVG function for echo integration is $20 \log r + 2\alpha r$ in the logarithmic domain, where r is the range and α is the absorption coefficient. Knowledge of the sound speed profile enters the TVG through the relation $r = ct/2$, where c is the speed of sound and t is the echo time.

EQUIPMENT USE

The acoustic equipment must be properly mounted, mechanically protected, and insulated from extraneous electromagnetic interference. It should be calibrated at regular intervals and at least once immediately before, during, or after an abundance survey.

MEDIUM

The influence of the medium is conspicuous, as through the absorption term in the TVG function. If air bubbles are present beneath the transducer, there may be an excess absorption requiring compensation if not outright avoidance.

SCATTERER IDENTIFICATION

Identification of the acoustic scatterers is necessary for matching the echo data with causative target. This may occur by physical capture, optical registration *in situ*, or both, depending on the particular application and possible need to distinguish among different kinds of organisms.

SURVEY PLANNING

The time and geographical region of the survey need to be decided. Ideally these are chosen on the basis of biology, specifically life history, but the critical accessibility of the organism to acoustic surveying also depends on weather and the availability of sufficient ship time. Given time and place, the manner of coverage of the area of occurrence needs to be defined. Ideally, this is done as uniformly as possible in order to minimize the effects of autocorrelation, and thus maximize the amount of information acquired per unit effort.

INTERPRETATION

The echo record is allocated to individual scatterer classes on the basis of the identifying data and subjective factors derived from an understanding of the underlying biology. Availability of a post-processing system is often very convenient for expediting this work.

DENSITY MEASUREMENT

At the end of the allocation process, values of area or volume backscattering coefficient are associated with location for each target species. Division of these quantities by the respective characteristic backscattering cross section yields the numerical density referred to unit area or volume.

INTERPOLATION

Sampling, along transects, is necessarily partial. To supplement this knowledge, inter-

polation between the transects must be performed. This can be done on an objective basis, as by kriging, or by assumption, especially in the implicit case when distribution maps are not prepared.

ABUNDANCE ESTIMATION

The end result of an acoustic abundance survey is a number or a set of numbers describing the total number of animals over an area or in a given volume. This number may be further quantified for each species by its distribution with respect to age or size classes.

6.3.3 System deployment

As with any underwater system, there are a variety of ways in which acoustical systems can be deployed. Specific to zooplankton acoustics is the fact that the higher frequency signals (low megahertz) required to detect some of the smaller animals can only travel distances of the order of meters. Thus such high frequency systems must be deployed very near the animals.

Acoustical systems and associated deployment types can be categorized as long range or short range.

Long-range acoustic systems and associated deployments typically involve lower acoustic frequencies (about 200 kHz and lower) that travel nominally 100 m or more. These frequencies can detect fish, larger zooplankton (>1 cm in length), and gas-bearing zooplankton. Deployment of long-range acoustical systems can be effected by transducers mounted on the hull or keel of the ship, on a vehicle that is towed near the surface, on surface buoys, and on a seafloor platform.

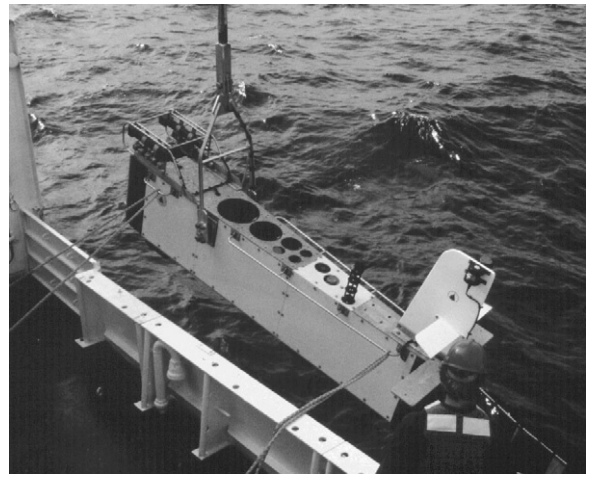
Short-range acoustical systems and associated deployments typically involve higher acoustic frequencies (above about 200 kHz) for detection of smaller zooplankton (<1 cm in length). The acoustic signal at these higher frequencies can only travel tens of meters or less. For these types of deployments, the transducer is typically mounted on a tow body that undulates through the water column, on a remotely operated or autonomous vehicle, vertically profiling mounts, cast systems, and moored arrays of systems (Figure 6.6).

There are exceptions to each of the above sets of examples. For example, the upper layer of small zooplankton can certainly be surveyed with a high-frequency hull-mounted system, while fish can be surveyed at higher frequencies within the short range. At the same time, it must be admitted that such designs may not be efficient for the particular survey problems.

6.3.4 Evaluation of sonar performance

Some examples are presented to illustrate how the sonar equation (equations 6.9 and 6.10) presented in section 6.2.2 can be evaluated.

The transducer is assumed to be resonant at 200 kHz and circular with a full beamwidth of 10° between opposite -3-dB levels. Assuming sea water with temperature 5 °C, salinity 35 ppt, and pH 7.7, the sound speed at the surface is 1470.7 m s^{-1} (Mackenzie 1981). The transducer diameter, determined by the frequency and beamwidth for a given sound speed, is 43.4 mm. The transmitting and receiving directivity indexes are assumed to be identical, with values given by the ideal case,



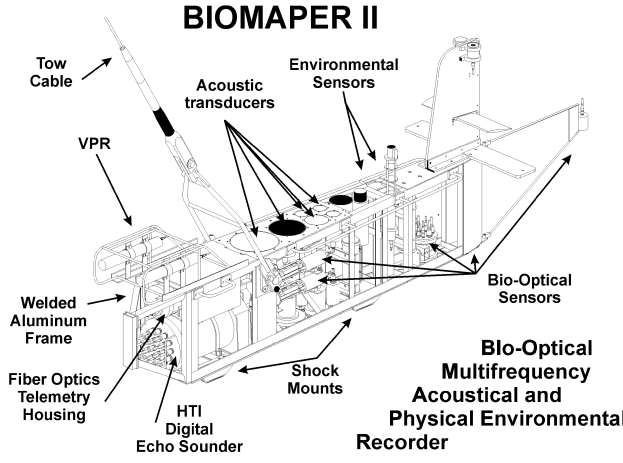


Fig. 6.6 A towed vehicle that contains upward- and downward-looking acoustic transducers, a video plankton recorder (VPR), and various environmental sensors. The system can be towed at fixed depth or in an undulating pattern so that the short range transducers and other sensors can sample much of the water column. From Austin *et al.* (1998).

hence $DI = 25.4\,\text{dB}$, $\psi = 0.0168\,\text{sr}$, and $\Psi = 10\log\psi = -17.7\,\text{dB}$. At a nominal acoustic intensity of $10\,\text{W cm}^{-2}$, the total acoustic power is 148 W. Thus, $SL = 217.9\,\text{dB}$ with respect to $1\,\mu\text{Pa}$ at 1 m. The absorption coefficient for the same conditions is $0.0456\,\text{dB m}^{-1}$ (Francois and Garrison 1982). The ambient noise level can be determined from equation 6.11 in section 6.2.2, assuming a given sea state. In the computations, each of two sea states is considered. The transmit pulse duration τ is assumed to be 0.1 ms and the receiver bandwidth 20 kHz, that is, 10% of the transmit frequency. In addition, the receiver noise was assumed to exceed the thermal noise by 10 dB.

Example 1. SNR for detection of a single on-axis target at 200 kHz
The sonar equation in its first form (equation 6.9) is applied for a range of values for the target strength *TS*.

	SNR (dB)					
	r(m) for $n_{ss} = 0$			*r*(m) for $n_{ss} = 6$		
TS (dB)	10	50	100	10	50	100
−120	−4.6	−36.2	−52.8	−6.8	−38.4	−55.0
−110	5.4	−26.2	−42.8	3.2	−28.4	−45.0
−100	15.4	−16.2	−32.8	13.2	−18.4	−35.0
−90	25.4	−6.2	−22.8	23.2	−8.4	−25.0
−80	35.4	3.8	−12.8	33.2	1.6	−15.0
−70	45.4	13.8	−2.8	43.2	11.6	−5.0
−60	55.4	23.8	7.2	53.2	21.6	5.0

Example 2. SNR for detection of multiple targets at 200 kHz
The sonar equation is applied in its second form (equation 6.10), assuming a range of values for the mean volume backscattering strength S_V.

	SNR (dB)					
	r(m) for $n_{ss} = 0$			*r*(m) for $n_{ss} = 6$		
S_V (dB)	10	50	100	10	50	100
−120	−13.6	−31.3	−41.9	−15.8	−33.5	−44.1
−110	−3.6	−21.3	−31.9	−5.8	−23.5	−34.1
−100	6.4	−11.3	−21.9	4.2	−13.5	−24.1
−90	16.4	−1.3	−11.9	14.2	−3.5	−14.1
−80	26.4	8.7	−1.9	24.2	6.5	−4.1
−70	36.4	18.7	8.1	34.2	16.5	5.9
−60	46.4	28.7	18.1	44.2	26.5	15.9

Example 3. Maximum detection range of a single on-axis target at 200 kHz
The sonar equation in its first form (equation 6.9) is solved assuming a detection threshold $DT = 20$ dB. The conditions are otherwise the same as in Example 1.

	r(m)	
TS (dB)	$n_{ss} = 0$	$n_{ss} = 6$
−120	2.5	2.2
−110	4.4	3.9
−100	7.8	6.9
−90	13.4	11.9
−80	22.7	20.2
−70	37.4	33.6
−60	59.2	53.7

Example 4. Maximum detection range of multiple targets at 200 kHz
The second form of the sonar equation (equation 6.10) is assumed, with $DT = 20$ dB, with the parameter values as in Example 2.

	r(m)	
S_v (dB)	$n_{ss} = 0$	$n_{ss} = 6$
−120	0.2	0.2
−110	0.7	0.6
−100	2.3	1.8
−90	6.8	5.4
−80	18.9	15.3
−70	45.3	38.0
−60	89.8	78.5

6.3.5 Exemplary model computations

The following example illustrates average values predicted by models for acoustic backscattering by animals in the three gross anatomical groups; decapod shrimp (*Palaemonetes vulgaris*, fluid-like), gastropod (*Limacina retroversa*, elastic-shelled), and siphonophore (*Agalma* sp., gas-bearing) (Example 5). Although the sizes are quite different, the average scattering levels are similar at the higher frequencies. The similarities are due to the fact that the small gas inclusion and small elastic-shelled body have stronger material contrasts with respect to the surrounding water than that of the larger fluid-like shrimp. There are significant differences at the lower frequencies because Rayleigh scattering dominates the fluid-like and elastic-shelled bodies while resonance of the gas begins to be a factor for the siphonophore.

Example 5. Model predictions of average target strengths (dB) for 16-mm-long decapod shrimp, 1.9-mm-long gastropod, and 37-mm-long siphonophore (using equations and parameters given in Stanton et al. 1994)
The two-ray model was used for the shrimp while high-pass fluid models were used for the gastropod and siphonophore. The material for the gastropod was assumed to be a dense fluid. The resonance is taken into account for the gas high-pass model.

Frequency (kHz)	Shrimp	Gastropod	Siphonophore
38	−88	−108	−63
120	−75	−89	−66
200	−72	−81	−68
420	−75	−76	−72

The differences in scattering characteristics can be further illustrated by examining the target strengths over a continuous range of frequencies for both single echoes and averaged echoes.

Single echoes In modelling an echo from an individual animal it is important to take into account the complex structure of the scattering. The structure is due to interference

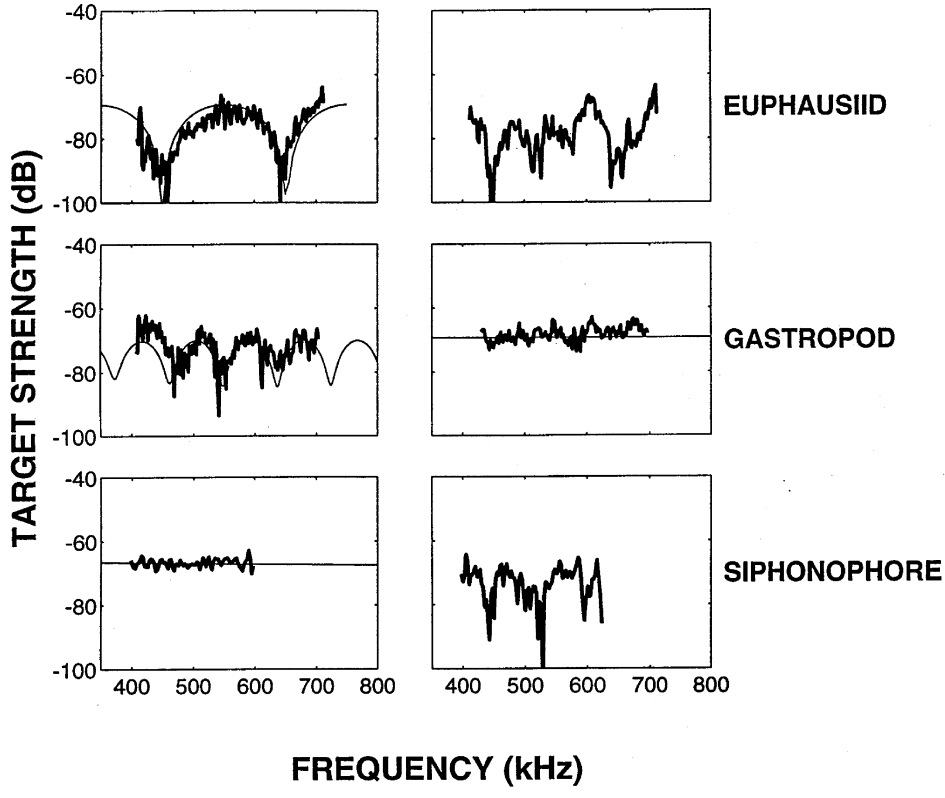


Fig. 6.7 Single echoes from individual euphausiid (34 mm), gastropod (2 mm), and siphonophore (48 mm long, with gas inclusion 1.3-mm-long by 0.5-mm-wide). Each echo (within a pair of echoes in a given row) is from a different orientation from the same animal or from a different animal. The ping-to-ping variability in the spectrum of the scattering is illustrated. The rough thick lines are data from live freshly caught animals and the smooth thin lines are from predictions using ray-based models taking into account the fluid-like body of the euphausiid, elastic-shelled body of the gastropod, and the gas-bearing body of the siphonophore. From Stanton *et al.* (1998a).

from different parts of the body. Ray models have shown success in qualitatively describing the structure of the scattering (Figure 6.7). For a fluid-like animal, two or more rays are used. Near broadside incidence, two rays may suffice (upper left corner of Figure 6.7). Under these conditions, one ray would be from the front interface of the animal while the other ray would be from the back interface. However, far away from broadside, more rays are required to describe the scattering from other parts of the body (upper right corner, model not shown). For an elastic-shelled body, a single ray may dominate (middle right plot) for some orientations. Under these conditions, the ray would be from the front interface. For other orientations, two or more rays may be required to describe the circumferential (Lamb) waves and possibly a ray from inside the opercular opening of certain types of animals (two rays used in middle left plot). For the gas-bearing body, only one ray may be required when the

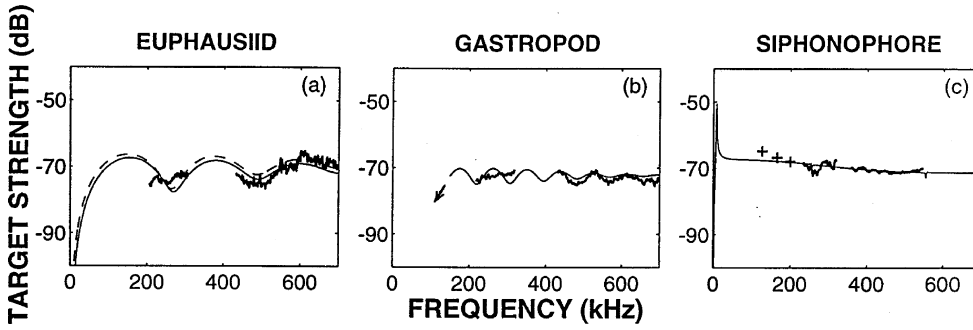


Fig. 6.8 Averaged echoes from the same or similar individual animals described in Fig. 6.7. The echoes were averaged over many pings over a wide range of orientation. The rough discontinuous lines and plus symbols are from live freshly caught animals and the continuous and dashed lines are from models. Two models are used for the euphausiid – a two-ray model and a DWBA-based model. The euphausiids were assumed to be smooth bent fluid cylinders in each model. The model used for the gastropod was a two-ray model taking into account the elastic properties of the shell (echo from the front interface and Lamb wave). Since this particular model is only valid for high frequencies, the line stops at about 200 kHz. The scattering levels are expected to drop rapidly below this frequency for this particular animal, as indicated by the arrow. The model used for the siphonophore involved the addition of two solutions: the modal-series-based solution for the gas and a two-ray model of fluid bent cylinders similar to the euphausiid ray model. From Stanton *et al.* (1998a).

scattering is dominated by the gas (lower left plot). Under other conditions when the tissue also contributes significantly, more rays may be required to account for the tissue (lower right plot, model not shown).

Average echoes Once echoes are averaged, much of the structure such as that shown in Figure 6.7 disappears and only a small amount remains (Figure 6.8). Because of the loss of structure in the averaging process, there is less importance in using a model that might predict the structure before averaging. This can be illustrated in the predictions for the euphausiid in Figure 6.8. Two different models were used, a two-ray model and one based on the DWBA. Each involves a smooth cylinder approach where the structure would not be predicted accurately, especially off broadside incidence. However, once averaged over a broad range of orientation angles, the inaccuracies are not important and there are reasonable comparisons with the data.

6.3.6 Echo abundance surveying of Antarctic krill

The echo integration method has been applied to Antarctic krill (*Euphausia superba*) in the Southern Ocean over a period of 20 years. The First International BIOMASS Experiment (FIBEX) was staged in 1981 in an attempt to acquire synoptic information about the distribution and abundance of the stock because of its key role in the Southern Ocean ecosystem (Everson 1977). Over the past decade a number of surveys have been performed, but with the intention of surveying stocks in rather limited geographical regions, for example, in the vicinity of Elephant Island (Hewitt and Demer 1994) and South Georgia (SC-CAMLR 1996, 1997).

The actual application of the method follows that described in section 6.3.2. Particular problems or challenges are (1) the frequent occurrence of the animal at shallow depths, requiring the use of an upwardly oriented transducer beam (Everson and Bone 1986), (2) separating the echoes of krill and salps (*Salpa thompsoni*), and (3) determining the target strength of the animal.

A new major echo abundance survey of *Euphausia superba* is being planned (SC-CAMLR 1997). The coordinating body is the Scientific Committee for the Conservation of Antarctic Marine Living Resources (CCAMLR 1993).

6.3.7 Target strength determination by caged-animal measurement: Antarctic krill

Caged-animal measurements have been employed for over two decades for the determination of target strength. There are many variants of these (Foote 1986). A particular approach that was applied to swimbladder-bearing fish in 1980 (Foote 1983) has been adapted for measurement of Antarctic krill (Foote *et al*. 1990).

Because of the weakness of individual krill as targets, an ensemble of krill was measured, and the echo energy was compared with the corresponding empty-cage value. At the same time, the range to the cage, with volume of 0.1 m^3, was chosen so that the cross section of the cage would lie well within the main lobe of the transducer, reducing effects of lateral movements of the krill on the echo strength.

The contribution of the cage to the observed krill echoes was removed in each of two ways, (1) a direct subtraction was performed in the energy domain, and (2) a least-mean-squares linear regression was performed of the echo energy on the number density of krill from a series of measurements of different ensembles. The empty-cage contribution was then inferred by extrapolation to vanishing density. The results are documented in Foote *et al*. (1990).

Support for the measurements was derived from computations based on the spherical model (Anderson 1950; Johnson 1977; Greenlaw 1979) using measurements of size, mass density, and sound speed (Foote 1990). Further support was derived by application of the deformed cylinder model (Stanton 1989) in Chu *et al*. (1993). By comparing simultaneous measurements at 38 and 120 kHz, the behavior as defined by the orientation distribution could be inferred.

6.3.8 Monitoring zooplankton with a fixed acoustic system

Diurnal migration is a major element in the behavior of zooplankton. It can be observed by an acoustic system fixed and in operation for a long period of time. The platform for this type of measurement could be a ship fixed at a station. If this approach is not practicable for the desired period, then various moorings could be used, such as a bottom-mounted system or a surface-moored system.

A fixed acoustic system was employed by researchers at the Bedford Institute of Oceanography, who mounted an acoustic Doppler current profiler on the seabed with the acoustic beam aiming in the upward direction for a period of 49 days (Cochrane *et al*. 1994). The echogram indicated distributions of sound scatterers periodically moving up and down the water column. Net samples showed fish and euphausiids occupying sections of the water column at different times of day. Estimates of abundance of euphausiids were made using cylinder-based scattering models.

6.3.9 Acoustic estimates of size distribution using a multi-frequency system

Holliday, Pieper, and colleagues developed and used a 21-frequency acoustic system for estimation of size distributions. The research was based, in part, on use of a modified version of Anderson's fluid sphere scattering model (Anderson 1950) and a least squares method (Holliday 1977). In practice, the method of non-negative least squares (NNLS) was used. The research is summarized in Holliday and Pieper (1995). The frequencies of the acoustic transducers spanned the range 100 kHz to 10 MHz, evenly spaced on a logarithmic scale. The frequency range was chosen so that zooplankton in the size range 0.1 mm to 10 mm could be sized. Because of the high frequencies and corresponding short detection ranges, the system needed to be cast. In addition to the acoustic transducers, there were other sensors to measure environmental parameters including temperature, conductivity, downwelling light, and fluorescence. Also, there were three pumps mounted on the system so that zooplankton could be pumped for later analysis and verification of the system.

The processed output of a single cast provided size distribution of zooplankton and properties of the water as a function of depth (Figure 6.9). Sizes were expressed in terms of the equivalent spherical radius, since the animals are being approximated as spherical. As a result of multiple casts in a region, contours of these parameters were constructed (Figure 6.10). As part of the analysis, the size distribution of the animals was converted into a measure of biomass density, specifically biovolume per unit cubic meter of water. The processed output therefore provided high-resolution size information on the animals coregistered with various properties of the water.

Sometimes, vastly simplified systems have also yielded useful numbers on size and numerical density. Mitson *et al.* (1996) are exemplary in this regard, basing their inferences on just two frequencies, 38 and 120 kHz.

Discrimination of zooplankton by multiple-frequency backscattering has also received much attention, as in the case of *Euphausia superba* and competing scatterers (Madureira *et al.* 1993a, 1993b). Again, just two frequencies have been employed, 38 and 120 kHz.

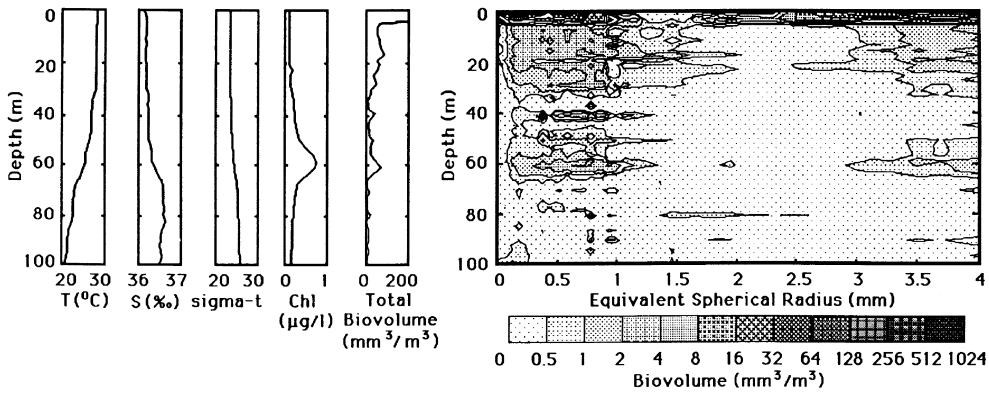


Fig. 6.9 Acoustic estimate of size distribution of zooplankton versus depth. Ancillary physical properties of water are also shown. Data collected from single cast. From Holliday *et al.* (1989, used with permission).

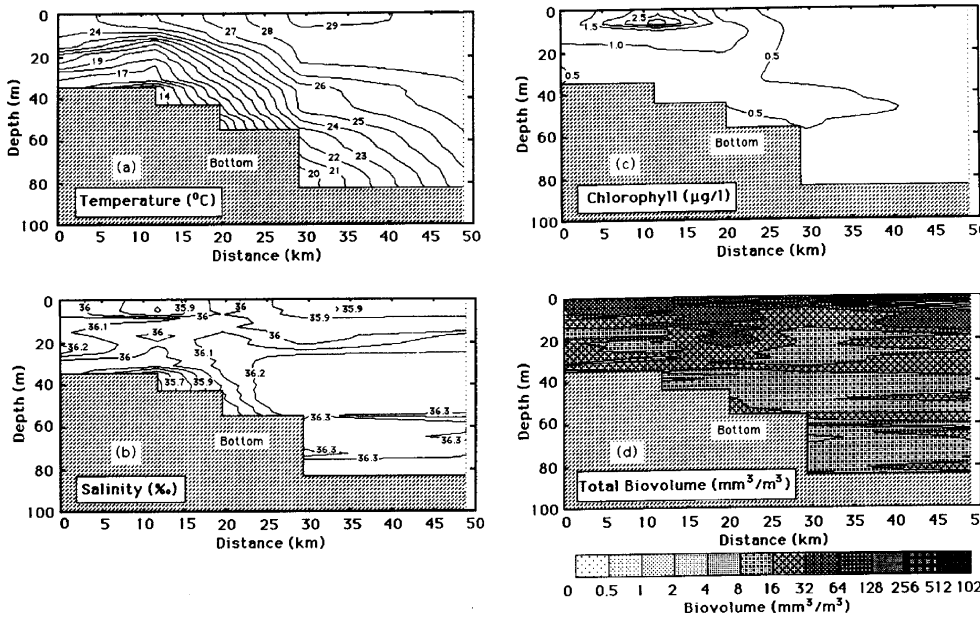


Fig. 6.10 Contours of acoustic estimates of zooplankton biomass as determined from data collected from multiple casts. From Holliday *et al.* (1989, used with permission).

6.4 ACKNOWLEDGMENTS

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7 Optical methods

K.G. Foote

7.1 INTRODUCTION

Optical methods have experienced an accelerated development over the past decade. In fact, when this manual was proposed (Anon. 1992), optical methods were not even included. They were incorporated the following year (Anon. 1993), with later specification (Anon. 1997).

Given the enormous progress that has been made in optical methods since 1992, much of which has been achieved by individual scientists or small groups of scientists, and which collectively span the range of development phases from the pre-prototype to the post-operational, the state of publication has been highly diverse. A series of journal papers has documented the design, development, testing, and applications of some systems; for some others, a single abstract has apparently constituted the entire publication record; in other cases, the publication record may consist of unpublished reports and manuscripts still in preparation.

The aim of this chapter is to describe those optical methods that are or could reasonably be made available to researchers. That is not to say that other methods might not contain the solution to particular problems, only that they are sufficiently specialized or relatively undocumented or unfinished to preclude immediate, wider use.

The primary methods considered here are light microscopy for the laboratory, silhouette photography, optical plankton counting, and video plankton recording as represented by four specific systems. Systems which are not discussed are: (1) generalized Schlieren systems (Strickler 1985; Schulze *et al.* 1992; Strickler and Hwang 1998), which are designed to observe small zooplankton including their prey and movements both *in situ* and *ex situ*; (2) Holocamera (Katz *et al.* 1999), which is a new submersible holographic camera still under development; (3) Mixed Light Imaging System (Widder 1992), which enables both bioluminescence and the light-emitting organism to be imaged simultaneously; and (4) Polarized Larval Recorder (Gallager *et al.* 1996b), which exploits the presence of birefringent crystals in some organisms, for example, the aragonitic structure in the shell of larval mollusks (Gallager *et al.* 1989).

To promote understanding of the methods described, as well as to provide technical background for understanding other methods, basic principles are presented in the first part (section 7.2) of this chapter. This is done through a discussion of phenomenology. Sources of variability are also discussed. Following presentation of the methods themselves, comparative issues are addressed.

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In the second part of this chapter (section 7.3), a specific measurement protocol is presented for the practice of silhouette photography in the laboratory. Measurements with both the optical plankton counter and video plankton recorder are illustrated through treatment of the following four topics: calibration with comparisons, limitations, operating scenario, and applications.

7.2 GENERAL DISCUSSION OF PRINCIPLES, TECHNIQUES, AND COMPARATIVE APPROACHES

7.2.1 Review

Scientific sampling of life in the ocean has a long history, as Hardy (1956) observes. Examples of net tows by the naturalist J Vaughan Thompson in the sea off Cork in 1828 and by Charles Darwin during the voyage of the 'Beagle' are cited.

As recently as 1984, when listing sampling devices for zooplankton, Yentsch and Yentsch (1984) could not name a single optical device, which reflects the state of development at that time. Five years later there was general recognition of the need for optical instruments to observe zooplankton *in situ* in studies of behavior, distribution, and abundance (Paffenhöfer *et al.* 1989). At that time, a Schlieren-based system (Strickler 1985), the optical particle counter (Herman 1988), and nascent applications of video technology could be cited.

Further impetus for developing optical methods was provided by the Global Ocean Ecosystem Dynamics (GLOBEC) program (US GLOBEC 1991a, 1991b, 1993; GLOBEC 1993). Included in these four GLOBEC references are reviews of optical, and integrated acoustical and optical technologies for application to zooplankton and other aquatic organisms.

The only other relatively recent reviews of optical methods for application to zooplankton seem to be those by Schulze *et al.* (1992) and Sprules *et al.* (1992). These address the respective technologies of video and non-video optical systems.

That there has been enormous activity since 1993 in the development and application of optical instruments to observe zooplankton both *in situ* and *ex situ* is evident from the literature, which is spread over many journals. It is an aim to draw together some of this literature to describe systems that are or have been operational, and that are or might be commercially produced for general use.

Inevitably some methods are neglected. The most accessible techniques, however, are discussed, namely light microscopy, silhouette photography, optical plankton counting, and video plankton recording. In the case of light microscopy, a range of microscope classes is described to indicate some powerful alternatives for laboratory examination of very small organisms.

Topics addressed in advance of the techniques are basic principles, including light phenomenology, illumination, water as an optical medium, light detection, magnification and resolution; and sources of variability, as due to effects of instrumentation, water medium, and animal properties and numerical density. Connections of the techniques are summarized after their review under the topic of comparisons, which also includes a review of characteristics of the Optical Plankton Counter and Video Plankton Recorder.

7.2.2 Basic principles

In order to be able to view a zooplankton specimen, there must be a visible optical contrast between it and the surrounding medium, i.e., sufficient light illuminating the specimen and a means of detecting light from the specimen. The surrounding medium must be free of extraneous matter so that its scattered light does not obscure the primary object. The imaging system itself must possess sufficient magnification to resolve the specimen and show fine details.

Already, even in a simple introductory paragraph, a number of concepts have been mentioned that may appear vague and have multiple definitions. To remove ambiguity from later discussion, some basic phenomenological elements of light are reviewed. The subjects of illumination, medium, and detection are treated separately, as are the topics of magnification and resolution. Two classic references on the basic principles are Jenkins and White (1957) and Born and Wolf (1970).

LIGHT PHENOMENA

Light

Light is an electromagnetic vibration whose frequency lies in the optical portion of the spectrum, with wavelength in the approximate range 400–700 nm, from blue to red. The ultraviolet range is 200–400 nm, and the near-infrared range is 700–1000 nm.

Wave-particle duality

Light may act as a wave under some circumstances and as a particle under others. When passing through an aperture, for example, light bends or diffracts as other waves do. When exchanging energy with matter, as in exciting electrons in silicon, light acts as a particle. This dual nature may appear contradictory at times, but it vastly simplifies conceptualization of diverse optical phenomena, such as those discussed below.

Light field

As an electromagnetic vibration, light has magnitude and direction. If coherent, it also has the property of phase; otherwise, incoherent light is composed of a myriad of waves of different phase, rendering the concept of phase meaningless. Light fields obey the principle of superposition: the result of interference is just the sum of the interfering waves, which can pass through each other in space without changing form.

Speed of light, refractive index, translucence, opacity

The speed of light *in vacuo* is 299 700 km s^{-1}, in other media it is less. In addition, light is generally absorbed. The real part of the refractive index describes the ratio of the speed of light *in vacuo* to that in the subject medium. The imaginary part describes the rate of absorption. If small, light is attenuated only slowly by absorption; this characterizes the state called translucence. If large, light is rapidly attenuated by absorption, describing the quality of opacity. The refractive index of air under standard conditions of temperature and pressure is 1.0003. The frequency dependence of the refractive index is called dispersion.

Scattering

Light that strikes a body extraneous to the medium will undergo some change. In certain very simple and special cases, this may result in either a slight retardation or advancement in its passage through the medium. In some other special cases, the light may be totally reflected or totally absorbed. In general, the redistribution or redirection of energy that occurs is called scattering. It is often called by the special names of reflection,

refraction, and diffraction, but the general name is truly encompassing, with the advantage of emphasizing the integrated nature of the phenomenon. The analytical approach of dividing the phenomenon into pieces often leads to a quagmire, while the integrated approach may point to the computationally easier and more general solution, namely integration over boundary surfaces in the case of homogeneous bodies or throughout bounded volumes in the case of heterogeneous bodies.

Reflection and refraction

Usually, reflection and refraction refer to particular scattering phenomena. Reflection refers to scattering in the medium of the incident wave when the wavelength is small compared to the characteristic radius of curvature and illuminated dimensions of the scattering body. Refraction refers to the bending of light when passing through the surface of a relatively large body whose radius of curvature is large compared to the wavelength.

Extinction

That which removes energy from a forward-propagating wave may be called extinction. It thus has two components: internal absorption and scattering.

Bioluminescence

Many organisms emit light either with or without stimulation. Both the emitted light and the process of light emission are called bioluminescence (Losee *et al.* 1985; Herring 1990).

Fluorescence

Certain materials can be excited by light of a particular spectral composition. The exciting light elevates electrons in the atomic or molecular structure, which then fall to a lower level that is still higher than the original state. The wavelength of emitted light is thus longer than that of the exciting light. This process and the emitted light are both termed fluorescence.

Polarization

Light is an electromagnetic vibration in which the constituent electric and magnetic field components vibrate in a plane transverse to the direction of propagation. If the direction of the electric field component of a unidirectional light field is constant, then the light is said to be polarized. Polarization can be induced by passing light through a grating or transparent material on which narrow parallel lines are scored.

Birefringence

Certain materials have two refractive indices, one for each state of polarization. Light falling on birefringent crystals will thus divide into two internal fields, each bending according to the respective index of refraction.

ILLUMINATION

There is a surprising range of light sources beyond that of natural light. Certainly for imaging very small objects or registering very faint objects, a strong controllable source of light is needed. Some of the basic sources of artificial light are mentioned here. Technical details can be found in Bass *et al.* (1995).

Tungsten-filament lamp

Passage of an electric current through a filament of tungsten excites electrons in the outer shell of the tungsten atom. Their return to more stable energy levels is accompanied by the emission of light. If the filament is sealed in a tube with an inert-gas atmosphere, the

constructed lamp becomes durable, hence stable at the same time. An advantageous characteristic of the lamp is that its spectrum is broadband, covering the same region as that of the sun. A major application is in light microscopes, described in section 7.2.4.

Gas-filled discharge tube

If a capacitor is discharged in a gas-filled tube, the rapid recombination of electrons and gas atoms generates a flash of light. The gas composition and voltage magnitude affect the spectral composition of the flash. The tube is frequently used in photography, for example, in an electronic stroboscope.

Laser

Certain media are capable of storing energy in a form which, when released, yields coherent optical radiation. Electronic stimulation of such media, which encompass gases, liquids, and solids, effects amplification, hence the acronym for Light Amplification by the Stimulated Emission of Radiation. The property of coherence is crucial in many applications: the emitted radiation is concentrated over an extremely narrow spectral bandwidth with very high intensity.

Light-emitting diode

The light-emitting diode (LED) is a semiconductor device in which electronic excitation is accompanied by the emission of light. The fact that this is a solid-state device with electronic control ensures repeatability in performance over a wide range of operating conditions. LEDs are used in the optical plankton counter, described in section 7.2.5.

WATER AS AN OPTICAL MEDIUM

The characteristics of optical media are divided into two classes: intrinsic and apparent. The three intrinsic properties are those of absorption, scattering coefficients of radiation, and volume scattering function. Apparent properties of optical media are derived from these and include, for example, the attenuation coefficient of radiation, transmittance, optical thickness, color index, color grade, and Secchi depth, i.e. the greatest depth at which a standard white disk can be seen.

The optical properties themselves are determined largely by three optically active components (Shifrin 1988).

1) Pure water The absorption spectrum is characterized by a minimum at 475 nm, accounting for the blue color of water.
2) Dissolved substances Dissolved oxygen absorbs far-ultraviolet radiation, and dissolved nitrates absorb ultraviolet radiation. 'Yellow substance' or 'Gelbstoff' is organic matter that absorbs blue light, 435–480 nm, giving rise to the complementary yellow color in the band 580–595 nm.
3) Suspended matter Particulates have a profound effect on the optical properties, as the overall size spectrum 10 nm to 50 μm spans the scattering regime from Rayleigh to geometric, with preferential scattering of blue light by the smallest particles and with broadband or white-light scattering by the largest particles. Phytoplankton contribute particles in the size range 1–50 μm.

Variability in the optical properties of naturally occurring bodies of water is associated with spatial and temporal differences in suspended matter. A simple source of such variation is phytoplankton, the degree of patchiness of which is typically so large that the optical plankton counter, described in section 7.2.5, uses a feedback loop to compensate for spatial fluctuations in ambient attenuance.

LIGHT DETECTION

In addition to the human eye, there are four types of sensors for detecting and also registering light, which are briefly described here. General reference is made to Bass *et al.* (1995) for technical details.

Photographic film

Special crystals, or grains, of silver halides are suspended in an emulsion of gelatin and coated on a substrate. When exposed to light, the crystals form what are technically called latent-image specks. When developed, those grains that are sufficiently exposed are reduced to metallic silver. Under fixation, remaining light-sensitive grains are washed away, yielding a permanent image. Two significant facts about photographic film are that it is very sensitive to light and its resolution is very high due to the small grain size, typically 0.1–3 μm.

Silicon-based image sensors

One of the properties of silicon is that a single photon may liberate an electron. The resulting imbalance in charge is accompanied by creation of a positively charged cavity or hole. The electron-hole pair may be registered, hence counted. At least four distinct silicon-based devices exploit this process of photoconversion, two significant characteristics of which are spectral sensitivity and quantum efficiency.

Charge-coupled device (CCD)

Individual silicon-based image sensors may be configured in a two-dimensional array. Charge is collected at the individual elements and converted to a voltage, which can be registered or 'read' through a scanning operation. The overall result is a two-dimensional image expressed in digital electronic form.

Image intensification

Under marginal conditions of detection, with either very low light levels or very brief exposure time, the amount of light falling on a CCD array or other self-scanned array is insufficient to provide good contrast. The number of photons is simply too low. This situation can be remedied by insertion of an electron multiplier at the output of each array element to amplify the signal level independently of the others. When configured in an array corresponding to that of a self-scanned array, the device is called an image intensifier (II).

MAGNIFICATION AND RESOLUTION

There are two measures of magnification (Jenkins and White 1957). The angular magnification is the ratio of the angles subtended by the image and object at the viewing distance, and the lateral magnification is the ratio of corresponding dimensions of image and object in the image plane, namely that normal to the optical axis. If unstated, magnification refers to the lateral magnification. In the simplest compound microscope, the overall magnification is the product of the magnifications of the two lenses, namely the objective and the eyepiece.

Resolution, or the capacity of an optical system to distinguish nearby points, is characterized by the least distance required to distinguish the points. If light from a single illumination point or very small facet were observed through a microscope, a pattern of alternating dark and light rings about a central bright spot would be observed. Mathematically, the pattern is described by a Bessel function of order one. The usual criterion for separation is that the central maximum due to one point falls at the first

minimum due to the second point, and vice versa. This Rayleigh criterion can be expressed as follows (Born and Wolf 1970):

$$\Delta s = 0.61\lambda/(n \sin \theta), \tag{7.1}$$

where Δs is the minimum separation distance, λ is the wavelength of light, n is the refractive index of the medium between object and lens, and θ is one-half the angular aperture in the object space. This formula applies to incoherent illumination and imaging by a circular object with matched objective and condenser lenses, as described in section 7.2.4. If the light in the same configuration is coherent, then the same formula applies but with the numerical factor replaced by 0.77 (Born and Wolf 1970). The term $n \sin \theta$ is so important that it is called the numerical aperture.

In the more general case that the objective and condenser lenses are not matched, then the formula for Δs becomes the following (Kapitza 1994):

$$\Delta s = 1.22\lambda/(NA_{\mathrm{obj}} + NA_{\mathrm{cond}}) \tag{7.2}$$

where NA is the numerical aperture of the corresponding lens.

7.2.3 Sources of variability

It is clear from the foregoing section that there are a number of sources of variability in optical observations. An attempt is made here to describe some of the principal effects.

INSTRUMENT EFFECTS

The possibility of making a measurement is defined by the basic properties of the instrument, which include resolution, sharpness and depth of focus, and sampling volume. The fact that the fundamental resolution is wavelength-dependent means that changes in illumination can affect the level of resolution. Changes in background, such as lighting, can affect the contrast, hence resolution too. This is also affected by the sizes of lens and apertures.

In so far as sharpness and depth of focus are related to the resolution, sampling volume also varies with resolution. Under good conditions, these factors may be relatively insensitive to small changes. Under marginal conditions, however, the small changes may be decisive in making a detection, where an identification is impossible.

In cases of high particle density, the number or rate of registration may vary simply because individual zooplankton are not always resolved as single objects and because of statistical fluctuations in the number of animals in the sampling volume. Both the optical plankton counter and video plankton recorder, described in section 7.2.5, are affected by similar factors. The possibility of obscuration of the illumination and scattered light by particles outside the sampling volume is a complicating factor.

WATER MEDIUM

The water medium itself is variable, as due, for example, to spatial fluctuations in plankton distribution. Optical properties such as attenuance are directly affected, which may in turn affect optical measurements of zooplankton *in situ*. This is the case with the optical plankton counter. Changes in the content and concentration of other suspended matter may also be a source of variability. The surface zone when disturbed by breaking waves contains variable concentrations of air bubbles, with an overall distribution pattern determined by Langmuir cells.

ANIMAL-DEPENDENT EFFECTS

Observation of single animals may be affected by such basic properties as pigmentation and degree of translucence or opacity. Pigmentation varies with individual and also changes with time, for example, the reddish copepod *Calanus finmarchicus* and its seasonal variations. The same is true of euphausiids, for example, *Euphausia superba*, whose pigmentation varies over quite short time periods, of the order of days, due to feeding on phytoplankton. Detection of single animals often depends on contrast, and the variable nature of translucence or opacity must affect optical registration.

The optical density of animal aggregations also depends on their translucence or opacity. Extremes of numerical density and translucence may suggest the effect of individual animal properties: a low numerical density of opaque organisms may be equivalent to a high numerical density of relatively translucent organisms. Alternatively, the path length for attenuation by absorption of light in an aggregation of translucent organisms may be a small fraction of that for opaque organisms in a similarly dense aggregation of the same overall size. Observation based on light attenuance, for example, with the optical plankton counter, will vary with animal translucence, everything else being constant.

7.2.4 Classes of light microscopy

Techniques of light microscopy fall into several categories. For instance, the techniques may be distinguished by the place of application: *ex situ*, as in the laboratory, or *in situ*, in the sea. Alternatively, the techniques may be distinguished by the aim of the observation, for example, imaging or quantification. Clearly some techniques can be used in both places or to fulfill both aims. Indeed, this is precisely the case of light microscopy, for this originated in the laboratory, where it is most widely used in zooplankton research, both for visualization and quantification. Similar microscopes have also been translated to the water column through ingenious adaptations.

In this section some basic techniques of light microscopy for the laboratory are described. In the next section, 7.2.5, specific adaptations for use in the laboratory and/or *in situ* are described.

IMAGING

In general, a microscope is an optical system that forms an image that is large compared to the original object and which itself can be viewed as an object (Rayton and Benford 1961). The simple microscope affords an immediate example. Its single lens may be used as a magnifying glass, reading glass, or dissecting microscope.

Multiple-lens systems define the compound microscope. Two lenses are essential, these are the objective, which has a short focus and is placed near the object to be magnified, and the ocular or eyepiece, which has a longer focus. The objective forms a real, magnified, intermediate image of the object. The ocular appears to be imaging the object at its focus, called the virtual image, but allows the light to be focused on the retina, where the final, real image is formed.

Microscopes may be binocular, in which case there are two eyepieces but a single objective. Interception of the light from the objective by a prism can split the light into equal halves.

Microscopes may be stereoscopic, in which case there is a double objective, with separate light paths to the respective eyepiece, or single objective but with oppositely polarized light beams.

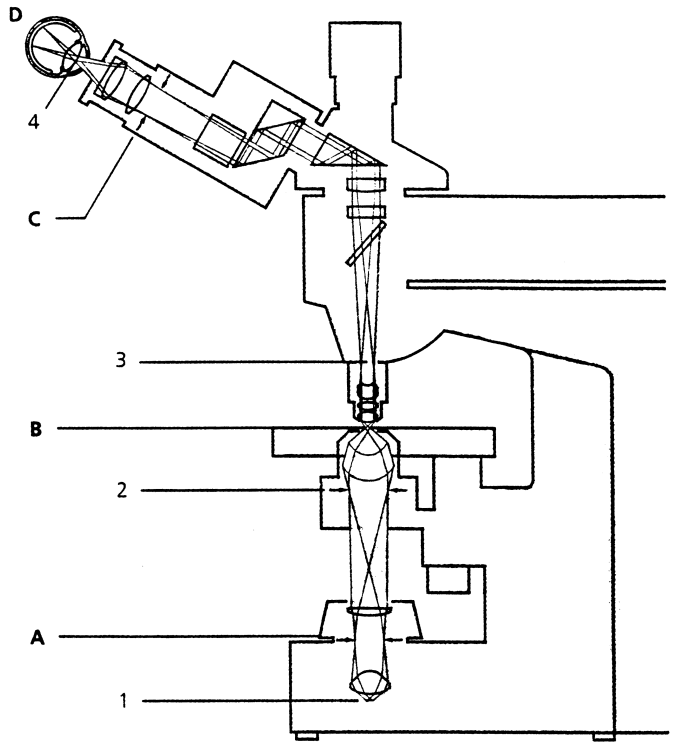

Fig. 7.1. Transmitted-light microscope in cross section (Kaptiza 1994). Used with permission.

No matter what the arrangement of lenses is, without proper lighting, imaging an arbitrary object may be futile. Attention to illumination and contrast is thus an essential part of microscope selection or technique, and is a recurrent theme in the following sections.

BRIGHT-FIELD MICROSCOPY

Both the object and its direct illumination are viewed together, which is suitable for opaque or highly absorbent objects. A transmitted-light microscope is shown in cross section in Figure 7.1 (Kapitza 1994). The illumination originating in the filament (1) is intercepted by the luminous field diaphragm at (A) and again by the aperture diaphragm (2), yielding a more or less constant illumination over the specimen plane (B). The aperture diaphragm is imaged on the objective pupil at (3). The intermediate image in the eyepiece (C) is focused by the eyepiece and pupil of the observer's eye (4) onto the retina (D).

Another major form of bright-field microscopy is achieved by the reflected-light microscope. This differs from the transmitted-light microscope in introducing the illumination to the object by the main path between objective and ocular by means of a beam splitter. The specimen is thus illuminated by light passing through the objective, and both image and illumination pass through the objective to the eyepiece.

CONTRAST TECHNIQUES

Translucent specimens have prompted the development of so-called contrast techniques.

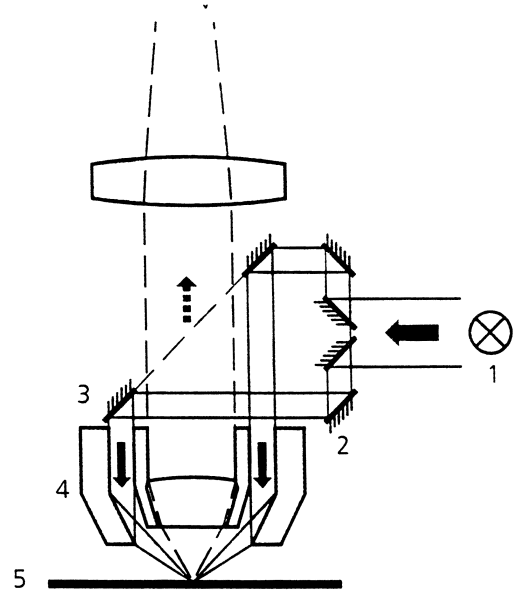

Fig. 7.2. Dark field in reflected light (Kapitza 1994). Used with permission.

These are especially important in zooplankton research because of the prevalence of translucence. *Ex situ* staining can be employed, but this is hardly a general method. In the following sections four non-staining contrast techniques are described.

Dark field

Oblique illumination can provide enough light for viewing some specimens, while leaving the background dark. An example of dark field is shown in Figure 7.2 (Kapitza 1994), where the illumination is achieved by reflected light. To prevent the source light from entering the objective–ocular path, the light from the source (1) is introduced by a mirror step assembly (2) and mirror with oval hole (3). The light that passes the outer sleeve of the objective reflects off a ring-shaped concave mirror (4), which directs the light onto the specimen plane (5), achieving oblique illumination. Dark field in transmitted light is achieved by using an annular aperture in the condenser, which then casts a hollow cone of light onto the specimen. The direct illumination can be achieved by placing the objective within the cone of transmitted light or by employing a second diaphragm after the objective.

Phase contrast

For essentially transparent specimens, the presence of matter will generally retard or advance the propagation of light in the specimen relative to that in the surrounding medium. If the specimen is observed *in vacuo*, then the light in the specimen will be retarded. This is usually the case for air too because of the nearness of the index of refraction to unity. The retardation, or advancement, can be measured by a change in optical path length. This property is exploited in the phase-contrast microscope, illustrated in Figure 7.3 (Kapitza 1994) for transmitted light. Use of an annular stop (1) in the condenser ensures dark-field conditions. Insertion of a so-called phase ring (5) after the objective increases the optical path of the transmitted light, while strongly attenuating this by absorption. Interference with obliquely scattered light in path (7) can

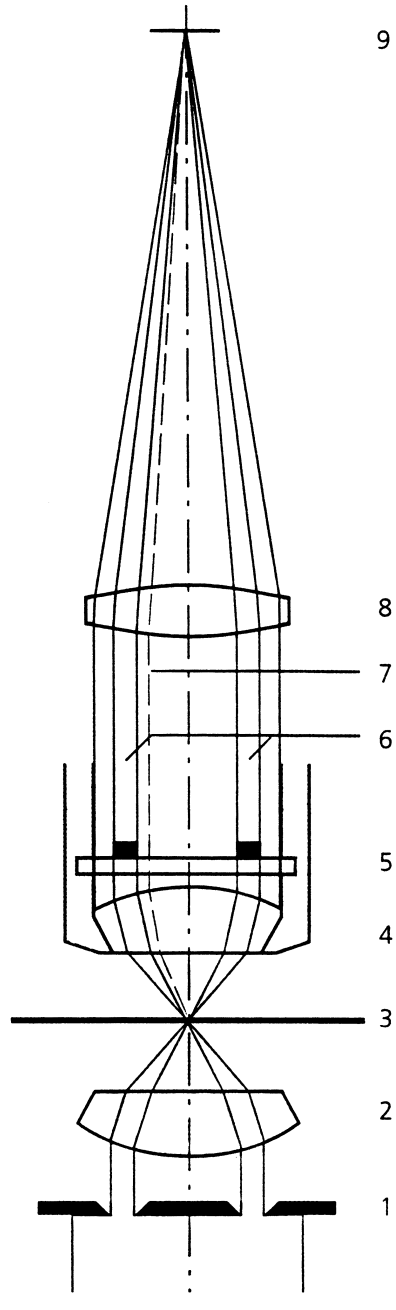

Fig. 7.3. Phase contrast in transmitted light (Kapitza 1994). Used with permission.

create the light and dark patterns characteristic of constructive and destructive interference, respectively.

Polarization contrast

Another form of contrast is based on sensing light that is polarized by the specimen. To distinguish this, incident light is polarized before the condenser, and light from the

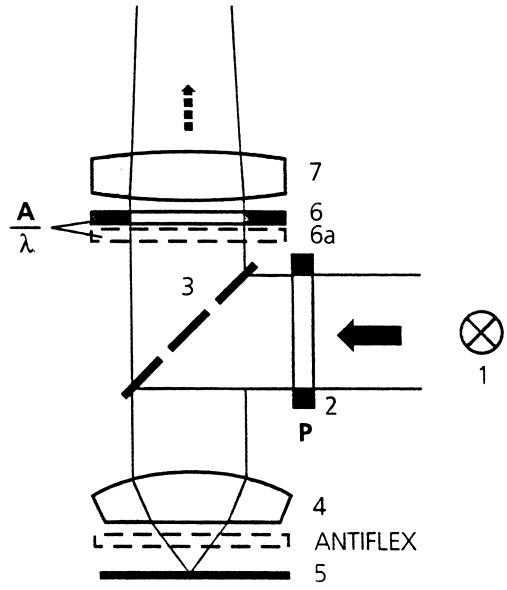

Fig. 7.4. Polarization contrast in reflected light (Kapitza 1994). Used with permission.

specimen with the same polarization as the incident light is blocked. This is typically achieved by linear polarization filters, described in section 7.2.2. In the case of polarization contrast in reflected light, illustrated schematically in Figure 7.4 (Kapitza 1994), the illuminating light is polarized at (2), split at (3), focused at (4) onto the specimen plane (5). The backscattered light that passes through the splitter is polarized by a second filter (6) with polarizing direction perpendicular to the first. Thus only light whose polarization direction is changed by the specimen reaches the tube lens (7), hence explaining the name given to the polarizer (6), the analyzer. To augment the image contrast further, a so-called lambda plate (6a) is sometimes inserted in the path before the second polarizer. Because of interference of multiple surface reflections in the lambda plate, the gray contrast becomes a color contrast by simple selection, as in Newton's rings. The thickness of the lambda plate is typically a multiple quarter wavelength of the dominant image color.

Differential interference contrast (DIC)

Another contrasting method is based on separation of the incident light into two components by a birefringent prism and recombination of specimen-scattered light by a similar birefringent prism. If polarizing filters are used as in the polarization-contrast microscope, then only relative changes in the polarization states of the incident light by the sample will be rendered visible. The technique is illustrated by DIC in transmitted light in Figure 7.5 (Kapitza 1994). The first birefringent prism (2) is placed after the polarizer (1) and before the condenser lens (3). The second birefringent prism (6) is placed after the objective (5) and before the analyzer (7). If a lambda plate (7a) is used, the gray-contrast image is converted to a color-contrast image.

FLUORESCENCE MICROSCOPY

Fluorescence in the specimen may be exploited by exciting this through the illumination

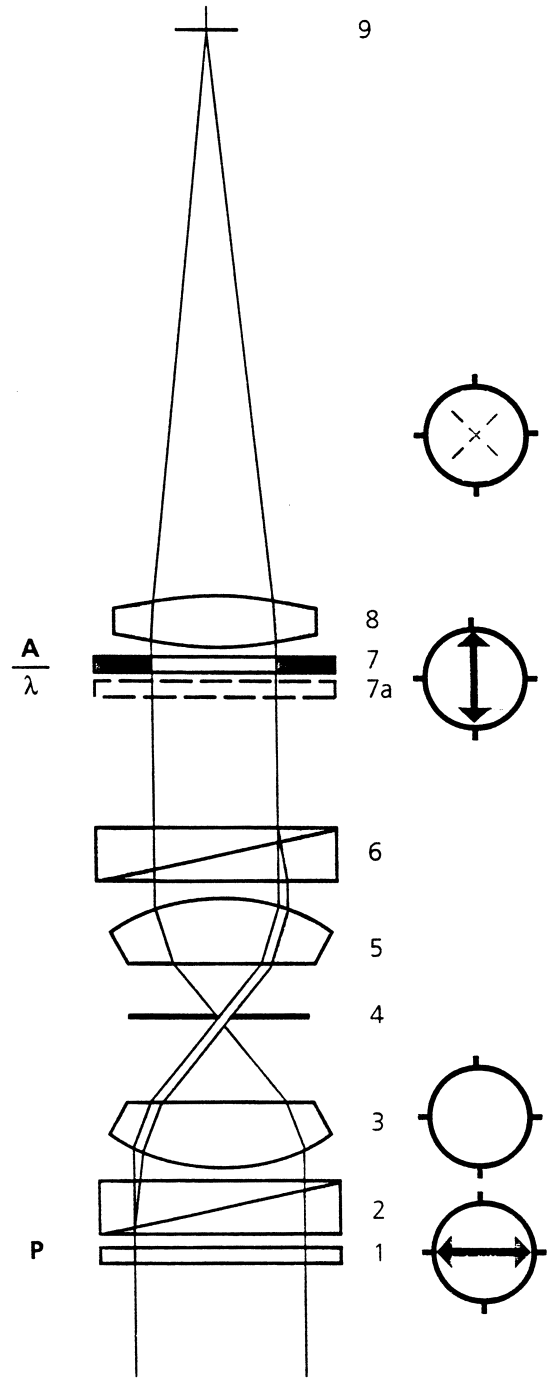

Fig. 7.5. Differential interference contrast in transmitted light (Kapitza 1994). Used with permission.

and detecting the emitted longer-wavelength light. To achieve this in a laboratory microscope, the illuminating source must contain the exciting spectral components but not components in the emission band. Following illumination of the specimen, residual source light must be blocked and the emitted light sought with a bandpass filter. The wavelength change in fluorescence is typically 3%–8% or 20–50 nm, requiring a fine filter tolerance. The need for intense exciting light must be countered by proper heat-shielding to protect the microscope. The technique is described in detail by Kapitza (1994).

QUANTIFICATION

Photomicroscopy and videomicroscopy

At least two major purposes are served by coupling a still camera or video camera to a microscope: a permanent image can be derived that is suitable for both archiving and measurement. A typical application is in morphometry of zooplankton specimens (Foote 1998a, 1998b), as for use in scattering models which require information on shape and structure. Inspection of successive frames of a video film may reveal important details of movement that may also be quantified (Kils 1992). Another form of quantification is enumeration of specimens by species. An application of this, namely silhouette photography, is discussed in sections 7.2.5 and 7.3.1.

Manual digitization is a preferred technique for reducing permanent images to numbers, especially when judgments must be made about the observed structure. Automatic recognition of features presented on digitally entered or scanned images can also effect quantification in some cases.

A technical matter that may have to be addressed in photomicroscopy or videomicroscopy is that of access to a proper intermediate image. Provision is generally made for such capture, often permitting simultaneous viewing of the object or its image.

Attenuance measurement

Light blockage by intercepting particles can also be registered. If the numerical density of sensed matter, such as an assemblage of zooplankton, is sufficiently low, then the light attenuance will directly yield a cumulative measure of quantity. A well-known device that exploits attenuance measurement is the optical plankton counter, treated in section 7.2.5.

7.2.5 Techniques

Three major operational techniques are reviewed in this section, silhouette photography, optical plankton counting, and video plankton recording. All three techniques are relatively advanced, with the second and third being steadily improved.

SILHOUETTE PHOTOGRAPHY

Zooplankton specimens are recorded as a shadowgraph on photographic film exposed by a flash of light. Details of the technique are given for use in the laboratory and *in situ*.

Laboratory application

Zooplankton are introduced in the dark into a shallow bath covering a sheet of positive, fine-grained film, the film is exposed by a flash light. The developed film reveals the outlines of the individual specimens. These can be classified and counted under a microscope, and their images can be digitized.

In the first documented study, Ortner *et al.* (1979) applied the technique to live specimens of zooplankton collected in the Sargasso Sea in February 1978. The bath was filled to 1 cm, distance to camera was 1 m, and flash duration was 3 μs.

In an application to zooplankton collected at the center of a warm-core ring of the Gulf Stream in spring 1982, Davis and Wiebe (1985) give many useful details on the technique. Some of these are included in the detailed description in section 7.3.1.

In situ application

Silhouette photography is also applied *in situ*, but generally using some form of concentration to increase the probability of capturing zooplankton on film. In the original system developed by Ortner *et al.* (1981), the concentrator was a funnel-shaped net without a cod-end. The camera was placed where the cod-end would have been with a nearby strobe.

In the first application (Ortner *et al.* 1979), the pulse duration was 0.5 μs, pulse period 3 s, and the sampled water volume was 0.26 l. In an early commercial version, the pulse period could be adjusted over the range 2–10 s. The film capacity was 800–1000 frames.

The technique has also been applied to fish eggs and larvae, as in the study by Houde *et al.* (1989). Recognized advantages are avoidance of shrinkage of eggs and the possibility of staging eggs. Disadvantages are the small size of the sampling volume and difficulty in distinguishing many species of eggs and larvae when the taxonomic diversity is high. Independent capture using a plankton net permitted comparison of numerical estimates of egg density.

In another, large-scale study, the results of *in situ* silhouette photography were compared against paired tunnel-net samples (Olney and Houde 1993). It was possible to classify animals according to genus or species in 55% of the cases. A number of rare or uncommon forms of zooplankton were observed, but others were missed, probably due to poor photographic qualities of the taxa or stalling of plankton along the walls of the camera net.

OPTICAL PLANKTON COUNTING

Optical plankton counter

The optical plankton counter (OPC) is a self-contained, operational system that is being widely used to quantify a range of mesozooplankton and macrozooplankton with characteristic sizes in the range 0.25–20 mm (Herman 1992). Not only is the device used as a primary instrument, but it is also used as an auxiliary instrument on multiple-sensor arrays.

Effective design aims of the OPC are to

1) determine numerical density, biomass, and distribution of target organisms by the automatic counting system
2) resolve sizes corresponding to different development stages, for example, later stages of *Calanus finmarchicus*
3) resolve detected zooplankton at distance spanning the range from millimeters to kilometers
4) assemble a system that can be towed up to 4 m s^{-1} at depths as great as 1000 m.

The principle of operation is based on light blockage. Because of natural variability in plankton-bearing water as an optical medium, the receiver output is maintained at a constant level by means of an electronic feedback loop. At a high towing speed, the ambient attenuance is tracked with a time constant of the order of seconds to minutes.

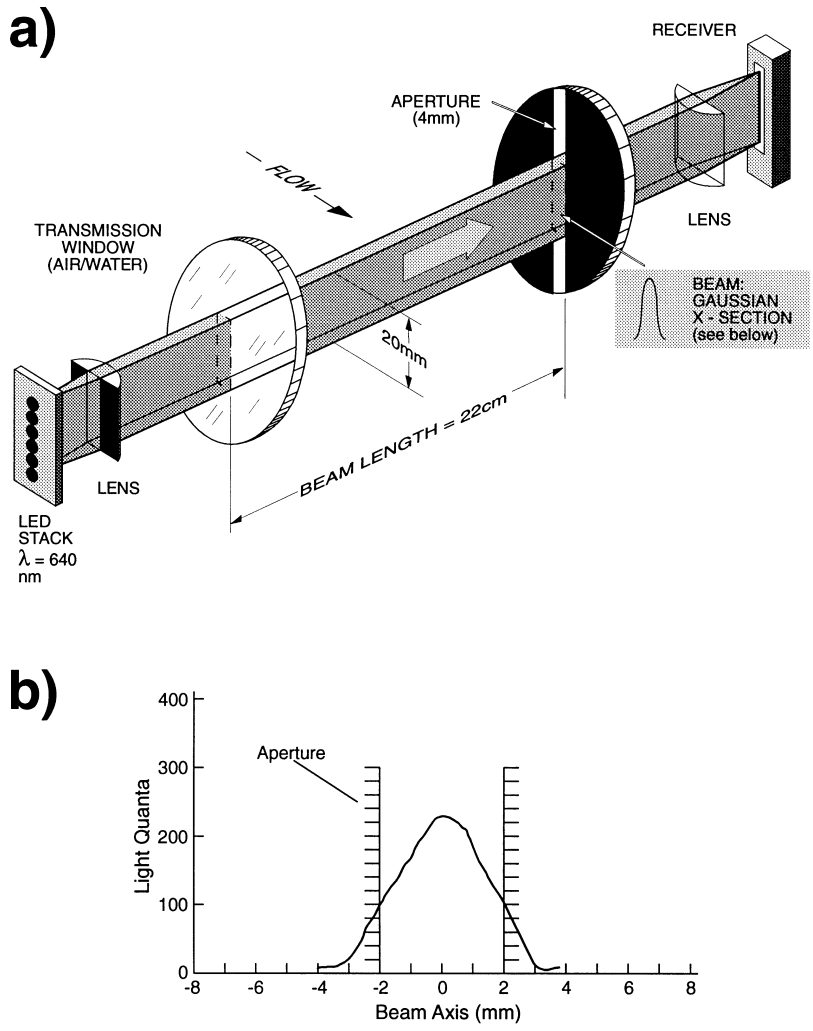

Fig. 7.6. Optical plankton counter: schematic diagram showing (a) basic design and (b) light beam cross section. Adapted from Herman (1992) by AW Herman. Used with permission.

The source intensity is varied on a similar time scale to maintain constant receiver output. When a particle passes through the sensing volume, the amount of light that falls on the detector drops, triggering a pulse-like drop in the receiver output. By AC-coupling this can be separated and counted.

In the first design, the source consisted of a bar-shaped light-emitting diode with operating wavelength 640 nm (Herman 1988). In the second design, shown in Figure 7.6, the source consisted of six LEDs at the same 640-nm wavelength (Herman 1992). Use of a cylindrical lens concentrates the otherwise diverging light in a beam that is roughly uniform over the rectangular cross section of the sensing volume. A second cylindrical lens focuses the light onto a photodiode detector. Use of rectangular apertures in the second design has rendered the beam Gaussian in shape, with enhanced detection in the central region and avoidance of signals in weak-field parts of the beam cross section.

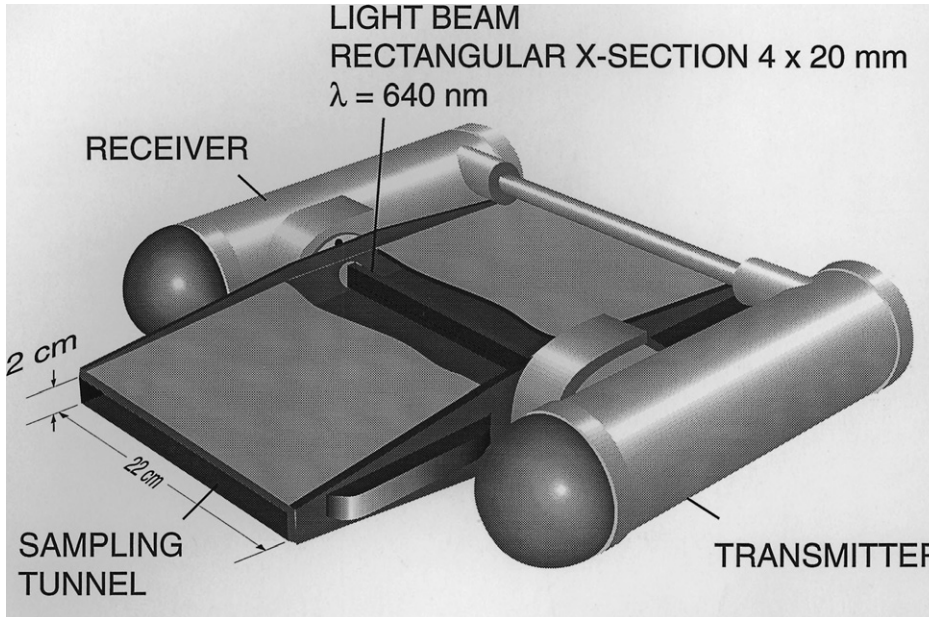

Fig. 7.7. Optical plankton counter: submersible version showing housing with sampling tunnel. Adapted from Herman (1988) by AW Herman. Used with permission.

Detection in the OPC occurs in a tunnel, indicated in Figure 7.7, that helps ensure uniform flow across the beam cross section. For the particular design parameters, it is often difficult to resolve and count individual zooplankton in fresh-water bodies such as lakes. In such cases, the sampling volume may be reduced by insertion of a block of absorptive material in the tunnel.

Continuous, underway fish egg sampler

The continuous, underway fish egg sampler (CUFES) is an operational system that pumps surface water from a depth of 6 m on board, concentrates organisms, and passes these through an on-board and on-line OPC for automatic sizing and counting (Checkley *et al*. 1997). A principal advantage is that physical samples can be taken for comparison with the OPC, as for calibration. This subject is discussed separately in section 7.3.2.

VIDEO PLANKTON RECORDING

Videomicroscopy is a major topic of relevance far beyond its application in zooplankton research, as shown in such texts as Inoue (1986) and Sluder and Wolf (1998). Four adaptations of videomicroscopy for the *in situ* observation of zooplankton are described.

Video plankton recorder

The video plankton recorder (VPR) is an operational system that allows both classification and enumeration of zooplankton on distance scales from millimeters to kilometers (Davis *et al*. 1996). A high degree of automation has been achieved in a system based on the following major components: video system to collect high-quality images of under-

water plankton, image processing and plankton registration system, and data processing and visualization system.

The design aims described by Davis *et al.* (1992a) are

1) automatic and continuous data collection over distance scales from millimeters to kilometers, with electronic storage of data
2) resolution sufficient for classification of individual particles by major group, for example, amphipods, copepods, chaetognaths, detritus including marine snow, euphausiids, fish eggs, ichthyoplankton, pteropods, *inter alia*
3) physical capture of actual particles for calibration of the electronic data
4) deployment by towing at speeds up to $5\,\mathrm{m\,s^{-1}}$
5) taxonomic analysis in near-real time.

Admittedly, the third aim was abandoned at an early stage (Davis *et al.* 1992b), when this was found to induce avoidance reactions. In particular, the modified Longhurst–Hardy Plankton Recorder (Haury and Wiebe 1982) that had been attached behind the optical sampling volume was removed from the VPR.

Operating principles of the different parts of the VPR are described.

Video system The underwater video system, shown in Figure 7.8, has optical components sufficient for resolving particles in the size range 10 μm to 2 cm. As many as four video cameras are configured to view concentric volumes at a range of 0.5 m under conditions of dark-field illumination at two or more magnifications, with the possibility of stereoscopic imaging. The cameras are synchronized at 60 frames per second to a red-filtered strobe with flash duration of 1 μs. Video data are telemetered to the surface by fiber-optic cable, where they are stored on videotape with time-code overlay, as indicated in Figure 7.8. Data derived from other sensors attached to the same platform that carries the VPR can be telemetered to the surface in the same way.

Image system The image processing and plankton registration system, together with other components and platform, is outlined in Figure 7.9. This may operate on the basis of live or recorded images. Significantly, operations of field-grabbing, convolution, edge detection, and export of extracted image coordinates can be executed at the collection rate of 60 frames per second. Determining whether or not a particle is present is done automatically, by examining the clarity of focus. A region of interest (ROI) is defined about each sharply focused particle. This is classified taxonomically by means of a neural network trained on the basis of 2000 images. The data rate achieved by the system as of 1996 is estimated as follows: at a collection rate of 60 frames per second, 1 to 3 ROIs are generated per second, with a resulting data rate of 3600 to 10 800 in-focus objects per hour.

Data system Results of taxonomy are stored together with data on the conditions of collection such as geographic position, depth and time. By relating the detection to the optical sampling volume, absolute measures of numerical density can be derived. These can be displayed graphically, using automatic visualization software, together with other variable fields such as temperature, salinity, fluorescence, and light absorption. Abundances of classified organisms can be derived by integrating the density fields over the survey region.

EcoSCOPE

EcoSCOPE is a small-scale video system for the observation of zooplankton behavior (Kils 1992). This system is integrated with a small sonar for detection of fish, and is used

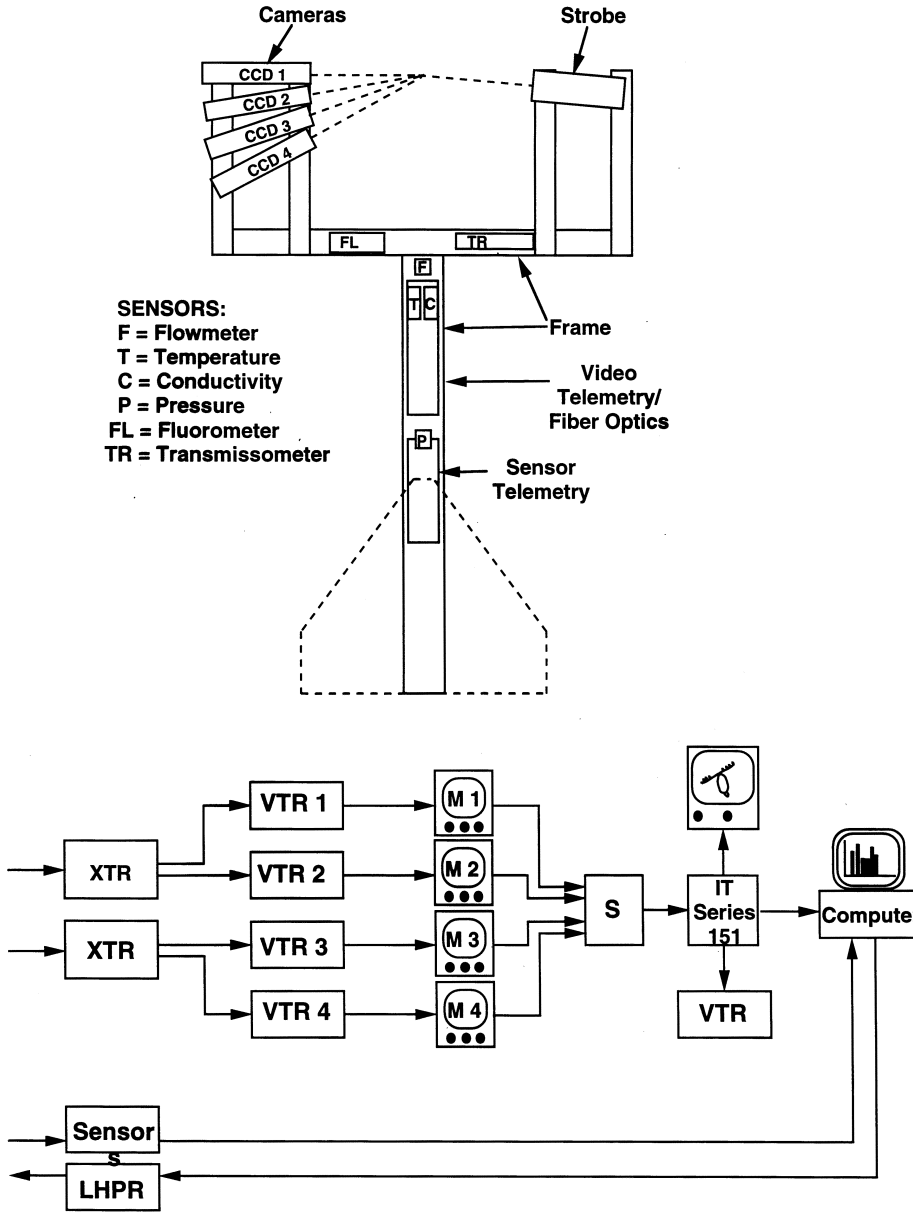

Fig. 7.8. Video plankton recorder: schematic diagram showing underwater components (upper part) and surface components (lower part). Adapted from Davis *et al.* (1992a) by CS Davis. Used with permission.

in studies of predator–prey interactions. It can be mounted on a remotely operated vehicle (ROV), and moved about to permit observation of, for example, juvenile herring feeding on copepods.

A schematic diagram is shown in Figure 7.10. The light source consists of strobed light-emitting diodes (LEDs), with maximum intensity at 700 nm. The lower endoscope conducts the light to ports where reflectors create sheets of light in the water volume. A

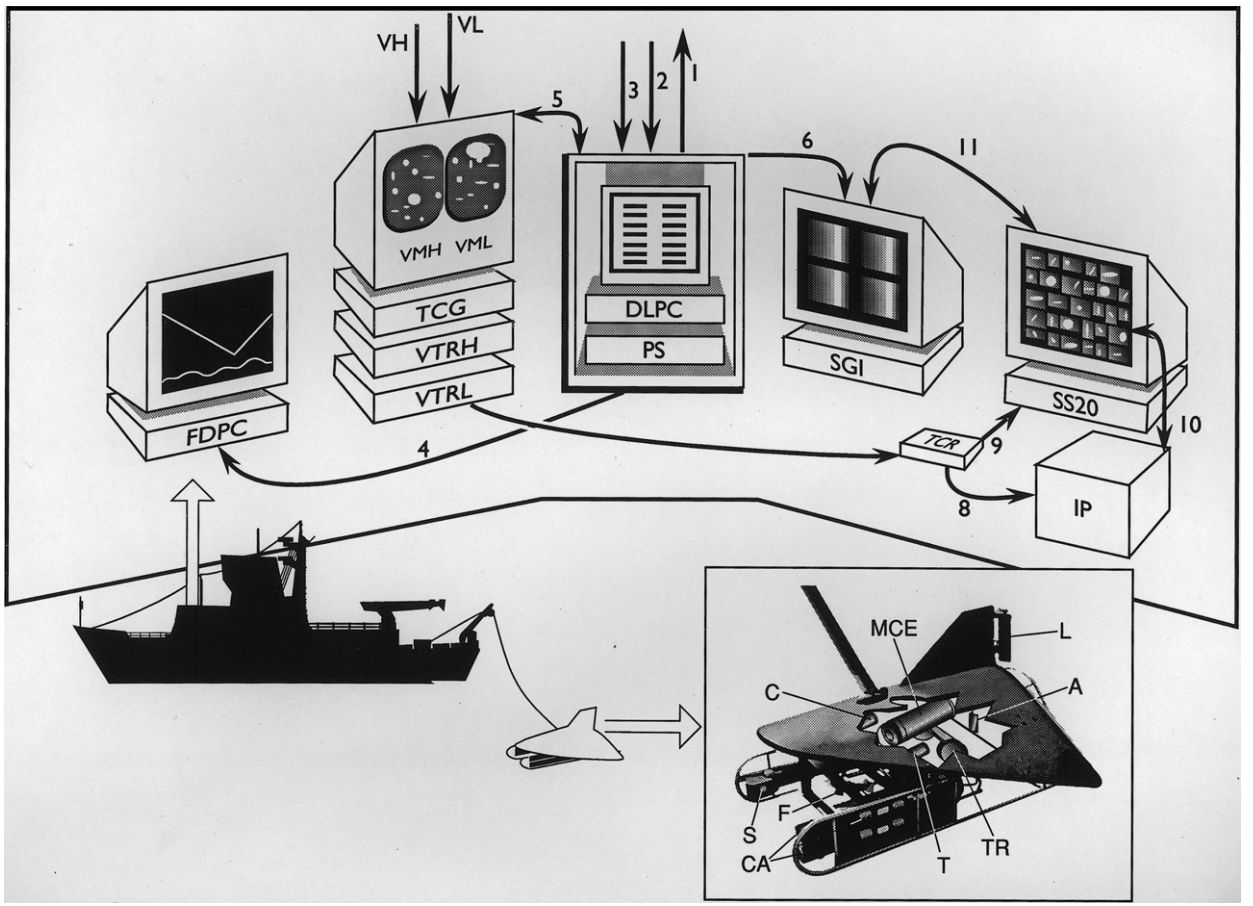

Fig. 7.9. Video plankton recorder: underwater video system on towed vehicle, image processing and plankton registration system, and data processing and visualization system. Prepared by CS Davis. Used with permission.

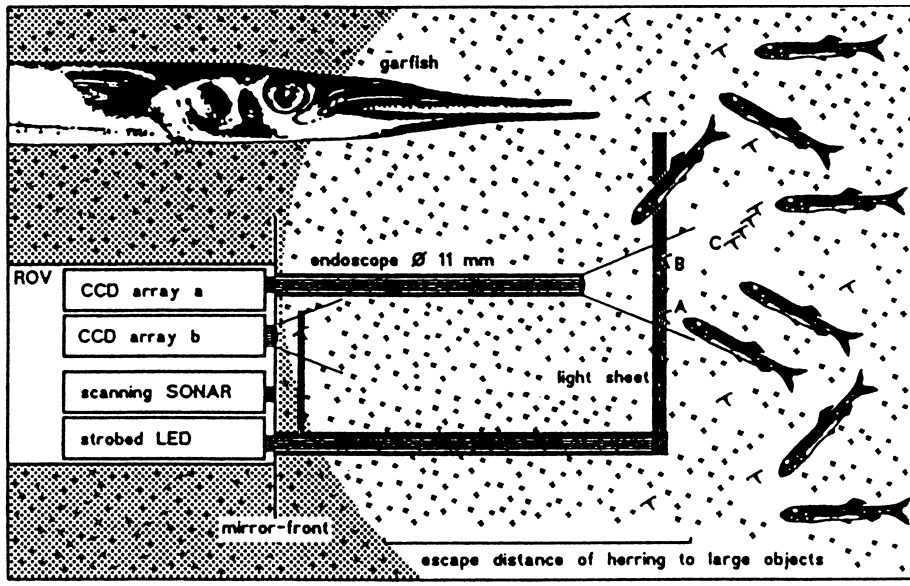

Fig. 7.10. EcoSCOPE: schematic diagram (Kils 1992). Used with permission.

second endoscope, the upper one, captures light from a region where both predators and prey can be imaged with a resolution of 100 μm, and conveys this to CCD array (a). CCD array (b) receives light directly from a near-source region of high magnification, with resolution of 5 μm.

Both CCD arrays are scanned, and their digital images are stored on a digital computer. Images can be observed in real time, but their analysis, as for species identification and measurement of interaction distance and time, is accomplished by post-processing.

Some spectacular images are presented in the cited paper (Kils 1992), which constitutes one proof of operation. The system itself does not seem to induce a behavioral response, and does not therefore bias measurements of production and prey. This may be because of the successful camouflaging of both the instrument and ROV.

Limitations are noted by the inventor. These include the presence of extraneous phytoplankton and particulate matter, which scatter light and reduce image contrast. Some organisms may be sensitive to the red light source, biasing observations of predator–prey interactions.

Ichthyoplankton recorder

The ichthyoplankton recorder (IPR) is an underwater video-based system that aims to image and quantify both fish larvae and prey in the size range 0.5–20 mm (Lenz *et al.* 1995). The practical difficulty of observing animals at low numerical density is addressed by use of a Gulf-III sampler with 200- or 335-μm mesh as a concentrator, indicated in Figure 7.11. The optical observation volume is aft of the net.

The detection unit of the IPR is shown in Figure 7.12. Dual arrays of strobed LEDs illuminate the animals in the flow chamber with adjustable pulse duration over the range 0.5–12 μs. A field 20 cm deep is scanned by a CCD array.

The performance of the IPR was established during a herring larvae survey in January

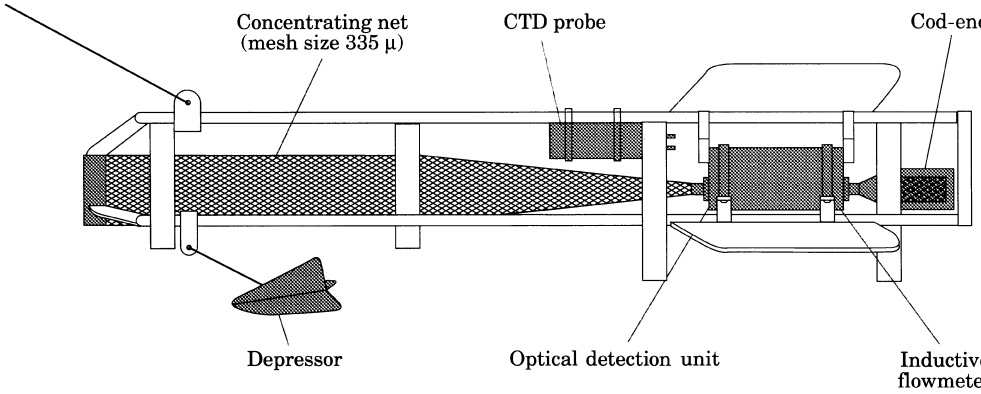

Fig. 7.11. Ichthyoplankton recorder: configuration with Gulf-III sampler for concentration of organisms (Lenz *et al.* 1995). Used with permission.

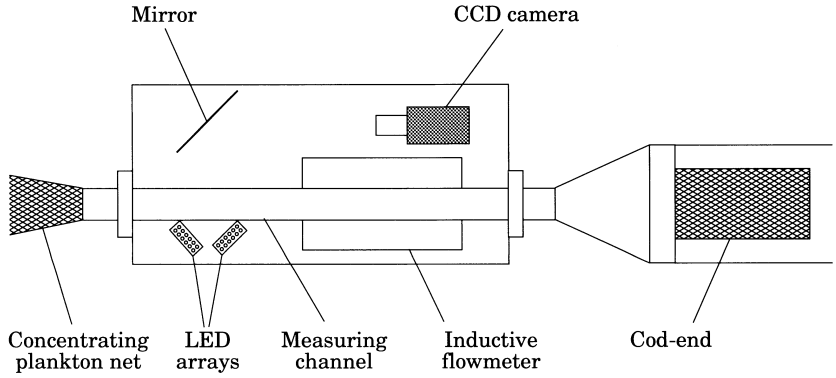

Fig. 7.12. Ichthyoplankton recorder: optical detection unit (Lenz *et al.* 1995). Used with permission.

1994. The instrument was compared against two other systems. Low densities of organisms prevented close comparison with gears that could not be deployed at the same time. None the less, the IPR and the 'Nackthai' were found to have similar sampling efficiencies, while the IPR was superior to the 'Messhai' by a factor of ten.

Some limitations of the IPR have been identified by Lenz *et al.* (1995).

1) Processing capacity Automatic software is necessary for processing the large volume of data that could be collected with the instrument.
2) Resolution At a sampling rate of 50 frames per second and towing speed of $2.5\,m\,s^{-1}$, the resolution is 5 cm.
3) Sampling bias Because of stalling of organisms on the wall of the Gulf-III net, the optical sampling must be biased.

The primary study documenting development of the IPR also describes an application for a herring larvae survey. Organisms registered by the system include fish larvae and eggs, but also polychaete, cephalopod, and chaetognath specimens.

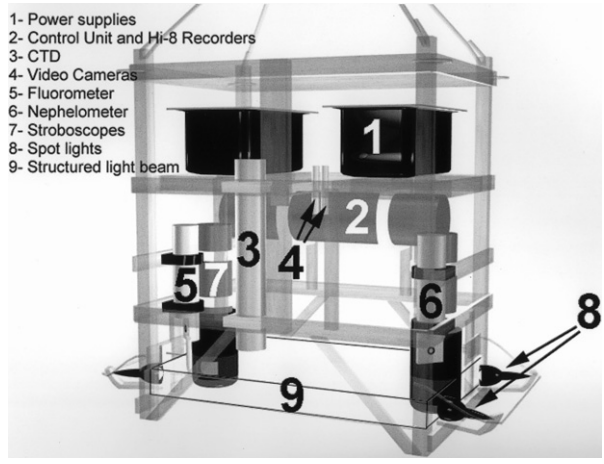

Fig. 7.13. Underwater video profiler: schematic diagram of Model III. Provided by G Gorsky. Used with permission.

Underwater video profiler

The underwater video profiler (UVP) has been designed to image and quantify the three-dimensional distribution of suspended particles and macrozooplankton in the water column (Gorsky *et al.* 1992). It has also succeeded in detecting marine snow and gelatinous zooplankton. Thus far, three distinct models of the UVP have been developed. Only the third model (Stemmann *et al.* 2000) is described here (Figure 7.13). The total system dimensions are $1.1 \times 0.9 \times 1.25$ m.

The optical sampling volume is defined by the illumination volume and the fields of two video cameras. Two stroboscopic lights are used with four mirrors to create a light beam that is 10 cm in breadth. Intersection of the fields of view of the cameras determine sample volumes of 1.3 and 6 l. It is also possible to use four 100 W spotlights for continuous observation of a larger volume. Some exemplary video photographs that include specimens of gelatinous zooplankton are shown in Figure 7.14.

Typically, the UVP is used as a sonde, with nominal data rate of 25 images per second. It can be lowered to 1200 m depth at speeds up to $1.5\,\mathrm{m\,s^{-1}}$ without affecting image quality.

Automatic processing of images includes detection of objects, enumeration of these, and determination of maximum length and area. The processing rate is five images per second.

Auxiliary instrumentation is integrated with the video system, if only by sharing the same vehicle, the UVP frame. The standard attachments are indicated in Figure 7.13, namely CTD probe, fluorometer, and nephelometer.

Calibration has been effected with biological particles spanning the size range 90 μm to 20 mm. The detection limit is at least 150 μm. Particles larger than 400 μm can be measured.

7.2.6 Comparisons

Comparative measurement may be necessary for identification of the primary optical data or for verification of the instrument, as when calibration is otherwise absent or

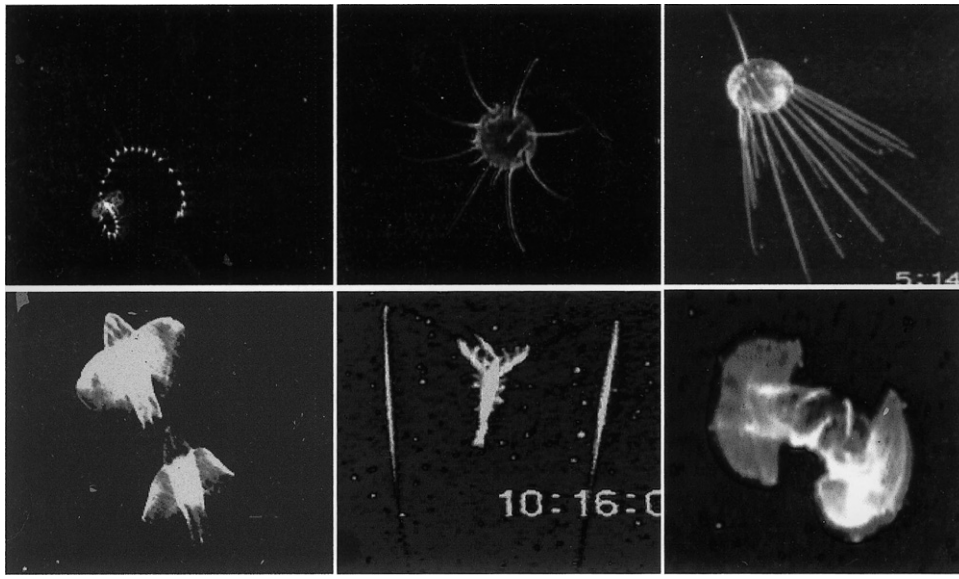

Fig. 7.14. Examples of zooplankton recorded by the underwater video profiler models II and III at depths in the range 200–1000 m. Upper row from left to right: feeding calycophore siphonophore from the Aegean Sea, medusa *Solmissus albescens* (3 cm in diameter) from the Alboran Sea, and an Antarctic medusa. Lower row from left to right: two mollusks *Cymbulia peroni* from the Alboran Sea (each 6 cm long), a decapod crustacean from the Ligurian Sea, and a lobate ctenophore from a Norwegian fjord. Provided by G Gorsky. Used with permission.

difficult to perform. These and related issues are discussed and illustrated in this section.

IDENTIFICATION

There is no substitute for independent identification of the organisms that are being optically registered. In the case of silhouette photography, reference to the actual organisms may establish how the shadowgraphs are to be interpreted and what their limitations are. For the optical plankton counter (OPC) independent identification of the detected organisms, by physical capture, is essential, for the instrument is literally blind. It requires such data to be collected at regular intervals for referencing purposes, to establish the connection between single-organism counts and the character of the organisms themselves, namely their species and size distributions. The video plankton recorder (VPR) and other video-based systems are powerful in their capacity to visualize, but physical capture is still advantageous for verifying the range of species and sizes of zooplankton that are actually present. Given the small size of the sampling volume, organisms at low concentrations may not be registered at all.

Automated recognition also requires independent identification of the organisms, if only to identify and avoid situations of ambiguity. Distinguishing among different stages of copepods, for instance, is sufficiently challenging, for the OPC and VPR alike, that automated recognition requires verification.

IMAGING VERSUS QUANTIFICATION

Optical systems may be used for imaging, quantification, or both. The need for data on the identity of the organisms has already been admitted. Measurement of numerical density also requires reference data, as on performance at both low and high densities. When calibration data are lacking, comparative data may provide a ready remedy.

VENUE AND DEPLOYMENT

A high degree of control is often possible when making measurements for it may be possible to acquire total knowledge about a sub-sample for comparison with the registration of the device itself *ex situ*. Performance of silhouette photography in the laboratory or use of the OPC on board a vessel, as in the continuous, underway fish egg sampler (CUFES), is accompanied by an extremely high degree of control.

For systems used *in situ*, control must be established in another way. Paired tows with a plankton net in the cases of *in situ* silhouette photography (Olney and Houde 1993) and the OPC (Herman, in prep.), plankton net with cod-end in the case of the IPR (Lenz *et al*. 1995), and MOCNESS in the case of the VPR (Benfield *et al*. 1996) are examples. It is important to note that in these cases, the comparative data are derived from integrated sampling stations, whereas the optical registrations themselves are essentially discrete, with high information content on small-scale patchiness.

While the present orientation has been towards that of the optical device, performance of other sampling gear can be examined by means of the same comparative data. This two-way use of such data explains why comparative data are to be distinguished from calibration data. It is often unclear as to which sampling tool sets the standard.

OPERATING RANGES OF TWO SYSTEMS

A brief comparison of the major *in situ* operational systems is attempted. The principal references given in section 7.2.5 for the OPC and VPR, are supplemented by Schulze *et al*. (1992) and Sprules *et al*. (1992).

The range of particle sizes that can be detected with the OPC is 0.25–20 mm in terms of equivalent spherical diameter. For the VPR the linear dimensions are 0.5–100 mm for taxonomy and 0.02–100 mm for counting. The sampling volume of the OPC is 17.6 ml; that of the VPR depends on the camera and can span the nominal range 1–1000 ml. Counting rates are theoretically limited to 200 particles s^{-1} for the OPC; and for the VPR by the number of particles that can be detected per frame, with a typical repetition frequency of 60 frames s^{-1} and possible extension to 200 frames s^{-1}. Nominal maximum operating depths are of the order of 1000 m for both instruments, but at least one OPC is rated to 2000 m. Nominal towing speeds are roughly 0.5 to 4 m s^{-1} for the OPC and 0.5 to 5 m s^{-1} for the VPR, at least according to specifications published in 1992.

It is reiterated that both the OPC and VPR have been under steady development in recent years, with continual advancement in performance.

7.3 MEASUREMENT PROTOCOLS, MODEL COMPUTATIONS, AND EXAMPLES

7.3.1 Silhouette photography in the laboratory

Silhouette photography is a standard laboratory technique. In this section, its practice by N Copley at the Biology Department, Woods Hole Oceanographic Institution, is

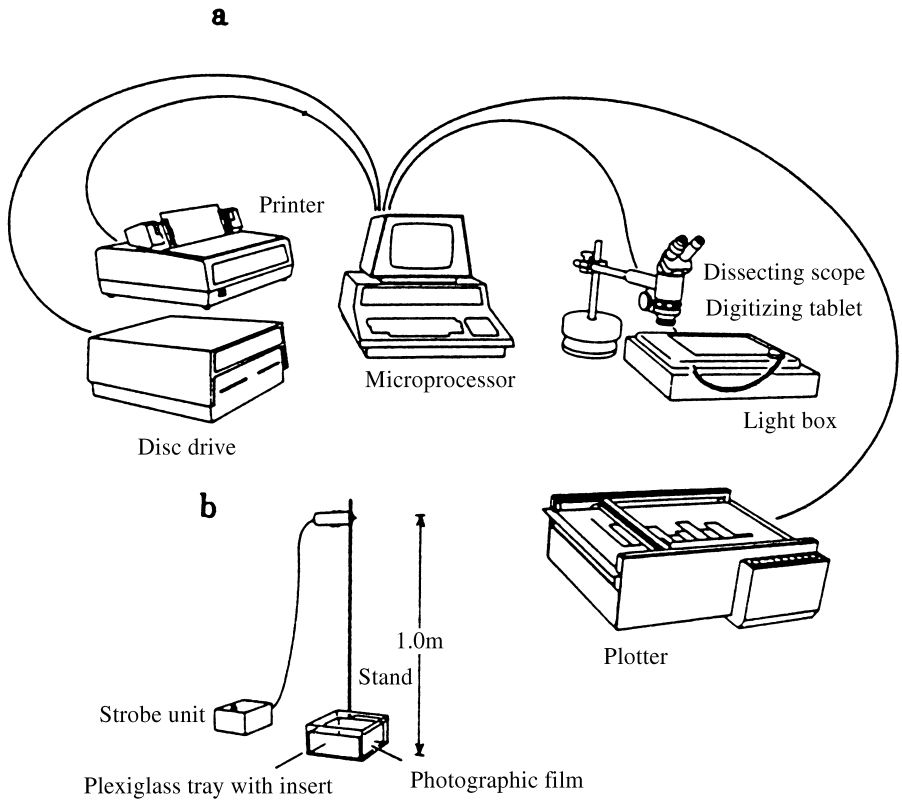

Fig. 7.15. A laboratory system for silhouette photography and digitization (Davis and Wiebe 1985). Used with permission.

described. This text represents a minor adaptation to that provided by N Copley, which is otherwise quoted. Reference is made to the laboratory system shown in Figure 7.15 (Davis and Wiebe 1985).

Silhouette photography is used to obtain counts and lengths of zooplankton individuals in a sample or sub-sample. In brief, the technique consists of the following steps. A sample of live or preserved zooplankton is poured into a plexiglass tray containing a sheet of photographic film. A strobe light positioned 25 cm above the film is flashed to expose the film. The film is rinsed off to remove plankton, and the sample is poured back into its container. The 20 cm × 25 cm film is processed using developer (1 min), stop bath (5 s), and fixer (1 min). The film is then rinsed for a minimum of 10 min in water and dried.

PROCEDURES

Preparation of the samples

1) Sieving Pass the preserved sample through a mesh equal to or smaller than the mesh size of the tow and transfer this to fresh water.
2) Splitting If the sample is dense, split it with the Folsom splitter. The aim is to avoid overlapping animals on the photograph. The number of animals depends on the mesh size. For 330 μm mesh, there should be no more than 5000 individuals on the film. The more the sample is split, the greater the error.

3) Temporary holding Put the sample in a jar with about 250 ml water, label the cover with the tow number, net number, degree of splitting, and other pertinent information.

Dark-room procedure

1) Materials and supplies Bring the following to the dark room: samples in jars, indelible marker to label film, two squirt bottles, film (Kodak no. 7302, fine-grain positive), photochemicals, forceps, plexiglass sample box, eye dropper for Photoflow, strobe-light assembly, trays and chemicals, tongs, small sieve, funnel.
2) Organization of materials Set up the dark room. Place three photographic trays on the counter, with the developer to one side, stop-bath in the middle, then fixer. Put tongs, labeled developer, and stop-fixer beside the corresponding tray. Beside the sink place a large tray for the wash. Attach a siphon to the faucet and place in the bath. The temperature of the water from this faucet should be about 20 °C. The strobe light should be 25 cm above the film. Put the plexiglass sample box under the strobe bulb.
3) Chemicals Prepare the chemicals on the day of photographing, especially the developer, as this does not maintain its potency very long. Check instructions on the bottles. Do this under a red safety light.
   (a) Sprint Quicksilver Print Developer Dilute 100 ml in 900 ml water. This is light- and air-sensitive. Cover when the lights are on. This can be disposed of in the drain.
   (b) Sprint Block Stop-Bath Dilute 100 ml in 900 ml water. This keeps well. Refer to the container for details.
   (c) Sprint Record Speed Fixer Dilute 200 ml in 800 ml water. This also keeps fairly well. Put 1 liter of each solution into the marked trays.
4) Handling light sensitive film Still under the safety light, remove a piece of film from the box. Find the notch and turn it to the top right. This ensures that the emulsion side is up. Using the indelible pen, label the film with the tow and net number and split (e.g. 1/4) about 5 mm down from the edge to ensure that it will not be covered by the collar insert that holds the film in place and prevents the sample from slipping underneath.
5) Adding film and sample to tray Squirt a little water into the sample box to prevent bubbles of air getting trapped under the film. Put the film in the box by putting one edge down first and slowly lowering the rest, watching for bubbles. Add more water if it is too bubbly. Slide the black collar insert on top of the film, checking that the label shows. Gently pour the sample onto the film, and rinse the container onto the film. Add 1–2 drops Photoflow to help floating particles sink. Check that the sample is spread out. Correct clumping by tipping the box up a little or use forceps to tease apart tight clumps. Let the sample settle for a few seconds before exposing.
6) Strobe Push the strobe button once. If pressed too hard it will flash twice and cause a double exposure. Remove the inner square, rinsing it into the box. Remove the film with fingers or forceps, rinsing both sides thoroughly into the box.
7) Developing and fixing the film Place the film in the developer for 1 min, or longer if the film is not dark. Slow developing indicates old chemicals, and new ones should be made. Rock the tray gently to encourage mixing and development. With the developer tongs, remove the film and place this in the stop-bath for 5 s. With stop tongs, move to the fixer for 1–3 min. Again, rocking the tray gently helps the process. Move the film to the bath and leave it there as long as possible, for a

minimum of 10 min and up to several hours if possible. Care must be taken that the films do not curl up and dry out at the edges. The longer the wash, the more thoroughly the fixer will be removed and the longer the films will last without bleaching.

8) Recovery of sample While the film is developing or washing, pour the sample from the box through a sieve, and pour the zooplankton sample back into its jar. Rinse the box well.
9) Rinsing, drying, and storing processed film Finally, dip the film into a solution of water with one capful of Photoflow and hang to dry. When the film is dry, slip it into a plastic page protector and store in a loose-leaf binder.

FURTHER PROCESSING OF THE FILM

The method of measuring the shadowgraphs of animals on the silhouette photographs must be very individualistic, since the digitizing device, controlling software, and analysis software are non-standard and often system-specific. A summary is therefore given, based on N Copley's text, but without details of image processing such as use of the digitizer or measurement of animals. An exemplary silhouette photograph taken by N Copley is displayed in Figure 7.16.

The processed film is placed on a digitizing table illuminated from below and overlaid with a grid of squares (in this case 22 by 18). A side-arm dissecting microscope is used to view the silhouettes of organisms on the film. Individuals are measured over the entire film if they are relatively large or not too numerous, otherwise, a group is selected for length measurements in randomly chosen squares in each of the four quadrants of the film. Length measurements are made with the digitizer in so-called 'point' mode for straight individuals, or in 'line' mode for curved individuals. Digitized coordinates of end points or center-line arcs are transferred automatically to the computer where they are reduced to body lengths. Lengths are stored in files according to taxonomic categories. After the data are acquired and stored, analysis can be performed to determine, for example, size frequency distributions, or to convert length data to wet weight or carbon mass, *inter alia*, by means of regression equations.

7.3.2 Optical plankton counter

This rather mature instrument continues to be developed on the basis of experience in measurements. It is essentially the instrument described in Herman (1992) that is considered in this review of measurement-related issues, namely calibration including comparisons, limitations, an operating scenario, and applications.

CALIBRATION INCLUDING COMPARISONS

Calibration has been thoroughly addressed apropos of sizing and sensitivity (Herman 1992). For example, use of beads of known diameter enabled the connection between apparent digital size as registered by the device and actual diameter to be established. Non-spherical shapes are addressed by use of an equivalent spherical diameter defined on the basis of equal volumes of actual and equivalent spherical shapes. The effect of orientation has also been examined for copepods, but differences due to size with calanoid stage over the range CIII–CVI are greater than differences due to orientation.

Sensitivity has been established partly by measurements on beads. The lower limit is about 250 μm. This has been confirmed by examination of rehydrated *Artemia* eggs, with diameters 240–290 μm.

Fig. 7.16. Silhouette photograph of an assemblage of zooplankton from slope water, taken by N Copley. Used with permission.

A missing measurement in the calibration work has been the operating characteristics linking OPC output with the numerical density of particles passing through the flow tunnel. Definition of this characteristic is important in applications over a range of sizes where the aim is to describe the density distribution. Whether aiming at relative or absolute determination of density, the calibration needs to be performed.

To verify the performance of the OPC on zooplankton, a large number of measurements have been performed on preserved specimens of copepods and euphausiids used in a recirculation bath. Examples of examined specimens include *Paracalanus parvus*, *Clausocalanus furcatus*, *Pseudocalanus minutus*, *Calanus finmarchicus*, *Metridia lucens*, *Calanus glacialis*, *Calanus hyperboreus*, *Thysanoessa* sp., *Euphausia eximia* and *Meganyctiphanes norvegica*.

The performance of the OPC on zooplankton has also been verified through a series of *in situ* measurements compared with plankton net samples. Agreement of vertical profiles of copepod concentration in the Grand Basin, May 1990, is nothing less than

outstanding, with the OPC showing fine-scale variations most likely due to variability in the spatial distribution itself.

LIMITATIONS

Development of the OPC, like that of other operational systems, has been characterized by an awareness of its shortcomings, which, after all, define the limits of performance. In the case of the OPC, limitations have been described in detail and where possible are being addressed. Some limitations mentioned in Herman (1988, 1992) are listed here.

1) Identification The OPC is blind. It counts objects but does not recognize form. Given other knowledge of organisms present in the sampling region, it can provide quantitative data on size and concentration, even allowing discrimination among different calanoid stages under suitable circumstances. The new laser-based OPC will provide some coarse information on form.
2) Organism translucence The translucence of zooplankton changes with season. For example, copepods are recognized to be relatively colorful during the grazing period because of ingestion of pigmented organisms, and relatively translucent during the wintering period. Changes in the translucence of the same organisms at the device wavelength of 640 nm will affect the registration, those of greater translucence giving the weaker signal and being assigned a smaller size.
3) Non-spherical shape and orientation effects Both effects are present but can be addressed through calibration. This has already been done successfully for *Calanus finmarchicus*.
4) Coincidence counts At relatively high numerical densities, not all individual particles can be registered. Groups or clumps of particles may be perceived as single, larger particles, thus biasing the measurement. Limits can be established by calibration, as described above. An operational solution to high counts is to use an absorptive spacer bar in the OPC tunnel. This reduces both the flow volume and signal intensity.
5) Sensitivity The lower limit under ordinary conditions is 250 μm, although there is evidence for detection to 150 μm or even to 125 μm. Device vibration currently limits the performance to 250 μm.
6) Ambient attenuance Inherent in the OPC design is use of a feedback loop to maintain constant output voltage in the presence of phytoplankton with a fluctuating attenuance. If the scale size of spatial variation in attenuance is less than about 1 to 2 m, compensatory changes in the device may trigger false counts.

OPERATING SCENARIO

During the first sea trial of a new broadband acoustic scattering system (Atkins *et al.* 1998; Foote 1998a), auxiliary measurements were made with the OPC model designed for use by the Marine Laboratory, Aberdeen. This self-contained device was attached to the frame of a MOCNESS in such a way as to ensure proper flow conditions. The unit was powered up in advance of deployment, and it was actually started immediately before deployment. Following deployment, its data were extracted and stored on a personal computer. As early analysis consisted of the extraction and display of the concentration profile by size class in a large matrix, with averaging over 2- and 10-m thick depth bins.

APPLICATIONS

Both of the principal works documenting the OPC design describe *in situ* measurements

of zooplankton (Herman 1988, 1992). These have been cited in connection with their validating aim, but they could also be cited for their applications.

In another work, Herman *et al.* (1993) established the capacity of the OPC for quantifying euphausiid distribution. This study was performed in the Emerald and Le Have Basins of the Scotian Shelf in autumn 1990. Both *Meganyctiphanes norvegica* and *Thysanoessa inermis* were caught with the Bedford Institute of Oceanography Net and Environmental Sensing System (BIONESS) (Sameoto *et al.* 1980). These observations, as well as acoustic measurements at 50 and 200 kHz, were related to the OPC observations of size and concentration. Agreement was reasonable when allowance was made for the very different sampling volumes and sensitivities of the several devices. A significant finding of the study was that elongated objects such as euphausiids could be measured reliably *in situ* with the OPC.

In a study performed between the Shetland and Faeroe Islands in winter 1992–1993, an OPC was used in conjunction with a CTD sonde to establish the connection between water-mass distribution and the vertical distribution of *Calanus finmarchicus* (Backhaus *et al.* 1994). The distribution of zooplankton in the non-Canadian waters of Lake Erie was determined using an OPC deployed along line transects, either with an undulating path in the water column or at a constant depth during different seasons in 1993 and 1994 (Stockwell and Sprules 1995). Biomass concentrations of zooplankton in a sea loch have also been measured by OPC in a study of seasonal variation (Heath 1995). The OPC has been used to determine zooplankton biovolume along the Atlantic Meridional Transect (ATM) from N50° to S50° (Gallienne and Robins 1998). The biovolume has also been distinguished by the following size classes: 0.25–0.5, 0.5–1, 1–2, and greater than 2 mm.

7.3.3 Video plankton recorder

Four issues associated specifically with measurement by the VPR are addressed. These are calibration including comparisons to verify its performance; limitations of the system; an exemplary operating scenario, including discussion of image and data analyses; and some exemplary applications. Key references are supplied.

CALIBRATION INCLUDING COMPARISONS

Performance measures for the VPR are being established through usage, by classification, sizing or staging, and determination of numerical density. While the achievements are impressive, conduct of an independent calibration would be useful.

One approach to calibration is by exposure of the system to concentrations of monodisperse beads, with identical sizes, or other particles with well-known distributional characteristics, as in a test tank or flow chamber. Checks on sizing and density determination would be immediate. Tests on small particles could define the sensitivity of the VPR, and show how this depends on particle color, for example. Tests performed at high numerical densities could define the onset of system saturation and perhaps suggest objective criteria for detecting and possibly compensating for the same.

A radically different approach to calibration was suggested in an early design described by Davis *et al.* (1992a). In this, a gauze recorder box in the form of a modified Longhurst–Hardy Plankton Recorder was placed just aft of the optical sampling volume. Physical capture of the observed organisms would allow the VPR registration to be linked directly to the particular organisms including their numerical concentration. However, as already noted (Davis *et al.* 1992b), the presence of the box induces

avoidance reactions in the zooplankton thus upsetting the primary measurement. Removal of the box has prevented the physical sampling believed necessary by the inventors.

An alternative to calibration is the comparison of VPR observations with those made by other sampling gear used in parallel, for example, as in Benfield *et al.* (1996). Accordingly, the Multiple Opening and Closing Net and Environmental Sensing System (MOCNESS) (Wiebe *et al.* 1976, 1985) was towed at each of five depths, where observations were also made with the VPR. Various organisms were identified, for example amphipods, copepods, euphausiids, larvacea, and pteropods, and their numerical densities were determined both by MOCNESS and by VPR. The concentrations of these organisms were sufficiently high that the total sampling volume of the VPR, $0.069\,m^3$, was sufficient for comparison with that of the MOCNESS, $149\,m^3$, at each depth. A general agreement was found in the results obtained with the two systems, with main discrepancies at low densities, where the VPR failed to register some of the organisms. It is noted that the resolution of the VPR is some tens of micrometers, while that of the MOCNESS is one square meter times the tow distance (149 m in the present case).

LIMITATIONS

Four limitations are mentioned.

1) Focus detection At high particle concentrations, in-focus particles are underrepresented, biasing estimates of numerical density in the same way.
2) Concentration threshold Organisms at low concentrations may not be registered by the VPR owing to the small size of the sampling volume.
3) Physical capture Removal of the gauze recorder box in the early design was necessary. Without a substitute means of physical capture, the need for *in situ* calibration remains unfulfilled.
4) Behavior-induced effects The VPR, its platform, or other instruments on the platform could induce avoidance reactions among the zooplankton to be observed. Tests might be performed to define or exclude such potential limitations.

OPERATING SCENARIO

An operating scenario is extracted from Benfield *et al.* (1998). This describes an application in the southwestern region of Georges Bank on 24 May 1992. The VPR was deployed from R/V *Endeavor*.

The system was operated continuously along a sawtooth or tow-yo trajectory between the surface and near-bottom zone. A total of four CCD video cameras were used, with imaging volume spanning the range $0.62–147\,cm^3$. Illumination was provided by a red-filtered strobe. Video data were telemetered to the vessel by optic cable, as were auxiliary data on depth, temperature, salinity, fluorescence, and transmittance.

Data were taken from a wide-field camera because of its relatively large sampling volume, $22\,cm^3$, and consequent ability to sample less dense concentrations. The image analysis system described by Davis *et al.* (1996) was used, although without full automation. Regions of interest (ROIs) were defined on the basis of focused images of zooplankton and their contents stored on hard disk with a time-code stamp. These stored images were classified and sized by means of standard software (MATLAB) on a workstation. Ultimately, organisms were categorized as belonging to one of six sound-scattering classes: amphipods, chaetognaths, copepods, euphausiids, pteropods, and

fish. Widths of identified organisms were used to determine length by means of length–width relationships estimated from ROIs with organisms in favorable orientation.

Finally, numerical concentrations were estimated for each sound-scattering class over 2-m depth bins. Near-surface data were excluded where daytime lighting effects biased density determination because of non-constant background lighting. Some effects were noticeable to depths in the range 6–8 m, and data in the upper 10 m were consistently excluded from further analysis.

The remaining part of the analysis involved other data, as the aim of the study was to combine VPR and acoustic data to estimate the spatial distribution of zooplankton biomass.

APPLICATIONS

Only a few applications are mentioned here.

In Davis *et al*. (1992b), patchiness of copepods, doliolids, and *Trichodesmium* were observed over distances of the order 1–100 m. Details of orientation and internal structure of individual specimens were also observed *in situ*.

Density distributions of *Calanus finmarchicus*, hydroid medusa and polyp stages of *Obelia* sp., *Limacina retroversa*, inter alia, were measured in vertical sections across the Great South Channel of Georges Bank by Gallager *et al*. (1996a). Measurements of temperature and salinity fields allowed association of zooplankton concentrations with water masses in some cases.

Norrbin *et al*. (1996) have observed zooplankton in different regions of Georges Bank in order to investigate the connection between mixing and stratification. Chaetognaths, hydroid colonies, *Pseudocalanus* sp., and Foraminifera were observed in a well-mixed area, while *Calanus finmarchicus*, *Limacina* pteropods, *Pleurobrachia*, and *Oithona* sp. were observed in a stratified area.

In Benfield *et al*. (1996) copepods, pteropods, and larvacea distributions were observed by the VPR in a stratified region of Georges Bank. Collateral observations with a MOCNESS allowed estimates of organism density to be compared favorably for sufficiently robust specimens at high numerical densities.

7.4 ACKNOWLEDGMENTS

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8 Feeding

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8.1 INTRODUCTION

Feeding is the main route for the transfer of energy and material from lower to higher trophic levels within communities, therefore its quantification will be a key factor when trophic interactions are studied. Food is also the source of energy and material for production and activity of zooplankton, so quantification of feeding behavior is fundamental in understanding the constraints of these processes. However, quantification is not necessarily straightforward, because the methods used must be appropriate for the specific organism studied. For example it is obvious that different methods are required when studying the feeding rate of ciliates compared to that of jellyfish and that different methods are required to study different levels of biological complexity. For example, to quantify the feeding of a developmental stage of a given species in a well defined environment, the most reliable results are achieved from an incubation experiment in as near to environmental conditions as possible. In contrast, quantification of feeding in a multi-species assemblage or a whole trophic level requires either a set of experiments where each species is investigated separately or the use of indirect methods. When selecting an appropriate method to measure feeding rate it is therefore important to define clearly the objective and the complexity level.

By definition, zooplankton comprises all free-living animals with mobility less than the physically driven water transport. The group is non-taxonomic and extremely diverse, representing body sizes from nanoplanktonic (2–20 μm) phagotrophic flagellates to giant medusae in dimensions of meters. The diversity of this group and their feeding mechanisms necessitate that a number of methods are available. Knowledge about the feeding mechanism of the studied species is therefore important when designing an experiment with a minimum of constraining factors.

8.2 FEEDING MECHANISMS OF ZOOPLANKTON

A broad division of feeding categories among marine invertebrates is *liquid feeders*, *microphages* and *macrophages* (Pandian 1975). The first group is not represented in zooplankton, although some macrophages may suck out the liquid content of a caught prey. Microphages use small-particle food and usually do not size each food particle individually, they can be further divided into *pseudopod*

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feeders, *ciliary feeders*, *mucus-filter feeders* and *setae-entrapping feeders*. The planktonic sarcodines (acantharians, radiolarians and foraminiferans) are examples of pseudopod feeders. Ciliary feeding is common among zooplankton groups and is used, for example, by ciliates and many larval forms, such as the veliger larva of gastropods and bivalves, the pluteus larva of echinoderms and the trochophora larva of annelids and echiuroids. Mucus-filters are used by, for example, thecosomate pteropods and appendicularians. Feeding by setae-bearing appendages is mainly used by the planktonic crustaceans.

The macrophages can be initially classified as either primary carnivorous or omnivorous. However, in the context of experimental methodology this is not sufficient. For example, the carnivorous group may be defined further according to (see Greene 1985; Bailey and Houde 1987):

1) mobility:
   (a) ambush
   (b) cruising
2) prey capture:
   (a) entangling
   (b) raptorial
3) prey detection:
   (a) visual
   (b) tactile
   (c) chemosensory.

Among herbivorous and omnivorous organisms we can distinguish between typical suspension feeding mode and raptorial feeding mode, but many organisms are able to use both. This is characteristic of calanoid copepods, where food particles above a certain size, for example 12 μm diameter for *Eucalanus pileatus* and *Paracalanus parvus* (Price *et al*. 1983), are detected individually and collected using at least four of their five pairs of feeding appendages (Paffenhöfer and Lewis 1989). Food particles smaller than 12 μm are collected by low amplitude movement of the second maxillae from a water current driven by the other appendages. In contrast, because they lack a fixture similar to the second maxilla, nauplii are not able to collect small particles passively like the copepods (Paffenhöfer and Lewis 1990). Thus, the larger copepods can utilize the lower range in food size better than the nauplii. This example demonstrates the importance of knowing the feeding behavior of the species or developmental stage under study in order to design an experiment to measure feeding. The morphology of the feeding structures may constrain the experimental design. For example, continuous mixing of water by air bubbling or use of a plunger should be restricted in experiments with organisms that use fragile structures to capture prey (e.g. appendicularian houses, pteropod mucus sheets etc.).

A classification according to these criteria, together with information on the size of prey and predator is important when designing an experiment. Feeding mechanisms within a taxon are more likely to be similar than those between taxa, although within taxon variability is considerable. Feeding mechanisms may also change between developmental stages (Paffenhöfer 1988).

This chapter emphasizes practical methodological approaches to feeding rather than attempting an extensive literature review. Mauchline (1998) reviews the literature on feeding of calanoid copepods, the best studied group. We refer readers to that source for more information and literature on the current state of knowledge.

8.3 EXPRESSION OF ZOOPLANKTON FEEDING RATES AND COMMON CONVERSION FACTORS

Zooplankton feeding is usually expressed as *clearance rate*, *ingestion rate*, or *daily ration*. There are many ways these feeding rates have been calculated, making it a potentially daunting task to compare the results of different investigations. In addition, scientists commonly use conversions between weight measurements and carbon, nitrogen, or energy content, amplifying the difficulty in comparisons. Below we give a strict definition of these three feeding rates and make recommendations for the use of conversion factors.

8.3.1 Clearance rate (*F*)

This was formerly known as filtration rate. It is defined as the volume of water cleared of food by a consumer organism per unit time and per consumer or consumer mass. This corresponds to the volume of water processed if we assume 100% capture efficiency and a homogenous food concentration in the experimental vessel. In a flow-through system a linear model can be used:

$$\boldsymbol{F = (1 - C_{out}/C_{in}) \times v/n} \tag{8.1}$$

where C_{out} and C_{in} is the food concentration leaving or entering the experimental chamber, n is the number of consumers and v is flow rate. In a closed environment (bottle type incubation) the processed water mixes continuously with surrounding water and a constant F assumes that a constant fraction of the instantaneous food concentration is taken away. The change in food concentration with time is described by an exponential model (Frost 1972):

$$\boldsymbol{C_t = C_0 \times \exp^{(-g' \times t)}} \tag{8.2}$$

where C_0 and C_t are the food concentrations at the beginning and end of the experiment, t is incubation time and g' is a coefficient defining the instantaneous change in food concentration. This is synonymous with instantaneous feeding coefficient only if feeding is the only factor acting on food abundance. If the control treatments reveal a change in food abundance that is not caused by feeding, then this must be corrected. Production of food items during the incubation is a common occurrence with food organisms which have high turnover rates, such as phytoplankton and protozoa. In this case, an exponential growth model is applied to the control treatments:

$$\boldsymbol{C'_t = C_0 \times \exp^{(k \times t)}} \tag{8.3}$$

where C'_t is the final food concentration in the controls (without predators) and k is the instantaneous growth coefficient of the food organism. The instantaneous feeding coefficient, g, is:

$$\boldsymbol{g = g' + k} \tag{8.4}$$

and the clearance rate, F, is:

$$\boldsymbol{F = V \times g/n} \tag{8.5}$$

where V is the volume of the incubation vessel.

Equations (8.2), (8.3) and (8.5) can be used for an alternative expression of F:

$$F = [\ln(C_t/C_0) - \ln(C_t/C_0)] \times V/(t \times n) = \ln(C'_t/C_t) \times V/(t \times n). \quad (8.6)$$

Thus, clearance rate can be calculated without measuring either initial prey concentration or the prey growth coefficient. However, it is prudent to measure both of these factors in order to maintain control of the processes involved. Equation (8.6) is useful for deriving clearance rates for the simple reason that it requires measurement of only two cell concentrations (final control and final experimental) rather than three, allowing more effort to be allocated to acquiring precise estimates of the two variables. We emphasize that, because clearance rate equations are based on ratios of concentrations, they are sensitive to measurement errors.

Numeric changes may also occur for the predators. For protozoan predators one must account for enumeration during the incubation. The term n in the formulae for individual clearance rates (equations 8.5 and 8.6) is adjusted according to Heinbokel (1978b):

$$n = (n_t - n_0)/\ln(n_t/n_0) \quad (8.7)$$

where n_0 and n_t are predator numbers at the beginning and end of the experiment.

Some predators may die during the experiment. In practice, this is difficult to correct appropriately, because mortality is not an inherent, continuous process like the cell multiplication. In an experiment with few predators, mortality may cause considerable bias. We recommend that experiments with predator mortality be repeated because the mortality indicates that the experimental conditions were not satisfactory. If this is not possible the following expression should be substituted for equation 8.5:

$$F = V \times g \times 2/\{(n_0 + n_t) \times t\} \quad (8.8)$$

The formulae for F assume that a fixed proportion of the instantaneous food concentration is removed per unit time and that the process is described by an exponential function. A linear model has also been suggested (reviewed by Conover 1978). Equation 8.5 will then change to:

$$F = (C_0 - C_t) \times V/([C] \times n \times t) \quad (8.9)$$

where $[C]$ is the average food concentration as given by Conover (1978):

$$[C] = C_0 \times (1 - \exp^{(-g' \times t)})/(t \times g'). \quad (8.10)$$

As with the exponential model, it may be necessary to correct for food growth, which is done by applying equation 8.3:

$$F = V/n \times \{(C_0 - C_t) + [C] \times (\exp(k \times t) - 1)\}/([C] \times t) \quad (8.11)$$

The difference between an exponential (equation 8.5) and a linear (equation 8.9) model is defined by:

$$\Delta F = \{\ln(C_0/C_t) \times [C]/(C_0 - C_t) - 1\} \times 100\% \quad (8.12)$$

where ΔF is the percentage difference between the two. In practice the difference is marginal provided that food concentration is not reduced heavily (Figure 8.1). Even with a 50% reduction in food, the difference is less than 4%. Thus, the choice between a linear or exponential model is not of vital importance as long as the reduction in food concentration is less than 50%.

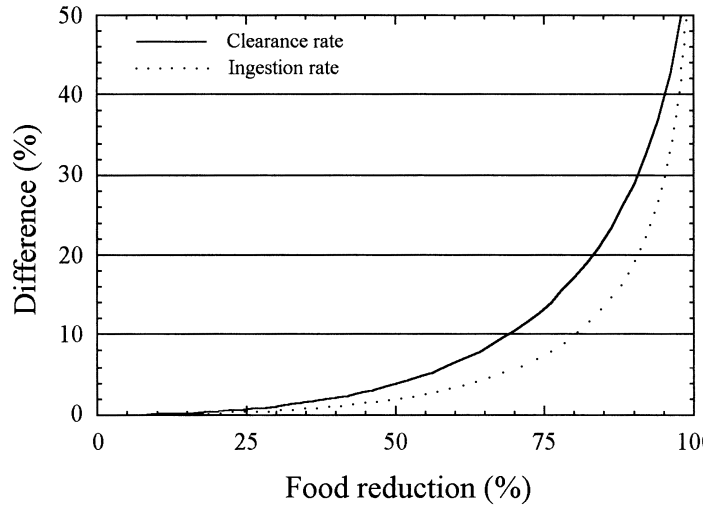

Fig. 8.1 Percentage difference in calculated clearance rate (*F*) and ingestion rate (*I*) between an exponential and a linear model. Exponential model for *F* defined by equation 8.5, for *I* by equation 8.13 and linear model for *F* defined by equation 8.9, for *I* by equation 8.15.

8.3.2 Ingestion rate (*I*)

Ingestion rate is the amount (number or mass units) of ingested food per unit of time and predator. If number of food items is used, information on mass per unit of food should also be given. We recommend using carbon as a general expression of mass. Alternatively, dry weight (DW), ash-free dry weight (AFDW) and nitrogen content are commonly used. Calculation of ingestion rate is based on its relationship with clearance rate, i.e.:

$$\boldsymbol{I = F \times [C]} \tag{8.13}$$

where $[C]$ is the mean food concentration. In an open-flow system this is:

$$\boldsymbol{[C] = (C_{out} + C_{in})/2} \tag{8.14}$$

and in a closed-bottle system it is defined by equation 8.10 above.

It is common to use the initial concentration (C_0) in equation 8.13, but this is reasonable only if the reduction in food concentration during the experiment is low. An arithmetic mean concentration has also been suggested (Rigler 1971) but it fails to reproduce true ingestion when food reduction is considerable. Figure 8.2 shows that in equation 8.13, an arithmetic mean is acceptable when food reduction is less than 50%, whereas the initial food concentration significantly overestimates ingestion rate even when food is reduced by 10%. There are several problems related to the choice of equation when analyzing feeding rate as a function of food concentration (Marin *et al.* 1986). We discuss this on page 371.

An alternative to equation 8.13 is to estimate *I* directly by applying a linear model to the experimental results:

$$\boldsymbol{I = (C_0 - C_t)/(n \times t)} \tag{8.15}$$

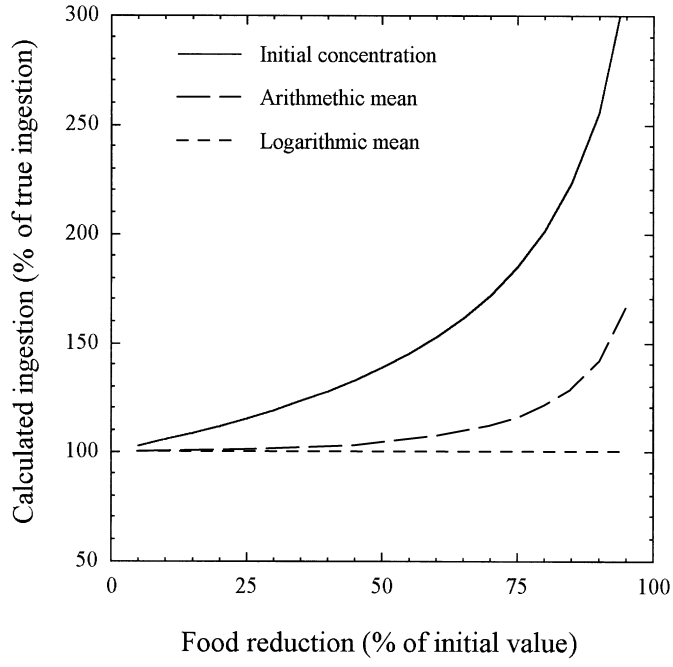

Fig. 8.2 Calculated ingestion rate as a function of percentage decrease in food concentration and using three different expressions of the food concentration.

The difference between this model and equation 8.13, where F is calculated from equation 8.5 is negligible provided the change in food concentration during the experiment is not dramatic (Figure 8.1). For example, only a 2% difference results from a food reduction of 50%. If food is produced during the experiment, this is corrected for by applying equation 8.3. Equation 8.15 then becomes:

$$\boldsymbol{I = \{C_0 - C_t + [C] \times (\exp(k \times t) - 1)\}/(n \times t)} \tag{8.16}$$

8.3.3 Daily ration (*DR*)

Daily ration (DR) is the mass of food ingested per day, expressed as a percentage of predator body mass (B) so that:

$$\boldsymbol{DR = I \times 24/(B \times t) \times 100\%} \tag{8.17}$$

where I is expressed as mass h^{-1} and both I and B have the same units (DW, AFDW, carbon, nitrogen, etc.). We recommend the expression of mass of biological material in carbon units primarily for convenience. The units chosen are a function of the goals and analytical methods of the study. The ingestion measurement should be made from 24 h incubations or a combination of shorter incubations covering the full 24 h period in order to eliminate bias due to diel feeding rhythms or short-term effects of pre-experimental conditions.

The daily ration is a valuable concept for two main reasons.

1) It is easy to compare the feeding potential of different sized predators by a direct ranking of their DR.

2) The total daily consumption may be calculated by multiplying the biomass of the predator population by its DR.

However, both of these approaches are meaningful only under identical conditions, because DR, like I, is modulated by a number of environmental factors, especially food abundance. Feeding experiments on different organisms at uniform temperature and under saturated food conditions, and subsequent calculation of DR, provides a means of ranking the potential impact of the different consumers in a zooplankton assemblage.

8.3.4 Conversions between units of mass and energy

Literature values are commonly used to convert from one mass unit to another and between mass and energy units (see Chapter 4). Such conversions are used in most calculations related to feeding. Because most of the dominant biochemical components included in zooplankton body constituents are not conservative, this practice can introduce considerable bias into the resulting estimates. The safest way to use such conversions is to investigate relationships on the same type of biological material, for example same species and developmental stage, same season, etc. However, this is not always feasible. Due to the large variability of most component ratios, we have not tabulated data for different taxa, but instead recommend that the investigator uses direct measurements, or locate appropriate published values for material that is as similar as possible to the target material of the investigation.

8.4 MICROZOOPLANKTON

Nano- and microzooplankton are heterotrophic organisms 2–20 μm and 20–200 μm respectively (Sieburth *et al.* 1978), which together constitute the 'protozooplankton' (Smetacek 1981). This operational group encompasses a diversity of taxa including flagellates, ciliates, dinoflagellates and certain metazoans which collectively perform a number of quantitatively important functions in pelagic ecosystems. Among their functional roles, the protozooplankton are major grazers of bacteria and phytoplankton in marine and freshwaters. Classically, the term 'grazing' refers primarily to consumption of phytoplankton (Stoecker and Evans 1985; Verity 1991). However, the prey of protozooplankton consumers is not restricted to autotrophs, and several of the methods described below are able to quantify consumption of bacteria and other microheterotrophs as well as autotrophic prey. Here we focus on methods commonly used to estimate protozooplankton grazing directly and indirectly, citing alternative methods where appropriate. Because the methods used in the laboratory and the field overlap in many cases, they are not separated here.

8.4.1 Methodological approaches

Protozooplankton grazing can be measured at either the level of individual organisms or the bulk micrograzer assemblage (Table 8.1). Typically, measurements of *per capita* grazing rates employ tracers, including various types of biological or inert particles, and radioisotopes. Alternatively, they may quantify the natural contents of protist food vacuoles, or monitor the disappearance of food under controlled experimental conditions. Several indirect and direct approaches have been developed to quantify grazing by

Table 8.1 Methods to measure microzooplankton grazing.

Method	Advantages	Disadvantages	Examples
Indirect methods: assemblage			
Correlation of natural consumer–prey cycles	Non-invasive	Correlations may not reflect natural relationships	Smetacek (1981) Sheldon *et al.* (1986)
Extrapolation of laboratory rates to the field	Non-invasive	May not represent *in situ* conditions	Beers and Stewart (1970, 1971) Taguchi (1976) Heinbokel (1978b) Rassoulzadegan and Etienne (1981) Capriulo and Carpenter (1983) Paranjape *et al.* (1985) Dolan and Marrase (1996) Takahashi and Hoskins (1978)
Pigment budget	Non-invasive	Invalid assumptions regarding conservation of pigments	SooHoo and Kiefer (1982) Welschmeyer and Lorenzen (1985)
Acid lysozyme assay	Non-invasive	Applies to bacterivores only; laborious calibration	Vrba *et al.* (1993) Gonzalez *et al.* (1993)
Direct methods: per capita			
Tracers of ingestion			
Particles	Quantitative; demonstrates ingestion directly	Selective feeding by consumers may affect results	Heinbokel and Beers (1979) Børsheim (1984) McManus and Okubo (1991) Dolan and Coats (1991a, 1991b)
Radioisotopes	Quantitative; sensitive	Alternate pathways of isotope uptake affect tracer cycling; highly manipulative; labor intensive	Lessard and Swift (1985) Neuer and Cowles (1995)
Disappearance of prey	Quantitative	Possible bottle effects	Jonson (1986) Verity (1985, 1991) Strom (1991) Jacobson and Anderson (1993)

Direct methods: assemblage			
Sea water dilution	Quantitative; minimally manipulative; simultaneous measurement of growth and mortality	Manipulation may alter natural assemblage	Landry and Hassett (1982) Gifford (1988) Gallegos (1989) Evans and Paranjape (1992)
Size fractionation	Quantitative	May be destructive to both consumers and prey; difficult to design true control treatments	Capriulo and Carpenter (1980) Verity (1986)
Metabolic inhibitors	Quantitative	Inhibitors may be non-specific	Campbell and Carpenter (1986) Sherr *et al.* (1986)

protozooplankton assemblages *in situ*. Indirect approaches include inference from *per capita* rates, in which the measured rates are applied to *in situ* grazer abundances to estimate the grazing impact of an entire taxon or assemblage, and correlation of natural consumer–prey cycles. The pigment budget and acid lysozyme assay are alternative indirect techniques. Direct methods involve manipulation of *in situ* protozooplankton assemblages or individual grazer organisms. The sea water dilution technique is currently the manipulative method used most commonly to measure grazing by the bulk protozooplankton assemblage. Alternative direct methods to measure grazing by protozooplankton assemblages include manipulations involving size-fractionation and use of metabolic inhibitors.

8.4.2 Indirect methods to measure assemblage grazing

The most commonly used indirect methods infer grazing by applying laboratory- or field-derived values of *per capita* protozooplankton grazing rates to *in situ* estimates of the numerical abundance of the organisms, or they infer grazing rates from biological or chemical assays such as phytoplankton pigments or digestive enzymes. They have the advantage of being non-manipulative and they may suggest hypotheses to be tested directly.

CORRELATION OF NATURAL CONSUMER–PREY CYCLES

In this approach, *in situ* numerical abundance or biomass of protozooplankton consumers and their putative prey over the annual cycle are evaluated in terms of positive and/or negative statistical relationships. The approach does not produce a quantitative estimate of grazing, but it can suggest trophic relationships for further examination. Smetacek (1981) studied the relationship between numerical abundances of protozooplankton and mesozooplankton over the annual cycle in the Kiel Bight. His analysis, using statistical methods, suggested a previously unsuspected negative correlation between heterotrophic protists and metazoan zooplankton, which eventually led to work by other investigators which established the importance of protozoan prey in the diets of suspension feeding calanoid copepods. Using a similar approach, Sanders (1987) observed a positive correlation of tintinnid ciliate abundance with the standing stock of chlorophyl smaller than 20 μm in the Damariscotta Estuary, suggesting that cells smaller than 20 μm are their major prey.

EXTRAPOLATION OF LABORATORY RATES TO THE FIELD

The studies of J.R. Beers and associates are classical examples of the indirect approach combining field abundances and laboratory rate data (e.g. Beers and Stewart 1970, 1971). This approach has the advantage that it is quantitative. It has the disadvantage that physiological rates measured in the laboratory may not reflect the norm *in situ*. Measured or hypothetical *per capita* rates are applied to numerical abundance and biomass data collected in the field, so that, for example, size-specific or taxon-specific estimates of microzooplankton grazing impact are calculated. Methods for collection, preservation and enumeration of protozooplankton are described in Chapter 5 and will not be repeated here. Other studies which follow this approach include Takahashi and Hoskins (1978), Burkill (1982), Rassoulzadegan (1982), Capriulo and Carpenter (1983), Paranjape *et al*. (1985), and Dolan and Marrase (1996). In such studies, if the goal is to express microzooplankton grazing impact in units of carbon, it is important to use appropriate carbon-to-volume conversion factors. Appropriate empirical conversion

factors include Strathmann (1967) and Montagnes *et al.* (1994) for phytoplankton, Putt and Stoecker (1989) for ciliates; Lessard (1991 and unpublished data; cited in Chapter 5) for heterotrophic dinoflagellates; and Børsheim and Bratbak (1987) for heterotrophic flagellates.

THE PIGMENT BUDGET

The pigment budget estimates grazing by micro- and macrozooplankton based on the fate of chlorophyl *a* and its degradation products (SooHoo and Kiefer 1982; Welschmeyer and Lorenzen 1989). The standing stock of chlorophyl in the euphotic zone is considered to represent a balance between production due to phytoplankton growth and removal due to grazing by micro- and macrozooplankton. Micro- and macrozooplankton are differentiated operationally on the basis of whether their fecal material sinks or not. It is assumed that macrozooplankton produce large, dense, rapidly sinking fecal pellets which leave the euphotic zone. Chlorophyl grazed by the microzooplankton compartment is converted to pheopigments in the form of fecal debris which has a negligible sinking rate and constitutes the pheopigments sampled by standard water bottles. This material is subject to photodegradation because of its low sinking rate. Photodegradation of pheopigments contained in macrozooplankton fecal pellets is ignored because of the pellets' short residence time in the euphotic zone.

The grazing impact of the two zooplankton compartments on the phytoplankton is determined from the depth distributions of pheopigments and a photodegradation decay coefficient. Three kinds of information are needed to calculate pigment-specific rates of phytoplankton growth, microzooplankton grazing and macrozooplankton grazing:

1) the vertical distribution of pigments derived from water sampled without manipulation using bottles
2) the downward flux of pheopigment measured using sediment traps
3) radiation flux through the euphotic zone, measured using a profiling optical sensor.

As Landry (1994) has pointed out, the pigment budget approach has the advantage of an elegant model framework to understand the balance between phytoplankton production and grazing. It has the severe disadvantage of flaws in several of its underlying assumptions. For example, in theory, chlorophyl is converted quantitatively to pheopigments during feeding (Shuman and Lorenzen 1975). In practice, pheopigment is not necessarily a conservative tracer of grazing by protozoa or metazoa (Klein *et al.* 1986; Conover *et al.* 1986; Barlow *et al.* 1988; Kiørboe and Tiselius 1987; Lopez *et al.* 1988; Penry and Frost 1991; Head and Harris 1996), and several factors, including light, may contribute to the destruction of pheopigments in the water column. We have therefore not given the details of the calculations. Interested readers should consult the original papers.

ACID LYSOZYME ASSAY

The acid lysozyme assay is a measure of the immediate past history of *in situ* ingestion of bacteria by protists (Gonzalez *et al.* 1993; Vrba *et al.* 1993). The method measures *in vitro* activity of an enzyme present in protistan food vacuoles at an instant in time, and is analogous to methods used to evaluate copepod feeding behavior (e.g. Hassett and Landry 1982; Harris *et al.* 1986; see also section 8.5.14). Rapid processing of large numbers of samples is the major advantage of this approach. Disadvantages include the method's specificity for bacterial but not phytoplankton prey and a laborious calibration using data collected in parallel particle uptake experiments. Acid lysozyme hydrolyzes

peptidoglycan, a structural polymer characteristic of eubacterial cell walls. The assay is based on the quantification of lysozome activity in cell lysates at acid pH. The basis of the method is determination of lysozyme activity present in protistan food vacuoles by using a fluorochrome-linked artificial substrate, 4-methylumbelliferyl(-D-*N*,*N*′,*N*″)-triacetylchitotriose (MUF-$[GlcNAc]_3$) as an analog of peptidoglycan. The rate of MUF-$[GlcNAc]_3$ cleavage from the substrate in sonicated samples at acid pH distinguishes the activity of enzymes present in protozoan food vacuoles from those present via extracellular or intracytoplasmic lysozyme activity.

The protocol of Gonzalez *et al.* (1993) follows. Sea water samples are disrupted using a sonicator with a tapered probe at 50 W for two 10 s bursts. The reactions are done in capped 20 ml glass or teflon vials. To each vial 1.8 ml of sonicated sample is added and mixed with 1.8 ml acetate buffer (pH 4.5; 0.05 mol l^{-1}) and 0.4 ml of substrate solution (5 μmol l^{-1} final concentration). The vials are incubated at *in situ* temperature for 4 to 48 h, depending on the intensity of enzyme activity. The enzymatic reaction is stopped by adding 2 ml of glycine ammonium hydroxide buffer (pH 10.3; 0.05 mol l^{-1} glycine in 0.2 mol l^{-1} NH_4OH). Fluorescence intensity is measured using a fluorometer equipped with a 365 nm excitation filter and a >450 nm emission filter. Fluorescence is converted to the concentration of MUF-$[GlcNAc]_3$ using a standard curve prepared from dilutions of 5 mM MUF-$[GlcNAc]_3$ stock solution over the concentration range 0.1–10 nmol l^{-1} MUF-$[GlcNAc]_3$. Nanoplankton may be concentrated by centrifugation to increase fluorescence yields in samples with low protist abundance and low rates of bacterivory. Gonzalez *et al.* (1993) used two kinds of controls: boiled, sonicated samples (control without active enzyme) and 0.2 μm filtered unsonicated samples (controls without microbes to test for the presence of extracellular digestive enzymes). The control which yielded the highest fluorescence is used to correct fluorescence values from the experimental samples. The method is calibrated by a series of experiments to follow uptake of fluorescently labeled bacteria in the same sea water samples. Fluorescence data are converted to grazing rates from the relationship between lysozyme activity and uptake of fluorescently labeled bacteria (FLB). Gonzalez *et al.* (1993) found that acid lysozyme activity and FLB uptake were significantly correlated over four orders of magnitude of bacterivory rates. However, the slope of the relationship differed depending on the source of the water.

8.4.3 Direct methods to measure per capita grazing rates

FOOD TRACERS: INERT PARTICLES

A variety of surrogate food particles have been used as analogs for heterotrophic bacteria, protozoan, and phytoplankton prey. These include starch grains (Spittler 1973; Heinbokel 1978a; Kivi and Setala 1995), fluorescent paint chips (McManus and Fuhrman 1986) and fluorescent microspheres (e.g. Børsheim 1984; Bird and Kalff 1986; Nygaard *et al.* 1988). Methods using tracer particles have the advantages that experiments are simple in concept, have short incubation times, and involve minimal sample handling. Labor-intensive data collection by microscopy is a disadvantage. Grazing rates determined from inert particle uptake experiments are often lower than rates measured using other methods. This has been explained by a number of factors including discrimination against inert particles, lack of prey motility, and changes in the properties of prey cell surfaces. However, Sieracki *et al.* (1987) observed that a variety of nanoflagellate species egest the contents of their food vacuoles upon fixation with glutaraldehyde and formaldehyde, a phenomenon that would account for the lower

grazing rates obtained using particle tracer methods. They recommended using van der Veer's (1982) fixative as an alternative.

Several important caveats apply to the use of particle tracer methods (McManus and Okubo 1991):

1) the particles must be quantifiable inside consumer food vacuoles following ingestion
2) they cannot change in abundance due to factors other than grazing during incubation
3) the concentration of particles added must be sufficient to resolve a grazing signal, but not so great as to affect grazing rates by increasing the standing stock of the target prey items
4) the particles must behave like natural food items with respect to capture by the grazers and endocytotic digestive processes. That is, the consumers must not discriminate for or against the tracer.

The first three criteria are relatively easily satisfied, but the question of discrimination is more complex. Some studies report selection against some kinds of particles (e.g. Pace and Bailiff 1987; Nygaard *et al.* 1988), while others show no significant differences between consumption of natural food items and tracer particles (e.g. Bird and Kalff 1986; Sherr *et al.* 1987; Gonzalez *et al.* 1990). Although specific experimental conditions may be a factor in these divergent results, independent studies using natural food items indicate that at least some heterotrophic protists are able to discriminate among different kinds of food (e.g. Sanders 1988; Verity 1991), so the diversity of results with tracer particles is not unexpected.

The basic approach is reviewed by McManus and Fuhrman (1988), McManus and Okubo (1991) and Sherr and Sherr (1993b): an aliquot of suspended tracer particles is added to the natural protozooplankton assemblage. A tracer concentration less than 10% of the total particle field is recommended to avoid increasing the prey concentration and thereby altering the functional response and influencing feeding rates. Because sufficient tracer particles must be added to generate a feeding signal, this may not be practical, particularly when studying assemblages from oligotrophic waters. The mixture is incubated and sub-sampled at intervals over a few hours to follow the uptake of labeled prey. The sampled material is preserved, concentrated onto filters or into settling chambers, and examined by inverted, compound or epifluorescence microscopy, as appropriate. The number of consumers and the number of particles inside each consumer are counted. Per capita clearance rates are calculated as (equations after McManus and Okubo 1991):

$$\boldsymbol{F = P_{ind}/(C \times t)} \tag{8.18}$$

where F is clearance rate (ml consumer^{-1} h^{-1}), P_{ind} is the number of tracer particles per consumer, C is the standing stock of tracer particles during the incubation (number/volume), and t is incubation time in hours. Clearance rate is plotted as a function of time and is determined from the linear portion of the uptake curve. Ingestion rate is calculated by:

$$\boldsymbol{I = F \times C_0} \tag{8.19}$$

where C_0 is the total concentration of particles (tracer + natural) at the beginning of an experiment. Note however the potential bias of high food reduction during the incubation (section 8.3.2).

FOOD TRACERS: PREY CELLS

Methods employing prey cells to monitor ingestion have the same conceptual framework as inert tracers when applied to field assemblages and may also be used to measure *per capita* rates. To measure particle uptake rates in natural assemblages, the protocols and equations described in the previous section are used. To measure *per capita* rates in laboratory experiments, the protocols and equations described on page 314 are used. The use of 'real' prey cells as tracers of ingestion addresses the question of discrimination for or against inert particles. A variety of live or heat-killed, stained or unstained prey cells have been used in experimental studies. Examples of each are reviewed below. A variation of the method includes the use of genetically engineered 'minicells' (Wickner 1993).

Live cells with distinctive characteristics (morphology, pigments) may be used without staining. For example, Nagata (1988) and Landry *et al.* (1984a) differentiated chroococcoid cyanobacteria in protist food vacuoles on the basis of the intense orange autofluorescence of their phycoerythrin pigments. Under appropriate conditions, prey items with distinctive morphologies may be used in a similar manner. Figure 8.3 shows cells of the autotrophic dinoflagellate *Alexandrium catenella* inside the food vacuoles of several taxa of heterotrophic protists. These particular organisms were collected from an

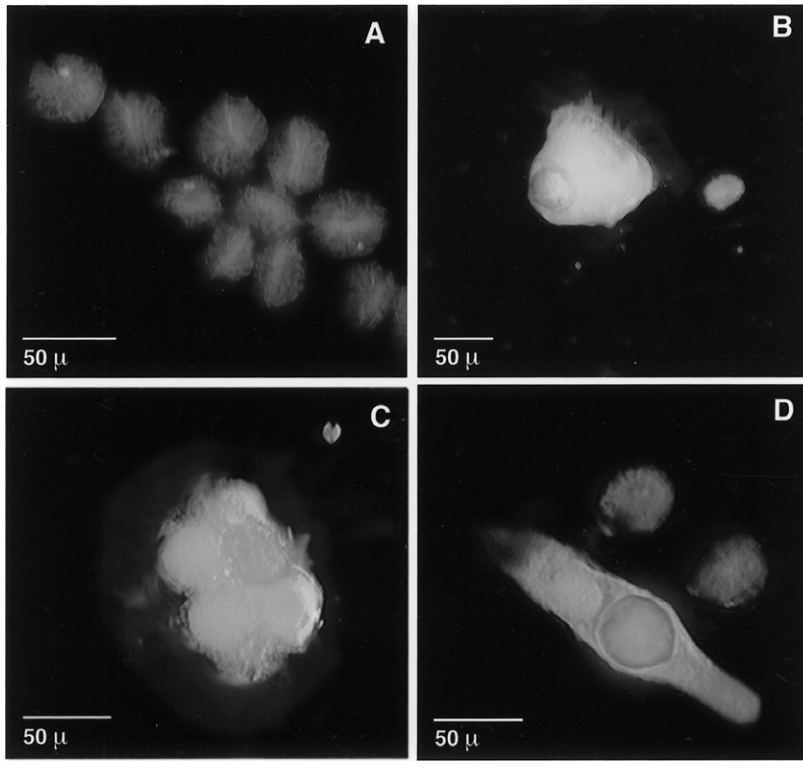

Fig. 8.3 Epifluorescence photomicrograph showing cells of the autotrophic dinoflagellate *Alexandrium catenella* (A), inside the food vacuoles of an oligotrich ciliate (B), the heterotrophic dinoflagellate *Polykrikos schwartzii* (C), and the litostomatid ciliate *Lacrymaria* sp. (D). Photographs: D.J. Gifford (see also Plate 6).

approximately 0.5 m thick layer of what was essentially a dinoflagellate monoculture, so that the potential prey field was extremely simple.

Bacteria and phytoplankton labeled with epifluorescent stain and then heat-killed have been widely used in particle uptake experiments. Methods to create fluorescently-labeled bacteria (FLB) and algal (FLA) cells were developed by Sherr and Sherr (1987) and Rublee and Gallegos (1989) respectively. Examples of studies employing these tracers to study grazing are cited in Table 8.1. Specific references should be consulted for details regarding the various stains and prey items used. The general procedure (Sherr and Sherr 1993b, 1993c) is to harvest the prey cells by centrifugation, suspend the pellet in buffer, add the epifluorescent stain and incubate with heat. The stained cells are concentrated by centrifugation, decanted and washed with saline, resuspended in buffer, and vortexed. Aliquots are transferred to plastic vials. The material is stored frozen or may be freeze dried. The concentration of FLB or FLA is determined by suspending a small aliquot in a small volume of buffer, sonicating, then collecting onto a black membrane filter and enumerating using epifluorescence microscopy.

The use of live, stained prey cells is an emerging technique which addresses the question of changes in the cell surface characteristics of dead prey. Li *et al.* (1996) used 5-chloromethyl-fluorescein diacetate (CMFDA) to stain laboratory cultures of two phytoplankton species and an oligotrich ciliate for use as prey items for natural assemblages of heterotrophic dinoflagellate predators. The prey cultures are stained with 1 μmol l^{-1} CMFDA for 45 to 60 min and an aliquot of labeled prey is added to the *in situ* assemblage containing consumers. The control treatment consists of the same volume of cell-free filtrate from stained cultures added to the assemblage. All treatments are incubated in the dark for a period of hours, then preserved with cold glutaraldehyde and stored in the cold and dark. Stained cells in consumer food vacuoles are enumerated from settled samples using an inverted microscope equipped for both transmitted light and epifluorescence. When using this method, one must determine the staining properties of the putative prey in advance: some cells require longer times to stain, while some require higher concentrations of stain.

FOOD TRACERS: RADIOISOTOPES

Single and dual radioisotope tracers have been used to measure per capita grazing rates of protozooplankters on bacteria and phytoplankton prey (Table 8.1). Single isotope methods follow uptake of one prey type by consumers. The dual isotope method has the advantage of discriminating between autotrophic and heterotrophic prey items. Both methods have the advantage of short incubation time, and their analytical sensitivity is high because prey can be labeled to high specific activities. Phytoplankton prey are typically labeled with ^{14}C-bicarbonate. In single isotope methods, heterotrophic bacteria may be labeled with ^{3}H-thymidine, ^{14}C-glucose or ^{14}C-amino acids; in the dual method they are labeled with ^{3}H-thymidine to distinguish them from photoautotrophs. The radioisotopes may be added either directly to raw sea water or to cultures, or natural prey assemblages may be prelabeled prior to addition to raw sea water.

The dual labeling technique is described below. Detailed protocols are found in Lessard and Swift (1985) and Neuer and Cowles (1995). The major disadvantages of the method are that it is extremely labor-intensive, and because of the need to sort grazers under the dissecting microscope, relatively few individuals are assayed. Both the dual label method and the single label method assume that (1) isotope recycling and excretion/egestion are negligible over experimental duration and (2) radioisotope label is neither adsorbed to the surfaces of grazer cells nor taken up osmotically by the

grazers. Neither Lessard and Swift (1985) nor Neuer and Cowles (1995) found these assumptions to be seriously violated in their applications of the dual label method. However, isotope cycling is problematic, even with very short incubation times, and trophic pathways may not be resolved unambiguously. Cycling of tracer is more likely to be a problem with the dual labeling method (Lessard and Swift 1985). For example, consumer uptake of ^{3}H, which is interpreted as grazing on bacteria, may result from secondary consumption of smaller bacterivores. Similarly, consumer uptake of ^{14}C, which is interpreted as grazing on phytoplankton, could result from feeding on bacteria secondarily labeled with the dissolved organic exudates of phytoplankton or grazers. The pre-labeling technique involving rinse and chase steps is intended to reduce the sizes of labile pools and minimize cycling problems (Hollibaugh *et al.* 1980; Taylor and Sullivan 1984). However, the intensive manipulation and handling of prey required before experimentation is likely to alter the natural assemblages. The magnitude of the isotope cycling problem may be substrate dependent: some labels are incorporated more rapidly and retained more efficiently than others (Nygaard and Hessen 1990; Turk *et al.* 1992; Caron *et al.* 1993).

In the dual label method, radioisotope labels are added to the natural assemblage, which is then incubated under *in situ* light and temperature conditions and sub-sampled at intervals over a period of several hours. Sub-samples collected include (1) aliquots filtered onto 0.2 mm polycarbonate filters to determine the amount of radiolabel taken up by the total particulate fraction and (2) aliquots pre-filtered through 20 μm mesh and then filtered onto 0.2 μm filters to determine the activity of the fraction smaller than 20 μm. The filters are rinsed several times with filtered sea water collected from the same source and placed into liquid scintillation vials. Radioisotope label in the protozooplankton grazers (typically ciliates and dinoflagellates) is determined by concentrating sub-samples of the labeled assemblage on a submerged 10–20 μm mesh, rinsing the mesh with several changes of filtered sea water, then sorting the grazers with a drawn pipette under a dissecting microscope. The grazers are sorted through three changes of filtered sea water and then placed into liquid scintillation vials containing perchloric acid to digest the grazer cells. Typically, three to five cells are placed into each vial, scintillation cocktail is added, and dpm (disintegrations per minute) of all samples are measured by liquid scintillation counting. Per capita clearance rates are calculated using Daro's (1978) three-compartment model:

$$\underset{q_1}{\overset{l_1}{\textbf{Water}}} \rightarrow \underset{q_2}{\overset{l_2}{\textbf{Prey}}} \rightarrow \underset{q_3}{\overset{l_3}{\textbf{Grazer}}} \rightarrow$$

where q is the concentration of isotope in each compartment and l is the rate of isotope exchange between compartments. If $q_1 \gg q_2$ and incubation time is short enough to reduce isotope recycling, the grazing rate is described by (equations after Neuer and Cowles 1995):

$$l_2 = 2 \times q_3/(q_2 \times t) \tag{8.20}$$

where t is time, the units of both compartments are dpm l^{-1}, and the unit of l_2 is h^{-1}. *Per capita* grazing rate is calculated by dividing both sides of equation 8.20 by the concentration of consumers, $n\,l^{-1}$ so that:

$$l_2/n = 2 \times q_3/(n \times q_2 \times t) \tag{8.21}$$

Setting $l'_2 = l_2/n$ and $q'_3 = q_3/n$,

$$\boldsymbol{l'_2 = 2 \times q'_3/(q_2 \times t)} \tag{8.22}$$

where l'_2 is the *per capita* clearance rate (volume cleared consumer^{-1} h^{-1}) and q_3 is the amount of label incorporated into each grazer cell (dpm cell^{-1}).

Variations on the method include manipulation of the size fractions of labeled material designated as 'prey'. Lessard and Swift (1985) used the particulate fraction smaller than 20 μm, reasoning that this fraction is the primary prey source for grazers larger than 20 μm. Neuer and Cowles (1995) argued that this size fraction is not appropriate for thecate heterotrophic dinoflagellates, which are able to consume a wide size range of prey, including items larger than themselves. Thus, they used the total particulate fraction as potential prey for the protozooplankton.

FOOD VACUOLE CONTENTS

The use of food vacuole contents to generate protozooplankton feeding rates is analogous to the gut fullness and gut fluorescence methods applied to crustacean zooplankton (e.g. Mackas and Bohrer 1976; Dagg and Wyman 1983). The method has the advantage that it involves minimal handling and is reasonably non-invasive. Laborious microscopical enumeration of consumers and prey is a disadvantage, as is the need for a high degree of taxonomic expertise when working with field assemblages. The vacuole contents method requires two kinds of information: (1) the average number of prey ingested per consumer and (2) the rate of prey digestion or vacuole turnover (Landry 1994). The first criterion is satisfied if the consumers can be sampled and preserved without significant loss of vacuole contents (Sieracki *et al.* 1987). The second is determined experimentally and may vary with environmental conditions (e.g. Dolan and Coats 1991a) and with prey type, size or quality (Bernard and Rassoulzadegan 1990; Gonzalez *et al.* 1990). In an early example of this method, Fenchel (1975) calculated ingestion rates of ciliates from an Arctic tundra pond from food vacuole contents of field populations and digestion rates of ciliates held in particle-free water in the laboratory. Kopylov and Tumantseva (1987) estimated *in situ* grazing rates for tintinnid ciliates from the average food vacuole contents of field-collected tintinnids and digestion rate data from individual ciliates held in a solution of sea water and detritus.

Dolan and Coats (1991b) used a variation of the method to study predacious hypotrich ciliates which feed on other ciliates. Ingestion rates were estimated using the appearance, inside the predator, of bacterivorous ciliates labeled with fluorescent microspheres. The method has the advantage that the prey remain mobile and presumably have unaltered surface characteristics. In Dolan and Coats (1991b) study, prey ciliates were exposed to fluorescent microspheres for 10 min, washed over a membrane filter with filtered sea water to remove excess spheres, and mixed into a culture of consumers. The labeled-prey mixture with predators was dispensed into a series of replicate incubation bottles. At 4 to 5 min intervals, the contents of an entire bottle were preserved with Bouin's fixative. Samples were analyzed by epifluorescence microscopy and the number of labeled prey in each cell counted. Ingestion rates were calculated as the slope of the linear regression of average number of prey ingested consumer^{-1} time^{-1}. Clearance rates were calculated as the number of prey ingested time^{-1} divided by the prey concentration.

PREY REMOVAL

Experiments to quantify grazing by protozooplankton which follow the disappearance of prey under controlled conditions employ the same design as classical experiments with crustacean zooplankton grazers (e.g. Frost 1972). In experimental treatments, consumer organisms are incubated with known concentrations of their putative prey. Control treatments consist of prey alone without grazers. All treatments are incubated for a time sufficient to resolve a statistically significant change in prey abundance. Examples of such experiments performed with a variety of protozooplankton taxa, including heterotrophic flagellates, ciliates, and dinoflagellates, are listed in Table 8.1.

Experiments are usually done using cultured protozoa and their prey. Culture methods for ciliates, heterotrophic dinoflagellates, and heterotrophic flagellates are given in Gifford (1985; 1993b), Lessard (1993) and Caron (1993), respectively. Protozoan consumers must be separated from their own prey by sorting through several changes of filtered sea water under a dissecting microscope. Some forms with hard parts can be concentrated onto mesh and resuspended (e.g. tintinnids). They are then added to the prey treatments (e.g. bacteria, cultured phytoplankton, other microheterotrophs) individually or as an aliquot of the sorted sample. Experiments can be incubated in any suitable container, but polycarbonate vessels are preferred because they can be cleaned thoroughly, and they are available in a wide range of sizes, from a few ml to many liters. Points to consider when performing experiments with planktonic protozoa include incubation without an airspace in the vessels to avoid damage and loss of delicate protozooplankton (Gifford 1985); bottle rotation to maintain consumers and prey in suspension (Stoecker and Guillard 1982; Jacobson and Anderson 1993); and the light regime (Strom 1998). Both Fenchel and Finlay (1983) and Verity (1985) have addressed the advantage of using consumer cultures in a state of balanced growth.

Grazing rates are calculated using the equations given in section 8.3. The correction for predator growth (equation 8.7) is especially important in this case, where the predator population may double over a 24-h incubation.

8.4.4 Direct methods to measure assemblage grazing rates

Direct methods involve manipulation of the protozooplankton assemblage in some way. They include sea water dilution, size-fractionation and use of metabolic inhibitors. In some cases one or more of the direct methods may be combined (e.g. dilution + metabolic inhibitors) or used in conjunction with indirect methods (e.g. dilution + tracer particles).

SEA WATER DILUTION METHOD

The sea water dilution technique (Landry and Hassett 1982), is the method currently in common use to measure grazing by the bulk protozooplankton assemblage. The method is attractive because it is simple in concept and execution. However, it is extremely labor intensive to collect and process the full suite of data from a dilution experiment. Because sea water is diluted rather than size-fractionated or concentrated, negative effects due to manipulation of the protozooplankton assemblage are minimized. The dilution method has the additional significant advantage of measuring phytoplankton growth and mortality due to grazing simultaneously. The method has also been used to quantify the impact of protozooplankton grazing on heterotrophic bacteria (e.g. Landry *et al.* 1984a; Ducklow and Hill 1985), but Landry (1994) cautions that the method is not

appropriate for this purpose because of the possible alteration of feedback relationships between dissolved pools and bacteria at high dilution levels.

The bulk protozooplankton assemblage is collected using water bottles and diluted with filtered sea water collected from the same source. A series of dilution treatments is set up, creating a gradient of grazer abundance. The treatments are incubated under *in situ* light and temperature conditions, typically for 24 h. Under reduced grazing pressure, the phytoplankton assemblage in each treatment grows at a rate which is a linear function of grazer density. The change in density of phytoplankton, P, with time, t, is similar to equations 8.2–8.4 earlier:

$$\boldsymbol{P_t = P_0 \times \exp(k - g) \times t} \tag{8.23}$$

or

$$\boldsymbol{(1/t)\ln[P_t/P_0] = k - g} \tag{8.24}$$

where k is the instantaneous rate of phytoplankton growth (d^{-1}) and g is the instantaneous mortality rate of phytoplankton due to grazing (d^{-1}). The term $\ln(P_t/P_0)/t$ is the 'apparent phytoplankton growth rate'. The apparent phytoplankton growth rate is plotted as a function of dilution factor. The y-intercept of this relationship is the 'true' phytoplankton growth rate, k, in the absence of grazing and the negative slope of the line is the grazing coefficient, g (Figure 8.4). The intercept (k) and slope (g) are calculated by linear regression analysis, with confidence intervals calculated for k, g, and the regression coefficient, r. The hypothesis that $g = 0$ is tested.

Phytoplankton growth may be monitored as changes in bulk chlorophyl (Landry and Hassett 1982; Gifford 1988), size-fractionated chlorophyl (e.g. Verity *et al.* 1993; Gifford

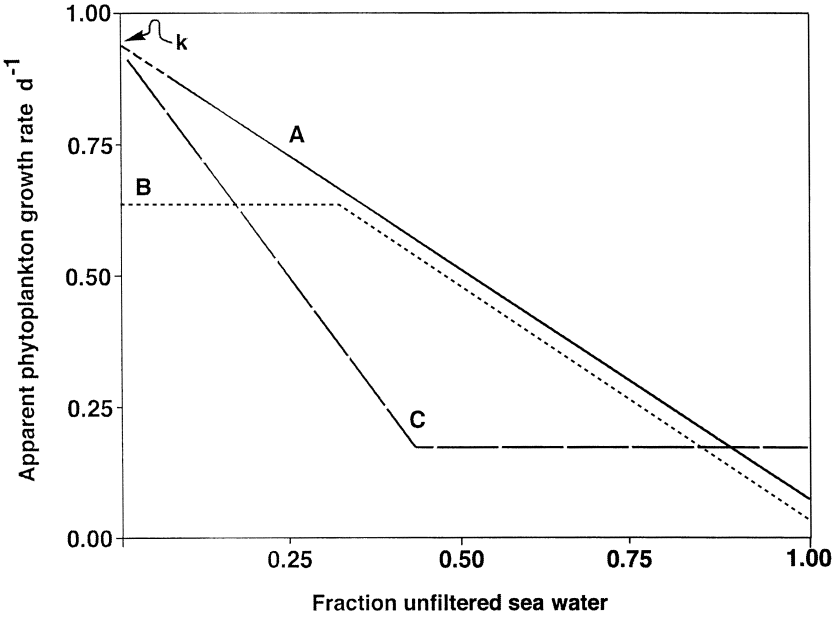

Fig. 8.4 Dilution curves. A, Linear curve, B, non-linear feeding with threshold at low food concentrations, C, non-linear feeding with threshold at high food concentrations.

et al. 1995), other pigments (e.g. Burkill *et al*. 1987; Strom and Welschmeyer 1991; McManus and Ederington-Cantrell 1992), cell counts from microscopy (e.g. Landry *et al*. 1984a; Weisse and Scheffel-Moser 1990) or flow cytometry (e.g. Landry *et al*. 1995). The latter technique is useful for rapidly quantifying grazing on cyanobacteria and other numerically abundant particles smaller than 20 μm, but flow cytometer design makes it difficult to examine sufficient volume to count larger cells with a high degree of statistical significance.

The method makes two important assumptions: (1) phytoplankton growth rates are density independent in the dilution series. That is, they are not affected by the presence or absence of other phytoplankton cells, and (2) phytoplankton mortality due to grazing is a linear function of grazer density in the dilution series. If experimental manipulation reduces prey densities below a feeding threshold, the method will overestimate both growth rate and grazing impact. In contrast, if *in situ* prey densities are saturating for ingestion, the per capita clearance rates of grazers may increase and growth and grazing impact will be underestimated. In practice, the method is quite robust to potential violations of its assumptions (Tremaine and Mills 1987a; Gifford 1988). Gallegos (1989) addressed the case where grazing rates were saturated at ambient conditions and non-linearly related to prey concentration: his three-point method evaluates phytoplankton growth rate at high dilution levels and can be used to evaluate the relationship between phytoplankton concentration and microzooplankton grazing. Evans and Paranjape (1992) proposed further iterations of the dilution model to fit experimental data under conditions of saturated grazing at high food concentrations.

Because this is a statistical method and errors are calculated for the instantaneous rates of growth and grazing, the relationship between k and g can be examined. There are three general cases for this relationship: $k > g$; $k < g$; and $k = g$. Note that it is possible that either k or g (or both) $= 0$. All of these cases have been observed in a variety of pelagic ecosystems. Under approximately steady state conditions, the relationship between k and g shifts over short time periods (order of days) (e.g. Landry *et al*. 1993), so that on average in the ocean, $k = g$ (Banse 1992).

WORKING PROCEDURES FOR THE SEA WATER DILUTION METHOD

We give here a description of the different steps in the sea water dilution method (Landry and Hassett 1982), which presently is the most widely used method to estimate protozooplankton community grazing. As Landry (1993) has emphasized, there is no standard protocol for a dilution experiment. The experimental design is adapted for the particular system under study, with consideration of factors such as the abundance of nano- and microplankton organisms, the volumes of water required to harvest the desired samples, and the number of replicate treatments.

Water collection

Collection and handling of water are done as described in Chapter 5. The bulk protozooplankton assemblage is collected using water bottles and then diluted with filtered sea water collected from the same source. Because of the time required to prepare filtered sea water for experiments and then set up the experimental treatments, it may be convenient to collect water and filter it on one day, maintain it at the appropriate temperature after filtration, then collect the microplankton assemblage and set up the experimental treatments the next day. Planktonic protists, particularly open ocean forms, may be sensitive to trace metal contamination. Thus, sea water for dilution experiments is collected using clean, metal-free bottles, such as teflon-lined Go-Flo

bottles, Niskin bottles outfitted with external springs; or Niskin bottles equipped with teflon-coated internal springs. All o-rings should be constructed of silicone or other material that has been tested and demonstrated to be non-toxic to microplankton organisms. It has been shown that the black tubing used in standard Niskin bottles is toxic to marine microplankton (Price *et al.* 1986). If such bottles must be used, the interior tubing should be replaced with silicone tubing and the o-rings should be replaced with silicone o-rings. Plastic powder-free gloves should be worn during all phases of water manipulation and experimental set-up in order to avoid trace contamination. This is particularly important when doing experiments with oceanic assemblages. All tubing, incubation vessels, carboys, etc. that contact sea water in a dilution experiment should be soaked in 10% HCl, rinsed copiously with high quality deionized water, and stored in clean plastic bags when not in use.

Filtration

Picoplankton, nanoplankton and microplankton can pass through filters commonly used to prepare 'particle free' sea water (Li and Dickie 1985; Li 1990; Stockner *et al.* 1990) and subsequently grow (Li and Dickie 1985; Li 1990). Because an overly vigorous filtration protocol may break up particles on the filter and increase the dissolved pool in the filtrate, promoting growth of any live cells that pass the filter, a 100% dilution treatment (i.e. 100% filtered sea water) should be done to check for growth of pigment-containing organisms that pass the filter and grow. The data can be corrected accordingly. A number of methods have been used to prepare filtered sea water for the experimental treatments. Filtration can be done by direct gravity flow from the collecting bottle or through a filter capsule or a large volume manifold constructed of teflon or other inert plastic material that can be thoroughly cleaned. Large (142 mm) methyl cellulose filters are recommended for this purpose because they produce minimal cell breakage and are less likely to add dissolved substances to the filtrate (Li and Dickie 1985; Li 1990). Filter capsules constructed of pleated methyl cellulose may also be used, provided they are presoaked for at least 1 h in 10% HCl made with high quality deionized water (e.g. Landry 1993).

Number of treatments and replicates

In principle, equation 8.24 can be solved for k and g using two dilution levels. In practice, most experiments include 4 to 6 dilution treatments with 2 to 3 replicates per treatment. The dilution treatments are distributed more or less evenly across a range spanning undiluted sea water (0% dilution) to 75% or greater dilution. A typical treatment series might include: 0%, 15%, 30%, 45%, 60% and 75% dilution treatments. Gallegos (1989) recommends adding 1 to 2 highly diluted treatments (95% to 99% dilution) to estimate k when the *in situ* phytoplankton concentration is sufficiently high to saturate micrograzer feeding rates. This situation pertains only in highly eutrophic systems and is unlikely to be necessary when working in oligotrophic or oceanic environments.

Filling the incubation bottles

The standard practice is to incubate dilution treatments in polycarbonate bottles. These have the advantage that they can be cleaned thoroughly. Dialysis bags and diffusion chambers have been used in some instances (Landry and Hassett 1982; Landry *et al.* 1984a), but these vessels are restricted to use with relatively small volumes of water. In extremely cold water, polycarbonate shatters (Stoecker, personal communication), and clean glass bottles may be used. Bottle volume should suit the purpose of the particular experiment. It should be large enough to minimize wall effects and to accommodate the

volume required for the samples to be collected. For example, samples collected for pigment analysis by HPLC may require several liters in oligotrophic waters.

The dilution treatments can be made in two ways. Measured amounts of filtered sea water and the microplankton assemblage can be siphoned into individual treatment bottles. Alternatively, it is easier to make a large volume stock of each treatment in an acid-cleaned polycarbonate carboy by siphoning the natural assemblage into filtered sea water which has been maintained at the appropriate temperature. The treatment is mixed gently with an acid-cleaned plastic spoon or paddle and then dispensed by siphon into the incubation bottles. Water should be transferred by siphoning through acid-cleaned silicone tubing, not poured, in order to minimize damage to planktonic protists (Gifford 1985). If water used to make dilution treatments must be passed through a mesh to exclude larger grazers, the mesh should be submerged, and the largest porosity mesh possible should be used. Such pre-filtering has to be used if meso/macro-zooplankton are abundant. To eliminate air pockets, which can have a detrimental effect on ciliates (Gifford 1985), incubation bottles are topped up, covered tightly with parafilm to exclude air, and capped.

The question of nutrient additions

Depressed phytoplankton growth rates have been observed when undiluted concentrations of phytoplankton are incubated in bottles without the addition of excess nutrients (e.g. Landry and Hassett 1982). It is best not to disturb the natural assemblage with additions of material. However, if nutrient levels are known or expected to be limiting, Landry (1993) recommends adding sufficient nutrient to allow 1 to 2 doublings per day of the phytoplankton assemblage. The addition should be done using as highly concentrated and as small a volume as possible. The nutrient additon is made to the stock of each dilution treatment before dispensing into treatment bottles as described above. Undiluted samples incubated with nutrients added are compared to undiluted treatments without nutrient addition to assess nutrient limitation.

The problem of initial treatments

In general, the same treatment bottles cannot be sampled at the beginning and end of an experiment because to do so would cause losses of some of the delicate microplankton organisms. Further, since the treatment bottles need to be incubated without airspace, replenishing the bottle volume after sampling is problematic. Different studies have used different 'initial' treatments. In some cases, a single 'pooled' sample is collected at the beginning of the experiment, and concentrations of the parameter being measured are calculated on the basis of dilution level in the remaining treatments. If this is done, it is important to evaluate the dilution level in all of the initial treatments by measuring something rapid and easy like chlorophyl as not all taxa dilute accurately (Gifford 1988). Alternatively, replicated initial treatments can be set up in parallel with the final treatments and incubated for 1 to 2 hours before harvesting the initial samples. The rationale for this is that most losses of planktonic protists due to handling occur immediately, and a period of stabilization produces better within-treatment replication.

Incubation conditions

Incubation can be done *in situ* or in on-deck (or on-land) incubators. *In situ* incubation takes advantage of ambient light and temperature conditions. The bottles are typically deployed on an anchored line (in shallow water) (e.g. Gifford 1988) or a floating array (in deeper water) (e.g. Landry *et al*. 1993). *In situ* incubation has the disadvantage that the bottles are not rotated. Rotation of incubation bottles maintains the microplankton

predators and prey in suspension. Not all studies using the sea water dilution method have rotated experimental bottles during the incubation period. However, gentle rotation is recommended to avoid two potential sources of error in the experiments. In the absence of rotation, prey organisms may settle during incubation and form an aggregate which renders them more susceptible to predation or allows them to escape predation by sinking to the bottom of the bottles. Gifford (1988) recommends that dilution experiments be incubated without an air space in the bottles because the combination of rotation and an air space is destructive to planktonic ciliates. Experimental bottles incubated on-deck are wrapped with an appropriate number of layers of neutral density mesh (plastic window screening) to simulate *in situ* light intensity. This is important because light adaptation by phytoplankton cells in dilution treatments can lead to underestimates of growth rate when pigments are used as a surrogate for cell abundance in well-mixed, eutrophic waters where the light field experienced by phytoplankton *in situ* is difficult to simulate (McManus 1995).

Data analysis

The data are analyzed as described on page 314. Equation 8.24 is solved for k and g, and the data are analyzed by linear regression, with confidence intervals calculated for k, g, and the regression coefficient, r. The hypothesis that $g = 0$ is tested. In experiments done under highly eutrophic conditions where the dilution curve is not linear, Gallegos' (1989) three-point method is used to solve for k and g. The apparent phytoplankton growth rate is plotted as a function of dilution factor. In the original presentation of the method, the initial dilution factor for phytoplankton was plotted on the y-axis (Landry and Hassett 1982). Landry (1993) later argued that dilution curves were more appropriately plotted as a function of *grazer* dilution. Gifford *et al.* (1995) compared the two methods and found no significant difference in the results. However, censusing all of the grazers in a dilution series is an extremely difficult undertaking: the grazers span a wide range of sizes and taxa, and several methods of preservation, visualization and enumeration are likely to be needed to enumerate them. In the end, the investigator will not know whether they have 'all' been counted. Thus, although Landry's recommendation is correct in theory, it is neither easy nor practical to put into practice and it has generally not been followed.

SIZE FRACTIONATION METHODS

Size fractionation is commonly used to study lower trophic levels (i.e., smaller organisms) within the microbial loop. The method is based on the assumption that filters or meshes of different porosity can separate protozooplankton consumers from their prey quantitatively. Grazing rates are then calculated by comparing prey growth rates in the presence and absence of predators (e.g. Capriulo and Carpenter 1980; Wright and Coffin 1983, 1984; Verity 1986; Kuuppo-Leinikki 1990; Kivi and Setala 1995). The method has the advantage that it is simple in concept. However, separation of consumers and prey is ambiguous, and the problems due to handling (Gifford 1985) and dissolved pool enhancement (Li and Dickie 1985; Li 1990) apply here. Further, concentration of protozooplankton on or against a mesh is biased toward retention or concentration of forms with hard parts, such as tintinnids. In concert with fixation artefacts, this may explain why both Verity (1985) and Capriulo and Carpenter (1983) concluded that the microzooplankton assemblages which they studied in Narragansett Bay, RI and Long Island Sound, NY were dominated by tintinnid ciliates; the soft-bodied aloricate ciliates which are typically more abundant than tintinnids were destroyed by size fractionation.

METABOLIC INHIBITOR METHOD

Metabolic inhibitor methods are most useful for measuring consumption of prokaryotes by phagotrophic protists. The premise of the method is to incapacitate a specific component or components of the microbial loop and measure other rate processes in its absence. Antibiotics which inhibit bacterial cell division include ampicillin, penicillin, vancomycin and chloramphenicol. Substances which inhibit grazing by eukaryotic consumers include colchicine, cyclohexamide, griseofulvin, and neutral red (e.g. Fuhrman and McManus 1984; Campbell and Carpenter 1986; Sherr *et al*. 1986; Tremaine and Mills 1987b; Weisse 1989; Caron *et al*. 1991). The method has the advantage of being simple in concept. Its disadvantages include non-specificity of the inhibitors, their potential toxicity to non-target organisms, and secondary effects on trophic pathways (e.g. Li and Dickie 1985; Sanders and Porter 1986; Sherr *et al*. 1986; Taylor and Pace 1987).

8.5 MESO- AND MACROZOOPLANKTON

Meso- and macrozooplankton are defined as being retained on meshes of 200 μm and 2000 μm, respectively, and they include metazoans varying in size from rotifers and copepod nauplii to large cnidarians within the classes Scyphozoa and Siphonophora. The high diversity within this zooplankton assemblage therefore makes it impossible to present methods suitable for all kind of organisms here and the investigator has to critically evaluate which constraints the target organism will give to the potential method.

A quick overview of methods discussed in this chapter is given in Table 8.2. The table focuses on the gain/effort aspect, i.e. the resources needed in relation to the quality of results from the different methods. It does not say anything about in which type of studies the different methods should be used, but we return to that question at the end of this chapter. We can see that the laborious methods also need well qualified professionals but that this does not mean that the accuracy and precision in the method is high. However, as a rule of thumb, the laborious methods also give more information about feeding, e.g. food selection, prey-size preferences etc.

8.5.1 Empirical relationships

Some general biological relationships roughly quantify feeding. From published data we know how environmental factors affect feeding rates of a given zooplankton species. These relationships are covered in section 8.8. Empirical relationships for closely related predator/prey combinations give rough estimates of feeding rate for a defined condition (e.g. food type and concentration, temperature). At the community level, precision will be very low due to the complexity. For example, general production/biomass (P/B) relationships have been published for different trophic levels, with zooplankton assigned a value of 10 (Dickie 1972). If this factor is combined with a growth efficiency of HO.33 (Kiørboe *et al*. 1985a; Peterson 1988; Båmstedt *et al*. 1999), total annual feeding can be calculated from the average zooplankton biomass multiplied by 10/0.33 (= 30). However, the value of such calculations is limited because they do not consider local conditions of trophic and taxonomic structure, length of productive season, age structure, etc. The same is true when applying the concept of 'transfer efficiency', i.e. the efficiency by which the production at one trophic level is transferred to production at

Table 8.2 Overview of common methods used to measure the feeding rate of meso and macrozooplankton; the columns should be understood as follows. Labor: time needed for analysis of a sample. Need of skill: practice needed to be able to do the analyses with maximum quality of the results. Accuracy/precision: variability due to the method. Uncontrolled problems: variations not caused by feeding and not possible to quantify. Information value: the amount of information in excess of the bulk feeding rate given.

Method	Labor	Need of skill	Accuracy/precision	Uncontrolled problems	Information value
P/B ratio and growth efficiency	Very low	Very low	Very low	None	Low
Gut fluorescence	Low	Very low	Low	Several	Low
Gut content analysis	High	Very high	Low	Several	High
Copepod mandibles in stomach	Very high	Very high	Quite high	Few	High
Balanced energy budget	Very high	High	Very low	Several	Low
Energy-budget relationships	High	High	Low	Several	Low
Radiotracer	Low	High	High	Few	Low
Food removal and microscopy	Very high	Very high	Very high	Few	High
Food removal and bulk analysis	Very low	Very low	Rather low	Few	Low
Digestive enzymes	Very low	Very low	Very low	Many	Low

the next (Begon *et al.* 1990). The 'pigment budget' (page 307) suffers from the same lack of resolution of food web components. Because these parameters blur the complexity of natural ecosystems, we caution against using 'bulk parameters' such as size fractions, total zooplankton, chlorophyl *a*, particulate organic carbon (POC), etc. as parameters in calculations of trophic relationships at the community level. However, use of published values and relationships for clearance or ingestion rate might provide a reasonable pilot approach to quantify feeding at the species level.

8.5.2 Field investigation on gut fluorescence

The development of the fluorescence method to measure chlorophyl pigments (Yentsch and Menzel 1963) provided scientists working on mesozooplankton feeding with a tool sensitive enough to measure chlorophyl *a* and derived pigments in zooplankton guts (Nemoto 1968; Mackas and Bohrer 1976). The principle behind the method is that the pigments from the ingested algae can be quantitatively recovered from the animal by extracting them in an organic solvent. This gives the amount of gut contents, and knowing the turnover rate of gut contents, ingestion rate can easily be calculated. Changes in the gut content (G) are determined by the balance between ingestion (I) and egestion (E), i.e.:

$$\mathbf{d}G/\mathbf{d}t = I - E \tag{8.25}$$

If we assume that the gut evacuation is described by an exponential model, i.e. that a constant fraction of the gut contents is evacuated per unit time then:

$$E = k \times G \tag{8.26}$$

where k is the gut clearance coefficient. If the feeding intensity is constant then a steady state will be established and gut content does not change, i.e.:

$$\mathbf{d}G/\mathbf{d}t = 0 \tag{8.27}$$

Combining equations 8.25–8.27 gives the working equation for the method, i.e.:

$$I = k \times G \tag{8.28}$$

The method is successful because of its analytical simplicity and the fact that it provides data directly from the field and does not require incubations. It has been especially useful to study diel feeding and to make quantitative comparisons of feeding rates on phytoplankton in the field. Uncertainty about pigment destruction during digestion, and its restriction to chlorophyl-bearing prey are the main weaknesses of the method (page 327). It provides a 'bulk' estimate of phytoplankton, and does not resolve the different pigment compositions of various phytoplankton taxa. However the use of the HPLC to separate pigments provides some basis to evaluate selectivity between phytoplankton groups (Kleppel and Pieper 1984; Buffan-Dubau *et al.* 1996). Dam *et al.* (1994) proposed that the simple gut fluorescence method applied to copepods can be combined with egg production measurements to calculate herbivorous and total feeding (section 8.7.2). Another modification of the gut-fluorescence method is to use it to study the time course of gut filling during 1–3 h experiments (Dagg 1983; Dam 1986; Head 1986). Copepods with empty guts are transferred to an algal food suspension and sub-samples taken at 5 to 10 min intervals to monitor the rate of pigment uptake. Short-term ingestion rates are calculated as the initial slope of the mean pigment content plotted

against time. Although this method combines the problems of bottle incubations and pigment destruction it can be designed to provide information on the dynamics of gut filling in relation to food type and quantity, time of day etc.

SAMPLING

Towing speed to collect zooplankton for gut fluorescence analysis reflects a compromise between reducing stress (requiring low speed) and reducing towing time (requiring high speed) in order to avoid gut evacuation during sampling. The capacity of the sampling gear is important, and a large plankton net can compensate for a tow of short duration. Stratified samples can be obtained with Longhurst Hardy Plankton Recorder (LHPR) type nets, where most animals are killed upon capture. Atkinson *et al.* (1992) showed that gut fluorescence measurements made on copepods collected using an LHPR were at least as high as those obtained from copepods collected from vertical tows of a conventional net. Stratified sampling devices where animals remain alive in the cod-end for long periods should be avoided. A formula for designing the actual sampling is given in equation 8.41. This can be especially useful when the target organisms are rare.

Many copepod species exhibit diel variations in feeding activity (reviewed by Haney 1988). If the goal of the investigation is to quantify grazing over a full day, the sampling schedule must be designed appropriately over 24 h. Higher sampling frequencies resolve more details of the diel variation. However, due to logistical constraints a diel sampling cycle seldom includes more than six time points, i.e. sampling every four hours. If the goal of the study is to examine short-term maximum grazing impact, then more effort should be put into night sampling. However, we caution against guessing the diel grazing cycle as the basis for an investigation, and recommend a pilot study first.

PREPARATION FOR ANALYSIS

Gut fluorescence can be reduced more than 50% during a 20 min sorting procedure with live animals (Kleppel *et al.* 1988), and even slight stress can cause copepods to defecate (Head 1988). The best way to reduce defecation losses is to freeze the copepods as soon as possible. To do this, the contents of the cod-end are filtered through a small piece of mesh, the mesh is folded double (without squashing it), wrapped in aluminum foil, and frozen immediately in liquid nitrogen. Liquid nitrogen is superior to ordinary freezing because it freezes material instantaneously. Sorting and preparation of the analytical samples are done later on thawed material. If manipulations have to be done before freezing the animals, the use of an anesthetic, such as MS-222 or soda water is advised (Kleppel *et al.* 1988). However Morales *et al.* (1990) showed that maintenance of copepods in soda water for periods longer than 2 h significantly reduced gut fluorescence.

GUT CLEARANCE COEFFICIENT

A good estimation of the gut clearance rate is a fundamental factor in order to have an accurate measurement of the ingestion by gut fluorescence (Peterson *et al.* 1990a). The content of the cod-end is transferred to a container of filtered sea water at ambient temperature. Samples are removed and frozen at different time steps, usually every 5 to 10 min. The gut clearance coefficient depends on both the model applied and on the time over which data are used (Morales and Harris 1990). Lack of knowledge about copepod gut dynamics makes the choice of an appropriate model difficult. The method of using animals in starved condition has been criticized because the gut dynamics of feeding and non-feeding copepods may not be the same (Baars and Helling 1985; Penry and Frost 1990). Nevertheless, when considering the initial 20 to 30 min of an experiment, gut

clearance rates seem to be similar for feeding and non-feeding copepods (Kiørboe and Tiselius 1987).

We recommend that experiments be run over 1 h and that the data scatter be evaluated before deciding to extend the initial period used for the calculation of k, since this depends on the species of consumer, prey species, and temperature. Stress induced by the high frequency of sampling in the sea water container may produce another source of error. This kind of error can be reduced by keeping the sub-samples in separate bottles and emptying only one at a time. In order to obtain a precise estimate of k we recommend at least eight points along the time axis. A certain number of replicates per sampling point are necessary in order to reduce the error inherent in the method (Tseytlin 1994). With a minimum of three replicates, the total number of samples for the experiment would be 24.

The gut clearance coefficient, k, is usually derived from a model of exponential decrease in gut fluorescence over time, assuming that a constant percentage of the gut content is evacuated per unit time (Baars and Oosterhuis 1984; Kiørboe *et al.* 1985b; Christoffersen and Jespersen 1986):

$$\boldsymbol{G_t = G_0 \times \exp(-k \times t)} \tag{8.29}$$

where G_t is the gut content at time t, and G_0 is the initial gut content.

An alternative linear model implies that a constant amount is evacuated per unit time (Kiørboe *et al.* 1985b; Christoffersen and Jespersen 1986):

$$\boldsymbol{G_t = G_0 \times (1 - k \times t)} \tag{8.30}$$

Huntley *et al.* (1987a) suggested that the most appropriate model to describe gut content after cessation of feeding is a power function of the form:

$$\boldsymbol{G_t = G_{max} \times t - \theta} \tag{8.31}$$

where G_{max} is the maximum gut fullness, and θ is a dimensionless constant, related to the gut clearance coefficient by:

$$\boldsymbol{k = \theta / t} \tag{8.32}$$

This model accounts for a gradual decrease of k with time (Head 1986; Wang and Conover 1986), where the exponential model assumes that k is constant. Using the power model, ingestion is calculated by:

$$\boldsymbol{I = \theta \times G / t'} \tag{8.33}$$

where t' is time since maximum gut fullness (Huntley *et al.* 1987a) and the other terms are as defined above. The major problem with this approach is that it assumes that maximum gut fullness equals the initial gut pigment in the gut clearance experiment, so that several experiments are necessary to determine when the animals' guts are maximally full.

Another method to estimate gut clearance rate uses the production rate of fecal pellets in incubation experiments (Dagg and Walser 1987; Peterson *et al.* 1990a). Animals are incubated in bottles for periods ranging from 5 to 24 h. Fecal pellets are collected and counted at the end of the experiment (Peterson *et al.* 1990a; Irigoien *et al.* 1996), or the total amount of chlorophyl pigments in fecal pellets is measured (Dagg and Walser 1987; Pasternak 1994). The gut clearance rate is calculated as:

$$\boldsymbol{k = P / (t \times n)} \tag{8.34}$$

where P is the total number of fecal pellets produced per individual, t is the incubation time (min) and n is the average number of fecal pellets in the copepod gut. When measured as chlorophyl derivatives the formula is:

$$\boldsymbol{k = cfp/(t \times G)} \tag{8.35}$$

where *cfp* is chlorophyl-related pigments from the fecal pellets (ng chl. ind^{-1}) and t and G are as defined above. Gut clearance rates obtained with this method are generally higher than those obtained from the decrease of gut fluorescence in non-feeding copepods (Peterson *et al.* 1990a).

Dam and Peterson (1988) observed that 70% of the variability in k is explained by temperature (T °C) and proposed an equation describing this relationship under saturated food conditions:

$$\boldsymbol{k = 0.012 + 0.001 \times T} \tag{8.36}$$

Other studies have given similar relationships (Uye and Yamamoto 1995; Irigoien 1998; Mauchline 1998). Dam (personal communication) has evaluated the original data by Dam and Peterson (1988) and suggests a modification that better fits a realistic Q_{10} value (see page 376). His new equation is:

$$\boldsymbol{k = 0.0124e \times \exp(0.07675) \times T} \tag{8.37}$$

As indicated earlier, food concentration also influences the gut clearance coefficient (Dagg and Walser 1987; Pasternak 1994) and the relationship with temperature may even disappear when the food is strongly limited (Irigoien *et al.* 1996). Reports also include presence (Baars and Oosterhuis 1984; Head 1986; Irigoien 1994) and absence (e.g. Wang and Conover 1986; Batchelder 1986; Head *et al.* 1988) of a relationship between initial gut fluorescence and the gut clearance coefficient. Because of the uncertainty about the importance of feeding level the practice of feeding animals with a phytoplankton culture prior to the gut clearance experiment should be avoided and freshly collected field animals should be used instead (Irigoien 1998). An alternative procedure could be to acclimate animals for 2 to 4 h before the experiment in water collected from the zooplankton sampling site (Kleppel, personal communication).

An increasing number of studies use values of k taken from literature. However, the gut clearance coefficient can vary significantly between species in the same community measured in the same experiment (Atkinson *et al.* 1996b). Unfortunately there is a paucity of studies about mechanisms controlling gut clearance rate. We recommend measuring the gut clearance coefficient in several experiments which include all species and the full environmental range of the community under study.

SORTING ANIMALS

Animals are sorted by species and stage (if necessary) under dim light. Morales *et al.* (1990) showed that a 40 min exposure to a dim light had no effect, but 5 to 10 min exposure to sunlight reduced gut fluorescence significantly. The number of animals per sample depends on the size and feeding activity of the animals, and on the sensitivity of the fluorometer used for the analysis. For large, actively feeding copepods, analysis of individuals is possible, but as a rule of thumb about 20 adult copepods of medium size should be pooled for an accurate measurement. Variability can be high, and a minimum of three replicates per sample is recommended.

EXTRACTION

Chlorophyl pigments from the guts are extracted in 4 to 10 ml of either 90% acetone or 100% methanol at 4 °C in the darkness. The extraction does not require homogenization (Huntley *et al.* 1987b; Morales *et al.* 1990) and nearly 100% of the pigments are extracted from whole copepods after 2 hours (Huntley *et al.* 1987b), although methanol was used by these authors. No significant differences have been found between overnight extraction (17 h) and nearly two days extraction (40 h) (Morales *et al.* 1990). Avoiding homogenization allows a recovery of the carcasses after analysis for accurate taxonomic identification and size measurement. However, large-sized copepods from Antarctic waters gave more efficient chlorophyl extraction when homogenized (Atkinson, personal communication), this cautions the procedure for larger zooplankton.

Kleppel *et al.* (1988) developed a more elaborate extraction technique to measure the gut fluorescence of individuals. The net gain in sensitivity with this technique is about 8-fold. Another method has been developed recently to measure individual gut fluorescence of small-bodied species (Takatsuji *et al.* 1997). Both methods are based on the use of small volume cuvettes for the measurement. The gain in sensitivity is about 2-fold over a traditional cuvette with 4 ml.

PIGMENT ANALYSIS

Measurement is done following the standard fluorometric procedure for chlorophyl *a*, with readings taken before and after acidification (Parsons *et al.* 1984). Commonly a large percentage of the chlorophyl-derived pigments in copepods are pheopigments, and it has been assumed that they represent pheophorbides (chlorophyl minus Mg atom and the phytol chain) which have been converted from chlorophyl with 100% efficiency on a molar basis (Shuman and Lorenzen 1975). Therefore a conversion factor of 1.51 has been applied to pheopigments by some authors because the molecular weight of pheophorbide is 66.3% that of chlorophyl (Dagg and Wyman 1983; Landry *et al.* 1984b). Copepod fecal pellets can contain not only pheophorbides (Jeffrey 1974; Bathman and Liebezeit 1986) but also pheophytin *a* (chlorophyl *a* minus Mg atom) (Hallegraef 1981; Gieskes *et al.* 1991). Gieskes *et al.* (1991) suggest that pheophorbides are formed after degradation of chlorophyl *a* to pheophytin and not to chlorophylides. Because pheophorbides and pheophytin are impossible to distinguish fluorometrically and have different molecular weights (869.16 for pheophytin *a* and 590.65 for pheophorbide *a*), the formula usually used to calculate pheopigments (Strickland and Parsons 1972) expresses pheopigments in terms of equivalent chlorophyl weight (Helling and Baars 1985; Conover *et al.* 1986). The problems of interference by chlorophyl *b* and *c* remain in the fluorescence method, but those problems are common to all estimations of chlorophyl *a* by fluorescence.

Some measurements on tracer pigments, such as fucoxanthin, peridinin, neoxanthin or others, have been done by HPLC providing data on selective feeding by copepods (Kleppel and Pieper 1984; Buffan-Dubau *et al.* 1996) and avoiding interferences by chlorophyl *b* and other chlorophyl pigments. HPLC measurements require higher number of animals, 50 to 150 (Kleppel and Pieper 1984), 400 to 500 (Buffan-Dubau *et al.* 1996; Meyer-Harms, personal communication). Nevertheless, as for chlorophyl *a*, a variable percentage of tracer pigments can be destroyed during the gut passage (Kleppel 1998). Problems could appear with the C18 column in the HPLC system with high latitude copepods because of their high lipid content (Meyer-Harms, personal communication). Also HPLC analysis is expensive and time consuming and in consequence not suitable for large numbers of samples.

Gut fluorescence measurements are generally given in ng chlorophyl *a* equiv. ind^{-1}. In order to facilitate comparisons it would be advisable to standardize results to μg chl *a* equivalents $\mu g\ c^{-1}$. However, when body size may be a confounding variable, the use of multivariate statistics or other statistical techniques is preferable when evaluating differences between results (Dam and Peterson 1991).

TRANSFORMATION TO CARBON

Carbon is often used as a general exchange unit in the calculation of fluxes and ecological models. The C:chlorophyl *a* ratio varies from 15 to 80 (Peterson and Festa 1984), but an average estimate is 40 to 50. Precautions have to be taken when using data from environments with extreme light conditions, where C:chlorophyl *a* ratios can be highly variable.

PIGMENT DESTRUCTION

Based on the work of Shuman and Lorenzen (1975) it has been generally accepted that chlorophyl pigments are not destroyed or degraded to non-fluorescent compounds during copepod digestion. Nevertheless, some authors report losses as high as 95% (Conover *et al*. 1986), but with a variability ranging from 0% to 95% (Lopez *et al*. 1988; Penry and Frost 1990; Head and Harris 1992; Mayzaud and Razouls 1992; Cary *et al*. 1992). Others report lower losses, in the range of 10% to 30% (Helling and Baars 1985; Dagg and Walser 1987; Kiørboe and Tiselius 1987; Pasternak and Drits 1988; Pasternak 1994; Dagg *et al*. 1997). On the other hand, experiments comparing different techniques to estimate feeding rates obtain reliable results with the gut fluorescence method (Kiørboe *et al*. 1982; Baars and Franz 1984; Kiørboe *et al*. 1985b; Peterson *et al*. 1990a).

Pasternak (1994) suggested that the very high destruction values measured in budget experiments are due to losses of fecal material and to destruction of pigments from fecal pellets related to bacterial activity, reducing pigment destruction by digestion to 10% to 20%. Those results agree with those of Gieskes *et al*. (1991) where the pigment destruction was negligible during the first 10 h of an experiment, but contradict the findings of Head and Harris (1996) who observed no pigment losses from fecal pellets over a 4-day period. The latter result may be due to reduced bacterial and protozoan activity at the low temperature (1 °C) of the experiment.

Another source of losses in budget experiments not related directly to digestion can be sloppy feeding (Conover *et al*. 1986; Kleppel 1998). Sloppy feeding can be an important mechanism releasing dissolved compounds (Dacey and Wakeham 1986) and very small particles (Roy *et al*. 1989). Few studies have quantified sloppy feeding, but the losses can be as high as 26% to 35% of the ingested material (Roy *et al*. 1989) and it seems reasonable that the results can be highly variable depending on grazer species, prey species and prey concentration. This would be in agreement with the results from Head and Harris (1996) who found that neither chlorophyl nor pheopigment was lost as copepods with food in their guts were allowed to defecate in filtered sea water, suggesting that most of the chlorophyl destruction occurred at an early stage of feeding.

Finally, in a very recent work Goericke *et al*. (1999) have found indications that about 40% to 70% of Chl *a* is degraded to a nonfluorescent compound, 13^2, 17^3 cyclopheophosbide (Cpp 516). However, depending on the extraction conditions (time and oxygen) Cpp 516 may partially degrade to compounds that fluoresce as 'normal' pheopigments. Consequently the variations in the pigment destruction level could be a consequence of the differences in the extraction protocol.

In any case, an important controversy remains about the importance of pigment

destruction during gut passage and further investigation is necessary to determine to what extent ingestion can be accurately estimated from gut pigment contents.

A practical method to check pigment destruction is to compare the disappearance of chlorophyl with the appearance of pheopigments during incubation experiments with copepods enclosed in bottles (Dagg *et al*. 1997). In that case it is recommended to filter all the content of the bottle to avoid losses of fecal material, as well as to use very fine filters (GF/F) to reduce the losses of the very small particles produced by sloppy feeding (Roy *et al*. 1989).

The gut fluorescence method only provides a coarse estimate of ingestion rate and it does not provide any information on the composition of the diet. The use of it is therefore restricted to studies where the precision and detailed information about food composition is of minor importance.

8.5.3 Working procedures for the gut fluorescence method

EQUIPMENT

The equipment necessary for gut fluorescence is divided in three parts.

Collecting the sample

To collect the sample a WP2 net is usually the best compromise due to its general availability and simplicity of use. Any other type of net can be used if the towing and manipulation time are not excessive. A non-filtering cod-end is advised. If samples have to be handled before freezing, animals should be anesthetized using soda water or MS-222.

Freezing the sample

We recommend that liquid nitrogen be used to freeze samples. This method has the advantage of instantaneous and complete freezing, and samples can be stored in the same liquid nitrogen. As a practical matter, a 10 l liquid nitrogen container is easy to carry even on small size boats and offers a good compromise between storage capacity and storage time. If liquid nitrogen is not available a deep freezer or freezing sprays can be used. In the latter case, the sample must be frozen quickly and the spray must not interfere with measurement of fluorescence.

Measuring gut fluorescence

The chlorophyl *a* content of the grazers is measured with a fluorometer. Any standard model instrument can be used. Some models adapted for field work provide additional flexibility to work under difficult conditions. When experiments to determine a gut clearance rate are done, a filtration unit is needed to produce filtered sea water.

SUPPLIES

Liquid nitrogen is required to freeze the samples and 90% 'analytical' or better (e.g. HPLC) grade acetone is used for fluorometric analysis of the samples. Use of 100% high purity grade methanol can be used as an alternative.

PROCEDURE

Gut content (Fig. 8.5a)

The contents of the cod-end are filtered through a small piece of net, the net is folded double (without squashing it), wrapped in aluminum foil and frozen immediately in liquid nitrogen.

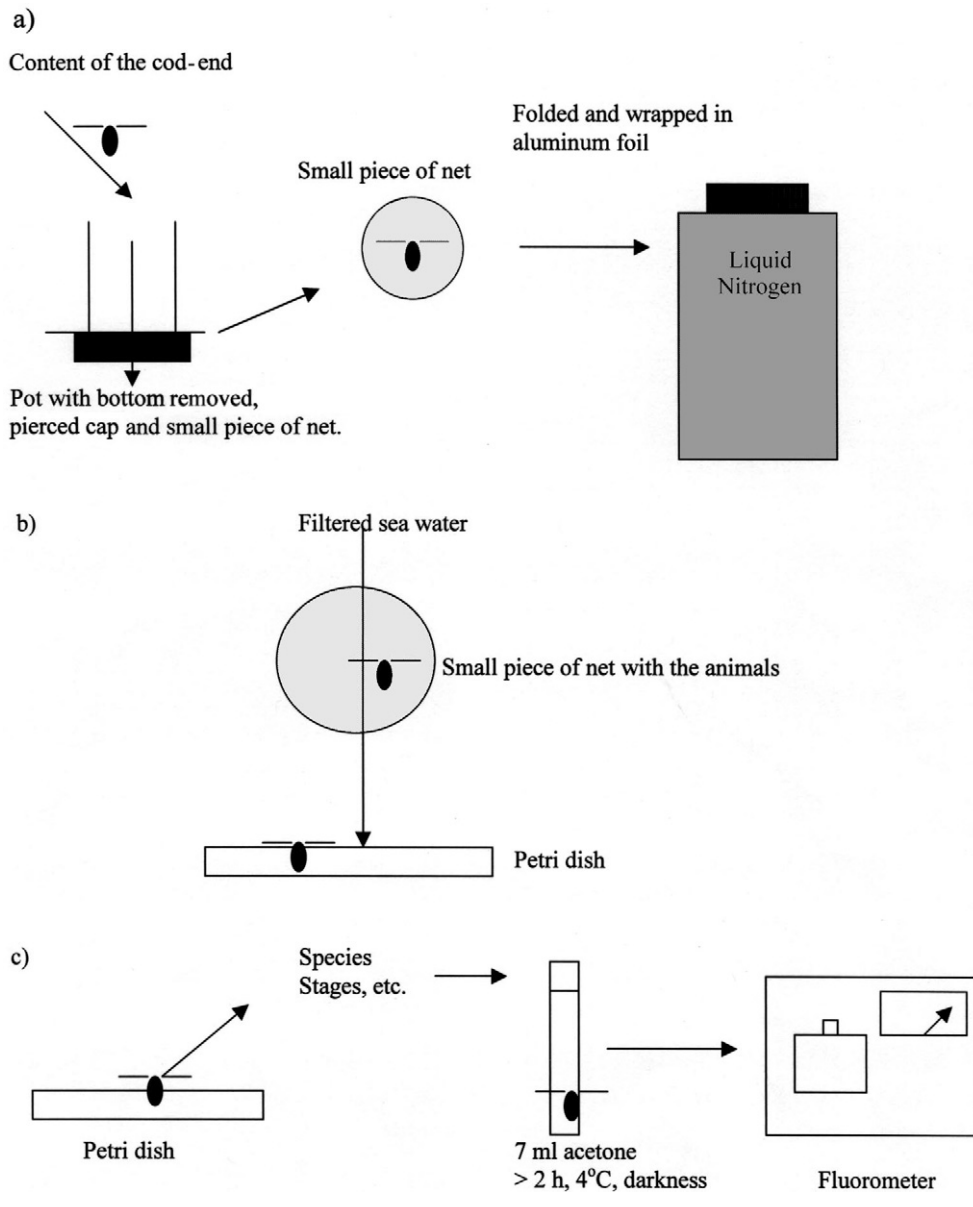

Fig. 8.5 Working principles for the gut fluorescence method.

Calculating the gut clearance coefficient

Immediately on retrieval of the net the content of the cod-end is transferred to a container of filtered sea water which is maintained at the field temperature. Samples are collected at different time steps (usually each 5 to 10 min after time 0) using the procedure described on pages 323 and 328.

Sorting animals (Fig. 8.5b)

The sample is rinsed from the small piece of net as quickly as possible into a petri dish with filtered sea water. Animals are sorted by species and stage (if necessary) under dim

light using a stereomicroscope. The number of animals needed is a function of animal size and phytoplankton concentration, but usually about 20 adult individuals of medium size copepods (> 1 mm) are sufficient. Because variability can be high, we recommend a minimum of three replicates per sample. If this analysis takes more than 10 to 15 min, then divide the material into portions that are defrosted and analyzed in turn, so that exposure to light and warmer conditions is minimal. Ice chips during the sorting procedure help keep the temperature low.

Extraction (Fig. 8.5c)

Sorted animals are placed into 4 to 10 ml of 90% acetone at 4 °C in darkness. Extraction does not require homogenization (Huntley *et al.* 1987b; Morales *et al.* 1990) and nearly 100% of pigments are extracted after 2 h (Huntley *et al.* 1987b). For larger ($\geqslant$ 10 mm) zooplankton species we recommend some trial tests to determine whether homogenization is necessary.

MEASUREMENT AND CALCULATIONS

Chlorophyl is measured by the standard fluorometric procedure, with reading before and after acidification with 10% HCl (Parsons *et al.* 1984). A high proportion of phytoplankton pigments in zooplankton guts is usually in the form of pheopigments. The amount of gut pigments is calculated as:

$$\textbf{Chl } \boldsymbol{a} = \boldsymbol{C} \times (\boldsymbol{F_o} - \boldsymbol{F_a})/\boldsymbol{n} \tag{8.38}$$

$$\textbf{Pheopigment} = \boldsymbol{C} \times (\boldsymbol{R} \times \boldsymbol{F_a} - \boldsymbol{F_o})/\boldsymbol{n} \tag{8.39}$$

where C is the fluorometer calibration constant, F_o and F_a are the fluorescence readings before and after acidification, R is the acidification ratio and n is the number of individuals. The gut content in units of chlorophyl a equiv. ind^{-1} is then calculated as

$$\boldsymbol{G} = (\textbf{Chl } \boldsymbol{a} + \mathbf{1.51} \times \textbf{Pheopigment}) \tag{8.40}$$

and equation 8.28 is used to calculate ingestion rate. The gut clearance rate constant, k, is derived from a model of exponential decrease in gut fluorescence in the filtered sea water through time, given in equation 8.29.

COMMENTS AND SPECIAL PRECAUTIONS

It is important to avoid unnecessary delays before freezing the sample once the net is on board. Labels written on aluminum foil are easily erased. A more reliable way to identify the sample is to pre-label by writing the identification characters directly onto the small piece of plankton mesh. Labels, data, etc. should be written in pencil that is not erasable by acetone. The usual safety precautions when manipulating liquid nitrogen and acetone should be taken.

8.5.4 Gut contents of field sampled consumers

The stomach and gut contents of an animal reflect its diet, and given that an appropriate digestion rate can be applied, they also provide the possibility of estimating ingestion rate. Analysis of stomach contents of field sampled zooplankton is therefore a common method for estimating ingestion rate of carnivorous zooplankton. Two items should be considered: (1) different prey types are not likely to be similarly recognizable in stomach contents and (2) different prey types may be digested at different rates. The first point means that the analysis of food selection may be biased, favoring prey types with

exoskeleton and easily recognizable structures. The second point means that predation pressure on different prey types may not be reflected accurately in the stomach contents.

The basic principle is otherwise the same as for the gut fluorescence method, with predation rate given by the product of stomach content (G) and stomach turnover rate in equation 8.28. However, there is an important difference between gut fluorescence method and that for carnivores, due to the actual measurement of k (page 333). For carnivores we measure the total time (t) needed for a food parcel from ingestion to total disintegration and define k as $1/t$. The gut-fluorescence method usually assumes an exponentially decreasing gut content, where k is the slope and only the initial 20 to 30 min are considered in the calculations (page 323). For carnivores, total digestion time is the only practical measurement we can use. However, because of the different definition of k for carnivores, their stomach contents should also be quantified differently to those of herbivores. For carnivores, each prey present in the stomach content, whether intact or partly digested, is assigned the mass of an intact prey. The rationale is that each prey item (or part of it) remains in the digestive tract for the full digestion time (t). The difference in estimated predation rate when using $1/t$ compared to an instantaneous rate constant, k, is not obvious and depends on the shape of the digestion curve. A linear digestion function gives the same result as $1/t$ and the more an exponential function deviates from linearity the more different the results will be.

GENERAL PROCEDURES

In general, planktonic carnivores have an upper body-size limit greater than planktonic herbivores, with giant scyphozoan jellyfishes and siphonophores as the largest members. Field sampling may require collection methods which differ from those used in traditional zooplankton sampling, especially if the purpose is to analyze stomach contents, where the duration of sampling may be critical. We emphasize two opposing sources of bias in this context: (1) decrease of stomach content because of digestion, egestion and stress-initiated regurgitation and (2) increase of stomach content because of cod-end feeding. In addition, care must be taken with the preservation procedure, where significant loss of stomach contents may occur due to regurgitation. Ideally, animals should be sampled individually with a dip net or collected with a bucket and preserved immediately, but such procedures are limited to surface dwellers. For smaller bodied carnivores, such as chaetognaths and crustaceans, the collection method used for general zooplankton may suffice. For larger and less abundant taxa, the choice of collection gear should be defined from information on abundance and statistical requirements according to:

$$\boldsymbol{A \geq n/(f \times v \times N \times t)} \tag{8.41}$$

where A is opening area of the net (m^2), n is the number of animals needed for statistical analysis of the results, f is the catch efficiency of the net (≤ 1.0), v is tow speed ($m\,h^{-1}$), N is abundance of animals (individuals m^{-3}), and t is the acceptable duration (h) of the tow. We suggest that the duration does not exceed 0.5 h, and preferably is much shorter. If we assume that the captured animals digest at a natural rate in the sampling gear, losses can be calculated. Results of such calculations are shown in Figure 8.6 for a linear digestion model and five exponential models with different rate constants (k). The linear model with a digestion time of 3 h, shows a loss of 17% during 0.5 h, the exponential models range from 10 to about 50%, with a rate constant (k) between 0.2 and 1.5 h^{-1}.

For example, to collect 20 individuals of the coronate scyphomedusa *Periphylla periphylla* in a fjord in Norway, where typical abundances are about 5 individuals 1000 m^{-3} (Fosså 1992), a tow speed of 1 knot (1852 $m\,h^{-1}$) requires collecting gear with

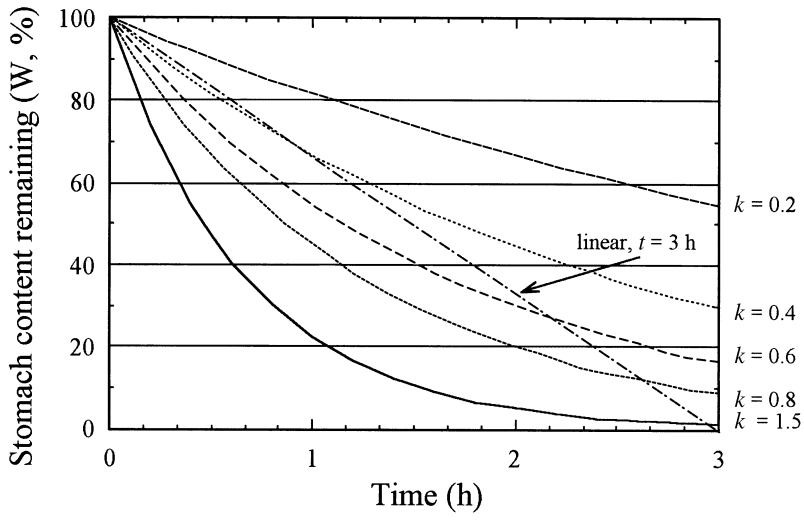

Fig. 8.6 Reduction in stomach content over time as defined by an exponential model, given by equation 8.29 with five different rate constants (k, h^{-1}), and a linear model with an evacuation time of 3 h.

a minimum opening area of 4 to 5 m^2, assuming 100% catch efficiency of the gear and 0.5 h sampling. We can see that the constraints related to sampling deep-living animals are severe, since the time needed for lowering and retrieving the sampling gear easily exceeds the suggested 0.5 h limit.

The risk of cod-end feeding varies and is species-specific. For example, krill can ingest prey in a non-filtering cod-end (Nicol 1984). Other predators, like medusae and ctenophores, might get prey into their stomach simply due to close packing in the cod-end. If cod-end feeding is suspected, prey contained in the esophagus of crustaceans and chaetognaths should probably be ignored.

The possibility of regurgitation during the preservation process should be evaluated. If there is a loss problem, the use of an anesthetic prior to preservation should be considered. Carbonated water (soda water) and MS 222 (ethyl *m*-aminobenzoate) are the most commonly used products for zooplankton work. Rapid freezing might also be an alternative. The use of liquid nitrogen ensures that there is minimum leakage through disrupted surfaces, where higher temperatures might give such problems. In all cases the preservation method should be tested on the specific material to be studied.

Diel variation in predation rate may be significant, and any extrapolations to daily or longer term periods from single time points is not advisable, unless data show otherwise. In diel studies we recommend a minimum sampling frequency of 4 h, i.e. six samplings per day.

SPECIAL CASE: COPEPOD MANDIBLES IN STOMACH CONTENTS

Because copepods can be the dominant prey of carnivorous macrozooplankton, there has been a great need for a reliable method to quantify copepods in predator guts. Many carnivores tear apart their prey during ingestion, so that the body shape of prey cannot be used for enumeration and size measurements. However, copepod mandibles can be ideal in this context. They are small enough to pass unbroken through the predator's mouth, they are inert to digestive enzymes, each prey always has two of them, and they

have a species-specific shape and a size directly related to the body size of the copepod (Karlson and Båmstedt 1994). Analysis of copepod mandibles has therefore been used as a quantitative tool on chaetognaths and euphausiids with great success, producing detailed information about the feeding biology of such predators (e.g. Stuart and Pillar 1990; Falkenhaug 1991; Gibbons *et al*. 1991; Båmstedt and Karlson 1998). Two precautions should be kept in mind when using this technique to estimate ingested prey biomass (Karlson and Båmstedt 1994). (1) Some copepod species, for example *Temora longicornis*, do not fit a general regression of mandible size versus body size. If a general equation is used for such a species, the estimated prey biomass is biased. (2) Individual prey body mass varies seasonally and with developmental stage. This is especially significant during overwintering of high-latitude species, where the decrease of body constituents may exceed 50%. Such a dramatic change is not reflected in the size of the mandibles, so that, unless a seasonal correction is given, a general transformation from mandible size to body mass of prey is not advisable. A procedure for euphausiids follows (Båmstedt and Karlson 1998).

Working procedure

The animals are collected with short net hauls, using the precautions for gut contents (see page 331), and frozen immediately. Preservation in formaldehyde is possible, although we prefer to avoid it because of the subsequent laboratory work. The animals are thawed in the laboratory, identified, and the length of carapace is measured. This is converted to body mass using an appropriate regression equation. Alternatively, the organisms can be weighed. The entire gut and stomach are dissected and placed into glycerine/water (1:1 by volume) for 20 min. Fullness is evaluated (e.g. 0, 0.25, 0.50, 0.75, 1.0), and the contents are stained with methyl blue. Copepod mandibles are then easily recognized and are collected, eventually identified, and their total width over the toothed edge measured using a compound microscope. With some practice, common species can be identified. The number of copepods in the stomach is simply half the number of mandibles present. The prosome length of copepods is estimated from species-specific regressions for prosome length (Y) versus mandible width (X). Because *Calanus finmarchicus* is such a dominant species in the North Atlantic we give the regression for this species below (from Karlson and Båmstedt 1994):

$$\boldsymbol{Y = -0.0690 + 0.0126 \times X} \tag{8.42}$$

where Y is in units of mm and X is μm. A general equation, based on seven species is given from the same publication:

$$\boldsymbol{Y = 0.0431 + 0.0112 \times X} \tag{8.43}$$

Karlson and Båmstedt (1994) also calculated mandible-width/dry-weight relationships. For *C. finmarchicus* this was:

$$\boldsymbol{Y = 0.00200 \times X^{2.215}} \tag{8.44}$$

with the dry weight (Y) in μg. The corresponding general equation, based on 7 species, was:

$$\boldsymbol{Y = 0.00235 \times X^{2.134}} \tag{8.45}$$

DIGESTION

The most difficult task involved in the method is applying a correct value for digestion rate. The procedure from the gut fluorescence technique cannot be reproduced, because

there is no specific chemical substance in the prey, like chlorophyl of algal food, and gradual digestion is thus difficult to follow quantitatively. Instead, one must determine the time elapsed from ingestion to empty gut. This is feasible for transparent animals, such as chaetognaths and jellyfish, and requires only frequent microscopic checking of the living animals. For non-transparent animals it is much more difficult, requiring a repeated sampling procedure, similar to the gut-fluorescence method, and microscopic evaluation of gut contents. By using lactic acid to make the animals transparent, predator guts can be checked for prey remains without dissection. Two other methods that do not necessitate repeated sampling of the animals are also available. The first is based on the defecation process, the second on the numeric relationship between stomach content and ingestion rate. Below we describe methods that are suitable for different types of predators. Methods based on the use of radioisotope tracers are not included here.

Transparent medusae and ctenophores

The prey is put directly into the mouth of the animals with a pair of tweezers to ensure a precisely defined starting time and the animal is maintained under constant conditions. The stomach content of the living animal is checked every 10 min, more frequently when digestion begins to be complete. Digestion time is defined as the elapsed time from start to no visable prey remains in the stomach. A sample size of at least five animals should be used.

Non-transparent predators

An experimental population is maintained with prey for a time sufficient to ensure a high frequency of fed animals. An initial sample is taken and the population is transferred to a prey-free environment for sampling over time. The initial sample gives the proportion of animals with empty guts, and this figure is subtracted from the subsequent samples. Since the time of last ingestion for each individual is unknown, i.e. the degree of digestion in the initial sample is variable, the average time to empty gut is an underestimate of the true digestion time, which will probably be closer to the maximum observed. Experimental duration depends on the physical environment and the predator/prey combination. The number of individuals in each sample depends on the variability of the target organism, but should not be less than ten. A higher number are needed than for analysis of transparent organisms because of the facts that the feeding time is uncontrolled and new individuals are collected at each sampling. The preserved samples are cleared with lactic acid and analyzed under the microscope. If possible, each individual is assigned a fullness index (e.g. 1.0; 0.75; 0.50; 0.25; 0) and the same procedure used in the gut fluorescence method is applied. This means that the plotted average fullness over time defines the instantaneous evacuation rate. If it is impossible to define a fullness index, the proportion (or number) of animals with food in their gut is plotted over time, indicating the underlying digestion time.

General method based on observed defecation

This method is based on the relationship:

$$DT = t_{ing} - t_{eg} \tag{8.46}$$

where DT is digestion time, t_{ing} is time at ingestion, and t_{eg} is time at egestion. The method involves transferring an experimental group of starved predators to an environment containing prey. The bottom of the incubation vessel is cleared of settled material at regular intervals, for example every 10 min. The time from the start of feeding to the first occurrence of fecal material corresponds to the minimum digestion time. An

alternative, but more laborious method, is to use feeding animals and at time zero add a new food, which is easily detected in the fecal material, to the experimental vessel. This eliminates bias due to the sudden change from starved to feeding conditions. This method has the disadvantage of time-consuming microscopic analysis of the fecal material. However, with an appropriate choice of food particle this does not have to be a major constraint.

General method based on constant ingestion rate and analysis of stomach contents

Assuming that the ingestion rate of a given population does not change over time, a simple experiment can be run in which predators are maintained with their prey for a period of time that exceeds their digestion time. If the number of prey in the stomach can be quantified, then the digestion time is calculated from:

$$\boldsymbol{DT = n/N \times t} \tag{8.47}$$

where n is the number of prey in the stomach, N is the total number of prey consumed, and t is the duration of the experiment (Båmstedt and Karlson 1998). An advantage of this method is that the animals are not starved and there is no need for repeated sampling over time. The method is synonymous to those of Peterson *et al.* (1990a), Irigoien *et al.* (1996), Dagg and Walser (1987), and Pasternak (1994). The method has been used successfully with euphausiids, scyphomedusae and ctenophores preying on copepods, with results comparable to those obtained using other methods (Båmstedt, unpublished data).

8.5.5. Methods based on budgets of material or energy

Ingestion represents the sole input term in the physiological budget of an animal. Thus, quantifying the other terms of the budget defines ingestion. The balanced growth equation for an individual organism is:

$$\boldsymbol{I = G + E + U + R} \tag{8.48}$$

where G is growth, including reproductive products, molts, mucus secretion, etc., E is egestion, U is excretion, and R is respiration. Theoretically this budget must balance over time. Because R is always greater than 0, zero feeding implies that G must be negative and stored body energy/material will be used. None of the budgetary terms are easy to quantify, and the low precision and accuracy associated with estimation of each term produces an even lower precision and accuracy in the ingestion estimate. When considering the sum of the right side of equation 8.48 as a method to estimate ingestion, the amount of work required to determine all four terms experimentally is disproportionate to the quality of the result. The method has an advantage in the fact that it includes all types of feeding. It can thus be used for omnivores in a very heterogeneous food environment. When calculating ingestion rates from chlorophyl *a* (based on gut fluorescence or bottle incubations) and converted to algal carbon using a carbon:chlorophyl *a* ratio, a common finding is that it does not fully support measured or estimated growth or respiration rates (equation 8.48). Although this may often be the case, it could also reflect either erroneously low grazing rates, the selection of an inappropriate carbon:chlorophyl *a* ratio or the fact that egg production/respiration rates are either erroneously high, out of phase with measured ingestion or an inappropriate time scale for the comparison. Thus the large numbers of possible errors with energy budgets makes this indirect method difficult to apply convincingly. However, the method can

also be broken down and specific relationships between the terms in the budget used as potential methods to quantify ingestion. Such a technique is easier to handle and we therefore consider each of the terms in this context.

GROWTH (*G*)

The ingested material provides energy for basic metabolic needs and is not used for growth until ingestion exceeds a certain level. After this point is passed, growth theoretically increases in parallel with ingestion, unless the assimilation efficiency changes. This means that there is a theoretical basis for expecting a fixed relationship between ingestion and growth. Gross growth efficiency (K_1) is a common way to express the quantitative relationship between growth and ingestion. This is defined as G/I. The theoretical upper limit for K_1 is about 0.7 (Calow 1977), but experimentally determined values average about 0.33 for copepods (Kiørboe *et al.* 1985a; Peterson 1988; Båmstedt *et al.* 1999). This value can then be applied, for example to population dynamic field studies, in which population feeding is calculated from a production estimate. However, such studies are constrained by great uncertainty in the production estimate due to factors such as mortality, age composition, reproduction and advection. It is safer to apply K_1 to studies of direct growth or production, for example egg production, where it is assumed that all growth potential is used for eggs. Results from this indirect method are comparable to those obtained using other methods (Kiørboe *et al.* 1985b; Peterson *et al.* 1990a). However, it must be emphasized that this method measures integrated feeding which preceded the actual measurement, implying that feeding history is important. For example, the copepod *Calanus finmarchicus* takes several weeks to fully recover its egg production rate after a period of low food (Båmstedt *et al.* 1999).

EGESTION (*E*)

Egested material is the part of the food that has not been absorbed in the digestive tract of the animal. This term defines the assimilation efficiency, AE, through the relationship:

$$I = E/(1 - AE) \tag{8.49}$$

If AE is known or a literature value can be used, quantitative measurements of fecal production (E) provide a method to estimate feeding. We must be very cautious when using this method, because of its sensitivity to the AE value used. This is especially important in the high range of AE. For example, if AE is underestimated, using 80% instead of the correct 95%, the calculated ingestion will be biased by a factor of 4, i.e. far outside an acceptable bias.

An overview of analytical methods to estimate AE is given in sections 8.5.7 to 8.5.9. For copepods which egest pellets covered with a peritrophic membrane quantitative collection of the fecal material is possible, although leakage and bacterial degradation must be considered when designing the experiment. For zooplankton groups without well defined fecal pellets, uncertainties in collection of fecal material may be so high that the precision and accuracy of the estimates become unacceptably low. Because this method also involves the use of an assimilation efficiency which by itself may be biased, the method is best suited for use in a relative way, for example to study diel variability or functional responses for a given species and developmental stage. Another promising method is to use an established empirical relationship between ingestion rate and the rate of fecal pellet production. This reduces the experimental procedure to include counting only fecal pellets. Several studies indicate that this method is suitable for copepods with a homogeneous food source (Gaudy 1974; Gamble 1978; Ayukai and Nishawa 1986;

Tsuda and Nemoto 1990; Båmstedt *et al.* 1999). It has also been used for euphausiids with great success (Clarke *et al.* 1988; Pakhomov *et al.* 1997). In order to use the method as a quantitative tool three criteria must be met:

1) Pellet size must be independent of feeding intensity.
2) Pellet size and pellet number must be independent of food composition in the environment studied.
3) Pellet number should show a strong relationship with feeding intensity.

EXCRETION (*U*)

Excreted material consists of metabolites from the animal's catabolism, especially nitrogenous components (mainly ammonium) from protein catabolism. Although excretion rate may be closely related to ingestion rate (Kiørboe *et al.* 1985a), its composition and amount depends on food type and feeding level. Thus, U is not appropriate to use to estimate I.

RESPIRATION (*R*)

Oxygen consumption is the integrated result of all aerobic energy-demanding processes in the body. The physical activity of feeding, as well as physiological digestion require energy, and the magnitude of these two processes is reflected in the respiration rate. Thus, on a theoretical basis this relationship could be used to estimate feeding rate from a measured respiration rate. However, we must advise against using a general relationship because respiration is the result of so many different processes, all characterized by high variability. The advantage of using such a relationship compared to a fixed ingestion per unit biomass is likely to be negligible.

8.5.6. Assimilation efficiency

Assimilation efficiency (AE) is the proportion of ingested nutritive substance (NS) in food that is absorbed by an animal during passage through the digestive system:

$$AE = (NS_{\text{food}} - NS_{\text{feces}})/NS_{\text{food}} \times 100\% \tag{8.50}$$

The review by Conover (1978), which also includes some benthic invertebrates, shows a range in average AE for different marine species from 15% to 99% and with highest values usually confined to the carnivorous diets. The basic unit of NS in the expression depends on the purpose of the study. For a general expression of AE, carbon, nitrogen, organic matter or energy can be used. We suggest that carbon be used whenever possible in order to be consistent with recommendations for other methods.

The assimilation of food material is a result of digestion of biochemical macromolecules (proteins, lipids, carbohydrates). Proteins must be degraded to amino acids, although dipeptides might be assimilated and hydrolyzed intracellularly (reviewed by Pandian 1975). Lipids are hydrolyzed to glycerol and free fatty acids, but uptake of monoglycerides is also important. Only small amounts of di- and triglycerides are assimilated (Pandian 1975). Carbohydrates must be degraded to monosaccharides, no uptake of polysaccharides occurs, and a small amount of disaccharides may be assimilated and degraded by disaccharases (Pandian 1975). Thus, AE is strongly dependent on the digestion efficiency of each food type. Factors such as the mechanical preparation of the food, the suite of digestive enzymes present and their efficiency are all important regulators of AE. Thus, different macromolecules in the food are likely to be

differentially degradable among species and have different assimilation efficiencies. There is no general rule for how this works, and there is a high interspecific variability in how efficiently different macromolecules or specific elements are assimilated (reviewed by Conover 1978). The investigator must decide what aspect of the assimilation efficiency is important in the proposed study and define the unit(s) to be measured in that context. If the objective is to define how much organic matter or carbon is extracted by the population, the use of organic carbon is recommended. However, if the aim is to explain constraints in nutrition, *AE* should be calculated for at least carbon and nitrogen separately, or preferably, for proteins, lipids and carbohydrates separately. A higher level of precision might be of interest in some cases, for example looking at the assimilation efficiency of some specific amino acids and fatty acids of essential importance for growth and development.

8.5.7 Measurement of assimilation efficiency: direct measurements

Equation 8.50 shows that quantification of total ingestion and defecation allows calculation of a precise *AE*. In practice, it can be very difficult to collect fecal material quantitatively, and leakage of soluble material may occur. The difficulties are especially severe when the sizes of the food particles and the fecal material overlap. Fragmentation of food and loss during ingestion (sloppy feeding) may also be a problem (e.g. Dacey and Wakeham 1986; Roy *et al*. 1989, see also page 340). Direct weighing of feces and consumed food cannot be used without including inorganic material, precluding proper conversion to any of the units suggested above. Labeling by ^{14}C of food and subsequent quantitative measure of ingested and defecated ^{14}C (e.g. Hargrave 1970) increases the sensitivity of the method, but otherwise has the same constraints as the gravimetric method. The use of radioisotope labeling has a number of constraints (Conover and Francis 1973; section 8.5.10). The gravimetric method is only suitable in situations where both ingestion and defecation are easily and precisely quantified. This usually requires that the consumers are large (e.g. salps, euphausiids), that the prey items are ingested whole and that fecal material is produced as pellets surrounded by a peritrophic membrane. Because collection of fecal material must be quantitative, this also means that the guts of the experimental animals must be completely emptied, a requirement that may be hard to meet after the animals have been removed from their food. The method has been used for tropical siphonophores feeding on copepods, where it gave results close to the ash-ratio method (Purcell 1983).

Direct measurements of incorporation of ^{14}C-labeled food into the body of a predator has been used to estimate assimilation (e.g. Pechenik 1979; Henning *et al*. 1991) although this does not account for respiration and excretion. Further, uncertainties about the exchange between different carbon pools (Conover and Francis 1973) render this application questionable.

8.5.8 Measurement of assimilation efficiency: indirect calculation

We can measure the components, respiration (R), excretion (U) and growth (G), which together make up the assimilated part of the food, and then use the balanced growth equation (equation 8.48) to calculate *AE*:

$$AE = (R + U + G)/I \times 100\% \tag{8.51}$$

where all components are expressed in the same units. In practice, this is a cumbersome

process, because it includes many experimental measurements and analyses, each with its own limitations. The end result may be an estimate with very low precision. Therefore, this method is not recommended for general use, although it has been used on marine copepods (Vidal 1980).

8.5.9 Measurement of assimilation efficiency: ratio methods

The principle behind ratio methods is to measure an absorbing component and an inert component that is unaffected by digestion both in food and feces. Food passing through the digestive tract gradually loses its nutritional content, while the amount of the inert tracer, by definition is constant. This causes an increase in the ratio (F) between the amount of inert tracer (IT) and nutritional substance (NS) from the food to the fecal material. Equation 8.50 can be rewritten:

$$AE = (1 - NS_{feces}/NS_{food}) \times 100\% \tag{8.52}$$

Because IT is the same in food and feces, we can divide both the terms NS_{feces} and NS_{food} by IT which gives:

$$\begin{aligned} AE &= [1 - (IT/NS_{food})/(IT/NS_{feces})] \times 100\% \\ &= (1 - F_{food}/F_{feces}) \times 100\% \end{aligned} \tag{8.53}$$

Thus, it is sufficient to analyze the ratio between the inert tracer and the nutritional substance in both the food and the feces, but it is not necessary to quantify total content of any of them. The formula introduced by Conover (1966) as the ash-ratio method can be reduced to this simple formula. Other applications using natural tracers (e.g. chlorophyl in phytoplankton, Landry *et al.* 1984b; silica in diatoms, Tande and Slagstad 1985) or radioactive tracers (e.g. double labeling with ^{51}Cr and ^{14}C, Calow and Fletcher 1972) are unified through this equation. Differences between methods reduce to a choice of material for the nutritional substance and the inert tracer. With few exceptions, they are subject to the same sources of potential bias, so the investigation should attempt to minimize these for the particular animal and food type under study. Tande and Slagstad (1985) evaluated how the relative error in the F factor of food and feces in equation 8.53 influences estimation of AE, showing that the method is robust to errors at high values of AE but sensitive when AE is low. Potential sources of bias in ratio methods are discussed on pages 339 to 341.

NON-HOMOGENEOUS FOOD MATERIAL

Both NS and IT should be distributed evenly in the food environment so that the ratio (F) is the same in the ingested material as in the samples analyzed for food. If NS and IT are natural components (e.g. organic matter and ash in the ash-ratio method), homogeneity is more probable than when using introduced tracers such as ^{14}C and ^{51}Cr. When using radioactive tracers, phytoplankton food should be exposed to the isotope(s) over several cell divisions in order to ensure homogeneity.

FOOD SELECTIVITY

In an assemblage of food particles, such as natural particulate matter, it is probable that food selectivity occurs. Then, if F varies between different types of particles, it biases the estimate of AE.

SLOPPY FEEDING

Losses during ingestion occur mainly when a predator ingests larger food items that have to be torn apart (Pechenik 1979; Deason 1980; Roy *et al.* 1989). Loss of fragments and leakage from tissues may cause considerable alteration of F in the ingested material compared to the intact food item, thus causing a bias in the estimated AE.

LOSSES FROM FECAL MATERIAL

Fecal material not occurring as membrane-bound pellets may be especially sensitive to this source of bias. Coprophagy and mechanical disturbance (e.g. through rotating incubation) may break pellets and cause loss of material with subsequent bias in the calculations. Use of stationary incubation jars with a false bottom of plankton netting is recommended to prevent coprophagy and mechanical disruption of pellets (Landry *et al.* 1984b), although use of non-motile algae may require plankton-wheel incubations (Tande and Slagstad 1985). Small amounts of liquid organic material from the food may remain after gut passage and leak into the water (Johannes and Satomi 1967). Direct tests of leakage of feces from a freshwater snail (Calow and Fletcher 1972) and a marine copepod (Tande and Slagstad 1985) indicate that this is not important within a time frame of 34 to 48 h. However, Johannes and Satomi (1966) found significant decreases in the organic component of shrimp fecal pellets over several days, especially when incubated in the dark. We therefore recommend recovery of feces within a few hours.

ABSORBANCE OF *IT* IN THE DIGESTIVE TRACT

Absorbance of IT in the digestive tract is a potential problem, especially for natural tracers like ash (Forster and Gabbott 1971; Prus 1971) and almost certainly for chlorophyl (see page 327), because AE will tend to be underestimated. Minerals are absorbed from food during gut passage and thereby reduce the ash content, although the actual amount of reduction can at times be negligible. The potential loss of chlorophyl-derived pigments during gut passage makes chlorophyl an inferior choice for the IT substance, although chlorophyl absorbance is still under debate (see gut fluorescence method). Biogenic silica as IT was not fully recovered (85%) in a methodological test (Tande and Slagstad 1985), but this could be attributed to sloppy feeding. Use of ^{51}Cr as IT produced a recovery of 90% (Calow and Fletcher 1972) but the authors noted that this might be a species-specific effect. However, if the proportional loss of IT during gut passage is known, it can be corrected (see below and equation 8.54).

PRODUCTION OF NON-FECAL MATERIAL MIXED WITH FECES

A certain amount of organic matter such as chitinous or mucus membranes or binding materials can be added to the ingested food during gut passage (Conover 1978), thereby increasing the organic content of the fecal material and causing an underestimation of AE. This bias is reported to be small. If NS is a natural part of the food, for example carbon or organic matter, a food origin and body origin cannot be distinguished. However, if NS is a tracer, such as ^{14}C, fecal NS is unaffected.

The double-labeling method of Calow and Fletcher (1972) does not seem to have been used by marine planktologists. The reason may be its complexity, necessitating analysis of both β-radiation and γ-radiation and the conflicting information on absorbtion of ^{51}Cr by different food organisms. The ash-ratio method (Conover 1966) is the only ratio method that has been used extensively, but like the other methods it requires that assimilation of IT is quantified for each predator–prey combination. This has to be done in incubation experiments where total ingestion (I_{tot}) is estimated precisely (page 341)

and all fecal material (E_{tot}) is collected quantitatively. Quantitative analysis of the *IT* component in both the food and the feces must be done with high precision. The factor F_{feces} in equation 8.53 is substituted by $k \times F_{feces}$, where k is:

$$\boldsymbol{k = 1 + (I_{IT} - E_{IT})/I_{IT}} \tag{8.54}$$

where I_{IT} and E_{IT} are the total content of the *IT* component in ingestion and egestion, respectively. Statistical principles should be adapted in the experimental design for this part as well as the actual assimilation experiments, including sufficient numbers of samples for a proper test of the results. The three most general methods are described next. The original papers should be consulted for further details.

ASH-RATIO METHOD

Samples of food and feces are filtered onto pre-ashed and pre-weighed glass-fiber filters, rinsed with isotonic ammonium formate to remove adventitious salt, and dried at 60–70 °C for at least 12 h. Blanks filtered with sea water only are treated in the same way. Filters as small as possible should be used for maximum precision in weighing. The dried samples are weighed, burned in a muffle furnace at 450–500 °C and finally weighed. Samples should always be stored in a desiccator because exposure to room conditions easily adds moisture (weight) to the samples. Weighings should be done immediately on removal from the desiccator. A microbalance with the highest possible sensitivity, preferably 1% or better of the smallest weight measured, should be used with fresh desiccant present in the weighing chamber. The organic content of the samples is:

$$\textbf{Organic content} = (\boldsymbol{W_{Dry} + Corr_{Dry}}) - (\boldsymbol{W_{Burned} + Corr_{Burned}}) \tag{8.55}$$

where W is weight and *Corr* is a correction factor defined by the average change in weight of blank filters after drying and ashing. Similarly, the ash content of the samples is:

$$\textbf{Ash content} = \boldsymbol{W_{Burned} + Corr_{Burned} - W_{Tared}} \tag{8.56}$$

where W_{Tared} is original weight of the filter used for the sample. Equation 8.53 is applied, where F is defined as the ratio Ash/Organic content.

CHLOROPHYL-RATIO METHOD

Chlorophyl *a* in phytoplankton ingested by zooplankton is degraded to pheophorbide *a*, which has a molecular weight 34% lower than the original chlorophyl molecule (Shuman and Lorenzen 1975). It can be analyzed quantitatively by fluorometry (Lorenzen 1966). In the method of Landry *et al.* (1984b) *IT* in equation 8.53 is chlorophyl *a* in food and *IT* is (pheophorbide *a*) × 1.51 in feces. *NS* in both food and feces is calculated separately for carbon and nitrogen. Since a small amount of intact chlorophyl *a* may remain in the fecal pellets this has to be added so that the formula for carbon is:

$$\boldsymbol{AE = [1 - (Chl_{food}/C_{food})/((Chl_{feces} + 1.51 \times Pheo_{feces})/C_{feces})] \times 100\%} \tag{8.57}$$

Landry *et al.* (1984b) used 50 to 100 *Calanus pacificus* in a 4-l glass cylinder equipped with a glass funnel in the bottom, with the two sections separated by 500 μm Nitex netting to prevent coprophagy. After about 16 h incubation fecal material was collected from a drain at the bottom of the funnel and separated from non-fecal material by screening and microscopy. Five samples for analysis of chlorophyl (pheophorbide), carbon and nitrogen were taken from each food environment tested and from the

resulting fecal material. Carbon and nitrogen were analyzed with an elemental analyzer, and chlorophyl and pheophorbide were analyzed as described in the section on gut fluorescence.

SILICA-RATIO METHOD

The method described by Tande and Slagstad (1985) uses ^{14}C as *NS* and biogenic silica (hydrated SiO_2 included in diatom cell walls, Parsons *et al*. 1961) as *IT* in equation 8.53. This requires that diatoms constitute a dominant component of the food environment. When using an assemblage of different phytoplankton groups or natural particulate material, it must be shown that there is no selectivity for or against the diatom component. Labeling of phytoplankton was done by adding 40 μCi $NaH^{14}CO_3$ l^{-1} to a culture and maintaining it in the light for at least one day before use. Different experiments with the copepod *Calanus hyperboreus* were performed in 2.2-liter Kilner jars or 1-liter PVC bottles mounted on a rotating wheel (1 rev min^{-1}) or in stationary PVC cylinders with 1000 μm Nitex netting in the bottom. Samples of phytoplankton and feces were hydrolyzed by adding 18 ml 0.5% Na_2CO_3 solution, heating to 85 °C for 2 h and cooling to room temperature (Paasche 1980). One sub-sample was used to measure ^{14}C activity and another was adjusted to pH 3 to 4 with 0.5N HCl and measured colorimetrically by the method of Strickland and Parsons (1972). This necessitates scaling down the volumes by a factor of two or more.

8.5.10 Radioisotope tracers

Radioactive tracers are a powerful tool to study marine zooplankton nutrition (Chipman 1959; Rice 1965; Sorokin 1966). However, many of the early isotope studies were flawed due to isotope recycling in the processes of excretion and respiration by bacteria, protozoa, phytoplankton and zooplankton (Conover and Francis 1973). With proper labeling techniques and controls to account for all of the isotope 'pools', labeling experiments can provide accurate, quantitative feeding data which, because of the sensitivity of the method, are particularly useful for studying zooplankton feeding behavior.

There are a number of advantages to using isotopes in zooplankton feeding studies. The enhanced sensitivity afforded by isotopes has been used to measure grazing rates of individual zooplankton (Marshall and Orr 1955a), species groups within a mixed zooplankton community (Griffiths and Caperon 1979; White and Roman 1992), and copepod nauplii (White and Roman 1992). Short-term grazing incubations with isotopes, often less than 1 h duration, minimize containment effects (Roman and Rublee 1980). Isotopes can be used to label particular foods or substrates and thus provide important information on plankton food-web dynamics (e.g. Marshall and Orr 1955a; Hollibaugh *et al*. 1980; Gottfried and Roman 1983; Mullin and Roman 1986). Investigators have used dual-labeling techniques to assess simultaneous zooplankton grazing on autotrophs and heterotrophs (e.g. Lampert 1974; Roman and Gauzens 1997). Longer term isotope feeding studies have been used to study carbon cycling in zooplankton (e.g. Sorokin 1966; Lampert 1975) as well as carbon incorporation into protein, carbohydrates and lipids in copepods (Roman 1991).

The use of isotopes in zooplankton feeding studies has a number of disadvantages. Radioactive isotopes can be expensive, safe handling procedures must be followed, and the radioactive waste must be disposed according to prescribed rules. There are several sources of error associated with isotope grazing studies. In ingestion experiments, the

methods assume that isotopes are not lost from the animals via respiration, excretion or fecal pellets. Thus experiments must be of short duration to minimize these losses. Another source of error that results in underestimating grazing on natural plankton communities occurs if the target animals select large cells (protozoa and larger phytoplankton) that may have a lower isotopic specific activity than smaller cells. Several types of isotope grazing applications are described in this section. In laboratory studies, phytoplankton cultures, protozoa and bacteria can be labeled isotopically and used for zooplankton feeding studies. In field studies, investigators have used pre-labeled cells as well as labeled the natural plankton community for grazing experiments.

METHODOLOGICAL COMPARISONS

Several investigators have used more than one technique to estimate zooplankton grazing, thus allowing comparison of the experimental approaches. There were no significant statistical differences between copepod clearance rates estimated in the laboratory by changes in chlorophyl concentration, Coulter Counter cell counts and ^{14}C-labeled phytoplankton, respectively (Hargis 1977). Clearance rates of natural assemblages of copepods estimated by Coulter Counter cell counts and the Daro (1978) isotope technique were not statistically different (Daro 1978; Cowles *et al*. 1987). *In situ* grazing incubations with isotopes (Roman and Gauzens 1997) gave similar zooplankton grazing estimates as the gut fluorescence technique (Dam *et al*. 1995) during March/April but not during October at a station in the equatorial Pacific.

WORKING PROCEDURES FOR LABORATORY EXPERIMENTS WITH ISOTOPES

Phytoplankton cultures may be labeled with a number of different isotopic tracers including ^{14}C (Marshall and Orr 1955c; Houde and Roman 1987), ^{32}P (Marshall and Orr 1955a; Roman 1977), ^{33}P (Napp and Long 1989), ^{131}I (Marshall and Orr 1955b), ^{35}S (Zimmerman 1973), ^{3}H (White and Roman 1991) and ^{65}Zn (Zillioux 1973). Generally, phytoplankton cultures are incubated with isotopes through at least two divisions so that the cells are labeled homogeneously (Hitchcock 1983). After labeling, the cells are separated from the non-incorporated label by either centrifugation or gentle filtration, then washed and resuspended in filtered sea water. Replicate samples of the labeled phytoplankton are taken for measurement of isotope activity (disintegrations per minute, dpm), cell counts, chlorophyl or dry weight in order to calculate the 'specific activity' of the phytoplankton, as dpm per cell or biomass. Zooplankton are added to treatments with labeled phytoplankton and separate zooplankton samples are taken for determination of zooplankton body mass (DW, AFDW, C, N, etc). Replicate bottles containing phytoplankton alone serve as controls. A subset of animals should be filtered immediately for 'time zero controls' to estimate adsorption of label to animals and filters. Grazing incubations are generally done on a rotating wheel (bottles oriented end over end) in the dark, for time periods less than the estimated turnover time of the gut contents, so there will be no isotope loss due to fecal production. After incubation, the animals are collected on sieves (mesh size small enough to retain the animals but large enough to pass labeled phytoplankton). The animals are rinsed with filtered sea water (some investigators also use 10% HCl), and the number of animals determined before isotope analysis. Samples of labeled phytoplankton are also filtered for determination of the radioactivity of the food suspension. Clearance rate (F in ml animal^{-1} h^{-1}) is calculated as:

$$F = (\mathbf{dpm}_{\mathbf{animal}} \times v)/(\mathbf{dpm}_{\mathbf{algae}} \times t) \tag{8.58}$$

where dpm_{animal} is the radioactivity of one animal, dpm_{algae} is the radioactivity of v ml of the phytoplankton suspension, and t is the incubation time in h. Ingestion rate (I in cell number, DW, AFDW, C, N, or chlorophyl animal^{-1} h^{-1}) is calculated by multiplying the clearance rate by the phytoplankton concentration during incubation (cell number, DW, AFDW, C, N, or chlorophyl ml^{-1}). Similar procedures were used by Hollibaugh *et al.* (1980) to measure the grazing of larvaceans on bacteria with the isotope methyl-^{3}H thymidine. Bacteria have been labeled to estimate zooplankton grazing on various types of detritus (Roman 1984; Mullin and Roman 1986) as well as zooplankton ingestion of protozoa (Baldwin and Newell 1991).

WORKING PROCEDURES FOR FIELD EXPERIMENTS

Isotope grazing rate determinations of natural assemblages of zooplankton have been used widely in lakes following the technique of Haney (1971). Pre-labeled yeast cells are used with *in situ* chambers which both trap zooplankton and simultaneously release isotopically labeled food. After short incubations (less than the gut turnover time of the zooplankton) the animals and particles are filtered and isotope activity determined. Clearance rate is calculated according to Equation 8.58, where v is the volume of the *in situ* chamber and dpm_{algae} is the total radioactivity added to the chamber. Ingestion rate is calculated by multiplying the clearance rate by the concentration of food (sum of labeled and non-labeled) in the chamber. The method assumes that there is no discrimination between the natural food particles and the added, isotopically labeled ones.

Another approach to estimating the grazing rate of natural assemblages of zooplankton was developed by Daro (1978). In this method ^{14}C-bicarbonate is added to natural plankton samples and so that zooplankton grazing rates on the surrounding phytoplankton and protozoa communities can be estimated. Daro (1978) chose a stationary 3-compartment model to express the exchange of radioactive tracers in her grazing experiments. This is a reasonable assumption considering the large magnitude of the initial isotope pool in the sea water relative to that taken up in the particulate matter and zooplankton during the short (<1 h) incubation. The short incubation also reduces feedback to the compartment via excretion and respiration by the phytoplankton and zooplankton. The calculations of grazing rate can be found in the original paper by Daro (1978) and these are explained in the earlier section on microzooplankton (see page 311).

Since the phytoplankton have to be labeled with ^{14}C in the light, this technique can only be used to estimate daytime grazing rates. Daro (1980) modified the procedure by pre-incubating sea water (poured through mesh to remove zooplankton) with ^{14}C under artificial light at night to label phytoplankton, then added zooplankton for dark incubations. Alternatively, other investigations have used isotopes such as ^{33}P (Napp and Long 1989) or [methyl-^{3}H]methylamine hydrochloride (White and Roman 1991) which are taken up by phytoplankton in both light and dark. Roman and Rublee (1981) combined the Daro (1978) and Haney (1971) approaches by releasing ^{14}C-bicarbonate and ^{3}H-thymidine in clear, Niskin-type bottles to estimate the *in situ* grazing rate of marine zooplankton.

8.5.11 Food removal methods

This method involves incubating zooplankton in bottles with food for a fixed length of time, measuring the decrease in food concentration compared to that in control bottles with no grazers, and thus calculating the feeding rate. The goal is to incubate

under conditions which mimic the natural environment as closely as possible, enabling laboratory-measured feeding rates to be applied to the natural environment.

This approach is the simplest direct method and the one longest in use (Gauld 1951; Paffenhöfer 1988). However, potential limitations of laboratory incubations include the stress of capture, handling and confinement of zooplankton, possibly unnatural food sources, turbulence, crowding of grazers and growth of prey assemblages. Such factors are known collectively as 'bottle effects' (Roman and Rublee 1980). Nejstgaard *et al.* (1997) describe one solution for alleviating bottle effects caused by the growth of micrograzers. Despite these problems and the often time consuming analysis involved, this method is simple and direct. It is the only method currently available which allows direct quantification of feeding rates on non-phytoplankton taxa. In the last 20 years it has become clear that phytoplankton, although easily measured by chlorophyl *a* (Chl *a*) assays, often represent only a part of zooplankton diet, and protozoans and metazoans often comprise an important fraction (reviewed by Stoecker and Capuzzo 1990; Gifford 1991; Kleppel 1993). This has led to an upsurge in the use of incubation methods, particularly in their simplest form, where zooplankton are incubated in natural sea water and feeding rates are calculated from microscope counts of the animals and plants in it (e.g. Gifford and Dagg 1991; Fessenden and Cowles 1994; Ohman and Runge 1994; Kleppel *et al.* 1996; Atkinson and Snyder 1997). The food removal methods described in section 8.5.12 emphasize incubations with copepod-size grazers and are suitable for quantifying omnivorous feeding behavior. Larger species require scaled-up incubation volumes (e.g. Price *et al.* 1988; Froneman *et al.* 1996) or through-flow techniques (see McClatchie 1992).

BOTTLE EFFECTS DURING INCUBATIONS

Bottle effects are defined here as the differences experienced by a grazer-food assemblage in a bottle as compared to its natural environment. For example there are differences in turbulence, light regime, crowding of grazers and interactions with the container walls. The fact that turbulence can influence copepod feeding behavior is now recognized, but most work has been done on a single copepod genus, *Acartia* (Saiz and Kiørboe 1995; Kiørboe *et al.* 1996). This genus has two feeding modes and can switch between them according to the extent of the turbulence. However it is difficult to examine the degree of turbulence a grazer encounters *in situ* or to recreate it. Saiz and Kiørboe (1995) suggested that turbulence might have a more severe effect on 'hop and sink' ambush predators than species which create a feeding current. Until a wider range of species is studied, a possible approach is to use a range of turbulence regimes for incubations, for example by using different mixing methods and speeds. However a useful standardized protocol, enabling intercalibration between workers, would include control and experimental replicates with no airspace which are rotated slowly (0.5 rev min^{-1}).

Two problems stem from the use of controls. First, feeding rates are calculated under the assumption that the cell growth term, k (see equation 8.3) is identical in both the grazed bottles and the controls. If nutrients are unlimiting this may be the case, but ammonium excretion by grazers may enhance phytoplankton cell growth in the bottles with phytoplankton (Roman and Rublee 1980). If nitrogen is suspected of being at limiting concentrations then all the bottles can be 'spiked' with 5–10 μmol l^{-1} ammonium to counteract this problem (Roman and Rublee 1980). The second problem with controls can arise where natural sea water is used as food. The timescales of growth and grazing within the natural assemblage are similar to the duration of the experiments. As well as the problem of cell growth, this gives the potential for 'food chain effects'. For

example if the zooplankton under study cleared heterotrophic dinoflagellates and diatoms at similar rates, but the dinoflagellates were also feeding on diatoms and keeping their numbers stable, then the effect seen would be an apparently lower clearance rate of diatoms than of dinoflagellates, because the zooplankton would have removed one of their major grazers, the dinoflagellates. Such effects are difficult to quantify without detailed knowledge of protozoan grazing rates and food selectivity. If, however, these are known (for example from concurrent dilution experiments) then the correction applied by Nejstgaard *et al.* (1997) can be used. This allows for the estimated grazing by the protozoans eaten by the copepods, and thus the corrected copepod grazing rate on phytoplankton is a higher value than the uncorrected for protozoan grazing. Another way around the problem, especially if the study is specifically concerned with feeding selectivity, is to shorten the incubation time to about 6 to 8 h. To achieve the desired depletion of food this requires roughly three times the stocking density of consumers as those used for a 24 h experiment. This reduces food chain effects by making the test animals the main grazers in the bottle, with less time available for growth and grazing among the prey assemblage. Another way around the problem is to incubate a range of grazers in each experiment, including a species which creates a feeding current and does not show carnivorous tendencies. For example *Calanoides acutus,* a Southern Ocean copepod, is one such 'baseline' species, which can be incubated as an internal control for comparison with other grazers (e.g. Atkinson *et al.* 1996a).

SLOPPY FEEDING

'Sloppy feeding' is the term coined for inefficient ingestion of large cells by zooplankton, which can produce small ungrazed particles. It appears to be most pronounced when animals are feeding on large diatoms under conditions of excess food (Deason 1980; Roy *et al.* 1989). In such situations counts of food particles may lead to overestimates of ingestion rate because the grazers turn cells into unrecognizable debris and ingest only a small portion. To check whether this is occurring, size fractionated chlorophyl *a* analysis can be used to determine whether there is a build up in the smallest size fraction. If long colonial diatoms are a feature of the food assemblage, both the number of cells and the number of colonies should be counted to check for breakage of colonies (Deason 1980; Stuart 1989). Likewise for long, rod-like diatoms such as some species of *Rhizosolenia*, all cells should be measured as well as counted, and clearance rates and ingestion rates based on the total measured length of cell encountered during the scanning procedure.

ESTIMATES OF COMMUNITY GRAZING RATE

A common objective is to measure the daily grazing impact of the whole mesozooplankton community, in order to examine whether they are responsible for cropping a significant fraction of daily primary production (e.g. Morales *et al.* 1991; Dam *et al.* 1993, 1995; Uitto 1996; Roman and Gauzens 1997; Swadling *et al.* 1997). Several studies have likewise estimated the predation impact of the copepod fraction of the community on protozoan populations (e.g. Gifford 1993a; Fessenden and Cowles 1994; Atkinson 1996). A variety of approaches have been used to assess community grazing, and all are time consuming if done carefully. Table 8.3 summarizes the three main methods, namely gut fluorescence, bottle incubations and the use of radiotracers. The choice is dictated by both the type of study, the time available for sampling and the characteristics of the grazer/food assemblage.

A major problem of measuring community grazing is that the dominant grazers are

Table 8.3 Summary of meso- and macrozooplankton feeding methods in relation to their suitability for some common study objectives. These objectives emphasize epipelagic crustaceans approximately up to the size of euphausiids.

Objective	Method	Main pros	Main cons	Notes
Community grazing impact on primary production	Gut fluorescence	Not too labor intensive aboard ship.	Difficulty of determining realistic values for pigment destruction and gut evacuation constant.	Can be used for sieve fractionated (bulk) size fractions or for selected numerical and biomass dominant species/larval stages.
	Food removal methods	Direct method, allowing inferences on size and taxon selectivity.	Bottle effects, technical problems if grazers and/or food are small. Potential problems with food chain effects.	Requires incubation of numerical and biomass dominant species and larval stages (e.g. both large and small animals). Image analysis or HPLC may be useful to enumerate a food assemblage dominated by small cells or detritus.
	Radiotracer method	Minimized incubation stress. Especially useful when grazers and/or food is very small.	Variable recycling of nutrients. Variable uptake rates in natural assemblages, causing problems in conjunction with selective grazing. Can be harder to measure large, rarer grazers.	For daily estimates repeated short-term experiments covering the full 24 h period are needed. If feeding rates on autotrophs and heterotrophs are compared, food-chain effects must be considered.
Community predation impact on protozoans.	Food removal methods	Minimized incubation stress. Especially useful when grazers and/or food is very small.	Bottle effects. Technical problems if grazers and/or food are small.	For daily estimates repeated short-term experiments covering the full 24 h period are needed. If feeding rates on autotrophs and heterotrophs are compared, food-chain effects must be considered.
Quantifying importance of omnivory	Radiotracer method	Minimized incubation stress. Especially useful when grazers and/or food is very small.	Bottle effects. Technical problems if grazers and/or food are small.	For daily estimates repeated short-term experiments covering the full 24 h period are needed. If feeding rates on autotrophs and heterotrophs are compared, food-chain effects must be considered.

continued

Table 8.3 *continued*

Objective	Method	Main pros	Main cons	Notes
Estimating importance of omnivory (energy budget methods additional to the above)	Gut fluoresence and experimental egg production	Uses relatively quick and commonly used techniques.	Indirect method, subject to numerous potential errors.	An energy budget approach to omnivory, additional to using food removal method described above.
	Gut fluorescence and egestion rate	Can be used on large species. Provides an estimation of total carbon ration, by monitoring defecation rate immediately after capture.	Indirect method, subject to numerous potential errors.	Another energy budget approach. Method is sensitive to assimilation efficiency, so this must be known fairly accurately from literature or else measured.
Predation rates by macro-plankton on metazoans	Large volume food removal methods	Direct method. Allows simultaneous comparison of feeding rates on other food sources.	Bottle effects. Difficult to manipulate large volumes of water. Difficult to know whether incubation is at environmentally realistic food concentrations.	Care needed in designing experiments. Pilot studies, acclimation periods and visual behavioral observations available.
	Gut content analysis (e.g. using copepod mandible method)	Semi *in situ* method.	Determining a realistic gut passage time can be problematic.	Can give species/developmental stages and sizes of copepod prey. Seasonal variation in prey body mass not reflected in mandible size.
Food web analysis	Gut content analysis	Genuinely *in situ*.	Not very quantitative. Underplays dietary role of soft bodied/easily digested foods.	Useful for baseline and comparative (e.g. seasonal) studies or for poorly studied environments. Easier for larger species.

Comparative (e.g. seasonal) studies of feeding activity	Digestive enzyme activity	Genuinely *in situ*. It might integrate recent feeding history. Can provide insights into species physiology.	Semi-quantitative. Requires careful calibration.	Useful in conjunction with other studies, e.g. gut fluorescence.
Mechanistic behavioral understanding of feeding	Food removal methods	Allows an individual-based scale of understanding of mechanisms, controls and behavior.	Bottle/containment effects. Possible problems of unnatural food sources or predators.	Video observation methods allow direct observations of feeding in conjunction with, e.g. swimming activity, variable food sources, turbulence or predators.
In situ diet variations in grazing behavior in relation to vertical migration, food, predators, etc.	Gut fluorescence	An *in situ* method. Large numbers of samples can be processed.	Day–night differences in pigment destruction may influence results. Only informs on herbivorous feeding.	A useful method to observe grazing activity and behavior *in situ*, but grazing rates can only be determined if concurrent gut evacuation rate constant and pigment destruction rates are determined.

not known beforehand, and could range in size from copepod nauplii to salps or euphausiids. Whatever method is used, obviously the major grazer species/larval stages should be included. Because communities are often dominated by a diversity of species of different size and stage of maturity, a requirement is also to measure a sufficient range of species and stages to reflect the whole community. Small grazers often dominate numerically but larger ones dominate biomass. Because small species can be dominant grazers, even when they are minor contributors to biomass (e.g. Morales *et al.* 1991; Atkinson 1996; Swadling *et al.* 1997), these should be assessed where necessary.

Table 8.4 suggests that the size structure of the grazer/food assemblage dictates to some extent the method employed. If grazers and food are small, both incubations and the gut fluorescence method become difficult due to physical difficulties of handling very small grazers and counting small food items. In these circumstances, radiotracer methods (e.g. Uitto 1996; Roman and Gauzens 1997; Swadling *et al.* 1997) may be the answer. However, depending on the method of capturing the grazers prior to the confinement, these may understimate the abundance and grazing impact of large or mobile species.

Several studies have used a biogeochemical approach to community grazing, involving sieve size fractionation of zooplankton and the gut fluorescence method. (e.g. Morales *et al.* 1991; Dam *et al.* 1993, 1995). Dam *et al.* (1993) pointed out that the approach of splitting the community into broad 'small', 'medium' and 'large' categories with sieves, and then measuring the average grazing rates of these, lost taxonomic resolution, but was defensible in some diverse assemblages where it would be difficult to measure representative species.

More taxonomically useful information is gained if the grazing rates of a range of species and stages is measured, and a community estimate constructed. This can be done either using the gut fluorescence method (e.g. Peterson *et al.* 1990b; Landry *et al.* 1994) or using food removal methods (e.g. Schnack *et al.* 1985; Perissinotto 1992). We do not recommend monitoring the feeding activity of bulk (unsorted) assemblages caught with a net, as this often contains a proportion of potential food cells, fecal pellets, debris and injured copepods. Species/larval stages selected to measure must include the numerical and biomass dominants, and if possible a large size range within each taxon. Community grazing can be estimated by constructing a relationship between grazer size and feeding rate for the animals incubated, and from this regression line, estimating grazing rate for the species not studied (e.g. Atkinson 1996a). This allows for the fact that small species tend to have higher weight specific feeding rates than large ones (section 8.8.4). Different groups, for example copepods and euphausiids have differing feeding capabilities, so such groups would warrant a separate allometric evaluation. Total grazing by the community can then be obtained by summing the mean grazing rates of all species and stages in the water column. All estimates of community grazing or predation impact are time consuming if done properly, and frequently the larger taxa are beyond the scope of a single study. Reliable grazing impact estimates also rely on intensive net sampling of defined depth strata (e.g. the upper mixed layer) to reduce problems of zooplankton patchiness and to define the vertical migration cycle.

8.5.12 Working procedures with food removal methods

COLLECTION OF ZOOPLANKTON

To be able to feed at natural rates zooplankton must be as undamaged as possible. Thus for collection fine mesh nets, 200 μm or finer, are required so that the delicate

setae of many copepod species suffer minimal abrasion. Towing speeds should be slow ($<0.2\,m\,s^{-1}$), and collections in rough seas avoided if possible due to the jerking motions of a rolling ship. A motion-compensating spring on the net, leader wire or winch may reduce this. If the catch still suffers abrasion, slow oblique tows from a drifting ship are a possibility, as is the use of a smaller diameter, finer mesh net hauled by hand. Stress on the catch is reduced further by using large volume impervious cod-end containers. Gelatinous species with delicate feeding appendages may require especially delicate collection (Deibel 1982). The catch is sorted as soon as possible at the same temperature as the water. For small and medium sized species such as copepods, this is usually done under a binocular microscope using either a wide-bore pipette or a small sieve. Experimental animals are transferred to bottles containing the same food assemblage and kept for up to 24 h under the same conditions as the experiment. If the food source offered differs from that *in situ*, this serves as an acclimation period (Huntley 1988).

THE FOOD SOURCE

The choice of food source depends on the question addressed. Studies concerned specifically with feeding mechanisms are well suited to the use of cultured food because this allows maximum control over experimental conditions and easier assessment of feeding rates. For example studies of food selection behavior (e.g. Price and Paffenhöfer 1984) or the effects of turbulence on feeding (e.g. Saiz and Kiørboe 1995) have tended to use cultured diatoms or flagellates while feeding behavior was monitored concurrently. For such cultures, automated particle counters such as the Coulter Counter greatly speed data collection (e.g. Donaghay 1980; Tackx *et al.* 1995; Meyer-Harms and von Bodungen 1997). However, the serious number of artefacts that these particle counters can generate in natural sea water assemblages (e.g. Harbison and McAlister 1980) means that their use is best restricted to cultured material.

Natural sea water is being used increasingly as a food source. Despite several drawbacks this allows zooplankton to feed on the wide range of particles they encounter in nature and provides quantitative insight into their primary food sources. Sea water can be collected from the same depth strata at which the copepods are feeding. This is not often known precisely unless a high resolution in the vertical sampling is used. An option is to collect water from the depth of the chlorophyl *a* maximum. If Niskin bottles are used, the interior tubing should be replaced with silicone tubing and the o-rings replaced with silicone o-rings. Alternatively, water can be collected with a clean plastic bucket lowered over the side of a ship or dock. In regions under partial iron limitation Gifford (1993a) recommends a rigorous metal-free cleaning protocol, as described by Fitzwater *et al.* (1982).

EXPERIMENTS

Suitable experimental containers for zooplankton up to the size of copepods range from 0.5 l (e.g. for <1 mm long copepods), up to 2 l or 5 l for larger species (Paffenhöfer 1984). These can be wide mouthed (3–5 cm) bottles of either polycarbonate or glass, although the latter must have a silicon lid seal. The container size, stocking density of zooplankters and the incubation time must be balanced so that their feeding reduces the favored taxon by about 30% to 40% (Gifford 1993a). If food removal is much greater than this, feeding activity may change during the experiment in response to low food concentration. For example, copepods may increase clearance rates by a factor of 6 to 8 to compensate for low food concentration (Paffenhöfer and Lewis 1990). If food

reduction is drastic an opposite effect, with reduced feeding or complete cessation may occur (Quetin *et al.* 1994). On the other hand, if grazers remove less than about 10% to 15% of a taxon, estimates of feeding rate on that taxon become imprecise due to the high variance often attached to estimates of food concentration (see sections 8.3.1, 8.3.2). To achieve roughly the desired depletion requires either some prior knowledge of the species' feeding capabilities derived from the literature, or a few trial incubations.

A complication occurs if the food is at saturating levels, for example during a bloom. Then the zooplankton may need to remove only a small fraction of the cells in order to satisfy their energy requirements (i.e. clearance rates are low but ingestion rates are high). This requires either higher than normal stocking densities or longer incubation times to achieve the desired depletion.

To set up an experiment, either natural sea water or a suitable concentration of culture should be obtained immediately beforehand, in a clean aspirator. It is then stirred gently and siphoned through acid-cleaned silicon tubing into the experimental bottles. If the natural sea water already contains large numbers of zooplankton it may need gentle screening through a submerged 100 μm or 200 μm mesh sieve. This however must be avoided unless absolutely necessary, as it can cause mortality of delicate ciliates (Gifford 1993a). Also in some situations, for example Antarctic phytoplankton blooms where very long diatoms predominate, screening through a 200 μm mesh can remove over half of the available phytoplankton (Atkinson *et al.* 1996a).

A typical setup might comprise a minimum of three initial bottles (incubation water with no zooplankton), three controls the same as the initials and three experimental replicates for each zooplankton species/larval stage under study. These bottles are filled nearly brim full, and if natural sea water is being used they should be left for about 2 h to stabilize the food assemblage. The zooplankton are then transferred from their acclimation bottles by pouring their contents gently onto a submerged shallow, coarse-meshed sieve and then washing them into the bottles with incubation water. All bottles are filled brim full and capped with no airspace. Contact with air and excessive turbulence can cause mortality of some delicate protozoan taxa, and should be avoided at all stages (Gifford 1993a). Either Parafilm®, clingfilm or silicon seals can be fitted over the bottle mouths to exclude air.

The duration of the incubations depends on the primary objective. If daily feeding rates are required, they should last 1 day to encompass a possible diel feeding cycle of the zooplankters. If the main aim is to assess diet from natural sea water assemblages, shorter incubations of about 8 h may be preferable (see page 345). Zooplankton sometimes feed at higher rates during darkness, and their feeding rhythm may also be under partial endogenous control (Head 1986; Stearns 1986). Therefore shorter duration incubations should be run during the hours when the grazers are feeding most intensely. Usually this occurs during the natural hours of darkness, but exceptions to this are common, for example copepod nauplii may feed most intensely during the daytime.

Incubations should simulate the natural environment as closely as possible. Temperatures both during setting up and during the incubations should be maintained at ambient values for the grazers using either a water bath, a temperature controlled room or cabinet, or by placing the bottles in the sea. Food must be kept in suspension, usually by rotating the bottles end over end slowly (0.5–2 rev min^{-1}) on a custom-built wheel. Large bottles or aspirators can be mixed either by a slowly rotating paddle (e.g. Price *et al.* 1988) with a peristaltic pump (e.g. Schnack 1985) or by intermittent stirring by hand. Bubble or magnetic stirrers should be avoided. Neutral density screening can be used to reproduce *in situ* light intensity for incubations done on deck or in the sea.

SUB-SAMPLING

The goals of the experiment dictate the sub-sampling methods. Feeding rates on total phytoplankton are measured by monitoring changes in chlorophyl *a* concentration. Duplicate or triplicate samples of 100 to 250 ml depending on chlorophyl *a* concentration, are filtered onto GF/F filters and extracted in 90% aqueous acetone for at least 12 h at 0 to 5 °C. Chlorophyl *a* concentrations are then measured fluorometrically (Parsons *et al*. 1984). Copepods generally tend to have low feeding rates on particles smaller than about 5 μm (Berggren *et al*. 1988). Therefore size fractionation of the water, for example through 20 μm nylon mesh, 5 μm polycarbonate filters or GF/F filters, involves little extra workload for the information it provides on size selective feeding (e.g. Perissinotto 1992).

HPLC analysis of the sub-samples is a more refined approach which can give insights into taxon specific selectivity (Head and Harris 1994; Meyer-Harms and von Bodungen 1997). If calibrated against traditional cell counting methods this seems a promising development (Meyer-Harms and von Bodungen 1997), being a compromise between simple chlorophyl *a* analysis and microscopic cell counts in both speed and taxonomic resolution. Although the equipment is expensive and complex, HPLC analysis may be especially useful in some circumstances, for example where most of the food is in very small cells and difficult to count.

The use of image analysis systems for glutaraldehyde preserved water samples is another option, providing information on feeding selectivity (Estep *et al*. 1990; Verity and Sieracki 1993; Verity and Paffenhöfer 1996). The method is as rapid as manual methods of counting and sizing cells under a microscope and is of optimal use when optical cell counts are difficult, for example in detritus-dominated estuarine systems (e.g. Tackx *et al*. 1995).

To count microplankton food items directly under a microscope, acid Lugol's solution (Sherr and Sherr 1993a) is the most widely used fixative. The concentrations of Lugol's used range from 0.5% to 20%, with the stronger solutions tending to preserve a higher proportion of protozoans but causing them to shrink more severely than 1% or 2% solutions (Leakey *et al*. 1994; Stoecker *et al*. 1994). We recommend that a stronger Lugol's solution (about 10%) be used as this preserves almost all protozoans, and the shrinkage of aloricate ciliates, by up to 50% in volume (Stoecker *et al*. 1994) can then be corrected for. A disadvantage of Lugol's solution is that it is not possible to determine whether cells are autotrophic or heterotrophic. If this information is needed, sub-samples can be preserved with glutaraldehyde for epifluorescence microscopy (Booth 1993; Sherr and Sherr 1993a; Sherr *et al*. 1993). See Chapter 5 for details.

To sub-sample, the desired volume of water is siphoned from the mixed contents of the bottles. The three initial bottles are sub-sampled on setting up the experiment and their remaining contents discarded. Sub-samples from control and experimental bottles are taken at the end, having first checked the zooplankton for mortality.

If grazers need further identification or weighing, the entire bottle contents can be filtered onto GF/F filters and the animals frozen. Mass specific feeding rates are expressed generally in units of carbon, so if facilities are available, carbon contents of grazers should be determined directly following earlier protocols (see Chapter 4). Failing this, dry masses can be obtained. For this, grazers can be thawed in filtered sea water, rinsed briefly in distilled water to remove salt, blotted dry and placed on small, pre-weighed aluminum foil boats. Depending on grazer size and precision of the balance, batches of 1 to more than 50 individuals can be pooled. The animals are then dried at

50 °C for 24 h (or 48 h for zooplankton larger than copepods), left to cool to room temperature in a desiccator and weighed immediately on removal.

MICROSCOPIC EXAMINATION OF SUB-SAMPLES

The determination of feeding rates using microscope cell counts is time consuming, particularly if used to estimate total carbon ingestion rates because this requires enumeration of all cell types in the food assemblage. Re-consideration of the aims of the experiment could pinpoint some time saving approaches. For example, if the aim is to compare the degree of omnivory among several species, only a few pairs of protozoan and diatom taxa (preferably of similar size and contribution to available carbon) may need to be counted. The goal of microscopic analysis is to count sufficient cells to obtain a reasonably precise estimate of feeding rate. This means counting a minimum of 100 cells from each of the grazed bottles (Lund *et al*. 1958; Venrick 1978). If depletion of a taxon is slight (e.g. $<15\%$) counting imprecision will probably mask the feeding signal unless many more cells are counted.

For Lugol's-preserved samples, the method of Utermöhl (1958) is used. This involves mixing the sample gently and pouring it into a settling column. Typical volumes of these are 10 ml, 50 ml or 100 ml, and the size used depends on the food assemblage. For a bloom, 50 ml is usually suitable, but samples from oligotrophic areas may require several aliquots from a 100 ml column to count sufficient cells. The time required for settling is proportional to the length of the column, and 50 ml columns should be left 24 h. To count the abundant small cells (5 μm or smaller), 10 ml columns are best, as small cells can stick to the walls of taller settling columns (see Chapter 5).

After settling is complete, a cover slip is slid over the receptacle to displace the column to one side. Counting under an inverted microscope is done most quickly by scanning different portions of the receptacle according to the abundance of targeted taxa. Rare taxa may require scanning the entire area from two or three separate 50 ml or 100 ml aliquots of the original sub-sample. Smaller and more abundant taxa may only require counting along several transects across the center of the receptacle under high magnification. Whatever the scanning protocol, cells tend not to settle uniformly, but collect around the edges, so if the whole slide is not analyzed, this should be taken into account. If cells selected for counting are so rare that larger volumes (e.g. >300 ml) need to be analyzed, the sample should be poured into a measuring cyclinder and the contents allowed to settle for an appropriate time. Most of the overlying water can then be siphoned off slowly through fine bore tubing, allowing the remainder to be washed with filtered sea water into an Utermöhl chamber for settling in the standard way. Successful counting of cells demands a standardized approach, so for example, counts should be performed by one person and done 'blind' to avoid subjective identifications.

FEEDING RATE CALCULATIONS

For definition of terms see section 8.3. If the laboratory-determined clearance rate on chlorophyll *a* is used to estimate *in situ* ingestion rate on phytoplankton, the results should first be converted to carbon ingestion rates by multiplying the chlorophyll *a* ingestion rate by the carbon : chlorophyll *a* ratio of ambient sea water, under the untested assumption that this is the same as that of ingested food. If the average chlorophyll *a* concentration in the incubation water is less than that reached in the chlorophyll *a* maximum layer, it is reasonable to use maximum *in situ* values rather than average experimental values in equation 8.13, as it is likely that zooplankton in the sea can find and exploit the richest phytoplankton layers (Harris 1988; Castro *et al*. 1991).

Quantifying total carbon intake due to feeding on protozoans is difficult. First, it requires knowledge of whether each of the non-diatom taxa are heterotrophic, mixotrophic or autotrophic (see Chapter 5). This can be achieved with epifluorescence microscopy of glutaraldehyde-stained cells, although the trophic status of some distinctive taxa is known. Second, the cell dimensions need to be measured, their volumes estimated and their carbon content further estimated from their volumes. Realistic estimates of volumes of non-spherical cells can be problematic. For example Tiselius (1989), estimating volumes of ciliates, found that an approximation to a cone yielded half the volume of an ellipsoid shape of similar length. As described in Chapter 5, carbon content can be estimated using a range of equations specific to microplankton groups (Strathmann 1967; Eppley *et al.* 1970; Borsheim and Bratbak 1987; Putt and Stoecker 1989; Ohman and Snyder 1991). See also page 306 for further details. Note that if 10% Lugol's solutions are used, then carbon contents of aloricate ciliates may require an upward revision due to shrinkage (Stoecker *et al.* 1994).

Total ingestion rates of microzooplankton by mesozooplankton can be obtained from the ingestion rates of the individual taxa. Because of the uncertainties outlined above, it is prudent to determine minimum and maximum likely ingestion rates based on the greatest and least conservative volumes and carbon contents of the various taxa. Many studies compare microzooplankton carbon ingestion with that of phytoplankton. To estimate phytoplankton carbon ingestion it is possible simply to multiply the clearance rate of chlorophyl *a* by the maximum *in situ* chlorophyl *a* value and then by the carbon : chlorophyl *a* ratio. However, we recommend using the same microscope method for both measuring and calculating cell volumes and carbon contents, despite the considerable extra workload.

8.5.13 Use of film and video to study feeding behavior

Since the first work by Alcaraz *et al.* (1980), high-speed filming and especially video recording have been used increasingly to study copepod behavior. The specific conditions required by the method (laboratory conditions, small volumes, etc.) limit its usefulness to behavior studies, with little application to date to quantifying feeding (but see Paffenhöfer *et al.* 1995). However, the use of these techniques has produced important advances in our understanding of feeding behavior. Handling and ingestion of different sized particles (Price *et al.* 1983), inter-specific differences in foraging behavior (Tiselius and Jonsson 1990), swimming behavior and time allocated to feeding under different conditions of food and turbulence (Saiz 1994), or influence of the presence of predators on time allocated to feeding (Tiselius and Jonsson 1997) are just a few examples of the power of this approach.

The methods are divided in two major types depending on whether the observations are done with tethered copepods (e.g. Price *et al.* 1988) or free-swimming copepods (e.g. Saiz 1994). Studies using tethered copepods are intrinsically limited in scope, but permit the use of high magnification, which allows detailed study of processes such as handling of prey (Price *et al.* 1988), individual variability in behavior (Turner *et al.* 1993) or heart beating rates (Alcaraz *et al.* 1994). Studies with free-swimming copepods, if technically more difficult, present a wider range of possibilities to study the influence of different factors on behavior. The primary technical difficulties of observing plankton in experimental aquaria are the transparency of the animals, the (usually) small size of the animals, and the need for sufficient space to avoid wall effects (Strickler 1998; see also Chapter 7 on optical methods for zooplankton research). The usual attraction of

copepods to visible light sources is another problem, usually solved by working in darkness with infrared light sources.

Currently the methodology is not standardized and there are probably as many technical approaches as there are groups working on the subject. However commercially available systems are beginning to appear in the market (Strickler 1998; Chapter 7).

8.5.14 Biochemical indices

The organic matter of ingested food consists of complex organic macromolecules, which must be degraded to simple low molecular weight compounds in order to be available as a source of energy and material to consumers. Digestive enzymes perform the chemical work in this process by hydrolyzing the ingested food to readily assimilable compounds such as amino acids, fatty acids and glucose. Enzyme production is regulated by ingestion and by the metabolic needs of the consumer. The former fact is the theoretical basis for enzyme activities as proxies of feeding activity. Mayzaud (1986) gives a comprehensive review of this subject. Although the review was restricted to copepods, this group is the only one well studied among zooplankton taxa. However, the results should be generally applicable because enzyme systems are similar in other taxa. Here we focus on a critical evaluation of the method to estimate zooplankton feeding rates from the activity of digestive enzymes. In order to understand the constraints in the method we must know how the digestive process works and the analytical principles.

The first phase of digestion occurs during ingestion and consists of mechanical preparation of food by the mouth parts. In crustaceans this fragmentation occurs when feeding on relatively large food items, while small food items can enter the esophagus unaffected. Other zooplankton groups ingest large prey whole. Hydrozoan and scyphozoan medusae, ctenophores and chaetognaths are examples of this category. Such predators are at a disadvantage compared to others because the digestive enzymes must first penetrate the prey surface and process an item with low area/volume ratio relative to a fragmented prey item. The next phase of digestion is enzymatic hydrolysis of complex biochemical components into simple, easily assimilable molecules. Since the biochemical organic constituents in the food can be classified into three principal groups – carbohydrates, proteins and lipids – the enzymes that hydrolyze them are classified similarly as carbohydrases, proteinases and lipases. This is a somewhat simplified view (see Mayzaud 1986) but suits our purpose. The last process in the digestion sequence is the intracellular phase where fully or partly digested food particles are absorbed into vacuoles of the gastrodermal cells and digested completely. When digestion is completed the gastrodermal cell ejects the digested material, which as compounds of low molecular weight can be reabsorbed by absorptive cells, distributed by the circulatory system and used in metabolism. Below we discuss three problems in using digestive enzyme activity as a general estimator of feeding rate.

1) The enzyme activity of zooplankton does not measure any of these three steps in the digestion process. Instead, it measures the amount of a digestive enzyme present at a given moment, although the result is usually expressed as the degradation rate of a substrate. Because a cell-free homogenate is used in the analysis, the continuous production of new enzymes that occurs in intact animals is eliminated. The amount of enzyme present generates a substrate degradation rate, but this rate may differ from that which occurs in nature where enzymes turnover continuously. The method is analogous to measuring the volume of a reservoir but not the rates of

inflow and outflow, when calculating how many households can get their water from it. Consequently, there is an important weakness in the methodological basis for comparison of ingestion rate and the measured activity of digestive enzymes.

2) For enzyme activity to be a good indicator of recent feeding activity, there must be a constant numeric relationship between the two, i.e. a change in feeding level should cause a proportional change in enzyme activity. However, this seems to be the exception rather than the rule (Mayzaud 1986). Båmstedt (1988) noted an exception, where *Calanus finmarchicus* exhibited an approximately tenfold diurnal variation in trypsin activity, closely correlated with feeding activity of the entire population. Although changes in the food environment are usually poorly reflected in enzyme activity, extremes in feeding are more clearly resolved. Thus, both Tande and Slagstad (1982) and Båmstedt (1988) documented dramatic increases in enzyme activity of *Calanus finmarchicus* during the winter–spring transition, and several authors have shown a strong negative relationship between the duration of starvation and enzyme activity (Mayzaud 1986). Båmstedt (1988) also showed that non-feeding adult males of *Euchaeta norvegica* and *Chiridius armatus* had almost no trypsin activity, while the active feeding stages had high activities. However, these differences were not reflected in the amylase activities of the same animals.
3) The method assumes that a given enzyme acts on many types of food, i.e. that changes in food composition do not cause variability related to feeding intensity. Theoretically this is not true and an individual enzyme as an estimator of overall feeding intensity will be inherently sensitive to changes in the quantitative relationship between carbohydrates, proteins and lipids. Because phytoplankton species commonly contain a major component of carbohydrates (Parsons *et al*. 1961) and zooplankton do not (e.g. Båmstedt 1986), any variation in the degree of carnivory would produce a bias in estimating feeding level based on carbohydrase activity. This rationale holds only if point (2) above applies, i.e. that there is a direct relationship between feeding and measured enzyme activity. From a seasonal survey of 19 zooplankton species, Båmstedt (1988) did not find any general inherent differences in either trypsin or amylase activity between herbivores and carnivores, supporting the idea that these enzyme activities are insensitive to trophic variability. However, significant differences were observed between the two dominant omnivorous copepod species, *Calanus finmarchicus* and *Metridia longa*, which have different life strategies. These copepods both exhibited a pronounced spring peak in enzyme activities related to the spring phytoplankton bloom. In contrast, two carnivorous copepods, *Euchaeta norvegica* and *Chiridius armatus* lacked such a peak. Kumlu and Jones (1997) found significantly higher trypsin activity in herbivorous crustacean zooplankton than in carnivores, with intermediate levels in omnivores. Mayzaud (1986) reported differences in laminarase activity between herbivorous and carnivorous zooplankton from contrasting environments. Thus, due to complex interspecific variability, activities of digestive enzymes from mixed zooplankton samples cannot be used as general indicators of zooplankton feeding intensity. Due to inconsistency in the results reported in the literature, we also caution against using the activity of a single enzyme as a measure of the degree of carnivory or herbivory, unless a pilot study on the target organism confirms its sufficiency.

Mayzaud (1986) lists the digestive enzymes used in zooplankton feeding studies and with their primary substrates and end products. Despite the important role of lipids in high-latitude marine food chains, studies of lipase are rare. In the next section we

describe analytical procedures for two of the most commonly used enzymes, amylase and trypsin, using the protocols of Båmstedt (1988).

8.5.15 Working procedures for measurement of digestive enzyme activity

Field collected material should not be stored for any length of time. If not processed within a few hours, it should be frozen, preferably at $-70\,^{\circ}C$ or lower (Mayzaud 1986). Further preparation is carried out with the material kept on ice. The material is homogenized in a Potter–Elvehjem homogenizer or a similar device, using either distilled water or a Tris-HCl buffer, 1–10 mmol l^{-1}, pH 7.0 or 7.5 (Head and Conover 1983). A sub-sample of this crude homogenate is used to analyze the protein content. This factor is used to calculate the enzyme activity, which is given per unit of protein.

AMYLASE

Amylase, or more correctly *α-amylase*, hydrolyzes starch via dextrin to maltose, which is finally hydrolyzed by the enzyme maltase to glucose. Although starch is not a dominant type of polysaccharide in the marine environment, amylase is commonly present in marine zooplankton (Mayzaud 1986). The analytical procedure is described by Street and Close (1956) and used on zooplankton by Samain *et al*. (1977).

Reagents

1) *1% amylose solution* 80 ml 0.1 mol l^{-1} NaOH is heated to 90 °C and added to 0.1 g amylose, suspended in 5 ml 96% ethanol. Chill and add 0.1 mol l^{-1} NaOH to 100 ml.
2) *Amylose working solution* Mix 10 ml (1) with 90 ml distilled water.
3) *Hydrochloric acid* 0.01 mol l^{-1}.
4) *Phosphate buffer* 0.02 mol l^{-1}, pH 7.0. Dissolve 1.735 g Na_2HPO_4 and 1.060 g KH_2PO_4 in distilled water to 1.0 l.
5) *J2/KJ solution* 0.1 mol l^{-1}. Dissolve 30.0 g KJ in 250 ml distilled water and then 13.0 g J2, and make up to 1.0 l with distilled water. Stored dark.
6) *J2/KJ working solution* 0.01 mol l^{-1}. Dilute 10 ml (5) with 90 ml distilled water.

Analysis

All procedures are performed on ice. Live zooplankton of about 2–3 mg dry weight (10–15 mg live weight) are homogenized in 3 ml distilled water and the crude homogenate is centrifuged to obtain a cell-free homogenate. Two ml of reagent (2), 2 ml of reagent (3) and 5 ml of reagent (4) are mixed in 50 ml vials warmed in a water bath (37 °C) for 3 min. One ml of the cell-free homogenate is added and mixed, then incubated at 37 °C for another 15 min. Four ml of reagent (6) and 15 ml distilled water are added and mixed, then read in a spectrophotometer at 660 nm against a blank, prepared by substituting the homogenate with 1 ml distilled water. The activity is given in mU (milli-units) and a standard curve is prepared by using amylase of known activity from a series of 0–160 mU.

TRYPSIN

Trypsin is a serine protease or endopeptidase which hydrolyzes lysine and arginine peptide bonds (Mayzaud 1986). Thus this enzyme represents only one step in the digestion of proteins. Complete digestion involves acid proteases such as pepsin, exopeptidases such as aminopeptidase, and di- and tri-peptidase. In contrast to terrestrial primary producers, phytoplankton have a high protein content. Thus, protein is a dominant food constituent for all zooplankton, irrespective of trophic position. The

analytical procedure was described by Erlanger *et al.* (1961) and used on zooplankton by Samain *et al.* (1977).

Reagents

1) Substrate buffer 0.1 mol l^{-1} Tris-buffer, pH 8.4. Mix 12.2 g Tris and 18.0 g $CaCl_2$ $2H_2O$ with distilled water to 1.0 l.
2) Substrate solution Dissolve 52 mg L-BAPA (*N*-benzoyl-L-arginine-4-nitroanilide) in 100 ml (1) by warming below 90 °C. This solution is prepared for each set of samples and is kept at 39 °C.

Analysis

All procedures are performed on ice. Live zooplankton of about 2–3 mg dry weight (10–15 mg live weight) are homogenized in 3 ml distilled water and the crude homogenate is then centrifuged to obtain a cell-free homogenate.

0.5 ml of the cell-free homogenate is mixed with 5 ml of reagent (2) and incubated at 39 °C for 10 min. Absorbance is read on a spectrophotometer at 405 nm and the sample is returned to the water bath, incubated for an additional 15 min and again read. The change in absorbance over 15 min corresponds to the enzyme activity. A distilled water sample is used as a blank. A calibration solution (*p*-nitroaniline calibration solution) is available from Merck (product no. 15912), with the activity given as the amount of *p*-nitroaniline per time unit.

The Arrhenius equation may be used to make temperature corrections, i.e. to transform enzyme activities measured at analytical temperatures (t_a) to *in situ* temperatures (t_i):

$$\mathbf{Activity}_{in\ situ} = \mathbf{Activity}_{\mathbf{analytical}} \times \exp[E_a/R \times \{1/(t_a + 276) - 1/(t_i + 276)\}] \quad (8.59)$$

where E_a is the energy of activation (kcal mol^{-1}), R is the gas constant, 1.987 cal K^{-1}, and the number 276 is the temperature in °C corresponding to 0 °K. E_a for polysaccharidases was given by Mayzaud (1986) as 8.76 for amylose as substrate, 14.33 for laminarin as substrate. E_a for trypsin, using benzoyl-L-tyrosine ethyl ester as substrate has not been given.

8.6 DIFFICULTIES WITH SPECIFIC ZOOPLANKTON GROUPS

Due to a number of factors, some types of zooplankton are especially difficult to survey. Factors such as large size, fragility, deep-water living, sensitivity to light and food specificity may restrict the possibilities of obtaining reliable information about their feeding rates and feeding behavior.

8.6.1 Stomach contents from field samples

General critical factors are considered on page 331, where sampling duration, feeding, egestion and regurgitation during sampling and handling are discussed. Problems related to specific groups are mainly due to depth of occurrence, abundance, size and fragility. Large, deep-living gelatinous organisms occurring in low abundance fall outside the reach of the methods reviewed here, but capture of individual specimens from a manned submersible or remotely operated vehicle (ROV) followed by immediate

Table 8.4 Methods to estimate feeding rate of salps. After Madin and Kremer (1995). X denotes if the method is appropriate for estimating clearance rate (F) and/or ingestion rate (I).

Method	*F*	*I*	Assumptions	Strengths	Weaknesses	References
Particle depletion	X		Normal feeding behavior in experiment, constant feeding rate	Standard, comparable method; depletion easily measured	Confinement; usually artificial diet; high particle concentrations	Andersen (1985) Deibel (1982, 1985) Harbison and Gilmer (1976) Harbison and McAlister (1980) Harbison *et al.* (1986) Reinke (1987)
Chlorophyl depletion	X		As above	As above; mixed diet possible	As above	Huntley *et al.* (1989) Madin and Kremer (1995)
Radiolabeled particles	X	X	Non-selective feeding and assimilation	Lower particle concentration	Confinement; respiration and defecation losses of label	Mullin (1983)
Gut pigment content	X	X	Gut-passage rate constant; pigment concentration known; background pigment known; pigment destruction known	No confinement; natural diet; simple and rapid analysis; time- and depth-specific data	Measuring gut-passage time; pigment destruction; variable pigment distribution in water	Madin and Cetta (1984) Madin and Purcell (1992) Madin and Kremer (1995)
Gut particle content	X	X	Trace particles conserved; randomly distributed	No confinement; natural diet; no pigment loss	Measuring gut-passage time; variable particle distribution in water; tedious quantification	Madin and Purcell (1992)

Defecation rate	X	X	Steady-state ingestion and egestion; normal defecation rates; conserved tracer; pigment concentration known	No gut-passage times; no background pigment	Confinement; abnormal feeding rates; tracer loss	Madin and Kremer (1995)
Calculated from metabolism	X	X	Metabolic rates and assimilation efficiency known; food availability known	Independent check on other methods; establishes minimum ingestion rate	Inverse calculation; may be very approximate	Cetta *et al.* (1986) Madin and Kremer (1995)
Calculated from swimming	X		Pulse rates known; feeding is continuous; all water passes through filter	No confinement; estimates filtration directly; establishes maximum clearance rate	Difficult to measure internal volume; pulse rate may be abnormal	Andersen (1985) Bone *et al.* (1991) Reinke (1987) Madin and Kremer (1995)

preservation has been an option for a decade or so (Marsh Youngbluth, personal communication). With the rapid technological evolution in acoustical detection methods, image quality and light sensitivity of video cameras, and data transfer to the mother ship or even to a land-based laboratory, together with new, remotely operated sampling devices, we can expect increased use of submersibles in nutrition studies of deep-sea zooplankton. However, because of the high operational costs associated with such techniques, the use of submersibles in deep-water environments is available at relatively few research institutions.

Fragility of organisms is a problem with gelatinous zooplankton, but within this group fragility varies. For example, among ctenophores we find extremely fragile forms, such as the lobate genera *Bolinopsis* and *Mnemiopsis*, whereas the cydippid counterparts, for example *Pleurobrachia* and *Mertensia* are quite robust. Collection of the most fragile forms requires a gentle sampling method in order to get whole specimens that can be measured and analyzed. Some of these forms might not remain intact in common preservatives like formalin and ethanol and may disintegrate when frozen and subsequently thawed. Even if the animals do not disintegrate during storage, we have to consider shrinkage when kept in the preservative. This factor is particularly significant for gelatinous zooplankton, because they have no exoskeleton and a watery body constitution that easily dehydrates and shrinks. For example, Båmstedt (1998) reported a 31% reduction in the oral–aboral diameter of *Pleurobrachia pileus* (Ctenophora, Cydippida) after one year in 4% formalin. If this is not corrected, the daily ration or weight-specific predation rate can be overestimated by a factor of three. If the specimens are relatively small, we recommend immediate morphometric measurements like length, diameter, height and wet weight or volume. Alternatively, a separate investigation can provide information on shrinkage over time and relate the measured dimension after preservation to the original carbon content or other relevant body-mass expression. If there is risk of disintegration in the preservative, measurements before preservation and storage in individual glasses will ensure that data are not lost. In this case it is particularly important to remove any prey adhering to the exterior of the predator, otherwise they will mix with prey in the stomach content when the animal disintegrates.

Some species of siphonophores may reach a length of several tens of meters and these may be very fragile, easily breaking into smaller pieces (Mackie *et al*. 1987). An example is the physonect siphonophore *Apolemia uvaria*, which is constructed of a sequence of cormidial groups along a central stem, reaching a length of up to 30 m. Each cormidial group can survive as a separate colony, and fragmentation into shorter lines of cormidial groups is so common that the majority of observations represent such incomplete colonies (Mackie *et al*. 1987; Båmstedt *et al*. 1998). The problem with such organisms is how to express feeding quantitatively. Ideally we should give the average predation rate per gasterozooid and define the abundance of gasterozooids per length unit of the colony. Such information can then be used to estimate predation of other colonies of various length.

Salps (Tunicata, Thaliacea) are a problematic group because they are large, consume primarily phytoplankton and are extremely fragile. Madin and Kremer (1995) conclude that no single methodological approach is best in all cases. We reproduce their recommendations in Table 8.4, since they are of general relevance for other fragile forms also.

8.6.2 Laboratory experiments

In an experiment where we want to replicate the feeding dynamics that occur in

nature, the quality of experimental animals is of utmost importance. Therefore, similar constraints as for field investigations on stomach contents exist, with limitations as a result of depth of occurrence, abundance, size and fragility. The organisms for which field data on feeding are most scarce are also the most difficult to use in laboratory experiments. However, there is no critical time limit to the sampling as there is for gut content analysis, and cod-end feeding is not a critical factor. Very slow towing and use of large cod-ends equipped with a closing device are factors that reduce stress on the animals. A closing device in the cod-end can be important with deep-water organisms, because they are not adapted to light conditions in surface water. For example, the visual function of euphausiids varies with their depth of occurrence (Hiller-Adams and Case 1984) and species with sensitive eyes may be injured by sunlight. Such seemingly healthy animals are likely to behave differently in an experiment than in their natural environment. Visually controlled collection by a submersible with a special sampling device is a possibility to bring very fragile animals up to the surface in intact condition, but again we must accept that this technique is not generally available.

Five factors should be considered in the design of a feeding experiment, depending on the type of consumer and the type of prey used:

1) volume and dimensions of the experimental vessel
2) number of consumers
3) number of prey
4) incubation time
5) water mixing.

These factors can be combined to enable an appropriate reduction in food over the experimental duration that can be easily quantified. Equation 8.56 can be used to dimensionalize incubation volume (V, litres), incubation time (t, hours), number of predators (n), and concentration of food items (C, food items litre^{-1}) for a given percentage of food reduction (FR), if we have an approximate idea of the daily ration (DR):

$$\boldsymbol{DR/100 \times n \times W_p \times t/24 = FR \times C \times W_f \times V} \tag{8.60}$$

where W_p and W_f are the body masses of an individual predator and food item respectively. Mass should be expressed in the same units for predator and food, preferably in carbon units. If we do not know the DR value, we suggest using 20% initially. The level of the factor FR must be decided for each type of experiment, and it is mainly constrained by statistical requirements. If food can be added in a precise amount, recovered easily, and enumerated precisely after the incubation, then FR can be very small, for example 5% to 10%. Variation between replicate experiments will then be explained by predator variability, not by random variability in sampling and enumeration procedures. This holds true in a predation experiment with, for example large copepods, euphausiids or small fish as prey, where all added and remaining prey are counted and there is no loss term other than predation mortality during the incubation and prey production does not have to be taken into consideration. In contrast, in an experiment with protists as prey, such as phytoplankton or ciliates, enumeration both at the beginning and end of the incubation is based on sub-sampling. In a typical experiment with a phytoplankton culture as food source we measure the cell concentration of a stock culture by sampling a small volume, dilute it and count the cells either under a microscope or with an electronic particle counter. In both cases we enumerate a small sub-sample of the original sample. The amount of food added to the experimental

treatments is determined from this information. The actual beginning and end concentrations are determined in the same way. In addition to the experiments with predators, we also have to consider prey production, estimated through the results of incubations without predators. Thus, a number of samplings and enumerations may form the base for the calculations, each with an inherent variability. In this case we need considerably higher differences between initial and final concentrations, simply due to the statistical requirements in a situation with high inherent variability. As a rule of thumb, we suggest that *FR* should be at least twice the standard deviation of replicate samples of the food concentration (Marin *et al.* 1986). If the variability inherent in the experiment is not known, we recommend using a value for *FR* of 20% initially.

In practice we know W_p and W_f, and we want to run the experiment with a given C. Based on the type of prey used we also define a value for *FR*. V is an important variable and a recommendation must consider the type of predator used in the experiment, with a large volume for large animals with extended catching structures or high mobility. We must also decide *a priori* whether to integrate over 24 h or assess diel feeding variability. Thus, from these requirements we define the number of predators to be used by inserting the selected values for the variables in equation 8.48, with n as the dependent (unknown) variable.

Water mixing in the experimental vessel has two purposes (1) to keep the food organisms in suspension and (2) to sustain homogeneity in food abundance. Both are important factors in quantitative feeding studies and deviation from these conditions may invalidate an experiment. As a complicating factor, some fragile organisms are sensitive to the mechanical stress caused by turbulence. Organisms like appendicularians, with delicate food-capture structures (Flood 1978), may be damaged easily and organisms with tiny tentacles or mucus sheets may be disturbed, resulting in unnatural feeding behavior. Thus, when there is a conflict between the opposing needs of mixing and undisturbed conditions, pilot tests should be performed in order to determine a suitable experimental design.

Large-sized gelatinous organisms are a problematic group in incubation experiments, particularly those whose hunting mode is 'ambush entangling'. Proper function of their feeding behavior requires a large volume, which may impose constraints in dimensions of the experimental vessel. For example, the same diameter medusae of two common scyphozoans from the Northeast Atlantic, *Aurelia aurita* and *Cyanea capillata* require dramatically different vessel volumes because the latter has tentacles at least ten times longer than its diameter, while the former has short tentacles (Costello and Colin 1995). The swimming behavior of different medusae must also be taken into consideration, because this may be an integrated part of the hunting technique (see Mills 1981; Madin 1988). Experiments performed on such organisms without prior knowledge of their feeding behavior are unreliable.

On the basis of the above discussion, it may appear that large experimental volumes should always be chosen. However, in addition to the obvious problems of access to large tanks or *in situ* enclosures, there can be other problems related to large volumes. First, when the volume increases more food is needed. If the food is a defined species that is sorted from a mixed assemblage, then the labor of sorting rapidly constrains the volume it is possible to use. Second, from equation 8.56, it may be necessary to use many predators to produce a significant reduction in prey concentration in a very large tank. This may then interfere with the spatial needs of the individual predator. Third, although a large volume is more stable in temperature than a small one, temperature can be difficult to manipulate in large volumes. Fourth, the difficulty of keeping the food

environment homogenous increases with volume. Fifth, quantification of prey abundance is increasingly difficult with increased volume. In practice one is bound to take sub-samples from a large tank, thereby introducing additional variability. Thus, experiments with predators that require large volumes of water and traditional incubation with enumeration of the predator removal of prey, cannot be recommended as a general method here. An attractive substitute is to use large tanks with defined prey concentration as the feeding environment, but analyze the stomach content of the predators instead of prey removal. Separate digestion experiments then complete the information required to use equation 8.28, where G is number or biomass of prey in the predator stomach. A single tank experiment can then give data from each individual predator, thus providing a statistically better base for the conclusions than with the prey removal technique.

8.7 OMNIVORY

Planktonic protozoa function as important trophic intermediaries in pelagic food webs by repackaging small bacterial and phytoplankton cells into prey accessible to larger consumers such as crustacean zooplankton. Correlations of decreases in protozoan stocks with increases in copepods in mesocosms (Sheldon *et al.* 1986) and in the field (Smetacek 1981) suggested that mesozooplankton control protozoan populations. Although a number of earlier studies suggested that they consume protozoa as well as phytoplankton (Mullin 1966; Zeistchel 1967; Harding 1974), until recently, suspension feeding copepods have been treated in both experimental studies and models as exclusively herbivorous. Several studies done during the mid- to late 1980s demonstrated that a number of mesozooplankton taxa consumed protozoa in controlled laboratory experiments (Robertson 1983; Stoecker and Sanders 1985; Ayuki 1987; Stoecker and Egloff 1987; Wiadnyana and Rassoulzadegan 1989; Sanders and Porter 1990; Wickham *et al.* 1993). It is now well documented that suspension feeding copepods and other meso- and macrozooplankton consume planktonic protozoa as well as phytoplankton under field conditions, and at times non-plant food constitutes an important component of their diet (Stoecker *et al.* 1987a, 1987b; Barthel 1988; Gifford and Dagg 1988, 1991; Tiselius 1989; Gifford 1993a; Fessenden and Cowles 1994; Ohman and Runge 1994; Atkinson 1995, 1996). The heterotrophic protistan prey of mesozooplankton includes flagellates, ciliates, and dinoflagellates.

Euphausiids can represent a significant fraction of the mesozooplankton biomass, especially in higher latitudes (Mauchline and Fisher 1969). These animals have traditionally been viewed as herbivores, but recent studies indicate considerable carnivorous feeding by several species. The principal type of animal prey seems to be copepods and in section 8.7.4 we report on a method that has been successfully used to estimate the degree of carnivory in euphausiids (Båmstedt and Karlson 1998), which can also be applied to other types of zooplankton.

8.7.1 A general method to estimate omnivory

The most commonly used method to measure omnivorous feeding involves monitoring the disappearance of prey. This is the classical method developed by Gauld (1951). Advantages are that it is conceptually straightforward and that all taxa of prey can be determined. Labor-intensive analysis using various microscopical methods can be a

disadvantage. The general experimental design is described in detail on page 314 and is reiterated only briefly here. Experimental treatments consist of the *in situ* microplankton assemblage with consumer organisms added; control treatments are the assemblage without consumers.

COLLECTION OF CONSUMERS

Copepod consumers are collected by gentle vertical hauls of a relatively fine-mesh ($\sim$200 μm) net in order to obtain animals with all their setae intact. Other consumers may have special collection requirements, for example salps and other fragile gelatinous zooplankton may need to be collected individually by divers.

COLLECTION AND HANDLING OF WATER

As open ocean forms of planktonic protists may be sensitive to trace metal contamination, sea water should be collected using clean, metal-free bottles, such as teflon-lined Go-Flo bottles, Niskin bottles outfitted with external springs, or Niskin bottles equipped with teflon-coated internal springs. All o-rings should be made of silicone. It has been shown that the black latex rubber tubing used in standard Niskin bottles is toxic to marine microplankton (Price *et al.* 1986). If standard Niskin bottles must be used, the interior tubing should be replaced with silicone tubing and the rubber o-rings replaced with silicone o-rings. Water containing the natural microplankton assemblage should always be siphoned rather than poured in order to minimize destruction of planktonic protists (Gifford 1985). If water must be passed through a mesh to exclude larger grazers, the mesh should be submerged. Rotation of incubation bottles maintains both consumers and prey in suspension. In the absence of rotation, prey organisms may settle during incubation and form a concentrate which is either more susceptible to predation or escapes predation by sinking to the bottom of the bottles. Gifford (1993c) recommends that bottles be incubated without an air space because the combination of rotation and an air space is destructive to planktonic ciliates. To eliminate air, incubation bottles are topped up, covered tightly with Parafilm to exclude air, and capped. The standard practice is to incubate dilution treatments in polycarbonate bottles, which have the advantage that they can be cleaned thoroughly. Bottles are typically incubated for 24 h in order to encompass any diel feeding cycle of the consumers. In the experimental design described by Gifford (1993a, 1993c) there are three sets of replicated treatment bottles: initial control bottles; final control bottles, and final experimental bottles. Because of the need to incubate without an airspace, it is not practical to sub-sample the same bottle for initial and final control data.

SAMPLE COLLECTION, PROCESSING AND ANALYSIS

A number of samples may be collected, including chlorophyl, nanoplankton (2–20 μm), and microplankton (20–200 μm). Water is collected by siphoning gently from the incubation bottle into the collecting vessel. Phytoplankton are analyzed by chlorophyl or other plant pigments and microscopic counts. Preservation depends on the target taxon (see Chapter 5 for details). Nanoplankton are enumerated using epifluorescence techniques. Microplankton are preserved with acid Lugol's solution and enumerated by inverted microscopy. If the objective of the experiments is to measure ingestion rates, the prey items must be measured and their carbon content calculated. Various manual, semi-automated and automated image analysis techniques may be used for both measurement and counting (e.g. Verity and Paffenhöfer 1996). In fact these are nearly as time-consuming as mechanical counting and few have access to the required equipment.

However, such methods render measurements for eventual volume estimation more consistent and precise.

DATA ANALYSIS

Grazing rates are calculated from the set of equations given in section 8.3, i.e. clearance rate from equations 8.2–8.5 and ingestion rate from equation 8.13. Several caveats apply when using this method. So-called 'bottle effects' can occur with any incubation method (Roman and Rublee 1980). While some of these may be overcome by addition of phytoplankton nutrients, particularly NH_4, trophic cascade effects need careful consideration (see page 345).

8.7.2 Gut fluorescence and experimental egg production

The gut fluorescence method (see sections 8.5.2, 8.5.3) provides direct information only on ingestion of phytoplankton. Nevertheless, Dam *et al.* (1994) and Peterson and Dam (1996) suggested a herbivory index (H) to estimate omnivory from gut fluorescence and egg production rates:

$$H = K_1/K_1' \tag{8.61}$$

where K_1' is egg production rate/pigment ingestion rate in terms of nitrogen or carbon and K_1 is gross growth efficiency (growth/ingestion) measured in the laboratory. Use of this index is subject to the uncertainty of pigment destruction, to the accuracy on the measurement of C:chlorophyll *a* or N:chlorophyl *a* ratio, and to the influence of different diets (herbivore *vs.* carnivore or omnivore) on the gross growth rate. Undoubtedly further information is required, but this index is a promising tool to estimate omnivory from two simple and commonly used techniques.

8.7.3 Gut fluorescence and egestion rate

One approach to estimate omnivory is to measure both total carbon ingestion and algal carbon ingestion and to quantify the degree of omnivory as the difference between the two (e.g. Pakhomov *et al.* 1997). These authors working on a large euphausiid species, calculated algal carbon ingestion from the gut fluorescence method, and multiplied this by a C:chlorophyl *a* ratio. Total carbon ingestion was estimated by measuring the time course of fecal pellet production by freshly caught euphausiids and adjusting this to ingestion using literature estimates of assimilation efficiency. This method can be used for large zooplankters with collectable fecal pellets, and does not require the animal to feed in captivity. However there are numerous assumptions and possible errors in both the gut fluorescence measurements and in egestion rate. As shown on page 336 the results are also sensitive to the exact assimilation efficiency value chosen to convert egestion rates to ingestion rates.

8.7.4 A method to estimate the importance of copepod prey for predators

This is based on the investigation by Båmstedt and Karlson (1998) where euphausiid stomach contents were analyzed for copepod mandibles. Gut fullness (relative scale from 0 to 1) is defined and the total mass of copepods in the stomach content calculated from equations 8.42–8.45 on page 333. In order to calculate the proportion of copepod mass

in the stomach, the weight of a full stomach must be known. There is no standard to do this, but direct weighing or volume estimation from size measurements may be used. An alternative, based on a large set of field data is described by Båmstedt and Karlson (1998). The weight of a full stomach is expressed as a proportion (S) of the body weight of the predator. Proportion of copepod mass in the diet is defined by:

$$\boldsymbol{C\% = W_{cop}/(SF \times S \times W_{pred}) \times 100\%} \tag{8.62}$$

where $C\%$ is the percentage of copepod mass in the diet, W_{cop} is the calculated weight of copepods in the stomach (see section 8.5.4), SF is the stomach fullness (range 0–1) and W_{pred} is the weight of the predator. All weights are expressed in the same units. The results by Båmstedt and Karlson (1998) and Nicol (1984) showed that a full euphausiid stomach was about 6% of the body weight, which can be used as a fixed constant of S, although its reliability for other species then included in the original papers is not documented. Alternatively the stomach can be dissected and weighed. The expression ($SF \times S \times W_{pred}$) in equation 8.62 is then replaced by the actual weight of the stomach.

8.8 FACTORS REGULATING FEEDING RATE

This section does not attempt to review how environmental factors affect feeding of various zooplankton taxa but focuses on those factors that should be considered in the design of feeding experiments. The overall approach of this book requires an emphasis on methodological problems rather than mechanisms.

8.8.1 Abundance of food items

The classical paper by Frost (1972) illustrates the basic principles, although later studies emphasized the importance of behavioral components that may modify the basic picture. The reason for abundance and particle size to be of such importance is shown by a simple model, the functional response, which explains the relationship between ingestion rate and food abundance. An individual zooplankter encounters its prey in direct proportion to prey abundance. Thus, in the absence of limiting factors, feeding rate increases linearly with food concentration. However, there are mechanical and physiological upper limits to the feeding rate, due to mechanical processing of food during capture and ingestion, the volumetric constraints of the digestive tract, and the time required for digestion. There is probably also a motivation factor operating to reduce the animal's interest in feeding with increasing food abundance. These factors produce a response that can be illustrated by one of four models (Holling 1959, type I, II, III or modified type II). Whether the governing factors act in a gradual or absolute way is debated, and among the four models, one response is linear (absolute) and three are hyperbolic (gradual). Usually we are interested in the way a group in a population, comprised for example of a given developmental stage, responds to different food concentrations. Inherent individual variability in size, gut capacity, physiological status, etc. certainly induce variability in this response. Variability in the results of feeding experiments is usually so high that the discrepancy between the models is difficult to detect, and it is a matter of taste which model one uses to describe the data. The four models give essentially the same information about the range in which ingestion is food limited, maximum ingestion rate, and the sensitivity of the ingestion rate to changes in food concentration. An example is given in Figure 8.7, where data from Båmstedt *et al.*

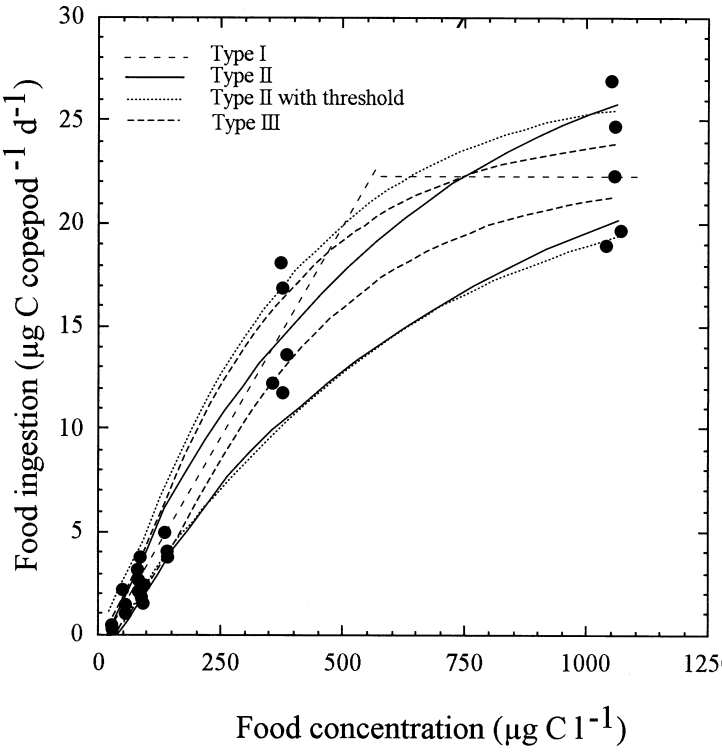

Fig. 8.7 Ingestion of *Calanus finmarchicus* with *Emiliania huxleyi* as food, showing actual measurements and fitted functional response models to the data. The two curves for each of model type II, modified type II, and type III represent upper and lower 95% confidence intervals in α and y_{max}. Original data from Båmstedt *et al.* (1999).

(1999) on *Calanus finmarchicus* feeding on the coccolithophoride *Emiliania huxleyi* is displayed, together with the calculated 95% confidence intervals for model II and modified model II, respectively, and a linear expression (model I). The two model II versions and the type III model are not statistically different, as seen from overlap of confidence intervals. The regression coefficients (R^2) are virtually identical: 0.93, 0.96 and 0.96. The main differences among the models occur at the lower food concentrations. This may pose a dilemma, because results at low food concentrations tend to be those with the lowest precision. An overview of different expressions for the models, with recommendations, is given in the next sections.

FUNCTIONAL RESPONSE. MODEL I

Model I is usually presented as two straight lines fitted by eye to the data. This is not a satisfying practice because it is subjective. Another method is to decide where along the x-axis the deflection point should be and then use two separate linear regression equations for the two data sets (below and above the deflection point, respectively) to calculate the position of the lines. With this approach the upper line may not be horizontal, obliterating the theoretical basis for the model, which assumes that the

ingestion rate is constant above a critical level. We recommend the following procedure.

1) Based on a scatter diagram of the data, decide where along the x-axis the deflection point (d) should be situated, defining two data sets.
2) Apply linear regression analysis to the data set with lower food concentration to define the line which describes ingestion rate in the food-limited region.
3) Calculate the average value for the data set that describes ingestion rate in the food-saturated region and draw a horizontal line at this level.
4) Combine the two straight lines into the type I model expression:

$$\begin{aligned} y &= a + b \times x \quad \text{for } 0 \leq x \leq d \\ y &= (\text{Sum } x)/n \quad \text{for } x > d \end{aligned} \tag{8.63}$$

In some situations data points located near the deflection point are likely to be contained in either data set, and such data can then be assigned to both, i.e. we allow some subjectivity. The procedure provides the usual statistical parameters for significance testing, although it is divided into two separate sets. Comparing two type I models implies that an ANOVA is applied to the two regression equations and that the two mean values for the horizontal line are compared, for example by using a simple Student's t-test. The first gives information about difference in slope and intercept in the food-limited region, and the second gives information about differences in maximum ingestion rate under saturated food conditions.

FUNCTIONAL RESPONSE. MODEL II

This is the most common model of the functional response and it has been expressed in two ways in its simplest form. Ivlev (1961) used the expression:

$$I = I_{max} \times [1 - \exp(-a \times C/I_{max})] \tag{8.64}$$

where I_{max} is asymptotic maximum ingestion rate, C is the food concentration, and a is a constant, defining the rate at which I approaches I_{max}. The level of I_{max} is set by the ability of the predator to handle and process the encountered prey, as well as the capacity of the stomach and gut and the turnover rate of gut contents. The constant a is defined by the volume of water processed and the efficiency with which food particles can be extracted from this water.

Model II has also been expressed by Crawley (1975) and Cushing (1978) as:

$$I = I_{max} \times C/(K_C + N) \tag{8.65}$$

where K_C is the half-saturation constant, i.e. the value of C where I has reached the level $I_{max}/2$. There are many other equations that produce the same picture of increasingly saturated ingestion with increased food concentration, but we recommend the use of either of the two presented above.

FUNCTIONAL RESPONSE. MODIFIED MODEL II

A modification of equation 8.64 was suggested by Parsons *et al.* (1967) on the basis of experimental results indicating a threshold food concentration below which feeding was not initiated. Mathematically this is expressed by substituting C in equation 8.64 with $(C - C_0)$:

$$I = I_{max} \times [1 - \exp(-a \times (C - C_0)/I_{max})] \tag{8.66}$$

Similarly, the Michaelis–Menten relationship (equation 8.65) was modified by Mullin *et al.* (1975) to account for a threshold feeding concentration as:

$$\boldsymbol{I = I_{max} \times (C - C_0)/[K_C + (C - C_0)]} \tag{8.67}$$

FUNCTIONAL RESPONSE. MODEL III

This model behaves similarly to the type II models at higher food concentrations, and the underlying explanation is the same. Due to its sigmoidal shape, model III differs from a type II response at lower food concentrations. This model has been applied especially to vertebrates, but studies on prey switching have provided arguments for its utility in invertebrates (Begon *et al.* 1990). This model is controversial for zooplankton, and there is no general explanation for the curve's S-shape. The model is not usually presented as an equation in published papers, but as a visual fit to the data, making statistical evaluation difficult. The original equation, given by Holling (1959) for small mammals preying on insects, also included a constant for handling time (h):

$$\boldsymbol{I = (a \times C^2/(1 + a \times h \times C^2)} \tag{8.68}$$

where a is a constant defining the instantaneous attack rate of the predator. In suspension-feeding zooplankton both constants h and a appear to be of little significance and an alternative form of sigmoid curve may be easier to accept. There are several different equations that generate a sigmoid shape. For example the Gompertz equation (Valiela 1984) is used to express development of body weight of an organism over time, and the logistic equation is used extensively to describe density dependent population growth. The differences in results produced by sigmoid equations are slight. Due to the already extensive use of the logistic equation in biology and its relative simplicity, we suggest that it also be used to calculate the model III functional response. This version is:

$$\boldsymbol{I = I_{max}/(1 + \exp[(K_c - C)/a])} \tag{8.69}$$

where I_{max} is the maximum ingestion rate, C is the food concentration, K_c is a constant defined as the food concentration for $I = I_{max}/2$, and a is a constant that defines the shape of the curve.

DESIGN OF FUNCTIONAL RESPONSE EXPERIMENTS

The traditional way to do a functional response experiment (e.g. Frost 1972) is to take end-point measurements from experiments with a suitable range of initial concentrations of a given food. Clearance rate (F) and ingestion rate (I) are calculated from equations 8.5 and 8.13, thereby assuming that F is constant throughout the experiment. Bias may be caused by a reduction of food concentration from saturated to non-saturated concentration over experimental duration, which by definition changes F. It may also be caused by a reduction of food below a threshold level where feeding ceases, and it can certainly also be caused by behavioral changes during the experiment. McClatchie and Lewis (1986) evaluated problems related to the end-point technique in functional response studies and concluded that a time-series approach is superior. Their argument for a type II model follows.

The rate of decline of food concentration in a feeding experiment is:

$$\boldsymbol{\partial C/\partial t = -I_{max} \times n/V \times [1 - \exp(-a \times C/I_{max})]} \tag{8.70}$$

where n is the number of predators and the other symbols are as defined above. Solving for C gives:

$$-C_0/C_c \times [C_t/C_0 + C_c/C_0 \times \ln\{(1-\exp(-C_t/C_c))/(1-\exp(-C_0/C_c))\} - 1] = a \times n \times t/V \quad (8.71)$$

where C_0 and C_t are initial and final food concentrations, C_c is the 'critical' food concentration, i.e. the lowest food concentration at which $I = I_{max}$ and other symbols are as defined above. When C_0/C_c approaches the origin, Equation 8.71 reduces to:

$$C_t/C_0 = \exp(-a \times n \times t/V) \quad (8.72)$$

This is the classical equation (equation 8.2), the term $a \times n/V$ being synonymous with g'. However, if $C_0 \gg C_c$ and $t \ll a \times n \times t/V$, then the ingestion rate is constant (I_{max}) during the experiment and the ratio C_t/C_0 is defined by:

$$C_t/C_0 = -I_{max} \times n \times t/V \quad (8.73)$$

In an end-point experiment using equation 8.71, if $C_0 \gg C_c$ there will be a significant error. For example, with $C_0/C_c = 5$ and $a \times n \times t/V \leq 2$, the true coefficient ($g'$) is underestimated from calculation of $a \times n/V$ by a negative factor of 0.8 (see McClatchie and Lewis 1986). The authors recommend use of a single experiment with an initial concentration $\gg C_c$ and a final concentration $\ll C_c$ with repeated analyses of food concentration over time. Several replicate experiments can be run in order to improve the statistical basis.

CALCULATING CURVE FITS IN FUNCTIONAL RESPONSE EXPERIMENTS

The model I fit is easily calculated using standard spreadsheet software or an advanced hand calculator, while the curvilinear models employ iteration techniques available in more advanced statistical software packages. Procedures for analysis of the non-linear regressions presented here are available from Statistical Analysis System (SAS) through their NLIN procedure and the modified Gauss–Newton method. The output is given as a scatter plot with the fitted curve, together with all statistical parameters needed to evaluate differences between curves. The iteration technique automatically generates the optimum estimates of all constants included in the models. The regression coefficient (r^2) for each fit can be used to decide which model provides the best fit to the data. Robinson (1985) reviews techniques used to calculate non-linear regressions of significance in biological processes.

One must also consider how the food concentration in a functional response is calculated. Initial concentration (C_0) as well as average concentration ([C]), in equation 8.10, have both been used. The latter is more common, because the average concentration mirrors the nutritional environment better throughout an experiment. In the case where ingestion is calculated according to equation 8.13, i.e. $I = F \times [C]$, the latter alternative generates a false high level of correlation because it is a compounding ratio, where $F \times [C]$ is correlated with its own denominator (Atchley *et al.* 1976). This effect is mainly related to the variance of C in relation to that of I. When the ratio of the coefficients of variation (CV) exceeds 2, i.e. $CV_C/CV_I > 2$, the correlation automatically exceeds 0.8 (McClatchie and Lewis 1986). This can be solved by plotting I against C_0, where I is calculated as $F \times [C]$. Use of [C] as the independent variable also causes an artificial increase in the degrees of freedom when using an ANOVA to test the

significance of the calculated regression equation (Marin *et al.* 1986). We therefore recommend using the last alternative, i.e. $I = F \times [C]$ plotted against C_0 in functional response calculations. This means that the independent variable (C_0) will deviate from the true average value ([C]). The reduction in food during the incubation should therefore be kept as low as possible in order to minimize this bias. However, this must be balanced by the need to obtain a statistically significant reduction in food concentration. Marin *et al.* (1986) suggest that a reduction corresponding to two standard deviations in the food enumeration technique should be the minimum. The corresponding incubation time to get this reduction is called *minimum analytical time* (MIT). This requires *a priori* knowledge of the variability in replicate feeding experiments. These authors also suggest that the experimental design be dimensionalized so that C_0 does not overestimate the mean food concentration by more than 10%. Thus, these two criteria define a time window in which the incubation time is enclosed. The window is rather narrow, especially for high grazing coefficients (Marin *et al.* 1986). Considering the potentially high variability in feeding experiments, MIT may even exceed the time corresponding to 10% overestimation of the mean food concentration, thereby eliminating the basis for using these principles.

If a time-series approach is used (McClatchie and Lewis 1986) we get measurements at gradually decreasing food concentrations from a single experiment, although this set of values must be considered as a single sample, since the individual points are not independent (i.e. all points are from the same set of experimental animals). Therefore, several replicate time series should be run and the statistical parameters from each of them are then used to calculate the mean parameters with their corresponding variance.

8.8.2 Size of food items

Food particle size is an important factor. The traditional view (*sensu* Frost 1972) is that increased size increases the slope of the functional response curve, i.e. maximum ingestion occurs at lower food concentrations. This implies that at a given food concentration (below saturation level) large food items are ingested more efficiently than small ones. Two factors account for this. First, retention efficiency decreases below a certain particle size. For most copepod species, this seems to occur between 5 to 10 μm (Berggren *et al.* 1988). Second, larger food items may be perceived from a distance and caught individually. This behavior increases the encounter rate. However, feeding strategies are complex and variable among copepod species, with the hydrodynamical signals both from the predator and the prey providing important regulating factors (Tiselius and Jonsson 1990). Some kind of food preference acts in most zooplankton species and optimal foraging theory offers a reasonable way to explain this preference. In its simplest form optimal foraging theory predicts that a predator should chose the prey that gives the optimum ratio of energy gain and energy expenditure (Begon *et al.* 1990). This usually implies that the larger the prey the higher the gain/expenditure ratio. Thus, prey as large as possible should be advantageous. However, dietary needs are not taken into account and the need for sufficient nutrition may necessitate a more mixed diet than indicated by optimal foraging theory. The theory further suggests that predators with short handling time of prey should be less selective that those with long handling times (Begon *et al.* 1990). Most zooplankton species can be classified in the former category. Thus theory does not provide any mechanisms to suggest that the size of the food item has any importance *per se,* other than the higher food mass per item and the higher detection radius, as explained in an encounter model (see section 8.9).

8.8.3 Turbulence

Small scale turbulence can increase the encounter rate of zooplankton with their prey (Rothschild and Osborn 1988; Visser and MacKenzie 1998; see also predation behavioral models, section 8.9). Consequenly, the zooplankton perceives a higher food environment and enhances its feeding rates accordingly. However, due to the functional relationship between clearance rate and food concentration, small scale turbulence has an enhancing effect on feeding rates mainly at below saturation food concentrations (Alcaraz 1997). Further, the influence of small scale turbulence depends on the size and behavior of the animal. Kiørboe and Saiz (1995) concluded that 'turbulence is most important for predators with low motility and large reaction distances above the Kolmogorov scales. Overall, this analysis suggests that turbulence is unimportant for very large and very small predators, being potentially significant only for meso-sized predators operating around the Kolmogorov length scale'. Small scale turbulence increases the metabolic rate of crustacean zooplankton through changes in swimming behavior (Alcaraz *et al.* 1994; Alcaraz 1997). The intensity at which metabolic rates are enhanced by small scale turbulence appears to be species specific and it is not clear whether enhanced ingestion compensates for it (Alcaraz 1997). Consequently, small scale turbulence can affect the gross growth efficiency differently as a function of the species (Saiz *et al.* 1992).

8.8.4 Consumer body size

It is well known that metabolic rate (R) is related curvilinearly to the body mass (W) of an organism, as described by the allometric equation:

$$R = \alpha \times W^{\beta} \tag{8.74}$$

where α is a scaling factor, depending on the units used in the expression of respiration rate and body mass and β is the body-mass constant, which is known to vary only moderately among different species, with a mean value around 0.7 (Zeuthen 1970). This rather stable value is explained by the fact that most cellular processes are controlled by the rate of transport through cell membranes. Cell mass is proportional to radius^3 and cell area is proportional to radius^2, thus the area/mass ratio is proportional to the radius raised to 2/3, i.e. about 0.7. Thus, a tenfold increase in body weight generates only a fivefold increase in metabolic expenditure. If metabolic rate is expressed per unit body mass, the ten times heavier individual will show only half the weight-specific metabolic rate as the small individual. This relationship holds for processes such as respiration, excretion and growth. Again, we can argue that because all the energy-demanding processes depend on body size, feeding should show the same relationship. The extensive review of feeding rates of marine calanoid copepods by Peters and Downing (1984) shows a range in allometric scaling from 0.37 to 0.75, supporting this suggestion reasonably well. Thus, when dimensionalizing experiments for an unknown organism of defined size, we can use information from a known species of the same type and apply the relationship above for a first approximation of its food requirements. However, the scaling factor (0.7) may differ significantly between assemblages. For example, in polar copepod assemblages Atkinson *et al.* (1996a) calculated a scaling factor between 0.49 and 0.65. These low values may reflect extensive storage of lipids in large polar copepods, and the results underline the caution required in applying literature data to specific assemblages.

8.8.5 Palatability/toxicity of food organisms

Feeding limitations due to physical characteristics of food items are relatively easy to understand and incorporate in mathematical expressions describing the trophic relationships between a consumer and its food. However, factors such as abundance, size, shape and swimming speed do not always explain the importance of a given food type for a given predator. Food quality or toxicity may be a factor. For example, it has been assumed that copepods select phytoplankton cells with high protein content as a consequence of their high nitrogen demand (Cowles *et al.* 1987; Houde and Roman 1987; Butler *et al.* 1989). Houde and Roman (1987) compared exponentially growing diatom food (high protein content) with stationary cultures (low protein content) in *Acartia tonsa*, but a similar experimental design with *Calanus finmarchicus* failed to show such effects (Nejstgaard *et al.* 1995). It has been suggested that the calcified coccoliths of *Emiliania huxleyi* function to deter predators (Young 1994). Observed low clearance rates on this food item by *Calanus finmarchicus* and *C. helgolandicus* (Harris 1994, Nejstgaard *et al.* 1995) might be interpreted as support for this idea. However, subsequent studies (Nejstgaard *et al.* 1995, 1997) have documented high clearance rates and efficient utilization of *E. huxleyi* for egg production. Paffenhöfer and Van Sant (1985) showed that the copepod *Eucalanus pileatus* preferred living phytoplankton cells before non-living particles, with 1.2 to 3 times higher ingestion on these. Non-food particles alone were not ingested and did not appear to reduce the feeding rate on food particles when occurring at typical environmental concentrations. We conclude that variation in the quality of live algal food, in terms of gross biochemical composition or as a consequence of cell surface texture, does not seem to be a strong factor for food selectivity and that detritus at typical environmental concentrations does not affect feeding. Quality certainly is important for growth and development, but with live algae of appropriate size it does not seem to be a strong mediating factor controlling feeding.

In contrast to food quality, toxicity may be a strong mediator of zooplankton feeding rate, as shown in both field studies (Estep *et al.* 1990; Nielsen *et al.* 1990; Hansen 1995) and laboratory studies (Huntley *et al.* 1986; Ives 1987; Buskey and Hyatt 1995; Nejstgaard and Solberg 1996). Tolerance of toxic algae varies among organisms, and copepods seem to be especially tolerant to haptophyte toxins, with low or no mortality at high concentrations (Valkanov 1964; Nejstgaard *et al.* 1995; Nejstgaard and Solberg 1996). Tolerance of dinoflagellate toxins is variable among copepods, but these usually affect feeding behavior (Sykes and Huntley 1987). Laboratory tests indicate that dinoflagellates act by physiological incapacitation of the consumer (Ives 1987). There can be significant sub-lethal effects with depression of feeding in environments with even non-blooming concentrations of toxic phytoplankton (Hansen 1995; Nejstgaard and Solberg 1996), and these effects may persist even when suitable phytoplankton dominate. Such effects vary with the species of grazer (cf. DeMott *et al.* 1991; Kirk and Gilbert 1992; Turriff *et al.* 1995; Mallin *et al.* 1995; Nejstgaard and Solberg 1996). Thus, feeding experiments using natural water containing an assemblage of food organisms may be affected by the presence of toxic algae in low or moderate abundance. When using natural water in feeding experiments, we therefore recommend using an appropriate monospecific cultured alga with a well documented high palatability as a control. Deviation in feeding between the control and natural water should then be evaluated regarding differences in cell size, abundance, etc., in addition to any suppressing (toxic) effect from components in the natural water. Direct experimental tests on the effects of isolated strains of potentially toxic phytoplankton should include analysis of toxicity of

the alga. For example, Nielsen *et al.* (1990) observed strong effects on grazers from a bloom of *Chrysochromulina polylepis* in the Kattegat, but no effects remained after isolation and cultivation in the laboratory. Larsen *et al.* (1993) showed increased toxicity of different geographic strains of *Prymnesium patelliferum* and *P. parvum* in phosphate-limited media and Aanesen *et al.* (1998) found that production of harmful chemical substances from *Phaeocystis pouchetii* was induced by high irradiance.

Irrespective of type of food used in an experiment, the acclimation to the food environment is important. The significance of feeding prehistory was emphasized by Huntley (1988) and recent studies on *Calanus finmarchicus* indicate that effects on production of eggs and fecal-pellets may remain for days and weeks rather than hours (Båmstedt *et al.* 1999). In feeding experiments acclimation time should therefore never be shorter than one full day.

8.8.6 Physical environmental factors

TEMPERATURE

Temperature exerts a fundamental control function on biological processes because of its influence on chemical reaction rates. Therefore, the same relationship that describes the temperature dependence of chemical reactions, the Arrhenius equation, can be used to describe the temperature dependence of metabolic activity. However, this formula is only suitable for a specific reaction since it includes a constant, E_a, *Energy of activation*, that varies among reactions. Metabolic activities usually involve many such reactions and a more usual way of expressing temperature dependence is the expression Q_{10}, defined as:

$$\mathbf{Q_{10}} = (\boldsymbol{r_1}/\boldsymbol{r_2})^{\mathbf{10}/(t_1 - t_2)} \tag{8.75}$$

where r_1 and r_2 are metabolic activities at temperatures, t_1 and t_2, respectively. Most biological rates have a Q_{10} of 2 to 3, which is also the typical range of chemical reactions given by the Arrhenius equation.

Due to the strong temperature dependence of metabolic activity, a gradual increase in mass-specific respiration rate occurs in zooplankton along a latitudinal gradient from boreal to tropical waters (Ikeda 1970). Activities such as growth rate and developmental rate of zooplankton are related directly to ambient temperature (McLaren 1963, 1966). Because all the energy-consuming processes are positively related to temperature, the organism requires more energy when the temperature is increased. Thus, feeding should follow the same temperature dependence as metabolic processes. Digestion rate follows the same rule, meaning that the turnover rate of stomach contents increases with temperature, thus increasing the feeding capacity. Finally, mobility level will also increase with temperature, helping the animals to search a larger water volume per unit time in warm than in cold environments (Thompson 1978). Since the viscosity also decreases with temperature it will be easier to swim around, especially for the smallest organisms. We will not go into this topic further, but refer to Chapter 10 on metabolism, emphasizing the importance of knowledge about temperature tolerance, acclimation, etc. when using an experimental temperature different from the natural one.

LIGHT

Most zooplankton organisms are non-visual feeders, i.e. they locate their food without vision. However, light-sensitive organs are common and can be important in determin-

ing an organism's depth of occurrence. Species with a pronounced diel migration pattern are probably governed by the cyclic change in light intensity. This factor must be considered in feeding experiments. Experiments done in full light may cause the organisms to aggregate towards the bottom, while complete darkness may cause the opposite. Directed light might also cause patchy distributions of both predators and prey in an experimental tank. Such problems may produce unreliable results because the food concentration experienced by the predator may be completely different from the average food concentration in the tank. Endogenous feeding rhythm may act, also in complete darkness (Stearns 1986). When using organisms (both predators and prey) whose feeding behavior is unknown, it is wise to perform a pilot test. This caution also includes experiments done outdoors, e.g. plastic bags immersed in the sea, where the organisms are prevented from migrating down in the water column.

SPATIAL CONSTRAINTS

Planktonic organisms occur by definition in the water column, and they are therefore usually not restricted in their swimming and feeding behavior by any physical objects in their natural environment. In an experimental situation, volume constraints may force organisms to make frequent contact with the walls or bottom of the experimental vessel. Any so-called wall effect that might be relevant should therefore be considered. For example, some large fast-swimming salps are extremely sensitive to confinement and require short incubations and large volumes, thereby eliminating particle depletion experiments (Madin and Kremer 1995). Smaller, slow-swimming species are better suited for such experiments. Some species, for example the euphausiid *Euphausia superba* frequently bump into container walls during the initial 2 to 3 days after capture, thereafter reducing contact with the walls and increasing feeding (Atkinson, unpublished data). Thus, large, highly mobile species should be given a 3 day period prior to the experiment, to allow acclimation to containment.

8.9 PREDATION BEHAVIORAL MODELS

Functional response models do not include behavioral factors explicitly, although such underlying factors help to explain why we obtain a certain type of functional response. It is possible to include such terms in the equation, and in Holling's so-called 'disk equation', which is a type II model, prey handling time is included (see Begon *et al.* 1990). In a predator–prey relationship, a mechanistic model can be used to describe the rate (E) at which a predator encounters its prey (Gerritsen and Strickler 1977):

$$\boldsymbol{E = C \times \pi \times R^2 \times (v_1^2 + 3 \times v_2^2)/(3 \times v_2)} \tag{8.76}$$

where C is the abundance of prey, R is the detection radius of prey and v_1, v_2 are the swimming speed of prey and predator where v_1 is the one that has the lowest swimming speed (prey or predator). In the natural environment there is usually a turbulence generated velocity component (w) that has to be considered, since this adds to the swimming-speed expression (Rothschild and Osborn 1988). In a turbulent environment the average speed by which two particles move in relation to each other defines this component. In equation 8.76 we replace v_1 and v_2 by $(v_1^2 + w^2)^{0.5}$ and $(v_2^2 + w^2)^{0.5}$ to give:

$$\boldsymbol{E = C \times \pi \times R^2 \times (v_1^2 + 3 \times v_2^2 + 4 \times w^2)/[3 \times (v_2^2 + w^2)^{0.5}]} \tag{8.77}$$

Turbulence is usually not expressed as velocity, but as a dissipation rate, in watts m^{-3}, but this can be converted approximately to velocity, given that we know the average distance (d) between the particles studied (Rothschild and Osborn 1988). If we assume a homogeneous prey distribution, $(C)^{-1/3}$ provides a good approximation of d (Kiørboe 1993), where C is the abundance (prey ml^{-1} = prey cm^{-3}). The distance, d is then expressed in cm. Thus, an abundance of 100 prey l^{-1} gives an average distance of 2.2 cm. The value for d can then be used to calculate w (from equation 4 in Rothschild and Osborn 1988):

$$w^2 = 3.62 \times (\varepsilon \times d)^{2/3} \tag{8.78}$$

where ε is the rate of turbulent kinetic energy dissipation. With the same prey abundance as above and a turbulent dissipation rate of 10^{-4} watts m^{-3}, w is 0.11 cm s^{-1}. In open waters this turbulence is generated by a wind speed of 4.2 m s^{-1}, i.e. relatively calm conditions. Rothschild and Osborn (1988) give further information on this matter. Direct experimental investigations have shown positive effects of turbulence on the feeding rates of some zooplankton, although these effects are not always predictable, due to differences in factors such as feeding mode, prey composition and sensitivity for high turbulence (Saiz and Kiørboe 1995; Kiørboe *et al.* 1996).

Vulnerability (V) is defined as the proportion of encounters leading to ingestion (Greene 1986), and ingestion rate (I) is then expressed as:

$$I = V \times E \tag{8.79}$$

If the prey is of considerable size for the predator, the predation process most likely includes a handling time of prey. The handling time is a constant (h) per food item. Equation 8.79 then generates:

$$I = (1 - h/t) \times V \times E \tag{8.80}$$

If the underlying mechanisms of the type II response are applied to this, i.e. gut capacity, decreased motivation of eating etc. (see section 8.8.1) we can define a model which includes more details about the behavior of the specific predator and prey:

$$I = (1 - h/t) \times (V \times E)/(a + b \times (V \times E)) \tag{8.81}$$

where a and b are constants defining the shape of the curve. In practice a number of separate experiments are required to define detection radius, swimming speed, vulnerability and handling time. When these have been defined, the results from feeding experiments are used to complete equation 8.81 and an iteration technique is applied to obtain values for the unknown constants a and b.

8.10 CONCLUDING REMARKS

As described in the Introduction, the choice of grazing rate method is dictated by the scale of the study objective (e.g. organism or community process) and by the type of zooplankton and food (e.g. carnivory or herbivory, degree of motility of grazer and food etc). Sections 8.5.1 to 8.5.14 cover a wide variety of feeding methods which have been used to address these problems. We have summarized some of the more common objectives of current feeding studies, methods suitable to address them and the main strengths and weaknesses of each (Tables 8.1–8.4). Many variations on the methods exist

to deal with particular problems: the individual sections provide further information on these.

Several grazing rate approaches, for example the gut fluorescence method, are erroneously described as *in situ* methods. However all approaches devised so far require confinement of grazers in containers at some stage. 'Bottle effects' (see page 345) which arise from this are various. For example modern radiotracer methods, although involving bottle incubation, minimize bottle effects as far as possible by use of non-stressful capture with confinement for short periods under identical light and depths to those *in situ*. Conversely the gut fluorescence method requires confinement at two stages, first to determine pigment destruction and second to measure the gut evacuation rate constant of freshly caught animals in filtered sea water. Traditional food removal methods require the grazers to feed during captivity. The extent to which these various types of containment (and indeed the methods themselves) affect the results is controversial and no real consensus has yet been reached. Undoubtedly the bias is worse for some grazer/food combinations than for others. However, despite the daunting list of problems associated with each approach and the numerous papers highlighting them, when the methods are followed carefully and fully, there can be a surprisingly good agreement between them (Kiørboe *et al.* 1985b; Peterson *et al.* 1990a).

Because each method has its specific problems, we recommend taking all possible steps to reduce artefacts and evaluate potential errors. This increases time requirements and less ambitious coverage may result, but the fact that a method is rapid is no justification for its use. It is more useful to have one good data point than 10 unreliable ones. Obviously there is always a tradeoff between degree of coverage (e.g. number of species/larval stages monitored) and quality of coverage. Because feeding methods are problematic, it may be worth considering devoting extra resources into measuring feeding with a second, independent method. Using two approaches is a powerful tool for two reasons. First, it provides an independently derived cross-check on the results. As an example, food removal incubations can be run concurrently with the gut fluorescence method. If, for instance, these provided algal carbon rations of 4% and 5% d^{-1} for a zooplankter, yet its egg production rate in that environment was 5% of body carbon d^{-1}, it is highly implausible that both methods simultaneously understimated ingestion by a factor of about three. An alternative explanation, for example that algae were not the major food source, is thus much more strongly supported.

A second advantage of using several methods is that they inform on contrasting aspects of feeding. For example gut fluorescence provides insights into diel periodicity and phytoplankton ingestion rates whereas incubations give *in vitro* ingestion rates of phytoplankton and indicate the importance of omnivory. Likewise, gut content analysis works well alongside incubations. The former gives semi-quantitative indications of the natural diet which are mainly free of experimental manipulation, whereas the latter give a quantitative but *in vitro* indication of feeding rates on these items. Incubations would complement radiotracer methods to evaluate commmunity grazing/predation impact, but this would require a big effort during the experimental phase.

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9 The measurement of growth and reproductive rates

J.A. Runge and J.C. Roff

9.1 INTRODUCTION: WHY MEASURE GROWTH AND REPRODUCTIVE RATES OF ZOOPLANKTON?

Mounting interest in marine population ecology and in the factors controlling secondary production in the sea has led to the development of a variety of approaches to the estimation of growth and reproductive rates of planktonic organisms. This chapter presents an overview of these methods, many of which have come into use only within the past one or two decades.

In keeping with the principal thrust of research into marine secondary production, the chapter focuses on the planktonic copepods. The methods for estimation of copepod growth and reproduction make use of their distinctive life-history characteristics, notably the presence of 13 life stages (egg, six naupliar stages and six copepodite stages) and the convenience that all growth can be assumed to be in the form of eggs during the final adult stage. These methods are not necessarily suitable for other taxonomic groups in the zooplankton, for example the gelatinous zooplankton, for which a review of current production methodology is not yet available. Nevertheless, several of the methods described here are suitable for application to other taxa, and examples are cited where appropriate. Specific examples of procedures for the measurement of egg production and growth of a planktonic copepod are given in sections 9.7 and 9.8 of this chapter.

Section 9.6 reviews biochemical and radiochemical methods for the measurement of processes related to zooplankton growth and development. These methods are still regarded as largely exploratory or under development. Therefore, the discussion is more general in nature and no specific protocols are provided.

It is important at the start to consider the theoretical context and potential applications for the measurement of zooplankton growth and reproduction. In some early studies the measurement of secondary production was an aim in itself, but more recent studies of growth and reproduction address particular questions or hypotheses that have broader implications for the understanding of how pelagic populations and ecosystems function and respond to environmental variability and stress. The applications of measurements of growth and reproduction can be grouped into five general themes which are discussed in the next five sections.

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9.1.1 Factors controlling the dynamics of copepod populations

The estimation of birth or recruitment rates into copepod populations requires measurement of egg production and egg hatching rates, either by incubation techniques (described in sections 9.3.2 and 9.4.2) in conjunction with determination of egg mortality rates (addressed in Aksnes *et al.* 1997), or by the egg ratio method (section 9.4.1). Recent studies of the dynamics of copepod populations, either in the field (e.g. Peterson and Kimmerer 1994) or by modeling (e.g. Miller and Tande 1993) illustrate the importance of quantitative birth rate measurement for understanding variation in zooplankton populations.

9.1.2 Variability in the production of the prey field for fish larvae

An estimate of the daily input of eggs (in the case of broadcast spawners see section 9.3) or nauplii (for egg-carrying species see section 9.4) is obtained from the product of the abundance of females (in terms of females m^{-2}) and female-specific egg production or egg hatching rates (eggs $female^{-1}\,d^{-1}$). In many environments, only a few copepod species dominate the prey field for fish larvae (e.g. Bainbridge and McKay 1968; Michaud *et al.* 1996). Estimates of egg production rates of dominant copepods have been employed in studies of sources of variability in prey production and its potential impact on growth rates of fish larvae (e.g. Kiørboe *et al.* 1988; Runge and de Lafontaine 1996). It is now feasible to construct maps of prey field productivity (at least the copepod contribution to it), using techniques described in section 9.3 (e.g. Runge *et al.* 1997).

9.1.3 The influence of food availability on growth and egg laying rates, including the linkage between copepod spawning and primary production cycles

A recurrent theme in zooplankton ecology concerns the relationship between food concentration, however defined, and the productivity of planktonic copepods (e.g. Huntley and Boyd 1984; Frost 1985; Peterson *et al.* 1991; Huntley and Lopez 1992). Egg production rate measurements (section 9.3) have been employed to measure precisely the response of copepods to the onset of the spring phytoplankton bloom (e.g. Runge 1985a; Diel and Tande 1992; Plourde and Runge 1993; Kiørboe and Nielsen 1994). The sensitivity of growth and egg production rates to changes in food concentration, composition and nutritional quality has been studied in controlled laboratory experiments as well as by observations of egg laying in relation to indices of food availability at sea (e.g. Kleppel 1992; Jónasdóttir 1994; Ohman and Runge 1994; Pond *et al.* 1997). These studies are providing a better understanding of the extent to which variability in the food environment may influence copepod growth and spawning rates. Moreover, the direct measurement of growth and reproduction (sections 9.3 and 9.4) may be a more feasible approach to estimate the copepod contribution to total grazing on microplankton than attempts to estimate *in situ* feeding rates directly (Peterson 1993).

9.1.4 Evaluation of environmental impacts

Growth and reproduction of zooplankton influence both rate processes controlling biogeochemical cycles and productivity at higher levels of the marine food web. Thus,

methods for direct determination or for measurement of biochemical indices of *in situ* growth and reproduction (sections 9.3 to 9.6) provide new tools for evaluation of immediate and long-term impacts of anthropogenic activity on key processes in the pelagic ecosystem. For example, Cowles and Remillard (1983) showed that very low water column concentrations of Louisiana crude oil influence production of viable offspring of planktonic copepods without having any lethal maternal effect. Measurement or indices of growth and reproduction (including hatching success) may prove useful for assessment of the impact of organochemical or trace metal contaminants in coastal ecosystems (e.g. Buttino 1994) or for evaluation of increases in water column UV-B irradiance due to ozone depletion over temperate and boreal oceans. These sublethal impacts may have important and hitherto unmeasured consequences on the pelagic ecosystem.

9.1.5 Estimation of secondary production

Historically, the measurement of secondary production has been a primary goal of zooplankton research. The methods for estimating secondary production are described elsewhere (Omori and Ikeda 1984; Rigler and Downing 1984; Poulet *et al.* 1995a). For most marine populations, growth rate is assumed to be exponential, in which case daily secondary production is calculated as

$$\boldsymbol{PR} = \sum[\boldsymbol{G_i B_i}] + \boldsymbol{G_f B_f} \tag{9.1}$$

where G_i and G_f are the weight specific growth rates (d^{-1}) of stage i and egg production rate of females, respectively, and B is the biomass ($B_i = N_i W_i$). The estimation of G is the subject of this chapter. The theory of secondary production calculations for continously reproducing populations is discussed by Kimmerer (1987). An example of production estimates using equation 9.1 is provided by Peterson *et al.* (1991). Estimates of total secondary production may eventually find application in models that partition primary production into benthic and water column components and address supply to fish and other predators.

The choice of which of the methods reviewed in the sections that follow depends on the research question. In some cases, a global model of growth (section 9.2) may be a sufficient approximation of growth and reproductive rates. Direct measurements will be needed for more precise estimates; for example in evaluating the extent to which food and female age structure negatively limit rates of egg production and hatching success.

9.2 MODELS OF GROWTH AND FECUNDITY

In some situations, for example the study of the influence of advection on the distribution of planktonic organisms or the study of the dynamic behavior of a marine ecosystem, a first-order approximation of the growth of zooplankton may be sufficient, given the magnitude of error inherent in other variables. Several empirically or allometrically derived approaches to the estimation of planktonic growth have been put forward.

9.2.1 Physiological or laboratory-derived budgetary models

Huntley and Boyd (1984) proposed to estimate growth rate in field populations by a modified von Bertalanffy equation:

$$dW/dt = A - R \tag{9.2}$$

where W is copepod body weight (μgC), A and R are variables describing rate of assimilated food input and respiratory loss, respectively. The feeding term is the product of the assimilation efficiency, a (assumed to be constant at 0.7), the food concentration in the environment, C (μgC ml^{-1}) and the individual clearance rate, F (ml water cleared of food per animal per hour):

$$A = aCF, \tag{9.3}$$

where F is a function of weight:

$$F = bW^n. \tag{9.4}$$

The respiratory loss term is also a function of W:

$$R = kW^m. \tag{9.5}$$

The species-independent coefficients, b, k, n and m were determined empirically, all as functions of environmental temperature, using data from previous studies. Theoretically, *in situ* secondary production of a mixed assemblage of copepods can be calculated from equation 9.2, a measure of food concentration, C, and the biomass of copepods, by applying equation 9.1. Huntley and Boyd (1984) provide an example of this calculation using synoptic data collected over a 320 km transect with automated instrumentation.

The drawback of the physiological method, and of its variations (see citations in Huntley and Lopez 1992), lies in the difficulty in accurately parameterizing the components of the modified von Bertalanffy equation, particularly the feeding terms. A criticism of the method is provided by Huntley and Lopez (1992) and Huntley (1996). In essence, for a given body weight, there is a large variation in F which is exceedingly complicated to resolve, given the multitude of factors that may influence feeding rate (e.g. Huntley 1988). In addition, the determination of aC, the appropriate concentration of assimilated food, is extraordinarily complicated, given the influence of food quality on growth, the difficulty in determining concentrations of both heterotrophic and autotrophic components of the food supply, and the age-dependent variation in copepod vertical mobility and distribution. Because of the nature of equations 9.3 to 9.5, the errors associated with each of the coefficients are multiplicative. For first-order approximations of growth, a much simpler approach is to employ an empirical model as described in the following two sections.

An alternative physiological model was developed by Ikeda and Motoda (1978, cited in Hirst and Sheader 1998). They estimated respiration rate as a function of body weight (equation 9.5) and converted oxygen consumption to carbon using a respiratory quotient of 0.8. Instead of estimating ingestion rate directly, they assumed a gross growth efficiency of 0.3 and an assimilation efficiency of 0.7. The resulting equation predicting growth was:

$$g = (7.714)(10^{0.0254T-0.126})(W^{-0.0109+0.892})(W_c)^{-1}. \tag{9.6}$$

Where T is temperature and Wc is individual dry weight (μC).

9.2.2 Temperature-dependent empirical model

Huntley and Lopez (1992) have proposed a temperature-dependent model of copepod growth, for which temperature is the primary forcing function. They assumed a constant exponential growth rate over all life stages:

$$W_a = W_e e^{g\tau}, \tag{9.7}$$

where W_a and W_e are weights of adults and eggs, respectively, and τ is the generation time (days from egg to adult) for a non-diapausing population or phase of the copepod's life cycle. An extensive data base (181 data points, 33 species) for habitats ranging from polar regions to the tropics ($-1.7\,^{\circ}$C to $31\,^{\circ}$C ambient temperature) was compiled. A nonlinear regression analysis of the pooled data yielded a significant relationship between weight-specific growth rate, g (d^{-1}), and temperature, T ($^{\circ}$C):

$$g = 0.445e^{0.111T}, \tag{9.8}$$

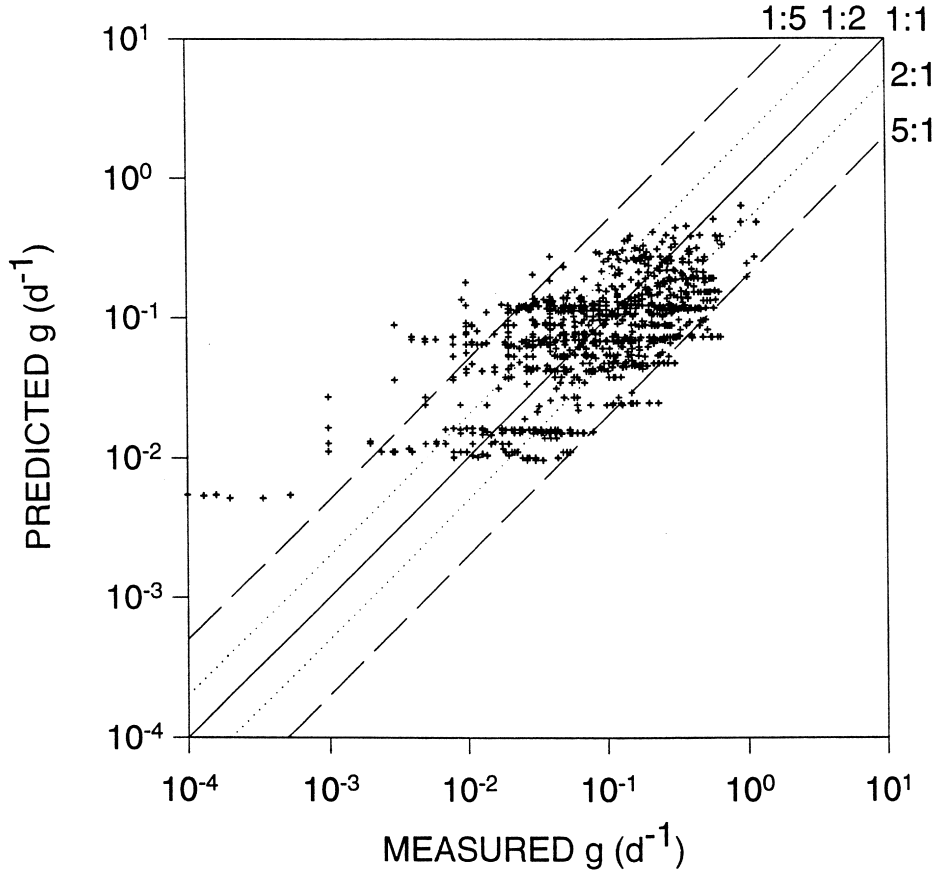

Fig. 9.1 Predicted weight-specific growth rates (from Table 9.1, equation describing relationship for all species, adults and juveniles) compared to the actual measurements used to construct the multiple linear regression. Lines representing ratios of predicted : measured values are shown to illustrate degree of scatter (adapted from Hirst and Lampitt 1998, with permission).

with an $r^2 = 0.91$. From the same data set, the general relationship between generation time and temperature was:

$$\tau = \mathbf{128.8}e^{-\mathbf{0.120}T}, \tag{9.9}$$

with a correlation coefficient (r^2) = 0.91 as well. There was no significant relationship between growth rate and adult dry weight and, in three out of four temperature ranges, between generation time and adult dry weight. Hence, Huntley and Lopez described a single temperature-dependent function for all copepod species that is independent of body size.

Implications of the Huntley and Lopez findings are that temperature is the dominant influence on copepod growth rates, food is not a primary factor limiting growth in the natural environment (i.e. non-diapausing life stages of all species are capable of finding sufficient food in their environment to sustain the predicted growth) and the physiological responses of all copepod species to temperature are similar. Of the two terms in the calculation of P, therefore, the biomass of copepods, rather than growth rate, is likely to be the dominant factor controlling spatial and temporal variability. From literature analysis, Huntley and Lopez conclude that variation in biomass on scales of 10 or more km and several days to months in time is on the order of 100, whereas variation in the predicted growth rate over a typical ambient temperature range (for example 6–9 °C for *Calanus finmarchicus* in May) is on the order of a factor of two or less.

9.2.3 Global model of *in-situ* weight-specific growth

Since the Huntley–Lopez model, published data on experimentally-determined copepod growth rates (using methods described in this chapter) has been accumulating. These data have recently been analyzed for the purpose of re-examining relationships among growth, temperature and body weight in marine planktonic copepods (Kiørboe and Sabatini 1995; Hirst and Sheader 1997; Hirst and Lampitt 1998). The resulting equations (Table 9.1) provide the most recent empirical relationships among these variables. Copepods were categorized according to spawning type (broadcast or egg-carrying) and life stage, i.e. whether growth represents reproduction (adults) or weight gain (juveniles). Over all categories, weight-specific growth rate is dependent on both temperature and body weight (Hirst and Lampitt 1998). However, for some individual categories (e.g. egg-carrying species), growth is dependent only on temperature.

Hirst and Lampitt analyzed a data set (Fig. 9.1) that is substantially larger ($N = 935$) than available for the previous models. It should be kept in mind, however, that the majority of the actual measurements are derived from measurement of egg production of broadcast spawners, as there are still relatively few *in situ* measurements of juvenile copepod growth. Moreover, the relationships are based on copepods normally residing in the upper 200 m of the water column. Predicted growth rates from both the Huntley–Lopez and Ikeda–Motoda models were significantly different from measured values in the new data set (Hirst and Sheader 1997). The Hirst–Lampitt equations therefore provide the best representation to all available data. These equations have obvious applications, for example in the spatial mapping of secondary production (P), where $P = GB$ (equation 9.1). New technologies for synoptic measurement of copepod abundance, such as multifrequency acoustic systems or the optical plankton counter provide data on variations in abundance at high spatial resolution. Combined with high resolution temperature data and equation 9.9, maps of spatial variability in community secondary production can be generated. In models coupling physical processes with

Table 9.1 Multiple linear and least-squares linear regression equations relating weight-specific growth (g, d^{-1}) to temperature (T, °C) and/or body weight (*BW*, μg C $individual^{-1}$) for epi-pelagic marine planktonic copepods. Relationships provided for species that broadcast eggs into the water column, species that carry eggs in an egg sac until hatching, and for all species combined. Number of observations on which relationship is based is provided in parentheses. Logarithms to base 10.

Broadcast spawning species	
Adults only (671)	$\log g = -0.6516 - 0.5244(\log BW)$
Juveniles only (105)	$\log g = 0.0111(T) - 0.2917(\log BW) - 0.6447$
Adults and juveniles (776)	$\log g = 0.0087(T) - 0.4902(\log BW) - 0.7568$
Egg-carrying species	
Adults only (54)	$\log g = -1.7726 + 0.0385(T)$
Juveniles only (99)	$\log g = -1.4647 + 0.0358(T)$
Adults and juveniles (153)	$\log g = -1.7255 + 0.0464(T)$
All species	
Adults only (726)	$\log g = 0.0133 - 0.3569(\log BW) - 1.0475$
Juveniles only (209)	$\log g = 0.0370(T) - 0.0795(\log BW) - 1.3840$
Adults and juveniles (935)	$\log g = 0.0208(T) - 0.3221(\log BW) - 1.1408$

copepod population dynamics or simulating food web linkages for geochemical flux studies, this first order approximation of copepod growth rate may be very useful.

As the authors of these studies point out, these methods have important limitations, which must be considered before applying the equations in a particular situation. First, they can only be applied in situations where the copepod population is actively growing. Clearly, a model predicting positive growth does not apply to a population that is in an overwintering diapause or resting state. Knowledge of the life histories of the copepod community in question would help identify time periods and regions where application of the model would be appropriate. Secondly, the Hirst–Lampitt equations represent epi-pelagic copepods (0–200 m) and not meso-pelagic populations. Finally, the equations represent a general, empirical model which may generate considerable error in any particular case (Fig. 9.1). Therefore, they may not be sufficiently accurate to answer specific questions requiring description of growth of a particular species in its natural environment.

9.3 DETERMINATION OF EGG PRODUCTION RATE: BROADCAST SPAWNING COPEPODS

9.3.1 The basic method

Egg production rate of broadcast spawners (or female-specific birth rate, as defined in Chapter 12, with units of eggs $female^{-1}\ d^{-1}$) is estimated by:

$$G_f = E \times 24/N_f t \tag{9.10}$$

where E is the number of eggs released during incubation time t (in hours) and N_f is the number of females under observation. Many species release eggs in batches or

'clutches' during a discrete spawning event, and it is sometimes convenient to express G_f as:

$$G_f = CS \times s \times 24/t \tag{9.11}$$

where CS (eggs female^{-1}) is the mean clutch size or mean egg output, when females are laying more than one clutch in 24 h, s is the ratio of number of females releasing eggs to the total number of females in the population and $(s \times 24/t)$ is the daily spawning frequency (d^{-1}). The egg production rate is often expressed as a fraction of female body carbon or nitrogen per day, by multiplying G_f by the ratio of egg carbon or nitrogen mass to female carbon or nitrogen mass.

The method, which is sometimes called the 'egg production' or 'direct observation' method, has its origins in the early work of Marshall, Orr and coworkers, who studied egg laying of *Calanus* sp. by observing females placed singly into small dishes containing sea water (Harding *et al.* 1951). However, Marshall and Orr (1955) did not use their observations to estimate *in situ* egg-laying rates. Subsequent research on egg production by broadcast spawners was restricted primarily to laboratory studies until Dagg (1978) described direct observations of egg laying of *Centropages typicus* in the New York Bight. Following closely on that study were observations of egg production of *Paracalanus parvus* (Checkley 1980a), *Bestiolina similis* (formerly *Acrocalanus inermis*: Kimmerer 1984), and species of *Acartia* (Landry 1978; Durbin *et al.* 1983; Saint-Jean and Pagano 1983; Ambler 1985) and *Calanus* (Runge 1985a, 1985b). Checkley's (1980a) study, however, did not employ direct observations, but rather adapted the Edmondson egg ratio method to broadcast spawners. This method, however, has not been generally adopted because of the additional sources of error, inherent when estimating the egg ratio from egg densities in the water column (Beckman and Peterson 1986). The egg production method has since been employed in studies of productivity of many copepod species (eg. Tiselius *et al.* 1991; Saiz *et al.* 1997).

Underpinning the egg production method is the postulate that the egg laying observed immediately after capture accurately reflects the spawning behavior of counterparts in the sea during the same period. Marshall and Orr (1955) suspected that a physiological stress associated with capture and handling affected egg release of *Calanus finmarchicus* and therefore ignored the first 24 h of egg-laying data. There is some evidence that female *Calanus* may be stimulated to spawn by prodding or exposure to light (Runge, unpublished data) but this only applies to females carrying very mature oocytes that would in any event have been released within a matter of hours. Other investigators have concluded that egg laying observed in the first 24 h is a valid reflection of *in situ* behavior for several reasons. First, as shown originally by Harding *et al.* (1951) and subsequently observed for other species (e.g. Runge 1985a; Stearns *et al.* 1989) females of several species release eggs on a diel cycle immediately after capture; if stress of capture stimulates egg release, as Marshall and Orr (1955) suspected, it should also disturb the diel spawning pattern. Second, daily spawning obtained by direct observation is predictable from the proportion of females identified as carrying mature oocytes (Runge 1985a; Plourde and Runge 1993; Niehoff and Hirche 1996). Third, in a study in which egg production rates, female abundance, and egg concentrations in the water column were simultaneously measured, the observed population egg production rate (the product of the female-specific birth rate and the female abundance) was similar to the abundance of eggs in early morning, the time of day thought to be most representative of the daily spawning output (Runge and de Lafontaine 1996). Finally,

maximum egg production rates measured by direct observation are consistent with the maximum, temperature-dependent egg production rates measured in laboratory experiments (Runge and Plourde 1996; Hirche *et al*. 1997). This fundamental postulate for the direct observation method has been probed in greatest depth for species in the genus *Calanus*, but is assumed to hold for species of other genera as well.

9.3.2 Procedures: know your species

As spawning behavior is quite variable among species, even those that are morphologically similar, it is imperative to conduct preliminary experiments and checks in order to ensure that the conditions during incubation after capture are well-suited for the target organism. What works well for a subarctic species like *Calanus finmarchicus* may not work at all for a subtropical species like *Acrocalanus gibber*. The following discussion is intended to serve as a guideline for development of protocols for the measurement of egg production rate of broadcast-spawning species. An example of a specific procedure (for *C. finmarchicus*) is provided in section 9.7.

CAPTURE AND HANDLING

In most cases, procedures for gentle capture and handling of plankton, which minimize the physiological stress of removal of females from their natural environment, have been followed. Techniques for capture of live plankton are described in Chapter 3. In populations where there is a possibility of a diel or diurnal spawning cycle, females should be captured several hours before peak spawning, in order to avoid starting the observation in the middle of egg laying and to minimize any short-term stimulatory effects of capture and handling on ripe females. Immediately after the catch has been diluted into receiving sea water, females should be sorted for incubation, a process which, depending on catch abundance and the number of species targeted, may require between 0.5 and 3 h (but typically less than 1 h). Sorting is done with an eyedropper or a modified pasteur pipette with a rubber bulb attached to the straight end. The distal end can be cut and tempered over a flame to provide a diameter that is just large enough to allow the copepod to enter the pipette without bending its first antennae. Several pipettes of different entrance diameters can be fabricated in this way and the appropriate one selected for the target organism. Ideally, sorted females will have furcal and antennal setae intact, as this is a sure indication that females were gently captured and uncrowded in the holding containers. However, if most females have broken furcal setae but are otherwise healthy-looking and active, it is not usually necessary to redo the tow, as there is no evidence that their egg-laying rates are any different. During sorting, care should be taken to maintain sea water temperature within the range of ambient water and to protect animals from bright sun or laboratory light. Dishes containing animals for sorting should not be left under microscope lights for periods longer than several minutes. Anesthetics (e.g. MS-222) should be used only if absolutely necessary, in which case the investigator should be prepared to show that this treatment does not influence short-term spawning behavior.

The observation of egg laying usually begins immediately after females are sorted from the catch. A notable exception is the study by Durbin *et al*. (1983), in which *Acartia tonsa* females were first acclimated in ambient sea water for 24 h. In this particular case, the water was well-mixed to the bottom, so that the acclimation water was representative of water anywhere in the water column. Moreover, acclimation volume was large relative to female density, so that there were only minor changes in particle concentra-

tion over the acclimation period. In most situations, however, acclimation of females in laboratory conditions identical to their environment is not feasible, so that incubations must commence directly after capture.

DURATION OF INCUBATION

The duration of incubation is normally 24 h. Shorter durations run the risk of biasing daily estimates of egg production rates if the females spawn on a diel or diurnal cycle, as observed in many species (e.g. Fig. 9.2 and Harding *et al.* 1951; Runge 1985a; Marcus 1988; Stearns *et al.* 1989; Checkley *et al.* 1992; Uye and Shibuno 1992; Laabir *et al.* 1995). Egg production of females incubated for periods longer than one day becomes increasingly dependent on the incubation conditions and therefore would be less representative of the *in situ* rate. Nevertheless, some studies include measurements of egg laying during the second 24 h period after capture (e.g. Mullin 1991), which may provide additional useful information about the *in situ* feeding history and spawning behavior.

Females should be checked for survival at the end of the incubation. For some species, like *Calanus finmarchicus*, mortality is rare. In other species, however, mortality may be as high as 10%. These females should be discounted; where there are several females per replicate, the entire replicate may have to be rejected.

INCUBATION CONTAINERS AND DENSITY OF FEMALES

Cannibalism is potentially a major source of error in the estimation of egg production rates. The extent to which females prey upon their eggs during the incubation period varies among species and incubation conditions. Cannibalistic behavior has been confirmed in species of *Centropages* (Dagg 1977; Ianora *et al.* 1992), *Paracalanus* (Checkley 1980b), and *Calanus* (Runge 1984; Kiørboe *et al.* 1985; Peterson 1988; Laabir *et al.* 1995). Dagg (1977) reported that *C. typicus* ingested at least 40% of the daily egg production when eggs were collected only once every 24 h. Runge and Plourde (unpublished data) found that, under certain conditions at sea, cannibalism can result in underestimation of egg production rates by a factor of 5–10 (Fig. 9.3).

Several studies have reported an inverse relationship (not necessarily linear) between observed egg production rate and density of females in the incubation containers (Kimmerer 1984; Ambler 1985; Kiørboe *et al.* 1985; Laabir *et al.* 1995). In most cases, this effect can be attributed to higher predation rates on eggs as the number of females in the container increases. However, Kimmerer (1984) provides evidence that overcrowding may directly inhibit egg laying, although the female densities at which this occurred ($>500\ l^{-1}$ of *Bestiolina similis*) are unrealisitic and would not normally be used in the direct observation method. There is no evidence of an effect of incubation volume *per se* on egg laying. Runge and Plourde (unpublished data) found that the mean egg production rate of single *Calanus finmarchicus* incubated in 25-ml capacity dishes (examined every 8 h during a 24-h incubation) is no different than the mean rate of single females incubated in 1 liter jars.

Error due to cannibalism can be minimized by

1) use of incubation containers equipped with a screened partition
2) use of incubation containers in which the volume/female ratio is substantial (a consensus guideline is >1000 ml female^{-1} for larger copepods and >200 ml female^{-1} for smaller copepods)

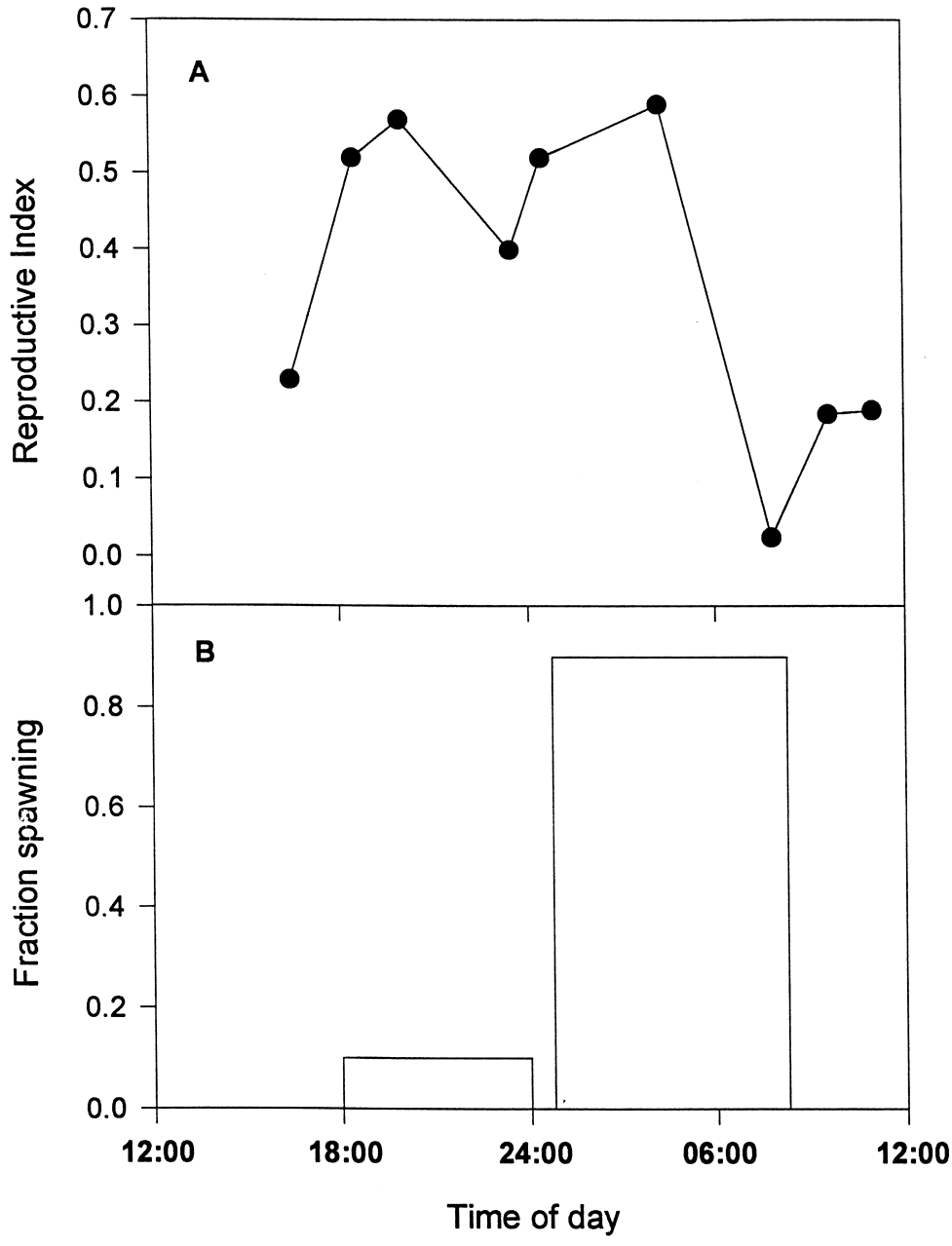

Fig. 9.2 Examples of evidence for diel spawning behavior in species of *Calanus*. (A) Time series of the proportion of females carrying very mature oocytes (stage 7) in a population of *Calanus finmarchicus* from the sea off southwest Nova Scotia; the drop in early morning suggests synchronous spawning (adapted from Runge 1987). (B) Egg laying in dishes by female *Calanus pacificus* captured in early morning from Dabob Bay in May, showing that most females released eggs during the following night (adapted from Runge 1985a, with permission).

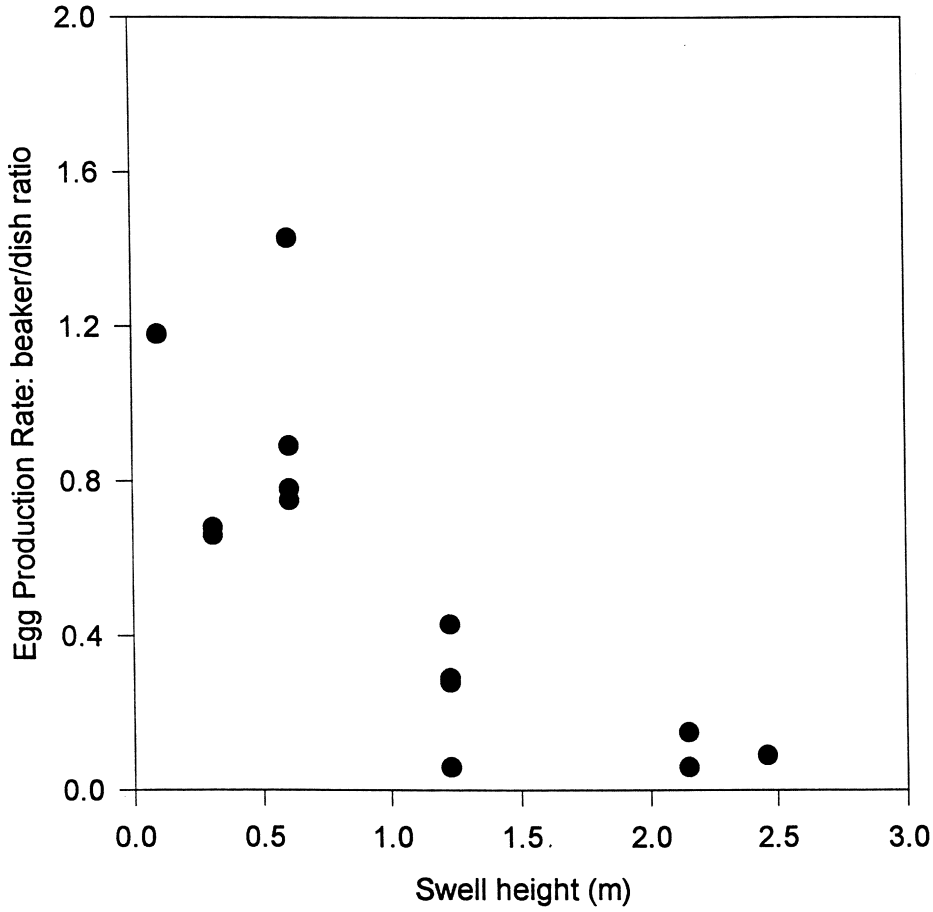

Fig. 9.3 Example of incubation conditions as a source of error in the shipboard estimation of egg production rate. Egg production estimates from a subsample of females incubated in groups of 10–15 in 1.5 l egg separation containers (beaker) and from a subsample of females incubated individually in 35 ml dishes (dish). Each point represents ratio of beaker/petri estimates on same sample as a function of sea conditions during incubation. Increasing mixing in beakers caused by pitching and rolling of the ship is hypothesized to maintain eggs in suspension, greatly increasing cannibalism (Runge, unpublished data).

3) incubation in dishes that are periodically checked during the incubation period, or by
4) enumerating crumpled egg membranes that may be left behind by females after ingestion of the egg's contents (e.g. *C. typicus*: Dagg 1977).

No solution is entirely effective in all situations. Eggs may remain suspended in incubation containers for relatively longer periods on moving platforms at sea and consequently may be subject to significantly higher predation rates depending on the sea state (e.g. Fig. 9.3). Incubation of eggs in dishes requires more frequent attention during the 24 h period, which may not always be possible. Females of at least some species (e.g.

Calanus finmarchicus) ingest the entire egg, leaving behind no identifiable trace of their prey, in which case it is not possible to assess how many eggs have been eaten during the incubation period.

The recommended procedure, where feasible, is to incubate females singly in either dishes that could be inspected at regular intervals during incubation, or in spawning chambers that allow eggs to sink rapidly away from maternal contact (e.g. Hirche 1990; Armstrong *et al*. 1991; Burkart and Kleppel 1998). Cannibalism in dishes is inhibited by the rapid sinking of eggs to the bottom, where it is apparently more difficult for copepods to capture and handle an egg compared to one that is suspended in the water. Spawning chamber volumes are typically 250–1000 ml. Dish capacity may be as small as 15–45 ml (polystyrene petri dishes) for larger species such as *Calanus pacificus* (Runge 1985a). Multiwell tissue culture plates have been used for smaller species such as *Microcalanus pygmaeus* (Kurbjeweit 1993), but the volume should not be so small that it promotes rather than hinders egg predation. Runge and Plourde (unpublished data) have found that inspection at 8 h intervals during a 24-h incubation is adequate for accurate clutch size estimation of *C. finmarchicus* held in 35–45 ml dishes. The procedure is not as time consuming as it may seem, as inspection of individual dishes and takedown at the end of the incubation period can be performed quickly. Observation of individuals, in addition to lessening the chances of cannibalism, minimizes loss of replicates in the case of mortality discussed above, gives a more precise estimate of the variance in egg-laying rate, and provides ecologically useful information on clutch size and individual variability.

In some situations, for example when observing copepods that are easily trapped at air–water interfaces, there may be no choice but to incubate in bottles. Whatever the container, care should be taken to ensure that it is clean and free of contaminants.

TEMPERATURE

Protocols for egg production measurements call for incubation of females at ambient temperature. In stratified water, where there may be large differences between temperature in the warmer surface mixed layer and in the colder deep water, the choice of which temperature to use is not always evident, especially for vertically migrating species. Moreover, when shipboard refrigerators or specially designed controlled-temperature incubators are employed, temperatures may unavoidably fluctuate by several °C during the incubation period. In subtropical environments, successful measurements have been made using a deck incubator with continuously running surface water. The small temperature variations are tracked with a temperature logger (D. McKinnon, personal communication).

For *Calanus* species, there is evidence that immediate, post-capture egg laying is unaffected over a wide range of incubation temperatures. Laabir *et al*. (1995) observed no difference in egg-laying rates among *Calanus helgolandicus* females captured from the western English Channel and incubated at 5, 16 and 22 °C. Runge and Plourde (unpublished data) obtained a similar result with *Calanus finmarchicus* captured from the Laurentian Channel and incubated at 6, 12 and 15 °C. However, immediate post-capture egg laying in the same population of females was inhibited by incubation in very cold temperatures (0–1 °C) that are characteristic of the cold intermediate layer in the Gulf of St Lawrence. These results imply that the spawning of *Calanus* sp. is not sensitive to temperature over a broad range about the ambient, mixed layer temperature, but that female copepods can exhibit control over release of eggs in unfavorable conditions.

Egg laying in other species, however, may be more affected by incubation tempera-

ture, especially in subtropical or tropical environments, where high ambient temperatures accelerate growth and metabolic rates. For example, Saiz *et al.* (1997) found that egg production of *Acartia grani* incubated in a high food regime increased 3–4-fold between incubation temperatures of 13–23 °C. For the selection of incubation temperature, therefore, it is prudent to conduct preliminary experiments to determine the temperature range over which egg laying is stable, or to carefully select one or more incubation temperatures representing the ambient temperature range of females, particularly for previously unstudied species living in thermally stratified waters.

LIGHT REGIME

Although there is evidence that diel or diurnal periodicity in spawning is a common occurrence (e.g. Stearns *et al.* 1989), there is little indication that the light regime during incubation influences the 24 h egg-laying rate. Runge (1985a) kept female *Calanus pacificus* in continuous darkness, apart from periodic examination under the microscope, but still observed a strong diel pattern in clutch production. Laabir *et al.* (1995) found no difference in egg production rate among female *Calanus helgolandicus* incubated in 24 h darkness, 24 h light or 12 h light:12 h darkness. At present, most investigators simulate the ambient light cycle during incubation; others employ continuous darkness or dim light. Because some species (e.g. *Centropages typicus*, Valentin 1972) may be more productive in light than continuous darkness, preliminary experiments are once again warranted for species whose spawning behavior is not well understood.

FOOD SUPPLY

The maxim 'know your species' applies particularly to the choice of food conditions during the incubation. It may be possible in some cases, particularly for small copepods, to simulate natural food conditions by carefully (so as not to damage the food organisms) collecting ambient water and using a container that is relatively large relative to the size of the female, such that the female does not have the opportunity to clear a substantial fraction of the volume before the end of the observation. If most of the small food particles are autotrophic and heterotrophic flagellates, mixing may not be necessary. Care must be taken (by use of controls or by pre-screening) to take into account the introduction of eggs and nauplii in the ambient water, which could be counted as part of the production at the end of the experiment. Many investigators use screened (typically 80–120 μm) ambient water with good results (e.g. Durbin *et al.* 1983), in this study, sources of error associated with the use of ambient water (improper selection of depth of water collection, depletion of food by grazing during incubation) were kept to a minimum.

However, it may not be feasible to accurately simulate natural food conditions during the incubation period. This is not necessarily problematic because in some species the female's feeding history prior to capture determines the egg-laying rate in the first 24 h after capture. For example, Plourde and Runge (1993) and Laabir *et al.* (1995) found that *Calanus finmarchicus* and *C. helgolandicus*, respectively, released equivalent numbers of eggs in filtered sea water and in sea water enriched with algae during the 24 h incubation. In some situations, egg production rate in enriched medium may be greater than in filtered sea water because the predation rate of females on their eggs is lower in the presence of alternative food (Runge and Plourde, unpublished data). However, if this source of error can be controlled, filtered sea water allows a more precise standardization

of the direct observation method for comparison of egg-laying rates among stations and sampling dates.

The lag time between ingestion of food and its assimilation into eggs varies with temperature and among species. In some copepods, like *Acartia tonsa*, ingested food may have an influence on egg production rates after 5–10 h (Tester and Turner 1990). Saiz *et al.* (1997) observed significant differences in egg production rate of Mediterranean copepods between incubations with filtered and food-enriched sea water. They concluded that food conditions during incubation can seriously bias the estimate of egg production rate in small, neritic copepods functioning at high ambient temperatures; the use of filtered sea water as the incubation medium would lead to an underestimation of the daily egg-laying rate. In these situations, they recommend incubating copepods in a large volume of water coming from the depth strata in which they feed. Alternatively, it is possible to conduct direct observations of egg production in two incubation media, filtered sea water and sea water enriched with suitable food, which together would bracket the *in situ* rate. Ancillary measurements of potential food concentrations in the water column may allow interpretation as to whether the actual egg laying rates are closer to the ambient or superabundant food treatment.

9.3.3 Statistical considerations

When females are incubated in groups, the female-specific egg production rate, G_f, is calculated by equation 9.10. In many situations the number (n) of replicates needed to obtain a mean egg production rate with acceptable confidence limits is 5 to 6. Given the assumption that the means are normally distributed, the confidence interval about the mean is calculated from the standard error and the Student's t distribution, with $(n-1)$ degrees of freedom (Sokal and Rohlf 1969).

The recommended procedure, however, is to incubate females (sorted at random from the catch) individually whenever possible. The central limit theorem justifies approximating the distribution of G_f with a normal distribution when N_f, the total number of females in the incubation, is sufficiently large. In practice 'sufficiently large' is taken to be when N_f is larger than 30; for smaller values of N_f the validity of the normal approximation is in doubt (Freund 1992). The daily female-specific egg production rate is therefore the mean of all individual observations over a 24 h incubation period, whether or not a female produced a clutch during that period. In other words

$$\boldsymbol{G_f = \sum CS_i \times 24/N_f t,} \tag{9.12}$$

where CS_i is the clutch size of female (i). This is equivalent to equation 9.10. A female may produce more than one clutch during the incubation period; all clutches should be included in the calculation. The variance about the mean is σ_B^2/N_f, where σ_B is the standard deviation of all n observations. One can use the theory of normal distribution to construct confidence limits about the unbiased estimate of the mean; the 95% confidence interval would be $-1.96\sigma_B/\sqrt{N_f}$ to $+1.96\ \sigma_B./\sqrt{N_f}$. As an extreme example, if in a sample size of 40 females only one female produced a clutch of 40 eggs in a 24 h period, the mean egg production rate would be 1 egg female^{-1} d^{-1}, the standard error would be 1.0 and the 95% confidence interval would be ± 1.96.

When reporting mean population egg production rate, all incubated females should be included in the calculation, whether or not they spawned. Mean egg output averaged only over spawners estimates clutch size (if each spawner produced a single clutch during

the incubation), but not egg production rate unless all females released eggs. Exclusion of the zero observations would bias estimates of population egg production determined from the product of the egg production rate and female abundance in the water column. Egg production based on spawners only is not necessarily an estimate of the 'true' egg production rate (since reproductively immature or spent females do not contribute to egg output, regardless of food concentration) as the females that do not spawn during the incubation may or may not be reproductively active. Production of a clutch of eggs may require more than 24 h; hence a female that did not spawn may simply be between clutches. As discussed in the following section, it may be possible to identify immature or spent females by visual inspection, from which a more accurate estimate of 'true' egg production rate (for use, say, in food limitation studies) could be determined.

9.3.4 Estimation of spawning frequency from preserved samples

Marshall and Orr (1955) recognized that the state of maturity of gonads can be distinguished through the transparent integument of the female. While oogenesis is a continuous process, stages in the development of a clutch of oocytes, corresponding, for example, to the start of vitellogenesis, can be identified. Several investigators have devised classification schemes that similarly describe stages of gonad development (e.g. Tande and Hopkins 1981; Runge 1985a; Kurbjeweit 1993; Norrbin 1994; Niehoff and Hirche 1996). Runge (1987) proposed that there is an empirical relationship between the *in situ* egg production rate and an index of reproductive maturity based on the proportion of females in a population that have attained a nominal level of gonadal development. In essence, the reproductive index is an indicator of daily spawning frequency (see equation 9.11). The relationship between spawning frequency and egg production rate would be linear in the case where clutch size is constant. Criteria for the reproductive index of Runge (1997) are based on analysis of females preserved in 4% formaldehyde. Niehoff and Hirche (1996) describe gonad maturity stages based on staining of females with 2% borax carmine in ethanol; with this method, they also were able to predict clutch size from histological sections. Significant relationships between egg production rate and a reproductive index have been obtained for populations of *Calanus finmarchicus* (e.g. Fig. 9.4 and Niehoff and Hirche 1996). Such relationships, when calibrated against direct observations of egg laying, allow estimation of egg production rates at a much greater resolution in space and time than is possible with labor-intensive incubations alone.

9.3.5 Egg viability

Recent studies have indicated that certain diatom species contain inhibitory compounds, or are deficient in essential nutrients, and that when ingested by female copepods, significantly reduce the viability of their eggs and cause malformations in the nauplii that do hatch (Poulet *et al.* 1994, 1995; Ianora *et al.* 1995; Laabir *et al.* 1995; Jónasdóttir and Kiørboe 1996). Because of the implications of this source of mortality for the understanding of recruitment rates and the production of prey for fish larvae, measurement of hatching success and naupliar viability are increasingly a standard part of egg production measurements.

The method is straightforward. After the incubation period, the females are removed and a known quantity of eggs are left for a period sufficient to allow hatching, which may be up to 72 h or more, depending on the temperature (e.g. Laabir *et al.* 1995). Specific

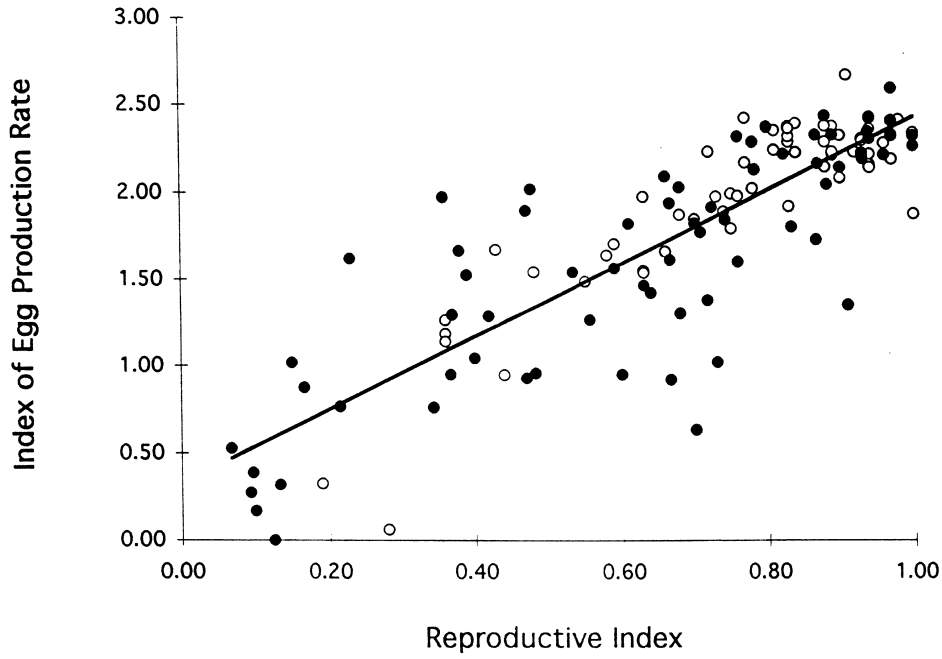

Fig. 9.4 Relationship between the reproductive index (proportion of females with mature oocytes) and an index (ln (1 + CSG_f)) of carbon-specific egg production rate ($CSG_f\ d^{-1}$) for *Calanus finmarchicus* in the Gulf of St Lawrence–Gulf of Maine system. Filled circles are from Georges Bank/Scotian Shelf and open circles from the Lower St Lawrence Estuary (data from Runge *et al.* 1997, and Runge, Plourde and Joly, unpublished).

techniques vary. For example, in one study, eggs from individual spawning events were pooled, and replicate subsamples of 50 eggs were incubated in 20 ml scintillation vials (Campbell *et al.*, in press). This involved two transfers, which are performed with the use of an automatic pipette in order to minimize egg damage. In other studies, eggs spawned in dishes were allowed to hatch undisturbed without transfer (e.g. Burkart and Kleppel 1998). While comparison of the two techniques yielded very similar hatching success (Runge, unpublished data), it is advisable to minimize disturbance, as delicate, non-viable eggs may disintegrate with handling. At the end of the incubation, nauplii and any remaining eggs may be preserved in formaldehyde or counted immediately after addition of acetic acid (white vinegar). Because eggs without properly formed membranes may disintegrate quickly, the number of eggs at the beginning of the incubation, rather than the sum of eggs and nauplii at the end, more accurately represents the initial number in the calculation of hatching success. In some cases it may be advantageous to employ stains such as trypan blue or Hoechst 33342, which specifically color dead and live embryos, respectively (Poulet *et al.* 1994). The proportion of hatching nauplii with obvious deformities (e.g. Poulet *et al.* 1995b) could also be determined.

In order to attribute hatching failure to nutrient deficiency or accumulation of toxins in females, the possibility of unfertilized or resting eggs must first be ruled out. Many copepod species, particularly members in the families Acartiidae, Centropagidae, Pontellidae, Temoridae, and Tortanidae produce diapause or quiescent eggs that would

not be expected to hatch under normal incubation conditions (Laabir *et al.* 1995). Unfertilized, or parthenogenic, eggs are typically recognizable by their gray or opaque color and by the absence of an operative cell membrane. Whereas females of some species, like members of the genus *Calanus*, store sperm in spermathecae and may therefore require only one mating in their lifetime (Marshall and Orr 1955), others, like *Acartia tonsa* (Wilson and Parrish 1971) may require frequent insemination. The causes of low hatching success, therefore, should be ascertained before interpreting the results of the egg viability observations.

9.4 EGG PRODUCTION RATES OF EGG CARRYING COPEPODS

9.4.1 Egg ratio method

A number of prominent calanoid and cyclopoid species carry their eggs until hatching. The eggs are held in an egg sac attached to the first urosomal segment of the female, although sometimes they are attached as a cluster or mass, adhering to one another without a surrounding membrane (Corkett and McLaren 1978). We define egg production rate (G_f) as the number of eggs extruded (usually as a clutch) per adult female per day. Egg hatching rate (H_f) is the number of eggs that hatch per adult female per day.

The measurement of egg hatching rate is founded on the egg-ratio method developed for rotifers by Edmondson (1960, 1968). It has been applied to freshwater cladoceran and copepod populations (e.g. Edmondson *et al.* 1962; Hall 1964) and more recently to marine copepods (e.g. Ohman 1985; Sabatini and Kiørboe 1994). The egg ratio is the abundance of eggs (E eggs m^{-2}) divided by the abundance of females (A females m^{-2}). The original egg ratio refers to eggs per total female abundance (adults and juveniles), but for copepod populations A refers to adult females only. If the age distribution of eggs is uniform and all eggs are viable, the egg hatching rate and egg production rate are equal and can be estimated as the egg ratio divided by the egg development time (D days):

$$\boldsymbol{H_f = E/AD.} \tag{9.13}$$

The abundance of eggs and females may be estimated from the same sample, provided that the sampling device captures both life stages with the same efficiency. The egg sacs or egg masses of many species become detached and may break apart during capture, in these cases the mesh size of the plankton net must be small enough to retain individual eggs. The detached eggs must be properly identified and included in the estimate of the total number of eggs in the sample. Siefert (1994) estimated H_f of *Pseudocalanus* sp. from published relationships of clutch size to prosome length (Corkett and McLaren 1978; Ohman 1985) and of egg development time to temperature (Corkett and McLaren 1978). She pointed out that gear type, towing speed, and mesh size are significant sources of error in the estimation of abundance of *Pseudocalanus* females. Sabatini and Kiørboe (1994) collected *Oithona* eggs and females with a submersible pump (400 l min^{-1}) and 45 μm mesh net, which may be the most efficient way to collect both eggs and adults, at least for the smaller copepod species.

The egg development time is a function of temperature and must be determined for each species. Laboratory protocols and functional relationships between temperature and egg duration for freshwater and marine species are provided by McLaren (1966), Edmondson (1971) and Landry (1975). The calculation of stage duration is discussed further by Aksnes *et al.* (1997).

The choice of which temperature most appropriately represents ambient temperature may be problematic, particularly for vertically migrating species in a thermally stratified water column. Detailed knowledge of the vertical distribution of females carrying eggs would be useful in this situation, nevertheless, the value of egg development time may be a significant source of error in the calculation of egg hatching rate. The problem of calculating egg hatching rates in thermally stratified environments is discussed by Prepas and Rigler (1978).

The estimation of egg hatching rate from the egg ratio/D may be used to interpret fecundity patterns for investigation of phytoplankton–zooplankton interactions, to determine naupliar production for evaluation of the prey field of fish larvae and to calculate birth rates for studies of the population dynamics in a given region (see Taylor and Slatkin 1981). Whatever the application, care must be taken to separate effects on the egg ratio/D of food limitation on egg production (e.g. Sabatini and Kiørboe 1994), size of females on clutch size (Corkett and McLaren 1978), temperature on egg development (references provided above) and mortality rate on the proportion of reproductively active females (e.g. Ohman *et al*. 1996).

9.4.2 Incubation method

In principle, egg production rates of egg-carrying copepods can be measured by immediate post-capture incubation of females, similar to the method for broadcast spawners. Here,

$$G_f = CS\ s\ 24/t, \tag{9.14}$$

where G_f (eggs female^{-1} d^{-1}) is the product of the mean observed clutch size (CS eggs female^{-1}) and the fraction of the sample population extruding a clutch (s) during an incubation of duration t (h), which should be long enough to be representative of the entire daily production (i.e. close to 24 h).

This approach has not been widely used. Dagg *et al*. (1984) estimated mean clutch size of *Pseudocalanus* from observations of females incubated in natural sea water over an 8 day period, but did not measure the frequency of clutch production in the period immediately after capture. Instead, they estimated egg hatching rate from the quotient of the mean observed clutch size divided by the egg development time for the ambient water column temperature, similar to the egg ratio method. Paul *et al*. (1990) estimated egg production rates of *Pseudocalanus* in Auke Bay, Alaska from incubations of individual females in vials containing natural sea water over a 48 h period.

The procedure follows the method outlined for broadcast spawners. Immediately after the catch, females of the targeted species are sorted at random as they are encountered. Thirty or more females are placed individually into separate vials containing sea water and incubated at an appropriate ambient temperature for a 24 h period (or perhaps longer). Effects of incubation conditions (food, light, temperature) are not known but suitable conditions presumably could be determined for each target species. At the end of the incubation period, the mean clutch size and proportion of females producing clutches is determined. Dagg *et al*. (1984) report that *Pseudocalanus* females may produce a new clutch within 12 h after hatching, so care should be taken to check for nauplii in vials containing females with clutches at the end of the observation period.

The incubation method yields an estimate of the egg production rate in the population during a given 24 h period. Changes in ambient temperature, food supply and mortality rates of females will result in changes in the daily rate. Daily measurement over a period

of time of clutch size and proportion of extruding females would provide insight into the constancy of egg production or the sources of variability. Assuming a uniform and constant mortality rate and 100% egg viability, a consistent proportion of females producing a new clutch each day implies a uniform age structure, in which case the egg production rate from the incubation technique would be equal to the egg hatching rate. However, if the population age structure is changing or mortality rates on egg carrying females are significantly different than other females, estimates of egg hatching from the egg ratio method could be expected to diverge from the egg production rate. A thorough understanding of the relationship between egg production and egg hatching rates under changing population characteristics requires careful study using population dynamics models (see also Aksnes *et al.* 1997).

9.5 THE DETERMINATION OF GROWTH RATE

9.5.1 Estimation of growth rate from preserved samples and demographic information

Data from preserved samples of field populations have been employed to estimate growth rates (e.g. Landry 1978; McLaren *et al.* 1989). The basic approach is to assume exponential growth

$$W(i+1) = W(i)e^{g(i)DT(i)} \tag{9.15}$$

where $W(i)$ is the weight of stage i at midpoint of development, $DT(i)$ is the stage duration, and $g(i)$ is the instantaneous growth rate. Some investigators (e.g. McLaren *et al.* 1989) prefer to calculate the finite daily growth rate:

$$G(i) = e^{g(i)} - 1 = (W(i+1)/W(i))^{1/DT(i)} - 1 \tag{9.16}$$

(note correction of typographical error in their equation 4). The calculation of growth rate by this method therefore requires estimation of (1) mean weight of each life stage and (2) development time (i.e. stage duration).

ESTIMATION OF DEVELOPMENT TIME

Depending on the data available, development time may be estimated directly from repeated sampling of a cohort or indirectly from laboratory measurements of the development time–temperature relationship. Landry's (1978) extensive data set allowed him to credibly identify eleven cohorts of *Acartia clausi* over the 2 year period of his study. To estimate growth rate, he plotted the log of weight of each stage against the development trajectory (in days) for each cohort (Fig. 9.5). The slope of the line is the instantaneous growth rate, which was constant over all developmental stages within a cohort but varied among cohorts. In cases where sampling is inadequate to identify development trajectories or where cohort structure does not exist, laboratory data, in the form of Belehradek's equation (see Chapter 12), has been used to infer stage duration from estimates of ambient temperature experienced by the developing population (e.g. McLaren *et al.* 1989).

ESTIMATION OF MEAN WEIGHT

The mean stage weight may be estimated directly from field samples or indirectly from length–weight relationships. For example, McLaren *et al.* (1989) measured dry weights

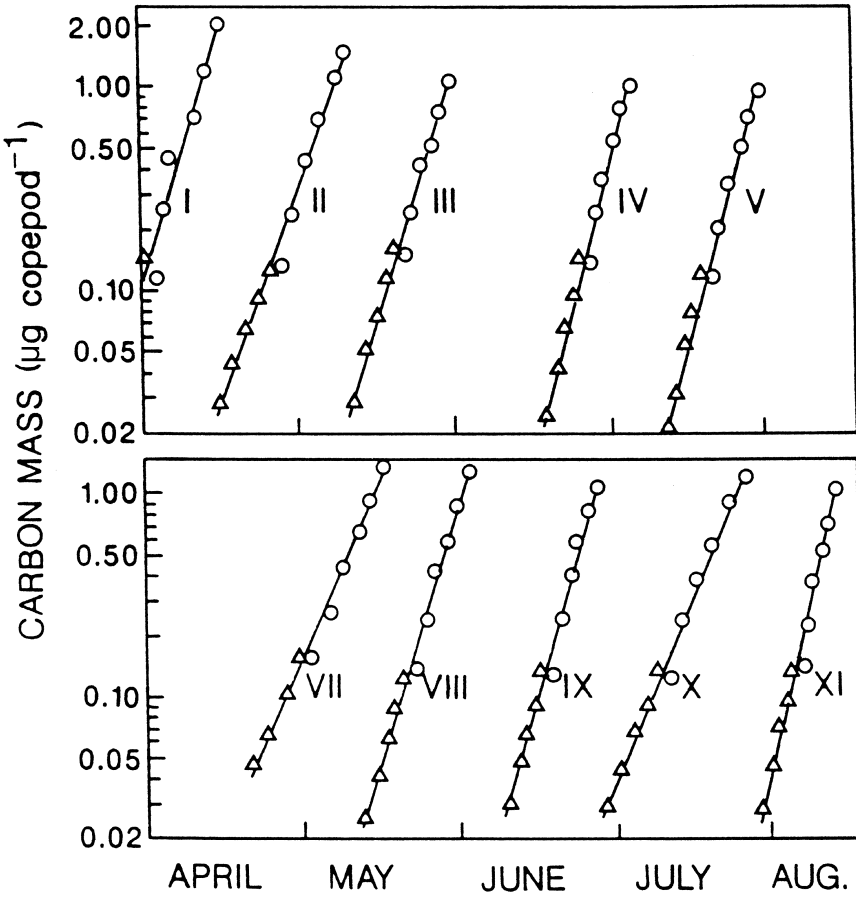

Fig. 9.5 Growth trajectories in 11 cohorts of *Acartia clausi* in Jakle's Lagoon. Development times from stage-frequency analysis of field samples, stage weights estimated from field samples and length, weight and carbon relationships, triangles and circles represent naupliar and copepodite stages respectively (adapted from Landry 1978).

of life stages in samples, correcting for preservation by assuming a 25% loss. For some species, all life stages were not present in samples, in which case they used various approximations to estimate mean stage weight. Landry (1978) determined the length–weight relationship for the population in Jakle's Lagoon, which he used in constructing growth trajectories for the individual cohorts.

LIMITATIONS AND SOURCES OF ERROR

Growth rate may be reliably estimated from demographic parameters in situations where the same population can be repeatedly sampled over time and cohorts are distinguishable (e.g. Landry 1978). However, where sampling frequency is inadequate, advection is important, or cohort structure is indistinct, development trajectories will be subject to error. For example, McLaren *et al.* (1989) were not able to estimate development rates of *C. finmarchicus* on the Scotian Shelf in summer. Even with greater sampling frequency, unequivocal identification of development trajectories on the

Scotian Shelf would be difficult, because of the possibility of advection of sub-populations of *Calanus* having a different cohort structure in the Nova Scotia Current.

There are also important sources of error inherent in estimating mean stage weight from field samples and development times from laboratory data. If the age structure within a stage is not uniform in the field population, estimates of growth based on weights of stages in a sample may be inaccurate. Application of Belehrádek's relationship to obtain development times requires knowledge of the temperature regime of the developing population. In a water column that is thermally stratified or undergoing rapid warming during spring, assigning the appropriate temperature to describe development is problematic. Moreover, it must be assumed that development is proceeding at a maximum rate, a situation which is not always observed, particularly when a life stage has entered diapause (e.g. Runge *et al.* 1985).

9.5.2 Direct measurement of growth rate

THE BASIC METHOD

Similar to the direct measurement method for egg production rate, the premise underlying the approach is that the growth rate of freshly captured zooplankton maintained in near-ambient conditions for short periods of time accurately reflects *in situ* growth during the same period. Heinle (1966) incubated *Acartia tonsa* for studies of its growth rate in Chesapeake Bay. Tranter (1976) proposed the approach as a general method for estimation of planktonic production. Copepods for incubation may be sorted individually by stage (e.g. Diel and Klein-Breteler 1986; Peterson *et al.* 1991) or, for small species for which sorting by stage is not feasible, by size fractions (termed 'artificial cohorts': e.g. Kimmerer and McKinnon 1987; Peterson *et al.* 1991). Molting rates of copepodite stages have also been estimated by incubations of individual stages (e.g. Falkowski *et al.* 1983; Miller *et al.* 1984; Runge *et al.* 1985). A more complete history of references to the method is provided in Berggreen *et al.* (1988) and Peterson *et al.* (1991).

Assuming that growth is exponential during incubations, the instantaneous growth rate, g (day^{-1}), for a given species may be calculated by:

$$g = 24/T \ln(B_t/B_0) \tag{9.17}$$

where T is the duration of incubation (h) and B_0 and B_t are the initial and final biomasses, respectively. Peterson *et al.* (1991) determined biomass at time (t) as

$$B_t = \sum N_{i,t} W_{i,t} \tag{9.18}$$

where $N_{i,t}$ is the abundance of stage i (expressed as percent total abundance of the species at time (t), to reduce sources of error due to mortality) and $W_{i,t}$ is the mean mass of stage i at time (t). A graphical method, in which the slope of the regression of mean log-transformed biomass on time is the instantaneous growth rate, is described in Kimmerer and McKinnon (1987). High mortality may be a significant source of error in the calculation of B. Kimmerer and McKinnon (1987) excluded copepods that died during the incubation and eliminated replicates with greater than 15% mortality.

$W_{i,t}$ for equation 9.18 should be determined directly from animals used in the experiment. While mass is traditionally expressed in terms of dry weight, determination of mass in terms of carbon and particularly nitrogen may be easier and more informative, as nitrogen may be a proxy for structural weight.

Another approach to the estimation of growth is by measuring the daily fraction

molting from stage i to stage j in incubations (see molting references above). The growth rate of each developmental stage is then calculated as follows:

$$g_i = \ln(W_{i+1}/W_i)MR_i, \tag{9.19}$$

where MR_i is the molting rate of stage i (d^{-1}) and W_{i+1} and W_i represent the mean weights of the successive stages (Peterson *et al.* 1991). This method assumes a uniform age distribution within stage, such that a constant fraction of the stage population is molting each day. The mean weights should be estimated from an unbiased field sample of each stage, taken at the time of the incubation. If growth is exponential, weights of newly molted individuals will not be representative of the mean stage weight of the population. Note that for the same data, equations 9.17 and 9.18 do not necessarily yield the same estimate of g, depending on the cohort age structure and how W is estimated. Miller and Tande (1993) have criticized the use of the molting rate method (which they term 'Kimmerer experiments') in populations with a strong cohort structure, because the age distribution within a stage at any given time is unlikely to be uniform, leading to serious errors in the estimation of g. The molting rate method should therefore be considered for use only in populations where there is strong evidence of continuous recruitment and uniform age structure within the stages for which g is estimated.

Kimmerer and McKinnon (1987) and Peterson *et al.* (1991) conducted incubation experiments using artificial cohorts, in which stage weights were estimated from literature values (Peterson *et al.* 1991) or from field samples collected closest in time to the experimental date (Kimmerer and McKinnon 1987). Experiments of this type are in essence molting rate experiments and are consequently subject to error if the age-within-stage distribution of the population under study is not uniform.

A variation of the size fractionation technique has been successfully used to estimate growth rates of larvaceans in tropical waters (Hopcroft and Roff 1995). The selection of screen sizes allowed mostly egg stages to pass into the experimental microcosms, which greatly reduced error associated with handling of post-hatch animals. Growth rates were determined from progression of the artifical cohorts as shown by daily length-frequency histograms. The exponential growth rate, g, was calculated as the natural log of the ratio of weights represented by the cohort modes divided by time.

PROCEDURES

Estimation of growth rate by short-term incubations is a relatively new method and as such is subject to revision as knowledge of appropriate and efficient techniques expands. Like the egg production method, an understanding of the life history, vertical distribution and behaviors of the target species will increase the likelihood that the incubations are representative of natural conditions and that the results are correctly interpreted. The following techniques are offered as guidelines for application of the method to particular environments and species assemblages. A study comparing methods for the estimation of molting rates was conducted by Shreeve *et al.* (1998). A detailed protocol for the measurement of growth and development of a planktonic copepod (*C. finmarchicus* is used as an example) is provided in section 9.8.

Capture

Techniques for the gentle capture and handling of zooplankton, described in Chapter 3 and in section 9.3.1 of this chapter, must be used to collect copepods for the incubations. Peterson *et al.* (1991), for example, collected zooplankton by lowering a plankton net to 10 m and slowly retreiving it while the ship drifted. The cod-end was a 5 l plastic bag in

which a small hole was made to allow air to escape. Duration of tows was about 5 min. Care should be taken, however, to ensure that the sample of a given stage in the net is representative of that stage in the entire water column. The catch should be protected from light and thermal shock, diluted and sorted or size-fractionated immediately after the net is retrieved.

Sorting

Although any stage may be sorted individually to produce a stage- and species-specific cohort, normally this is practical only for older, larger stages. To ensure accurate identification of stage, it may be necessary to double sort, i.e. pick individuals for experiments from a preliminary selection. There must be a sufficient number of individuals to yield a statistically valid estimate of initial and final body mass, and more if the incubation is of several days duration and daily samples for body mass are to be collected. The advantage to sorting is the possibility of not only directly measuring growth, but also estimating molting rates.

Size fractionation for creation of artificial cohorts can be used to isolate any developmental stage or stage grouping. The procedure must be done carefully, as damage to the exoskeleton of copepods during this process may prevent successful completion of ecdysis. Kimmerer and McKinnon (1987) immersed a large PVC cylinder with the bottom covered by mesh of appropriate size into a container of freshly captured plankton, then gently siphoned from inside the cylinder, creating two size fractions. The process was repeated until a fraction concentrating the targeted species and life stages was obtained. Peterson *et al.* (1991) passed diluted plankton samples through sieves of various sizes, keeping the sieves immersed at all times. Both methods yielded size fractions suitable for growth determinations. The selection of mesh sizes for plankton nets and sieves may require preliminary trials. Peterson *et al.* (1991) noted that all life stages and species may not be sufficiently abundant at a given time and station for determining their growth rates by this method. The advantage of the method is that it can be used for naupliar stages or copepodite stages of small species, which are difficult to sort individually and must be obtained in large numbers for adequate CN measurements. The disadvantage is that, because size fractionation typically yields a mixture of stages, stage-specific growth rates cannot be measured, only total cohort growth.

Incubations

The duration and conditions of incubation for measurement of growth or molting rate vary among studies. To obtain molting rates of *Calanus finmarchicus* copepodites, Runge *et al.* (1985) incubated individuals in 15 ml petri dishes in a refrigerator at ambient surface layer temperatures for 24 h. Kimmerer and McKinnon (1987) used 4 l plastic jars or acrylic boxes moored at 5 to 8 m for up to 50 h. Peterson *et al.* (1991) incubated *Calanus* copepodites and artificial cohorts in 1.2 and 10 l jars, respectively, both of which were maintained for 24 h in deck incubators cooled by continuously circulating sea water. Campbell *et al.* (submitted) employed 100 to 300 l plastic barrels placed on deck in holding tanks, also cooled by running sea water from the pump system of the ship. Whereas Kimmerer and McKinnon (1987) and Peterson *et al.* (1991) used 35- or 120-μm filtered sea water, Campbell *et al.* (in press) filled their mesocosms with sea water containing a natural assemblage collected with a diaphragm pump in order to minimize damage to delicate microplankton. The copepod concentrations in the mesocosms were dilute so that daily grazing impact was low, allowing the experiment to continue over several days with daily subsampling for estimating changes in weight and stage composition. In all deck incubations, care must be taken to protect

experimental animals from the harmful impact of UV-A and UV-B radiation, which could cause either mortality or sublethal effects on copepod growth processes.

9.6 BIOCHEMICAL AND RADIOCHEMICAL METHODS

There have been persistent attempts to relate biochemical properties of zooplankton to growth or development rates. These methods should be regarded as still largely exploratory or under development. Hopefully, in future years they will become as standardized as the ^{14}C technique for measurement of primary production. Marine ecologists have frequently been overenthusiastic in taking fledgling methods to the field, where their proper calibration and evaluation may be impossible (Leftley *et al*. 1983). As suggested by Conover and Poulet (1986), '... we must go back to the laboratory ... under carefully controlled conditions' in developing and calibrating these techniques.

The biochemistry of growth is complex and a full consideration of this subject is beyond the scope of this chapter. Growth is the net difference between all anabolic and all catabolic processes; it manifests as the rate of change of biomass. Any biochemical quantity or process can potentially be used to document the rate of change of biomass, but we should distinguish between more predictable 'structural growth' (*sensu* McLaren 1986) and more variable 'storage', e.g. of lipids, if body composition is changing seasonally or ontogenically. Ratios of biochemical quantities, titre or activity of hormones or growth factors, activities of enzymes in anabolic pathways, and radiochemical rates of synthesis of specific macromolecules, are all potential measures of growth rates.

Where growth is not exponential, biochemical quantities or indices may appear correlated with growth rates purely as a function of ontogenic change. The true test of a measure of growth rate is that it should vary among animals of the same size, and/or ontogenic stage of development, which are growing at different rates. Unfortunately this crucial test is rarely applied. Growth in planktonic animals may be a function of both temperature and food concentration. A true measure of growth rate must therefore also reflect changes in growth as a function of both these variables.

9.6.1 Ratios of biochemical quantities

Since the early investigations on RNA and DNA in invertebrates (e.g. Sutcliffe 1965), there has been continuing interest in the potential of these nucleic acids, or their ratios, as measures of growth rates. Relationships were initially investigated on the assumptions that the amount of DNA per cell is constant while RNA fluctuates in proportion to metabolic (including growth) rate. These assumptions are not strictly correct. RNA and DNA are readily assayed on whole tissue homogenates by the ethidium bromide fluorometric method (Karsten and Wollenberg 1972, 1977), although many variations and modifications have been used (see other references in this section). Perhaps partly because of the relative simplicity of these assays, interest in nucleic acids has been continuous.

There is often a positive correlation between RNA content and growth rate, but in several studies the relationship has been shown to lack sufficient specificity to reliably predict growth rates (e.g. Dagg and Littlepage 1972; Ota and Landry 1984). RNA content is predominantly a function of body size, as is growth rate in many taxa (Båmstedt and Skjoldal 1980; Banse and Mosher 1980). DNA content per unit biomass

generally decreases with increased body size (e.g. Sulkin *et al.* 1975), and with increased growth rate (as a function of diminished cell volume, e.g. Ota and Landry 1984; Bulow 1987), although relationships are generally weak. The positive relationship between DNA C-value (haploid DNA content) and development duration is well known in a diversity of eukaryotes (see e.g. Cavalier-Smith 1978). Such relationships are also found within the copepods, where genome size is a correlate of temperature corrected embryonic duration as well as body size (McLaren *et al.* 1987, 1988).

A positive correlation between the RNA/DNA ratio and somatic or reproductive growth rate may be observed (e.g. Nakata *et al.* 1994; Westerman and Holt 1994), but it is often too weak to be of predictive value (see e.g. Bulow 1987). Even under controlled laboratory conditions there may be no overall correlation between this ratio and growth rates (Ota and Landry 1984). A mixed model of temperature and RNA/DNA ratios (Buckley 1984) explained 92% of the variation in growth of eight species of larval fish, however temperature alone accounted for twice the contribution of the biomass ratio. Temperature itself is therefore the dominant factor in growth variation as was shown for copepods by Huntley and Lopez (1992).

In more recent literature, largely concerning benthic invertebrates and larval fish, the emphasis has (more appropriately) been to accept RNA/DNA ratios as indices of condition factor or of the impact of toxicants, rather than as measures of growth itself (e.g. Barron and Adelman 1984; Steinhart and Echman 1992). Other biomass ratios, such as protein/chitin are also potentially useful as indices of condition.

Quantities of nucleic acids and their ratios require careful interpretation because they are known to vary substantially between even closely related taxa (e.g. McLaren *et al.* 1988); with age, body size and stage of ontogenic development within species, because body composition changes irrespective of food climate (e.g. Robinson and Ware 1988; Jones and Roff, unpublished data); in relation to the phase of the molt cycle (Sulkin *et al.* 1975). Additional complications are: only part of the RNA complement – mRNA and tRNA – will vary with metabolic rate, while rRNA is more conservative; somatic polyploidy and polyteny of DNA varies both seasonally and among tissues within species (Brodsky and Uryvaeva 1985); non-growing animals (e.g. in diapause) still have a normal complement of RNA and DNA.

All biomass ratios must have strict limitations as growth indices. Further fundamental objections are: (1) such ratios are dimensionally incorrect, a ratio is dimensionless, a growth rate has dimensions $M.T^{-1}$ or T^{-1}; (2) such ratios cannot take into account the effects of temperature which, on a global scale, is the simplest overall predictor of growth rate in copepods (Huntley and Lopez 1992). In summary, biomass ratios may be useful as species-specific and age or stage-specific condition factors. While such ratios may reflect the past character of tissue accumulation, and may even show correlation with current or past growth rate, they are not measures of growth rate itself.

9.6.2 Hormones and growth factors

The control of growth in vertebrates involves complex and poorly understood interactions of many hormones and growth factors, which may be general or tissue specific in their actions (e.g. Kelly 1990). Despite more limited knowledge in invertebrates, there is promise that such molecular studies could produce reliable indices of growth rates.

For example, cellular activity and rate of synthesis of many biochemicals are correlated with ecdysteroid levels in arthropods (Dean *et al.* 1980). The titre of ecdysone

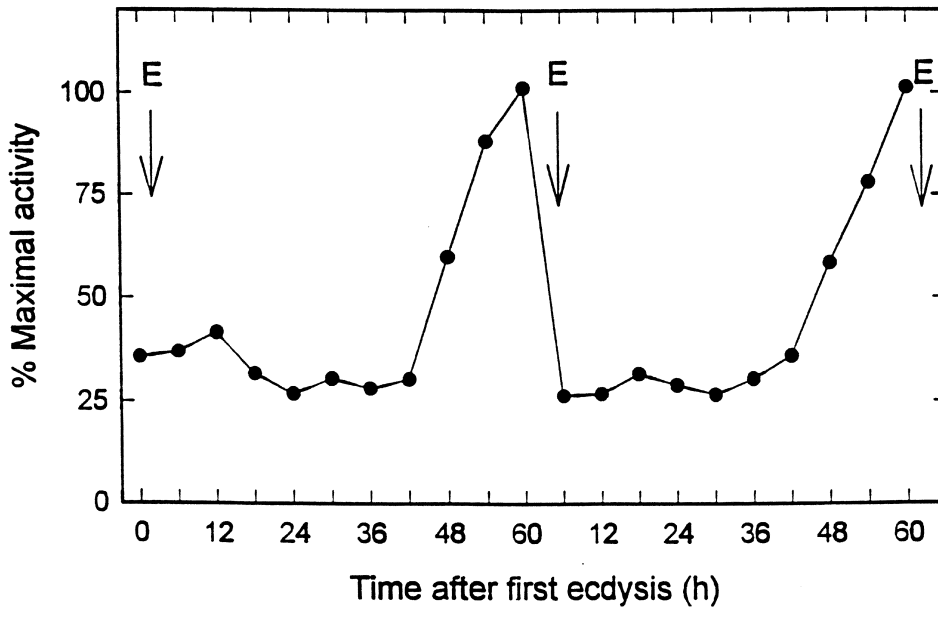

Fig. 9.6 Relative activity of chitobiase from *Daphnia magna* over the molt cycle (mean values for triplicate samples, each of ten animals are shown; standard errors, not shown, are from less than one to two times symbol size. E = ecdysis). The mean duration of the molt cycle for the population is 59.4 $\pm$ 4.7 h (Espie and Roff 1995b, with permission).

rises sharply at premolt in both insects and crustacea (e.g. Spindler-Barth *et al.* 1986; Funke and Spindler 1987; Van Beek *et al.* 1987). Thus the mean titre of ecdysone in a population, or the frequency of animals with hormone above a basal level could provide an index of molt rate in a manner analogous to the frequency of dividing cells (FDC) method of Heinbokel (1988). Modulation of such biochemical concentrations over a molt cycle forms the basis of the chitobiase and frequency of animals in apolysis (FAA) methods of Espie and Roff (1995a, 1995b) and Roff and Espie (unpublished data, see Fig. 9.6).

Other approaches should be exploited as our knowledge of biological regulation improves. Thus telomerases and other cell regulation factors (e.g. cyclins) may modulate over the cell cycle, and the average titre over the cell cycle, or the frequency of cells above some basal level could be used as an index of cell division rate (e.g. as in the FDC method of Heinbokel 1988). New assays of cell proliferation, e.g proliferating cell nuclear antigen, are now being applied to planktonic organisms (Lin *et al.* 1994; Moore *et al.* 1994), and show promise as descriptors of growth rate. However, again such approaches can only yield indices or correlates of growth or development rates rather than growth rates *per se*.

9.6.3 Enzyme activities

Enzyme activities (EAs) are attractive as potential indices of metabolic rates for several reasons: (1) they *are* the chemical basis of all metabolic rate processes, (2) as rate functions themselves they are dimensionally correct (MT^{-1} or T^{-1}), (3) they are rapid

and cheap to measure, (4) assays can be conducted under defined conditions, and (5) they may afford high precision and good replicability. Thus EAs have received increasing attention as indices of metabolic activity in zooplankton although, unfortunately, replicability between EA assay sets may be poor for unknown reasons (Dean *et al*. 1986). General methods for enzymatic analyses are compiled in the series of volumes edited by Bergmeyer *et al*. (1983), but precise reaction conditions vary among researchers.

Several approaches to the development of growth indices based on EAs are possible, however some basic principles in the application of enzyme assays must be appreciated. Unfortunately, many investigators have failed to acknowledge the limitations and dangers in the interpretation of EA data, despite repeated cautions (e.g. Båmstedt 1979; Mayzaud 1986a; Newsholme and Crabtree 1986; Berges *et al*. 1990, 1993). These limitations and cautions include the following.

1) When examined over several orders of magnitude in body mass, EAs may show high correlations with metabolic rates, even among species (see e.g. King and Packard 1975). However, while relationships between EAs and body mass may be similar among species (e.g. Berges and Ballantyne 1991; Espie and Roff 1995b), they are not coincident, and when examined over a narrower range of body mass they are often very different, both in scaling exponents and intercepts (Espie and Roff 1995b, Fig. 9.7). Perspectives on such differences are only just emerging (e.g. Childress and

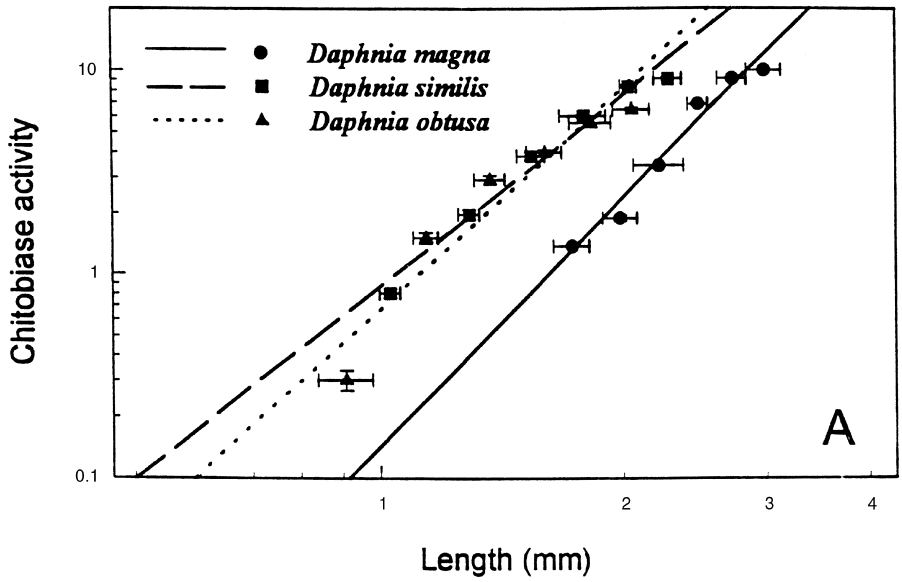

Fig. 9.7 (A) Relationship between chitobiase activity (nmol MBF (methylumbelliferone) liberated 10 min^{-1}) and body length for three *Daphnia* spp. Regression parameters for the three species are: (*D. magna*) $a = -8.98$, $b = 4.05$, $r^2 = 0.97$; (*D. similis*) $a = -5.05$, $b = 3.57$, $r^2 = 0.98$; (*D. obtusa*) $a = -6.33$, $b = 3.57$, $r^2 = 0.91$; $P < 0.001$ for all regressions. Bars represent standard errors of mean lengths and chitobiase activities in triplicate samples each of 30 animals (Espie and Roff 1995b, with permission).

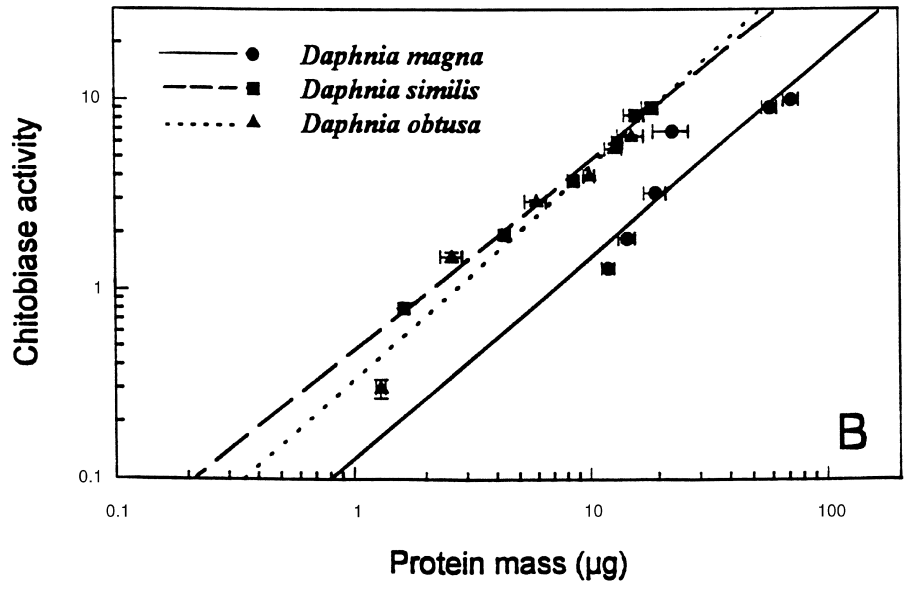

Fig. 9.7 (B) Relationship between chitobiase activity (nmol MBF liberated 10 min^{-1}) and protein mass for three *Daphnia* spp. Regression parameters for the three species are: (*D. magna*) $a = -2.08$, $b = 1.04$, $r^2 = 0.85$; (*D. similis*) $a = -0.88$, $b = 1.03$, $r^2 = 0.99$; (*D. obtusa*) $a = -1.42$, $b = 1.13$, $r^2 = 0.94$; $P < 0.001$ for all regressions. Bars represent standard errors of mean protein mass and chitobiase activities in triplicate samples each of 30 animals (Espie and Roff 1995b, with permission).

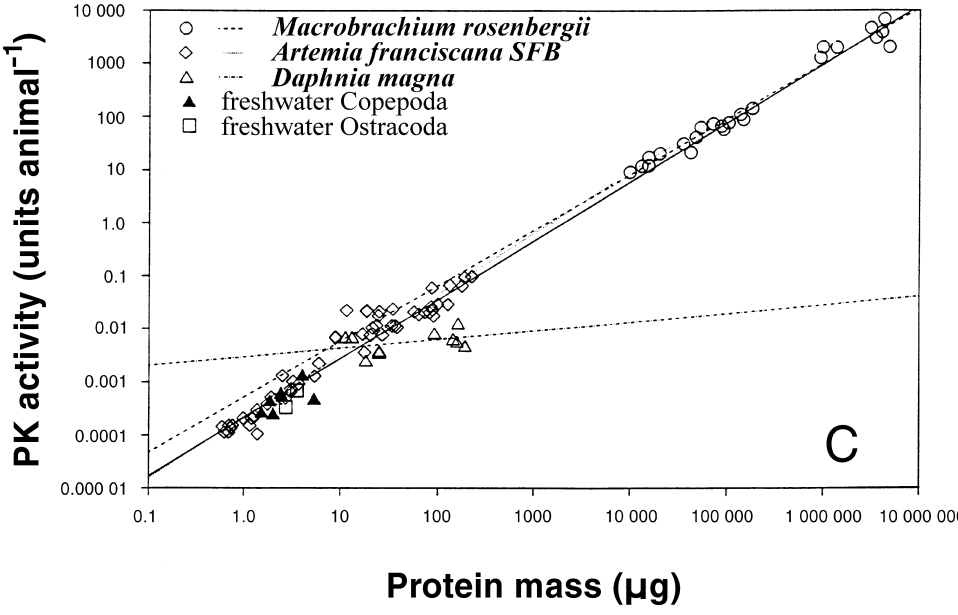

Fig. 9.7 (C) Whole animal pyruvate kinase (PK) activity versus protein weight for five crustacean species (Berges and Ballantyne 1991, with permission).

Somero 1990). K_m (the Michaelis constant) and EA_{max} (maximum enzyme activity) values for a given enzyme also differ between species (e.g. Espie and Roff 1995a). As the species composition of the zooplankton community changes, so must the relationship between the measured EA and the metabolic process which it purports to index. Thus EAs assayed on whole zooplankton communities may be only a crude approximation of the corresponding actual metabolic rate. If the size structure of the zooplankton community is also varying, relationships are further confounded (see next paragraph). Such composite relationships will generally have poor predictive power.

2) The major correlate of EA is body size (Berges *et al.* 1990, 1993). Of eight enzymes assayed by Berges *et al.* (1990), representative of several anabolic and catabolic pathways, only one, NDPK (nucleoside diphosphate kinase, an enzyme in the nucleotide synthesis pathway), showed a demonstrable relation between growth rate and EA (see Fig. 9.8). To have meaning as indices of metabolic processes therefore, EAs should be assessed on both a species- and size-specific basis. This is especially important where growth rate is not exponential, because growth rate itself is then automatically confounded with body size.
3) There have been repeated warnings of the misuse of ratios and scaling relationships in the physiological-ecology literature (e.g. Packard and Boardman 1988; Berges *et al.* 1990 etc.). Conversion from an EA to a metabolic rate is often done with both variables expressed on a mass-specific basis without regard to scaling exponents. In such cases EA indices are simply surrogate measures of plankton biomass, biased according to the size-spectrum of the community sampled (see Berges *et al.* 1993).
4) Different isozymes within species will have different K_m and V_{max} values, and the isozyme complement may vary between populations of a species (e.g. Alayse-Danet 1980). Relationships between EA_{max} and metabolic rate will therefore vary in an unspecified manner both seasonally and spatially. Despite the fact that isozymes are widely investigated in population genetics studies, their existence is rarely acknowledged in metabolic-ecological studies (see Espie and Roff 1995a for example).
5) Reaction conditions *in vitro* are optimized to stabilize or maximize EA (EA_{max}, Packard *et al.* 1971), essentially a measure of enzyme concentration. The relation between such rates and those *in vivo* are generally unknown, because *in vivo* enzymes are subject to regulation and may operate at a variable level below EA_{max}, and because *in vitro* EA_{max} is often optimized to some arbitrary level. An EA can yield an index of metabolic rate if EA_{max} *in vitro* is a good estimate of flux through a pathway *in vivo* (e.g. Newsholme and Crabtree 1986). Thus EA_{max} can greatly (and variably) overestimate the actual metabolic flux (by up to 100 × e.g. Newsholme and Crabtree 1986; Espie and Roff 1995a). In practice, experimental calibration of the EA to an independently measured rate is necessary, but is surprisingly seldom performed (see Berges and Harrison 1995 for example).
6) Calibration factors between EAs and metabolic rates may vary greatly and unpredictably, even between assay trials, depending *inter alia* on the precise reaction conditions, physiological state of animals and time before assay commences. Standard calibration factors between EA_{max} and a metabolic rate may therefore be unattainable. However, unless they are calibrated, measures of EAs cannot provide reliable or generalized estimates of metabolic rates (King and Packard 1975).

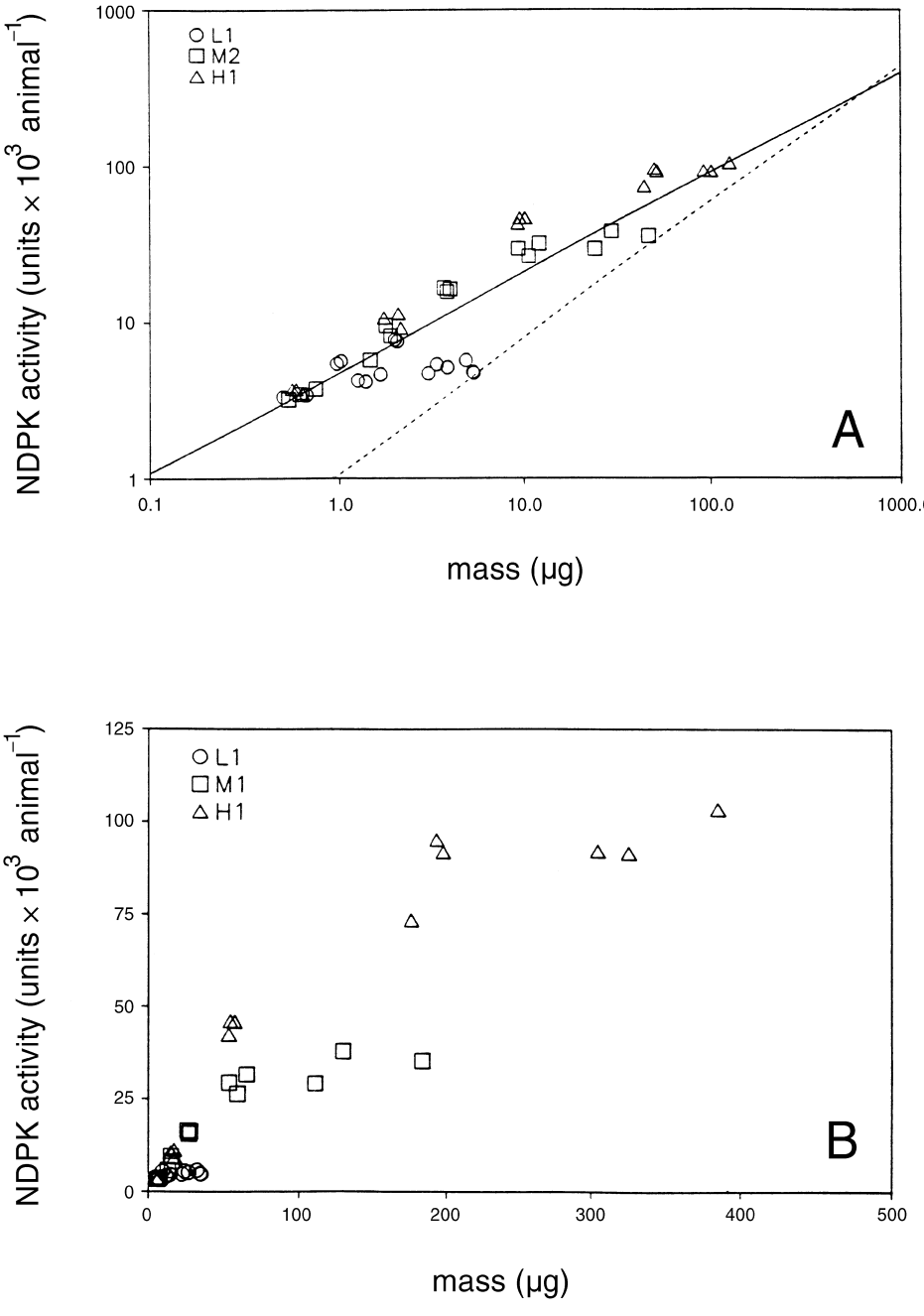

Fig. 9.8 (A) Log-transformed nucleoside diphosphate kinase (NDPK) activity versus log-transformed dry mass and protein mass in *Artemia franciscana* SFB under different culture treatments (L = 2000, M = 10 000 and H = 50 000 cells ml^{-1} of *Dunaliella salina*) for trial 1 data. Solid line represents protein mass regression, dashed line represents dry mass regression. Data points are presented only for protein mass. (B) Linear plot of the same data, dry mass only (Berges *et al*. 1990, with permission).

7) It must be shown that the EA actually does modulate with variations in the metabolic or development rate it is purported to index; surprisingly this is rarely adequately demonstrated (see Espie and Roff 1995a, 1995b, and Fig. 9.6).

There is clear evidence that digestive EAs modulate in response to food concentration, and during diel, molt, ontogenic and seasonal cycles (see review by Mayzaud 1986b; Harms *et al.* 1991; Roche-Mayzaud *et al.* 1991). In contrast, the modulation of other EAs in relation to other metabolic functions, including growth rate, is far less clear. Many relationships proposed in the literature (e.g. respiration and excretion) are in fact confounded with body mass as warned by Båmstedt (1979) and Berges *et al.* (1990, 1993).

Several studies have reported correlations between EAs and growth rates in larval or juvenile fish (e.g. Dean *et al.* 1986; Goolish and Adelman 1987; Walzem *et al.* 1991; Clarke *et al.* 1992; Mathers *et al.* 1992; Pelletier *et al.* 1993), although other studies have failed to confirm such relationships (e.g. Pasdar *et al.* 1984). Growth in fish is typically not exponential, and in several of these studies, the confounding effect of body size on growth rate has not been adequately separated; effects of starvation may also dictate some relationships. However, when body size and mass effects are statistically controlled, significant relationships between EAs and growth rates may remain (e.g. Clarke *et al.* 1992; Mathers *et al.* 1992).

Significant declines in activity of citrate synthase have been reported in unfed *Temora* and *Acartia* sp. (Clarke and Walsh 1993). However these authors suggested this enzyme as an index of diminished (physiological) condition, rather than as an assay for growth. This interpretation is reinforced by Berges *et al.* (1990, 1993). In their studies, seven of eight enzymes (including citrate synthase) showed a strong relationship to body size, but no relation to growth rate. NDPK was an exception, and showed clear differences in animals of identical size growing at different exponential rates (Fig. 9.8). The relationship to growth is complex, and subsequent investigations (Jones and Roff, unpublished data) failed to develop these relationships into reliable predictors of growth rate. One reason for this may be that NDPKs 'are a family of relatively unspecific enzymes' (that catalyze the transfer of terminal PO_4 groups between diphosphate nucleotides). There is marked electrophoretic heterogeneity of this enzyme between tissues (up to five different isozymes, Parks and Argawal 1973).

Another enzyme involved in nucleotide synthesis is aspartate transcarbamylase (ATC). Bergeron (1983, 1986, 1990, 1992, 1993) has proposed a relationship between ATC and mesozooplankton production. However, the inverse seasonal relationship between biomass and mass-specific ATC activity (Bergeron 1983) suggests a confounding effect of body size. Although a relationship between ATC and growth rate was suggested by Alayse-Danet (1980), that relationship was admittedly confounded with body size. If ATC is an index of growth rate, then it should vary between animals of the same species and same size, growing at different rates. This has not yet been shown (see Bergeron and Alayse-Danet 1981).

Even though relationships between EAs and growth rates may be established, their use as predictors of growth rates for field populations still presents difficulties. For example, enzyme kinetic properties (EA_{max} and K_m) differ significantly even between strains of the same species (Alayse-Danet 1980). Also, EAs are highly temperature dependent. Although EAs may be readily converted from one temperature to another (by Arrhenius plots), such relationships are isozyme specific and EAs may increase or decrease with temperature (e.g. Mathers *et al.* 1992). It is therefore essential to examine

for the existence of isozymes in the populations studied (see e.g. Espie and Roff 1995a, 1995b), and even more important to do so if attempting to compare or extrapolate EAs between communities across temperature ranges. It is disturbing that, although analysis of isozymes is a routine tool in population genetics studies, in ecological studies their existence is essentially ignored.

Most of the above problems, involved in demonstrating relationships between EAs and growth rates, may be overcome where EAs modulate in response to cyclic physiological changes, such as those that occur during periods of feeding and at molt in crustacea. The chitobiase method of Espie and Roff (1995b) is an example of such an approach, as is the DNA polymerase method of Sapienza and Mague (1979). In planktonic crustacea, the activity of chitobiase increases sharply from intermolt to premolt (Fig. 9.6), when animals are in apolysis, and there are strong linear relationships between chitobiase activity and duration of the population molt cycle (Fig. 9.9). The average titre of chitobiase in an asynchronously developing population is therefore a measure of the frequency of animals in apolysis. More simply, where a population is not perfectly asynchronous, temporal variations in chitobiase activity will define the duration of the molt cycle (D) directly. Here an EA is being used as a 'quantitative marker of the frequency of a cyclic phenomenon' not as a direct measure of a quantity. Following determination of D, growth rate is calculated by conventional means, as the product of molt rate ($1/D$) and the growth increment (see e.g. Chisholm and Roff 1990).

In summary, there may be enzymes whose activity modulates directly with growth rate of animals, and which may quantitatively represent the flux of biochemicals through metabolic pathways. In the case of phytoplankton, nitrate reductase appears to do just this (Berges and Harrison 1995). In zooplankton, the challenge remains to identify such enzymes, and properly calibrate their activity, taking into account the basic principles and limitations outlined above. The large body of recent literature on relationships between EAs and growth, chiefly in the disciplines of clinical pharmacology and microbiology, should be a rich source of inspiration for plankton biologists seeking to develop enzymatic methods.

9.6.4 Radiochemical methods

In contrast to EAs, which may only provide indices of rate functions, isotope methods can produce estimates of the rates themselves. Radiochemical techniques for measurement of rates of production by primary producers (Steeman-Nielsen 1952) and bacteria (Azam *et al.* 1983) have been in existence for some years, and have undergone many modifications and improvements. Despite attempts to establish radiochemical methods for zooplankton secondary production by Russian workers (e.g. Shushkina *et al.* 1974), no methodology was published until Roff *et al.* (1994). Several approaches to the problem are now documented in various groups of metazoa, but they have received little attention yet in zooplankton, perhaps because of past disappointments. The possible approaches include: *in vitro* rate of incorporation or production of label using whole body homogenates or body tissues; *in vivo* uptake of labeled compounds from the environment; *in vivo* infusion, perfusion or flooding dose injection of labeled compounds; *in vivo* ingestion of labeled food.

IN VITRO INCORPORATION

Many of the methods which belong in this category are simply radioisotope modifications of EA methods (e.g. the ^{14}C ATC method of Bergeron 1983). The method of

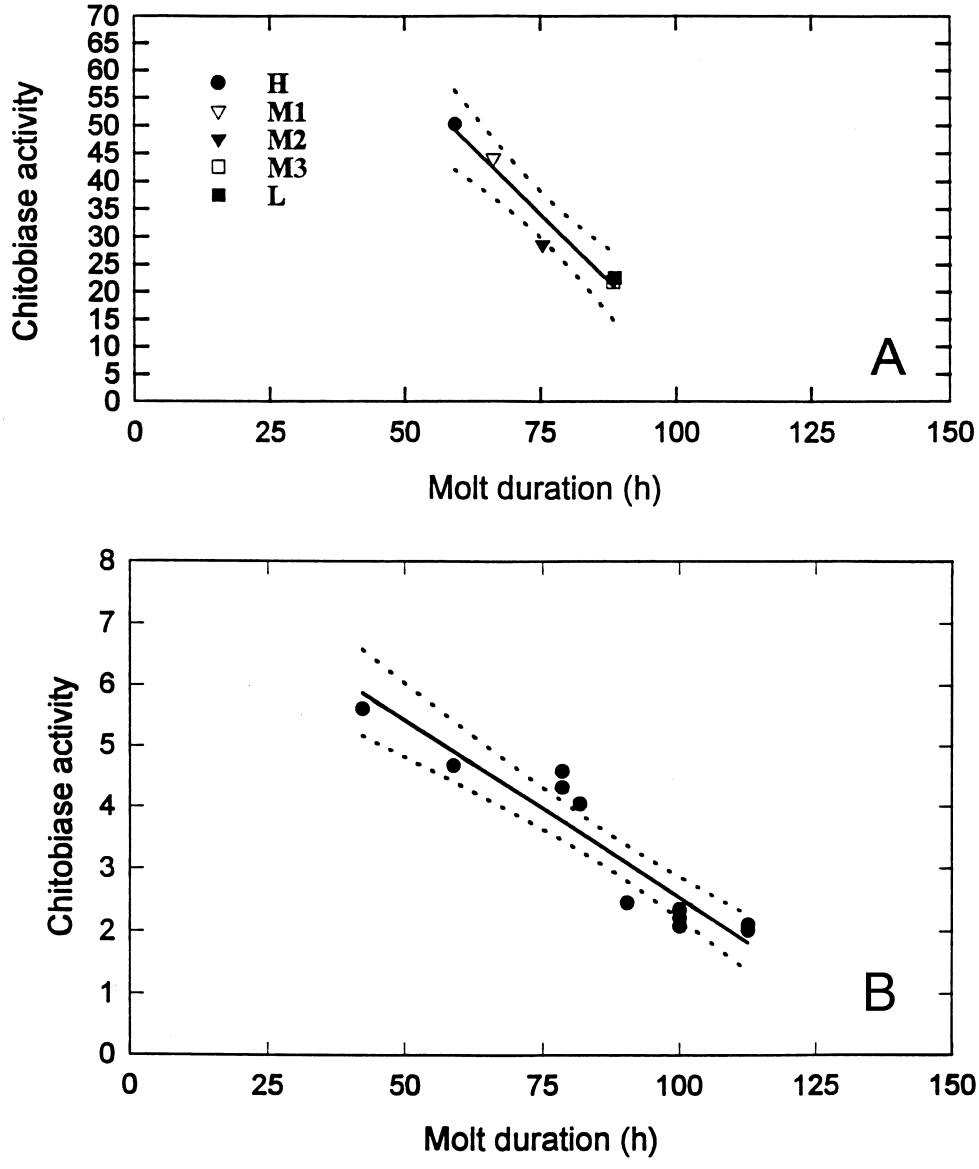

Fig. 9.9 (A) Relationship between chitobiase activity (nmol MBF liberated (mg protein)$^{-1}$ 10 min^{-1}) and duration of the molt cycle of *Daphnia magna* raised on *Chlorella vulgaris*: H = 12 500, M1 = 4150, M2 = 1 050, M3 = 450 and L = 150 cells ml^{-1} at 20 °C. Regression parameters are: $a = 106.4$, $b = -0.96$, $r^2 = 0.95$, $P < 0.001$. Dotted lines are 95% confidence limits of standard errors for mean chitobiase activities from triplicate samples each of 35 animals, and molt durations. (B) Relationship between chitobiase activity (nmol MBF liberated (mg protein)$^{-1}$ (10 min)$^{-1}$) and duration of the molt cycle for a field population of *Daphnia rosea*. Regression parameters are: $a = 8.31$, $b = -0.06$, $r^2 = 0.88$, $P < 0.001$. Dotted lines are 95% confidence limits of standard errors for mean chitobiase activities from triplicate samples each of 200 animals, and molt durations (Espie and Roff 1995b, with permission).

Sapienza and Mague (1979), which assays the activity of DNA polymerase by incorporation of [^{3}H] thymidine triphosphate, is essentially a measure of the proportion of cells in S-phase (the period of DNA replication), and is therefore an example of an EA method used as a 'quantitative marker of the frequency of a cyclic phenomenon'. Although the method has been often cited since, it has apparently not been repeated, nor has it been calibrated as a workable measure of growth rate. This is unfortunate because far more is now known about crustacean DNA polymerases (e.g. McLennan and Miller 1987), and a simple enzymatic method for continuous monitoring of DNA polymerase activity now exists (Nyren 1987).

Successful incorporation of label from UDP-N-acetyl-D-[^{14}C] glucosamine into crustacean chitin, by chitin synthetase, *in vitro* was accomplished by Carey (1965). The process was further investigated and methods refined by Horst (1981, 1983), but the technique has not been quantified in terms of rate of chitin synthesis or growth rate. Again, because chitin synthesis and recovery during the molt cycle is cyclic in nature, the enzymes involved should all show significant relationships to growth and development rates.

An *in vitro* technique to index growth rate, by measuring uptake of [^{14}C]-glycine into fish scales, has been successfully developed (Ottaway and Simkiss 1977), although there are complications in accounting for effects of environmental temperature (Adelman 1980) and other problems (for review see Adelman 1987). The technique of Horst (1981, 1983) could probably also be modified in this way to measure growth in isolated exoskeleton parts of crustacea (e.g. pleopods of euphausiids, whole dismembered copepods etc.) and could result in a direct measure of growth.

The rate of protein synthesis can be measured *in vitro* or *in vivo*. Protein synthesis rate can be measured *in vitro*, in preparations of ribosomes from various tissues which incorporate ^{14}C amino acids such as lysine or phenylalanine (Henshaw *et al*. 1971; Rosenlund *et al*. 1984; Hansen *et al*. 1989). The rate of protein synthesis, although it may be significantly correlated with growth rate, is not a direct measure of growth itself, because the rate of protein turnover must be accounted for. This technique has been used primarily as a means of assessing fish diet under aquaculture or holding conditions.

All these *in vitro* methods potentially suffer from the same objections as raised above for conventional EA assays. It is unlikely that rates measured by any *in vitro* technique will correspond precisely to the rate *in vivo*. However, these methods deserve further evaluation, and the potential exists to calibrate them as growth indices. A common feature of several of the above methods is that they quantify some anabolic process which is cyclic, rather than continuous, in animal tissues. Within a population therefore, the mean activity of some quantity increases as the frequency of animals occupying some synthetic phase of a cycle increases.

IN VIVO UPTAKE

A variety of marine invertebrates and their planktonic larvae are known to absorb DOM (dissolved organic matter) including FAA (free amino acids) (Stephens 1981; Manahan 1990). Freshwater invertebrates and arthropods in general have rather limited capacity for DOM uptake. Even juvenile fish may take up significant quantities of FAAs. Fauconneau (1984) used this property to trace the *in vivo* rate of synthesis of proteins from external ^{14}C FAAs. The method proposed is essentially the same as that for single animal injection techniques. Although uptake of FAAs from an external source is taxonomically widespread, its penetration may be limited to the epidermal layer (Stephens 1981). Quantification of the rate of whole body protein synthesis may

therefore prove problematic. Notwithstanding such potential difficulties, this approach has potential for the development of a direct method to measure growth rates, especially in non-arthropod meroplanktonic larvae.

IN VIVO INJECTION

Techniques for measuring the rate of protein synthesis in larger metazoa (vertebrates) are now well established (see e.g. reviews by Waterlow *et al.* 1978; Waterlow 1984). A radio- (^{14}C or ^{3}H) or stable (^{15}N or ^{13}C) isotope of an amino-acid (usually leucine or phenylalanine) is introduced to the whole animal (or perfused tissue) by continuous infusion, by flooding dose or by preliminary feeding. The amount of label incorporated into proteins can then be assayed by recovery from the whole animal or specific tissues, by following the kinetics of the isotope in the blood stream, by biopsy of specific tissues, or by following the appearance of the label in excreta. Such methods successfully measure the rate of total protein synthesis in live animals. This rate however, is an imprecise correlate of growth rate, not a measure of growth rate itself, because net growth equals synthesis rate minus degradation rate. Degradation (turnover) rates of body proteins vary greatly, from minutes to years. The relationship between growth rate and rate of total protein synthesis must therefore define this turnover rate which can be expected to vary among species, among tissues and ontogenically. The turnover rate of specific proteins can however be established, e.g. as shown by Eising and Suselbeck (1991).

In mammals and fish, whole body protein synthesis rates are at least twice the rate of protein accretion (Reeds *et al.* 1985; Houlihan *et al.* 1988), and may be up to 20 times the accretion rate in specific tissues (Houlihan *et al.* 1986). The relationship between total protein synthesis rate and growth rate may therefore be weak in vertebrates (e.g. Garlick *et al.* 1973). In invertebrates, it appears that the proportion of protein synthesized which is retained as growth is much higher than in vertebrates; 90% or greater (Hawkins 1985; Houlihan *et al.* 1990). This proportion itself decreases as growth rate diminishes however, and there has not yet been a systematic examination of the relationship between rate of protein synthesis and growth rates. However, there is no doubt that such methods can lead to direct measures of growth in terms of protein.

IN VIVO INGESTION

The technique of individual infusion is clearly not practical for most planktonic animals, and crustacea at least show little propensity for uptake of DOM (Stephens 1981). This leaves acquisition via the diet as the only practical means of introducing radio-label for *in vivo* studies. Earlier attempts to interpret growth rates based on this approach (i.e. rate of incorporation of non-specific ^{14}C label from phytoplankton), led to data which were really a mix of feeding and assimilation rates. Following Conover and Francis (1973), who showed that these techniques could not lead to quantifiable production and transfer rates, the approach fell into disuse.

These problems can however be overcome by appropriate techniques. For example, Hawkins (1985), using a modification of the isotope techniques of Waterlow *et al.* (1978), has shown how the rates of protein synthesis and breakdown can be calculated from ^{15}N introduced in the diet, and how this can be used to quantify the 'efficiency of protein synthesis' (= net synthesis/overall synthesis). From this, a measure of instantaneous growth as protein could be derived. However, the 'efficiency of protein synthesis' can be expected to vary considerably in marine organisms, depending on nutritional state and seasonal and ontogenic effects, and a knowledge of this is critical to derivation of a growth rate based on measurement of total protein synthesis.

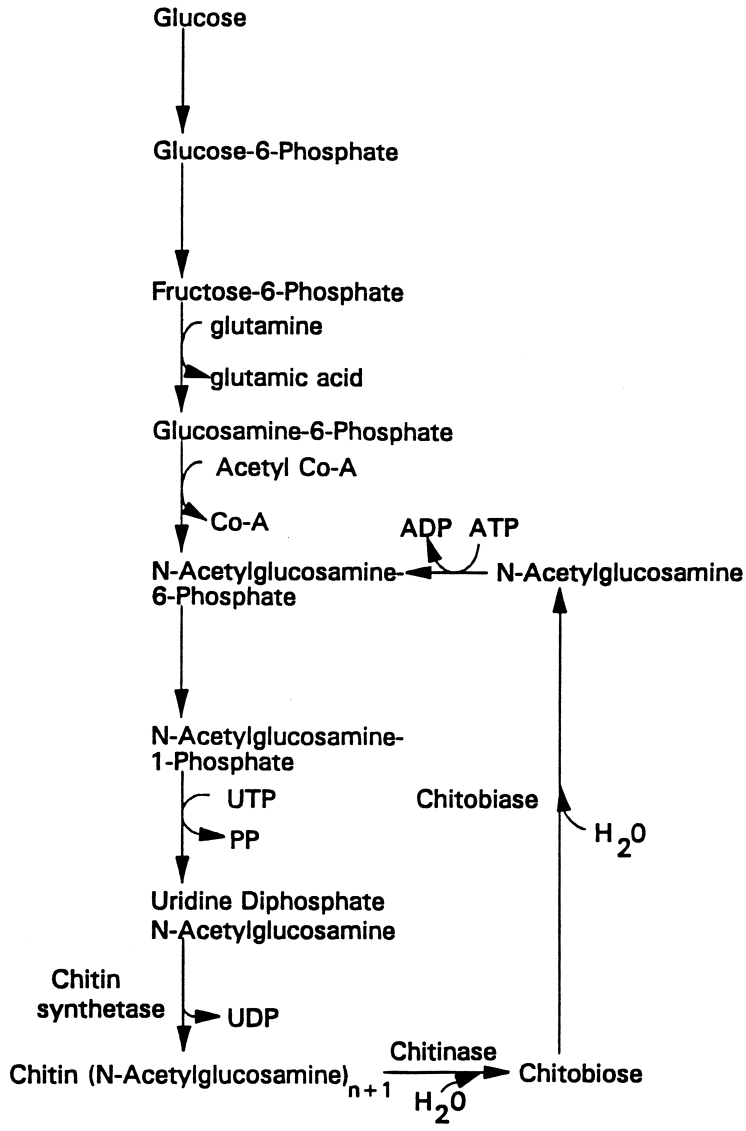

Fig. 9.10 Chitin synthesis and recovery pathways, modified from various sources (Roff *et al.* 1994, with permission).

Ideally, a growth rate based on radiochemical techniques would follow the rate of incorporation of a defined labeled precursor monomer of known specific activity, into a defined macromolecular product which is not subject to catabolism, or which has a measurable turnover rate. The net rate of synthesis of the defined macromolecule can then be converted into tissue or whole organism growth by simple proportion. This is the basis for example of the [^{3}H] thymidine technique for measuring bacterial production (e.g. Azam *et al.* 1983). There are inherent advantages in defining growth from the rate of synthesis of a specific macromolecule, rather than in terms of an array of biochemical pools which have widely varying turnover rates (see e.g. Redalje and Laws 1981).

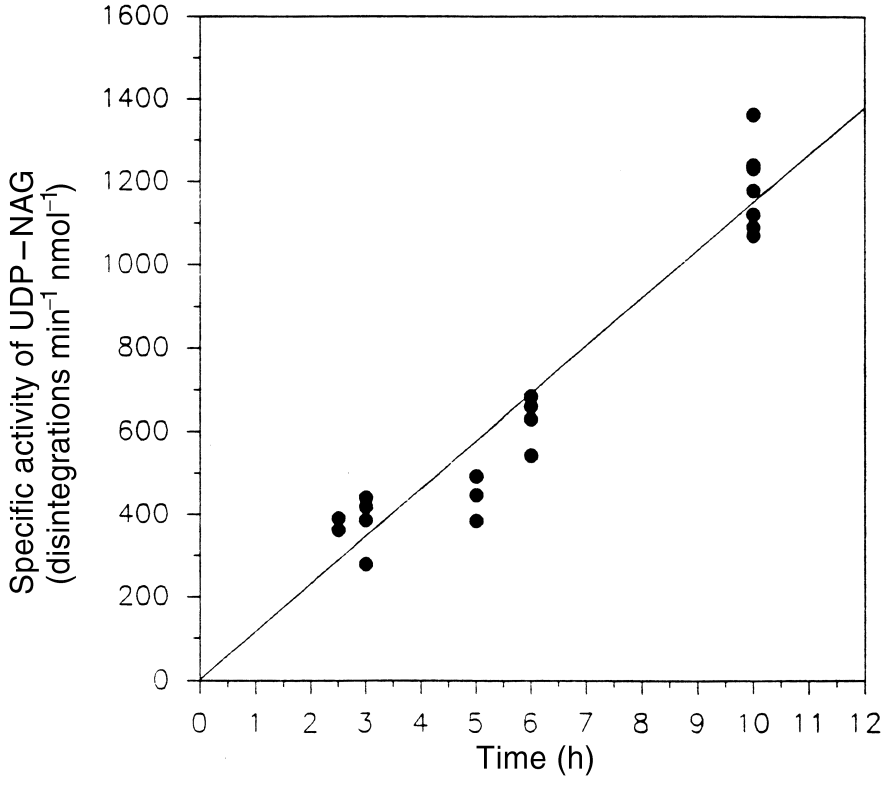

Fig. 9.11 Relationship between specific activity (dpm nmol^{-1}) of uridine diphosphate n-acetylglucosamine (UDP-NAG) of the precursor pool, and time from start of feeding in ^{14}C labeled *Chlamydomonas reinhardtii* ($n = 21$, $r^2 = 0.93$, $P < 0.01$, intercept not significantly different from zero) (Roff *et al.* 1994, with permission).

The chitin synthesis method of Roff *et al.* (1994) is an application of such concepts to planktonic crustacea. This method is based on following the rate of incorporation of [^{14}C]NAG from [^{14}C]UDP-NAG into the chitinous exoskeleton (Fig. 9.10). The specific activity of the [^{14}C]UDP-NAG precursor pool increased linearly with time after feeding on label (Fig. 9.11), as did the rate of [^{14}C]NAG incorporation into the exoskeleton. From this data, growth rates can be calculated directly in a manner analogous to those used in the [^{14}C] 'primary production' technique (Roff *et al.* 1994).

The two quantities required to calculate a rate of chitin synthesis are the average specific activity (SA) of the precursor NAG, and the total amount of [^{14}C]NAG incorporated into the exoskeleton during exposure to labeled food. By analogy to the rate of carbon fixation by primary producers, the amount of chitin synthesized can be determined from:

$$\frac{[^{12}\text{C}]\text{NAG incorporated (nmol)}}{[^{12}\text{C}]\text{NAG available (nmol)}} = \frac{[^{14}\text{C}]\text{NAG incorporated (dpm)}}{[^{14}\text{C}]\text{NAG available (dpm)}} \tag{9.20}$$

where dpm is the number of disintegrations per minute. Therefore, the amount of [^{12}C]NAG incorporated during an experiment will equal:

$$[^{14}\text{C}]\text{NAG incorporated} \times [^{12}\text{C}]\text{NAG available}/[^{14}\text{C}]\text{NAG available}$$

The expression [^{12}C]NAG available/[^{14}C]NAG available is the reciprocal of the mean SA of the precursor pool of NAG during the experiment.

A simplification of this method is now under development. This basic method could also be modified to follow the rate of synthesis of a single protein; actin of muscle tissues, which is readily separated and quantified, is an obvious choice. There is also no fundamental reason why growth as DNA should not be measured in a manner analogous to that of Roff *et al*. (1994) for chitin.

9.7 MEASUREMENT OF EGG PRODUCTION RATE OF A MARINE PLANKTONIC COPEPOD (*CALANUS FINMARCHICUS*)

9.7.1 Facilities

If measurement is made at sea, the ship must have an enclosed laboratory with a bench for microscope work and either a cold room or space and power capacity for installation of one or more incubators. Hot water (for washing) and salt water taps are desirable but not absolutely necessary, as sea water for washing and incubations may be obtained from pumps or water bottles. For coastal and fjord work, inshore vessels in the order of 20 m are feasible; in the open ocean, the larger and more stable the vessel, the greater the range of weather conditions under which reliable measurements may be obtained.

If measurements are made at a shore-based laboratory, the location should be within a short distance of the station location. Time between capture and sorting should be minimized. Consideration should be taken as to time of capture in relation to possible night spawning cycles; the closer to spawning, the greater the likelihood for release of eggs during transport. In the lower St Lawrence Estuary, *Calanus* females are routinely captured at approximately midday and sorted in a shore-based laboratory within 4 h of capture without any apparent bias.

9.7.2 Equipment and supplies

1) Net vertical ring net, 0.5–1 m diameter, 150–500 μm mesh, 4:1–3:1 ratio (finer meshes tend to minimize breakage of setae and caudal rami). The cod-end should be restricted flow (i.e. a limited number of screened holes at the top of the cod-end) with large (in the order of 8 l) volume capacity for retention of catch during tow.
2) Reception, sorting and counting materials.
   - 10–20 l clean plastic bucket or 6 to 9 4-liter glass or plastic (e.g. polycarbonate) jars for reception of catch. Clear receptacles are preferred, for visual control of catch dilution.
   - Insulated coolers and ice or icepacks for temperature regulation of catch prior to sorting.
   - Stereo microscope with optical micrometer.
   - Glass pipettes, customized tip, and 100 mm diameter petri dishes for sorting.
   - Counters for keeping track of females and egg counts.

- Basin, ice or ice packs for temperature control during sorting.
- Thermometers (preferably digital).
- Plastic petri dishes (45 ml) or spawning chambers for incubating individual females (for egg production rate) and eggs (for hatching success/viability). Examples of spawning chambers are a plexiglas cylinder with 300 μm mesh bottom immersed in a 250 ml polycarbonate beaker or egg incubation system as described in Burkart and Kleppel (1998).
- Apparatus for filtering seawater to 1 μm (e.g. bucket and submersible pump with in-line filters).
- 20 ml scintillation vials, for use in egg viability measurements and preservation of females.

3) Incubators upright temperature-controlled incubators or walk-in cold rooms are desirable, but ordinary refrigerators may also be used, as ambient temperatures where *Calanus* are distributed are typically in the range of the temperature control. Expect, however, temperature variation with refrigators. When there are multiple egg production experiments, it is preferable to have two or more incubators, in order to minimize disturbance (hence temperature fluctuation) during incubation.
4) Preservatives 4% buffered formaldehyde. An anesthetic (MS-222) may be used to facilitate post-incubation measurements of female prosome length.

9.7.3 Procedure

CAPTURE

Zooplankton are captured with a slow (< 0.5 m s^{-1}) oblique or vertical tow. The type of tow depends on the abundance of females; the object is to sample the female population in as short a time as possible (typically, the upward part of the tow lasts no longer than 10–15 min). If oblique tows are necessary, the ship may be allowed to drift if windy; otherwise, the ship should be instructed to proceed at approximately 1 knot. As a rule, the angle of the wire to water surface should never be less than 45°. For an unbiased estimate, the entire female population should be sampled, implying tow depths from near bottom to surface or, in deep waters, a depth of 200 m to the surface is considered sufficient. For estimates of water column egg production rate (i.e. eggs m^2 d^{-1}) the live tow should represent the same population sampled by the quantitative tows for female abundance. A portion of the live catch may be preserved in 4% formaldehyde, for later analysis of reproductive index.

SORTING THE CATCH

Immediately upon arrival on deck, the cod-end is removed (before rinsing the net) and its contents are gently diluted into ambient, preferably subsurface sea water. The catch should be evenly distributed among the receiving containers, i.e. pour a small amount into each container, then repeat. Use ice and thermometers (or a warm water bath in freezing temperatures) to maintain sea water at or below ambient surface-layer temperatures; temperature control is facilitated by dilution into gallon jars immersed in water or ice in coolers. With clear jars, the dilution can be easily checked: there should not be dense aggregations of plankton on the bottom of the container, as females tend to reside there. Sort immediately from the gallon jars, using a large bore pipette to transfer plankton to sorting dish and customized pipette for picking females. During this entire

process, care must be taken to maintain females at or below ambient surface layer temperature and not to expose females to high light intensity.

INCUBATION

Between 30 and 40 females are transferred to filtered sea water (see page 414 for discussion of feeding conditions during the incubation) in the egg chambers or petri dishes, one female per container. The females are held for 24 h at ambient, surface-layer (typical benchmark is 5–10 m) temperature. Studies have not revealed that light conditions influence egg laying during a 24 h incubation, although a dim light/dark cycle is recommended. If females are kept in dim light, the intensity should be less than ambient daytime intensity at 5 m. Petri dishes should be checked for eggs every 8 h, at which time eggs on the bottom of the dish are counted, recorded, and removed. Viable and non-viable (darkly opaque, deformed or lacking egg membrane) eggs should be distinguished. Egg chambers with mesh bottoms may be kept for 24 h, after which eggs are screened off and counted or preserved for later counting. In some experiments, a second 24 h incubation may be carried out. After observations are finished, females are preserved in 4% formaldehyde.

DATA ANALYSIS

The mean, daily egg production rate (epr = eggs female^{-1} d^{-1}) is expressed as the sum of all eggs released divided by the total number of females (n) incubated. The standard deviation (s) is calculated using all observations of the number of eggs in each container, including all zeros (discounting any container in which the female is missing or dead). The 95% confidence intervals about the mean are calculated by mutiplying the standard error ($s/\sqrt{n}$) by ± 1.96. The weight-specific egg production rate is calculated by multiplying epr by the egg carbon mass (μgC) and dividing by the female body mass (μgC). Mean and standard error for clutch size is obtained from the observations of egg number in dishes where a female has spawned. Note that when eggs are not checked during the incubation interval but rather are collected at the end of the 24 h period, some females may have spawned two (on rare occasions, three) clutches.

9.8 DIRECT DETERMINATION OF COPEPOD MOLTING AND GROWTH RATES IN THE FIELD

9.8.1 Facilities and equipment

- running sea water waterbath
- large volume tanks (20–300 l) for mesocosm experiments
- 150-μm mesh ring net equipped with large-volume cod-end
- 30 l Go-Flo water bottle (for smaller tanks) or diaphragm pump (for larger tanks)
- CHN analyzer

9.8.2 Supplies

- neutral density screening
- nitex screening for creating artificial cohorts (variable size: 200 μm for Calanus-sized nauplii)
- beakers

- sorting petri dishes and pipettes (for copepodites)
- buckets or jars in coolers (for receiving contents of cod-end)
- sieve (150 μm mesh, sieve diameter depends on tank size), for mesocosm sampling
- labeled tin boats for C and N analysis (preweighed if also for dry weight)

9.8.3 Procedure

'ARTIFICIAL COHORT METHOD' (used here for naupliar molting and growth rates)

Mesocosms (recommended minimum number of replicates: 4) are filled with 100 μm-prescreened water collected from 2 to 5 m (or from chlorophyl *a* max if there is one) with the diaphragm pump or Go-Flo bottles. The mesocosms are covered with two layers of neutral-density screening in order to reduce light levels by 75%, then placed inside a water bath of circulating surface sea water. To collect nauplii, a fine-mesh (70 or 150 μm mesh) net with large-volume cod-end is slowly towed (approximately 10 m min^{-1}) vertically from 50 to 100 m to the surface. The catch is immediately screened (without rinsing net) through a 200 μm mesh nitex immersed in a bucket of sea water at ambient water temperature. This creates a <200 μm size fraction (artificial cohort) of stage N3 to N6 nauplii (note that the mesh and net sizes are for *C. finmarchicus* N3 to N6; other sizes are needed for older stages or other species). The cohort is divided among the mesocosms to a density of approximately 2000 nauplii per basin. The tanks are very gently aerated to promote vertical mixing. Each mesocosm is sampled daily (duration dependent on temperature; typically 3 to 5 d but up to 21 d in Arctic waters) with the use of a small plankton sieve towed the entire vertical length of the basin. The samples are usually stored in 4% formaldehyde for later stage identification and enumeration. This procedure estimates molting rates, the inverse of yields development times, assuming constant age within stage. To measure cohort growth rates (which is more difficult and laborious because of the small size of the nauplii), 30 nauplii of each stage from the initial cohort and from each of the replicate mesocosms are measured and conserved in a dessicator in labeled, preweighed tin boats for C and N analyses.

'SORTING METHOD' (used here for copepodite molting and growth rates)

Copepodites are collected with a slow vertical tow using a 150 μm mesh net, as described above. The contents of the cod-end are diluted into clean, preferably transparent, 4 to 8 l jars filled with surface water and kept in a bath in coolers on deck to maintain ambient temperature. Sorting of copepodites is made under a dissecting microscope; no anesthetic should be used, as this may interfere with short-term timing of growth processes. All sorting is done as quickly as possible to minimize handling, and the animals are kept in beakers maintained at near-ambient temperatures with an ice water bath. Care is taken to select only copepodites in very good condition, i.e. no damage to the setae on the first antennae or caudal ramii. Damage to setae, especially on the first antennae, during collection and handling can lead to incomplete molting; while this would not affect molting rate estimates during short term incubations, it would reduce long term growth rates. To ensure accuracy of identification, copepodites are sorted first into beakers containing copepods of the same stage. From these beakers, a minimum of 30 to 40 copepodites (more if there are great size differences withing stage) are sorted again into replicate (four recommended) beakers.

The four beakers containing sorted copepodites are then randomly assigned, one for an initial sample and three for triplicate experimental treatments. They are added to

mesocosms (up to 20 l) filled with either ambient surface water that is gently reverse-filtered through a 150-μm nitex screen (to ensure that no additional copepodites are added to the tank), or with ambient water enriched with phytoplankton cultures. Enriched treatments can be used as well, but it is found that molting rate does not respond as fast to improved feeding conditions as growth; hence even if the animals are severely food limited there is little increase in molting rate in the short incubations. The tanks are protected in two layers of neutral-density screening and placed in a water bath (temperature controlled with circulating, surface sea water), normally for 48 h in temperate–subtropical environments and up to 21 d in Arctic environments. The contents of the bottles are then sieved through 150 μm nitex screen, processed for final size measurements and preserved in 4% formaldehyde for later analysis.

9.8.4 Data analysis and interpretation

MOLTING RATES

Naupliar molting rates using the artificial cohort technique can be determined from the molting rate equation given in Campbell *et al.* (submitted), a slight modification to the equation in Peterson *et al.* (1991). The nauplii in the preserved mesocosm samples are enumerated and stage-identified. To estimate the molting rate for N4, for example, the proportion of nauplii equal to or greater than N5 within a replicate mesocosm is plotted with time (i.e. day of incubation). For each replicate, the proportion equal to or greater than N5 is regressed on time; the slope of the regression line divided by the initial proportion of N4 represents the molting rate (d^{-1}). Molting rates may also be expressed as relative molting rate, calculated as the observed molting rate divided by the maximum rate predicted from laboratory experiments at the appropriate ambient temperature. This relative molting rate indicates the degree to which ambient conditions may be limiting naupliar development.

Molting rates of a given copepodite stage using the 'sorting' method are determined from the daily fraction that had molted from that stage.

GROWTH RATES

Measurements are taken for initial body size (length and C and N mass). Final size measurements (for copepodite stages, noting at the same time whether molting had occurred for the molting rate calculations) are made at the end of the incubation. Growth rates are calculated from the time change in copepod mean carbon or nitrogen mass. The enriched treatment is used to determine if development and/or growth rates were food limited and, as such, could be enhanced with the addition of the mixed phytoplankton diet.

A relative molting or growth index may be calculated as the observed rate divided by the maximum rate determined from laboratory experiments at the ambient temperatures.

9.8.5 Notes and comments

CREATION OF ARTIFICIAL COHORTS: ALTERNATIVE TECHNIQUES

Selection of net mesh sizes and screens for fractionation of the catch depends on the species or species assemblage under investigation (see references). It may be necessary to test a number of different screen combinations in order to optimize selection of the targeted size or stages. The method can also be used to estimate growth rates of

zooplankton other than copepoda, such as larvacea in tropical waters (Hopcroft and Roff 1995).

CHANGING THE WATER

In mesocosms, water should be changed periodically by removing water through reverse filtration and replacing it with fresh sea water collected with Go-Flo water bottles or the diaphragm pump. For short 2 to 3 d incubations, water change may not be necessary. For longer durations the decision to change water may be based on daily monitoring of chlorophyl *a* concentrations. To reverse filter, a larger diameter plexiglass tube with a screen on the bottom (same size as used to initially screen water) is placed into the mesocosm. Water is siphoned out through a much smaller diameter tygon tube placed within the plexiglass tube. In this way the animals are not sucked up against the screen and damaged, which may result if water is simply siphoned out directly with screened, small-diameter tubing.

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10 Metabolism

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Review

10.1 OXYGEN CONSUMPTION AS AN INDEX OF METABOLISM

'Metabolism' may be defined as the sum of all processes through which protoplasm is formed from food (anabolism) and broken down into waste matter (catabolism), with release of energy. Energy gained through the processes is stored exclusively in the form of adenosine triphosphate (ATP), and used for various activities (locomotion, production of new tissue, ion pumps etc.) of living organisms. ATP is generated from the tricarboxylic-acid (TCA) cycle and oxygen is required to drive the cycle. Therefore metabolic rate may be estimated by measuring oxygen consumption rate. Exceptions are anaerobiosis in intertidal organisms and burst escape activity, but the oxygen debt incurred is usually repaid later when oxygen is once more available (cf. Clarke 1987). 'External respiration' is the mechanism by which oxygen is brought into and carbon dioxide expelled from the organism, whereas 'internal respiration' refers to the sum of enzymatic reactions, both oxidative and nonoxidative, by which energy is made available for biological work (Prosser 1961). In most ecological studies dealing with intact organisms, 'external respiration' is of special interest and is simply referred as 'respiration'. For this reason, oxygen consumption rate is referred to as respiration (or respiratory) rate or *vice versa*. In this sense, either oxygen consumption or carbon dioxide production can be used as an index of metabolic rate of marine zooplankton. However, accurate determination of carbon dioxide in sea water is hampered by the strong buffering action of sea water and has been used in only a few studies, i.e. *Neomysis integer* by Raymont and Krishnaswamy (1968) and *Rhincalanus gigas* by Rakusa-Suszczewski *et al.* (1976). In contrast, accurate determination of dissolved oxygen in sea water became possible with the advent of the Winkler titration method in the late 1800s (Winkler 1888). In the early 1960s, oxygen-electrodes were introduced to the study of marine zooplankton metabolism as a simple tool for measuring dissolved oxygen in sea water (Teal and Halcrow 1962; Halcrow 1963).

Examples of very early studies on the oxygen consumption rates of marine zooplankton may be found in the work of Vernon (1896) on salps, followed by that of Ostenfield (1913, cited in Marshall 1973) on the copepod *Calanus hyperboreus*. Since then voluminous data have been accumulated on the metabolism of various zooplankton species living in many regions of the world's oceans (for review, see Marshall 1973; Ikeda 1974; Conover 1978; Raymont 1983). Part of the data on selected zooplankton species are shown in Table 10.1. Omori and Ikeda (1984) and Le Borgne (1986) reviewed methodologies for studies of marine zooplankton metabolism.

The metabolic rate of animals is defined with respect to the activity of animals as follows: 'standard' (or 'basal') metabolism is the oxygen consumption rate for main-

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Table 10.1 Rates of oxygen consumption, ammonia excretion and inorganic phosphate excretion of various marine zooplankton species determined by incubating specimens in filtered sea water. — = no data, indiv = individual.

Zooplankton species	Location	Experimental temperature (°C)	DW (mg)	Oxygen consumption ($\mu l\ O_2\ indiv^{-1}\ h^{-1}$)	Ammonia excretion ($\mu gN\ indiv^{-1}\ h^{-1}$)	Phosphate excretion ($\mu gP\ indiv^{-1}\ h^{-1}$)	Source
Coelenterata							
Aglantha digitale	Barents Sea	1.1	14.0	2.27	0.082	0.035	Ikeda and Skjoldal (1989)
Ctenophora							
Mnemiopsis leidyi	Narragansett Bay	20	299	30.35	2.93	1.00	Kremer (1977)
Thecosomata							
Cavolinia longirostris	GBR inshore water	27	8.9	16.7	1.81	0.30	Ikeda and Skjoldal (unpubl.)
Limacina helicina	Barents Sea	0.0	0.593	0.451	0.0072	0.0052	Ikeda and Skjoldal (1989)
Gymnosomata							
Clione limacina	Barents Sea	−0.1	16.47	1.65	0.034	0.021	Ikeda and Skjoldal (1989)
Copepoda							
Calanus finmarchicus, C6♀	Barents Sea	0.1	0.387	0.328	0.013	0.0050	Ikeda and Skjoldal (1989)
C. glacialis, C6♀	Barents Sea	3.5	0.410	0.630	0.026	0.010	Ikeda and Skjoldal (1989)
C. hyperboreus, C6♀	Barents Sea	1.3	3.95	1.49	0.049	0.0050	Ikeda and Skjoldal (1989)
Neocalanus cristatus, C5	off Hokkaido	6.3	1.59	1.67	0.164	—	Ikeda (1974)
N. plumchrus, C5	off Hokkaido	7.3	0.785	0.68	0.017	—	Ikeda (1974)
Eucalanus bungii, C6♀	off Hokkaido	6.0	1.01	0.80	0.058	—	Ikeda (1974)
Metridia pacifica, C6♀	off Hokkaido	7.7	0.182	0.383	0.019	—	Ikeda (1974)
Acartia australis	GBR inshore water	24.5	0.0091	0.056	0.0054	0.0018	Ikeda and Skjoldal (1980)
Undinula darwini	Indian Ocean	28.5	0.15	1.13	0.137	0.044	Gaudy and Boucher (1983)
Euchaeta marina	Indian Ocean	28.5	0.22	1.30	0.131	0.052	Gaudy and Boucher (1983)
Temora discaudata	Indian Ocean	28.5	0.034	0.23	0.020	0.010	Gaudy and Boucher (1983)

Mysidacea							
Acanthomysis pseudomacropsis♀	off Hokkaido	14.9	2.88	4.36	0.630	—	Ikeda (1974)
Amphipoda							
Themisto libellula	Barents Sea	−0.1	2.98	2.53	0.053	0.018	Ikeda and Skjoldal (1989)
Euphausiacea							
Thysanoessa inermis	Barents Sea	1.9	34.78	13.77	0.855	0.33	Ikeda and Skjoldal (1989)
Euphausia pacifica	off Hokkaido	10.2	12.95	16.10	0.781	—	Ikeda (1974)
E. lucens	off SW Africa	12.5	5.00	10.47	0.850	—	Stuart (1986)
Chaetognatha							
Sagitta elegans	Barents Sea	−0.3	4.50	1.41	0.345	0.028	Ikeda and Skjoldal (1989)
S. enflata	GBR inshore water	25	0.71	1.67	0.12	0.021	Ikeda and Skjoldal (unpubl.)
Appendiculata							
Oikopleura dioica	Lab culture	24	0.0032	0.209	0.0125	0.0012	Gorsky *et al.* (1987)
Thaliacea							
Salpa thompsoni, sol.	Antarctic water	−1.0	114.63	12.0	0.806	0.39	Ikeda and Mitchell (1982)
Thalia democratica, sol.	off S. Africa	17.3	2.97	1.77	0.18	—	Ikeda (1974)

tenance only, 'routine' metabolism is the oxygen consumption rate measured with uncontrolled but minimum motor activity, and 'active' metabolism is the oxygen consumption rate with enforced activity at a maximal level. When the oxygen consumption rate is measured at different activity levels, the rate extrapolated to zero activity is the standard metabolism. In most studies on zooplankton metabolism, no attempts have been made to relate the oxygen consumption data to the level of activity of animals on the premise that the measured rates are close to routine metabolism. As notable exceptions, Torres and Childress (1983) and Buskey (1998) established the relationship between oxygen consumption rates and swimming speed in the migrating euphausiid *Euphausia pacifica* and swarming copepod *Dioithona oculata* respectively. According to their results, standard metabolism is 0.7 and active metabolism is 2.7 times routine metabolism in *E. pacifica*, and respective figures are 0.5 and 3.3 times routine metabolism in *D. oculata*. As an alternative approach, net cost of swimming for several crustacean plankton has been calculated on the theoretical grounds of mechanical power dissipated as drag (cf. Morris *et al.* 1990, and references therein).

10.1.1 Conversion of oxygen consumption to carbon and calorific units

The molar ratio of carbon dioxide produced to oxygen consumed is called the 'Respiratory Quotient' (*RQ*). In theory, the *RQ* varies from 0.71 to 1.0 depending on metabolic substrates (lipid, 0.71; protein, 0.80; carbohydrate, 1.0; from Prosser 1961). According to Gnaiger's (1983) re-calculation the *RQ* value for carbohydrate remains unchanged (1.0), but for lipid, it is 0.72 and for protein it changes as a function of the excretory end-product, i.e. 0.97 for ammonia and 0.84 for urea. Since marine zooplankton are considered to be primarily ammonotelic (see Nitrogen and phosphorous metabolism in section 10.2) an *RQ* of 0.97 may be more appropriate to convert their oxygen consumption data to carbon units;

$$\mathbf{ml\ O_2\ (individual\ h)^{-1}} \times RQ \times \mathbf{12/22.4 = mg\ C\ (individual\ h)^{-1}} \quad (10.1)$$

where 12/22.4 is the weight (12 g) of carbon in 1 mole of (22.4 l) of carbon dioxide. Oxygen consumption rate expressed in units of carbon represents the carbon requirement for zooplankton metabolism, and can be used as an index of minimum food requirements when assimilation efficiency and growth are not taken into account.

The caloric equivalent to a unit volume of oxygen consumed varies depending on the metabolic substrate utilized. According to Gnaiger (1983), there are 440, 447 and 471 kilo joules (kJ) per mole of oxygen consumed for lipid, protein (ammonia as end-product) and carbohydrate, respectively. Since 1 mole of oxygen is 22.4 l and 1 calorie is equivalent to 4.1868 J, the oxycaloric equivalents can be expressed in calories as 4.69, 4.76 and 5.02 kcal per liter of oxygen consumed for lipid, protein and carbohydrate metabolism, respectively.

10.2 NITROGEN AND PHOSPHORUS METABOLISM

Animals produce various substances as end-products of metabolism. Although zooplankton excreta include both liquid and solid forms (fecal pellets) we will only consider here liquid forms. Nitrogen compounds have been measured in terms of total N, ammonia-N, amino-N and urea-N, and phosphorus compounds in terms of total-P, inorganic-P and organic-P. Among these, ammonia and inorganic phosphorus are of

special interest because of their importance as immediately available nutrients for phytoplankton (cf. Corner and Davies 1971). Excretion rates of ammonia and inorganic phosphate of selected zooplankton species are shown in Table 10.1.

A major end-product of nitrogen metabolism in aquatic animals is ammonia, in contrast to urea or uric acids in terrestrial animals (Wright 1995). Ammonia is present in two forms in sea water: unionized ammonia (NH_3) and ammonium ion (NH_4^+). The form of ammonia excreted by zooplankton is not certain (for crustaceans see Regnault 1987). The forms of ammonia in sea water may be expressed by the equilibrium equation:

$$\mathbf{NH_4^+ \leftrightarrow NH_3 + H^+} \qquad (10.2)$$

The equilibrium reaction is influenced by temperature and pH. In the usual pH range (7.5–8.2) of sea water and at 18 °C, less than 5% of ammonia is in the form of NH_3. This percentage increases as temperature or pH increases. Because of the lack of information about ammonia forms excreted by marine zooplankton and complex equilibrium of ammonia in sea water, ammonia excreted by marine zooplankton has been referred as either ammonia (NH_3) or ammonium (NH_4^+) (note that NH_3-N and NH_4^+-N are comparable to each other in terms of N). In the following no distinction is made to between the two forms of ammonia.

Ammonia is well documented as the major form of dissolved nitrogen excreted by marine zooplankton. For *Neomysis rayii* and *Euphausia pacifica*, ammonia-N accounted for 75%–85% of the total-N excreted, followed by 10%–24% as amino-N and 0%–1% as urea-N (Jawed 1969). Ikeda and Skjoldal (1989) determined excretion rates of ammonia-N, urea-N and dissolved free amino acids (DFAA)-N of five zooplankton species (*Clione limacina, Calanus glacialis, C. hyperboreus, Themisto libellula* and *Sagitta elegans*) and noted that ammonia-N was the most important (78%–93% of the total), followed by urea-N (1%–23%) and DFAA-N (6%–25%). Corner and Newell (1967) reported that 60%–100% of the total-N excreted by *Calanus helgolandicus* was ammonia and the rest was urea. Corner *et al*. (1976) confirmed this result for the same species fed barnacle nauplii; only 9%–10% of total-N excreted was urea. Ammonia is also the dominant form of nitrogen excreted by the ctenophore *Mnemiopsis leidyi* (Kremer 1977). While all these studies were made using the sealed chamber method mentioned below (Measuring metabolic rate on live zooplankton, section 10.3), Gardner and Paffenhofer (1982) determined ammonia and amino-N excretion by *Eucalanus pileatus* every 10 minutes with a flow-through system (see measuring metabolic rate on live zooplankton, section 10.3). According to Gardner and Paffenhofer's results, the amount of nitrogen excreted in the form of ammonia was far greater than that excreted as amino-N for this copepod. The time course of amino-N excretion was characterized by a discontinuous pattern. Although the mechanism of amino-acid release is not clear, it may come from food during feeding ('sloppy feeding'), from diffusion across cell membranes, from catabolism of food, or from osmoregulation (Gardner and Paffenhofer 1982; Forward and Fyhn 1983; Williams and Poulet 1986).

In contrast to studies showing ammonia as the major nitrogenous excretory product, Webb and Johannes (1967) reported abnormally high excretion of dissolved free amino-acids by mixed zooplankton. The results of Webb and Johannes were considered to be an artifact caused by an extremely high density of zooplankton in their experiments, i.e. amino-N leaking from the injured specimens and possible cannibalism during incubation, although Webb and Johannes (1969) suggested that the contradictory results of other workers were due to overlooking the bacterial uptake of amino-acids during the incubation (see page 471).

Dissolved phosphorus compounds in zooplankton excreta can be separated into inorganic and organic fractions (technically, the organic fraction is not measured directly but calculated from the difference between the total and inorganic fractions). Pomeroy *et al.* (1963) reported that 33%–55% of the total-P excreted by mixed zooplankton was inorganic. Later, Hargrave and Geen (1968) found a high percentage in the organic fraction (up to 74% of the total-P excreted) for three copepod species. According to the seasonal study of Butler *et al.* (1969, 1970), the percentage of the organic fraction in the total-P excreted by *Calanus finmarchicus* was highest (70%) in spring when phytoplankton food was most abundant, but the inorganic fraction dominated during the food-depleted winter. It should be noted that this seasonal change in the relative amounts of organic and inorganic-P was not seen for nitrogen excretion, in which ammonia comprised a constant 88% of the total-N excreted by the copepod. For *Euphausia superba* maintained with and without food for 7 months, Ikeda and Dixon (1982) found that while both organic and inorganic fractions of both nitrogen and phosphorus excreta were determined in fed specimens, only the inorganic fraction was detected for starved specimens.

As was the case for nitrogen excretion, discrepancies seen in the results of phosphorus excretion experiments by previous workers may be caused by dissimilar experimental designs including the use of individual or mixed species (see page 462). Feeding conditions of zooplankton prior to or during the experiments may be considered prime factors influencing the proportion of organic fraction in total phosphorus excretion (see page 467).

10.3 MEASURING METABOLIC RATE ON LIVE ZOOPLANKTON

Determination of metabolic rate is straightforward in theory and rather simple in practice. However, a number of experimental conditions are known to affect the results. Techniques currently in use for determining oxygen consumption and excretion rates of ammonia and phosphorus on live zooplankton can be classified largely into two types.

1) Sealed chamber method Specimens are confined in containers filled with sea water for a certain period and the decrease of oxygen or increase of excretory products during the period (several hours to a day) are monitored throughout, or determined at the end of the incubation. Despite the possible detrimental effects of oxygen depletion and accumulation of excreta, the method is simple in practice and has been used extensively. The technical problems associated largely with this method are reviewed in detail in the following section (Technical problems, section 10.3.1).
2) Flow-through method Oxygen consumption and accumulation of excreta are measured continuously on the specimens placed in a flow-through system. This method avoids some of the problems associated with method (1) but it often requires that specimens be placed in a very small cell. In addition, it requires a rigorous calculation taking into consideration flow-rate, cell size and metabolic rates of zooplankton to obtain reliable data (Northby 1976; Niimi 1978; Propp *et al.* 1982). Application of this method has been limited to the study of Gerber and Gerber (1979) for simultaneous measurements of ingestion and oxygen consumption rate of tropical lagoon zooplankton, that of Gardner and Scavia (1981) for nitrogen excretion of *Daphnia* sp., and that of Gardner and Paffenhofer (1982) for nitrogen excretion of *Eucalanus pileatus*.

10.3.1 Technical problems

In the following, various sources of potential error in measuring metabolic rates of zooplankton collected from the field are reviewed and discussed. For this purpose, only key references were selected. While most of the references concern zooplankton, benthic species and fishes are also referred to when appropriate.

CAPTURE STRESS/STARVATION

A common problem with experimental studies of zooplankton is the delay between actual capture of specimens and subsequent measurements. Such delays may range from several hours to several tens of hours; they are often unavoidable due to the logistics of field sampling and laboratory routines. Some studies have noted decreases in metabolic rates of zooplankton with the increase of time after capture, and a question naturally arises as to what causes this. The validity of measurements taken at varying times after capture may be called into question as well.

The first observation of this nature was made by Marshall *et al*. (1935), who reported a rapid fall in the oxygen consumption rate of *Calanus finmarchicus* soon after capture. Since then, similar results have confirmed this phenomenon, not only in oxygen consumption rate, but also in the excretion rates of ammonia and inorganic phosphorus of several zooplankton species (Ikeda and Skjoldal 1980; Skjoldal *et al*. 1984 and references therein). Compared with that of oxygen consumption rate, ammonia and phosphorus excretion rates in zooplankton just after capture are more variable due to the switch in metabolic substrates (e.g. from protein to lipid, cf. Skjoldal *et al*. 1984).

Stress incurred during the course of sampling and possible starvation of specimens in captivity may be considered as part of the cause of the rapid fall in metabolic rates after capture (Figure 10.1). The magnitude of both effects on metabolic rates may vary from species to species, depending on the depth of occurrence of zooplankters, the sampling gear used, weight-specific metabolic rates, temperature, etc. From this scheme, a higher oxygen consumption rate just after capture may be explained by 'oxygen debt' brought about by the extra exercise during capture; this capture stress would be greatest at the beginning of an experiment. It is difficult to provide the quantity and quality of appropriate food during experiments, thereby the effect of starvation predominates during the progress of experiments.

Capture stress has been effectively demonstrated in the oxygen consumption measurements of sockeye salmon (*Oncorhynchus nerka*) by Brett (1964). Most of the energy needed during heavy extra exercise of sockeye salmon is supplied by anaerobic processes, not through the TCA-cycle; this is demonstrated by excessive accumulation of lactic acid in the blood (Black *et al*. 1962, 1966). For zooplankton, adenylate energy charge (EC) ratio has been used as an index of capture stress for the initial decreases in metabolic rate of freshly caught specimens of the copepod *Acartia australis*, the decapod shrimp *Acetes sibogae australis* (Ikeda and Skjoldal 1980), and the copepod *Euchaeta norvegica* (Skjoldal *et al*. 1984). However, no appreciable capture stress was detected from the results of this index in both studies. In addition, lactic acid levels in *E. norvegica* just after capture were low and not indicative of any oxygen debt (Skjoldal *et al*. 1984). Thus, the results of Ikeda and Skjoldal (1980) and Skjoldal *et al*. (1984) indicate that starvation was the primary cause for the decrease in metabolic rates of these zooplankters.

A progressive decrease in metabolic rates of starved specimens for hours, days or weeks has been demonstrated in various zooplankton species (Conover 1968; Mayzaud 1976; Ikeda 1977a, 1977b; Båmstedt and Tande 1985; Kremer and Reeve 1989). The

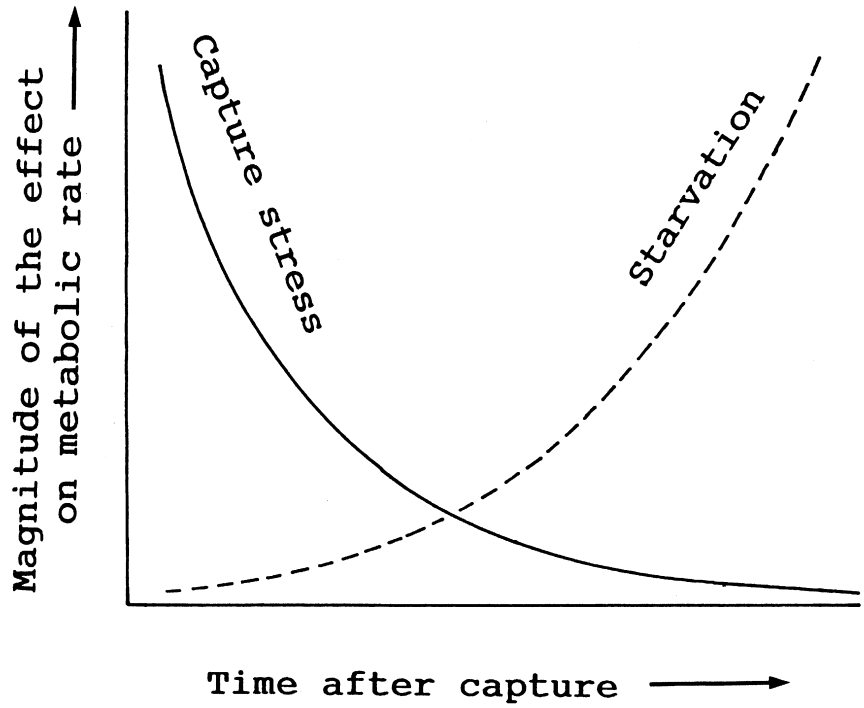

Fig. 10.1 Schematic presentation of the sequential changes of the effects of capture stress and starvation on the metabolic rate of zooplankton after capture.

effect of starvation can be minimized by the use of zooplankton immediately after capture, provided there is no capture stress. Provision of appropriate food during the time between capture and experimental manipulation may be an alternative, but prolonged maintenance of wild zooplankton in the laboratory may change its behavior, swimming activity, and nutritional condition, all of which could affect the metabolic rates.

Biggs (1977) and Cetta *et al.* (1986) enclosed gelatinous zooplankton directly in glass jars while Scuba-diving for the measurements of oxygen consumption and ammonia excretion rates. For oxygen consumption measurements Smith (1982), Youngbluth *et al.* (1988) and Bailey *et al.* (1994) adopted a gentle suction of animals into a chamber equipped with an oxygen sensor and incubated animals in *in situ* conditions on board submersibles. All these procedures are considered to be superior to that of the conventional catch–transfer–incubation method (i.e. sealed chamber method) to minimize the effects of handling and laboratory conditions. However, application of these methods is costly and it is difficult to control contamination by bacteria and microzooplankton in the incubation containers. Presently, *in situ* methods have been limited to large zooplankton which are recognizable by the unaided eye.

CONTAINER SIZE/CROWDING

The use of smaller containers or high densities of specimens may be needed to bring a measurable difference in oxygen and/or nutrient concentrations between experimental and control chambers. Marshall and Orr (1958) examined the effect of container size on the oxygen consumption rate of *Calanus finmarchicus* using various container sizes (30–

160 ml) and found no appreciable differences between the results. Zeiss (1963) compared oxygen consumption rates of *Calanus finmarchicus* and *Daphnia magna* placed under several densities of specimens. While the rates of *C. finmarchicus* were not affected in the range of 2.5 to 10 specimens ml^{-1}, the rate of *D. magna* increased at higher densities. Razouls (1972) measured the oxygen consumption rate of *Temora stylifera* and *Centropages typicus* at densities from 0.1 to 2 individuals ml^{-1}. The highest oxygen consumption rates were obtained at 0.5 individual ml^{-1} and these rates decreased at lower and higher densities in both species.

Some density effects have been reported in the excretion rates of nutrients by zooplankton. In *Acartia tonsa* the phosphorus excretion rate decreased when the density of individuals exceeded 400 specimens ml^{-1} (Hargrave and Geen 1968). In contrast, Nival *et al.* (1974) obtained anomalously high ammonia excretion rates for *Temora stylifera* incubated at densities greater than 200 specimens ml^{-1}.

Container size and crowding are analogous in terms of volume available per specimen, i.e. density, but the former is largely interaction with container wall and the latter interaction between individuals. Therefore, the effect of these parameters on metabolic rates could vary depending on species-specific differences in swimming behavior, perception of stimuli, tolerance to a rapid depletion of oxygen, and associated accumulation of excreta. For carnivorous species, cannibalism could easily be induced by the increase in density. Thus, metabolic rates of zooplankton could be either enhanced or depressed by higher densities as described above. As a general guideline for designing metabolic studies, experiments using higher densities of specimens should be avoided to obtain the best results. Information about natural density of test zooplankton species should be useful to design experimental densities of specimens for their metabolic measurements, and check the validity of the results whenever necessary.

INJURY/DEATH

Physical injury of specimens (such as broken legs or rupture of a part of the body) incurred during collection at sea or handling in the laboratory prior to their use for experiments can easily happen, but is often difficult to eliminate, especially for small zooplankton. Including injured or dead specimens during incubation are possible sources of error in determining metabolic rates of zooplankton. Mullin *et al.* (1975) found that both ammonia and phosphorus excretion of mixed zooplankton always overestimated the sum of excretion rates for healthy specimens, and considered that overestimation of the former was due to the release of nutrients by injured specimens. Ikeda *et al.* (1982) demonstrated that artificially injured copepods (*Acrocalanus gibber* and *Tortanus gracilis*) released phosphorus at a rate 6 to 9 times that of non-injured copepods (control). In the same set of experiments, artificial injury caused a twofold increase of ammonia excretion in *T. gracilis*, but no significant increase was seen in *A. gibber*. The death of a euphausiid, *Euphausia pacifica*, during incubation was observed to cause an eightfold increase in phosphorus excretion when compared to healthy specimens, but little increase in ammonia excretion was observed (Table 8.3 in Omori and Ikeda 1984).

Thus, it is clear that the effect of injured or dead specimens is most serious in determining phosphorous excretion rates, followed by ammonia excretion rates. The rapid release of phosphorus (PO_4^{-3}-P) from injured or dead specimens is considered to be due to hydrolysis of leached body fluids. While no relevant information is available at present, reduced oxygen consumption rates are expected in experiments where injured or

dead specimens are incubated together with healthy ones. In any case, if specimens die during an experiment, the data are best abandoned.

OXYGEN SATURATION

Solubility of oxygen decreases with increasing temperature and increasing salinity. Oceanic surface waters are nearly saturated. The oxygen concentration in sea water is expressed as ml per liter of sea water (1 ml $O_2\,l^{-1}$ = 1.43 mg $O_2\,l^{-1}$ = 44.6 μmol $O_2\,l^{-1}$ = 89.2 μg-at $O_2\ l^{-1}$), percent saturation against calculated saturation value from temperature and salinity, or barometric pressure (in mm of mercury, or Torr). The latter expression is commonly used in respiratory physiology and is 760 (gas pressure of 1 atmosphere) × 0.2095 (partial pressure of oxygen at STP) = 159 (mmHg, or Torr) for air-saturated sea water at 1 atmosphere.

In general, the oxygen consumption rate of marine animals is affected by the oxygen saturation of the surrounding sea water. For some animals (metabolic conformers) oxygen consumption rate is proportional to the concentration of oxygen, while for others (metabolic regulators) a constant oxygen consumption rate is observed as the oxygen concentration is reduced down to some critical level (Pc), below which the rate declines rapidly (Prosser 1961). However, intermediate types are also seen.

Marshall *et al.* (1935) observed a rapid decrease in the oxygen consumption rate of *Calanus finmarchicus* when the oxygen concentration decreased to below 3 ml $O_2\ l^{-1}$ (about 50% saturation). On the other hand, oxygen consumption rates of two mysid species (*Archaeomysis grebnitzkii* and *Neomysis awatschensis*) were not affected until the oxygen concentration decreased to 30% saturation, below which the rates decreased (Jawed 1973). An extreme capacity for respiring at low oxygen concentrations is reported for some midwater crustaceans (e.g. *Gnathophausia ingens*) from the oxygen minimum layer of the eastern Pacific (Teal and Carey 1967; Childress 1971, 1975). Ikeda (1977a) studied the relationship between oxygen consumption rate and oxygen saturation level for seven zooplankton species from Saanich Inlet, British Columbia. He observed no distinct Pc concentration for the seven species, but the effect of lowered oxygen concentration was less pronounced in *Calanus plumchrus* and *Holmesiella anomala*, collected from oxygen-deficient bottom water, than in species from oxygen-rich surface water. In comparing his oxygen consumption measurements on *Euphausia pacifica* with those of earlier workers, Paranjape (1967) found that his results were very low, although he used oxygen-saturated water, and concluded that they reflected the low oxygen concentration in the natural habitat of the *E. pacifica* used in his experiments.

For a realistic estimate of the oxygen consumption rate of zooplankton in the field, it is important to take into account not only the oxygen saturation in the experimental containers but also that in the field where the specimens were collected. In most oxygen consumption measurements with zooplankton from oxygen saturated environments, a reduction of the oxygen concentration to no less than approximately 80% saturation by the end of the incubation will safely avoid the effect of low oxygen.

TEMPERATURE

The relationship between various biological rate processes and temperature is frequently described by the Arrehenius relationship and the van't Hoff rule. The Arrehenius relationship describes the relationship between reaction rate (k) and absolute temperature (T);

$$k = A\exp(-E_a/RT) \tag{10.3}$$

where A is constant, E_a is the Arrehenius activation energy, and R is the gas constant (1987 kcal mol^{-1}). A plot of 1n k against T^{-1} is therefore linear, with a slope of $-E_a/R$. The Arrehenius plot is a standard way of estimating activation free energies in enzyme kinetics, assuming strictly that the reaction involves only a single rate-limiting step (cf. Clarke 1987). For enzymes involved in the electron-transfer-system (ETS, see ETS Activity, section 10.4.1), E_a has been estimated as 16 kcal mol^{-1} for epipelagic zooplankton and 13.2 kcal mol^{-1} for bathypelagic zooplankton (Packard *et al.* 1975). The van't Hoff rule, which is described by the Q_{10} approximation, is the most commonly used way to describe the relationship between metabolic rates and temperature in marine zooplankton;

$$Q_{10} = (k_1/k_2)^{10/(t_1-t_2)} \tag{10.4}$$

where k_1 and k_2 are the rates corresponding to temperatures t_1 and t_2 (Prosser 1961). Q_{10} may be solved graphically, plotting the logarithm of k against t. Q_{10} is 2 to 3 for most biological rate processes (Prosser 1961).

The response of rate processes to temperature is defined according to the time scale involved as 'acclimation', 'acclimatization' and 'adaptation' (Clarke 1987). 'Acclimation' is the adjustment of rate processes to a new temperature in the laboratory, and 'acclimatization' is the adjustment of the rate processes to changes in environmental temperature (diurnal, seasonal). It should be noted that in laboratory acclimation it is usual to modify only temperature, keeping all other conditions constant. In contrast, acclimatization involves adjustment to the whole range of environmental variables characteristic of the field situation. Adaptation is an evolutionary adjustment, or genetic change accomplished at the population level to a daily or seasonal variation in temperature requiring acclimatization.

From an ecological viewpoint, there is a great deal of interest in the question of whether it is possible to predict the acclimatized or adapted metabolism of animals from laboratory acclimation experiments. Halcrow (1963) measured the oxygen consumption rate of *Calanus finmarchicus* acclimated to various temperatures and found that the rates were highly variable depending on the previous thermal history, time for acclimation, and season of collection. For specimens transferred acutely to higher temperature, 'overshoot' of the rate was observed before reaching the rate at a new stable level. Seasonal fluctuations of the oxygen consumption rate–temperature curves (so called R–T curves) have also been demonstrated in several neritic copepods acclimated in the laboratory (Anraku 1964; Gaudy 1973). Thus, accurate extrapolation of oxygen consumption data (and nutrient excretion data) of zooplankton obtained at field temperatures to rates at other temperatures in different seasons and locations is difficult using acclimation experiments. Clearly, maintenance of zooplankton at dissimilar temperatures between capture and experiment should be avoided to obtain valid metabolic rates of field zooplankton. Kinne (1964) reviewed diverse patterns of metabolic response in animals subjected to rapid temperature changes.

LIGHT

A large body of data indicates that full sunlight can be lethal to zooplankton and many other marine organisms (cf. review by Segal 1970) so that extreme care should be exercised when collecting zooplankton during daylight hours. Collection of zooplankton at night is the method of choice for use in metabolic experiments whenever feasible.

While most determinations of metabolic rate have been made in the dark or under subdued light to avoid excess activity of animals, illumination may affect the oxygen

consumption rate during daylight hours. Marshall *et al.* (1935) suspended glass bottles with *Calanus finmarchicus* at various depths in the sea and found an appreciable (100% or more) increase in its oxygen consumption rates when suspended above 5 m depth. For the specimens suspended below 5 m, no effect of sunlight was detected. Conover (1956) reported an increase in oxygen consumption rates of *Acartia tonsa* exposed to a single 20W fluorescent lamp in the laboratory, but he failed to find any light effect on *A. clausi* under the same conditions. *Euphausia pacifica* placed under light ($1.2-1.6 \times 10^2$ μW cm^{-2}) and dark ($< 1.8 \times 10^{-5}$ μW cm^{-2}) did not show any difference in oxygen consumption rate (Pearcy *et al.* 1969), but *Euphausia superba* consumed 30% more oxygen under the light (650 lx) than in complete darkness (Kils 1979). Fernández (1977) conducted extensive experiments on the rates of oxygen consumption, ammonia excretion and phosphorus excretion of several copepods under various intensities of sunlight (eight graded levels between 100% and 0%). According to his results, all rates increased in proportion to the amount of light above a certain threshold, which varied in different species, depending on the illumination at the depth where they were captured.

These results suggest that metabolic rates measured in the dark may underestimate rates during daylight hours for some epipelagic zooplankton. Whether or not to take into account light conditions depends on the vertical distribution pattern of zooplankton species of interest in the field. From a technical viewpoint, a drawback inherent with experiments in natural light conditions is the difficulty of reproducing day-to-day changes in weather. To best estimate metabolic rates in light sensitive zooplankton, laboratory experiments establishing the relationship between metabolic rate and light intensity, together with a field survey on natural light regimes of zooplankton, would be a good first step.

Evaluation of the effect of sunlight on zooplankton metabolism is becoming more important than before because of the reduction in the measurable ozone layer in the past several years and the resulting increase in incident solar mid-ultraviolet (UV-B, wave length 320–400 nm) radiation which damages DNA. The potential for deleterious effects on organisms living at the surface of both land and ocean is profound. To date, experimental study on this topic in marine zooplankton has been limited to the influence of UV-B dose/dose-rate on survival, fecundity and egg hatching success in several neritic copepods (Damkaer *et al.* 1980; Karansas *et al.* 1981; Dey *et al.* 1988). Surprisingly, the eyes of deep-sea pelagic crustaceans have been demonstrated to be sensitive to very low intensities of near-UV light (Frank and Widder 1994a, 1994b).

SALINITY

In offshore water, salinity is nearly constant in most locations. Therefore, in experiments on offshore zooplankton it is not necessary to consider salinity effects. However, for metabolic studies on zooplankton inhabiting near shore waters where spatial and temporal changes in salinity prevail, salinity may be one of the variables affecting the results.

Oxygen consumption rates of the copepods *Calanus finmarchicus* (Marshall *et al.* 1935) and *Centropages hamatus* (Anraku 1964) were depressed when individuals were placed in lowered-salinity water. In contrast, oxygen consumption rate of a typical estuarine copepod, *Acartia tonsa*, was found to increase in diluted sea water (101% increase at 30% sea water, Lance 1965). Dissimilar metabolic responses to lowered-salinity sea water may be explained by species-specific differences in tolerance to the reduced salinity. *Calanus finmarchicus* and *Centropages hamatus* are stenohaline species

and the decrease in oxygen consumption rates may have been due to physiological damage caused by low salinities below their tolerance limits. On the other hand, *A. tonsa* is a euryhaline species and its increased oxygen consumption rate may reflect higher energy requirements for osmotic and ionic regulation at low salinities. Increased oxygen consumption and ammonia excretion rates in diluted sea water have been reported on the Arctic under-ice amphipod *Onisimus glacialis* (Aarset and Aunaas 1990). In *O. glacialis*, increased ammonia excretion was interpreted as being due to increased use of NH_4^+ as a counter ion for Na^+ exchange during hyposmotic stress. The effect of salinity on nitrogen metabolism in crustaceans varies depending on the osmoregulatory capabilities of species. Nonetheless, it would be fairly safe to say that ammonia excretion rate increases when animals are hyper-regulating, and conversely, decreases when they are hypo-regulating (Regnault 1987).

FEEDING

The increased oxygen consumption rate in association with feeding is commonly called 'Specific Dynamic Action' (SDA) (Prosser 1961). Kiørboe *et al.* (1985) made detailed calculations of the energy budget of a copepod, *Acartia tonsa*, and concluded that the increased metabolism associated with SDA was largely related to biosynthesis and transport. Energy cost of feeding, gut activity, amino-acid oxidation and urea excretion were minor contributors to the total SDA. In other words, the SDA represents the 'cost of growth' rather than the 'cost of feeding' for the copepod. To better extrapolate metabolic data of zooplankton obtained in the laboratory to the field population, SDA and associated nutrient excretion need to be taken into account.

Technically, GF/C or GF/F filtered sea water is commonly used for the determination of zooplankton metabolic rates to avoid production or consumption of oxygen (or nutrients) by microorganisms in the sea water used for incubations. Since little is known of the natural foods and nutritional history of zooplankton at the time of capture, use of filtered sea water provides a common basis for interspecific comparison of various zooplankton from the sea. However, metabolic rates measured in non-feeding zooplankton may underestimate rates in the field.

Oxygen consumption rates of three copepod species incubated with algae (food) increased in proportion to their ingestion rates (Gaudy 1974). A positive correlation between oxygen consumption rate and ingestion rate has also been reported on *Euphausia superba* (Ikeda and Dixon 1984). Conover and Lalli (1974) measured the rate of *Clione limacina* in the presence of the prey (*Spiratella retroversa*) and found the rate to increase 2.0 to 3.5 times during feeding. Vidal (1980), however, failed to find any significant effect of the presence of food on the oxygen consumption rate of *Calanus pacificus*.

To determine nutrient excretion rates of zooplankton feeding on phytoplankton, a correction for the simultaneous uptake of zooplankton excreta by phytoplankton must be made. The magnitude of this correction will vary depending on the concentration of phytoplankton and excreta. Figure 10.2 shows the results of excretion experiments in which *Metridia pacifica* was incubated in sea water containing various amounts of phytoplankton (*Skeletonema costatum*). Apparent excretion rates of ammonia and phosphorus tended to increase with increasing phytoplankton concentration, but both rates dropped rapidly at the maximum phytoplankton concentration. Phosphorous uptake by phytoplankton was so great that the apparent excretion rate of phosphorus became negative. To estimate the real excretion rate of *M. pacifica* during feeding, Takahashi and Ikeda (1975) corrected for phytoplankton uptake of ammonia and

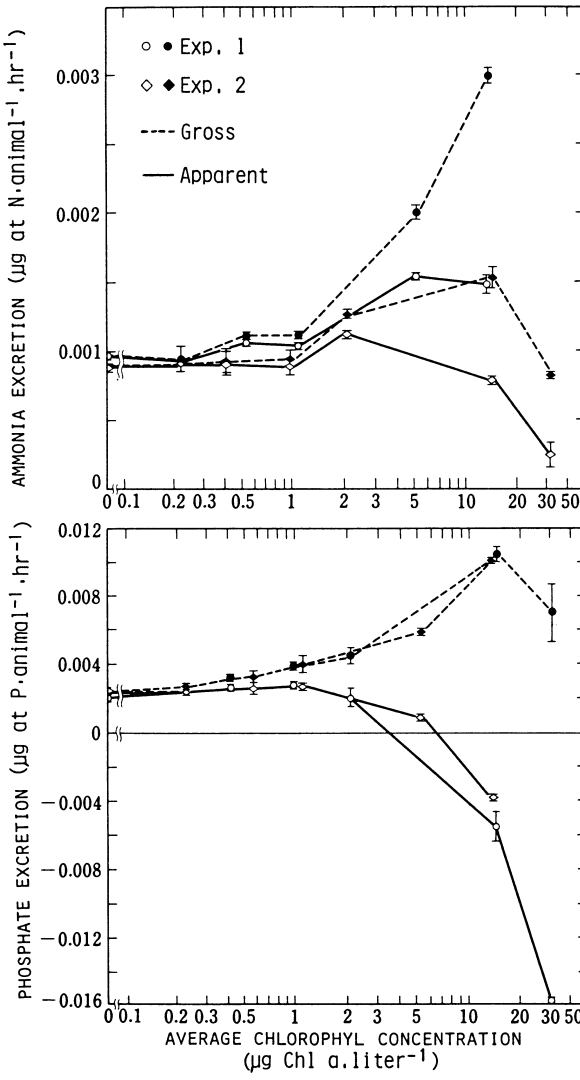

Fig. 10.2 Apparent and gross rates of ammonia and inorganic phosphorus excretion by *Metridia pacifica* incubated in bottles (900 ml) with different concentrations of phytoplankton (*Skeletonema costatum*) for 24 h at 8 °C. About 100 specimens of *M. pacifica* were used in each bottle. Concentration of phytoplankton is expressed as chlorophyl *a*. Gross excretion rates are calculated by correcting for the uptake by phytoplankton of ammonia and inorganic phosphorus during incubation (After Takahashi and Ikeda 1975, with permission).

phosphorus by applying Michaelis–Menten uptake kinetics for the phytoplankton they used. Excretion rates thus corrected are also shown in Figure 10.2 as gross excretion rates. The increase in the gross excretion rate in relation to phytoplankton concentration reflects a proportional increase of zooplankton feeding on phytoplankton. In a similar study, Lehman (1980) estimated ammonia and phosphorus excretion rates of *Daphnia pulex* feeding on *Chlamydomonas reinhardtii*. To correct for nutrient uptake by

Chlamydomonas, he added both nutrients in excess to the incubation medium of *Daphnia* so as to achieve a constant nutrient uptake by *Chlamydomonas*. Michaelis–Menten kinetics saturate at high nutrient concentrations, thus nutrient uptake by *Chlamydomonas* becomes independent of nutrient concentration. Adopting Lehman's (1980) procedure, Miller and Landry (1984) determined the ammonia excretion rate of *Calanus pacificus* feeding on *Thalassiosira weissflogii*, and concluded that the excretion rate of *C. pacificus* was independent of ingestion rate.

Caperon *et al.* (1979) measured ammonia excretion of microzooplankton in natural sea water using a ^{15}N isotope dilution technique. In this method, ammonia labeled with ^{15}N is added to natural sea water. The decrease in ^{15}N ammonia as a fraction of the total ammonia ($^{15}N + {}^{14}N$) is due to its dilution by microzooplankton excretion. This method is an alternative approach to the analysis of zooplankton–phytoplankton interactions in zooplankton excretion studies.

In an attempt to simplify the experimental design for assessing the effects of feeding on nutrient excretion by *Daphnia pulex*, Vanderploeg *et al.* (1986) used heat-killed algae which does not take up or release nutrients. However, application of this procedure requires caution, as Conover *et al.* (1988) found that heat-killed algae were less acceptable as food for the copepod *Pseudocalanus* sp., resulting in depressed rates of oxygen consumption and ammonia excretion.

Clearly, metabolic rates in zooplankton show considerable variability with feeding. Results obtained using various methods show little consistency. Considering the complex interactions between prey and predators it is conceivable that these dissimilar results may partly be due to differential experimental methods, different kinds of prey organisms, and species-specific differences in nutritional and metabolic status of predators.

DIEL RHYTHM

Biological rhythms are composites of an endogenous physiological pacemaker which generates the basic frequency, coupled to an environmental cycle which adjusts the phase of the internal clock to local time (DeCoursey 1983). 'Circadian' is the term for the endogenous rhythm repeating about every 24 h (from *circa dia* meaning 'about once a day'). From technical viewpoints, a diel rhythm in zooplankton metabolism, if it existed, would be a possible source of error in estimating daily metabolism from short-term experiments. Diel metabolic rhythmicity is a complex phenomenon that may couple feeding, swimming behavior and spawning to diel cycles of light, temperature, oxygen concentration and other environmental variables. Biological rhythms are often difficult to distinguish from purely exogenously controlled behaviors without experiments in which environmental factors are strictly controlled (cf. DeCoursey 1983).

Endogenous biological rhythms are characterized by a great 'inertia' and therefore may persist for quite a long time after an organism has been isolated from its natural environment (Mezykowski and Rakusa-Suszczewski 1979; DeCoursy 1983). On this premise, Pearcy *et al.* (1969) placed individual *Euphausia pacifica*, a species which is known as an oceanic diel vertical migrator, in Warburg flasks and monitored its oxygen consumption rate over 32 h in continuous darkness. The results showed no diel rhythm in the oxygen consumption rates of this euphausiid; this finding is further supported by the absence of a light–dark effect on the rate of this species. However, similar experiments on mixed freshwater zooplankton and *Diaptomus kenai* by Duval and Geen (1976) detected a common diel rhythm in the oxygen consumption rates in each; the rates were bimodal, with maximum values at dawn and dusk. Pavlova (1994) determined oxygen consumption rates at different times of the day on copepods that

normally perform a diel vertical migration (*Pleuromamma xiphias, Calanus euxinus*), and found significantly higher rates in those specimens collected at night. In the same study, Pavlova (1994) noted that no day–night differences were seen in copepods in which diel vertical migration was less evident (*Acartia clausi, Temora discaudata*). Checkley *et al.* (1992) investigated diel rhythmicity in ammonia excretion rates of the migratory copepods *Acartia* sp. and *Centropages furcatus*. Specimens collected at various times within a day were used in incubation experiments (this was in contrast to the use of a stock of specimens as in the experiments of Pearcy *et al.* 1969; Duval and Geen 1976), and found that the daytime rates were 2.5 to 5 times greater than the night-time rates (Figure 10.3).

Evaluation of endogenous diel rhythmicity in metabolic rates may not be necessary for the studies in which the major emphasis is daily metabolism (metabolic rate integrated over one day). In theory, if starvation is not a problem, this can be achieved by

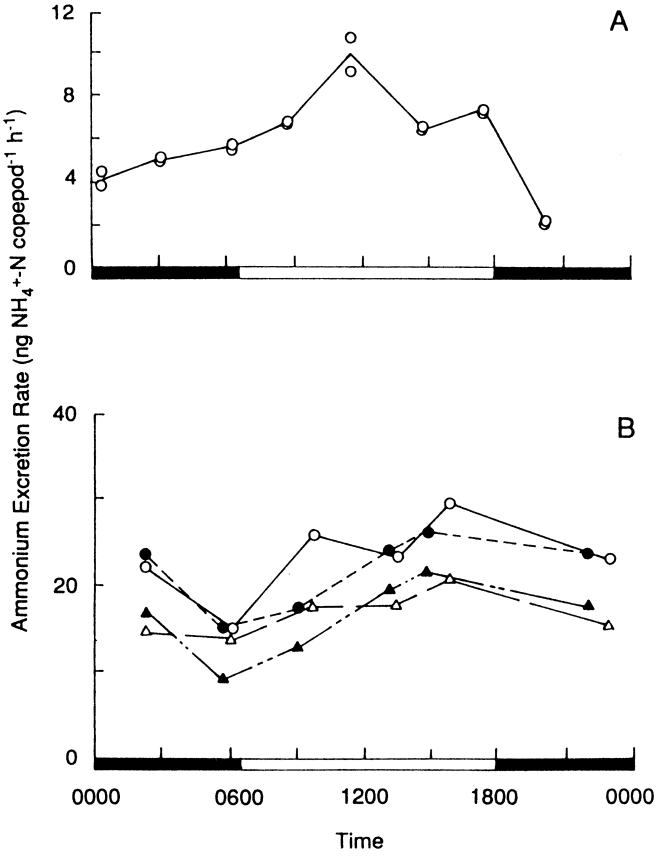

Fig. 10.3 Ammonia excretion rate of adult female copepods collected at ca. 3 h intervals for 24 h, and incubated in filtered sea water for 3–4 h under simulated *in situ* conditions of light and temperature. (A) *Acartia* sp. at 25 °C. Symbols represent duplicate ammonia analyses. (B) *Centropages furcatus* at 22–30 °C. Symbols: open circle, specimens from shallow warm; closed circle, those from deep warm; open triangle, those from shallow cold; closed triangle, those from deep cold waters (after Checkley *et al.* 1992, with permission).

incubating zooplankton for time periods of one half day or one full day. The diel cycles of some important environmental variables will then be accomplished within the laboratory experiments. The following is an example in which only cyclic changes in ambient temperature are taken into account in the estimation of daily metabolism.

The hyperiid amphipod *Themisto japonica* is a typical oceanic diel vertical migrator. During the course of its migration from the surface to 350 m depth everyday, the amphipod encounters a change in temperature from 15 °C to 1 °C in summer. Using a programmable water bath, Ikeda (1992) simulated the daily fluctuation of temperature for migrating *T. japonica*, and compared oxygen consumption rates of specimens incubated in this fluctuating temperature bath for 1 day (= 1 cycle of temperature change) with rates of those maintained in a constant temperature bath. The constant temperature bath was set to 8 °C (integrated daily mean temperature). The daily oxygen consumption of *T. japonica* placed in the fluctuating temperature bath did not differ significantly from that of *T. japonica* in the constant temperature bath. While the associated change in hydrostatic pressure encountered by *T. japonica* was ignored in the experiment, previous findings (e.g. Torres and Childress 1983) suggested that effects of pressure on metabolism over the depth range of *T. japonica* would be minimal (see page 472). Thus, daily metabolism of migrating *T. japonica* can be estimated from the constant temperature experiment in which the temperature was carefully adjusted to its daily mean. Similar results were obtained for *Euphausia pacifica* by Torres and Childress (1983). Apparently, life in fluctuating temperature serves mainly to eliminate the short term metabolic overshoots and undershoots typically seen in the metabolism of acclimated individuals when temperature is acutely raised or lowered (cf. Prosser 1961).

BACTERIA

Bacterial growth during incubation is unavoidable and may be a source of error when measuring rates of oxygen consumption and/or nutrient excretion using the sealed chamber method. Marshall and Orr (1958) used antibiotics, such as streptomycin and chloromycetin (50 mg l^{-1} of each), to minimize bacterial oxygen consumption during their experiments on oxygen consumption and feeding in *Calanus finmarchicus*. Presence or absence of antibiotics did not affect the oxygen consumption rate of the copepod, but some copepods ceased feeding after addition of antibiotics. No appreciable effect of antibiotics (streptomycin, 50 mg l^{-1}) was seen in the oxygen consumption rates of *Neocalanus plumchrus* (Ikeda 1970).

For nutrient excretion experiments, Hargrave and Geen (1968) incubated several zooplankton species with and without antibiotics (penicillin and streptomycin), to measure excretion of phosphorus (inorganic and organic). In all species the excretion rates were lower for the specimens incubated without antibiotics, thus showing bacterial uptake of phosphorus during incubation. Mayzaud (1973) observed similar results for organic nitrogen excretion by *Meganyctiphanes norvegica*, but not in *Acartia clausi* in the same experiment. Jawed (1969) maintained *Neomysis rayii* and *Euphausia pacifica* with antibiotics prior to nitrogen (ammonia, amino acids, urea and total nitrogen) excretion measurements in autoclaved sea water and bottles. The excretion rates of all forms of these treated specimens were not significantly different from the rates of untreated specimens.

Bacteria may be either a net regenerator or consumer of nutrients, depending on their growth rate and physiological state (Goldman *et al.* 1987). In this light, not only the differences in zooplankton species and in experimental designs, but also the physiological condition of bacteria involved in each experiment may contribute to inconsistent

results between studies. As in the oxygen consumption experiments above, the use of antibiotics requires caution concerning appropriate dosage. Some antibiotics interfere with the ultraviolet method for nitrogen analysis (Butler *et al*. 1969). Overall, the degree to which bacteria affect the measurement of nutrient excretion by marine zooplankton is presently unclear.

MOLTING

Molting, when it occurs during the incubation of individual crustacean zooplankton, may result in overestimates of routine metabolism. In euphausiids, molting is known to accelerate oxygen consumption rates (Paranjape 1967; Ikeda and Mitchell 1982). Bulnheim (1972) made detailed measurements of increased oxygen consumption (2.2–3.9 times the rate at non-molting) during the course of molting in benthic gammarid amphipods. Not only oxygen consumption, but also phosphorus excretion rate in euphausiids (Ikeda and Mitchell 1982), and ammonia excretion rate in decapod crustaceans (Regnault 1987) has been observed to increase when specimens molt during incubation.

HYDROSTATIC PRESSURE

Hydrostatic pressure increases progressively with increasing depth (1 atmosphere or 101.3 kPa per 10 m depth). Early interest in the effect of hydrostatic pressure on zooplankton metabolism mainly concerned diel vertical migrators, particularly residents of the deep scattering layer that experience large pressure changes ($\geq$20 atm) twice daily as a result of their vertical excursions. Oxygen consumption rates of diel vertical migrators such as euphausiids (Teal and Carey 1967; Pearcy and Small 1968), decapods (Teal 1971), and thecosomatous pteropods (Smith and Teal 1973) determined under various combinations of temperature and hydrostatic pressures in the laboratory yielded a consistent result that the rates were affected by temperature but not by pressure. The lack of appreciable hydrostatic pressure effects on oxygen consumption rates has recently been confirmed on bathypelagic chaetognaths, hydromedusae and a polychaete by comparing rates at 1 atm and at 100 atm in the laboratory (Childress and Thuesen 1993). All these results suggest that estimation of oxygen consumption rates of zooplankton living at depth is experimentally feasible at 1 atm, provided that live-specimens are recovered successfully to the surface without damage. In this regard, Bailey *et al*. (1994) noted a loss in motor activity of delicate mesopelagic gelatinous zooplankton due to decompression, which was reflected in reduced oxygen consumption rates.

It is well documented that the metabolism of pelagic crustaceans, fishes and cephalopods declines with the increase of depth of occurrence (Childress 1975; Quetin *et al*. 1980; Ikeda 1988; Torres *et al*. 1979, 1994; Childress 1995). This depth-related decline of crustacean and fish metabolism is not due to the increase of hydrostatic pressure as mentioned above, nor to the decrease of temperature; it is an adapted characteristic of many species of deeper-living fauna (Childress *et al*. 1980; Childress and Mickel 1985; Ikeda 1988). A comparison of oxygen consumption rates and intermediary metabolic enzyme activities in chaetognaths living at various depths by Thuesen and Childress (1993a, 1994), revealed that a depth-related decline in metabolism was not the case for chaetognaths and medusae. Thuesen and Childress (1993a, 1994) and Childress (1995) postulate that a depth-related change of metabolism would be selected for in visual predators that require a well-developed musculature to chase down prey that can be visually targeted in well-lit surface waters, but not in visual predators in the light-

limited deep-sea. The limited distances for visual detection afforded by the low light levels at depths inhabited by deeper living species has resulted in a loss of locomotory musculature and a concomitant decline in metabolic rates. The same argument does not apply to non-visual predators such as chaetognaths whose methods of prey detection are not appreciably altered with depth. In light of the diversity of pelagic fauna in the deep-sea, more data are needed to prove or disprove the present hypothesis for reduced metabolic rates of deep-living zooplankton.

TURBULENCE

In the sealed chamber method for measuring metabolic rate, zooplankton are typically confined in standing (turbulence-free) water during the experiment. In nature this is not the case, and zooplankton living in the shallow layers of the sea are more or less under the influence of small-scale turbulence at all times. Small-scale turbulence can affect zooplankton metabolism in two ways: (1) by increasing the encounter probability between zooplankton and food particles, and (2) by increasing the frequency of escape reaction of zooplankton, a metabolically expensive swimming behavior (Saiz and Alcaraz 1992).

Saiz and Alcaraz (1992) placed *Acartia* sp. in 25- to 50-ml screw-cap polyethylene vials three-quarters filled with GF/F filtered, air-saturated sea water, and the vials were placed on a reciprocal shaker (90–100 strokes min^{-1}, 2.5 cm amplitude) for 24 h to determine their ammonia and phosphorus excretion rates. As a control, *Acartia* sp. was placed in standing vials. The excretion rates of *Acartia* sp. placed on the shaker were 1.6 times higher on average than the rates of controls. Saiz and Alcaraz (1992) noted that the intensity of turbulence generated by shaking in their experiments was not quantified, but was probably higher than that found in the field, at least in oceanic waters. Clearly, oxygen consumption is superior to nutrient excretion for evaluating the effects of small-scale turbulence on zooplankton metabolism. In a later study, Alcaraz *et al.* (1994) determined heartbeat rates, instead of oxygen consumption rates, of several zooplankton species (both marine and freshwater) under quantified turbulence (ca. 5 $mm^2\ s^{-3}$) and observed increases ranging from 5% to 93%. However, a poor correlation between heartbeat and oxygen consumption rates was reported for *Euphausia superba* and *Parathemisto gaudichaudi* by Opalinski (1979). More investigation is needed to evaluate and generalize the effect of small-scale turbulence on zooplankton metabolism. Clearly, development of new experimental techniques and quantitative evaluation of turbulence levels encountered by zooplankton in the field will help to resolve the interactions between turbulence and metabolism in zooplankton.

10.3.2 Body size and temperature as bases of metabolic comparison

Metabolic rate (M) of animals is known to vary as a function of body mass (W) intraspecifically and interspecifically (Zeuthen 1947, 1953; Hemmingsen 1960). The relationship is expressed as

$$M = aW^b, \tag{10.5}$$

where a and b are constants. According to Zeuthen's (1953) recapitulation theory the mass exponent b varies successively with the increase of body mass, from 0.75 to 1.0, then to 0.75, where the middle 1.0 is typical in very small metazoans phylogenetically or mid-development stages ontogenetically. However, Banse (1982) re-analyzed published data of metabolism–body mass relationships of very small invertebrates, concluding that the b

for this group of animals was near 0.75 instead of 1.0. Thus, Zeuthen's theory appears not to be warranted any more (Banse 1982). For marine planktonic metazoans of which body mass ranges five to six orders of magnitude at most, the mass exponent b is characterized as less than 1 and usually in the range of 0.7 to 0.9. Within narrow body mass ranges the relationship between metabolic rate and body mass is often masked by the scatter of the data, i.e. the confidence interval of b is too wide to judge $b < 1$. From a statistical viewpoint, a geometric (GM) regression model, rather than arithmetric (AM) regression model (= the least-square regression), is appropriate for calculating b (Ricker 1973; Laws and Archie 1981). A body mass exponent calculated from AM regression (b) and that (d, i.e. $M = cW^d$, where c and d are newly designated constants) from GM regression are the same when the correlation coefficient (r) is 1.00, but the former is greater than the latter when $r < 1.00$, and $r < 1.00$ is usually the case (i.e. $d = b/r$ cf. Ricker 1973).

For metabolic comparison between species with dissimilar body masses, a common constant b needs to be established or assumed (or else the conclusion will vary depending on the choice of body mass). The results of metabolic comparison are also affected by the expression of body mass when the body composition of the species to be compared is different. Ikeda and Mitchell (1982) compared oxygen consumption rates of *Euphausia superba* and *Salpa thompsoni* on the basis of equivalent body mass and noted that the rate of the former was greater than the latter by a factor of 17 on a wet weight, 4 on a dry weight, and 0.8 on a carbon or nitrogen basis. Carbon or nitrogen units appear to be superior to wet and dry weight to reduce the phylogenetic differences in body composition for the purpose of interspecific metabolic comparison. Schneider (1990) reached the same conclusion from the body carbon based comparison of ammonia excretion rates between gelatinous and nongelatinous zooplankton. Ikeda (1988) proposed 'Adjusted Metabolic Rate' (AMR) for metabolic comparison between mesopelagic and epipelagic zooplankton. AMR is defined as M divided by (body nitrogen)$^{-b}$. The mass exponent b used in the comparison of Ikeda (1988) was 0.8505 for oxygen consumption rate, 0.8361 for ammonia excretion rate, and 0.8704 for phosphorus excretion rate, all of which are derived from statistical analysis of comprehensive data sets describing epipelagic zooplankton metabolism collected from several regions of the world ocean (cf. Table 10.2).

The relationship between metabolic rates (M) and temperature (T°C) is frequently described by Q_{10} (see Temperature, section 10.3.1). When the Q_{10} is constant over a given temperature range, the relationship is re-expressed as $M = \alpha\beta^T$, where α and β are constants. Since it is known already that temperature effects are difficult to analyze using laboratory acclimation experiments (see Temperature, section 10.3.1), a comparison of metabolic rates of zooplankton living in dissimilar thermal regimes is most appropriate for drawing generalized conclusions regarding temperature effects. Intraspecific comparisons would be ideal, but the data presently available are not sufficient for this analysis. Interspecific comparison is the only alternative.

Ikeda (1985) compiled oxygen consumption data of 143 zooplankton species, ammonia excretion data on 131 species, and phosphorus excretion data on 52 species (general size range: 10^{-3} to 10^3 mg dry weight) from tropical, subtropical, temperate, subarctic and Antarctic waters (temperature range: -1.4 to 30 °C) and analyzed the data as a function of body mass and habitat temperature, assuming a constant mass exponent b in metabolism–body mass relationship and a constant Q_{10} over the temperature ranges investigated. His results revealed that the 94% to 95% of the variation in oxygen consumption rates could be ascribed to habitat temperatures and body sizes of zooplankters (Table 10.2). Excretion rates of ammonia and inorganic phosphorus were

Table 10.2 Regression statistics of metabolic rates (Y = oxygen consumption, ammonia excretion, or inorganic phosphorus excretion rates) on body mass (X_1 = dry (DW), carbon (CW), nitrogen (NW), or phosphorus (PW) weight) and habitat temperature (X_2) for marine zooplankton. Q_{10} calculated from a_2 are shown on the right-hand side of the table ($Q_{10} = \exp(10 \times a_2)$) (modified from Ikeda 1985).

			$\ln Y = a_0 \ln X_1 + a_2 X_2$				
Metabolic rate	**Body mass unit**	**N**	a_0	a_1	a_2	R^2	Q_{10}
Oxygen uptake	DW	721	−0.2512	0.7886	0.0490	93.9	1.632
	CW	721	0.5254	0.8354	0.0601	95.5	1.824
	NW	721	1.7412	0.8505	0.0636	95.1	1.889
	PW	721	3.7890	0.8167	0.0552	94.0	1.737
Ammonia excretion	DW	1 186	−2.8900	0.7616	0.0511	85.4	1.667
	CW	1 186	−2.1763	0.8293	0.0648	86.5	1.912
	NW	1 186	−0.9657	0.8361	0.0656	86.2	1.927
	PW	1 186	1.0708	0.8063	0.0562	84.5	1.754
Phosphate excretion	DW	749	−4.3489	0.7983	0.0285	86.4	1.330
	CW	749	−3.6031	0.8622	0.0438	86.5	1.550
	NW	749	−2.3490	0.8704	0.0441	86.9	1.554
	PW	749	−0.1839	0.8569	0.0376	84.2	1.443

more variable; 84% to 87% of their variability was explained by changing temperature and body mass. Because of the wide variability in the characteristics of the studied zooplankton species, Q_{10} varied as a function of the body mass unit chosen for comparison. General ranges were 1.63 to 1.89 for oxygen consumption rate, 1.67 to 1.93 for ammonia excretion rate, and 1.33 to 1.55 for phosphorus excretion rate. The combination of body mass-specific Q_{10} with AMR can allow standardization of metabolic rates in terms of body mass and temperature as bases for metabolic comparison of zooplankton.

The data analyzed in Table 10.2 do not include metabolic data of Arctic zooplankton. Because of the interest in historical 'metabolic cold adaptation', oxygen consumption rates of Arctic species are of special interest, i.e. despite a similar sub-zero habitat temperature the rates of Arctic zooplankters are expected to be lower than the rates of Antarctic zooplankters because the Arctic ecosystem is younger. For a detailed historical account of this hypothesis see Holeton (1974) and Clarke (1983). An intra-generic comparison of oxygen consumption rates in selected zooplankton species from the Arctic and Antarctic revealed no significant difference between the two (Ikeda 1989a). Thus, the regression equation in Table 10.2 can be used as the basis for metabolic comparison of zooplankton living over world oceans.

As an obvious divergence from the regression equation in Table 10.2, extremely reduced metabolic rates have been reported for overwintering copepods in 'diapause'; i.e. *Calanus pacificus californicus* in the Santa Barbara Basin (Alldrege *et al*. 1984), *Calanus finmarchicus* and *C. helgolandicus* in Norwegian fjords (Hirche 1983), *Calanoides acutus* in the Weddel Sea (Drits *et al*. 1994), *Neocalanus cristatus, N. plumchrus* and *Pseudocalanus minutus* in the Japan Sea (Ikeda and Hirakawa 1998). These copepods are primarily herbivores living in cold water, and their diapause stages (mostly late copepodite stages) are characterized by their occurrence from the mesopelagic zone, motionless in behavior and a large accumulation of lipids (stored energy) in the body. For a recent account of dormancy (including diapause) of planktonic copepods, see the review of Williams-Howze (1997).

10.3.3 Metabolic quotients

Simultaneous measurements of oxygen consumption, ammonia excretion and inorganic phosphorus excretion allow the ratios of oxygen consumption to ammonia excretion (O:N), ammonia excretion to inorganic phosphorus excretion (N:P), and oxygen consumption to inorganic phosphorus excretion (O:P) to be compared on an atomic basis. These ratios, called metabolic quotients, change depending on the metabolic substrates of the animal. Nitrogen and phosphorus contents of animal protein, lipid, and carbohydrate, together with the amount of oxygen required for combustion of each class of biomolecule are summarized in Table 10.3. The O:N, N:P and O:P ratios for each class of organic matter are also calculated. It must be noted that these ratios will change slightly, because of diversity within a class of biomolecule, particularly in the make-up of protein and lipid, and no precise information is available for zooplankton material. Even so, consistent variations are observed in these ratios depending on whether the metabolic substrate is protein, carbohydrate or lipid. It is apparent from Table 10.3 that a carbohydrate-dominated metabolism causes high O:N and O:P ratios. Low N:P and O:N ratios are characteristic of lipid- and protein-oriented metabolisms respectively.

Raymont and Krishnaswamy (1960) and Raymont and Conover (1961) observed little

Table 10.3 Average nitrogen and phosphorus composition of organic matter, the oxygen required to oxidize each class of organic matter in an animal body, and calculated metabolic quotients (O:N, N:P and O:P, by atoms) (modified from Ikeda 1974).

	Carbohydrate	Lipid	Protein
N (g/g)[a]	0	0.0061	0.178
P (g/g)[a]	0	0.0213	0.007
O (l/g)[b]	0.83	2.01	1.02
O:N	∞	412	7.2
N:P	—	0.63	56
O:P	∞	261	403

[a] *From Rogers (1927)*
[b] *From Gnaiger (1983)*

change in the carbohydrate content of starved zooplankton. In conjunction with these observations, the carbohydrate content of zooplankton was found to be only a few percent of the dry weight (5% at most). Even if all the carbohydrate in a zooplankter was metabolized, it would not be large enough to support the animal's metabolic requirement for one day. Therefore, protein and lipid are considered the major metabolic substrates of starved zooplankton. According to Conover and Corner (1968), the O:N ratio of some boreal zooplankton is high at the end of summer but declines throughout winter, with a corresponding decrease in lipid. During the spring phytoplankton bloom, zooplankton feed actively on phytoplankton and store lipid, but the O:N ratio remains low during this period. The O:N ratio increases only after the zooplankton have deposited a large amount of lipid, indicating a close association of the O:N ratio to the lipid content of zooplankton.

Assuming that the respective nitrogen content of protein and lipid is 16% and 0%, and the oxygen required for complete combustion per 1 g for protein and lipid is 1.04 and 2.02 l, Ikeda (1974) calculated an O:N ratio of 24 when protein and lipid are metabolized in equal quantities at the same time; hence an O:N ratio less than 24 indicates protein-oriented metabolism and a ratio greater than 24 indicates lipid-oriented metabolism (from the data in Table 10.3 this is re-calculated to be 20.6). O:N ratios were used to compare the metabolic substrates of zooplankton inhabiting a large geographical area extending from tropical through subarctic waters. As a result, he concluded that zooplankton living in tropical, subtropical and temperate waters were characterized by protein metabolism, while those living in subarctic waters showed a wide range in the O:N ratio. In addition to season (Conover and Corner 1968) and specific differences (Ikeda 1974; Ikeda and Mitchell 1982; Gaudy and Boucher 1983; Quetin *et al*. 1980; Ikeda and Skjoldal 1989), O:N ratios are known to change as a result of starvation (Mayzaud 1976; Ikeda 1977b), recent feeding conditions of animals (Ikeda and Dixon 1984), and acclimated temperature (Mayzaud and Conover 1988). Extremely low O:N ratios (lower than the theoretical minimum of 7.2, see Table 10.3) seen in the results of some workers in Table 10.4 may be caused by artificially high excretion rates of animals, the result of severe stress either in the field or in the laboratory (Mayzaud and Conover 1988). Mayzaud and Conover (1988) reviewed the O:N ratio in marine zooplankton and noted that while the O:N ratio reflected changes in the biochemical composition of the body for zooplankton with large energy reserves (lipid), the ratio for

Table 10.4 The O:N, N:P and O:P atomic ratios from the measurements of oxygen consumption, ammonia excretion and phosphorus excretion rates being reported by previous workers.[a]

Zooplankton	O:N	N:P	O:P	References
Mixed zooplankton (UFW)	7.7	7.0	54.0	Harris (1959)
Mixed zooplankton (UFW)	41.0	9.98	222.0	Martin (1968)
Calanus cristatus (UFW)	5.7	19.0	110.0	Taguchi and Ishii (1972)
Calanus plumchrus (UFW)	6.8	13.0	89.0	Taguchi and Ishii (1972)
Mixed zooplankton (UFW)	13.48	10.33	142.4	Le Borgne (1973)
Sagitta hispida (FW)	—	11.3	—	Beers (1964)
Mixed zooplankton (UFW)	—	—	72.0	Satomi and Pomeroy (1965)
Calanus helgolandicus (FW)	9.8–15.6[b]	—	—	Corner *et al.* (1965)
Boreal zooplankton (10 species) (UFW)	6–200[b]	—	—	Conover and Corner (1968)
Calanus finmarchicus (FW)	—	10.8	—	Butler *et al.* (1969)
Calanus finmarchicus (FW)	—	11.0[c]	—	Butler *et al.* (1970)
Sagitta hispida (FW)	6.8	—	—	Reeve (1970)
Calanus helgolandicus (FW)	—	16.5[c]	—	Corner *et al.* (1972)
Temora stylifera (FW)	7–15[d]	—	—	Nival *et al.* (1974)
Mediterranean zooplankton (4 species) (FW)	1.6–12.1	—	—	Mayzaud (1973)
Boreal, temperate, subtropical and tropical zooplankton (81 species) (FW)	4–115	—	—	Ikeda (1974)
Mixed zooplankton (UFW)	—	6.8	—	Mullin *et al.* (1975)
Mesopelagic crustaceans (14 species) (FW)	9.1–91.0	—	—	Quetin *et al.* (1980)
Antarctic zooplankton (14 species) (FW)	7.0–19.8	2.5–24.7	43–304	Ikeda and Mitchell (1982)
Tropical zooplankton (27 species) (FW)	2.7–28.6	2.3–44.5	19.6–410	Gaudy and Boucher (1983)
Calanus glacialis (FW)	3.0–9.9	7.7–12.9	38.7–76.5	Båmstedt and Tande (1985)
Tropical ctenophores (4 species) (FW)	10.2–15.8	—	—	Kremer *et al.* (1986)
Salps (10 species) (UFW, FW)	13–28	—	—	Cetta *et al.* (1986)
Antarctic mesopelagic zooplankton (7 species) (FW)	14.1–73.9	1.8–15.9	67–290	Ikeda (1988)
Arctic zooplankton (10 species) (FW)	7.0–19.8	2.5–24.7	43–304	Ikeda and Skjoldal (1989)

[a] Use of unfiltered (UFW) and filtered (FW) sea water for measurement is noted. — = no data. [b] Ninhydrin N. [c] Total N/total P. [d] Taken from Nival et al. 1974, Figure 10.

small neritic zooplankton with few energy reserves reflected the quality and quantity of food in the environment.

The N:P and O:P ratios are not as sensitive as the O:N ratio for assessing metabolic substrates of zooplankton, though in earlier studies interest centered around the constancy of N:P and O:P values. Satomi and Pomeroy (1965) compared the O:P ratio of mixed zooplankton from three different sites and obtained quite similar values (63–75, with a mean of 72). From a seasonal study of total nitrogen and total phosphorus excretion by *Calanus finmarchicus*, Butler *et al*. (1970) found the atomic ratio of N:P was stable through spring (11.0) and winter (14.6). If the N:P and O:P ratios are constant, estimation of oxygen consumption, nitrogen excretion and phosphorus excretion rates can be made by measuring only one of these variables. However, subsequent studies by other workers revealed that there is a large variation in these ratios (Table 10.4). The N:P and O:P ratios are now known to change not only between species (Ikeda and Mitchell 1982; Gaudy and Boucher 1983; Ikeda and Skjoldal 1989) but also within the same species, depending on temperature (Le Borne 1982), time for incubation (Le Borne 1979; Ikeda and Skjoldal 1980; Båmstedt and Tande 1985), quality and quantity of food given (Ikeda and Dixon 1984), and prolonged starvation of animals over weeks (Ikeda 1977b). Since an abnormally high phosphorus release has been observed in damaged zooplankton (see Injury/death in section 10.3.1), extremely low N:P and O:P ratios may be used as indicators of damage in experimental animals.

Redfield *et al*. (1963) proposed the average C:N:P composition of marine phytoplankton and zooplankton as 106:16:1 by atoms. Richards (1965) constructed a model of the organic matter composition of phytoplankton and zooplankton based on Redfield's ratio and a sequence of decomposition:

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 106O_2 = 106CO_2 + 16NH_3 + H_3PO_4 + 106H_2O \quad (10.6)$$

$$16NH_3 + 32O_2 = 16HNO_3 + 16H_2O. \quad (10.7)$$

Hence,

$$(CH_2O)_{106}(NH_3)_{16}H_3PO_4 + 138O_2 = 106CO_2 + 122H_2O + 16HNO_3 + H_3PO_4. \quad (10.8)$$

From this scheme, the O:N, N:P, and O:P ratios are predicted to be 17, 16, and 276, respectively. When biological oxidation by zooplankton is considered, the nitrogenous end product is not NHO_3 but NH_3. Thus, the appropriate ratios would be 13, 16, and 212. Application of these ratios to individual zooplankton species is cautioned, since a large departure of the C:N:P ratio from that of Redfield has been noted (cf. Corner and Davies 1971).

10.4 METABOLIC RATE AND ENZYMATIC INDICES

The search for physiological indices to characterize field-caught specimens has been slowly gaining momentum in biological oceanography for the last 30 years. Physiological indices may be thought of as biochemical proxies for a physiological rate that is either difficult to measure or where time constraints make sufficient data collection difficult in the time frame of a single cruise. The development of reliable enzymatic indices will help resolve the large hysteresis between the data gathering efforts of physical and chemical

oceanographers and zooplankton biologists. In a modern oceanographic survey the CTD-rosette and automated nutrient analyzers allow physicists and chemists to complete much of their data-gathering and often some analysis before leaving the ship. Similarly, phytoplankton biologists are now able to map chlorophyl biomass in real-time. In contrast, zooplankton biologists often leave the ship with a wagon-load of samples that take months to analyze and little if any information on the physiological well-being of the zooplankton they have collected, even in a relative sense throughout the survey area. New methods such as optical plankton counters (see Chapter 7) may help to resolve the time-lag in analysis of distribution and abundance of zooplankton. Likewise, enzymatic methods may help to describe physiological status of zooplankton more quickly than traditional incubation methods, and further, will work on frozen specimens. As is the case with optical plankton counters, enzymatic methods will also result in some loss of resolution. It will become obvious as the field develops whether the loss in resolution is acceptable.

Biochemical indices are best grouped by the physiological rate that they are acting as a proxy for. Four physiological rates have been addressed by investigators using biochemical proxies: ingestion rate, growth rate, oxygen consumption rate, and excretion rate. Ingestion and growth rates are covered elsewhere (Chapters 8 and 9, respectively) leaving us to consider proxies for respiration and excretion in this chapter. Biochemical indicators of respiration in zooplankton include succinate dehydrogenase, first described by Curl and Sandberg (1961), electron transfer system (ETS) activity, developed by Packard (1969), lactate dehydrogenase (LDH) and pyruvate kinase (PK) activity (Berges *et al.* 1990, Berges and Ballantyne 1991), citrate synthase (CS) activity (Berges *et al.* 1990, 1991; Clarke *et al.* 1992; Clarke and Walsh 1993) and, malate dehydrogenase (MDH) activity (Thuesen and Childress 1993a, 1993b, 1994). By far, ETS activity is the most widely employed index of respiration in oceanography today, although the enzymes of intermediary metabolism (LDH, CS, PK, MDH) show considerable promise as physiological indices as well. Ammonia excretion can be estimated using the activity of glutamate dehydrogenase (GDH, Bidigare and King 1981) a key enzyme in the transamination reactions of the cell. Mayzaud (1986) provides a nice review of enzyme methods as proxies for metabolism up to the time of the article's publication.

Successful application of enzymatic methods to estimate metabolic rates of zooplankton relies on a constant relationship between the two. Packard (1985) calculated a theoretical value of 0.5 for the ratio of respiration rate to ETS activity (R/ETS), assuming that Michaelis–Menten kinetics could be applied to respiratory chemistry and that the concentration of the respiratory regulator (ADP) was maintained near the Michaelis constant (K_m). The R/ETS ratios determined on size-fractionated mixed zooplankton from diverse oceanic systems at various seasons of the year by Hernández-León and Gómez (1996) are shown in Figure 10.4. It is clear that the ratio varies from near 0 to 2.8 with its mode around 0.5. The R/ETS ratios of marine zooplankton are known to be independent of temperature and little affected by body size (King and Packard 1975).

Much less work has been done on the ratio of GDH to ammonia excretion (GDH/NH_4). Bidigare and King (1981) showed a close relationship between GDH activities and ammonia excretion rates ($r = 0.92$, $n = 7$), which was confirmed by Park (1986a) ($r = 0.94$, $n = 5$) and Park *et al.* (1986) ($r = 0.98$, $n = 10$). However, Park (1986b) presented data on the variability of GDH/NH_4 for well fed and starved zooplankton. The GDH/NH_4 ratios determined on size-fractionated mixed zooplankton from waters

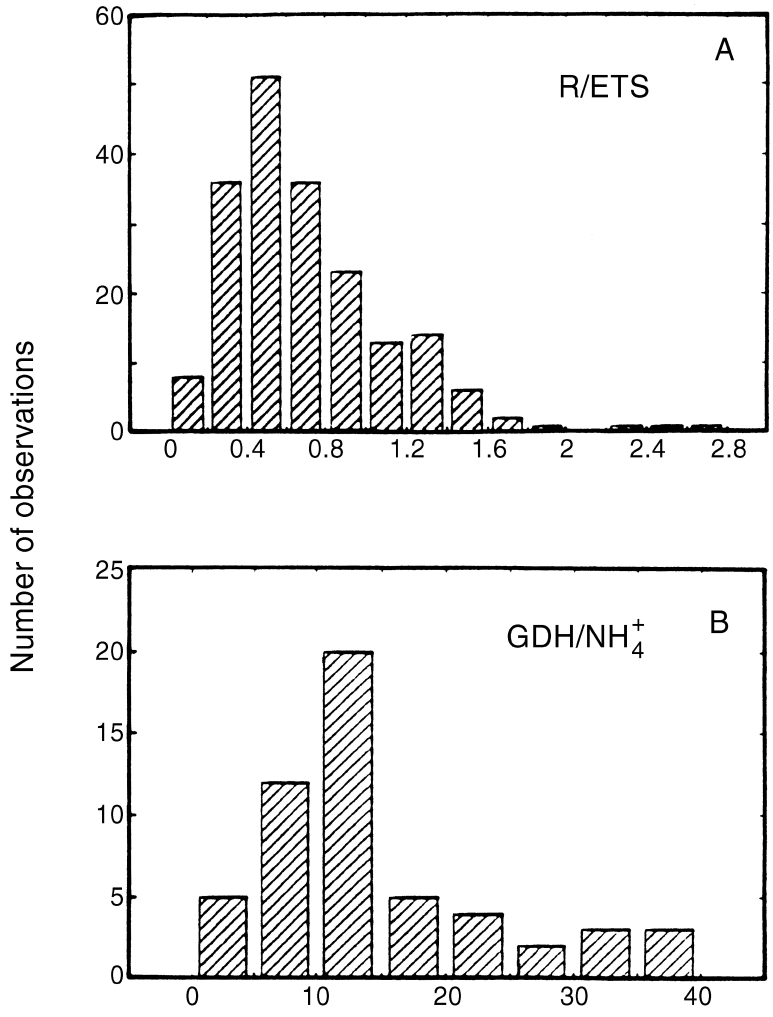

Fig. 10.4 Variations in the metabolic rate and enzyme activity ratio. (A) Ratio of respiration rate to ETS activity (R/ETS, $N = 202$) at experimental temperatures of 0.2–28 °C (redrawn from Hernández-León and Gómez 1996). (B) Ratio of GDH to ammonia excretion rate (GDH/NH_4, $N = 54$) at experimental temperatures 18–22.4 °C (Hernández-León and Torres 1997, with permission).

around Gran Canaria Island in spring and winter seasons by Hernández-León and Torres (1997) are summarized in Figure 10.4. It is apparent that, like the R/ETS ratio, the GDH/NH_4 ratios are also variable with a mode between 10 and 15. At present, accuracy of enzymatic methods to predict metabolic rates is seriously limited by inherent variabilities in the relationships with metabolic rates.

10.4.1 ETS activity

Enzymatic reaction rates follow Michaelis–Menten kinetics when substrate concentrations limit the rate of reaction. When enzymatic activity is measured, we are indirectly

measuring the amount of enzyme because activity is obtained at saturating substrate, i.e., at V_{max}. In contrast, we measure the metabolic rate *in vivo* in whole organisms whose cells and mitochondria may be subject to substrate limitation. If the enzymatic activity is only measured at V_{max}, a high variability in the ratio between metabolic rate and enzyme activity must be expected. This variability is found in the R/ETS ratio and Packard's argument invoking Michaelis–Menten kinetics, as stated above, has been used to explain it. This would be a biochemical explanation but a physiological one must also be sought.

Respiration rates of the copepod *Acartia tonsa* increased with increasing ration to a level three to four times standard metabolism (Kiørboe *et al*. 1985), the variability normally observed in the R/ETS ratio. It may be that, for a given ETS activity, a copepod's metabolism can vary from routine or active metabolism (high R/ETS) to standard metabolism (low R/ETS). Thus, ETS activity measures the potential of organisms to use their biochemical machinery to meet the physiological demands associated with different levels of activity. If physiological demands exceed the biochemical potential, the cells may adapt to the new situation by *de novo* synthesis of enzymes to increase the activity of the ETS. This would explain part of the variability in the R/ETS ratios found by Hernández-León and Gómez (1996) (cf. Figure 10.4). It has been recently observed (Hernández-León and Gómez 1996) that despite the possible variations in the size structure of zooplankton the R/ETS ratio of zooplankton varies with chlorophyl and primary production during development of the late winter bloom in the Canary Islands. They found higher R/ETS ratios coinciding with higher values of autotrophic production. Clearly, the organisms showed higher respiration rates coinciding with better food availability in quantity and/or quality. These results led Hernández-León and Gómez to believe that animals have a characteristic enzymatic level and that the respiration process does not use all the enzymatic capacity of cells (the Michaelis–Menten argument of Packard 1985). Animals can suddenly increase the locomotory activity or ingestion rate (and therefore the specific dynamic action) without changing the ETS activity level.

To test the Michaelis–Menten argument of Packard (1985), Hernández-León and Gómez (1996) designed an experiment using a flow-through system in which respiration rates and ETS activities of the copepod, *Calanus finmarchicus*, were measured simultaneously. Respiration rate was parallel to ETS activity (Figure 10.5B) when the copepods were fed synchronously. Increased respiration rates and ETS activities lasted only 4 h. They explained the response as respiratory control. Respiration in the mitochondria, the activity of its enzymes and the velocity of ATP production are driven by the relative concentrations of ADP, ATP and phosphate in the mitochondria and not by the

Fig. 10.5 Comparison of ETS activity and respiration rate in *Calanus finmarchicus* using a flow-through system. The experiment was run at 6.5 °C. (A) Respiration rate (open circles) and ETS activity (closed circles) of specimens under a continuous food supply. (B) Respiration rate and ETS activity of specimens provided low (LF) and high (HF) concentrations of food. The black bar indicates the dark period coinciding with the natural light–dark cycle. It is noted that a strong parallelism is seen between respiration rate and ETS activity. (C) Relationship between respiration rate and ETS activity from the experiments of (A) and (B). Points 1 and 2 in (C) are uncoupled data as indicated by an arrow in (A) and the first value of the same experiment. Units in (C) as in (A) and (B). Redrawn from Hernández-León and Gómez (1996, with permission).

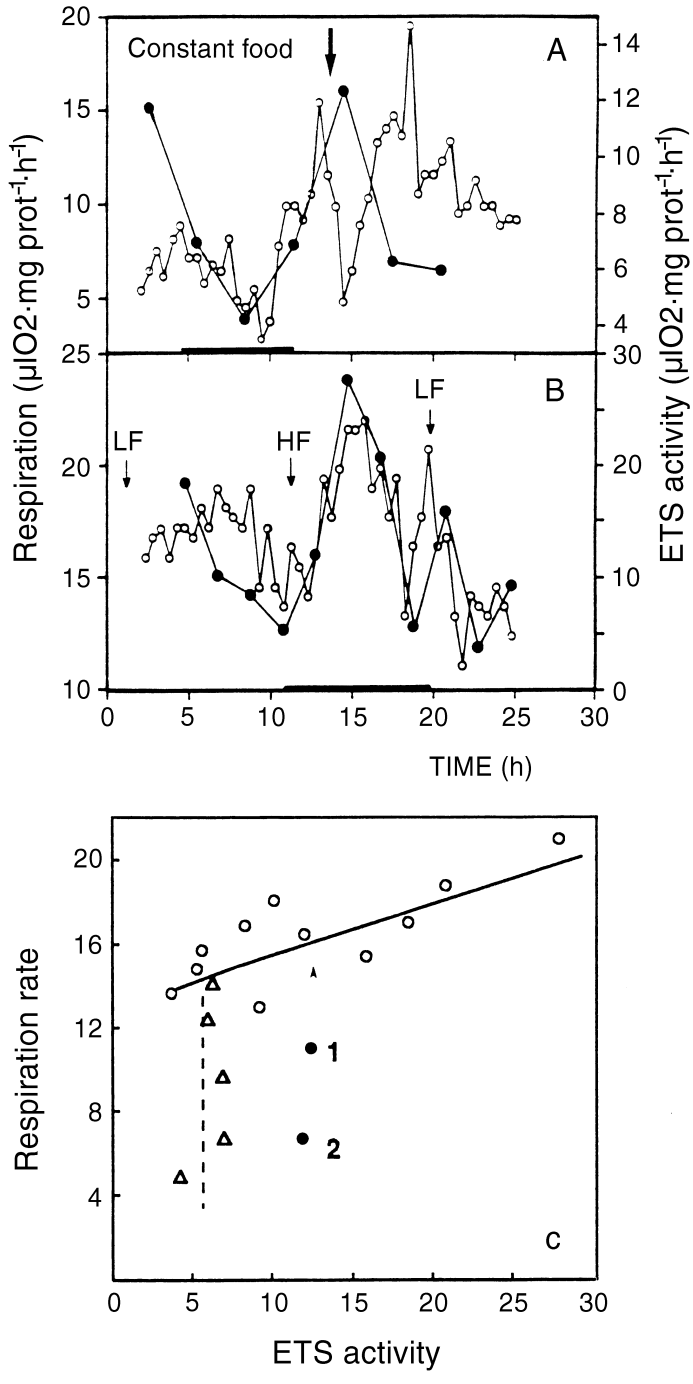
Constant food
A
B
LF
HF
LF
Respiration (µlO2·mg prot-1·h-1)
ETS activity (µlO2·mg prot-1·h-1)
TIME (h)
Respiration rate
ETS activity
1
2
c

Fig. 10.5

concentration of the respiratory substrates. When substrates are not limiting, the ADP and phosphate concentrations are high, and ATP is low, the oxygen consumption rate reaches its maximum. They suggested that ETS activity is measuring respiration when there is no substrate limitation (continuous line in Figure 10.5C). At low food concentrations, or when the copepods are incubated in filtered sea water, substrate limitation can be observed and then respiration is not predictable from ETS because the latter represents V_{max} (dashed line in Figure 10.5C). When an enzyme has a regulatory role, there is variability in K_m and therefore this parameter cannot be taken as constant. It is now evident that because of the variable nature of both enzymatic activity and metabolic rate on short time scales, search for a single conversion factor between the two variables does not make sense.

The coupling between respiration rate and ETS activity is corroborated by the results of Båmstedt (1980), Ikeda and Skjoldal (1980) and Skjoldal *et al.* (1984) who found ETS activities and respiration rates decrease in parallel during starvation. Finlay *et al.* (1983) also observed that respiration rates and ETS activities of protozoans vary in parallel except during starvation and re-feeding experiments. Recently, Packard *et al.* (1996) developed a bisubstrate enzyme kinetics model which enables the estimation of *in vivo* ETS activity for the marine bacterium *Pseudomonas nautica*. *In vivo* ETS activity thus estimated tracked well with respiration rate throughout all growth phases (including nutrient-starved senescent phase) of the bacterium. To estimate *in vivo* ETS activity using this bisubstrate enzyme kinetics model, intracellular substrates (nicotinamid adenine dinucleotide (NADH) and nicotinamide adenine dinucleotide phosphate (NADPH)) were determined, in addition to conventional *in vitro* ETS activity.

10.4.2 Enzymes of intermediary metabolism

Two assumptions underlie the use of metabolic enzymes as physiological indicators. The first is whether or not the concentration of enzymes within the aerobic and anaerobic pathways is modulated up or down by the cell in accordance with the cell's energy requirements, which dictates the carbon flow through those pathways. The second is that activity of the chosen enzyme accurately represents the activity of the pathway it is derived from. As with the ETS measurements, activities are measured under saturating conditions of substrate and cofactors so that what is obtained is an estimate of V_{max}. Comparisons of activities, or V_{max}, between individuals give good indication of differences in enzyme concentration, and therefore differences in physiological condition or metabolic poise (Hochachka and Somero 1984). A useful reference that discusses the rationale for choosing V_{max} as a basis for comparison is Newsholme and Crabtree (1986).

Use of intermediary metabolic activities (as distinguished from the ETS assay treated in ETS Activity, section 10.4.1) as indicators of physiological status, or as proxies for metabolism, is fairly new to zooplankton biology. With a few exceptions (e.g. Curl and Sandberg 1961; Packard and Taylor 1968) all the published work dates from 1990. However, much groundbreaking had already been done with fishes (Childress and Somero 1979; Somero and Childress 1980; Sullivan and Somero 1983; Kaupp 1987; Lowery *et al.* 1987; Torres and Somero 1988; Kaupp and Somero 1989) so the fact that metabolic enzyme activities had good potential as physiological indicators was already known; what was required was the transfer of the techniques to planktonic animals.

Clarke *et al.* (1992) described the influence of ration level on activities of LDH and CS in two species of very young fish larvae (ichthyoplankton): red drum (*Sciaenops ocellatus*)

and lane snapper (*Lutjanus synagris*). It was found that growth in the ichthyoplankton correlated quite positively with activities of LDH and CS. A similar result was obtained for the copepods *Acartia tonsa* and *Temora longicornis*; copepods starved over a 36 h period showed a significant drop in CS activity when compared with well-fed controls. The results of Clarke and Walsh (1993) echo much of the early work on succinate dehydrogenase (SDH) in zooplankton in which SDH activity was observed to correlate well with oxygen consumption rate in *Artemia* (Packard and Taylor 1968) and with seasonal changes in condition in the mysid *Neomysis integer* (Raymont *et al.* 1967).

Recent work by Thuesen and Childress (1993a, 1993b, 1994) has greatly expanded the phyletic range in which intermediary metabolic enzyme activities (CS, MDH, LDH, PK) have been examined. Their work describes enzyme activities and metabolism in nemerteans, annelids, chaetognaths and cnidarians. For our purpose, it is important on two main fronts. First, it shows that enzyme activities can be measured readily even in the very watery (> 95% of wet weight) gelatinous zooplankton. Second, there is a strong correlation of oxygen consumption, enzyme activity, and life habit in the zooplankton examined. Scaling coefficients ('*b*') for aerobic enzymes were species-specific and varied between 0.67 and 1.0.

Intermediary metabolic enzymes show excellent promise as indicators of condition and as proxies for metabolic rate. However, no published work has attempted to correlate intermediary metabolic enzyme activities and short-term, biologically important, environmental characteristics such as primary production or chlorophyl biomass. Preliminary data generated by one of the author's (J.J. Torres) laboratories during an Antarctic field study fills this need in addition to strongly supporting the use of metabolic enzymes as indicators of condition of copepods. Adult *Rhincalanus gigas*, a dominant copepod in the Antarctic system, were collected during the annual spring bloom of phytoplankton at the edge of the retreating pack-ice in the Scotia–Weddell Sea region. Three locations were sampled: a low productivity area deep within the pack-ice, the highly productive area within the ice-edge bloom, and the less intense, but still productive area seaward of the bloom. Oxygen consumption rates were measured at sea (Figure 10.6A) and CS activities were determined in the laboratory (Figure 10.6B) on *R. gigas*, the latter were frozen in liquid nitrogen and maintained continuously at $-80\,^{\circ}C$. There was a significant difference ($P < 0.05$, ANOVA) in oxygen consumption rate and in CS activities between the low productivity pack-ice zone (zone 5) and the higher productivity ice edge (zone 3) and open water stations (zone 1). The fact that oxygen consumption and CS activity covary with chlorophyl biomass suggest first, that CS has considerable promise as a biochemical proxy for metabolism, and second, that it is responsive to conditions of the copepod in the field.

10.4.3 Potential sources of error

In addition to the quality and quantity of food in a zooplankter's diet (see Feeding, section 10.3.1) several other variables may give rise to errors in enzymatic estimation of metabolic rate.

GROWTH CONDITIONS

Little information is available for zooplankton describing the influence of growth conditions on the relationship between enzyme activity and metabolic rate. A higher R/ETS ratio associated with active growth and a lower R/ETS ratio associated with senescence have been observed in bacteria (Christensen *et al.* 1980) and microalgae

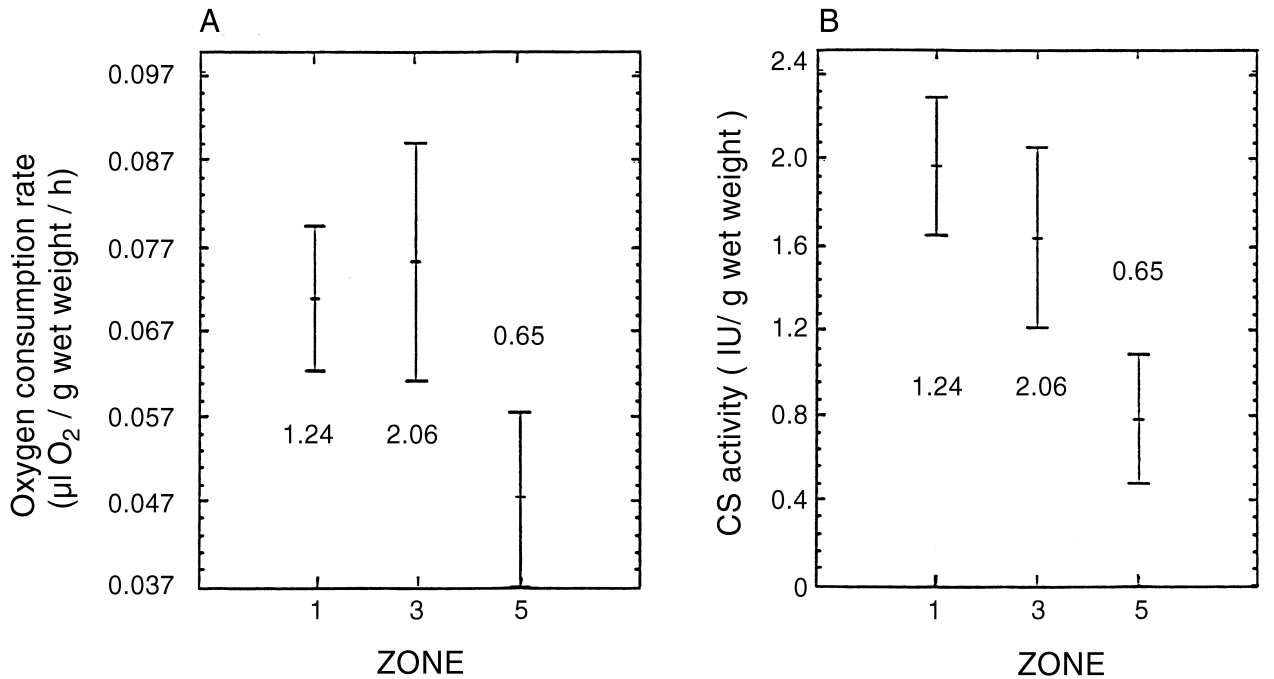

Fig. 10.6 Oxygen consumption rate and citrate synthase (CS) activity, the enzyme catalyzing the initial step in the Krebs Cycle, of the Antarctic copepod, *Rhincalanus gigas*, with relation to the Antarctic ice edge. Zone 5 is consolidated pack-ice, zone 3 is the highly productive marginal ice zone, zone 1 is the open water seaward of the ice-edge bloom. Oxygen consumption rates are expressed as μl O_2 g wet weight^{-1} h^{-1}. Enzyme activity is expressed in international units per gram wet weight (IU g^{-1}: IU = μmol substrate converted to product per minute). Error bars are 95% confidence limits. Numbers within the figure are the values for chlorophyl biomass (μg l^{-1}) corresponding to each zone. Oxygen consumption rates were significantly ($P < 0.05$; ANOVA) lower in the pack ice (zone 5) than at the ice-edge (zone 3) or open water (zone 1) locations. CS activities were significantly ($P < 0.05$; ANOVA) lower in the pack ice (zone 5) than at the ice-edge (zone 3) or open water (zone 1) locations. Oxygen consumption data contributed by Helena Kawall, and CS activity data by Steve Geiger.

(Kenner and Ahmed 1975). As mentioned in Enzymes of intermediary metabolism, section 10.4.2, Clarke *et al*. (1992) found that growth of ichthyoplankton (larvae of *Sciaenops ocellatus* and *Lutjanus synagris*) correlated well with activities of LDH and CS. In contrast, Berges *et al*. (1993) observed that activities of CS and GDH did not correlate well with specific growth rate in laboratory-raised *Artemia franciscana* and cautioned against their use in the field. These inconsistent results may reflect the physiological dissimilarities of experimental organisms between studies, but evaluation of growth conditions may be a necessary adjunct for obtaining reliable correlations between metabolism and biochemical proxies in field-caught plankton.

BODY SIZE

A series of two papers (Berges *et al*. 1990; Berges and Ballantyne 1991) described scaling of metabolic enzyme activities with body size at different ration levels and in different species (*Artemia franciscana, Daphnia magna*). The authors concluded that scaling with body size was enzyme dependent and often differed from the classic '*b*-value' of 0.75 in the allometric equation between metabolic rate (M) and body mass (W) (i.e. $M = aW^b$, where a is a constant, see also Body size and temperature as bases of metabolic comparison, section 10.3.4, and equation 10.5), hence scaling must be considered in any field application of enzyme activities as biochemical proxies.

A large amount of scatter in the R/ETS and GDH/NH_4 data in Figure 10.4 may be due to the mixture of zooplankton with different body sizes (= body mass). Christensen *et al*. (1980) compared R/ETS ratios of various marine organisms including bacteria to zooplankton (body mass: 10^{-7} to 10^3 mg dry weight) and noted only a slight dependence of the ratio on the body mass (mg DW) of organisms:

$$\log_{10}(\mathrm{R/ETS}) = -0.0485 \log_{10}\mathrm{DW} + 0.191. \qquad (10.9)$$

Packard (1985) summarized the R/ETS ratio to be 0.17 for phytoplankton, 1.1 for bacteria, 0.25 for protista and 0.49 for zooplankton. Analyzing the relationship between respiration rates and ETS activities, a linear regression, instead of a mean, has been suggested to improve the precision in estimation of the former from the latter (King and Packard 1975; Arístegui and Montero 1995).

MISMATCH BETWEEN METABOLIC RATE AND ENZYME ACTIVITY

Båmstedt (1980) noted a delayed response of ETS activities to increased respiration rates in *Acartia tonsa*. Finlay *et al*. (1983) found a time-lag between respiration rates and ETS activities on ciliates which were starved and then fed. Finlay *et al*. explained the cause of the time-lag as a limitation in the amount of enzymes involved in the ETS. This uncoupling would be a source of variation in the relationships between enzymatic activities and metabolic rates of organisms collected in the field, where food availability may change over short time scales as a consequence of physical instabilities of water columns. The time-lag observed by Finlay *et al*. (1983) on ciliates was on an hourly time scale. While the application is presently limited to the bacteria bisubstrate enzyme kinetics model proposed by Packard *et al*. (1996) should provide a better insight to the uncoupling problems between respiration and ETS activity.

CALIBRATION DESIGN

Present calibration procedures for the relationship between enzyme activity and metabolic rate appear inadequate. Figure 10.7 illustrates a typical calibration of ETS activities against respiration rates using the sealed chamber method as practiced by

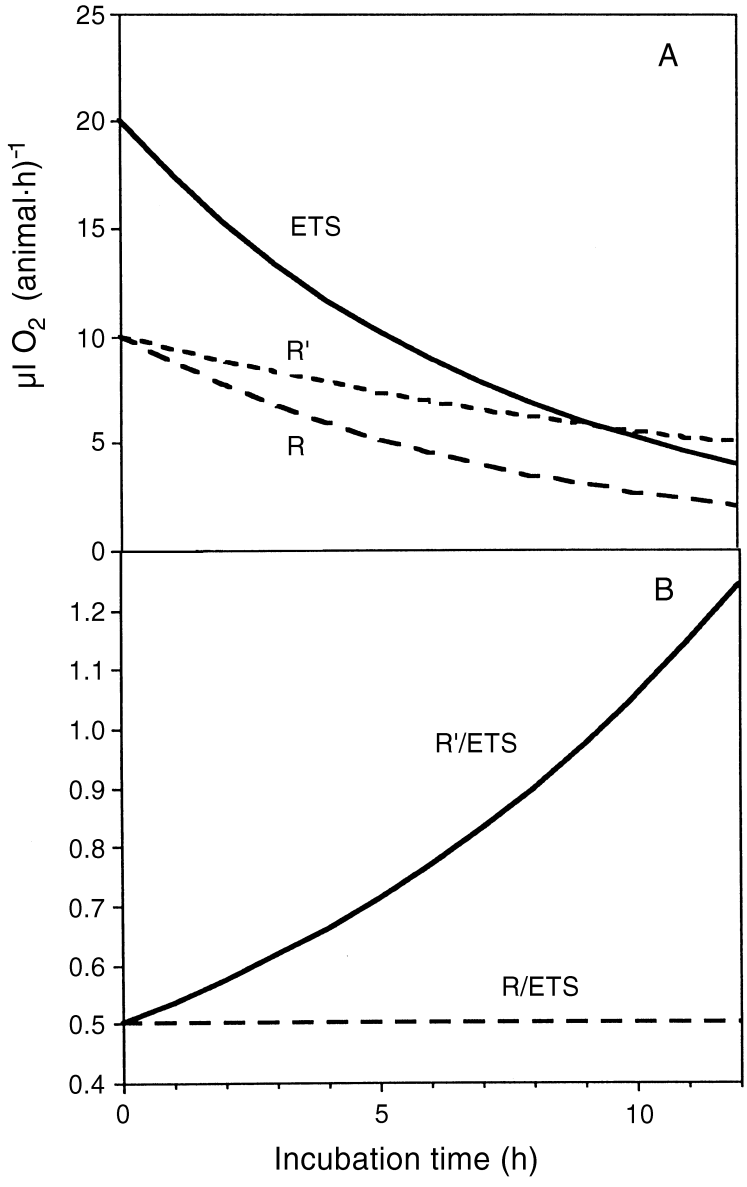

Fig. 10.7 (A) Model run for the likely changes in the ETS activity and respiration rate (R, and its integrated mean over incubation time R′) of zooplankton placed in a closed bottle filled with filtered sea water. (B) Resultant R/ETS (and R′/ETS) ratio. In this run, ETS and R at the start ($t = 0$) is designated as 20 and 10 μl O_2 (animal h)$^{-1}$, respectively, and decrease exponentially to 4 and 2 μl O_2 (animal h)$^{-1}$, respectively, at the end of incubation lasting for 12 h, maintaining a constant R/ETS ratio of 0.5. Note that the decrease with the progress of incubation time of R′ is much slower than that of R, which reflects an increase of R′/ETS during the incubation. Assumed equations for the change with time are; ETS = $20e^{-0.1341t}$, $R = 10e^{-0.1341t}$, and $R' = \int_0^t R\,dt = 10(1 - e^{-0.1341t})/(0.1341t)$.

previous workers (Båmstedt 1980; Ikeda and Skjoldal 1980; Skjoldal *et al.* 1984). In the sealed chamber method, respiration rate is determined from the difference in the oxygen concentration between experimental and control bottles at the end of the incubation (i.e. R′: integrated mean respiration rate over the incubation period in Figure 10.7), and ETS activity is measured on zooplankton retrieved from experimental bottles at the end of incubation. Because of this methodological constraint, the R′/ETS ratio obtained from the sealed chamber method is subjected to error unless there is a constant respiration rate and ETS activity over the incubation period. In the model shown in Figure 10.7, a rapid decrease of ETS activity and a less pronounced decrease of integrated mean R′ reflected a progressive increase of the R′/ETS (1.24 at the end of 12 h, in contrast to a designated ratio of 0.5). The problem highlighted in the model is not limited to the ETS activity, it applies to the activity of other enzyme systems as well. To overcome this problem, a continuous recording system for metabolic rate, such as the flow-through system (Measuring metabolic rate on live zooplankton, section 10.3) is more appropriate for the purpose of calibration.

10.5 CONCLUDING REMARKS

While the determination of metabolic rates of live zooplankton is easily accomplished with either the sealed chamber method or the flow-through method, extrapolation of measured rates to a species population in the field is not straightforward. One obvious problem is the lack of a clear index to judge whether or not the specimens collected from the field and brought into the laboratory are 'damaged'. Part of the problem may be overcome by experience and by checking morphology, coloration, swimming behavior, orientation patterns, and response to stimuli (light, touching with needle, etc.). However, this sort of quick screening prior to an experiment may not always detect physiological damage.

The second problem associated with experiments on live zooplankton is the difficulty in reproducing natural environmental conditions for zooplankton in a laboratory experiment. Environmental and nutritional conditions of zooplankton in the laboratory affect the metabolism of zooplankton. Our knowledge about the quantitative metabolic responses of zooplankton to environmental and nutritional conditions is quite limited. Most previous studies concern one or two potential parameters affecting metabolism; few attempts have been made to examine the combined effects of various parameters. Newell *et al.* (1976) analyzed a combination of six variables (exposure temperature, body weight, starvation period, feeding period, acclimation temperature, and lipid content) on the oxygen consumption rate of an intertidal isopod, *Ligia oceanica*, in the laboratory. They found that 85% of the observed variation in the rates could be attributed to the six parameters. An approach of this sort is of great importance for the extrapolation of laboratory results to field populations of zooplankton.

The third problem is the lack of adequate techniques to control swimming activity of test zooplankton during experiments. It has been assumed that the change in swimming activity of animals brought in the laboratory and confined in containers for metabolic measurements is minimal. This may be the case for small, less active zooplankton. For larger and more active zooplankton such as euphausiids, determination of metabolic rate under well defined activity levels of animals (i.e. standard, routine, active metabolism) is needed for valid comparison of the data between workers and for better estimation of metabolic costs for diel vertical migration in the field (Torres and Childress 1983).

The fourth problem is associated with zooplankton themselves; an assemblage composed of highly diverse groups of animals (11 phyla at least, cf. Omori and Ikeda 1984) poses a real problem in establishing standardized methods for measuring their metabolic rates. High phylogenetic diversities are inevitably accompanied by a wide body-size range, and dissimilarities in behavior, locomotory activity, physiology and nutrition of species are involved. It is almost certain that inconsistent experimental results on zooplankton between workers reflect the complexities of the animal groups they have studied.

Contrary to earlier expectations, enzymatic methods for estimating metabolism are not very simple, and are not necessarily free from the problems discussed here. In addition to requirements for proper calibration with metabolic rate measurements on live zooplankton under controlled laboratory conditions, information about nutritional state, growth condition and size of animals may also be required for the better estimation of metabolic rates of zooplankton in the field. In this regard, direct determination (sealed chamber method or flow-through methods) and indirect determination (enzymatic methods) will complement each other as we work toward establishing better methodologies for assessing zooplankton metabolism.

Practice (T. Ikeda and J.J. Torres)

10.6 COLLECTION AND HANDLING OF ZOOPLANKTON

Most zooplankters are fragile, and for use in metabolic experiments it is important that the specimens remain undamaged. Figure 10.8 shows two examples of collecting buckets designed for such samples (see also Reeve 1981), their volumes (2 to 30 l) are several times larger than that of an ordinary cod-end bucket and they have mesh windows in the anterior part.

The cod-end bucket is filled with sea water prior to sampling so that the net will sink promptly when deployed. A vertical haul or short horizontal tow with a fine-mesh net is preferable in order to avoid damage to the plankton during the haul. The towing speed

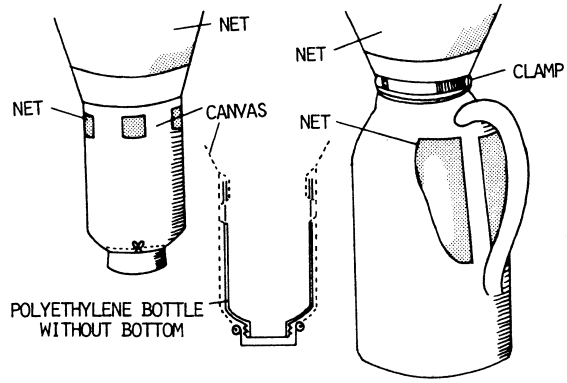

Fig. 10.8 Two examples of cod-ends for collecting live zooplankton (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

should be about 0.3 m s^{-1} or less, and when sampling from a large ship, it is better to stop the engine and make use of the drift of the ship caused by the wind. A long tow weakens the plankton in the net. In tropical waters, where the water temperature is high, very short tows should be used. After retrieving the net, the bucket is quickly placed in a container filled with a large amount of sea water, after which the samples are gently transferred to the container. The plankton that remain attached to the net should be considered damaged and not transferred into the container. As plankton are damaged from direct exposure to the sun, the best sampling time is after sunset (Light, section 10.3.1).

To sample actively swimming macroplankton, a net with a large mouth and good filtration efficiency, such as the ORI-C net (Omori 1965), should be used. The posterior part of the ORI-C net is made of mesh gauze which is finer than the anterior section to minimize damage to the organisms during the tow. Additional protection for specimens may be achieved by using specialized cod-end buckets that are designed to close at the end of the tow, thereby creating a parcel of protected water for bringing animals to the surface unharmed. For example, the thermal cod-end described by Childress *et al.* (1978) has been used to successfully recover deep-living oceanic zooplankton. Johnson and Attramadal (1982) devised another type of protective cod-end bucket for use on sledges to collect epibenthic animals. This simply designed bucket can be modified for midwater horizontal tows to avoid the mechanical damage of specimens and prevent exposure to large fluctuations in temperature, salinity, and light during recovery.

Gelatinous plankton are best captured by divers who introduce them into wide-mouth containers, but some may be obtained with net hauls in the manner described above. To transfer specimens to another container, they should be scooped gently with a small beaker or specially designed net (Figure 10.9A).

When transporting the living zooplankton to the laboratory, their density should be kept as low as possible. Polyethylene containers with airtight lids are excellent for this purpose. To reduce damage of organisms due to agitation of water during transport, the container should be completely filled. Also, every effort should be made to avoid direct sunlight and change in water temperature. It is therefore advisable to cover the containers with dark cloth. For certain species aeration (gentle bubbling of sea water with air from air compressors) may be needed if transportation takes a long time.

For sorting specimens, a portion of the sample is gently poured into a transparent container of appropriate size and healthy animals are removed by pipette, spoon or scoop. The pipettes are made of glass or clear acrylic tubes of various diameters. Simple apparatus and methods to aid sorting of live zooplankton are shown in Figure 10.9 (B and C). In some cases the sample may be left for 10 to 30 min after having been transferred to a container, and the differences in the phototaxis or geotaxis of different species used to good advantage in sorting. Small transparent organisms are easily seen if the background of the container is darkened and light enters through the side. For some species, their characteristic swimming behavior as well as body coloration may distinguish them for sorting. Selection of experimental specimens is based on appearance. Any evidence of physical damage such as bruising or opaque patches on an otherwise transparent individual constitutes grounds for rejection. With crustaceans, any fouling of limbs or unnaturally limited range of motion on the swimming legs would be a basis of rejection. It is worth the energy to observe living zooplankton in beakers, buckets, or petri-dishes to get a feeling for how they move and what a normal range of limb motion is. There is no substitute for knowing your animal!

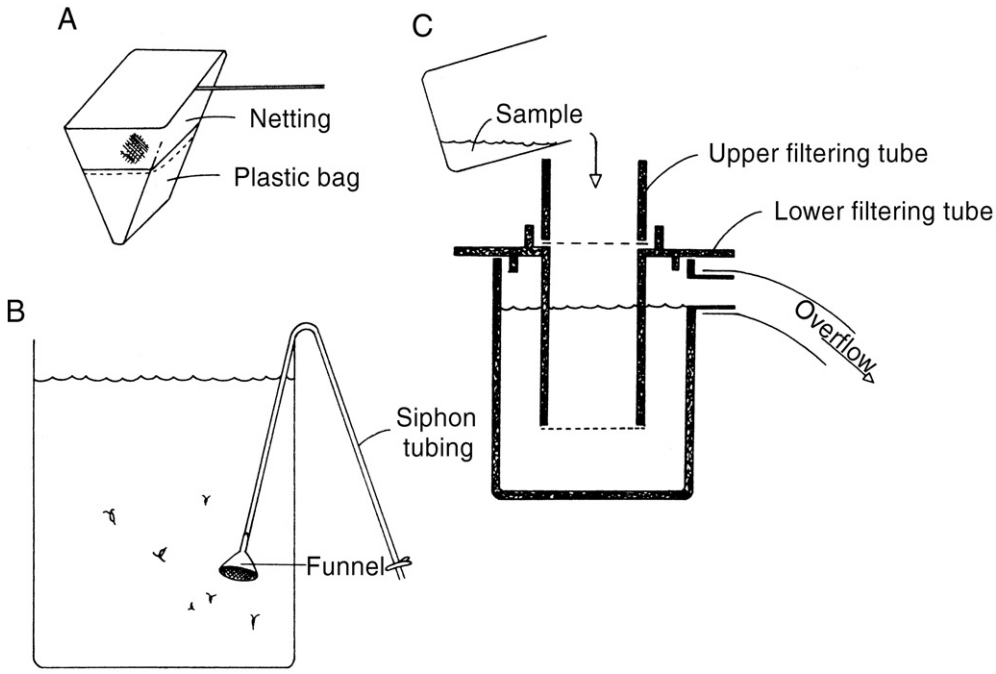

Fig. 10.9 Simple apparatus to aid sorting of living zooplankton. (A) net of which the lower part is replaced with a plastic bag. With this net large zooplankton are transferred with a certain amount of water from one container to the next without exposing them to air. (B) A siphon system to remove unwanted small specimens. On one end of the system is fitted a funnel covered with a mesh screen. A gentle siphoning out of the water is essential to avoid damaging larger specimens. (C) A double filtering system to remove undesired larger and smaller zooplankton simultaneously. A zooplankton sample is gently poured into the upper filtering tube through a coarse mesh screen and passes into the lower filtering tube through a fine mesh screen into a container filled with sea water. The desired intermediate size zooplankton are retained in the lower filtering tube. Upon completion, the upper filtering tube is removed and zooplankton in the lower tube are transferred to the container with a pipette. A whole set is made with Plexiglass or Perspex (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

With regard to the time delay between actual capture of zooplankters and subsequent measurements, there is a trade-off between stress and starvation (Capture stress/starvation, section 10.3.1). Specimens should be allowed to recover from the trauma of capture for a period of time but not for so long that the metabolism drops significantly due to starvation. Different investigators use different recovery periods varying from 2 h to greater than 24 h. A few things should be kept clearly in mind when deciding for oneself how long to allow animals to remain in the unnatural environment of the laboratory before using them in experiments. First, any manipulation of the experimental subject is a potential source of stress or excitement. If the investigator is concerned with accumulation of oxygen debt during capture, similar concern should be extended after the introduction into the respirometer. Second, the animal is never going

to be healthier and happier than it was when swimming in the ocean. Even if it can be maintained in the laboratory and fed for days or weeks it is unlikely in the extreme that it is improving in health. Thus, in principle, the sooner after capture that a metabolic rate is taken, the better the result is likely to be.

10.7 RESPIRATION

10.7.1 Oxygen consumption – Winkler titration (T. Ikeda)

The method described below largely follows Omori and Ikeda (1984). In the sealed chamber method, bottles with and without experimental zooplankters are prepared simultaneously and the difference in concentrations of dissolved oxygen after a period of incubation is attributed to respiration of the zooplankters. This method has been the most popular since the original oxygen consumption measurements on *Calanus finmarchicus* by Marshall *et al.* (1935).

The major advantage of the water bottle method is its simplicity in that it requires no specialized equipment. However, the method requires relatively long incubations, therefore starvation cannot be completely eliminated as a potential influence on the rate (Technical problems, section 10.3.1). Since the changes in oxygen consumption rate of zooplankton during the incubation period cannot be determined, the method assumes that the oxygen consumption rates of zooplankters are constant during the entire incubation period.

INCUBATION BOTTLES

BOD bottles are ideal, but ordinary glass reagent bottles fitted with airtight lids may also be used. The capacity of each bottle should be measured prior to its use in an experiment. The capacity may be obtained from the difference in the weight of each bottle with lids before and after filling it with distilled water (1.00 g = 1.00 ml). Prior to the experiment bottles and pipettes for the transfer of specimens from one container to the other should be cleaned with acid solution (6*N* HCl), then rinsed well with pure water and dried.

WASHING/EXPERIMENTAL SEA WATER

Water sampled from the site from which zooplankters were obtained is used to wash and subsequently incubate the zooplankters. In order to remove other organisms and particles, water should be filtered through GF/C, GF/F or Nuclepore filters with equivalent pore-size (0.4 μm). To remove smaller oceanic bacteria, Nuclepore filters with 0.2 μm are best. Vacuum filtration usually reduces the dissolved oxygen content in water. Therefore, aeration of the water is necessary to readjust the oxygen content to near saturation level. Most experiments start at oxygen saturation. Special attention may be required for experimentation on animals from low oxygen habitats. To remove particles from low-oxygen water without appreciable changes in oxygen content, a gentle filtration system (see Figure 10.9B) using a combination of a siphon system and funnel covered with fine mesh netting (10 or 20 μm mesh openings) may be used.

Three typical washing procedures are illustrated in Figure 10.10 A–C. The best washing method depends on the character of the zooplankters and the experimental design. Generally, the washing procedure is not so critical for oxygen consumption rate measurements with oxygen saturated water, but it is a very critical source of error for

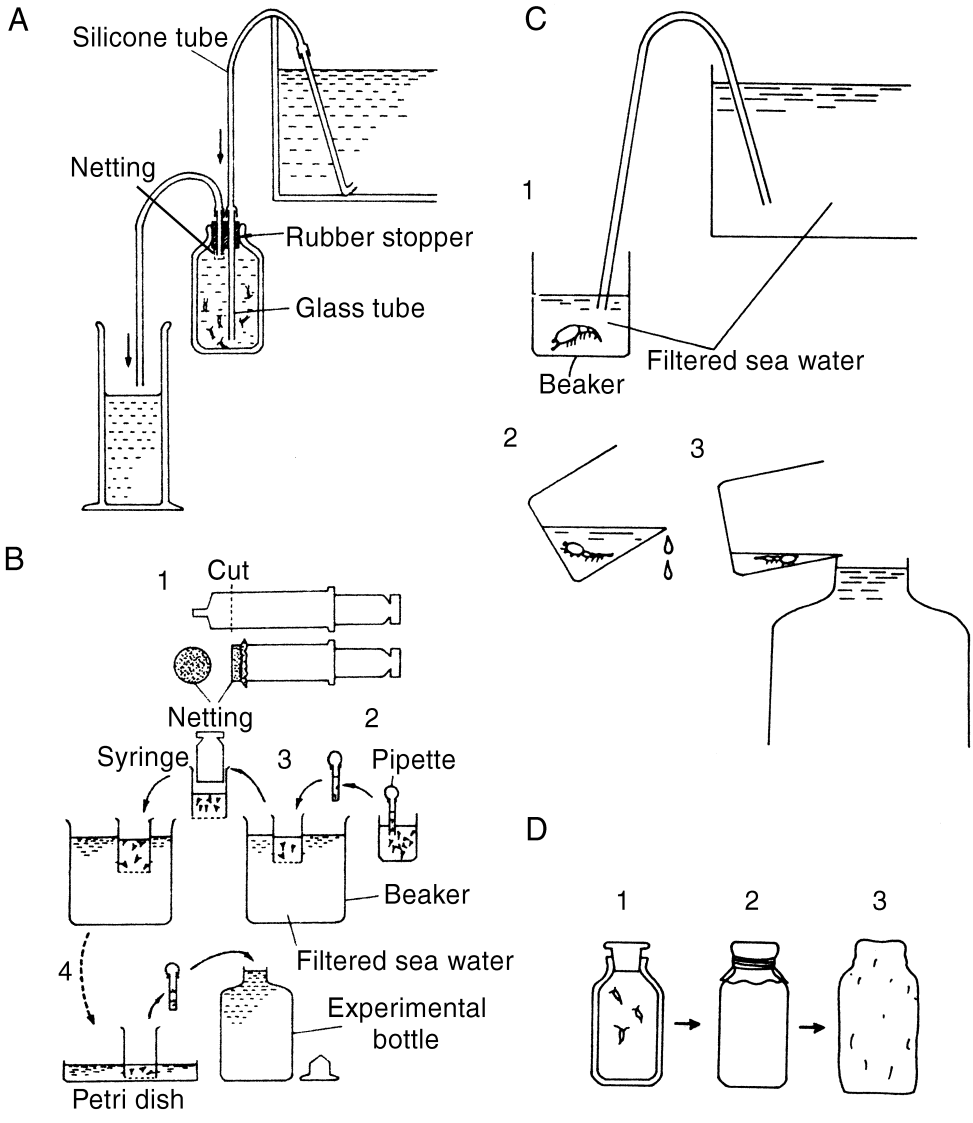

Fig. 10.10 Three procedures for washing zooplankton for metabolic experiments, and preparation of bottles for incubation after washing. (A) A siphon system, originally designed by Marshall *et al.* (1935) for oxygen consumption measurements. In their original method, sea water volume needed for flushing is 6–7 times the bottle volume. (B) Transparent plastic syringe. Place animals in the syringe with a pipette, and rinse them in several beakers. Then, place the syringe into a petri-dish and introduce the animals into bottle with pipette. A convenient size of syringe is 20 ml (2 cm in diameter), but larger ones can be used when fine netting is placed over the tip. (C) Glass beaker for relatively large zooplankton. Gently add filtered sea water in the beaker and decant carefully. Repeat this treatment 3 to 4 times, and then place the animals into a bottle. (D) Preparation of a bottle for incubation. See text (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

ammonia and phosphorus excretion measurements mentioned in section 10.8. It is very important not to damage the specimens while washing them. The number of zooplankters should be counted before incubation (and again at the end of incubation). The bottles are filled with experimental water using a siphon system, placing one end of the siphon onto the bottom of the bottles. After zooplankters have been washed and placed into the bottles, lids should be fitted so as to avoid trapping any air bubbles. The lids are firmly wrapped with a plastic sheet and rubber band to reduce the risk of introduction of air bubbles in the bottles during incubation. Control bottles without zooplankters should be prepared concurrently using exactly the same procedure. In a typical experiment with ten experimental bottles, two control bottles are prepared before the first experimental bottle and two after the last experimental bottle. An additional control bottle may be inserted between the fifth and sixth experimental bottles (15 bottles in total). The bottles are ready for incubation after wrapping with aluminum foil or black plastic sheet (Figure 10.10D). A water bath with a temperature control unit is best for incubating the bottles.

INCUBATION

The incubation period will vary depending on the experimental temperature, density of zooplankters relative to the volume of the bottle, and rates of oxygen consumption of the zooplankters. For medium to large sized zooplankters, 12 or 24 h is recommended to eliminate the effect of diurnal rhythm on the metabolic rates. The period may be shortened to only a few hours for very active zooplankters that are less tolerant to prolonged starvation during the incubation period. The length of incubation period should first be based on the size of zooplankton (i.e. tolerance to starvation). Decision on the size of bottles used in the incubation and the number of specimens in each should be based on the need to minimize accumulation of excreta and oxygen depletion at the end of the incubation (see Oxygen saturation, section 10.3.1).

Antibiotics, such as streptomycin and chloromycetin, are often used to minimize bacterial activity in the water. The difficulty with using antibiotics is the determination of dosages, which vary among different zooplankters. Unfortunately, little is known about the appropriate dose of antibiotics for different zooplankton, and excess antibiotics may seriously affect the activity of experimental animals. Some antibiotics may interfere with other chemical analyses (Bacteria, section 10.3.1).

To determine dissolved oxygen, a volume of water is removed from the bottle after incubation. Figure 10.11 shows a siphon system that we use to transfer water from incubation bottles into small oxygen bottles. Duplicate water samples are sufficient when the analyst is skilled in the technique.

At the end of the incubation, the activity of zooplankters in the experimental bottles should be checked. These zooplankters may then be transferred to a petri-dish (larger zooplankters) or directly onto a piece of mesh (smaller zooplankters). Specimens can be counted either with the unaided eye or, for smaller zooplankters under a dissecting microscope. Zooplankters should then be weighed (wet weight) and/or weighed after drying (dry weight). Prior to weighing, water adhering to the body of a specimen should be removed by placing the specimen on filter paper. For smaller zooplankters that are difficult to handle individually, this can be achieved by placing the filter paper on the other side of the mesh. Although a brief rinse with distilled water to remove salts is desirable it is sometimes accompanied by loss of integrity, and organic matter, due to body lysis from osmotic shock (see Omori 1978).

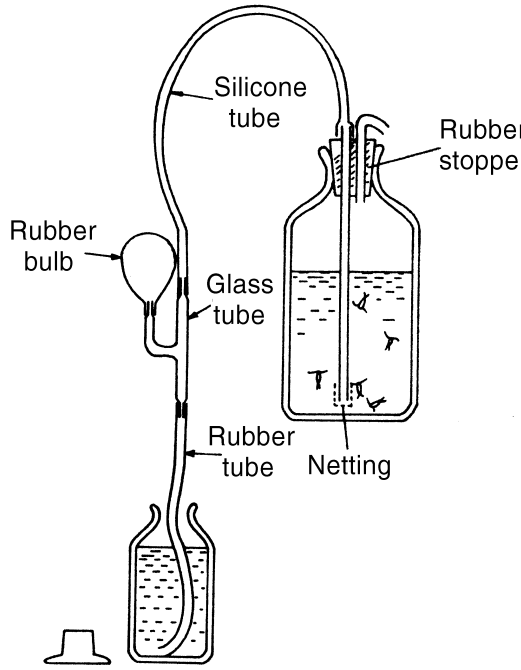

Fig. 10.11 A siphon system to transfer sample water from experimental bottles into oxygen bottles at the end of incubation (after Omori and Ikeda 1984, 'Methods in marine zooplankton ecology' (ISBN: 047/-80/070), John Wiley & Sons, Inc., reprinted by permission of John Wiley & Sons, Inc.).

TITRATION

Numerous minor modifications have been made for the original titration method of Winkler (1888), and the following is one by Strickland and Parsons (1972).

Analytical apparatus

300-ml BOD bottles, 10-ml titration burette (with 0.05-ml scale divisions), 1-ml pipette (dispensing type), 50-ml pipette, 125–250-ml conical flasks or beakers (it is easier to discriminate between the colors during titration if the entire bottom and half of the sides are painted white on the outside), As a titration burette, the Metrohm piston burette (cylinder volume: 10 ml) is accurate and easy to handle, especially for shipboard measurements.

Reagents

1) Manganous sulfate reagent: 480 g of manganous sulfate tetrahydrate $MnSO_4 \cdot 2H_2O$ or 365 g of $MnSO_4 \cdot H_2O$) is diluted in distilled water to make a volume of 1 l.
2) Alkaline iodide solution: 500 ml of sodium hydroxide NaOH (analytical reagent grade) is dissolved in 500 ml of distilled water. 300 g of potassium iodide KI (analytical reagent grade) is dissolved in 450 ml of distilled water. Mix the two solutions together. A great deal of heat will be liberated.
3) Approximately 0.01*N* sodium thiosulfate: 2.9 g of high-grade sodium thiosulfate $Na_2S_2O_3 \cdot 5H_2O$ and 0.1 g of sodium carbonate Na_2CO_3 are dissolved in 1 l of distilled water and one drop of carbon disulfide CS_2 per liter is added as a

preservative. The solution should be prepared 24 h before use and is stable over a long period if stored below 25 °C in a dark well-stoppered bottle.

4) 0.0100*N* iodate solution: A small amount of potassium iodate KIO_3 (analytical reagent grade) is dried at 105 °C for 1 h and exactly 0.3567 g weighed out after cooling. This is dissolved in 200–300 ml of distilled water by warming slightly. After cooling, distilled water is added to make a solution of 1 l. The solution is stable indefinitely if kept in a brown bottle in a dark cool place.
5) Starch indicator solution: 0.1%–0.2% of soluble starch solution is used. For long-term use preservation solution can be made as follows: 2 g of soluble starch is suspended in 300–400 ml of distilled water and a 20% solution of sodium hydroxide is gradually added with vigorous stirring until it becomes clear. After 1 to 2 h concentrated hydrochloric acid is added until the solution becomes slightly more acidic to litmus paper. Then 2 ml of glacial acetic acid is added. Finally, distilled water is added to make a solution of 1 l.

Determination of the factor for the sodium thiosulfate solution

A 300-ml BOD bottle is filled with sample water, add 1.00 ml of concentrated sulfuric acid and 1.00 ml of alkaline iodide solution and mix thoroughly. Then 1 ml manganous sulfate solution is added and mixed well. A 50-ml aliquot is poured into each titration flask. Use one or two flasks for blank determinations. Exactly 5.00 ml of iodate solution is added with a 5-ml pipette. Allow iodine liberation to proceed for 2 to 5 min at a temperature below 25 °C and out of direct sunlight, then titrate the iodine with the thiosulfate solution. The starch indicator is added when the yellow color of the liberated iodine fades; the first moment of the disappearance of blue color is the end point. If V is the titration volume of sodium thiosulfate solution in milliliters, the calibration factor f is:

$$f = 5.00/V. \tag{10.10}$$

The titration should be performed with at least three flasks and the average value obtained becomes f. A blank determination is made by titrating without adding iodate solution. When the blank correction exceeds 0.1 ml of sodium thiosulfate, the reagents are suspect and should be made afresh.

Analytical procedure

1) Remove the stopper from the 300-ml BOD bottle containing the sample water, and, by placing the tip of a pipette a little below the water surface, add 1.0 ml of the manganous sulfate solution followed by 1.0 ml of alkaline iodide solution. Then carefully restopper the bottle immediately so that bubbles are not trapped in it and shake the bottle well. After 2 to 3 min, shake the bottle again. Then allow the BOD bottles to stand quietly for several hours at room temperature until the precipitate has settled at least one-third of the way down the bottle, leaving a clear supernatant solution. Immersion of the bottles into water is recommended to prevent introduction of air bubbles prior to titration.
2) The titration is performed within several hours to one day after fixation. Remove the stopper from the bottle and add 1.0 ml of concentrated sulfuric acid by placing the tip of a pipette just below the water surface. Then restopper the bottle and shake the bottle well in order to dissolve the precipitate.
3) Transfer 50.0 ml of this solution into the specially painted flasks or beakers by means of a pipette. Titrate immediately with standard 0.01*N* thiosulfate solution. During titration the water should be stirred by a magnetic stirrer. When the straw color of the iodine becomes very pale, add 5 ml of starch indicator. The titration is

continued until the blue color disappears; this moment is the end point. Read the volume of sodium thiosulfate used up to the end point.

If the volume of the BOD bottle is Y (ml) and that of sample water used for titration is X (ml) and the titration volume of $0.01N$ sodium thiosulfate solution is expressed as V (ml) and the blank correction as V' (ml), the dissolved oxygen concentration in the sample water can be obtained by the following formula:

$$\text{mg-at } O_2\, l^{-1} = Y/(Y - 2) \times 5.00/X \times f \times (V - V') \tag{10.11}$$

where 2 in the denominator $(Y - 2)$ of the first term of the right-hand side is the total volume of the manganous sulfate solution (1.0 ml) and alkaline iodide solution (1.0 ml) added.

Thus, when a 50-ml aliquot is taken from a 300-ml BOD bottle,

$$\text{mg-at } O_2\, l^{-1} = 0.1006 \times f \times (V - V')$$

The milliliters or milligrams of oxygen in a liter of water can be calculated from the expressions

$$\text{ml } O_2\, l^{-1} = \text{mg-at } O_2\, l^{-1} \times 11.2 \tag{10.12}$$

$$\text{mg } O_2\, l^{-1} = \text{mg-at } O_2\, l^{-1} \times 16.0 \tag{10.13}$$

Note: When BOD bottles or similar glass bottles less than 300 ml are used, the amounts of reagents may be reduced proportionally. As an example, if 30-ml bottles are used, the amounts of reagents are 1/10 these given above. The titration of the entire contents in a titration flask is recommended rather than taking aliquots. Use the following formula for calculating the dissolved oxygen concentration

$$\text{mg-at } O_2\, l^{-1} = 1/(Y - 0.2) \times 5.00 \times f \times (V - V') \tag{10.14}$$

The author (T. Ikeda) has applied this scaled-down Winkler method successfully to quantities of sample water as small as 5 ml (though some loss in precision is inevitable). For even smaller quantities of sample water (1–2 ml), the use of a special version of this method, the so-called micro-Winkler method, may be recommended (for details, see Barnes 1959).

CALCULATIONS

Oxygen consumption rate (R) may be calculated using the following equation:

$$\begin{aligned} R &= [(C_o - C_t) - (C_o - C_{t'})] \times (V_c - V_z)/[t \times (N \text{ or } W)] \\ &= C_t - C_{t'}) \times (V_c - V_z)/[t \times (N \text{ or } W)], \end{aligned} \tag{10.15}$$

where C_o is the oxygen concentration at the beginning of incubation; C_t and $C_{t'}$ are oxygen concentrations in control and experimental bottles, respectively, at the end of incubation; V_c is the volume of experimental bottles; V_z is the volume of zooplankters; t is the incubation time; N is the number of specimens, and W is the mass of specimens used. Note that C_o is canceled out in the calculation, it is therefore not necessary to determine it in the experiment in which filtered sea water is used. V_z is estimated from the wet weight of the zooplankters assuming 1 ml = 1 g wet weight, but this is negligible in most experiments.

R is expressed in two ways: per individual and per unit mass (mass-specific or, formerly more common, weight-specific rate) of zooplankters. For the purpose of this

manual we will use the terms mass and weight interchangeably. Specimen total mass is often not mentioned in publications where the mass-specific expression is used. In either expression of R, data on the mass of zooplankters should be provided with R so that other researchers can interconvert if necessary, but also because body mass has a considerable influence on metabolism (see Time-course method in section 10.9.1)

10.7.2 Oxygen consumption – electrodes (J.J. Torres)

Oxygen electrodes provide a valuable alternative to the Winkler technique for measuring dissolved oxygen. The basic principles of taking the respiratory determination remain the same as those used in bottle incubations, i.e. the experimental subject is confined to a known volume for a period of time determined by the researcher and, of course, the oxygen requirements of the organism. The advantage to the investigator using O_2 electrodes is that oxygen within the experimental vessel is continuously monitored. This is not a trivial matter, as it allows the investigator to monitor oxygen consumption with respect to other variables, for example, the animals' locomotory activity, or time after feeding, without the need for taking many sequential sub-samples of the volume of water the animals are residing in. In addition, since oxygen electrodes produce a flow of electrical current, the investigator can choose among the large number of hardware and software alternatives currently available for recording data. With the current generation of personal-computer-assisted data acquisition systems, the investigator can move directly from the raw data into a spreadsheet or database program and then into statistical analysis without ever having to touch a sheet of paper. Alternatively, data from the oxygen electrode can be recorded on a strip chart recorder and analyzed by hand.

Oxygen electrodes are, in the author's (J.J. Torres) opinion, the unequivocal best choice for determinations of metabolism in marine organisms from the size of small copepods to large fishes. However, they do not give the level of precision one can expect from the Winkler method in the hands of a well-trained marine chemist. Chemists participating in WOCE (World Ocean Circulation Experiment) are expected to show results reproducible to $\pm$ 0.010 ml l^{-1} (0.04 μmol kg^{-1}-sea water, about 0.2% of air saturated O_2 concentrations; Culberson 1991). In contrast, a good, well calibrated electrode is accurate to $\pm$ 1% of the oxygen reading at air saturation and should give highly repeatable numbers. Realizing from the outset that O_2 electrodes require a special patience in handling will go a long way toward successfully employing them. They are not as reliable or trouble-free as pH electrodes and should not be considered as such.

The most widely used oxygen electrode is the polarographic design of Clark (1956). For detailed information on the construction of Clark-type electrodes the compendium edited by Gnaiger and Forstner (1983) is a good source. A brief description of how Clark electrodes work is appropriate here, as it will aid the user in selecting the commercial electrode most appropriate for their application, or for the more adventurous, the diameter of platinum wire most appropriate for the electrodes they manufacture in their own laboratory.

The sensor tip of a Clark electrode is constructed of a platinum cathode and a silver–silver chloride anode (Figure 10.12). The anode and cathode are bathed in a 1 mol l^{-1} solution of KCl and the tip is sealed by an oxygen permeable polypropylene membrane about 25 μm in thickness, usually held in place with an O-ring. The polypropylene membrane effectively segregates the electrode tip from the outside medium; the pores in the membrane allow gases into the sensor tip but prevent interference from the larger

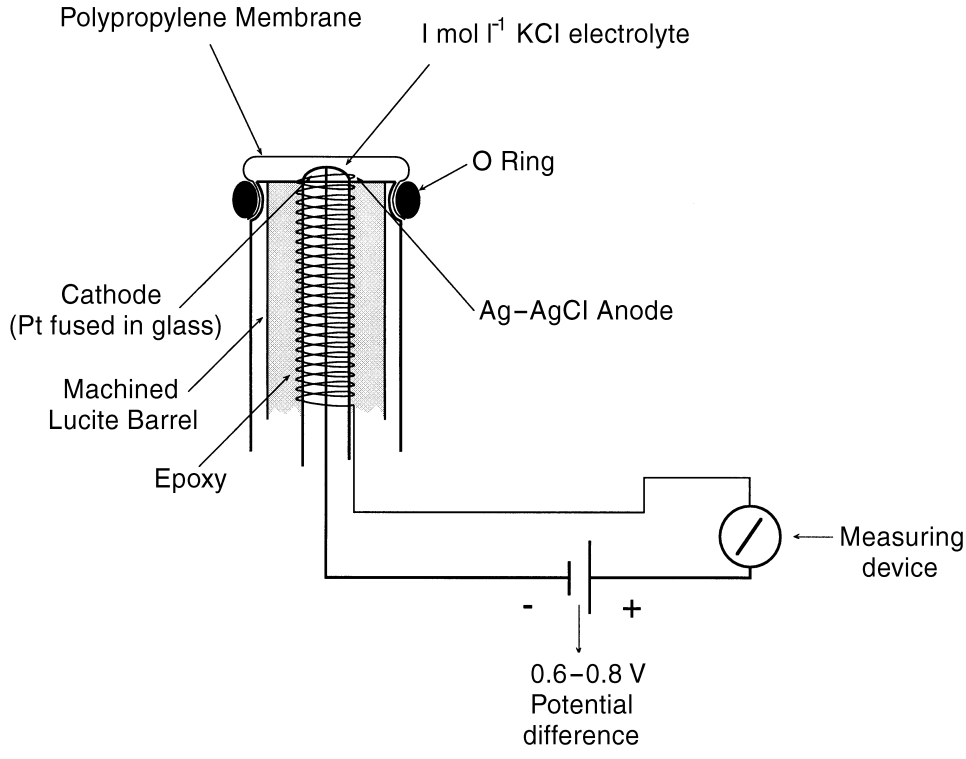

Fig. 10.12 A Clark oxygen electrode showing its major components. Platinum is fused in glass to make the cathode and is exposed only at the tip of the electrode. The silver–silver chloride anode and the cathode are bathed in an electrolyte composed of 1 mol l^{-1} KCl and sealed to the aqueous environment with a polypropylene membrane. See text.

hydrated ions of the salts in solution. Oxygen diffuses through the polypropylene membrane and is reduced at the platinum cathode, a current flow is induced by a five-step reaction that occurs continuously at the electrode tip (Figure 10.13):

At the Pt cathode (negative pole)	(1) $O_2 + 4e^- \rightarrow 2O^=$
Within the 1 mol KCl electrolyte	(2) $2O^= + 2H^+ \rightarrow 2OH^-$
	(3) $2OH^- + 2KCl \rightarrow 2KOH + 2Cl^-$
At the Ag–AgC1 anode (positive pole)	(4) $2Cl^- \rightarrow 2Cl + 2e^-$
	(5) $Ag + Cl \rightarrow AgCl$

The oxygen ions created at the cathode tip combine with available hydronium ions in the electrolyte to form hydroxyl radicals. These in turn combine with the KCl in the electrolyte to release chloride ions. Chloride ions give up their electrons at the anode, yielding a current flow and an AgCl precipitate on the Ag–AgCl anode.

The reaction occurring at the electrode tip is interesting, easily understood, and underscores two important properties of the Clark electrode. First, the electrical current produced by the electrode is directly proportional to the partial pressure of oxygen within the medium. Second, by its very nature, the oxygen electrode itself consumes

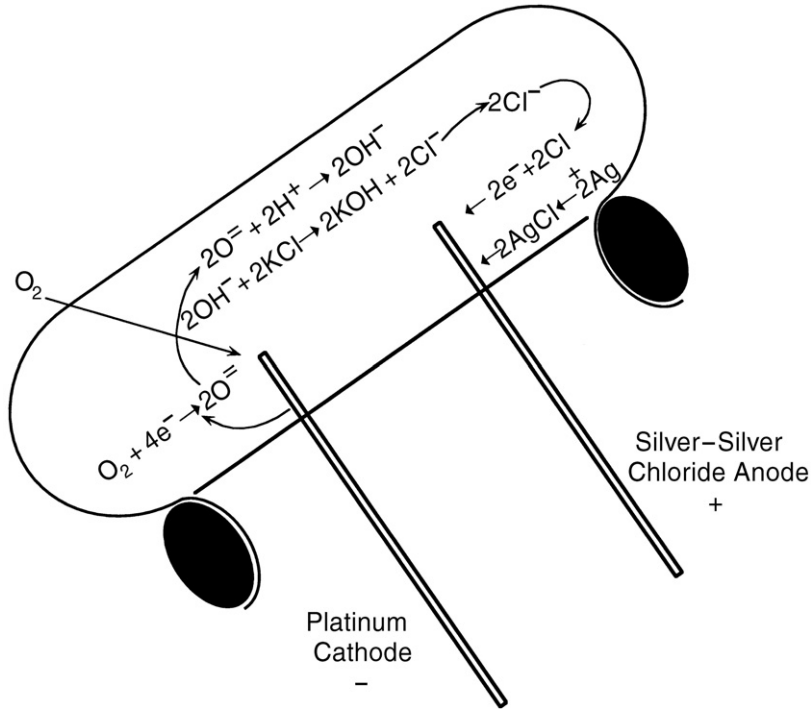

Fig. 10.13 Electrolyte dissociation at the tip of a Clark electrode. Oxygen diffuses through the polypropylene membrane and is reduced at the cathode, forming the oxygen ion. This initiates a series of reactions within the electrolyte that produces an electrical current in direct proportion to the oxygen in solution. See text.

oxygen. This second property of oxygen electrodes is important in their employment as a research tool and bears further examination.

At any interface of a fluid and a surface, such as that at the surface of the electrode membrane in sea water, a stagnant boundary layer forms due to the physics of fluid flow. If simple diffusion of oxygen through the boundary layer and membrane is not adequate to meet the oxygen demands of the electrode, the boundary layer will become depleted in oxygen. The electrode will then need to be stirred to minimize the thickness of the boundary layer and to facilitate diffusion of oxygen into the electrode tip. As we can discern from Figures 10.12 and 10.13 and the five-step reaction given above, the oxygen consumed by the cathode is directly proportional to its size, i.e. the diameter of the wire that is exposed at the tip. If the cathode is small enough, diffusion alone will provide sufficient oxygen to the electrode to obviate the need for stirring. Ideally, the cathode diameter should not be greater than 15 μm to eliminate the need for stirring.

A disadvantage of oxygen electrodes this small is the very low current output which requires substantial amplification. The need for amplification can make the electrodes more susceptible to electrical noise if they are not used with a well designed circuit. Another way of addressing the problem of stirring is to pulse the polarizing voltage of the electrode (Langdon 1984). To allow the electrolytic dissociation to proceed at the electrode tip as described above, a potential difference of 0.6 to 0.8 V must be maintained between the anode and the cathode of the electrode (Figure 10.12). If the polarizing voltage is turned off, the reaction at the tip will cease and the boundary layer at the tip of

the electrode will no longer be depleted in oxygen by the consumption of the electrode itself. In this manner, an electrode with a larger cathode can be used without the need for stirring. There is no reading taken during the time the electrode is off between pulses, making this design a little reminiscent of sequential sampling using the Winkler technique. Nevertheless, the electrodes employing this circuit design are widely used and have a good reputation for accuracy.

CHAMBERS

Apart from three basic requirements, the design of chambers for sealed jar respirometry is limited only by the needs and creativity of the investigator. The three basic requirements are, first, that the chamber be able to accept the electrode tip; second, that the chamber–electrode combination are sealed to the atmosphere; and third, that there is some means for rigorously controlling temperature within the chamber. Two useful chamber designs are shown in Figures 10.14 and 10.15 (cf. Quetin and Mickel 1983). In Figure 10.14 the tip of a syringe is cut off and the electrode itself forms an opposing plunger to the syringe plunger. In Figure 10.12, a water-jacketed lucite chamber has a chimney that mates with an O-ring on the barrel of the electrode. The syringe barrel chamber can be submerged within a water bath for temperature control while the water-jacketed chamber is connected to a circulating refrigerated water bath.

The design shown in Figure 10.14 has been successfully employed for respiratory determinations on individual copepods and fish larvae as small as 2 mm using unstirred electrodes. The design in Figure 10.15 is more suitable for larger species such as euphausiids and amphipods and can be used with stirred or unstirred electrodes. The cut-off for the minimum size of animal that is amenable to being an experimental subject

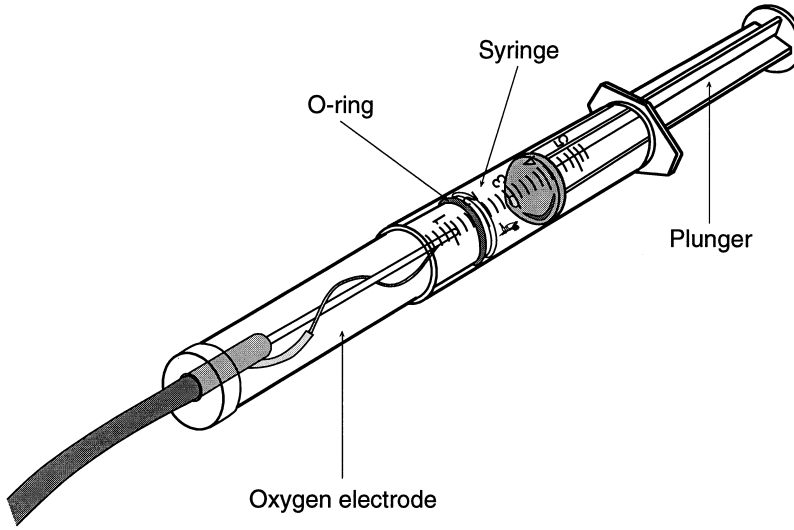

Fig. 10.14 A microrespirometer using an oxygen electrode and a cut syringe. The same O-ring that seats the electrode membrane seals the respirometer to the atmosphere. The assembled syringe–electrode combination as shown is placed in a water bath for temperature control. Alternatively, one can use the same principle with a luer-lock valve on the end of the syringe and using the electrode in place of the syringe plunger. The valve obviates the need for cutting the syringe.

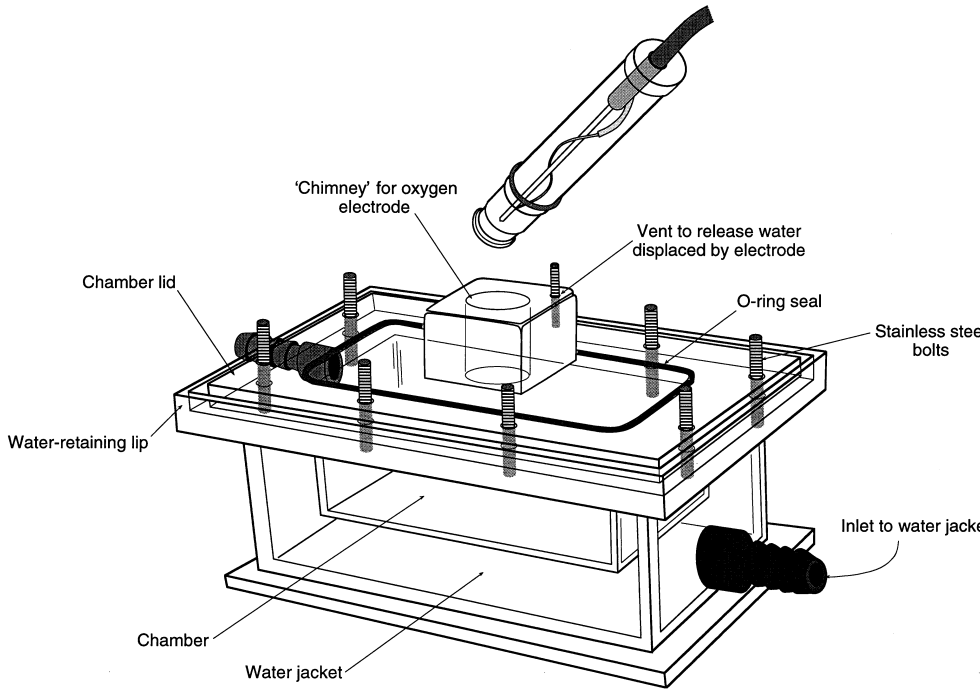

Fig. 10.15 A water-jacketed chamber for measuring oxygen consumption in larger zooplankton. The O-ring midway up the oxygen electrode makes a gas-tight seal on the walls of the chimney. A water-retaining lip allows the chamber to be overfilled to aid in eliminating bubbles. The chamber lid seats on an O-ring seal and is held down with wing nuts. The entire chamber is manufactured from lucite. Design by Joe Donnelly.

with stirred electrodes must depend on the good judgment of the investigator. Clearly, if an animal is swimming hard to maintain a position within the respiratory chamber against the current created by the stir bar, stirred electrodes are not appropriate. In no case is it appropriate to use stirred electrodes with gelatinous species.

Most investigators will be using electrodes available from commercial sources. Many come with choices of chamber types that are designed to mate with their electrode; some are very low volume systems that are suitable for use with zooplankton. If the investigator has just spent a considerable sum of money on an electrode system, he or she will be loathe to put their electrode in a lathe to modify it. In those cases the creativity and machine shop work will have to be restricted to the respiratory chamber. The investigator should, where possible, opt for flexibility in their chosen system.

PITFALLS

Temperature is a critical factor in respiratory determinations with the oxygen electrode, not only because of the effect of temperature on the metabolism of ectotherms but because oxygen electrodes are very sensitive to temperature. In fact, electrodes are more sensitive to temperature than they are to oxygen because the electrochemical dissociation occurring at the electrode tip is governed by the same Q_{10} rule as any biochemical reaction. Thus, while the solubility of oxygen at 10 °C is 123% of that at 20 °C, the current

flow generated at the electrode tip at 10 °C will be about 50% and so will the electrode output. Some commercially available electrodes are available with temperature compensation but keeping experimental and calibration temperatures identical is the simplest and best procedure.

When using the small volumes typical of experiments with individual zooplankton, bubbles can be a large potential problem in respiratory determinations. Oxygen is about 30 times more concentrated in air than in sea water; a very small volume of air can perturb the accuracy of a respiratory determination made in, for example, 2 ml of sea water. The investigator must be meticulous in removing all bubbles from the respiratory chamber. The best way to prevent bubbles is to avoid introducing them into the chamber to begin with. Siphoning water, using a large syringe, or pouring gently into your respiratory chamber will prevent introduction of bubbles. Once bubbles are in, microbubbles can be chased with larger bubbles or brushed out with small artist's brushes. Do not introduce cold water into a respiratory chamber and allow it to warm up. Bubbles will form as the solution degasses due to the reduced solubility of gases at warmer temperatures. Allow the water to equilibrate to experimental temperature first.

EXPERIMENTAL PROTOCOL

Electrodes are best calibrated immediately prior to, and directly after, experiments. In most cases, a high calibration is obtained by gentle aeration of the electrode in a calibration bath at experimental temperature or by using a combination of stirring and aeration. A low calibration is obtained by using a more violent bubbling with nitrogen gas in the calibration bath to drive off the oxygen in solution. Sodium sulphite is sometimes used to obtain a low calibration, but there is potential for poisoning a platinum cathode (see the five-step reaction given above) and it should be used with caution. Nitrogen gas, though inconvenient, does nothing but purge the oxygen from solution. Electrodes should ideally come to within 3% of electrical zero at the low end. Electrodes should be stable at the low and high ends and there should be little or no difference in high and low calibrations before and after the run. Differences of greater than 5% in the pre- and post-run high calibrations constitute grounds for rejection of a run. Linearity is assumed between the low and high points on the calibration.

The sea water to be used as a respiratory medium should be well filtered to remove microorganisms. The author (J.J. Torres) uses membrane filters (0.45 μm pore size) and has had little problem with control rates. Experimental subjects should be transferred to filtered water at experimental temperature prior to introduction into the respirometer as a rinsing step. After a satisfactory calibration has been obtained, the experimental subject is introduced to a bubble-free respiratory chamber and, taking extreme care not to introduce bubbles or disturb the subject more than absolutely necessary, the chamber is sealed, the electrode inserted, and the run is allowed to begin. It is best if visual stimulation is kept to a minimum by shading or isolating the animal in some way. If stirring is to be used, insuring that the magnetic stirring motor is fully warmed up prior to the run will prevent the magnetic stirrer speed-up that can result in homogenization of your experimental animal. It is best to avoid stirrer–hotplate combinations because both malfunctions in the hotplate and oversights due to fatigue can result in an unintended bouillabaisse instead of an experiment.

Control runs are used mainly to check for contributions by microorganisms to the total measured oxygen consumption rate. Since most microorganisms will be introduced by the experimental subject, it is best to take control rates at the termination of a run by removing the subject, replacing its volume with fresh filtered sea water, and

re-starting the run. Keeping respiratory chambers clean and using only filtered sea water for electrode calibrations and respiratory runs will go a long way to preventing errors when measuring control rates. Other uses for control runs are to check for electrode drift and oxygen consumption by the respiratory chamber or respiratory medium. In the latter two instances runs would be set up as normal, but without an animal.

Antibiotics are sometimes added to the filtered sea water employed as a respiratory medium to prevent microbial growth in the respiratory chamber. Those most commonly used are penicillin, streptomycin, and neomycin at concentrations of 25 to 50 mg l^{-1}. The authors' (J.J. Torres) experience has been that microbial growth is a negligible problem within the time frame of a typical run (8 to 12 h) when working with filtered sea water at temperatures less than 15 °C. Microbial growth needs to be more carefully monitored at higher temperatures and antibiotics can help to control this. Runs greater than 12 h in duration need to be checked carefully for microbial contributions at any temperature but especially at temperatures above 15 °C.

The recording apparatus should be consulted frequently. Notes on animal activity and condition should be made at intervals as unobtrusively as possible. Low oxygen becomes stressful to most pelagic species at about 25% of air saturation (e.g. Donnelly and Torres 1988; Torres *et al.* 1994); they can no longer maintain a constant oxygen consumption rate below this level. It is best if the chamber volume allows the animals to remove 5% to 10% of the oxygen in the chamber each hour.

CALCULATIONS AND TREATMENT OF DATA

Calculations of metabolism with data from oxygen electrodes use the same principles regardless of the recording system. Two leaps of faith are required when equating the electrical output of oxygen electrodes to quantities of dissolved oxygen. The first is that the electrical current produced by the electrode varies directly and linearly with the dissolved oxygen in the range from zero to air saturation. The second is that the high calibration point is equivalent to the oxygen solubility at the temperature and salinity in the calibration bath.

The assumption of linearity can be checked by testing the output of the electrode at air saturation as you would during a normal calibration and then at oxygen saturation by using a tank of pure oxygen. This will then give you the output at 0%, 21% and 100% O_2, enough points to check for linearity of response. Alternatively, you can use the Winkler method or a gas chromatograph to check at various points between zero and air saturation. The author (J.J. Torres) has never noted a problem with linearity.

The assumption that the high calibration point of the electrode is equivalent to the oxygen solubility at the calibration temperature and salinity is necessary to convert the electrical output of the electrode to the units of dissolved oxygen that are of use to us. Values for oxygen solubilities over a wide range of temperatures and salinities are available from the exacting research of marine chemists. The most widely accepted numbers for oxygen solubility are those from the equation of Weiss (1970), who, using the data of Carpenter (1966) and Murray and Riley (1969), described the relation between temperature, salinity and oxygen solubility. Weiss's equation, as reported in Kester (1975; equation 8.11) is:

$$\ln c^* = A_1 + A_2(100/T) + A_3 \ln(T/100) + A_4(T/100) + S‰[B_1 + B_2(T/100) + B_3(T/100)^2] \quad (10.16)$$

for c^* = O_2 solubility in μmol kg^{-1} based on the data of Carpenter (1966) then: A_1 = −173.9894, A_2 = 255.5907, A_3 = 146.4813, A_4 = −22.2040, B_1 = −0.037 362, B_2 = 0.016 504, and B_3 = −0.002 0564, and for c^* = O_2 solubility in $cm^3\ l^{-1}$ based on the data of Murray and Riley (1969) then: A_1 = −173.4292, A_2 = 249.6339, A_3 = 143.3483, A_4 = −21.8492, B_1 = −0.033 096, B_2 = 0.014 259, and B_3 = −0.001 7000.

Values assume an atmosphere of 20.94% O_2 at 760 mmHg total pressure and at 100% relative humidity. As an alternative to using the equations above, one can look up the tabulated values in either of the original papers and use those. The differences in solubilities between the original papers and Weiss's improved numbers are small enough ($\leq 0.03\ cm^3\ dm^{-3}$) for our purposes to make the error an acceptable one. Reading any of the original papers on oxygen solubility and especially Kester's treatment will familiarize the investigator with units, accuracy, and precision in determining values for dissolved gases.

The electrode output at the low and high calibration points is assumed to be 0% and 100% of air saturation respectively. The O_2 solubility determined from Weiss's equations or from the original tabulated data at the experimental temperature and salinity yields the total dissolved oxygen at air saturation. The investigator can then compute the oxygen consumption from the percent change in total electrode output with time. For example:

- electrode output at high cal is 100 mV which equals O_2 solubility at air saturation: 7.10 ml l^{-1}
- electrode output at low cal is 0 mV
- total volume of respirometer: 5 ml
- total oxygen in system: 35.5 μl
- wet mass of experimental subject: 10 mg

After 1 h the electrode reads 90 mV, 10% of oxygen in the system has been removed. Oxygen removed from system is 3.55 μ1, oxygen consumption rate is 0.355 μl O_2 mg wet mass $^{-1}\ h^{-1}$.

10.7.3 Enzymatic method – electron transfer system (S. Hernández-León)

The Electron Transfer System (ETS) is the pathway responsible for transfer of electrons to oxygen, the final electron acceptor. As a consequence, its activity is responsible for oxygen consumption by both the cell and organism, and can be used as an index, or biochemical proxy for zooplankton respiration in the sea (section 10.4.1). ETS may be characterized as a multi-enzyme, multi-substrate system, and its activity is determined in substrate saturating conditions, i.e. at the maximal rate (V_{max}). The substrates are nicotinamide adenine dinucleotide (NADH), nicotinamide adenine dinucleotide phosphate (NADPH), and succinate, which saturate microsomal and mitochondrial ETS. The tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) is used as an artificial electron acceptor to measure the electron transmission rate: a mole of oxygen consumed will be equivalent to two moles of INT reduction. The slightly yellow INT is reduced to the pink colored formazan and the intensity of the color is read with a spectrophotometer.

The enzymes involved in the electron transfer system are NADH dehydrogenase (EC 1.6.99.3), NADPH dehydrogenase, (EC 1.6.99.6), NADPH-cytochrome reductase (EC 1.6.2.4) and succinate dehydrogenase (EC 1.3.99.1). The activity of these enzymes are recorded in the same assay and the ETS activity is not necessarily the addition of the

activities of the different enzymes of an enzyme complex (Savenkoff *et al*. 1995; Gómez *et al*. 1996). NADH dehydrogenase is the most active dehydrogenase in zooplankton material.

Since the work by Packard (1971) the assay method has been modified by the addition of Triton X-100 (Owens and King 1975), and by changes in pH of the buffers (Kenner and Ahmed 1975). The assay method described here is essentially the same as that of Kenner and Ahmed (1975) and Packard and Williams (1981), modified slightly by Gómez *et al*. (1996) for zooplankton.

REAGENTS

1) Homogenization buffer 0.05 mol phosphate buffer, pH 8.0, containing 6.72 g Na_2HPO_4, 0.362 g KH_2PO_4, 1.5 g polyvinyl pyrrolidone (PVP), 18.5 mg $MgSO_4.7H_2O$ and 2 ml Triton X-100, in 1 l of deionized-distilled water (DDW). Store at $-20\,^{\circ}C$.
2) Daily homogenization buffer Add, immediately before use, an appropriate amount of 0.1 mol NaCN (e.g. 0.735 g NaCN in 150 ml of DDW) to obtain a final concentration of 2 mmol NaCN (e.g. 1 ml NaCN solution in 49 ml of the homogenization buffer).
3) Substrate buffer Prepare a solution of 0.05 mol phosphate buffer, pH 8.0, containing 6.72 g Na_2HPO_4, 0.362 g KH_2PO_4, 36 g disodium succinate hexahydrate and 2 ml Triton X-100. Store at $-20\,^{\circ}C$.
4) Daily substrate solution Just before the assay, dissolve 15 mg NADH and 5 mg NADPH in 24 ml of the substrate buffer.
5) Blank substrate buffer Prepare the substrate buffer (3) without substrates as a blank. Store at $-20\,^{\circ}C$.
6) INT solution Prepare a solution of the tetrazolium salt 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrasolium chloride (INT) in DDW up to a concentration of 4 mmol (e.g. 1 g INT in 500 ml DDW). Filter the solution and store at $-20\,^{\circ}C$ in the dark.
7) Quench solution 50% formalin (36%) plus 50% 1 mol H_3PO_4, pH 2.5. Store under refrigeration (0–4 $^{\circ}C$).

Bacterial contamination should be kept to the absolute minimum when mixing up solutions. To achieve this, working in very clean conditions is essential, and solutions should not be kept for long at room temperature.

SAMPLE PREPARATION AND PRESERVATION

Sorted live zooplankton is ready to use for the assay after removing excess water with blotting paper. For mixed zooplankton, samples in the net cod-end are poured onto a 100 μm mesh sieve and washed with filtered sea water. The mesh sieve can then be put over blotting paper to remove excess water. If samples are too large to apply this procedure, they may be gently vacuum filtered. Samples are scraped off the filters using a spatula for the assay. Fresh samples are best, but freezing samples may be inevitable in the experiments at sea.

Ahmed *et al*. (1976) observed that although a cell-free extract of the copepod *Calanus finmarchicus* frozen at $-2\,^{\circ}C$ lost considerable ETS activity in 24 h, no measurable loss was found when intact specimens were stored at $-20\,^{\circ}C$ or below for at least one week. No significant loss of ETS activity during storage for 34 days at $-20\,^{\circ}C$ has been reported for two fish species (Ikeda 1989b). On the other hand Båmstedt (1980) found

that frozen intact specimens of *Acartia tonsa* lost 50% of their ETS activity within 30 h at −20 °C and virtually no activity was detected after 72 h.

Recently, Gómez *et al.* (1996) observed no significant loss in ETS activity on zooplankton samples preserved in liquid nitrogen (−196 °C) for at least 2 months. Therefore, preservation of zooplankton samples in liquid nitrogen is recommended for the ETS assay, whenever the immediate assay is not practicable.

ASSAY PROCEDURE

1) The zooplankton sample is homogenized with or without GF/C filters (Notes for the assay procedure and calculations, section 10.7.3, part a) in a teflon-glass tissue grinder (Notes for the assay procedure and calculations, 10.7.3, part b) for 2 min at 0 to 4 °C in a known volume of homogenization buffer (approximately 10–20% (v/v) homogenate) (Notes for the assay procedure and calculations, section 10.7.3 part c)
2) The homogenate is then poured into a chilled graduated cylinder in order to record the volume. The difference between this volume and the volume of buffer used for homogenization is the wet volume of the sample.
3) Centrifuge (Notes for the assay procedure and calculations, section 10.7.3 part d) for 10 min at 1000 *g* at 0 to 4 °C. Because the method is very sensitive the supernatant must be diluted in homogenization buffer. A 3 to 61 × dilution is often used depending on the concentration of sample in the homogenate.
4) Take a 1-ml aliquot of diluted supernatant, mix with 3 ml of the daily substrate solution and 1 ml of INT solution, and incubate for 20 min at the *in situ* temperature in the dark. The color of the reaction mixture changes during the incubation period to pink due to the reduction of the INT to formazan. Red color is indicative of excess sample. Concurrently, another 1 ml of the diluted supernatant is incubated in the blank substrate buffer (substrate blank). Get another blank to account for the reaction between substrate and INT (reagent blank). Take 1 ml of daily homogenization buffer and incubate using the same procedure as with the sample.
5) Stop the reaction with 1 ml of the quench solution.
6) Read absorbance with a spectrophotometer at 490 nm and at 750 nm as the turbidity base line. The increase in absorbance can be monitored continuously by using a temperature-controlled cuvette placed in a spectrophotometer. New technologies such as microplate readers and robotic samplers can also be used to save time when a large number of samples has to be processed.

In order to obtain specific rates, measure protein contents of the homogenized samples obtained at step 3, by the method of Lowry *et al.* (1951) using bovine serum albumin as the standard, or modified Lowry *et al.*'s method by Peterson (1977, 1983) for samples with very low protein content.

Please note that Savqenkoff *et al.* (1995) suggested the use of a yeast (*Saccharomyces cerevisiae*) as a reference to standardize ETS assays. The yeast is available in dried form which can be weighed and stored. The procedure consists of weighing five samples of the yeast from 1 to 5 mg with an ultramicrobalance using its large grains. Follow the steps given in the assay procedure but homogenize together with GF/C filters (diameter: 25 mm) in order to disrupt mitochondria walls. Plot weights against ETS activity. ETS activity data from dissimilar assay procedures by different workers could be compared with the reference material.

CALCULATIONS

ETS activity is calculated as:

$$ETS = 60 \times H \times AS \times COD/(\mathrm{INT} \times T \times L \times f), \tag{10.17}$$

$$COD = [(AOD \times AS) - (BOD \times BS) - (FOD \times FS)]/AS, \tag{10.18}$$

where H is the homogenization volume, AS is the assay volume, BS is the blank volume, FS is the reagent blank volume, AOD is the difference between the spectrophotometer readings at 490 and 750 nm of the assay (BOD and FOD are the same differences for substrate blank and reagent blank, respectively), T is the incubation time (minutes), f is the volume of homogenate used in the assay (ml), L is the path length of the spectrophotometer cuvette cell used (usually 1 cm), 60 is the factor to convert minutes to hours, and INT is the standardization factor (see below) which converts the absorbance of the produced INT-formazan. The ETS activity is given in μl $O_2 h^{-1}$ but could be expressed in electron equivalents (μEq e^-) by dividing the ETS activity by 5.6 (1 mol of O_2 = 4 Eq e^- and $ETS \times 4/22.4 = ETS/5.6$).

ETS assays are normally run at one temperature during a set of experiments, or during a cruise. The experimental temperature may not be the *in situ* temperature. ETS activity obtained at a given incubation temperature may be converted to the activity at *in situ* temperature using the following equation:

$$ETS_{in\ situ} = ETS_{incu} \times \exp[E_a(1/T_{incu} - 1/T_{in\ situ})/R]$$

The value of the Arrhenius activation energy (E_a) is about 15 kcal mol^{-1} in *Calanus finmarchicus* and in the range of 13 to 16 kcal mol^{-1} for a variety of other plankton. The symbol R is the gas constant (1.987×10^{-3} kcal mol^{-1}) and T is the absolute temperature (Packard *et al*. 1975).

Convert ETS activity to respiratory oxygen consumption rate using the correlation between respiration rate and ETS activity (R/ETS ratio). The R/ETS ratio is theoretically around 0.5. Problems associated with the R/ETS ratio are detailed in ETS activity, section 10.4.1.

INT STANDARDIZATION

Because of different qualities (or different trade marks) of INT, it is highly recommended that you standardize it using the procedure given below.

REAGENTS AND SOLUTIONS

1) 0.1 mol phosphate buffer Add 13.6 g KH_2PO_4 to 1 l of DDW. Add 14.2 g Na_2HPO_4 to 1 l of DDW. Mix 808 ml of the Na_2HPO_4 solution with 192 ml of the KH_2PO_4 solution. Add 10 ml Triton X-100 to the mixed solution. Store at $-20\,^{\circ}C$.
2) INT color reagent Add 8 mg phenazine methosulphate (PMS) and 0.4 ml Triton X-100 to 16 ml of the INT solution to be standardized.
3) 0.05 mol phthalate buffer Dissolve 2.55 g phthalic acid in 172 ml of DDW. Add 51 ml of 0.1 *N* HCl and 22 ml Triton X-100. Adjust to pH 3.0 if necessary. Dilute to 250 ml.

Standardization procedure

1) Prepare a solution of 0.043 μmol ml^{-1} of NADH in 0.1 mol phosphate buffer, pH 7.4 (e.g. 2 mg NADH in 60 ml of 0.1 mol phosphate buffer).
2) Prepare a series of dilutions to obtain different concentrations between 0 and 0.258 μEq e^- in a total volume of 3.0 ml.

3) Add 2.0 ml of INT color reagent to each tube and incubate in darkness for exactly 1 min at room temperature. Stop the reaction with 1.0 ml of 0.05 mol phthalate buffer. Total volume of the assay is 6.0 ml. Read absorbance at 490 and 750 nm with a spectrophotometer.

The factor used in the standardization is calculated converting the μmol NADH ml^{-1} to μEq e^- (multiply μmol ml^{-1} by 2). Plot absorbance against the μEq e^- and obtain a slope from a least square linear regression. Then, multiply the slope by 1.24 obtaining the value to convert the absorbance of the INT-formazan produced in the calculation of ETS. This value should be around 1.42 because the INT-formazan has a molar extinction coefficient (A_{490}) in 0.133% Triton X-100 solution of 15.9×10^3 mol^{-1} cm^{-1}. INT requires two electrons to be reduced to formazan and oxygen requires four electrons to be reduced to water. Therefore, the extinction coefficient of INT-formazan is equivalent to a 0.5 molar solution or 11.2 l O_2 1^{-1}. Because the solvent volume is taken into account in the ETS assay, the equivalent absorptivity of 1 μl O_2 per ml of solvent will be $15.9 \times 10^3/(11.2 \times 10^3) = 1.42$.

NOTES FOR THE ASSAY PROCEDURE AND CALCULATIONS

a) The use of GF/C filters is to facilitate disruption of mitochondria walls. However, Gómez *et al.* (1996) found no significant difference between ETS activities of mixed zooplankton samples processed with and without GF/C filters, and suggested that chitin exoskeleton of crustacean zooplankton had the same function as GF/C filters for crustacean zooplankton samples or mixed zooplankton samples which include crustaceans.

b) A Polytron ultrarapid homogenizer may be used instead, as Gómez *et al.* (1996) noted no significant differences between the resultant ETS activities of samples processed with teflon-glass grinder and the Polytron ultrarapid homogenizer. Sonication may also be an alternative, but the author (S. Hernández-León) has not tested this yet.

c) Crude homogenates of zooplankton can be maintained in a water-ice bath (0–4 °C) for up to 90 min without any significant loss in ETS activity (Båmstedt 1980; Gómez *et al.* 1996) thus allowing preparation of several samples before the start of the assay.

d) While Gómez *et al.* (1996) observed no significant differences in ETS activities of samples centrifuged before and after the incubation, the procedure given here (centrifuge before the incubation) is recommended to avoid an increment of turbidity in the assay.

10.7.4 Enzymatic method – lactate dehydrogenase and citrate synthase (J.J. Torres and S.P. Geiger)

Lactate dehydrogenase (LDH) and citrate synthase (CS) catalyze key reactions of anaerobic and aerobic intermediary metabolism respectively. LDH is the terminal enzyme of anaerobic glycolysis as the pathway is conventionally presented; it catalyzes the conversion of pyruvate to lactate and is one of the three rate-limiting steps in the pathway. CS catalyzes the condensation reaction between acetyl-CoA and oxaloacetate to form citrate, which is the first and rate-limiting step in the Krebs cycle. The rationale behind using LDH and CS as indicators of metabolism is, first, that the activity of each enzyme is indicative of the metabolic poise (cf. Hochachka and Somero 1984), or activity, of its respective pathway, and second, that the activity of the pathway relates

directly to the metabolism and therefore the overall physiological condition of the whole organism (see Enzymes of intermediary metabolism in section 10.4.2).

LDH is best applied as a physiological indicator in ichthyoplankton (Clarke *et al.* 1992; Brightman 1993). The data available suggest that LDH activities in ichthyoplankton correlate well with growth and physiological condition, and are further supported by years of research on the biochemical physiology of vertebrates (Hochachka and Somero 1984). Less is known about the function of LDH in crustaceans and other zooplankton. It is present in detectable quantities (e.g. Thuesen and Childress 1993a, 1993b, 1994; Geiger *et al.* 1996; Thuesen *et al.* in press) but its function in invertebrate metabolism is less well described and variability even between species of a general taxonomic group, e.g. calanoid copepods, can be very high. For example, results on LDH activities in Antarctic copepods (S.P. Geiger and J.J. Torres unpublished data) using the techniques described in Lactate dehydrogenase, section 10.7.4 suggest low, but detectable, levels of LDH, whereas results described in Thuesen *et al.* (in press) suggest very high activities in deep-living copepods of the California Current. Invertebrates exhibit considerably more creativity with their glycolytic endpoints than do vertebrates (Hochachka and Somero 1984). Thus, the meaning of LDH activity should be evaluated for invertebrate preparations on a case-by-case basis.

CS, while it is a slightly more difficult assay, seems to work equally well on both vertebrate and invertebrate subjects and is especially useful in copepods. We recommend it as a first choice for assaying condition in copepods and LDH as a first choice in ichthyoplankton.

TISSUE PREPARATION AND GENERAL PROCEDURES

Enzyme activities are most stable when tissue is frozen initially in liquid nitrogen, and then stored at $-80\,^{\circ}C$. No loss in enzyme activity occurs for at least several months and for most enzymes, storage at $-80\,^{\circ}C$ allows nearly indefinite storage. Both LDH and CS are fairly stable enzymes and if the ideal situation cannot be achieved, then investigators should do their best to approach them and be wary of deterioration. If a choice must be made, the initial (liquid nitrogen) freezing step is the most critical one and the samples can usually then be stored in a conventional ($-20\,^{\circ}C$) deep-freeze with little loss of activity for a few months. A word of caution: tissues and enzymes do vary in their sensitivity to storage, so these procedures should be viewed as general guidelines and not gospel. Gelatinous organisms and small copepods are particularly sensitive to desiccation with long term storage. It is best to determine empirically the sensitivity of your own preparations to storage. Once homogenized, samples should be used within several hours and not be refrozen.

Homogenize tissue in ice-cold 50 mmol imidazole buffer (pH 7.2 at $20\,^{\circ}C$) using either a sonicator or conventional homogenizer. The dilution factor to be used varies with the tissue and its inherent enzyme activity. It is imperative that the dilution factor be known precisely because it is an important element in calculating enzyme activity and expressing it in standard units (see Calculations, section 10.7.4). For tissues with low activity, a dilution factor of 1 to 4 (1 g tissue to 4 ml buffer) is useful, for tissues with more enzyme a 1 to 24 dilution is more appropriate. Large tissue samples (e.g. *Euphausia superba* abdominal muscle) should be ground in chilled ground glass homogenizers, and then briefly sonicated. For small tissue samples (e.g. calanoid copepods, or young < 10 mm fish larvae) sonications usually provide adequate homogenization. Large tissue samples are best centrifuged at 2500 to 5000 *g* for 10 min to reduce particulate interference during the assay.

In some cases it is better to initially homogenize tissues in ice-cold distilled water. If your sample is too small or too delicate to obtain a reliable initial weight (e.g. a small copepod frozen in a cryovial) and you wish to normalize your activities to protein, you can add a known volume of distilled water to the vial and homogenize 'in place' with a sonicator. We have found no significant loss in enzyme activity when compared to homogenizing in buffer, and homogenizing in water allows you to perform multiple assays on single samples. Some buffers, notably Hepes buffer, interfere with the Lowry protein assay and with the Ethidium Bromide technique for determination of nucleic acids. An initial homogenization step in distilled water will allow you to obtain protein concentration in your homogenate, thereby giving you a standard for comparison with other investigators and with other values in your own data set.

Enzyme activity is very temperature-sensitive, a change of 1 °C in the cuvette will result in a 10% change in reaction rate. Thus, a spectrophotometer with thermally controlled cuvette holders (or a plate reader with thermal control) is imperative for determinations of enzyme activity. Cuvettes should be allowed to equilibrate to experimental temperature to ensure a stable, comparable reaction rate.

PITFALLS

It is *extremely* important to measure accurately the volume of homogenate that you add to the reaction cuvette. Use the smallest pipette available for adding homogenate and be certain to wipe off any small drops from the outside of the pipette before dangling it over the cuvette. It is often easiest to use the pipette to stir thoroughly the homogenate into the assay buffer, stirring is important. Properly maintained (calibrated!) automatic pipettes such as the Pipetman are fine for enzyme assays. Some old-timers (such as the senior author and his mentor) prefer to use Lang–Levy mouth pipettes to add the homogenate to the reaction cuvette. A gentle exhalation after dispensing homogenate into the cuvette produces a small flow of bubbles; this, when followed by hand stirring, mixes the homogenate in very thoroughly.

It is important to keep track of the daily order of assays as preparations may change in activity during the day. Protease activity may cause your homogenate to deteriorate with time, cocktails may evaporate, resulting in an increased concentration of reactants, and a host of other things can potentially affect your results. Keep good notes; if you observe changes you may be able to address them later. *Remember that enzyme assays using NADH should be kept shielded from light as much as possible. NADH is very photoreactive*. Wrapping all beakers in aluminum foil works well. Also try to keep the beakers covered, either with foil or parafilm, to prevent excessive evaporation.

Imidazole buffer changes pH at a rate of -0.02 units/$^{\circ}C^{-1}$. This rate of change is very similar to that observed in animal tissue. All buffers have a characteristic range of change in pH with temperature. You must account for this change if you are mixing up buffers at room temperature and plan to run them at a different temperature. Be sure that you are using a pH electrode that is suitable for use with organic solutions.

The pHs chosen for LDH and CS in the protocols given below (in section 10.7.4 on Lactate dehydrogenase and Citrate synthase) are optimal, or very close to optimal, for the broad array of fishes and crustaceans, including copepods, that have been examined in our (J.J. Torres and S.P. Geiger) laboratory. CS is a mitochondrial enzyme and requires a slightly more alkaline milieu than LDH, a muscle enzyme, to more accurately mimic the conditions in the mitochondrial matrix. We recommend the pHs given below as an excellent place to start your enzyme studies, and if further optimization for your

experimental organism is warranted by the character of your study, a pH series can be run to fine tune the assay. pH is important, for example, the direction of the LDH assay can be reversed at too high a pH.

Other potential sources of error that are easily addressed are dilution effects in your homogenate, i.e. is your homogenate homogeneous? If you suspect problems, a test for linearity by using half and twice the homogenate concentration in successive activity determinations can be performed to test for potential problems in the dilute homogenates typical with zooplankton samples. If you obtain a non-linear result, you can adjust your homogenate volume until reproducibility is obtained.

The substrate concentrations given below are greatly in excess, which will provide you with V_{max} numbers for your homogenates. None the less, if you suspect substrate limitation in your enzyme, testing for linearity by halving and doubling substrate concentrations in the cocktail is a good way of verifying that substrates are not limiting.

LACTATE DEHYDROGENASE (PYRUVATE + NADH + H^+ ↔ LACTATE + NAD^+; ABSORBANCE AT 340 NM DECREASES AS NADH IS OXIDIZED TO NAD^+)

All solutions should be mixed up to achieve pH 7.2 at 20 °C. The final assay medium should contain the following:

- 80 mmol imidazole buffer
- 0.15 mmol NADH
- 5.0 mmol Na-pyruvate.

To make 50 ml of assay cocktail follow these procedures.

1) Make up a stock of 200 mmol imidazole buffer (Sigma No. I-0125): 3.4 g 250 ml^{-1} H_2O.
2) Make up a stock of 50 mmol imidazole buffer, 0.85 g 50 ml^{-1} H_2O. Refrigerated stock of 200 mmol and 50 mmol imidazol buffer can be stored for several weeks.
3) Make 3.25 mmol NADH fresh daily (Sigma No. N-8129), 0.0115 g 5 ml^{-1} 50 mmol imidazole.
4) Make up 100 mmol Na-pyruvate fresh daily (Sigma No. P-2256), 0.110 g 10 ml^{-1} of 50 mmol imidazole (Note: NADH and Na-pyruvate need not be mixed to exactly 5 or 10 ml. Simply weigh out a small amount, then add enough buffer to reach the desired concentration. This will be 0.435 ml buffer mg^{-1} NADH, and 0.091 ml buffer mg^{-1} Na-pyruvate).
5) To make the assay cocktail (make it fresh daily) add 20 ml of imidazole buffer (solution 1), 2.5 ml each of NADH (solution 3) and Na-pyruvate (solution 4), and 25 ml of H_2O.

Add 10 to 20 μl homogenate to 2 ml of the assay cocktail. Observe the change in absorbance at 340 nm for 30–90 s. The slope will decrease rapidly after an initial linear interval. Adjust the homogenate volume to allow the slope of the reaction during the initial linear phase to be easily determined.

CITRATE SYNTHASE (ACETYL-COA + $OXALOACETATE^{2-}$ + H_2O ↔ $CITRATE^{3-}$ + COASH + H^+)(− SH DISSOCIATES FROM COASH AND REACTS WITH DTNB)

All solutions should be mixed up to achieve pH 8.0 at 20 °C. The final assay medium should contain the following:

- 50 mmol imidazole buffer
- 0.1 mmol acetyl-Coenzyme-A (Boehringer-Manheim No. 10197, preferred over Sigma Acetyl CoA)
- 0.2 mmol DTNB (5,5* Dithio-bis(2-Nitrobenzoic acid)
- 0.5 mmol oxaloacetic acid.

To make 50 ml of assay cocktail follow these procedures.

1) Make up a stock of 50 mmol imidazole buffer (Sigma No. I-0125, 0.85 g 50 ml H_2O). This stock can be refrigerated for several weeks.
2) Make 4 mmol DTNB fresh daily (Sigma No. D-8130, 16 mg 10 ml^{-1} 50 mmol imidazole).
3) Make 40 mmol oxaloacetate fresh daily (Sigma No. 0-4126, 53 mg 10 ml^{-1} 50 mmol imidazole). Adjust pH to near 7.0 before bringing total volume to 10 ml (approximately eight drops of 2*N* NaOH or KOH).
4) To make the assay cocktail mix 47.5 ml of imidazole buffer (solution 1), 2.5 ml DTNB (solution 2), and 5 mg Acetyl-CoA (Acetyl-CoA is very expensive, roughly one US dollar per 2 ml assay, so do not make up more assay buffer than you need for that day. By reducing the assay to 1 ml cuvettes you can reduce this cost by 50% and also use less homogenate, allowing smaller tissue samples to be used. However, this may increase the noise in the assay slightly).

Add 10 to 50 μl homogenate to 2 ml assay cocktail to observe the background change in absorbance at 412 nm for 1 to 3 min. Once the slope has flattened out, add 25 μl oxaloacetate and observe the change in absorbance for an additional 2 to 5 min. This slope will often require a 30 to 60 s lag time after the addition of oxaloacetate before becoming linear.

CALCULATIONS

The rate of change of reactant to product during steady state conditions at the initial velocity of an enzyme assay is a measure of the activity of an enzyme. International units of enzyme activity (IU) are μmoles substrate converted to product per minute. IUs of enzyme activity are most often standardized to either the weight of tissue being assayed (e.g. IU per mg wet weight) or to protein concentration (IU per mg protein). If the enzyme is saturated with substrate, and not inhibited by product, then under constant conditions the rates of enzyme reaction reduces to the following:

$$\textbf{Rate} = k[E].[s] \tag{10.20}$$

where k is specific to most species and assay conditions, $[E]$ is the concentration of enzyme, and $[s]$ is the concentration of substrate. Remember that you are not measuring exact *in vivo* rates but only consistent *in vitro* rates.

The Beer–Lambert law describing the relation between the optical density of a solution and its concentration says:

$$A = e \times C \times L \tag{10.21}$$

where A is absorbance, e is the extinction coefficient of solute in $l\,mol^{-1}$-cm (also seen as $l = 1000\ cm^3$, making the equation $cm^2\ mol^{-1}$), for NADH, $e = 6.22 \times 10^6$ at 340 nm, and for DTNB, $e = 13.6 \times 10^6$ at 412 nm, C is the concentration of substance in $mol\,l^{-1}$, and L is length of light path in cm (generally 1 for the most common cuvette size).

Setting the equation above equal to concentration we get: $C = A/(e \times L)$. The enzyme activity is the change in concentration of substrate per unit time, which in turn is described by the change in absorbance per unit time. To express enzyme activity in μmole product converted per minute:

$$\begin{aligned}(\Delta A/\mathbf{min}^{-1})/(e \times L) &= (\Delta A\ \mathbf{min}^{-1})/[(6.22 \times 10^6\ \mathbf{cm}^2\ \mathbf{mol}^{-1}) \times 1\ \mathbf{cm}] \\ &= (\Delta \mathbf{mol\ ml}^{-1})\ \mathbf{min}^{-1} \end{aligned} \quad (10.22)$$

The Δmol ml^{-1} min^{-1} can then be multiplied by the concentration of tissue in the homogenate to standardize to wet mass.

For example, you have added 10 μl of a 1:4 homogenate to 2 ml of reaction cocktail to determine LDH activity. You obtain a rate of change in absorbance of 0.026 absorbance units per min. Thus, $0.026/[(6.22 \times 10^6\ \text{cm}^2\ \text{mol}^{-1}) \times 1\ \text{cm}] = 4.2 \times 10^{-9}$ mol min^{-1} ml^{-1} $= 4.2 \times 10^{-3}$ μmol min^{-1} ml^{-1}. For 2 ml of cocktail, total activity is: 4.2×10^{-3} μmol min^{-1} ml^{-1} $\times$ 2 $= 8.4 \times 10^{-3}$ μmol min^{-1}. For a 1:4 dilution the concentration of tissue is 0.2 g 1000 μl^{-1}. You used 10 μl of the 1:4 homogenate, so to obtain the activity for 1 g of tissue, (1000 μl 0.2 g^{-1}) $\times$ 1/10 μl = 500, and $500 \times 8.4 \times 10^{-3}$ μmol min^{-1} = 4.2 μmol min^{-1} g^{-1} wet mass.

More simply, for LDH: (2 ml cocktail/6.22) $\times$ (ΔAbsorbance/min) = No. of μmoles substrate converted to product per minute. For CS simply substitute 13.6 (extinction coefficient) for 6.22.

FURTHER READING

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CS and LDH assays were taught to Jose Torres by George Somero of Stanford University, who refined them and initially applied them to marine species as indicators of

metabolic status. Discussions with Pat Walsh and Liz Clarke of the University of Miami were instrumental in initially applying the techniques to individual plankters. The text has been distilled from our own experience as well as from numerous handouts and protocols that we have accumulated through the years. We gratefully acknowledge the help of our colleagues in passing on their knowledge, a tradition we are continuing here.

10.8 EXCRETION (T. Ikeda)

Live zooplankton release a variety of substances into the surrounding water. Those substances may be classified into solid forms (feces, molts for crustaceans, gelatinous 'houses' for appendicularians, etc.) and liquid forms. We will consider only the liquid forms in our treatment of 'excretion'. Zooplankton excretion has been measured as dissolved nitrogen (total-N, amino-N, urea-N and ammonia-N) and dissolved phosphorus compounds (total-P, organic-P and inorganic-P). Filtered sea water (see Washing/experimental sea water, section 10.7.1) has been used in most of the previous determinations of zooplankton excretion. From a technical viewpoint, the use of filtered sea water is simple in design and free from complicated corrections for simultaneous uptake/release of nitrogen or phosphorus by prey organisms. However, excretion rates determined on zooplankton placed in filtered sea water (i.e. no food) may provide conservative estimates (Feeding, section 10.3.1) Not only food, but various internal and external conditions of zooplankton are also known to affect the results (Technical problems, section 10.3.1). These methodological constraints need to be kept in mind for extrapolating laboratory-determined excretion data to field populations.

The two methods commonly used to measure excretion rates of marine zooplankton, are described in sections 10.8.1 and 10.8.2.

10.8.1 Single end-point method

Preparation of bottles and filtered sea water, and procedures of washing and incubation of zooplankters are fully described in Oxygen consumption – Winkler titration (T. Ikeda), section 10.7.1. Sample water withdrawn at the end of experiment is used for the analysis of nitrogen and phosphorus compounds instead of dissolved oxygen analysis. Excretion rate (E) of nitrogen and/or phosphorus compounds is calculated as;

$$\begin{aligned} E &= [(C_{t'} - C_0) - (C_t - C_0)] \times (V_c - V_z)/[t \times (N \text{ or } W)] \\ &= [(C_t - C_t] \times (V_c - V_z)/[t \times (N \text{ or } W)] \end{aligned} \tag{10.23}$$

where C_0 is the concentration of the compounds at the beginning of incubation; C_t and $C_{t'}$ are the concentrations in control and experimental bottles respectively, at the end of incubation; V_c and V_z are the volumes of experimental bottles and zooplankton, respectively, t is the incubation time; N is the number of specimens, and W is the mass of specimens used. Note that C_0 is canceled out in the calculation. It is not necessary to determine it in the experiment in which filtered sea water is used. V_z is estimated from wet weight of specimens assuming 1 ml = 1 g wet weight, but is usually negligible.

E is thus expressed per individual or per unit weight (weight-specific rate) of zooplankters. Weight is often not mentioned in publications where the weight-specific

expression is used. In either expression of E, the weight of zooplankters should be provided with E so that other researchers can convert the results into both expressions.

Simultaneous analyses of dissolved oxygen and ammonia/inorganic phosphorus allow calculation of 'metabolic quotients' such as O:N (oxygen consumption:ammonia excretion), N:P (ammonia excretion:inorganic phosphate excretion) and O:P ratios (oxygen consumption:inorganic phosphate excretion) as indices of metabolic substrate (Metabolic quotient, section 10.3.3). This is an advantage of the 'Oxygen consumption – Winkler titration' method for excretion measurements.

10.8.2 Time-course method

The single end-point method described above assumes a constant excretion rate for the incubation period, but this may not always be the case. Time-course measurements can detect any changes in excretion rates during the course of an experiment. In a typical time-course measurement of ammonia and inorganic phosphate excretion, glass or polycarbonate bottles or beakers of appropriate volume may be used since the filling of sea water to the top of the container (i.e. no air space) is not necessary. These bottles or beakers should be cleaned with an acid solution (6N HCl). Containers are filled with a known volume (2.00 l in this example) of filtered sea water. Unlike the 'single end-point method' (Single end-point method, section 10.8.1), washing of specimens prior to incubation is not very critical in this method. After introduction of the zooplankton, each container is sealed with lids (for bottles) or parafilm (for beakers) to avoid contamination. The experiment is ready to run. A water bath with a temperature control unit is best for incubating the containers, which should also be kept in dark or dim light. During the course of the experiment, duplicate water samples for both ammonia and inorganic phosphate (10 ml $\times$ 4 = 40 ml) are withdrawn first at the start and then every 1 h up to 6 h. Because of this design, the volume of sea water in the container gradually declines during the course of the experiments. Since the density of specimens may affect the excretion rates (Container size/crowding, section 10.3.1), excessive depletion of sea water ($>70\%$ of the initial volume) is highly undesirable. A plot of ammonia or inorganic phosphate versus time will reveal whether the excretion rates are constant with time or not. Since the volume of sea water decreases during the course of an experiment using this method, the best way of expressing the data is not as concentration versus time but as actual amount per container (concentration $\times$ volume of sea water) versus time. When the increase of ammonia or inorganic phosphate with time is linear, excretion rate E can be calculated from the slope of the regression line (i.e. the increase in the amounts of ammonia or inorganic phosphate per unit of time) divided by the number of specimens or by the mass of specimens incubated.

10.8.3 Ammonia and inorganic phosphate analysis

The following is a slightly modified analytical method for ammonia and inorganic phosphate from the manuals of sea water analysis authored by Strickland and Parsons (1972) and Parsons *et al.* (1984). The sample water (50 ml) needed for the original method is reduced to 10 ml. Details of analytical methods for other nitrogen compounds (urea, dissolved free amino acids) and phosphorus compounds (total phosphorus, organic phosphorus) may also be found in these manuals.

AMMONIA-N

Reagents

1) Deionized water Remove the ammonia from distilled water by passing it through a small column of cation exchange resin in the hydrogen form just before use and store the water in a tightly stoppered glass flask.
2) Phenol solution Dissolve 20 g of crystalline phenol (analytical reagent grade) in 200 ml of 95% (v/v) ethanol.
3) Sodium nitroprusside solution Dissolve 1.0 g of sodium nitroprusside $Na_2Fe(CN)_5NO{\cdot}2H_2O$ in 200 ml of deionized water. The solution is stored in a brown bottle. It is stable for about a month.
4) Alkaline solution Dissolve 100 g of trisodium citrate and 5 g of sodium hydroxide (analytical reagent grade) in 500 ml of deionized water. This solution is stable for a long period.
5) Sodium hypochlorite solution Use a solution of commercial hypochlorite (e.g. Chlorox) which should be at least 1.5*N* (see Note a).
6) Oxidizing solution Mix 100 ml of reagent (4) and 25 ml of reagent (5). It is best to prepare this solution immediately before the analysis.

Analytical procedure

Add 10 ml of the sample water (see Note b) to a test tube with an accompanying screw cap and then 0.4 ml, 0.4 ml and 1 ml of solutions (2), (3) and (6) respectively. Mix well after each addition. Fit the cap to the test tube in order to avoid contamination from ammonia in the air and allow to stand at a temperature between 20 to 27 °C for 1 h. Then measure the extinction at 640 nm relative to distilled water in a spectrophotometer using 10-cm cells. It is best to conduct the reaction in a constant-temperature water bath. The reaction requires a full 60 min for completion. During that time the samples should never be placed in direct sunlight or near a window. The detrimental effect of sunlight on the reaction has been pointed out (Liddicoat *et al.* 1975).

Ammonia-N concentration of the sample water can be obtained from the following equation (see Note c):

$$\mu\text{g-at N l}^{-1} = (E - E_b) \times F \tag{10.24}$$

where E is the extinction of the sample water (a mean of duplicate readings), E_b is the extinction of reagent blank, and F is the factor. For E_b and F, see Calibration, section 10.8.3. 1 μg-at N is equivalent to 14 μg N.

Calibration

Dissolve 0.6607 g of ammonia sulfate (analytical reagent grade) in 1 l of deionized water (1 ml $\equiv$ 10 μg-at N). Add 1 ml of chloroform and store in a dark place with a stopper. At the time of use dilute the standard solution 100 times with distilled water to make a secondary solution, and dilute this solution further with filtered sea water containing as little ammonia as possible to make the standard solution. If sea water is added to 10 ml of this secondary solution to make 1 l of the standard solution, the resulting ammonia concentration is equivalent to 1.0 μg-at N l^{-1} of ammonia-N. Pipette 10 ml of dilute standard into each of three test tubes and carry out the ammonia determination described in Analytical procedure, section 10.8.2. Calculate the factor, F, as

$$F = 1.0/(E_{std} - E_b)$$

where E_{std} is the average extinction of three standards and E_b is the average extinction of

the reagent blank. To obtain E_b, use deionized water in place of dilute standard solution and carry out ammonia determination.

NOTES FOR THE AMMONIA ANALYSIS

a) To check on the strength of hypochlorite, dissolve 12.5 g of sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) in 500 ml of distilled water. Add a few crystals (*ca.* 2 g) of potassium iodide (KI) to about 50 ml of distilled water in a small flask and pipette in 1.0 ml of hypochlorite solution. Add 5 to 10 drops of concentrated hydrochloric acid (HCl) and titrate the liberated iodine with the thiosulfate solution until no yellow color remains. Discard the hypochlorite when less than 12 ml of thiosulfate is used.

b) Temporary storage of sea water prior to analysis appears satisfactory in glass or polyethylene bottles, but analysis should not be delayed for more than 1 to 2 h at the most. If the analysis cannot be performed in this time period, samples should either be frozen at $-15\,^{\circ}C$ or stored unfrozen in the presence of 0.4 ml of phenol solution (Reagent (2)) per 10 ml of sample . Samples may be stored in either manner for up to 2 weeks (Degobbis 1973).

c) It assumes that the extinction is linear over the range of ammonia concentrations analyzed. For a linearity cheek of extinctions against ammonia concentration, a serial dilution of the standard solution should be made once during the experiment. The range of extinctions should cover the expected range of readings of experimental values.

INORGANIC PHOSPHATE-P

Reagents

1) Ammonium molybdate solution Dissolve 15 g of ammonium paramolybdate (NH_4) $Mo_7O_{24} \cdot 4H_2O$ (analytical reagent grade, preferably fine crystals) in 500 ml of distilled water. It should be protected from direct sunlight and be preserved in a polyethylene bottle. This solution is stable indefinitely.

2) Sulfuric acid solution Add 140 ml of concentrated sulfuric acid (analytical reagent grade, specific gravity of 1.8) to 900 ml of distilled water. Allow the solution to cool and preserve in a glass bottle.

3) Ascorbic acid solution Dissolve 27 g of good quality ascorbic acid in 500 ml of distilled water. Store the solution frozen in a polyethylene bottle. Thaw for use and refreeze at once. This solution can be kept for only about 1 week at room temperature but it is stable for several months if frozen.

4) Potassium antimonyl-tartrate solution Dissolve 0.34 g of good quality potassium antimonyl-tartrate $C_2H_2(OH)_2COOKCOO(SbO) \cdot 1/2H_2O$ in 250 ml of warm distilled water. The solution should be preserved either in a glass or polyethylene bottle. It is stable for several months.

Analytical procedure

Immediately before the analysis, the above solutions (1), (2), (3) and (4) are mixed in the ratio of 2:5:2:1 (v/v) respectively. Use this reagent for one-batch samples and discard any excess; it should not be kept for more than 6 h. To 10 ml of sample water (see Note a) in a test tube, add 1 ml of the mixed reagent and mix immediately. After 5 min or at most within 1 to 2 h, measure the extinction of the solution relative to distilled water in a 10-cm cell at a wavelength of 885 nm.

Phosphate-P concentration of the sample water can be obtained from the following equation (see Note b):

$$\mu\text{g-at P l}^{-1} = (E - E_b) \times F \tag{10.25}$$

where E is the average extinction of the sample water (a mean of duplicate readings), E_b is the average extinction of reagent blank, and F is the factor. For E_b and F, see Calibration, section 10.8.3. 1 μg − at P is equivalent to 31 μg P.

Calibration

Dissolve 0.816 g of anhydrous potassium dihydrogen phosphate KH_2PO_4 in 1 l of distilled water (1 ml ≡ 6.0 μg-at P) and store in a dark bottle with 1 ml of chloroform. The solution is stable for many months. Dilute 10 ml of the standard solution to 1 l with distilled water (1 ml ≡ 6.0×10^{-2} μg-at P). Pipette 0.5 ml of dilute standard into each of three test tubes and make up to 10 ml with distilled water (3.0 μg-at P l^{-1}). Carry out the phosphate determination described in Analytical procedure, section 10.8.3. Calculate the factor, F, as

$$F = 3.00/(E_{std} - E_b) \tag{10.26}$$

where E_{std} is the average extinction of three standards and E_b is the average extinction of the reagent blank. To obtain E_b use distilled water in place of dilute standard and carry out the phosphate determination.

NOTES FOR THE INORGANIC PHOSPHATE ANALYSIS

a) As the storage of sample water for inorganic phosphate analysis is always associated with uncertain errors, immediate analyses are recommended (Gilmartin 1967).

b) It assumes the extinction is linear over the range of phosphate concentrations analyzed, and this is usually the case for phosphate analysis. For linearity check of extinctions against phosphate concentration, a serial dilution of the standard solution should be made once during the experiment. The range of extinctions should cover the expected range of readings of experimental values.

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11 Methods for population genetic analysis of zooplankton

A. Bucklin

11.1 BACKGROUND

For a population geneticist, the amount of genetic diversity and the degree of population genetic structuring (i.e. division into genetically distinct sub-populations) are essential characteristics of a species. Both diversity and structure reflect the evolutionary history and presage the possible futures, including ability to respond to environmental perturbations. For a biological oceanographer, levels of genetic diversity may provide a rough estimate of the age of a lineage or species. Patterns of genetic diversity within and between species may be used to identify cryptic species, to reconstruct the evolutionary history of a group, and to accurately estimate biodiversity. Population genetic structure may be useful to provide quantitative estimates of dispersal (gene flow) across a species' geographic range and to understand the extent to which dispersal is determined by ocean currents.

Zooplankton are not merely passive passengers in the ocean's currents. Many species are active swimmers, and may control their vertical position and influence how and where ocean currents transport them. Despite the obvious influence of swimming on advective transport, many biological oceanographers have assumed that zooplankton populations are virtually homogenized by ocean currents, and constitute a single, well-mixed population across their entire distributional range. The extent of sub-division of a species reflects the patterns and pathways of dispersal across the distributional range, the effects of differing types of natural selection in different portions of the range, and random variation in gene frequencies caused by genetic drift.

Although dispersal is relatively easy to model, it is very difficult to measure. Inferences from population genetic structure are useful, particularly for marine zooplankton. The population genetic basis for this estimate is that the exchange of individuals among conspecific populations will act to genetically homogenize the population (assuming the transported individuals eventually reproduce in the destination population, which is the proper definition of dispersal). Gene flow acts to decrease population genetic structure (the genetic differentiation of conspecific populations). Thus, population geneticists are able to infer levels of gene flow from descriptions of population genetic structure. The

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inferential method is described in Statistical analysis of gene flow (dispersal), section 11.3.3.

When population genetic analysis is integrated into ecological, evolutionary, and oceanographic studies, patterns of genetic diversity may improve estimations of population dynamic parameters (birth, death, immigration, emigration); dispersal and gene flow; effective population size and reproductive variance; and systematics, taxonomy, and accurate assessment of biodiversity. The molecular data can be used to design rapid, molecularly based protocols to identify cryptic species, assess physiological condition and growth, and identify species remains in the gut contents of predators.

11.2 TECHNICAL APPROACHES TO DETERMINING GENETIC DIVERSITY

Population geneticists use a variety of heritable characteristics to assess genetic diversity. Since the 1970s, biochemical genetic variation has been assayed by electrophoretic separation of enzymes based on their charge density (Harris and Hopkinson 1977). More recently, geneticists have employed a variety of techniques to reveal molecular genetic variation (i.e. differences in the structure of DNA itself). Valid and widely used techniques include Restriction Fragment Length Polymorphisms (RFLP) of mitochondrial DNA, direct sequencing of DNA, analysis of microsatellite DNA, DNA fingerprinting, Randomly Amplified Polymorphic DNA (RAPDs), and Amplified Fragment Length Polymorphisms (AFLPs) – among others. New molecular techniques are developed every year, and some will undoubtedly prove useful to zooplankton biologists.

This chapter provides a brief overview of the principles and practices behind some of the technical approaches used by population geneticists, marine biologists, and biological oceanographers, to assay genetic diversity in natural populations. Each section includes a cursory consideration of the advantages and disadvantages of each technique in addressing questions about the ecology and evolution of marine organisms.

11.2.1 Allozymes

Electrophoretic separation of allelic variants of enzymes (allozymes) has been used to study the population genetics of a wide variety of species for many years (see Avise 1994). Although electrophoretically evident variation is only a small portion of the genetic variation of a gene, many enzymes have been shown to exhibit high levels of allozymic variation. Planktonic crustaceans seem particularly variable (Nelson and Hedgecock 1980). Although allozymes have been largely replaced by molecular characters as indicators of population genetic structure, they remain useful tools, and may be particularly useful for studies of breeding structure.

A number of previous studies on planktonic copepods include the use of protein electrophoresis to resolve allozymic variation among copepod populations (Battaglia *et al*. 1978; Bucklin and Marcus 1985; Sevigny and Odense 1985; Sevigny and McLaren 1988; Bucklin *et al*. 1989; Sevigny *et al*. 1989; Bucklin 1991; Cervelli *et al*. 1995; Kann and Wishner 1996). Two series of studies demonstrated strong population genetic differentiation associated with environmental variability: Burton's studies of the tidepool copepod *Tigriopus californicus* (Burton and Swisher 1984; Burton 1986, 1994) and Battaglia's examination of *Tisbe* sp. (Bisol *et al*. 1976; Battaglia *et al*. 1978).

A number of euphausiid species have been assayed for allozymic variability, including an examination of small-scale patchiness (Bucklin and Wiebe 1986) and a comparison of several species with different latitudinal ranges (Ayala and Valentine 1979). Two species are particularly well-studied: *Euphausia superba* (Fevolden 1986, 1988; Fevolden and Schneppenheim 1989) and *Meganyctiphanes norvegica* (Fevolden 1982; Sundt and Fevolden 1996). Allozymic variation has also been used to identify marine invertebrate larvae (Hu *et al*. 1992).

11.2.2 Restriction fragment length polymorphisms of DNA

One means of investigating molecular variation is through digestion of the purified DNA with restriction enzymes that cut the DNA strand wherever a recognition site sequence (usually 4 to 8 base pairs) occurs. Restriction Fragment Length Polymorphisms (RFLPs) assay DNA sequence variability at multiple sites without respect to function or position in the genome, and have generally high levels of intraspecific variability. In some cases, RFLPs may yield a molecular fingerprint that is unique to a particular individual. RFLPs are also useful markers of population genetic structure (see Avise 1994).

A frequent population genetic approach is the use of RFLP analysis of the entire, intact mitochondrial genome, a circular molecule located in a cytoplasmic organelle and encoding genes involved in central metabolic processes (Fig. 11.1). Mitochondrial DNA

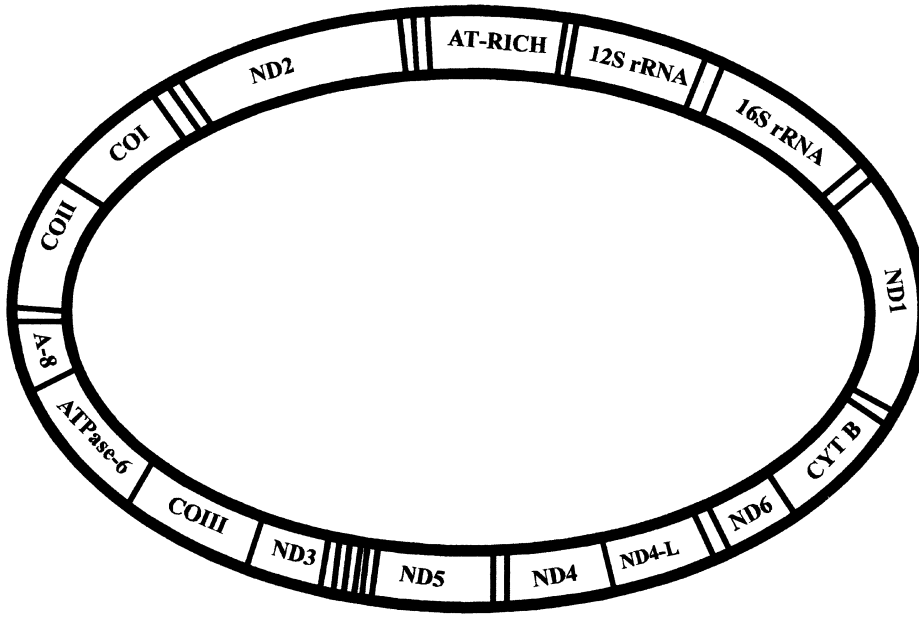

Fig. 11.1 Diagram of the gene arrangement in arthropod mitochondrial DNA (mtDNA). MtDNA is a circular molecule associated with cytoplasmic organelles, encoding twelve proteins and two rRNAs. See Avise (1994) for explanations of gene abbreviations and functions. Patterns of mtDNA sequence evolution are becoming well known, making it a useful marker for molecular systematics and population genetics.

(mtDNA) has the distinct advantage that it is inherited only from the maternal parent and without recombination in most organisms, unlike nuclear genes which experience recombination each generation (see Avise *et al*. 1987). Since time scales of zooplankton dispersal in the ocean may be far longer than the life-span of individual zooplankton, it is advantageous to use as tracers genetic characters that are identical from one generation to the next. Mitochondrial traits provide conserved markers for large-scale and/or long-term studies of dispersal. Also, mitochondrial genes frequently exhibit greater population differentiation than nuclear genes (Birky *et al*. 1989), because male dispersal does not cause exchange of mitochondrial genes.

Because most zooplankton species have small individual size, extraction of the intact mtDNA molecule is technically challenging. In some cases, selected regions of mtDNA have been amplified and the DNA sequence determined. Restriction digestion protocols can be designed based on the known patterns of intraspecific sequence variation, or restriction enzymes that yield diagnostic RFLPs can be determined empirically. Examples of the amplify-and-restrict technique may be found in Bucklin and Kann (1991); Silberman and Walsh (1992); Dixon *et al*. (1995); Kann and Wishner (1996); and Bell and Grassle (1998).

11.2.3 DNA sequence analysis

It is now also possible to rapidly assess molecular variation at the highest level of detail: that of the nucleotide base sequence of the DNA molecule. A variety of technical advances have made feasible the use of DNA sequence data for population genetic analysis. Direct sequencing of gene portions amplified by the Polymerase Chain Reaction (PCR) without an intermediate cloning step is primary among the technical advances (Innis *et al*. 1988). Also of importance are the automation of sequencing protocols (Smith *et al*. 1986; McBride *et al*. 1989) and the use of consensus primers (i.e. primers whose design is based on alignments of homologous sequences from numerous, frequently diverse taxa; Kocher *et al*. 1989) for amplification and sequencing, obviating the need for prior sequence data from the species under study (Innis *et al*. 1990). Detailed analysis of DNA sequence variation within species will reveal the biology of the species (including reproductive variance, dispersal, and survivorship) and may also reveal the processes by which genes and species evolve.

Population genetic analysis based on DNA sequence variation begins with characterization of each individual's genotype (diploid nuclear gene sequence) or haplotype (haploid mitochondrial gene sequence). Nuclear genes may be either sequenced from denatured DNA (i.e. single-stranded templates) or the genotype may be statistically inferred from patterns of sequence ambiguity in the double-stranded template (see Clark 1990). Sequence variation may be readily recognized by multiple sequence alignment (using various software programs, see Computer methods and software sources, section 11.3.4). Population genetic analysis is based on the frequencies of each genotype or haplotype in samples collected across the geographic range of the species.

There exist sequence data for a wide variety of zooplankton taxa and genes. Among chaetognaths, a portion of the nuclear 28S rRNA has been sequenced for 18 species (Telford and Holland 1997). A portion of the same gene was sequenced for *Panulirus* sp. larvae by Silberman and Walsh (1992). Nuclear 18S rRNA sequences are available for branchiopods *Bosmina* and *Artemia* (Kim *et al*. 1993a); the cumacean *Diastylis* (Kim *et al*. 1993b); and several decapods (Moon *et al*. 1994; Garcia-Machado *et al*. 1996; Geller *et al*. 1997). Several cDNA regions of genes encoding glycolytic enzymes have been

sequenced for the copepod *Calanus* (Crawford 1995). Commercial prawns of the genus *Penaeus* have received considerable attention; sequences are available for cDNAs of chitinase (Watanabe and Kono 1997), amylase (Van Wormhoudt and Sellos 1996), and trypsin (Klein *et al*. 1996).

The only complete mitochondrial sequence for marine zooplankton is that of *Artemia franciscana* (Ramon Valverde *et al*. 1994). Partial mtDNA sequences are available for shrimp species (Palumbi and Brand 1993; Shanks *et al*. 1998). Sequences for the mitochondrial gene, cytochrome oxidase I (COI), are available for the amphipod *Gammarus* (Meyran *et al*. 1997); the copepods *Diaptomus* (Guarnieri 1996; Bucklin *et al*. 1996a), *Pseudocalanus* (Bucklin *et al*. 1998b), and *Tigriopus* (Burton and Lee 1994); and the euphausiid *Meganyctiphanes* (Bucklin *et al*. 1997). A region of the mitochondrial 16S rRNA has been sequenced for species of the copepods *Calanus* and *Metridia* (Bucklin *et al*. 1992, 1995, 1996b; Bucklin and Kocher 1996), *Nannocalanus* (Bucklin *et al*. 1996a), and *Acartia* (Caudill 1995), and the euphausiid *Euphausia superba* (Patarnello *et al*. 1996). Interestingly, *E. superba* has been the subject of many molecular studies, including purification of one tRNA (Oshima *et al*. 1981) and two ribonucleases (Van *et al*. 1982); chromosomal analyses (Yabu and Kawamura 1984; Ngan *et al*. 1990); and a provocative study of UV-B-induced DNA damage (Malloy *et al*. 1997).

DNA sequence data can be used to design rapid protocols for population genetic analysis (Fig. 11.2), including Restriction Fragment Length Polymorphisms (RFLPs, see Restriction fragment length polymorphisms of DNA, section 11.2.2), oligonucleotide probe hybridization (see Oligonucleotide probe hybridization, section 11.2.4), allele-specific PCR amplification (see Allele-specific PCR, section 11.2.5), and Single-Strand Conformation Polymorphism (SSCP; Sheffield *et al*. 1993). SSCP resolves haplotypes differing by a small number of base substitutions, and has been shown to resolve a high proportion of sequence variation of mtDNA, depending upon base composition and sequence length (Ostellari *et al*. 1996). Also, SSCP analysis is rapid and inexpensive enough to allow assay of large sample sizes, thus increasing statistical power and resolution. SSCP has been used to examine recruitment patterns in marine invertebrate larvae (Li and Hedgecock 1998).

A related approach to identifying DNA sequence variants differing by only a few base substitutions, based on their tertiary structure, is Denaturing Gradient Gel Electrophoresis (DDGE). DDGE has been used for discrimination of bacterial species (Teske *et al*. 1996), among other groups.

11.2.4 Oligonucleotide probe hybridization

Direct comparison of homologous DNA sequences may reveal short, approximately 20 base-pairs (bp), regions that are diagnostic of each species. These regions can be used to design sequence-specific oligonucleotide hybridization probes to identify each species in the assemblage. Hybridization protocols may vary but usually involve an initial PCR amplification using consensus primers, immobilization of the amplification product on a membrane, and hybridization with a sequential series of labeled probes to discriminate the species.

Oligonucleotide probes have been used to identify phytoplankton cells (DeLong *et al*. 1989) and marine invertebrate larvae (Olson *et al*. 1991; Banks *et al*. 1993; Medeiros-Bergen *et al*. 1995; Bell and Grassle 1998). Sequence-specific hybridization may also be used to determine the presence or absence of particular species in pooled samples and in the gut contents of predators (Fig. 11.3).

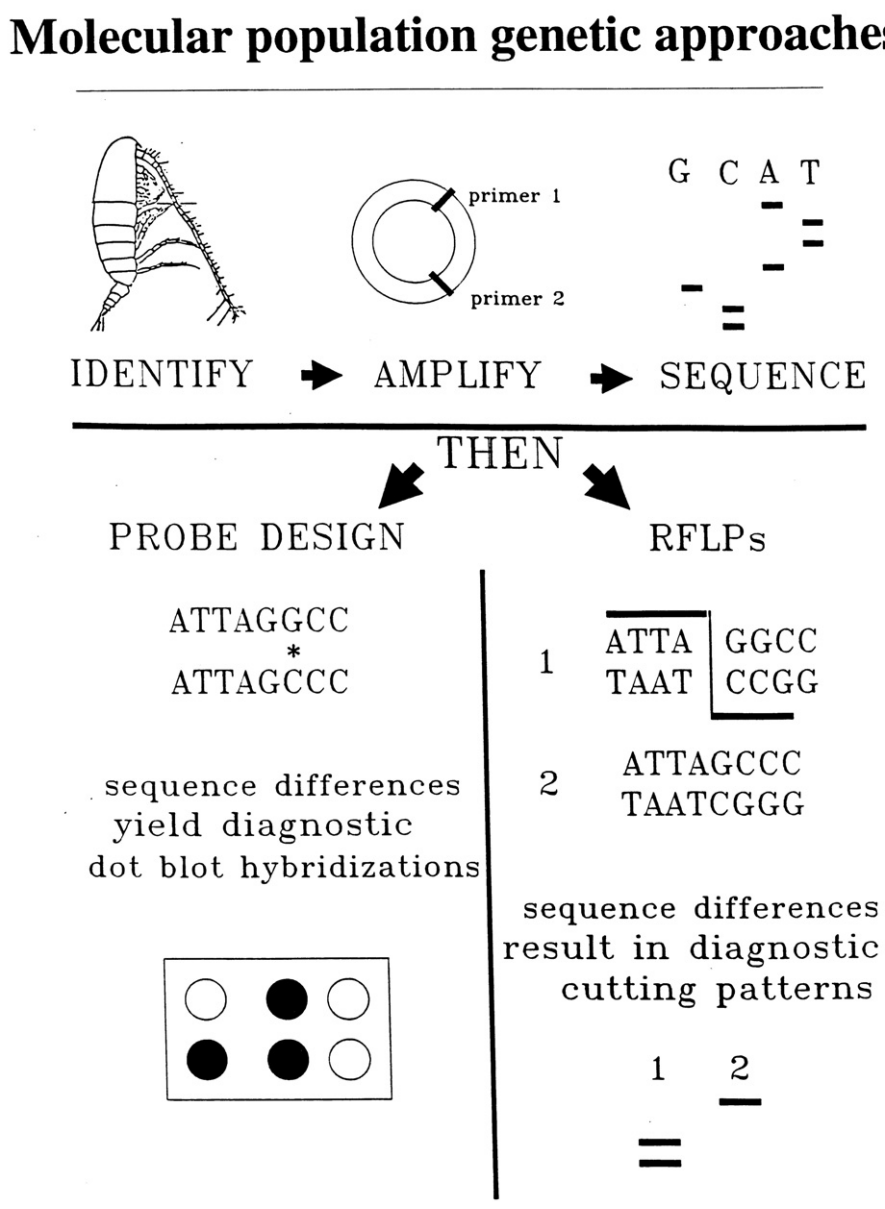

Fig. 11.2 Diagram of molecular population genetic techniques for marine zooplankton. The fundamental information is the DNA sequence of a selected region, which may now be determined by direct sequencing of the products of a PCR amplification. Based on the DNA sequence, oligonucleotide probes can be designed that are specific to a given allele, population, or species. These can be used in hybridization reactions to determine the presence or absence of the target sequence in an unknown sample. Alternatively, the DNA sequence can be used to select restriction enzymes that can discriminate between sequences by the presence or absence of the target site. Restriction Fragment Length Polymorphisms (RFLPs) are revealed by gel electrophoresis.

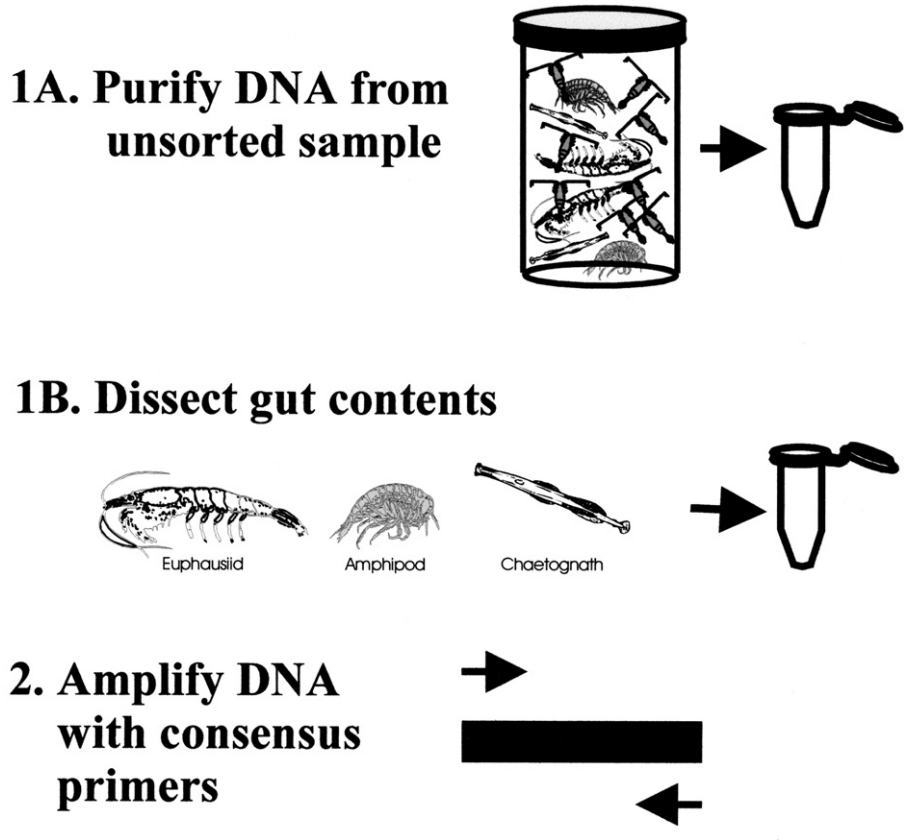

Fig. 11.3 Oligonucleotide probe hybridization may be used to determine the presence or absence of targeted zooplankton species in unsorted samples (1A) and amid the gut contents of predatory zooplankton (1B). DNA is purified from the homogenized sample or dissected gut contents and amplified using conserved PCR primers for a selected region (2). A suite of species-specific probes is designed based on known DNA sequences and used in sequential hybridizations of the membrane with the attached sample DNA (3). A dark spot indicates that the fluorescently labeled probe has found its target sequence, and exposed the X-ray film. See Medieros-Bergen *et al.* (1995) for techniques.

11.2.5 Allele-specific PCR

DNA sequence variation may also be used to design rapid and inexpensive protocols to discriminate species based on allele-specific amplification by the polymerase chain reaction (PCR; see Charlieu 1994). Individuals of any size and life stage may be identified by a suite of PCR reactions, each using a common primer and a species-specific primer. Allele-specific amplification has a further advantage: multiple reactions can be 'multiplexed', that is carried out simultaneously and competitively in a single tube (Gibbs *et al.* 1989; Fig. 11.4). In some cases, the competitive reaction may increase the accuracy and reliability of molecular systematic identifications (Bucklin *et al.* 1998b). Competitive species-specific PCR has been used for a variety of marine organisms (Banks *et al.* 1993; Dixon *et al.* 1995; Fell 1995; Bucklin *et al.* 1998b, 1999).

In some cases, the same PCR primers yield different products, especially different-sized products in different species. The different lengths of the ITS-2 region were used to develop PCR-based identification protocols for mussels by Dixon *et al.* (1995).

11.2.6 Microsatellite DNA

A variety of nuclear traits are currently in use in studies of population structure in marine organisms. Of greatest interest now are Variable Number Tandem Repeats (VNTR), also called microsatellite DNA, that are highly variable within species (see Wright 1993). Similar to allozymes, microsatellites are allelic variants of nuclear genes. Unlike allozymes, the sequences do not encode functional gene products; these non-coding regions are often highly variable. Despite higher levels of variation, VNTRs may not reveal population structure as well as mtDNA, because the VNTRs will experience recombination with the production of each new generation. Among crustaceans, microsatellite DNA protocols exist for the American lobster (Tam and Kornfield 1996) and the freshwater crayfish (Imgrund *et al.* 1997), and are under development for copepods and euphausiids. The microsatellite DNA assay may be multiplexed for efficiency; colorimetric detection systems have been developed that allow partial automation of the protocols (Olsen *et al.* 1996).

11.2.7 RAPDs

Randomly amplified polymorphic DNA (RAPD) is a PCR-based approach that generally reveals extensive variation between individuals (Williams *et al.* 1990). There have been persistent concerns about reproducibility and accuracy of the results of RAPD analysis, which have been shown to vary with template concentration (Davin-Regli *et al.* 1995) and amplification reaction parameters. Several modifications of the technique, including longer PCR primers (Gillings and Holley 1997) and careful standardization of protocols have resulted in a higher level of confidence in the technique. RAPDs have been used to examine phylogenetic relationships among species and population genetic structuring within species. Among zooplankton, RAPD variation was analyzed for *Artemia* sp. by Badaracco *et al.* (1995) and for *Pandalus borealis* by Martinez *et al.* (1997) and has been used to discriminate species of marine larvae (Crossland *et al.* 1993; Coffroth and Mulawaka 1995).

One advantage of population genetic analysis using RAPDs is that numerous markers – ranging widely in levels of variation – are generated from one survey. Even for high gene flow species like zooplankton, it may be possible to select RAPD characters that

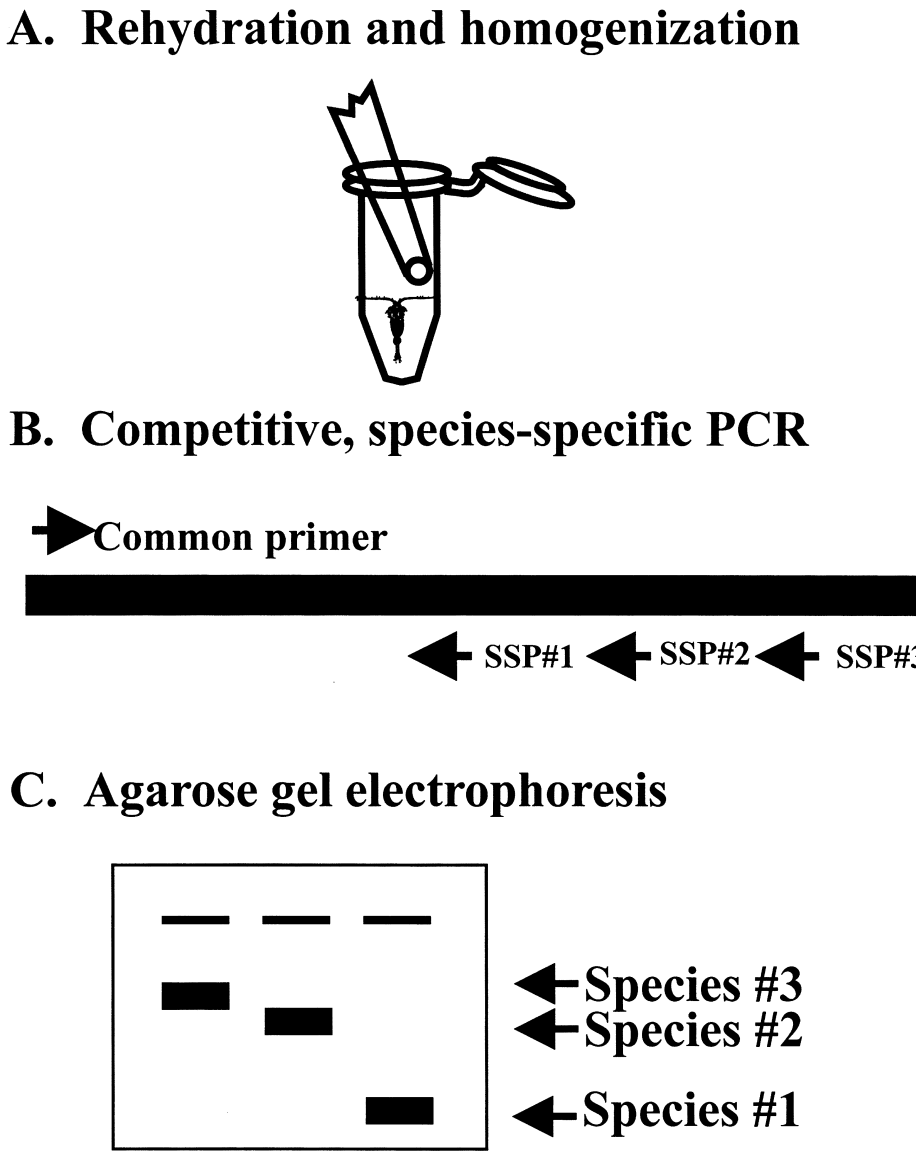

Fig. 11.4 Steps used to identify zooplankton species using multiplexed, species-specific PCR. Individual organisms are homogenized in the PCR reaction cocktail with several competing primers (each specific to one species and amplifying a diagnostic length of DNA fragment) and one common primer. The species is identified by electrophoresing the PCR products on an agarose gel. SSP no. 1, no. 2, and no. 3 indicate the relative positions of the competing, species-specific primers. Figure modified from Bucklin *et al.* (1998a).

show significant frequency differences among geographic populations and to ignore characters that are either nonvariable across the species or have unique patterns in every individual.

11.2.8 New and emerging techniques

In recent years, population geneticists have employed a variety of techniques to examine genetic structure and estimate gene flow. There is a growing body of work that explicitly compares the advantages and disadvantages of two or more techniques. In several studies of genetic discontinuity in populations of the copepod, *Tigriopus californicus*, using nuclear and mitochondrial gene genealogies and allozymes (Burton and Lee 1994) and mating experiments (Ganz and Burton 1995), all characteristics revealed significant differences between adjacent tide pool populations on the California coast. Kann and Wishner (1996) found that neither allozymic nor mtDNA variation discriminated samples of the copepod *Calanus finmarchicus* collected from the Gulf of Maine. Statistical analysis of population genetic diversity and structure using multiple data sets has been detailed by Michalakis and Excoffier (1996). In order to understand how the choice of genetic marker affects our perceptions of population genetic diversity and structure, additional comparative studies, using multiple genetic techniques for the same species, are much needed.

New techniques, and new modifications and applications of existing techniques, are appearing all the time. DNA fingerprinting by amplified fragment length polymorphism (AFLP) has shown much promise for revealing genetic diversity using DNA of any origin or complexity (Vos *et al.* 1995). AFLP analysis has been used for genome mapping in numerous plants and, more recently, in animals.

Other technical frontiers include the use of old, degraded, or improperly preserved tissues for molecular analysis. DNA has been extracted and sequenced from ancient human remains (Handt *et al.* 1996), so it is perhaps not surprising that zooplankton preserved in formalin and other noxious chemicals may be analyzed for molecular diversity. France and Kocher (1996) demonstrated that usual methods of PCR amplification and direct sequencing may be used for zooplankton tissue preserved in formalin and stored in alcohol. Short-term storage in formalin (up to periods of months) does not prevent molecular assay (Bucklin, unpublished data), but longer-term storage may fragment and denature the DNA, preventing some or all types of molecular analysis. Formalin-preserved zooplankton samples have also been used for biochemical analysis (Fudge 1968).

11.3 STATISTICAL APPROACHES TO ASSESSING GENETIC DIVERSITY AND STRUCTURE

Population genetic structure is quantified based on the frequencies of individual traits in geographic populations of a species (see Wright 1978, for a comprehensive treatment). It should be noted that population genetics, despite the term, is a study of individual characteristics and cannot be done using groups or pools of individuals. Dispersal between populations may be inferred from population genetic structure based on the fundamental principles and theory of population genetics (see reviews by Avise 1994, and Wilson *et al.* 1985). Genetic characters have the advantage that they are unambiguous identifiers of an individual or lineage, but a population genetic approach will yield

statistical rather than deterministic conclusions about the dynamics of zooplankton populations in the ocean. Although it is unlikely that it will be possible to predict an individual zooplankton's destination, it may be possible to determine the proportion of immigrant individuals in a given region on an oceanographically relevant time scale.

The truly enormous population sizes and geographic ranges of many zooplankton species present a daunting sampling problem. Statistically sound sampling strategies must also take into account the lack of geographic and temporal stability of populations and the various spatial scales of interest. The number of individuals required per sample is in part a function of the genetic diversity of the sample. Replicate samples are necessary at some stations to evaluate sampling error; frequent sampling at some locations will help to establish any temporal variation in the genetic character of the samples at a given geographic location. A nested sampling approach is frequently employed to reveal small-scale structure and to avoid aliasing at particular spatial scales. Zooplankton have been shown to exhibit genetic heterogeneity at small scales and over short time periods, termed 'chaotic patchiness' by Hedgecock (1994), which does not confound the significant spatial patterns seen at larger spatial scales. In general, population genetic analyses of marine zooplankton have revealed structure at large spatial scales (e.g. across an ocean basin, Bucklin *et al*. 1996b, 1997), although some coastal (Burton 1994) and estuarine (Caudill 1995) species are significantly structured at much smaller scales (see Bucklin *et al*. 1998b).

11.3.1. Statistical measures of genetic diversity

The next step in the measurement of genetic diversity is to calculate one of the numerous statistical tests that take into account the numbers and/or types of variants of a gene (Fig. 11.5). A simple indication of molecular diversity is the number of variants (i.e. alleles or genotypes of nuclear DNA; haplotypes of mitchondrial DNA) of a gene. There are two fundamental types of genetic diversity statistics: one is based on the numbers of differences between variants and one is based on the number of variants of a gene. An example of the first type is nucleotide diversity (π), which is calculated by the formula:

$$\boldsymbol{\pi} = [\sum \boldsymbol{p_{ij}}]/\boldsymbol{n_c} \tag{11.1}$$

where p_{ij} is the proportion of different nucleotides between the ith and jth variants and n_c is the total number of sequence comparisons $[n(n-1)/2]$ (Nei 1987). An example of a statistic based on the number of variants is the diversity measure, h, which is calculated by the formula:

$$\boldsymbol{h} = \mathbf{1} - \sum \boldsymbol{f_i^2} \tag{11.2}$$

where f_i is the frequency of the ith variant (Nei 1987).

Studies of zooplankton – as well as many other marine species, both vertebrate and invertebrate – have revealed skewed mtDNA haplotype frequency distributions, with numerous identical and unique haplotypes. This skewed distribution yields low values of haplotype diversity, h, which are lower than expected for such numerous organisms (see Avise 1994; Bucklin and Wiebe 1998; Table 11.1) Observed values of nucleotide diversity, π, for zooplankton are within the range $\pi = 0.0005$ to 0.020 that is typical for a wide variety of organisms (Stephan and Langley 1992).

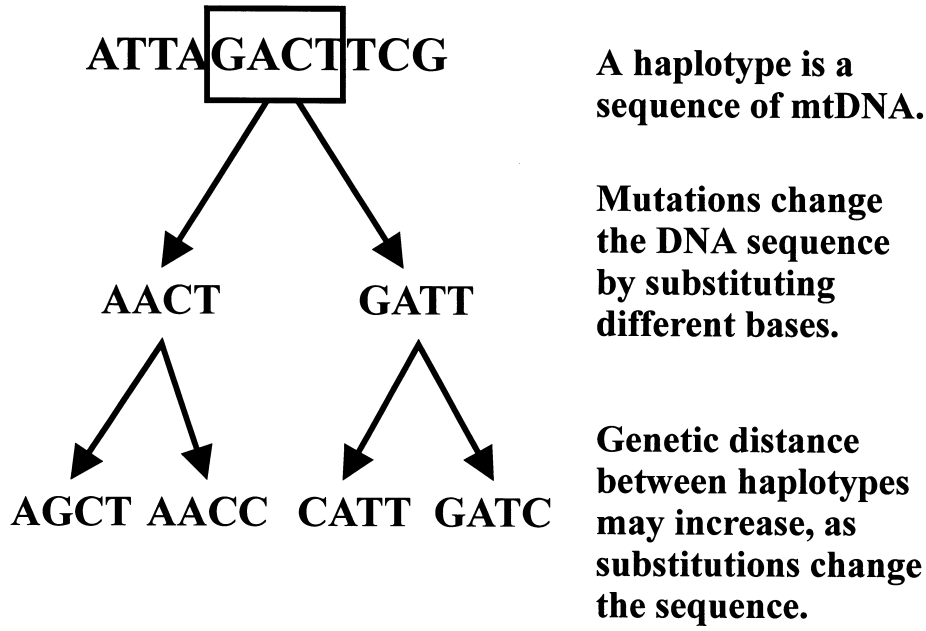

Fig. 11.5 Molecular genetic diversity arises from successive base substitutions in a DNA sequence to generate new haplotypes, which may become increasingly divergent with subsequent substitutions. The number, frequency, and pairwise difference between haplotypes (in the case of mtDNA) are the basis of molecular genetic diversity indices.

Table 11.1 Values of molecular genetic diversity indices and statistics of population genetic structure for three species of copepods, *Calanus finmarchicus*, *Nannocalanus minor*, and *Acartia tonsa*, based on a 350 base-pair region (220 base-pairs for *A. tonsa*) of mitochondrial 16S rRNA. Table columns are: numbers of individuals sequenced (N), numbers of different haplotypes (k), haplotype diversity (h), nucleotide diversity (π), Monte-Carlo chi-square test of haplotype frequency differences (χ^2), and percent of variance due to comparisons between populations within groups based on Analysis of Molecular Variation (V_B). The statistical significance of the χ^2 test is indicated by p. (Modified from Bucklin *et al.* 1998b.) NS indicates lack of statistical significance.

Species	N	k	h	π	χ^2	V_B
Calanus	216	31	0.37	0.003	$p < 0.001$	NS
Nannocalanus	158	51	0.77	0.005	$p < 0.550$	2%
Acartia	270	22	0.66	0.066	$p < 0.0001$	33%

11.3.2 Statistical measures of genetic structure

GENOTYPIC FREQUENCIES

The first step in the analysis of population genetic structure and gene flow is the determination of frequencies of the variants (hereafter called alleles) of individual traits (genes) in each geographic area. Each area is ideally characterized on the basis of multiple samples; the samples may be pooled after preliminary evaluation indicates that they do not differ significantly in the traits of interest. Whether the geographic area sampled constitutes a 'population' in the formal sense is difficult – if not impossible – to determine for zooplankton. However, the pooling of samples to characterize a geographically defined population, and the comparison among populations within and between regions, begins with characterization of genotype (or, in the case of haploid mitochondrial genotypes, haplotype) frequencies for each sampled area (Fig. 11.6).

CHI-SQUARE TESTS OF HETEROGENEITY

A very simple and straightforward means of assessing population genetic structure is to statistically compare the frequencies of the different alleles of a gene in different populations. Whether genetic variation is measured by assaying allozymes or molecular variation of DNA, the critical parameter is the frequency of each allele. Statistical differences in the frequencies of each allele in samples collected across the domain are evaluated by a chi-square test, using a Monte Carlo simulation (Roff and Bentzen 1989). Significant differences in allele frequencies between geographic populations provide evidence of population genetic structure.

F-STATISTICS

A usual statistical approach to the description of population genetic structure is the use of *F*-statistics (Wright 1978), which allows hierarchical analysis of the distribution of genetic variation within and between populations. The statistic, F_{ST}, reflects the proportion of the observed genetic variation that can be explained by partitioning between populations. Values of F_{ST} range from 0 to 1.0, where 0 indicates no genetic differences between populations and 1.0 indicates complete reproductive isolation between them. F_{ST} is calculated by:

$$F_{ST} = \sum [V_p/(p(1-p))]/n \tag{11.3}$$

where V_p is the variance of the frequency of allele p, p is the mean allele frequency across all samples, and n is the number of alleles (Wright 1978).

Values of F_{ST} for marine zooplankton vary widely, and depend upon the environment, the species, and spatial scale of genetic structuring examined. Populations of the copepod *Calanus finmarchicus* on opposite sides of the North Atlantic may be quite divergent based on mtDNA haplotypes ($F_{ST} = 0.188$; Bucklin *et al.* 1996b). At smaller spatial scales zooplankton may be genetically patchy: samples of the copepod *Metridia pacifica* collected in the California Current ranged from very similar in allozymic frequenices ($F_{ST} = 0.011$) to quite different ($F_{ST} = 0.141$; Bucklin *et al.* 1989).

ANALYSIS OF MOLECULAR VARIATION

Classical population genetic analyses consider only haplotype frequency differences, but more recent adaptations for analysis of molecular variation include consideration of the divergence among the haplotypes. One such analysis is Analysis of Molecular

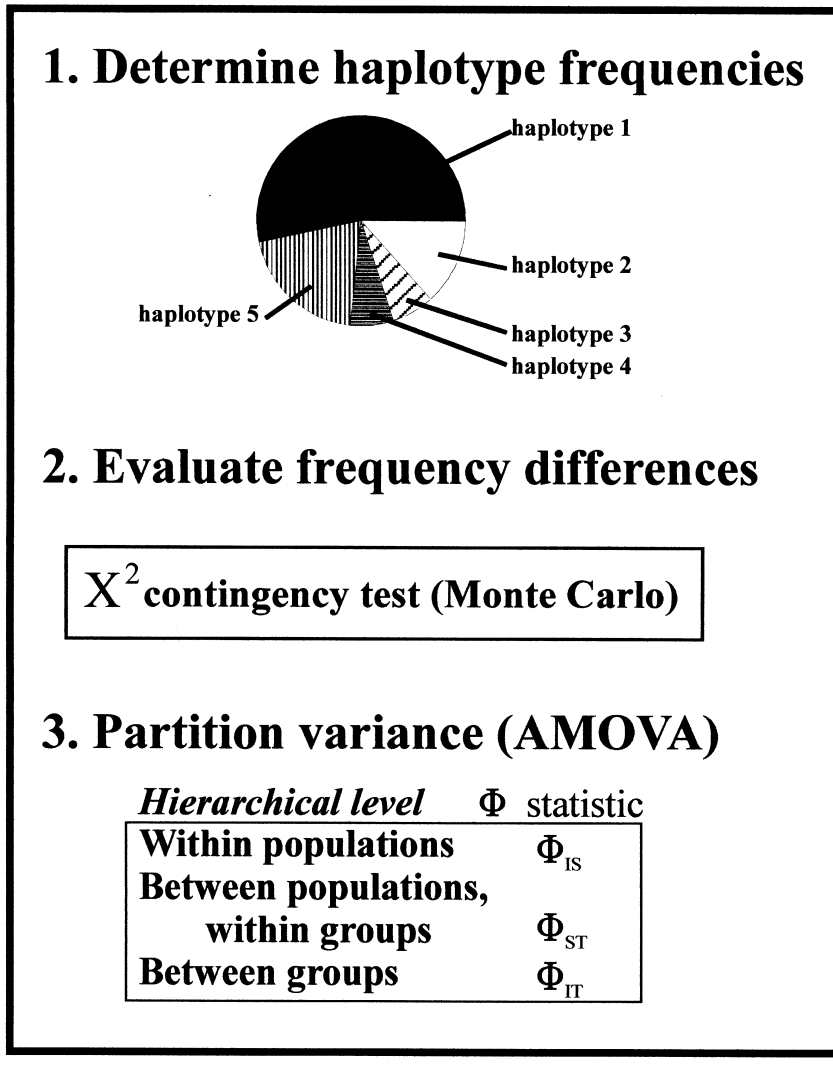

Fig. 11.6 Quantification of population genetic structure begins with determination of haplotype frequencies in the population. Haplotype frequency differences are statistically evaluated by a Monte-Carlo chi-square test (Roff and Bentzen 1989), and hierarchical analysis of variance determines the proportion of variance due to comparisons among populations within groups (Excoffier *et al.* 1992). See text for further explanation.

Variation (AMOVA; Excoffier *et al.* 1992). AMOVA may be used to partition variation among comparisons within populations, between populations within groups, and between groups (Fig. 11.6). Each variance component may be quantified. For examination of population genetic structure, the most important component is Φ_{ST} (representing the proportion of genetic variance resulting from partitioning of a species into genetically distinct populations; Weir and Cockerham 1984). Φ_{ST} is equivalent to F_{ST}, with the additional consideration of genetic distances between alleles. Based on the estimates of variance, pair-wise genetic distances between populations may be

estimated by a modified co-ancestry coefficient, D (Weir and Cockerham 1984), which is calculated by:

$$D = -\ln(1 - \Phi_{ST}) \tag{11.4}$$

D increases approximately linearly with time. The significance of D may be evaluated by the AMOVA program by comparison with null distributions of the statistic (Excoffier *et al*. 1992).

11.3.3 Statistical analysis of gene flow (dispersal)

ESTIMATES BASED ON *F*-STATISTICS

Based on the statistic of genetic structure, F_{ST}, the number of individuals exchanged between two populations per generation may be estimated by:

$$Nm = 1/(F_{ST} \times 4) \tag{11.5}$$

where N is the number of individuals in a given population and m is the proportion of those individuals resulting from immigration (Wright 1978). As a benchmark, values of Nm greater than 1 (corresponding to $F_{ST} < 0.25$) are typical of 'high gene flow' species, which are expected to become genetically homogeneous in the absence of counter-acting forces. However, counter-acting forces, especially differential selection, may be very strong and may maintain population differentiation in the presence of gene flow far in excess of $Nm = 1$.

For open-ocean zooplankton, some population genetic studies have estimated $Nm \gg 1$ (Bucklin *et al*. 1998b). However, dispersal across very large distances may be less frequent: $Nm = 1.3$ for *Calanus finmarchicus* populations across the North Atlantic (Bucklin *et al*. 1996b). In addition, the very high mortality rates ($\gg 99\%$) that are characteristic of marine zooplankton may allow strong selection to maintain genetic differences among populations, despite high gene flow with ocean mixing.

PHYLOGEOGRAPHIC APPROACHES

There are also a number of new statistical approaches that are currently used in the analysis of gene flow. Among the new approaches, phylogeographic analysis uses the techniques of phylogenetic reconstruction of the evolutionary history of organismal groups to examine intraspecific patterns of genetic and geographic variation (Avise 1994). This approach is based on the reconstruction of a tree showing the molecular relationship among the individuals assayed (Fig. 11.7). The grouping of individuals from the same geographic region on the same branches of the tree provides evidence of population genetic structure. Statistical evaluation of tree topology is by a bootstrap test (Kumar *et al*. 1993). Other tree-based approaches seek to incorporate both differences in genotype frequency and genetic distances between the genotypes in the analysis of population structure (Slatkin and Maddison 1989).

11.3.4 Computer methods and software sources

OLIGONUCLEOTIDE PRIMER AND PROBE DESIGN AND EVALUATION

Oligonucleotide primers and probes can be evaluated and optimized using the software programs *OLIGO* (Rychlik 1992) and *Amplify* (Engels 1992). Analysis of primers using the software package *OLIGO* (Rychlik 1992) can indicate their suitability: primers should have guanine (G) and cytosine (C) together making up at least 35% of

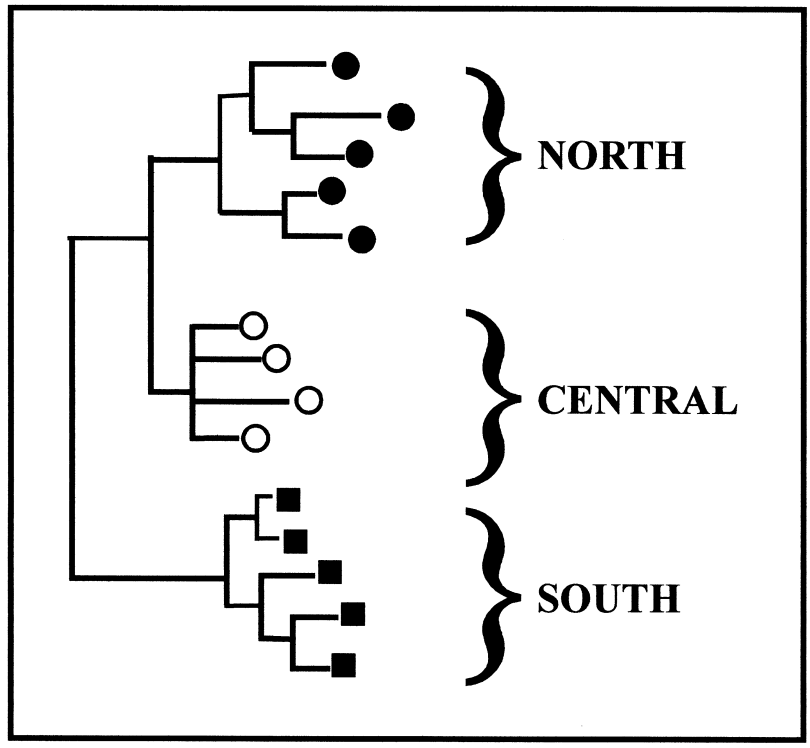

Fig. 11.7 Population genetic structure may be revealed by phylogenetic reconstruction of within-species variation. Significant differentiation of geographically distinct, genetically isolated populations is indicated by geographical sorting of the individuals on the phylogeographic tree.

the base composition; temperatures of dissociation (T_d) should allow PCR annealing temperatures in the typical 45–55 °C range; and suitable primers should have no stable dimers or hairpins (i.e. $\Delta G < -3.5$; see Rychlik 1992). Evaluation of the likelihood of PCR amplification with designed primers can be done using *Amplify* (Engels 1992).

DNA SEQUENCE ANALYSIS

There are several software packages available for DNA sequence analysis, including multiple sequence alignment and phylogenetic evaluation. The Genetics Computer Group (GCG, Madison, WI, USA) Sequence Analysis Software Package includes programs based on Smithies *et al.* (1981), published by Devereaux *et al.* (1984), and now commercially available. The multiple sequence alignment program is PileUp (based on Feng and Doolittle 1987). DNASTAR (DNASTAR, Inc., Madison, WI, USA) is a comprehensive package for sequence analysis and bioinformatics. These and several similar commercial software packages are expensive but comprehensive. The best software package for any particular study will depend upon the size of the dataset, the type of data, and the most frequent statistical analyses to be performed. For studies with very numerous (more than 100 individuals) and/or long (more than 1000 base pairs) sequences, the GCG software package may be preferable.

Perhaps the most valuable resources for molecular genetics work are the databases and search algorithms available through the World Wide Web from the National Center

for Biotechnology Information (NCBI), which can be found at the internet address: http://www.ncbi.nlm.nih.gov/. The search engines at this site allow examination of a literature database, PubMed, and a comprehensive database of DNA and protein sequences, the GenBank Sequence Database. Entirely free of cost, it is possible to search the databases for DNA and protein sequences by species or taxon using the Entrez Browser and by actual sequence using BLAST, a sequence-similarity search tool. At the website, there are also instructions for submitting sequences to GenBank.

POPULATION GENETIC ANALYSIS

Statistical evaluation of population genetic structure, based on most types of genetic characters, may be performed using the Restriction Enzyme Analysis Package (REAP; McElroy *et al*. 1992) and Analysis of Molecular Variation (AMOVA; Excoffier *et al*. 1992), as explained in Chi-square tests of heterogeneity and Analysis of molecular variation, section 11.3.2. Another package, Arlequin (copyrighted freeware available at http://anthropologie.unige.ch/arlequin/about.html) is touted as the first 'exploratory population genetics software environment'. Arlequin does allow a wide variety of analyses for diverse types of molecular datasets (DNA sequence, RFLP, RAPDs, etc.) without reformatting the input file, but the early versions have been plagued by software bugs. Analysis of one data set by numerous statistical tests and software packages is strongly recommended.

PHYLOGENETIC ANALYSIS

Phylogenetic reconstruction may be used to examine population genetic structure within a species, or evolutionary relationships between species or higher taxa. There are numerous possible methods of phylogenetic reconstruction (Swofford and Olsen 1990; Felsenstein 1982, 1988). A typical solution to the problem posed by the many possible methods of phylogenetic reconstruction is to use a number of different approaches and evaluate their topological similarity (see Saitou and Imanishi 1989, for discussion). Phylogenies may be reconstructed using

1) parsimony analysis in the software package, PAUP (Phylogenetic Analysis Using Parsimony; Swofford 1991)
2) the Molecular Evolutionary Genetics Analysis (MEGA) package (Kumar *et al*. 1993), which is based on distance measures (Felsenstein 1991), including neighbor joining (Saitou and Nei 1987) and clustering (Devereaux *et al*. 1984)
3) a comprehensive package using several methods, Phylogenetic Inference Package (PHYLIP; Felsenstein 1991).

The full range of software for phylogenetic analyses may be appreciated by searching the Internet. The website, http://www.ucmp.berkeley.edu/subway/phylo/phylosoft.html, will provide addresses and access for a number of programs. A particularly useful link is a comprehensive summary of phylogeny programs, which is available at http://evolution.genetics.washington.edu/phylip/software.html.

11.4 STRATEGIES FOR PRESERVATION OF ZOOPLANKTON SAMPLES FOR GENETIC ANALYSIS

The best means to ensure that the zooplankton samples collected today will be suitable for a variety of types of analyses in the future – some not yet developed – is to

Table 11.2 Recommended preservation strategy for zooplankton samples that are intended for diverse uses and methodologies. Splits of the samples are preserved in a variety of ways. This recommended method is based on that routinely used in the US GLOBEC field program over Georges Bank, in the northwest Atlantic during 1995–1999.

Split	Preservative	Intended use
Half	Buffered formalin	Taxonomy, distribution, abundance
Quarter	95% Ethyl alcohol	Molecular genetics, fish otoliths
Quarter	Liquid nitrogen	Biochemical assays, molecular genetics

preserve portions of each sample in different preservatives. A typical protocol, used for the US GLOBEC Georges Bank Study zooplankton collections and other recent cruises, involves splitting the samples before preservation, and then preserving splits of the sample using a variety of methods for a variety of purposes (Table 11.2).

11.4.1 Preservation and storage in ethanol

Ethanol is an ideal preservative for nearly all protocols targeted at genomic DNA. Ethanol will not preserve intact mtDNA molecules or any species of RNA, but genomic DNA and fragmented mtDNA will be preserved indefinitely for molecular analysis. A genuine disadvantage of alcohol as a preservative is that it is much less effective than formalin, and spoilage of overcrowded or high-biomass samples is a distinct likelihood. Sample volume should be only one-quarter to one-third of the volume of alcohol, and round clear-glass jars should be used to minimize the risk of poor preservation. Jar tops with plastic or foam liners (rather than cardboard) will minimize evaporation. It is essential that the alcohol be changed within days of the initial preservation, with continued changes until the alcohol remains clear. Removing fish and jellies will also help ensure good preservation. Alcohol-preserved samples will require careful curation throughout their life-span: they should be checked periodically for evaporation and putrification.

11.4.2 Quick freezing in liquid nitrogen

Flash freezing in liquid nitrogen (or as a less-desirable alternative, in an ultra-cold freezer set to $-85\,^{\circ}C$) is necessary for preservation of proteins (e.g. enzymes), mRNA, and intact mtDNA. Flash freezing is a suitable alternative to alcohol preservation for most molecular analyses, but any advantages of flash freezing for molecular analysis are usually outweighed by the expense and difficulty of obtaining and transporting frozen samples. Liquid nitrogen cannot be shipped by air, dry-shippers may be purchased to allow safe transport of deep-frozen samples at $-80\,^{\circ}C$ without the use of liquid nitrogen. In remote locations, liquid nitrogen may occasionally be found in manufacturing areas, it is used in iron smelting.

Zooplankton samples that will be frozen should be handled rapidly (before all others), since many species, especially crustaceans, have significant DNAase, RNAase, and protease activities.

11.4.3 Formalin, glutaraldehyde, and other bad things

Zooplankton samples in oceanographic collections are typically preserved in 5 to 10% w/v formalin. Protocols usually call for pH buffering of the formalin before use, but this is typically not done or is done ineffectively. The ideal method of pH buffering is to allow the diluted formalin to equilibrate for at least 10 days as a super-saturated suspension of sodium carbonate. Samples should be re-buffered by addition of sodium carbonate to super-saturation after 24 h. Thereafter, the samples should be checked periodically to maintain pH values between 8 and 9.

Zooplankton preserved and stored in buffered or un-buffered formalin (and presumably glutaraldehyde) yield usable DNA if assayed within several months (see France and Kocher 1996). Damage to DNA from formalin storage appears to be cumulative, extraction of usable DNA will become increasingly difficult over time. The low pH of un-buffered or poorly buffered formalin may accelerate DNA damage, and effective pH buffering seems to prolong the usefulness of the samples (Bucklin, unpublished data). Preferably, any samples of genetic interest should be switched out of formalin and into 95% ethanol as soon as possible after preservation.

11.4.4 Dehydration

DNA may be extracted from dried tissue and used for molecular analysis (Aguilera and Karel 1997). Similar to formalin preservation, the DNA will consist of shorter and shorter fragments over time.

11.4.5 *In situ* molecular analysis

There have been several recent breakthroughs in the design of oceanographic instrumentation to allow remote collection and/or identification of planktonic species. Molecular techniques can probably be integrated into a variety of oceanographic collection devices. In some cases, the devices have been designed specifically to make this possible. Molecular protocols (RFLPs and probes for 18S rDNA) are being incorporated into oceanographic instrumentation by Scholin and Anderson (1996) to allow remote detection of planktonic dinoflagellates that cause harmful algal blooms. A device to sample planktonic larvae for molecular analysis has been designed by Dixon *et al.* (1998) and dubbed PLASMA (Planktonic Larval Sampler for Molecular Analysis).

Incorporation of molecularly based identification protocols on multi-sensor platforms for remote species identification may be modeled on numerous recent efforts to allow *in situ* analysis of biological parameters on submerged instrumentation. Moored, tethered, benthic, and drifting devices that analyze a variety of biological moieties have already been deployed on experimental and operational bases. *In situ* analysis of collected samples has been used to analyze nutrient concentrations, fluorescence, and phytoplankton 'pigment taxonomies'. Automated *in situ* analysis of photosynthesis and micro-nutrient concentrations has been done by Craig Taylor *et al.* (Woods Hole Oceanographic Institution) using instruments of his design, the Continuous Flow Chemical Analyzer (CFA) and the Submersible Incubation Device (SID; Taylor and Doherty 1990; Taylor *et al.* 1993). Other *in situ* measurements have been made, including nitrate concentrations in hydrothermal vent fields (Johnson *et al.* 1986a, 1986b, 1989) and continuous real-time measurement of ocean chemistry, including

carbon dioxide, methane, and hydrogen sulfide (Jannasch *et al.* 1994; Johnson and Jannasch 1994).

Many of the numerous molecular protocols currently in use to discriminate species are robust, accurate, and reliable and highly suitable for oceanographic application. In addition, many of the protocols are simple enough to be adapted for high throughput and/or real-time sampling, and some of them may be automated for remote applications. The source of innovation for multi-sensor sampling platforms for remote species identification may come from teams of oceanographers and engineers, and may also involve borrowing from the commercial successes of biotechnology firms producing agricultural diagnostic tests and kits for use by the non-specialist.

11.5 GENERAL RECOMMENDATIONS

Population genetic diversity and structure are fundamental characteristics of a species, which may help reveal the underlying population dynamic, ecological, and evolutionary patterns and processes of marine zooplankton. For biological oceanographers, population genetic studies may be useful to understand dispersal and transport in ocean currents, delimit biogeographic domains in the ocean, reveal cryptic species, allow accurate estimation of biodiversity, and infer evolutionary relationships within and among taxa. A varied suite of molecular protocols has been and may be applied to marine zooplankton for diverse studies ranging from marine ecology to molecular evolution. The technical approach should be appropriately matched to the goal of the study. Random assays (i.e. assay of multiple sites without respect to function) such as RAPDs and RFLPs tend to be highly variable and may be particularly useful for studies of breeding structure and individual fingerprinting; species-specific primers and probes are useful for species identification and analysis of species presence or absence in samples; DNA sequencing is preferable for phylogenetic reconstructions. The primary limitation to the application of molecular population genetic protocols for zooplankton is individual size; individual zooplankton – the fundamental focus of population genetic studies – may be too small for some molecular techniques (e.g. RFLP analysis of intact mtDNA).

Biological oceanographers should consider including molecular protocols in their techniques toolbox. In many cases, molecular protocols may be easily followed by the non-specialist, and molecular approaches may be successfully integrated into oceanographic studies. Some caution is necessary, however. Expert advice should be sought during the planning phases of any experimental or observational program, to identify the molecular approach that is most appropriate for the project. Expert advice is required again in the interpretation of results.

Biological oceanographers have only recently begun to study the population genetics of zooplankton. There are many important and interesting questions demanding our attention, and many species for which no genetic data exist. The best means to address this comprehensive lack of information is to integrate molecular population genetic analyses into multi-disciplinary oceanographic field programs, and to continue to educate biological oceanographers about the particular strengths of population genetic analysis of marine zooplankton.

11.6 MEASUREMENT PROTOCOLS

11.6.1 Introduction

There are many excellent references and protocol books for molecular genetic analysis. Among the most useful for the recent initiate are: Sambrook *et al.* (1989) *Molecular cloning: a laboratory manual*; Ausubel *et al.* (1995) *Short protocols in molecular biology*; Innis *et al.* (1990) *PCR protocols: a guide to methods and applications*; Innis *et al.* (1995) *PCR strategies*; Hillis *et al.* (1996) *Molecular systematics*; and recent issues of *BioTechniques: The Journal of Laboratory Technology for Bioresearch* (see section 11.7 for the complete references). All basic protocols and recipes in molecular population genetics are explained in exhaustive detail in these books, in terms that are useful and readable by the non-specialist. In addition, most published studies include detailed descriptions of the techniques employed, with references to the primary literature describing the technique.

This brief summary of useful protocols for population genetic analysis of zooplankton will be restricted to specific modifications of established protocols that our laboratory at the University of New Hampshire has used to make molecular analyses cheaper, faster, easier, and more reliable for crustacean zooplankton, especially copepods and euphausiids.

11.6.2 Facilities and equipment

Many molecular analyses can be performed with a minimum of special laboratory equipment. Standard laboratory equipment that is assumed to be available includes:

- pH meter
- water baths
- heating and stirring bases
- centrifuge (refrigerated, up to 14 000 rpm)
- vortex
- 200 V power supplies
- microwave oven
- convection oven; incubator
- refrigerator and non-frost-free freezer
- ultra-cold ($-80\,^{\circ}C$) freezer
- chemical fume hood
- dark room equipped for film development.

Special equipment required for most molecular analyses includes:

- PCR machine
- UV light table
- gel camera
- vacuum pump
- DNA desiccator.

Some molecular protocols require:

- hybridization oven
- hybridization gel manifold.

There are many more pieces of equipment needed for more ambitious molecular studies. For students or researchers on a tight budget, it is possible to set up a laboratory to assay variability by simple methods (PCR amplification and RFLP or species-specific PCR) using only a few critical pieces of equipment. It is possible to send PCR products to commercial molecular laboratories for DNA sequence determination, use the sequence data to design cheap and easy protocols, and assay molecular diversity of zooplankton at sea or in a standard laboratory. Costs of molecular techniques vary widely. Supplies costs may run at $5000 to $20 000 per project per year, depending on the techniques and the sample sizes.

This summary of protocols will focus on specific inventions and modifications of standard molecular protocols for marine zooplankton. It should not be used as or considered to be a comprehensive treatise on any technique.

11.6.3 General laboratory rules

These simple recommendations are used as guidance for students new to molecular analyses and laboratory work.

1) Write down the date when any chemical was first used or container first opened.
2) When measuring liquid or solid chemicals, pour out the correct amount carefully. If you pour out excess, *do not* return it to the container. *Throw it away!*
3) Consult the protocol book if you are at all uncertain about a procedure.
4) If you know what you did wrong it may still *sometimes* be worth continuing the procedure as an experiment. If you make an error in a procedure, decide whether to continue or start over by asking
   - will I learn anything if I complete the experiment as is?
   - have I already done the most expensive portion of the procedure?
   - how much time will it take to redo the procedure?
5) *Admit* all mistakes – you will not be blamed, only educated.
6) Be ultra-careful of ethidium bromide contamination. Do not touch light switches, counters, phone, etc. with gloved hands. Small spills can be cleaned by laboratory personnel (using protocols available in standard references); professionals should be called in to handle large spills of this and any toxic materials.
7) Wear gloves! Do anything involving solvents, acids, and strong bases in the chemical fume hood.
8) Report broken equipment or apparatus immediately.

11.6.4 Procedures

SAMPLE PRESERVATION

Preservation in 95% ethyl alcohol

Preservation and storage of zooplankton samples in 95% ethanol is cost effective and appropriate for many molecular analyses. Alcohol will preserve genomic DNA, but will not retain the circular structure of mitochondrial DNA (mtDNA), and will not preserve mRNA, tRNA, or proteins. Alcohol is not a powerful preservative, a large excess of alcohol and frequent alcohol changes immediately after preservation are needed to achieve good sample quality. Samples should be preserved immediately upon collection since biochemical and molecular degradation (by proteases, DNAases, and RNAases) is rapid.

Protocol steps:
1) Drain off excess sea water from the sample using a sieve of the same size or smaller than the collecting net mesh size.
2) Wash sample into jar using a squirt-bottle filled with 95% ethyl alcohol. Place label inside jar; all information should be written in pencil. For convenience, we also use jar-top labels.
3) Add 95% *undenatured* ethyl alcohol. Denatured alcohol cannot be used for preservation of genetic samples since it will destroy the DNA. 100% ethanol (usually including benzene) is acceptable for preservation, but less desirable.
4) Make sure the tissue volume is one-quarter to one-third of the sample volume; mix well during the first 24 h.
5) Change alcohol after 24 h; continue to change the alcohol until sample liquid appears clear.

Flash-freezing in liquid nitrogen

Preservation in liquid nitrogen is necessary for tissues and individuals that are intended for biochemical or molecular analysis of proteins, mRNA, tRNA, or other ephemeral moieties. Care should be taken to preserve the samples as soon as possible after collection, using short or shallow plankton tows, preventing warming of samples on deck (by dropping plastic-wrapped ice into buckets with living plankton and sea water if necessary), and working quickly to preserve samples. For some types of assays (e.g. mRNA extraction), it is preferable to freeze individual organisms in cryo-preservation vials. In this case, zooplankton samples should be kept in beakers in ice baths; sub-samples should be examined quickly under a dissecting microscope to identify and remove the targeted individuals, which should be immediately frozen in cryo-preservation vials with isotonic, filtered buffer solution or filtered sea water to cover. Label vials with felt-tip pen.

Please note that it is preferable not to submerge the sample in the liquid nitrogen, but to suspend it above the liquid phase. Nitrogen is dehydrating and will damage small individuals in particular. Do not allow the samples to thaw out before the assay.

We describe here our usual procedure for block-freezing unsorted samples.

Protocol steps:
1) Drain sample on a sieve; spoon the sample into a plastic bag with the label facing outward. Plastic will fracture at $-85\,^{\circ}C$, so the sample cannot be preserved only in plastic.
2) Wrap or roll the sample in the plastic bag in aluminum foil. The foil can be labeled on the outside with a felt-tip marker pen.
3) Place the sample in an ultra-cold freezer or liquid nitrogen carboy.
4) Chip fragments from the frozen block for homogenization with a mortar and pestle partially filled with liquid nitrogen. For some protocols, individuals of particular species can be chipped from the block. In other cases, identified individuals should be frozen separately in cryo-preservation vials.

DNA PURIFICATION

Phenol extraction of DNA

For larger organisms, such as euphausiids, amphipods and decapods, DNA must be extracted before molecular analysis can be done. The usual approach is a phenol extraction followed by an ethanol precipitation.

Protocol steps:

1) Mix up the extraction buffer solution (see Extraction buffer preparation, section 11.6.5). The extraction buffer may be made up in advance and stored at the temperature, but the proteinase K enzyme must be kept frozen and added just before use. Our protocol is for small organisms or tissue samples ($\leqslant 0.4$ cc tissue volume); for larger organisms or samples, consult one of the recommended protocol books. We do both the homogenization and the extraction in 1.5 ml centrifuge tubes.
2) Wearing gloves, dispense 500 μl extraction buffer preparation (see Extraction buffer preparation, section 11.6.5) reagents into each labeled 1.5 ml reaction tube. Place the tissue sample in the 1.5 ml reaction tubes. Tissue volume should be less than the volume of extraction buffer preparation. Using a clean glass rod or melted pipette tip, crush the organism in the extraction buffer solution until the tissue is well broken up, and the buffer becomes milky. (We make custom-fit homogenizers by melting pipette tips over a gas flame and molding them into a centrifuge tube filled with a drop of mineral oil.)
3) Close the tops of the centrifuge tubes and seal them with parafilm. Lay the tubes on an agitator or orbital mixer in an incubator or oven at 37 °C. Agitate for at least 2 h. Times may vary up to 24 h depending on the amount and type of tissue to be extracted.
4) Remove tubes from oven and add 500 μl equilibrated phenol to each tube. Mix by smoothly rocking the tube horizontally for 1 min, centrifuge at 14 000 rpm for 5 min, remove immediately from the centrifuge. Pipet the aqueous (top) layer from the tubes, being very careful not to include any of the phenol (lower) layer, and dispense into new 1.5 ml tubes. Label tubes with felt-tip marker.
5) Add 250 μl phenol, 240 μl chloroform, and 10 μl isoamyl alcohol to the tubes with the aqueous layer from the previous step. Mix by smoothly rocking the tube horizontally for 1 min, centrifuge at 14 000 rpm for 5 min, remove immediately from centrifuge. Pipet the aqueous (top) layer from the tubes, being very careful not to include any of the phenol/chloroform (lower) layer, and dispense into new 1.5 ml labeled tubes.
6) Add 500 μl chloroform to the tubes with the aqueous layer from the previous tubes. Mix by smoothly rocking the tube horizontally for 1 min, centrifuge at 14 000 rpm for 5 min, remove immediately from centrifuge. Pipet the aqueous (top) layer from the tubes, and dispense into new 1.5 ml tubes. Do not forget to label. At this final step, it is essential that you *do not include any of the chloroform (lower) layer*! Any remaining phenol or chloroform will extinguish the subsequent molecular assays.
7) Add 130 μl 10 mol l^{-1} ammonium acetate and 870 μl ice-cold 95% EtOH to the tubes, mix gently, and leave in the freezer at -20 °C overnight. If you are in a hurry, you may precipitate the DNA at -80 °C for 20 min. Do not leave longer at ultra-cold temperatures or the alcohol will freeze.
8) Remove tubes from freezer and centrifuge for 30 min at 4 °C. Decant (i.e. slowly pour off) all liquid, pouring toward the side away from the DNA. You may be able to see DNA pellets in the bottom of the reaction tube (white globs), but not always. Make sure these clumps stay in the test tube – pour slowly.
9) Carefully add 1 ml ice-cold 80% EtOH, being careful not to disturb the DNA pellet. Centrifuge for 15 min at 4 °C. Decant alcohol – *slowly* – and drain tubes on paper towels. Dry the DNA samples in a desiccator or leave to drain on paper towels until

dry (several hours). Tap the sides of the reaction tube to make sure that no liquid is left in the tubes.

10) Resuspend the DNA in 50 μl 10 mmol l^{-1} Tris with 2 mmol l^{-1} EDTA (ph 8.0) buffer. Add buffer to each tube, do not vortex or mix, and let the tubes sit at 4 °C for at least 2 h. Vortex slowly or mix. Once fully resuspended, keep the DNA at −20 °C for long-term storage; keep the DNA at 4 °C if it will be used frequently (every few days) to avoid numerous freeze–thaw cycles.

Concentration of DNA

A DNA solution may be concentrated by re-purifying the DNA by phenol extraction. A disadvantage of this approach is that the salts used to help precipitate the DNA will also be concentrated, and may hinder some reactions. One alternative is to use ammonium acetate, in place of sodium acetate, since the former sublimates under vacuum.

Protocol steps:

1) Add to the re-suspended DNA (in 50 μl of TE buffer)
   - 5 μl 3 mol l^{-1} sodium acetate
   - 100 μl 95% ethanol (cold).
2) Allow to precipitate overnight in freezer.
3) Centrifuge at 4 °C for 30 min at 14 000 rpm.
4) Decant the ethanol solution.
5) Add 100 μl of 75% ice-cold ethanol.
6) Centrifuge at 4 °C for 15 min at 14 000 rpm.
7) Decant the ethanol solution.
8) Dry the precipitate in a dessicator, and resuspend in 10 μl of TE buffer. Store at 4 °C.

PCR AMPLIFICATIONS

Preparation of zooplankton for PCR

Very small individuals ($\leqslant$1 mm) can be prepared for molecular analysis without DNA purification or extraction. For copepods, we routinely rehydrate ethanol-preserved copepods by soaking in distilled water for a few hours (do not let them sit for more than 24 h). Place copepods in 250 μl distilled water in the PCR reaction tube. After the copepod sinks to the bottom of the reaction tube, remove the water from the reaction tube and proceed with the PCR reaction.

It is also possible to rehydrate copepods and remove ethanol preservative by boiling. Place copepods in 250 μl distilled water in the PCR reaction tube and close the tube tightly. Suspend tube in a floating rack in rapidly boiling water for 5 to 15 min. Either the copepod or the water it has been boiled in may be used for the PCR reaction.

PCR amplification of small zooplankton

We typically use 50 μl reaction volumes for PCRs with copepods. Reaction volumes of 25 μl or less usually work just as well; they save money but yield less product for sequencing, hybridization, or purification.

Protocol steps:

1) Make up a pool (Pool A) of the following reagents, using volumes calculated for one more reaction than you will do. For each reaction use:
   - 35 μl of dHOH
   - 5 μl of 10 × PCR buffer (from the manufacturer)
   - 4 μl of 25 mmol l^{-1} $MgCl_2$ solution

2) Quick-spin the tube containing the pool, and aliquot 44 μl of the pool into each PCR reaction tube holding a copepod. Use a pipette tip to crush the copepod against the side or bottom of the tube in order to release the DNA.
3) Make up another pool (Pool B) of the following reagents, using volumes calculated for one more reaction than you will do. For each reaction use:
   - 5 μl of a 2 mmol l^{-1} dNTP solution (equimolar mix dATP, dCTP, dGTP, dCTP)
   - 0.5 μl of a 10 μmol l^{-1} solution of Primer A
   - 0.5 μl of a 10 μmol l^{-1} solution of Primer B
   - 0.25 μl (1/4 unit) of TAQ polymerase enzyme.

   Always add the TAQ polymerase last, pipetting the enzyme slowly.
4) Aliquot 6.25 μl of Pool B to each tube. Add two drops of mineral oil to each tube and quick spin. Put a drop of oil into each well of the PCR machine that you will be using and place the tubes. (Be sure that the covers are snapped down tightly.)

PCR amplification using extracted DNA

Protocol steps:

1) Make up a pool of the following reagents, using volumes calculated for one more reaction than you will do. For each reaction use:
   - 35 μl dHOH
   - 5 μl of 10 $\times$ PCR buffer (from the manufacturer)
   - 4 μl of a 25 mmol l^{-1} $MgCl_2$ solution
   - 5 μl of a 2 mmol l^{-1} dNTP solution (equimolar mix dATP, dCTP, dGTP, dCTP)
   - 0.5 μl of a 10 μmol l^{-1} solution of Primer A
   - 0.5 μl of a 10 μmol l^{-1} solution of Primer B
   - 0.25 μl (1/4 unit) of TAQ polymerase.

   Always add the TAQ last, pipetting the enzyme slowly.
2) Aliquot 49 μl of the reagent pool to each tube.
3) Add 1 μl of each DNA solution, being very careful not to contaminate the tubes, and leaving a final tube without DNA (as a negative PCR control). Thaw the DNA thoroughly, if frozen, but do not allow it to sit at room temperature. Vortex slowly (or finger vortex) and quick-spin the DNA before use.
4) Add two drops of mineral oil to each tube and quick spin. Put a drop of oil into each well of the PCR machine that you will be using and place the tubes. (Be sure that the covers are snapped down tightly.)

PCR protocols for zooplankton

PCR protocols (i.e. the temperature and duration of each step: denaturation, annealing, and elongation) vary widely. Which protocols will work well depends upon many factors: the quality and concentration of the template DNA, the template/primer ratio, the sequence and base composition of the primers, and the degree to which the primers are perfect matches for the DNA. Our laboratory's general strategy for PCR protocol optimization (using consensus primers with template DNA of unknown sequence) is to begin with a 'forgiving' PCR protocol: denaturation at 94 °C for 1 min; annealing at 37 °C for 2 min; and elongation at 72 °C for 3 min for 40 cycles. We optimize the reaction to the highest annealing temperature that produces a bright, clear, sharply defined band of amplified DNA after agarose gel electrophoresis. For consensus primers based on vertebrate sequence with crustacean zooplankton, the optimum annealing temperature may be 40 to 42 °C. For 'perfect fit' primers (i.e. designed from the sequence of the

species we hope to amplify), optimum annealing temperatures may be 50 to 55 °C or higher.

Another important parameter is the number of cycles of amplification. We generally use fewer cycles (30) for perfect-fit primers spanning shorter regions (100–200 base-pairs), and more cycles (40) for primers we suspect of being quite different from the template DNA and those spanning longer regions (1000–2000 base-pairs).

GEL ELECTROPHORESIS

Running an agarose gel

Protocol steps:

1) Make up a 1.5% solution of agarose in 1 × TAE (see 50 × TAE, section 11.6.5) i.e. 1.5 g agarose per 100 ml TAE, for the volume of your gel apparatus. Stir well by swirling and heat the mixture in the microwave oven until the solution is clear, swirling occasionally and avoiding prolonged boiling.
2) After removing from the oven, add 5 μl of a 10 mg ml^{-1} ethidium bromide solution for every 100 ml agarose solution (the ethidium bromide concentration in the gel should be 0.5 μg ml^{-1}), mixing well. Be very careful with ethidium bromide, wear gloves at all times, and be sure to thoroughly wash anything that comes in contact with it. Cool the solution (if needed) in a water bath to about 60 °C and pour into the gel mold with the toothed comb in place.
3) Let the gel sit for about 30 min or until the gel solution is opaque. (You can test by gently poking the gel with a blunt tip.) Make sure the gel mold is in the correct position in the electrophoretic apparatus and pour TAE buffer to just cover the gel. The buffer level should be only 1 to 2 mm above the gel. Remove the comb(s).
4) Mix 2 μl loading dye and 8 μl of the DNA sample in spots on a piece of parafilm laid on the bench. Swirl with a pipette tip and pipette all 10 μl into one well of the agarose gel. Load all samples in succession, as well as molecular weight markers.
5) Carefully place the cover on the gel box and plug in the electrodes so the samples will be migrating toward the positive (red) electrode. Turn on the power supply, and set the voltage to about 10 volts per 1 cm migration distance in the gel (e.g. if the gel migration distance is 6 cm, set the voltage to 60 volts). Make sure the marker dye is migrating in the correct direction. Do not allow the gel to sit for long periods without power. To increase the running time, turn the voltage to very low, but do not turn off entirely until you are ready to photograph the gel.

GEL PURIFICATION OF DNA

Running a preparative gel

Preparative gel electrophoresis is done using a low-melting point agarose, such as Nusieve (FMC BioProducts, Rockland, ME, USA) or similar. This agarose liquifies at about 37 °C, so the DNA may be more readily removed from the agarose after electrophoretic separation.

Protocol steps:

1) Make a 1% solution of low-melting point agarose in 1 × TAE, using the recommended volume for the gel mold. Stir well by swirling and heat the mixture in the microwave oven until the solution is clear, swirling occasionally and avoiding prolonged boiling.
2) After removing from the oven, add 5 μl of a 10 mg ml^{-1} ethidium bromide solution

for every 100 ml Nusieve agarose solution (the ethidium bromide concentration in the gel should be 0.5 μg ml^{-1}), mixing well. Be very careful with ethidium bromide, wear gloves at all times, and wash anything that comes in contact with it in a dilute chlorine bleach solution. Cool the solution (if needed) in a water bath to abut 60 °C and pour into the gel mold with the toothed comb(s) in place. For preparative gels we use larger-toothed combs capable of holding up to 50 μl.

3) Let the gel sit for about 30 min or until the gel solution is opaque. We usually gel low-melting point agarose in the refrigerator. Make sure the gel mold is in the correct position in the electrophoretic apparatus and carefully pour TAE buffer to just cover the gel. The buffer level should be only 1 to 2 mm above the gel. Remove the comb(s) being very careful not to tear the gel.
4) Add 10 × loading dye to each sample; use 2 μl loading dye for each 10 μl of DNA solution. Mix the loading dye and DNA solution, and load the desired volume (40–50 μl) into the wells, being careful not to add oil to the wells. You may *carefully* wipe excess oil off the outside of the pipette tip using a clean KimWipe.
5) Place the cover on the gel apparatus, attach the electrodes so the DNA will migrate toward the positive (red) electrode. Turn on the power and set the voltage to 10 volts per 1 cm of distance to be migrated (e.g. 60 volts for a 6 cm gel).

Excision of DNA from the preparative gel

Protocol steps:

1) Very carefully remove the gel block from the apparatus and place on a piece of saran wrap and/or a paper towel.
2) Gently slide the gel onto the saran wrap on the UV-light surface and observe the gel. When working with the UV light be sure to wear a shield, gloves, and long sleeves.
3) Using a single-edge razor blade, cut the selected band as tightly as possible, trimming the top and bottom of the sample as well as the sides, to concentrate the sample. The volume of agarose should be as small as possible, and no more than 100 μl. Make sure that a clean razor blade is used for each band to avoid mixing or contaminating templates.
4) Place the bands into 1.5 ml tubes. Keep refrigerated if they are not used immediately. Do not allow to dry out and never freeze DNA in agarose.

Agarase digestion for DNA template preparation

The low-melting agarose may be removed from the DNA by digestion with the enzyme agarase (Sigma Chemical Co., St Louis, MO, USA). The protocol involves an overnight digestion. Alternatively, the DNA can be purified by phenol extraction and ethanol precipitation which takes longer and gives lower yields.

Protocol steps:

1) Place the 1.5 ml centrifuge tubes with the Nusieve band cuts in floating racks in a 65 °C water bath for 20 min to completely liquify the agarose. A dry bath may also be used.
2) After 20 min, quick-spin the tubes and replace in 65 °C bath for 2 min.
3) Move the tubes to a 37 °C water bath and add 1 to 1.5 μl agarase solution (made by rehydrating 1000 units in 200 μl) after the temperature falls below 50 °C. Finger vortex but do not allow the solution to cool below 37 °C, or the agarose will re-gel and the agarase digestion will not work effectively.
4) Incubate the tubes at 37 °C for at least 1 h; incubation can be done overnight. Store under refrigeration.

11.6.5 Recipes and safety information

BUFFERS AND FREQUENTLY USED SOLUTIONS

Extraction buffer preparation

Make up a solution as follows:

- 10 mmol l^{-1} Tris-HCl, pH 8.0
- 2 mmol l^{-1} EDTA, pH 8.0
- 10 mmol l^{-1} NaCl
- 1% sodium dodecyl sulfate (SDS).

The buffer may be made up in advance and stored at room temerature. Just prior to use add:

- 8 mg ml^{-1} DTT (stock: 1 mol l^{-1} dithiothreitol (DTT) in 0.01 mol l^{-1} NaOAc, pH 5.2; store at $-20\,^{\circ}C$)
- 0.4 mg ml^{-1} proteinase K (stock: 20 mg ml^{-1}; store at $-20\,^{\circ}C$).

50× TAE

Note: work in a fume hood and wear gloves. For 1000 ml measure:

- 242 g Tris base
- 57.1 ml glacial acetic acid
- 100 ml of a 0.5 mol l^{-1} EDTA solution (pH 8.0).

Add reagents to a 1000 ml graduated cylinder, add dHOH to 1000 ml. Add a stir bar and stir until the solution is clear.

Loading buffer (10×)

For 10 ml, to be aliquoted in 1 ml tubes and stored at $-20\,^{\circ}C$:

- 2 ml 0.5 mol l^{-1} EDTA
- 7 ml dHOH
- 1 ml 10% SDS solution
- 2 g sucrose
- 25 mg of 0.25% bromphenol blue solution.

Agarase solution

Make up storage buffer and autoclave:

- 30 mmol l^{-1} NaCl
- 10 mmol l^{-1} Tris pH 7.5
- 1 mmol l^{-1} EDTA
- 50% glycerol.

Resuspend 1000 units of enzyme in 200 μl of buffer and store at $4\,^{\circ}C$.

SAFETY INFORMATION

Chloroform

Health hazard rating 3 (severely carcinogenic)

- If swallowed and the casualty is conscious induce vomiting!
- If inhaled the casualty should go outside into the fresh air. If the casualty is having difficulty breathing give them oxygen.
- In the case of skin contact immediately flush the eyes or skin with plenty of water for at least 15 min.

Ethidium bromide

- Health hazard rating 2
- Use the same first aid procedures as for chloroform.

Formaldehyde
Health hazard rating 3–4 (carcinogenic)
- There is no first aid information for this chemical as the effects are not immediately apparent.
- If you think you have been exposed to formaldehyde contact a physician at once.

Formalin, 10% v/v
Health hazard rating 3 (carcinogenic)
- If swallowed and the casualty is conscious give large amounts of water and induce vomiting.
- If inhaled the casualty should go outside for some fresh air. Give oxygen if the casualty is having difficulty breathing.
- In the case of eye or skin contact flush with water for at least 15 min.

Hydrochloric acid
Health hazard rating 3 (poisonous)
- If swallowed *do not* induce vomiting. If the casualty is conscious give them water, milk or milk of magnesia.
- If inhaled the casualty should go outside into the fresh air. If breathing is difficult give the casualty oxygen.
- In the case of eye or skin contact, immediately flush with water for at least 15 min while removing contaminated clothing. Wash clothing before wearing it again.

Phenol
Health hazard rating 3 (life threatening)
- Use the same first aid procedures as for hydrochloric acid.

Sodium hydroxide
Health hazard rating 3 (poisonous)
- If swallowed *do not* induce vomiting. If the casualty is conscious give them large amounts of water, follow this with diluted vinegar, fruit juice or egg whites beaten with water.
- If inhaled the casualty should go outside into the fresh air. If breathing is difficult give the casualty oxygen.
- In the case of eye or skin contact, immediately flush with water for at least 15 min while removing contaminated clothing. Wash all contaminated clothing before wearing again.

11.7 FURTHER READING

Ausubel, F.M., 1993 *Current protocols in molecular biology*, CD-Rom version. John Wiley and Sons, New York.

Formerly known as 'The Red Book', this CD-Rom version is an encyclopedic treatment of molecular techniques that is the best source for explaining the fundamental principles behind each approach. This is an ideal reference for trouble-shooting, and includes even very recent, innovative techniques. The last book version was published in 1989 and is no longer available. The CD-Rom version can be updated more easily.

Ausubel, F.M., Brent, R., Kingston, R.E. and Moore, D.D., 1995 *Short protocols in molecular biology*. John Wiley and Sons, New York.

A useful and more affordable reference based on the encyclopedic treatment of the CD-Rom version.

Sambrook, J., Fritsch, E.F. and Maniatis, T., 1989 *Molecular cloning: a laboratory manual*. Cold Spring Harbor Laboratory Press, Plainview, NY.

This multi-volume reference is my favorite source for explanations for students and others who simply wish to make a particular technique 'work'. It is an excellent source of recipes for standard buffers and solutions, and provides simple and clear explanations for the more traditional molecular approaches.

Innis, M.A., Gelfand, D.H., Sninsky, J.J. and White, T.J., 1990 *PCR protocols: a guide to methods and applications*. Academic Press, New York.

Innis, M.A., Gelfand, D.H. and Sninsky, J.J., 1995 *PCR strategies*. Academic Press, New York.

These two collections of articles present very detailed and complete treatment of selected topics, including optimization of PCR protocols, primer and probe design, allele-specific PCR, and data analysis. The explanations are most useful for practitioners with some experience in the technique described, since most of the papers assume a fairly high level of knowledge and experience.

Hillis, D.M., Moritz, C. and Mable, B.K. (eds), 1996 *Molecular systematics*, 2nd edn. Sinauer Assoc., Sunderland, MA.

This book is a useful resource for molecular data analysis. The chapters provide good, balanced coverage of the interpretation and meaning of various types of molecular data. The range of possible analytical and statistical methods are discussed and well-referenced.

Mullis, K.B., Ferre, F. and Gibbs, R.A., 1994 *The polymerase chain reaction*. Springer Verlag, Berlin.

Edited by the person judged responsible for the molecular revolution that made possible the application of molecular biology to ecology and evolution, and oceanography. This book is intended as a handbook on PCR, providing a wide variety of methodological protocols and applications.

BioTechniques: The Journal of Laboratory Technology for Bioresearch. Eaton Publishing Co., Natick, MA.

This monthly magazine provides detailed advice and protocols on up-to-the-minute molecular and general laboratory techniques. The authors frequently include researchers from commercial laboratories, sometimes from the company that makes the product being used in the study. The information is useful to practitioners of all levels of experience.

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12 Modeling zooplankton dynamics

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12.1 INTRODUCTION

Modeling is a useful tool to relate abundance, distributions, fluctuations, and production of living organisms to variations in the abiotic environment, food conditions, and predation. Mathematical models integrate the dynamics of several variables into a single representation via interactions of processes (Wroblewski 1983). Nowadays models represent a continuum of complexity from simple response curves to complex marine food webs (Steele 1974; Walsh 1976; Kremer and Nixon 1978). Models are used prognostically or diagnostically, they serve to test different scenarios, and to understand the discrepancies between models and data. When models have been sufficiently tested and are considered robust, they can serve to predict future states.

Zooplankton models are built for three main objectives:

1) to estimate the flow of energy and matter through a defined ecological entity, for example an organism, a population or zooplankton community
2) to estimate the survival of individuals and the persistence of populations in their physical and biological environments, and to look at the factors and processes which regulate their variability
3) to study different aspects of behavioral ecology.

The core of this review is a description of models dealing with marine mesozooplankton. Models dealing with limnic zooplankton, or with fish larvae or meroplanktonic larvae are also partially described, and are only included to elucidate technical details or general principles. Models of microzooplankton are not described here. We aim to present detailed, specific applications of models on bioenergetics or demography of zooplankton rather than a general mathematical study of modeling. For general principles we refer the reader to papers and books listed in section 12.2.4.

Models dealing with a range of system components are reviewed in Table 12.1. The system components can be the internal components of the individual body, the individuals of a population, the populations of a single species, or the functional groups of zooplankton species. Process models dealing with physiological functions of individual organisms or specific links between physiological parameters and biological functions will also be presented. In section 12.2 we present general strategies for building

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Table 12.1 Overview of the different models and their objectives following the levels of organization.

Type of models	Objectives
Process models	to define rate expressions or parameterization
Models of individuals	to calculate individual budget and growth
Models of groups of individuals	to simulate their trajectories in the physical environment to simulate individual variability in behavior, growth and development
Models of populations	to simulate population dynamics, to simulate dispersion of a population in a physical structure
Models of metapopulations	to simulate interactions between species to simulate succession of species
Models of ecosystems including zooplankton communities	to simulate the role of the zooplankton community in the functioning of an ecosystem

models, writing the mathematical relations and applying numerical methods to integrate the equations of the mathematical expressions of the model.

The rest of this chapter is devoted to the presentation of models relating to different levels of biological organization: individual (section 12.3), population (section 12.4) and community (section 12.5). Models coupling biological entities (individual, population, functional groups of zooplankton) with the physical environment to simulate spatial dynamics are presented in section 12.6.

12.2 MODELING APPROACHES AND TECHNIQUES

12.2.1 Steps of model building

The first step in building a model is to be clear exactly what the objectives are. These objectives will determine the scope of the model, as well as the kind of model to use and the output required from the model. The general method for building a model of a complex system is to identify simpler components of the system and to describe the interactions among these components and external variables of the system and among the components themselves (Wroblewski 1983).

CHOICE OF STATE AND FORCING VARIABLES

The first step is the identification of the components (*functional units*, or *compartments* or *subsystems*) of the system, and depends on the purpose of constructing the model. The level of complexity in the representation of the system (number of components, number of interactions in the model) depends on what we really understand, on the available knowledge for building specific details of a model, and on the capacity to represent details at the same level for the different parts of the system. In choosing the components, modelers must attempt to reduce the complexity of nature to manageable portions, whilst retaining sufficient structure to model processes that are of interest to them. There is no golden rule for doing this and, to a large extent, success will be judged by the ability of the model to predict observations and increase understanding. Adding

more compartments to a model adds to the dynamic complexity and can make model interpretation difficult. Thus, a stepwise approach is recommended, beginning with a model containing the minimum number of compartments required to tackle the problem in hand, and only adding further components as necessity dictates.

Determining the appropriate model is sometimes more difficult than parameter estimation. The pertinent variables (quantities that may vary with time and space) fall into two categories. First, the *internal variables* (or *state variables*) are those related to processes governing the system dynamics, for example the internal compartments of the body of an organism, the developmental stages of a population, and the trophic levels of an ecosystem model. Second, the *external variables* control some of the internal variables of the system. Temperature, light, and food concentration are typical external forcing variables of biological systems.

CHOICE OF MODEL UNITS

The next choice that must be made is the units or currencies of the model. For example carbon, nitrogen, phosphorus, or dry weight can be used if the variable is mass, whereas densities or numbers of individuals can be used if the variable is abundance. The choice of units will depend on the problem being studied. Many models have mixed units. Element ratios are usually assumed constant but Anderson (1992) presents a model for studying the influence of the modeled C:N ratio.

CHOICE OF MATHEMATICAL FUNCTIONS TO MODEL THE INTERACTIONS BETWEEN VARIABLES

The study of interaction patterns is frequently helped by the use of various types of diagrams. Each variable is connected to other internal or external variables. The way in which each variable changes through the influence of one or more of the others must be known and the interactions described by mathematical functions. An important step is to determine the specific forms of these functions. In biological modeling, the relationships between variables are mostly established empirically, and the mathematical functions used to describe these relationships are not always derived from first principles. Empirical relationships should be used with caution. Each empirical relationship is only valid in certain space–time ranges, and should not be used outside these ranges without experimental tests. However, modelers frequently neglect this aspect. If several functions can describe a relationship between two variables, the simpler mathematical expression, i.e. the one with fewest parameters, should be chosen.

IDENTIFICATION OF PARAMETERS

An important process is the estimation of the values of the parameters (often called *calibration*). Such estimates often result from curve fitting of experimental data to the variables. If the model components represent large biological entities (e.g. the phytoplankton and zooplankton compartments of a pelagic ecosystem model), the function representing the relationship between the variables (e.g. the general grazing of phytoplankton by zooplankton) cannot be fitted from data. The form of the function is extrapolated from more specific studies, and possible parameter values are chosen from a large range of values due to the numerous species represented in the experiments. Most of the physiological parameters (e.g. ingestion rates, excretion rates, etc.) are experimentally obtained using small groups of animals of a given species.

Some parameters such as mortality rates are difficult to estimate experimentally. Because each situation is unique, one can only guess a mortality rate and examine the

model solution to see how well it fits the observations. This is called 'tuning' the model. It is a valid modeling technique when all but one set of parameters is well known. For instance, mortality can initially be set to zero to determine whether observed shifts in life-history stages of zooplankton could be simulated without mortality.

In more sophisticated methods models can be used for identifying values of parameters. In this case the properties of the model (its structure, the biological function used, etc.) need to be well understood. As an example, Jellison *et al.* (1995) present a comparison of cohort models for identifying demographic parameters.

12.2.2 The mathematical description of the system

SYSTEMS OF EQUATIONS

If the system is specified by the values of n state variables, the mathematical model of the system requires n equations. Equations are built by introducing rules for the interactions between variables of the system (state variables) and variables outside of the system (forcing variables). These rules are deduced from particular knowledge of the processes involved. There is no equivalent in biological models of physical laws such as the Navier–Stokes equation for fluid dynamics that provide the basis for physical oceanography; there is no set way to define how material is transferred between variables. Most of the models reviewed in sections 12.3–12.6 are dynamic simulation models, which allow time-dependent development of the state variables, either with no space dependence or with one to three space dimensions. Thus, most of the models considered here consist of time-dependent differential equations. The state of the system at any given time t depends upon what the state was at a previous time $t - \mathrm{dt}$ and upon the conditions that prevailed and influenced the direction and rate of change from $t - \mathrm{dt}$ up to but not including the instant t. In some cases, steady state conditions are considered by solving *time dependent* equations; usually the equations are not solved analytically, but numerically using computers. Ordinary differential equations (ODEs) are much more frequently used than partial differential equations (PDEs). This is a consequence of the availability of numerous routines for solving ordinary differential equations and of the greater simplicity of the equations. Reducing the dependence of the state variables to time or to one space dimension yields ordinary differential equations. Including more than one independent variable results in partial differential equations with greatly increased complexity for the numerical solving schemes.

In describing the way in which a dynamic system changes, two distinctions must be made: (1) continuous versus discrete state variables and (2) deterministic versus stochastic descriptions.

Biological state variables (e.g. the individual weight of an organism) are continuous, but many state variables of interest in zooplankton ecology are not continuous (e.g. the developmental stage of copepods). In the latter case, the value of the variable changes by discrete jumps. The treatment of discontinuous variables can be diverse, and they can be treated as though they were continuous if the scale at which we observe the system is coarse relative to the scale at which the jumps occur.

Biological functions can be built with two components: a deterministic component and a stochastic component in which the additional effect of variability due to randomness is added to the deterministic component. Stochasticity is used to represent known and not well understood effects on a process, and is added to a deterministic component. When the model is structured to account for such uncertainties in some of its functions, the model is referred to as a *stochastic model* (as opposed to a

deterministic model). They yield a probability distribution over the output sample space. In deterministic descriptions, the behavior of the system is completely determined by its state and by the specified conditions. As a result, a deterministic description of a dynamic system and its evolution through time usually gives a description of a particular trajectory. In a stochastic description, the additional effect of variability due to randomness is added to the deterministic component. This results in a distribution of probabilities for each set of possible behaviors. The connection between deterministic and stochastic descriptions is made by considering the expected or average behavior.

NUMERICAL METHODS

There are two ways of solving differential equations: analytical and numerical methods. Many differential equations cannot be solved analytically and must be solved numerically. Numerical techniques for solving differential equations are inexact, and involve approximations that allow a solution to be found by iterative calculations. There are many different numerical methods, but all can be implemented using a range of computer programs (e.g. Press *et al.* 1992). A good way to understand the principle of numerical methods is to use it for equations having a known analytical solution. The basis of the numerical solution of a differential equation is a difference equation that relates successive values of the solution at closely spaced intervals with the general form given by:

$$\frac{dN}{dt} = f(N(t), t) \tag{12.1}$$

with initial value $N(0)$ at time $t = 0$. f is the symbol of a function, N is the studied variable.

The numerical solution uses the difference equation:

$$\bar{N}(t + \delta t) = \bar{N}(t) + \frac{d\bar{N}}{dt}\delta t \tag{12.2}$$

where $\bar{N}(t)$ is a calculated value which is an approximation to the true value $N(t)$. The difference equation is iterated starting with the known value $N(0)$ at time $t = 0$ to produce the numerical solution $\bar{N}(\delta t), \bar{N}(2\delta t)$ and so on. Thus the numerical method produces an approximate solution to the differential equation which consists of a series of values at time intervals determined by the choice of the time increment. In practice the time increment δt is made small enough to achieve a satisfactory approximation.

One of the simplest approximations of an ODE by a difference equation is called Euler's method. The accuracy of the solution obtained by such a simple technique is related linearly to the step length δt, and generally can be improved by making δt smaller. However, the smaller the value of δt, the more calculations are required. This not only increases computer time, but the rounding errors of the computer can start to become significant, and introduce errors into the solution. There are other practical numerical methods for solving ODEs (see Press *et al.* 1992). An efficient recursive method approximating a Taylor series expansion is the Runge–Kutta technique.The fourth order Runge–Kutta method succeeds virtually always and is commonly used in many models.

For studying advection-diffusion processes PDEs have to be written. It is often difficult or impossible to find analytical solutions to PDEs, and we are forced to rely on

numerical approaches. There are many different techniques for obtaining numerical solutions to PDEs (see Press *et al*. 1992 for some that work reasonably well).

In some cases, it is possible to transform a PDE to an ODE (see for instance Sewell 1988, for a review of methods; and see Botsford *et al*. 1994, for an application). If not, finite difference methods are the most common applied numerical solution techniques (see Press *et al*. 1992). Many algebraic models are in fact discrete approximations to PDEs (Sinko and Streifer 1967), and certain integral formulations can also be shown to be equivalent to PDEs (Streifer 1974). The escalator boxcar train technique (Goudriaan 1986; De Roos 1988) is an example of an approximation to PDEs.

12.2.3 Computer programing and languages

The choice of which computer language to use for zooplankton modeling is usually determined by personal preference. However, there are a few practical implications associated with the choice. Relatively complex models can be developed using spreadsheet programs, where little active programing is required, and where good graphics are readily available. However, for increased flexibility and speed, it is often useful at some stage to write your own programs. The most commonly used programing languages in zooplankton modeling are BASIC, PASCAL, C, and FORTRAN. Existing computer routines for applying different numerical methods are available in these four languages. Many ocean models, particularly 3-D ocean models, are written in FORTRAN, so it is easier to link your zooplankton model to such a model if it is coded in a similar fashion. However, for developing individual-based models, an object-oriented language such as C++ or JAVA might be preferred.

There are various graphical programing languages available that are specifically designed to facilitate modeling of non-linear, dynamic systems. Among the most versatile of these languages is the graphical programing language STELLA II (Costanza *et al*. 1998; Hannon and Ruth 1997; Richmond and Peterson 1994). Some examples of models built with STELLA will be mentioned in this chapter.

12.2.4 Further reading

Several recent books are useful for introducing biological oceanographers to ecological models but the examples given are rarely taken from plankton:

Brown, D. and Rothery, P., 1993. *Models in biology: mathematics, statistics and computing*. Wiley, Chichester, 688 pp.

Edelstein-Keshet, L., 1988. *Mathematical models in biology*. Random House, New York, 586 pp.

Gold, H.J., 1977. *Mathematical modelling of biological systems. An introductory guidebook*. Wiley and Sons, New York, 357 pp.

Concerning the use of mathematical functions for fitting data for plankton processes:

Abramowitz, M. and Stegun, I.A., 1972. *Handbook of mathematical functions*. Dover Publications, New York, 1046 pp.

Concerning numerical methods and codes of these methods:

Press, W.H., Teukolsky, S.A., Vetterling, W.T. and Flannery, B.P., 1992. *Numerical recipes in*

FORTRAN. The art of scientific computing. Cambridge University Press, Cambridge, 963 pp. (This is also available for BASIC, PASCAL and C)

Sewell, G., 1988. *The numerical solution of ordinary and partial differential equations*. Academic Press, London, 271 pp.

Several books give examples of comparisons between stochastic and deterministic models of the same modeled system. In addition to those listed above we recommend:

Renshaw, E., 1991. *Modelling biological populations in space and time. Cambridge Studies in Mathematical Biology*. Cambridge University Press, Cambridge, 403 pp.

12.3 MODELS OF INDIVIDUAL BIOENERGETICS AND LIFE-HISTORY TRAITS

To construct a realistic population model one begins by formulating submodels for the individuals in the population. In order that a population model be predictive, it must represent the demographic effects of the physiological processes at the level of individuals (Streifer 1974).

12.3.1 Individual bioenergetics

BUDGET OF INDIVIDUAL ZOOPLANKTON

Several examples of trophodynamic formulations can be found in models of individuals, populations and ecosystems. The basis of many models of individuals and population-level individual-based models is the standard bioenergetic supply–demand function (Beyer and Laurence 1980, 1981; Batchelder and Miller 1989; Carlotti and Sciandra 1989; Caparroy and Carlotti 1996; Carlotti and Hirche 1997). In this function, growth is represented as the difference between the amount of food absorbed by an organism and the metabolic costs of its daily activities. Conover (1978) provides an extensive review of the different processes of the individual energetic balance of zooplankton (see also Mauchline 1998, for calanoid copepods). The principal functional forms used in models of biological energetics will be outlined in this section, and selected applications in recently published models will be provided.

The general balance equation of input and output fluxes of matter (or energy) in an organism is:

$$\begin{aligned}\textbf{Growth}\ (G) = {} & \textbf{Ingestion}\ (I) - \textbf{Egestion}\ (Eg) - \textbf{Metabolic losses}\ (ML) \\ & - \textbf{Release of gametes or eggs}\ (RG) \\ & - \textbf{Other losses (molts, etc.)}\end{aligned} \tag{12.3}$$

where

$$\textbf{Metabolic losses}\ (ML) = \textbf{Respiration}(R) + \textbf{Excretion}\ (Ex) \tag{12.4}$$

The assimilation efficiency is obtained as:

$$\textbf{Assimilation}\ (A) = (\textbf{Ingestion} - \textbf{Egestion})/\textbf{Ingestion} \tag{12.5}$$

Most models of individual bioenergetics consider input–output fluxes of matter. Ingestion is the process whereby animals acquire organic matter. Assimilation is the

proportion of the ingested matter that goes through the stomach epithelium. The difference is egested, usually in the form of fecal pellets. Usually assimilation is assumed to be constant. Metabolic losses are the products resulting from the catabolism of matter, which are eliminated from the body through the processes of respiration and excretion.

The apportioning of matter inside the body is rarely represented in models. A general equation for this is:

$$\begin{aligned}\textbf{Growth } (G) = {} & \textbf{Gut fullness } (GF) + \textbf{Somatic growth } (SG) \\ & + \textbf{Gonad and genital tract growth } (GG) \\ & + \textbf{Production of gametes } (PG)\end{aligned} \tag{12.6}$$

Most of the examples of processes detailed in the rest of this section are for copepods, but there are examples of budget models for organisms of all zooplankton taxa: for example ctenophores (Kremer and Reeve 1989), krill (Huntley *et al.* 1994b), and fish larvae (Laurence 1977).

INGESTION RATE

Allometric relation of ingestion rate to weight

Maximum daily ration (I_m) expressed as a percentage of body weight (w) tends to decrease as body size increases (Paffenhöfer 1971) following the relationship:

$$I_m = kW^c \tag{12.7}$$

Most values of the scaling exponent c range between about 0.6 and 0.9, and the value of 0.75 is commonly used (Moloney and Field 1989). The value of k depends on the species studied.

Ingestion functions related to food concentration

Marine copepods show an increasing ingestion rate as food concentration increases (Frost 1972 and references therein; also see chapter on feeding). The functional form of this response has generally been a Holling Type II shape (Holling 1959, 1965, 1966) showing a saturation of ingestion rate at high prey concentrations (C). Holling II is justified theoretically as a Michaelis–Menten function:

$$I = I_m \frac{C}{C + a} \tag{12.8}$$

where I_m is the maximum ingestion rate and a is the half-saturation coefficient.

Commonly, Ivlev's (1955) formulation is used to describe this functional response (Steele and Mullin 1977). The Ivlev formulation requires two parameters: the maximum ingestion rate I_m (with units of weight per unit time^{-1}), and α, the rate at which saturation is achieved with increasing food levels (with units of concentration^{-1}). The ingestion rate I is then:

$$I = I_m(1 - e^{-\alpha C}) \tag{12.9}$$

where C is the food concentration.

Generally, below a threshold concentration of food (C'), the observed feeding is zero. This can be easily introduced in equations 12.8 and 12.9 as:

$$I = I_m \frac{(C - C')}{C + a} \tag{12.10}$$

$$I = I_m\left(1 - e^{-\alpha(C-C')}\right) \tag{12.11}$$

The functional form of the response showing both a saturation of ingestion rate at high food concentrations and a threshold at low food concentrations is named the Holling Type III shape.

Other functions used for copepods are presented in Table 12.2. Several models have been built to explain certain parts or the whole shape of the functional response. Lam and Frost (1976) developed a model taking into account the energetic costs of filtration to predict changes in filtering response under various conditions of food concentration and food size. Sensitivity studies in ecosystem models have shown that these models are sensitive to parameters of the grazing function (Frost 1987; Fasham 1995).

Table 12.2 Models of the functional response. (I_{max} maximal ingestion rate; C food concentration; C' food threshold; K, a, b, d, α, γ parameters).

Formulation	Type	Authors	Species
$I = aC$	I	Gauld (1951) Gamble (1978) Frost (1972)	Several species *Calanus finmarchicus* *Calanus pacificus*
$I = aC + b$	I	Dagg and Grill (1980) Dagg *et al.* (1980) Huntley (1981)	*Centropages typicus* Several species *Calanus* sp.
$I = dC^2 + aC + b$	II	Mullin (1963)	*Calanus* sp.
$I = I_{max}(1 - e^{-\alpha C})$	II	Peruyeva (1976) Paffenhöfer and Van Sant (1985) Saiz (1993)	*Calanus glacialis* *Eucalanus pileatus* *Acartia clausi*
$I = I_{max}\frac{C}{K + C}$	II	Cushing (1968)	Theoretical
		Saiz and Kiørboe (1995) Yen (1983) Bartram (1980)	*Acartia tonsa* *Euchaeta elongata* *Paracalanus parvus* *Acartia tonsa*
$I = I_{max}(1 - e^{-\alpha(C-C')})$	III	Mullin *et al.* (1975)	*Calanus pacificus*
$I = I_{max}(1 - e^{-\alpha/C})$	III	Kiørboe *et al.* (1982) Kiørboe *et al.* (1985)	*Centropages hamatus* *Acartia tonsa*
$I = I_{max}(1 - e^{-\alpha C})^{\gamma}$	III	Thébault (1985)	*Temora stylifera*
$I = I_{max}\frac{(C - C')}{K + (C - C')}$	III	Mullin *et al.* (1975)	*Calanus pacificus*
$I = I_{max}\frac{C^2}{K^2 + C^2}$	III	Steele (1974)	Theoretical
	III	Wickham (1995)	*Cyclops kolensis*
$I = I_{max}\frac{C^n}{K^n + C^n}$	III	Real (1977)	Theoretical

Ingestion related to fluctuations of food concentration

Mayzaud and Poulet (1978) demonstrated a near linear response of five neritic copepod species to changes in food levels. This lack of saturation was thought to be due to variations in gut enzyme levels in response to varying phytoplankton concentrations. Franks *et al.* (1986) gave a mathematical formulation of this grazing response derived from Mayzaud and Poulet (1978) as:

$$I = I_m \alpha C\left(1 - e^{-\alpha C}\right) \tag{12.12}$$

As C gets larger, the formulation becomes linear with slope $I_m\alpha$, which is the initial slope of the Ivlev formulation. When C is large, the Ivlev curve shows saturation while the Mayzaud–Poulet curve does not. When C is small, the herbivore grazing rate is lower using the Mayzaud–Poulet formulation than the Ivlev. Franks *et al.* (1986) compared the effect of the choice of such ingestion functions on the dynamics of herbivore-zooplankton interaction in an N–P–Z (nutrient–phytoplankton–zooplankton) model.

Ingestion related to food quality – grazing on several resources – switching behavior One severe limitation of studies in which only a single species of food is utilized is the possibility that the results obtained do not represent the complete spectrum of feeding behavior of copepods. The effect of food size on grazing efficiency has been considered in some models (Steele 1974; Steele and Mullin 1977; Bartram 1980). Bartram (1980) developed a model involving a general filtration rate and an efficiency of retention, itself a function of cell size, and tested it experimentally for two copepods. He applied the model to a population of copepods of different age classes feeding upon an assemblage of food particles of different sizes. Based on optimal foraging theory, Lehman (1976) proposed a model for filter-feeding behavior in mixtures of particle types and used it to evaluate selective ingestion of particles based on their comparative abundances, size and digestibility. Both these examples illustrate how food quality can be as important as food quantity in determining ingestion rates of copepods.

In their ecosystem model, Fasham *et al.* (1990) discuss the problem that arises when zooplankton modeling deals with parameterizing grazing on multiple prey of different types. For example in their model, the zooplankton graze on phytoplankton, detritus and bacteria, and it is necessary to specify how much of each prey is grazed at any time. One approach to this problem is to define a measure of total food, as:

$$F = p_1\mathbf{P} + p_2\mathbf{D} + p_3\mathbf{B} \tag{12.13}$$

where p_1, p_2, p_3, are constants determining the zooplankton preferences for various food types. P is phytoplankton, D is detritus and B is bacteria. A Michaelis–Menten expression can be used to define the grazing rate on, for example, phytoplankton as:

$$G_1 = g\mathbf{Z}\frac{p_1\mathbf{P}}{K + F} \tag{12.14}$$

where g is the maximum specific grazing rate, Z is the zooplankton biomass, and K is the half-saturation constant for grazing.

Zooplankton preferences can be constant, but Fasham *et al.* (1990) chose to assign preference as a function of the relative proportion of the food based on Hutson's (1984) switching expression:

$$p_i = \frac{p_i'\mathbf{f}(X_i)}{\sum_k p_k'\mathbf{f}(X_k)}$$

where X_k is the concentration of kth food type and p_i' is defined as the preference for each of the different food types when the concentrations of these foods are equal. In their model, the simplest functional expression used is $f(X_k) = X_k$ which results in the model zooplankton selecting the most abundant food organism. Another possible functional type is the normalized limitation function describing zooplankton ingestion of the ith food type as suggested by Pace *et al.* (1984). Fasham *et al.* (1990) outline the properties of the non-prey-switching function and those based on Hutson's (1984) and Pace *et al.*'s (1984) switching expressions. Evans (1988) emphasizes that the model predictions can be very sensitive to the parameter values used in the switching function, whereas there are very few data on zooplankton feeding preferences to provide such values.

Ingestion related to predator and prey swimming activities and turbulence effects Several models have been developed to simulate the different steps of the feeding process of a swimming organism catching prey in the natural environment. The first models were developed for fish larvae, and then applications were extended to zooplankton in relation to the effect of turbulence. Most of the models described below refer to fish larvae. The amount of food ingested is a function of the number of prey encountered, captured and eaten, the levels of turbulence, light and prey aggregation.

The number of prey encountered and prey ingested are functions of the local prey concentration modified by local turbulence (Rothschild and Osborn 1988; MacKenzie and Kiørboe 1995). The behavior of the larvae (e.g. whether they exhibit cruise behavior or pause–travel behavior) also affects the encounter rate and these aspects are summarized next.

Rothschild and Osborn (1988) discussed the role of turbulence in affecting (enhancing) encounter rates with planktonic prey. Subsequent studies, for example Sundby and Fossum (1990), MacKenzie and Leggett (1991), Muelbert *et al.* (1994), explored the role of turbulence in oceanic conditions, finding an effective increase in contact rates of 2 to 10 times under various wind- and tidal-driven flows. With this formulation, an estimate of $N(i)$ the number of ith prey of concentration $p(i)$ encountered over a 24 h period in a turbulent environment is

$$N(i) = \sum_{24h} LA(i)D(i)p(i)\Delta t \tag{12.15}$$

The effect of the turbulent velocity ω enters in the determination of $A(i)$, the velocity of a larval fish relative to its prey

$$A(i) = \frac{[\sigma^2_{\text{prey}}(i) + 3\tau^2 + 4\omega^2]}{3(\tau^2 + \omega^2)^{1/2}} \tag{12.16}$$

where the larval fish swimming speed τ, and the ith prey swimming speed $\sigma_{\text{prey}}(i)$ are assumed to be on the order of one body-length per second. The parameter L is a binary day/night switch and

$$D(i) = (2/3)\pi\rho^2 \tag{12.17}$$

is the cross-sectional area of perception of the larva, where $\rho = (3/4)L$ is the prey encounter radius and is related to L the larval fish length (e.g. Werner *et al.* 1996).

The turbulent velocity (squared) is

$$\omega = 3.615(\varepsilon r)^{2/3} \tag{12.18}$$

where the separation distance r can be approximated as a function of the concentration $p(i)$ of the ith prey item (Rothschild 1992) as:

$$\boldsymbol{r = 0.55p(i)^{-1/3}} \tag{12.19}$$

and ε is the turbulent kinetic energy dissipation rate specified as a function of space and time. No single formulation has yet parameterized the precise nature of small scale turbulence affecting predator–prey encounters, and different formulations may better represent different limiting cases (Osborn 1996). The definition of the appropriate length scale to estimate the contribution of turbulence to predator–prey contact rates (e.g. equation 12.16) is still a matter of discussion (Dower *et al.* 1997). The length scale has been variously defined as the average distance between prey particles (Sundby and Fossum 1990; MacKenzie and Leggett 1991; Sundby 1995; Werner *et al.* 1996), the Kolmogorov scale (Muelbert *et al.* 1994), the eddy separation distance (Davis *et al.* 1991) and the larval fish reactive distance R (e.g., Evans 1989; MacKenzie *et al.* 1994; Denman and Gargett 1995; Kiørboe and MacKenzie 1995).

For larvae that are pause–travel predators, and defining the effective encounter spatial scale as the larval reactive distance, MacKenzie and Kiørboe (1995) formulated an expression for the encounters E_{p-t} (no. prey $\sec^{-1}$) as

$$\boldsymbol{E_{p-t}(i) = \frac{2}{3}\pi R^3 p(i) P_F + \pi R^2 p(i)(\tau^2 + 2\omega^2)^{0.5} P_F P_D} \tag{12.20}$$

where R is the larval reactive distance (e.g. a fraction of the larval body-length), P_F is the pause frequency (no. $\sec^{-1}$), P_D is the pause duration (sec), and $p(i)$ and τ are as given above. Finally, the estimate of prey encountered is

$$\boldsymbol{N(i) = \sum_{24h} L E_{p-t}^{(i)} \Delta t.} \tag{12.21}$$

A model for the influence of small-scale turbulence on post-encounter processes in larval fish indicated that turbulence can have an overall beneficial or detrimental effect on larval fish ingestion depending on the magnitude of the turbulence and on larval behavior (MacKenzie *et al.* 1994). A dome-shaped relationship is found where ingestion rates are maximum at intermediate rather than high levels of turbulence; the decrease in pursuit success in highly turbulent environments negates the increase in ingestion rate caused by the increase in encounter rate. The implementation of this formulation is achieved by scaling the number of prey encountered by the estimated probability of successful pursuit P_{sp}. The value of P_{sp} depends on the turbulent velocity ω, the pursuit time t_p and the larval reactive distance R. The intersection of the prey excursion sphere (of radius ωt_p) and the larval encounter sphere (of radius R) define appropriate values of P_{sp} (see MacKenzie *et al.* 1994 for details).

Some models have attempted to represent the effect of microscale turbulence on the ingestion of copepods (Davis *et al.* 1991; Saiz and Kiørboe 1995; Caparroy and Carlotti 1996). In their model, Caparroy and Carlotti take into account different processes implicit in the process of ingestion: encounter rate, capture rate, and ingestion *sensu stricto*, i.e. when a prey is in the mouth. The encounter rate is related to relative displacement between prey and predator, which is the consequence of swimming behavior and the microscale fluid motion (Rothschild and Osborn 1988). Different models have represented simple swimming behavior and derived encounter rate expressions for linear swimming (Gerritsen and Strickler 1977) or random-walk swimming (Evans 1989; Yamazaki *et al.* 1991). Kiørboe and Saiz (1995) introduced several types of swimming in their model.

Effect of temperature on ingestion Temperature has an important effect on all physiological functions. As the ingestion process is the input of matter into the individual, the effect of temperature on ingestion is crucial both at the individual level and also at higher levels of organization. Providing that energy and other resources are not limiting, physiological rates usually increase with temperature within the range normally encountered by the organism until a sudden decline near the upper limits when enzyme systems become damaged.

Most of the time only the increasing part of the curve is considered in models, and the function usually used is a power function of temperature:

$$Y_T = AB^T \tag{12.22}$$

Other functions which mimic the complete curve with the increasing and decreasing parts are presented in Table 12.3.

Responses of organisms to temperature have been expressed quantitatively in terms of the temperature coefficient Q_{10} generated from the Arrhenius equation which denotes the ratio of the rate of a metabolic process (e.g. ingestion) at one temperature to the rate at a 10 °C change in temperature, i.e.

$$Q_{10} = \frac{Y_{T+10}}{Y_T} = B^{10} \tag{12.23}$$

The Q_{10} can be calculated as soon as rates are measured for two temperatures as:

$$\log(Q_{10}) = \frac{10}{T_2 - T_1} \log\left(\frac{Y_{T2}}{Y_{T1}}\right) \tag{12.24}$$

By measuring the rate at successive temperatures, it is usually observed that Q_{10} is not independent of temperature over the temperature range in question. The Q_{10} values generally decline with increasing temperature. The Q_{10} of ingestion is generally found to be around two. The effect of temperature on gut clearance rate also may be represented by different models (Dam and Peterson 1988), and the Q_{10} is again found to be slightly above two (Ikeda 1985; Dam and Peterson 1988).

Combined effects of external factors on ingestion

In their copepod growth model, Carlotti and Nival (1992) considered the effects of food concentration, temperature and weight on ingestion and they multiplied the effects of the three functions. Such combinations should be made with caution because the biological responses of combined parameters can differ from the effects studied separately. For example, Thébault (1985) showed that the effect of temperature can differ with food concentration.

Light limitation

The effect of light on prey ingestion rates for certain larval fish has been studied in the laboratory by Huse (1994). For young larvae, ingestion rates were observed to decrease at low and at high light intensities (too much light reduces the required contrast for the larvae to sense their prey). The penetration of light in the water column and its modulation by cloudiness and suspended matter in the water can affect the vertical position of feeding organisms (as they seek adequate light levels for feeding). Combined with the vertical structure of the flow, the effect of light may have an indirect effect on the dispersal of organisms. The inclusion of the effect of light limitation on the capture of prey by recently hatched cod larvae is discussed in the modeling studies of Lough *et al.* (1997) and Quinlan *et al.* (1997).

Table 12.3 Formulation of temperature effects on metabolism.

Formulation	Type	Process	Species	Authors
$a + bT$	linear function	gut clearance rate	*Temora longicornis*	Dam and Peterson (1988)
$a + b\log(T + c)$	log-linear function	gut clearance rate	*Temora longicornis*	Dam and Peterson (1988)
$a(T - c)^b$	Belehrádek law	gut clearance rate	*Temora longicornis*	Dam and Peterson (1988)
$a(b)^T$	exponential law	gut clearance rate	*Temora longicornis*	Dam and Peterson (1988)
$k(T - T_{min})^a(T_{max} - T)^b$	similar to beta function			
$\left(\frac{T - T_{min}}{T_{max} - T_{opt}}\right)^{b\left(\frac{T_{max} - T_{opt}}{T_{opt}}\right)}$	modified beta function		*Temora stylifera*	Thébault (1985)
$k(1 - e^{-a(T - T_{min})})(1 - e^{-b(T_{max} - T)})$	exponential product	swimming	Dinoflagellates	Kamykowski and McCollum (1986)

ASSIMILATION AND EGESTION

In most models, the assimilation rate is usually assumed to be a constant fraction (usually ranging between 0.6 and 0.8) or some fixed function of the ingestion rate. This is probably an over-simplification of the digestive process; assimilation is linked to gut transit time and fecal pellet production. As several methods to measure ingestion based on gut content (gut fluorescence) or fecal pellet production have become available, detailed models of the processes of gut transit and gut evacuation have been built.

Gut content has been used extensively to infer feeding rates of aquatic herbivores (see review in Baars and Helling 1985) and various models have attempted to simulate the processes involved in the change in gut content for both zooplankton (Dam *et al.* 1991) and fish (Jobling 1994). The instantaneous amount of food in an animal's gut, the gut content (G) is a function of its ingestion rate (I) minus its evacuation rate (E) and the amount of food destroyed or undetected in the gut (U):

$$\frac{dG}{dt} = I - (E + U) \tag{12.25}$$

E and U are considered to be proportional to gut content:

$$E + U = KG \tag{12.26}$$

where K is the gut clearance rate constant min^{-1}. Dam *et al.* (1991) present a mathematical model with simple analytical solutions describing the time-dependent changes of gut content and discuss the short term variability in feeding behavior. A much more complex model based on chemical-reactor theory and comparing the gut to a plug-flow reactor has been developed by Penry and Jumars (1987).

Slagstad and Tande (1981) suggested a mathematical model of the assimilation process in copepods depending on the ingestion rate, the phytoplankton species composition and physiological state of the animal. This model predicts a decrease in assimilation efficiency with increasing ration.

Assimilation efficiencies will also differ according to the currency being used in the model. When more than one element is modeled, inconsistencies can arise if prey and predator have different element ratios. Moloney (1992) showed how matter could be created in models where element-ratio effects are not taken into account and constant assimilation efficiencies are used.

EXCRETION AND RESPIRATION – ENERGETIC COSTS

Excretion and respiration represent the metabolic losses in the nitrogen budget and in the carbon budget respectively. In the simplest formulation, respiration or excretion rates as a whole can be related to weight (Peters 1983; Vidal and Whitledge 1982; Ikeda 1985; Corkett and McLaren 1978). However, metabolic rates can be subdivided into several components because of the heterogeneous nature of catabolism: costs of locomotion activity, basic metabolism, assimilation, synthesis of somatic and gonad tissue, matter transformation for storage, etc. (see Clarke 1987). One major difficulty is the possibility that each of the separate components might vary differently with season and temperature.

The level of detail in the representation of the different components depends on the questions to be answered with the model.

The simplest formulation is to relate the whole metabolic process to body size. As already presented, most body size relationships are of allometric form. The scaling exponent is usually close to 0.75. The variable to be used as an index of body size in

scaling studies deserves careful consideration (Anderson and Hessen 1995). Dry weight is the traditional measure for ecological variables such as growth, but Vidal and Whitledge (1982) suggest that dry weight-based scaling relationships may be biased if animals have large proportions of metabolically inactive tissue, such as lipid stores. Others have chosen to express body size in terms of carbon (e.g. Ikeda and Skjoldal 1989; Schneider 1990). Schmidt-Nielsen (1984) and Cammen *et al*. (1990) have recommended the use of body nitrogen content as a mass variable, protein content provides an easily determined measure of body nitrogen. Because metabolism–size relationships can change with the size index selected (Berges *et al*. 1990), metabolic measurements based on different mass variables are not easy to compare, and need careful consideration before inclusion in models.

Integrating the metabolic budget of copepods during their lifetime, i.e. under various food conditions, Steele and Mullin (1977) identify three main components of respiration in zooplankton: basal or routine metabolism, the costs associated with foraging and capturing food, and the cost of assimilating and biochemically transforming the food. The last two components are often grouped as active metabolism. Carlotti and Sciandra (1989) suggest that the basal metabolism is related to weight, and the active metabolism is a proportion of the ingestion rate. In their growth model of a ctenophore, Kremer and Reeve (1989) use similar components.

Caparroy and Carlotti (1996) present a deterministic model of a copepod's energy budget to study the effect of turbulence on ingestion and on the related physiological processes. In such a model, taking into account the swimming speed of the predator, the specific cost of swimming is of primary importance in estimating the consequences of feeding strategies in different turbulent conditions. Several models evaluate the energetic cost of swimming activity by copepods and the effect of buoyancy on this cost (Vlymen 1970; Morris *et al*. 1985) and fish (Laurence 1985). Tiselius and Jonsson (1990) used theoretical hydrodynamic models to investigate costs and benefits of different feeding strategies (see also Haury and Weihs 1976). In his model of fish feeding, Laurence (1985) takes into account the cost of processing and utilizing the digested food.

Anderson (1992) presents a bioenergetic model of marine heterotrophs (zooplankton and bacteria) and determines food quality (in terms of C and N content) on growth and nitrogen excretion. This model illustrates the close link between nitrogen excretion and respiration, because excretion varies with the type of substrate respired.

The effect of temperature on metabolic rates can be represented by various functions, such as those presented in Table 12.3. The Q_{10} is generally found to be between two and four.

GROWTH AND EGG PRODUCTION MODELS

Individual growth is simulated by integration of the equation

$$G = \frac{dW}{dt} = \textbf{Ingestion} - \textbf{Egestion} - \textbf{Excretion} - \textbf{Respiration} \qquad (12.27)$$

Growth and egg production generally do not occur simultaneously; when the matter budget is positive, it is used for either growth or reproduction. A time step of 1 h is generally sufficient to simulate the dynamics of physiological processes and growth over several days. At each time step, the calculated growth increment is integrated over time.

Growth models based on allometric relations to weight

The rates of metabolic losses in non-limiting food conditions and constant temperature

are usually considered as allometric relationships (Laurence 1978; Beyer and Laurence 1980, 1981; Peters 1983; Huntley and Boyd 1984; Moloney and Field 1989; Kiørboe and Sabatini 1995; Hirst and Sheader 1997). Von Bertalanffy (1960) produced the gross equation that expresses the rate of growth (G) as the difference between anabolism and catabolism:

$$G = \frac{dW}{dt} = kW^c - jW^b \tag{12.28}$$

where W is the body mass, t is time, and k and j are indices specific to particular combinations of genotype and environment, and c and b are 'scaling exponents'. A scaling exponent of less than 1.0 means that larger animals demonstrate lower rates of metabolism per unit weight than do smaller animals.

If $c \neq b$ there is an optimal growth for the body mass M_{opt} and an optimal mass for a balance between losses and gains M_{max}:

$$M_{opt} = \left(\frac{jb}{kc}\right)^{\frac{1}{c-b}} \tag{12.29}$$

$$M_{max} = \left(\frac{j}{k}\right)^{\frac{1}{c-b}}. \tag{12.30}$$

The highly simplified growth equation 12.28 described by von Bertalanffy (1938) summarizes many different processes, with the influence of several external parameters. Temperature affects metabolism, and several empirical relationships (exponential, linear, power) relate the four parameters of equation 12.28 to temperature (Atkinson 1994). The exponential function of temperature is the most common (Huntley and Boyd 1984).

Allometric relationships, with or without their temperature-dependent effects, are based on regression models that can be derived from data sets using single species or groups of several species, and obtained under various conditions. Furthermore, not all authors proceed in the same way in calculating these regressions. For example, after log transformation of the values of rate processes and corresponding weight values, Moloney and Field (1989) fixed the values of the allometric exponents, rather than allowing these to be estimated by regression. Thus, allometric relationships should be used with caution in any model.

Growth and egg production models in changing environmental conditions

These models should take into account detailed physiological functions of those rates that vary with fluctuations of external variables. Food and temperature are usually taken into account in budget models of zooplankton (Kremer and Nixon 1978; Carlotti and Sciandra 1989; Carlotti and Hirche 1997). Table 12.4 presents a list of publications containing growth models for different zooplankton groups and species.

In Carlotti and Sciandra's (1989) model of the copepod *Euterpina acutifrons*, food and temperature affect ingestion which, in turn, influences the rate of excretion, so that the excretion rate is influenced indirectly by temperature and food. Because the physiological connections are numerous and non-linear, it is important to test the consequences of an hypothesis in relation to a given process. By comparing model output and data under different external forcing conditions, different scenarios in the hierarchy of the physiological processes can be tested. For example, Huntley *et al.* (1994a) developed a physiological model of growth of Antarctic krill *Euphausia superba* in terms of C and N

Table 12.4 Zooplankton growth models.

Species	Authors
Ctenophore	
Mnemiopsis leidyi	Kremer and Nixon (1978)
Mnemiopsis mcradyi	Kremer and Nixon (1978)
Meroplanktonic larvae	
Crassostrea virginica	Dekshenieks *et al.* (1993)
Pectinaria koreni	Carlotti (1996a)
Cladocera	
Daphnia sp.	Gurney *et al.* (1990)
CRUSTACEA	
Copepods	
Copepods	van den Bosch and Gabriel (1994)
	Omori (1997)
	McLaren (1997)
	Broekhuizen *et al.* (1994)
Euterpina acutifrons	Carlotti and Sciandra (1989)
	Carlotti and Nival (1992)
Calanus sp.	Steele and Mullin (1977)
	Steele and Frost (1977)
Calanus finmarchicus	Slagstad (1981)
	Carlotti and Radach (1996)
	Carlotti and Hirche (1997)
	Carlotti and Wolf (1998)
Calanus glacialis	Slagstad and Tande (1990)
Metridia pacifica	Batchelder and Miller (1989)
Metridia lucens	Batchelder and Williams (1995)
Mysidacea	
Mysis mixta	Gorokhova (1998)
Decapoda	
Hyas araneus	Anger (1990)
Euphausiacea	
Euphausia superba	Astheimer *et al.* (1985)
Fish larvae	
Engraulis mordax	Wroblewski (1984)
Gadus morhua	Werner *et al.* (1993)
Melanogrammus aeglefinus	Werner *et al.* (1993)
Melanogrammus aeglefinus	Cushing and Horwood (1994)
Theragra chalcogramma	Hinckley *et al.* (1996)
Theragra chalcogramma	Rose *et al.* (1996)
Theragra chalcogramma	Rose and Cowan (1993)
Theragra chalcogramma	van Winkle *et al.* (1993)

to study different scenarios of the life strategy of krill during winter. In this case, the time step was one day, and the simulations were run over a four month period.

Budget models can be used as a tool to test the importance of newly explored environmental factors such as microscale patchiness and turbulence to growth and

recruitment of planktonic consumers (Davis *et al*. 1991; Tiselius *et al*. 1993; Nonacs *et al*. 1994).

Egg production can be related to the mass budget of females. The simplest formulation is to consider that above a given mass (mass of mature females), all assimilated matter is used for egg production and not for growth (e.g. Carlotti and Nival 1992).

Carlotti and Hirche (1997) present a model of the individual bioenergetics of *Calanus finmarchicus* females with details of the oocyte maturation steps. Their model considers the transfer of matter to different parts of the body from the ingested matter in the gut. The matter is directed toward either the structural weight, the lipid reserves sac, or the gonads. Four steps of oocyte maturation are considered. By comparison with data, the model suggests that, for this species, egg production cannot be dependent only on external parameters but is also strongly linked to the state of internal compartments.

Egg production models related to external parameters

Several empirical relationships relating observed egg production directly to external variables have been proposed, mainly in relation to temperature and food. Corkett and McLaren (1978) defined a temperature dependent empirical equation

$$F(T) = \frac{E_s \cdot S}{t_1 + S_n \cdot t_S} \tag{12.31}$$

where $F(T)$ is the daily egg production per adult female, E_S is the number of eggs in one sac, S_n is the total number of sacs produced over life time, t_1 is the time from reaching adult to the appearance of the first sac, and t_S is the time between the appearance of successive sacs (Davis 1984b). The values of t_1 and t_S are expressed as a percentage of the embryonic duration which depends on temperature following a Belehrádek's equation (see page 590). Uye (1981) also defined an empirical relationship between copepod egg production and temperature and food. Checkley (1980a, 1980b) suggested a relationship between copepod egg production and female length and temperature. These empirical relationships are useful for population models which do not consider individual budgets (e.g. Davis 1984b).

12.3.2 Vital rates

Vital rates (e.g. development rates and mortality rates) can be obtained from cohort development studies either in laboratory controlled conditions, in mesocosms, or *in situ*. Aksnes *et al*. (1997) discuss obtaining data for life tables from cohort analyses of populations of copepods. Such data allow the estimation of durations of successive developmental stages of species under various environmental conditions (temperature, food, and salinity). In this section, we present empirical functions of development rates and mortality rates commonly used in population dynamics models. Inverse methods are described that estimate vital rates by fitting simulations of a population model to data.

Vital rates are usually linked directly to external parameters (e.g. temperature, food concentration) which are experimentally easy to control. Indeed, vital rates depend on physiological states of the organisms which vary with the external factors. The modeling of functional biological properties which modify vital rates (i.e. Carlotti and Sciandra 1989, their Figure 1) results in more efficient simulation of the dynamics of organisms if the external conditions of temperature and food are highly variable.

DEVELOPMENTAL STAGE DURATIONS OF CRUSTACEAN ZOOPLANKTON

In non-limiting food conditions, development time of stages from egg to C5 as a function of sea water temperature is commonly fitted using Belehrádek's equation (McLaren 1963, 1978; Mauchline 1998):

$$D_i = a_j(T + b)^c \tag{12.32}$$

where D_i is the development time of stage i (days) and T is temperature (°C). Parameter a_i (days $^\circ C^{-1}$) governs the mean slope, b (°C) allows for shifts in the temperature scale and c (dimensionless) determines the curvature of the response. The parameters b and c are considered as characteristics of the species and are equal for all the stages. Values of a_1, b and c are obtained by fitting embryonic durations (stage 1) at different temperatures. In the absence of sufficient data, c is often assumed to be -2.05 (Mauchline 1998, see his table 48), and b is set to a reference value (e.g. 13.87 for *Pseudocalanus*, Davis 1984a, 1984b; Corkett and McLaren 1970, 1978).

Parameter a_i depends on stage i and is determined by knowing stage durations D_i at a given reference temperature:

$$a_i = a_1 \frac{D_i}{D_1} \tag{12.33}$$

The adult duration also changes with temperature but it is more difficult to estimate, because it is fixed by mortality. Davis (1984a) considers the adult female life D_{12} to be twice the length of the reproductive period.

Several empirical models have related stage duration to temperature (Heip 1974; Guerrero *et al.* 1994). The latter authors carried out a comparative analysis of several equations (Belehrádek's equation, linear equation, hyperbolic and power equations, and exponential equations) used to describe the dependence of the development of organisms on temperature. McLaren (1995) and Blanco *et al.* (1995) discuss the biological significance of the different equations. These equations fit the observed development time in a range of temperatures that are not the extreme temperatures. At the optimal temperature a minimum development time is observed, and above this optimal temperature the development time increases for higher temperatures.

Under fluctuating temperature conditions, the molting cycle can be modeled by approximating the stage duration over short time steps with constant temperature. If the time step of the model is dt, the fraction of the molting cycle completed after dt at time t (MC_t) is:

$$MC_t = \frac{dt}{a_i(T + b)^c} \tag{12.34}$$

where T is the temperature during dt. By summing the molting fractions MC_t over the time since entry of the organism into that particular stage, the completed portion of the molting cycle is obtained. The molting cycle is completed when the sum is 1, and the stage duration can be calculated by summing the number of time steps. Miller and Tande (1993) modeled the development time for a single cohort of *Calanus finmarchicus* over one year in this way (see also section 12.4).

Stage duration also can be considered as the time period necessary for an organism to grow from a starting weight to a final weight, as modeled by Carlotti and Sciandra (1989). In doing so, there is an implicit hypothesis that development and growth are completely linked, which is not true in many cases. Nevertheless, models such as this allow the investigation of possible effects of external factors on development.

MORTALITY RATES

The representation of mortality in zooplankton modeling and the estimation of parameter values is difficult for population dynamics models as well as ecosystem models. The importance of emphasizing mortality when studying the dynamics of natural populations is expanded by Ohman and Wood (1995, 1996). The importance of the mortality term in bulk models is discussed in section 12.5.

Zooplankton mortality arises for a number of reasons. These can be classified as internal (developmental stage, senescence, genetic background), external (starvation, predation, parasitism) and the combination of external and internal factors (e.g. efficiency of enzymatic activity is a function of temperature) (see Ohman and Wood 1995 for a review). As a consequence there are a variety of formulations to represent mortality.

The simplest formulation is to consider a constant mortality for the whole population. This approximation neglects the fact that different stages may have different sensitivities during critical periods such as molt or starvation, and that individuals in a given stage may have different mortality rates depending on their physiological state. Numerous models consider constant stage-dependent mortality rates that decrease with life stage (i.e. Cushing 1975; Wroblewski 1980; Batchelder and Miller 1989). Mortality values used in population models are estimated from field data using different methods (see Aksnes *et al.* 1997; Wood and Nisbet 1991), and the range of estimated values is often large enough to simulate very different dynamics. By running simulations over successive years, it is possible to reduce the range to values giving stable population cycles. The use of different data sets to compare simulated and observed population abundance and structure is the best way to reduce the parameter range.

Mortality rates probably vary within stages for several reasons, and detailed formulations have been used in several models. Mortality caused by starvation can be related either directly to food concentration (Andersen and Nival 1986) or to an index of the physiological state of organisms in terms of their specific growth rates (Wroblewski 1984; Carlotti and Sciandra 1989; Bryant *et al.* 1997). An average value of specific growth rates over the previous few days could be introduced to represent the ability to buffer short starvation periods. Bryant *et al.* (1997) add a complementary effect of temperature in their mortality function.

Predation is often believed to be the major source of mortality for herbivorous zooplankton (Ohman 1986). Davis (1984a, 1984b) presents model simulations of copepod seasonal cycles on Georges Bank investigating the role of predation by chaetognaths (size selective), ctenophores (non selective) and carnivorous copepods, in the control of population growth. Mortality caused by each predator is represented as the product of predator abundance and the consumption rate (number of copepods eaten daily by one predator) which is temperature dependent. A formulation of the size selective chaetognath predation is developed in detail in Davis's (1984b) paper. The model runs were made with various consumption rates until the model output matched observed seasonal cycles.

Fiksen and Giske (1995) divide the contribution to mortality by predation into visual and tactile fractions. The visual component mainly consists of fish and the tactile component of invertebrate carnivores and omnivores like medusae, chaetognaths and predatory copepods. They use a process model of predation by visual predators on zooplankton (Aksnes and Giske 1993; Giske *et al.* 1994; see also Aksnes *et al.* 1997). The model consists of a set of equations that calculate the visual range of a planktivore from the prevailing light conditions, influenced by irradiance at the surface, the fraction

reflected at the air–water interface, turbidity, depth, prey characteristics and planktivore eye sensitivity threshold for prey recognition. In Fiksen and Giske's (1995) model, tactile predation is assumed to be a size-dependent mortality rate which can be represented by a negative power function of weight (Peterson and Wroblewski 1984; McGurk 1986). Hansen *et al*. (1994) present a synthetic study of the size ratio between predator and prey in zooplankton. The integrated effect is likely to be a decline in tactile predation pressure with age and stage (Ohman 1988), although this may not apply in all cases. Size dependent cannibalism is part of this tactile predation and is probably an important source of mortality for eggs and nauplii (Kremer and Nixon 1978; Peterson and Kimmerer 1994).

INVERSE METHODS TO ESTIMATE VITAL RATES

Population dynamics models presented in section 12.4 can be used to estimate stage durations (or development rates) and mortality rates by fitting the model to observed data. A wide variety of inverse methods can be used to do these fits (Manly 1989). Inverse methods estimate parameters by fitting simulations to data.

Although many methods employ analytical manipulations of the basic equations, most can be solved through parameter estimation techniques widely employed in system identification: Parslow *et al*. (1979) illustrate these techniques in a comparative analysis of four different cohort models. They found uncertainty in the estimates of both stage duration and mortality to increase with increasing sampling error and decreasing sampling intensity. Hay *et al*. (1988) also used a model to describe the birth, growth and mortality rates of small copepods reared in enclosures. They found that uncertainty in the estimates of mortality could be reduced by aggregating the stages. Jellison *et al*. (1995) use systems identification techniques to compare parameter estimates from stage-structured population models with different degrees of complexity to represent the development rate. Development and mortality rates were estimated in all three of their models using standard non-linear estimation techniques. The best values were determined by minimizing the weighted squared errors between modeled and simulated data using the Levenburg–Marquardt algorithm (Press *et al*. 1992). In a similar way, Rothschild *et al*. (1997) developed a numerical method for separating and estimating growth and mortality coefficients using a stage- or size-structured population model and an optimization formulation. A basic assumption when using inverse methods is that the rates of mortality and development are constant within each stage. Rothschild *et al*.'s (1997) method permits the incorporation of time-related functions of the rates.

Inverse techniques have been used to estimate vital rates from field data. Huntley *et al*. (1994b) used analytical solutions of a population dynamics model to estimate stage-specific mortality and development of *Calanoides acutus*, an Antarctic species, as the population emerged from overwintering diapause. Miller and Tande (1993) also compared simulations with an age-within-stage model to stage abundance data of *Calanus finmarchicus*. They determined stage durations by examining the temporal progression of fractional abundances of the stages, and discuss the quality of sampling in these estimations.

12.3.3 Evolutionary forces on the organism

Imagine a population of copepods or any other biological species. Within this population assume a limited number of genetic recipes: DNA-based codes for building a body, making enzymes for digestion, setting rules for allocation of surplus food to

body tissues and to reproduction and so forth. Let us term such a recipe a 'genetic strategy'. Offspring inherit the genetic codes from their parents; let us for simplicity assume they inherit everything from their mothers. We can calculate the instantaneous rate of increase ρ of each such strategy i by

$$\boldsymbol{\rho_i} = \frac{\ln\left(\frac{N_i(t)}{N_i(0)}\right)}{t} \tag{12.35}$$

where N is the number of individuals carrying i and t is the time period. If the environment remains stable, or if the time period t is sufficiently long, then ρ_i describes the growth rate of strategy i. As there always will be a maximum number of individuals possible in any population (either because of resources, competitors, predators, parasites, or a combination of all) the total population will gradually be dominated by those strategies that have the highest rates of increase:

$$N(t) = \sum_i N_i(0)e^{\rho it} \tag{12.36}$$

The fitness of a strategy may be defined as the difference between its rate of increase and the average rate of increase (r) of the whole population (the whole gene pool of strategies) (Giske *et al.* 1993):

$$\boldsymbol{\Phi_i} = \boldsymbol{\rho_i} - \boldsymbol{r} \tag{12.37}$$

Over evolutionary time only those strategies that on average produce the highest rates of increase may persist. Hence all strategies of life that exist among copepods or other species, are the results of natural selection, where the criterion for long term survival is maximization of the rate of increase ρ.

Evolution through natural selection introduces a biological *force* on the individuals (Dawkins 1995; Giske *et al.* 1998a). Although this is a completely passive and unconscious process, genetic codes will tend to produce individuals that live for the maximization of their reproductive rates (Sibly 1989; Figure 12.1). This also means that the lives of the individual organisms may be understood by the logic of natural selection, and sometimes also predicted from ecological factors. By resolving equation 12.37, we could find optimal decisions and trade-offs among developmental, physiological and ecological variables. This could enable us to model how an organism will lead its life.

The three main demographic variables controlling the rate of increase of the strategy are the fecundity (b), probability of survival from egg to reproduction (S), and the generation time (α):

$$\boldsymbol{\rho_i} = \frac{\ln(\boldsymbol{bS})}{\boldsymbol{\alpha}} \tag{12.38}$$

The survival can again be expressed as a function of the instantaneous mortality rate M

$$\boldsymbol{S} = \exp(-\boldsymbol{\alpha M}) \tag{12.39}$$

By substituting for S in equation 12.32, we can write

$$\boldsymbol{\rho_i} = \frac{\ln(\boldsymbol{b})}{\boldsymbol{\alpha}} - \boldsymbol{M} \tag{12.40}$$

which is an expression of what can be maximized through natural selection (Sibly 1989). From equation 12.40 we can evaluate the fitness value of an action, and we can decide

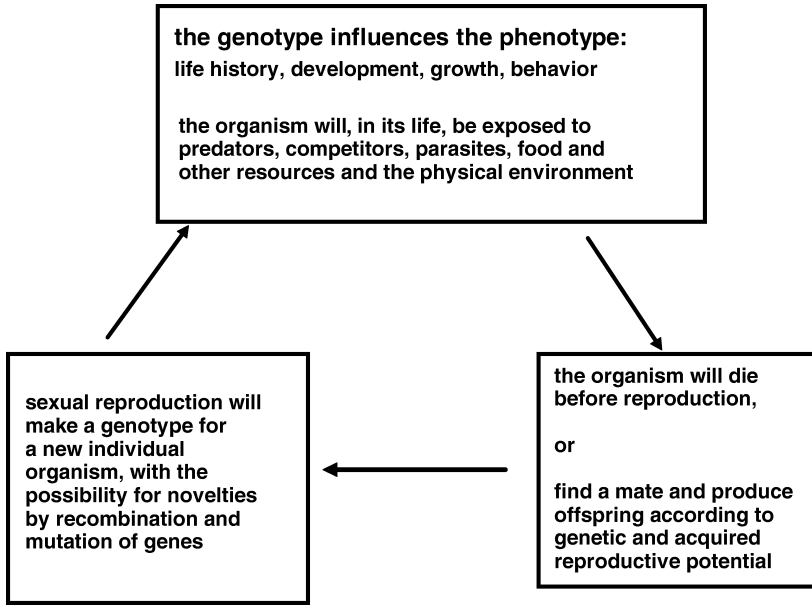

Fig. 12.1. The evolutionary premise for optimization theory in ecology: evolution leads to adaptation by natural selection. Although evolution cannot be sure to find the optimal solutions, it creates adaptations.

what to do among several alternatives. For instance, if a copepod could 'choose' among several depths with variable food concentrations (impacting fecundity), temperatures (impacting generation time) and light intensities (impacting mortality risk), then equation 12.40 can be used to calculate the fitness effects of the decisions. In many cases, the benefit of staying in warm, food-rich surface waters will be higher at night (with low light intensity and predation risk from visual predators) than during daytime, and there will be a benefit of diel vertical migration.

We can further split this equation into age-dependent processes by the Euler–Lotka equation (Euler 1760; Lotka 1907; Stearns 1992):

$$1 = \sum_{Y=1}^{\infty} m_Y l_Y e^{-eY} \tag{12.41}$$

Here we see that the optimal life-history strategy will depend on how behavior may impact fecundity (m_Y) or survival (l_Y) in this time period as well as in later periods. Heavy reproduction may in some instances impact both current mortality risk and future fecundity (e.g. by depleting energy reserves). Fitness maximization has implications far beyond these demographic variables. All aspects of life (biochemistry, morphology, anatomy, physiology, life cycle, and behavior) can be optimized by natural selection in order to maximize the expected rate of increase.

The Euler–Lotka equation will only find the precise value of ρ in an environment that repeats itself from generation to generation. In variable environments, fitness is maximized by a strategy that also minimizes intergenerational variation in the rate of increase (Yoshimura and Clark 1993; Tuljapurkar and Caswell 1997). But for ecological

modeling of zooplankton, the Euler–Lotka equation or a derivative of it will suffice in most cases.

12.3.4 Further reading

Books with descriptions of individual budget dynamics:

Kremer, J.N. and Nixon, S.W., 1978. *A coastal marine ecosystem. Simulation and analysis*. Springer-Verlag, Berlin, 217 pp.

Clark, C.W. and Mangel, M., 1999. *Dynamic state variable models in ecology: methods and applications*. Oxford University Press, Oxford, in press.

Kooijman, S., 1993. *Dynamic energy budgets in biological systems. Theory and applications in ecotoxicology*. Cambridge University Press, Cambridge, 350 pp.

Mangel, M. and Clark, C.W., 1988. *Dynamic modelling in behavioural ecology*. Princeton University Press, Princeton, NJ, 308 pp.

Metz, J.A.J. and Diekmann, O., 1986. *The dynamics of physiologically structured populations. Lecture Notes in Biomathematics 68*. Springer-Verlag, Berlin, 511 pp.

Tuljapurkar, S. and Caswell, H., 1997. *Structured-population models in marine, terrestrial, and freshwater systems*. Population and Community Biology Series 18, Chapman and Hall, New York, 643 pp.

For a review of knowledge on calanoid copepods, useful for parameter calibration, see:

Mauchline, J. 1998. *The biology of calanoid copepods*. Academic Press, San Diego, 710 pp.

12.4 POPULATION MODELS

12.4.1 Populations described by one variable

The simplest and earliest models describe populations in terms of one variable, the total number of individuals in that population. These models postulate that the rate of change of the population number, N, is proportional to N (Malthus 1798; Pielou 1969):

$$\frac{dN}{dt} = rN \tag{12.42}$$

Verhulst (1838) modified this equation by adding a non-linear term, giving the logistic equation (Pielou 1969):

$$\frac{dN}{dt} = rN\left(1 - \frac{N}{K}\right) \tag{12.43}$$

By adding a time delay term to the logistic equation, oscillations of the population can be represented (Cunningham 1954).

12.4.2 Populations described by several variables – structured population models

Life cycles of zooplankton species are complex with individuals developing through different life stages. They are relatively long compared with bacteria, phytoplankton and microzooplankton. Some modeling approaches use variables that structure the popula-

tion with respect to age (age structured population models – ASM), stage (stage structured population models – SSM), size (size structured population models – SiSM) and weight (weight structured population models – WSM).

DISCRETE-TIME DIFFERENCE EQUATION MODELS AND MATRIX MODELS

Matrix models constitute a class of population models that incorporate some degree of individual variability. In a recent overview, Caswell (1989) showed that they are powerful tools for analyzing, for example, the impact of life-history characteristics on population dynamics, the influence of current population state on its growth potential, and the sensitivity of the population dynamics to quantitative changes in vital rates. Matrix models are convenient for cases where there are discrete pulses of reproduction, but not for populations with continuous reproduction. They are not suitable for studying the dynamics of populations that live in fluctuating environments.

Age structured matrix models (ASMM)

One of the first representations of age-structured dynamics was obtained by working with discrete age-classes, often referred to as a *Leslie matrix* (see Caswell 1988, 1989, 1997). Suppose there are m age classes numbered $1, 2, \ldots, m$, each covering an interval τ. If $N_{j,t}$ denotes the number of individuals in age class j at time t and G_j denotes the fraction of the population in this age class that survive to enter age class $j + 1$, then

$$N_{j+1,t+1} = G_j N_{j,t} \tag{12.44}$$

Individuals of the first age class are produced by mature individuals from older age classes:

$$N_{1,t+1} = \sum_{j=1}^{m} F_j N_{j,t} \tag{12.45}$$

where F_j is the number of age class 1 individuals produced per age class j individual during the time step τ.

The system of equations 12.44 and 12.45 can be written in matrix form:

$$\begin{pmatrix} N_1 \\ N_2 \\ N_3 \\ \vdots \\ N_m \end{pmatrix}(t+1) = \begin{pmatrix} 0 & F_2 & F_3 & \cdots & F_m \\ G_1 & 0 & 0 & \cdots & 0 \\ 0 & G_2 & 0 & \cdots & 0 \\ \vdots & \ddots & \ddots & \cdots & \vdots \\ 0 & 0 & & G_{m-1} & 0 \end{pmatrix} \begin{pmatrix} N_1 \\ N_2 \\ N_3 \\ \vdots \\ N_m \end{pmatrix}(t). \tag{12.44}$$

Such models were originally formulated for populations in which the individual age was the main source of variability (Leslie 1945, 1948).

Stage- (SSM), size- (SiSM) and weight- (WSM) structured matrix projection models

The Leslie matrix has been modified to deal with size classes, weight classes and developmental stages as the key individual characteristics of the population. Organisms grow through a given stage or size/weight class for a given duration.

The population projection matrix, often referred to as a *Lefkovitch matrix* (Lefkovitch 1965), has the form:

$$\begin{pmatrix} N_1 \\ N_2 \\ N_3 \\ \vdots \\ N_m \end{pmatrix}(t+1) = \begin{pmatrix} P_1 & F_2 & F_3 & \cdots & F_m \\ G_1 & P_2 & 0 & \cdots & 0 \\ 0 & G_2 & P_3 & \cdots & 0 \\ \vdots & \ddots & \ddots & \cdots & \vdots \\ 0 & 0 & & G_{m-1} & P_m \end{pmatrix} \begin{pmatrix} N_1 \\ N_2 \\ N_3 \\ \vdots \\ N_m \end{pmatrix}(t). \tag{12.45}$$

where F_i is the size- (or weight- or stage-) specific fertility, G_i the probability of surviving and growing into the next size class, and P_i the probability of surviving and remaining in the current size class. G_i and P_i can be written in terms of the size-specific survival probability σ_i and the size-specific growth probability (or probability of molting) γ_i:

$$G_i = \sigma_i \gamma_i \tag{12.46}$$

$$P_i = \sigma_i(1 - \gamma_i) \tag{12.47}$$

The parameter γ_i can be estimated from the distribution of durations within each size class (or weight class or stage). Caswell (1988, 1989) presented the use of different stage duration distributions to estimate γ_i: geometric distributions, fixed stage durations for all individuals, variable stage durations, and negative binomial distributions.

For a stable population, the dominant eigenvalue λ of the matrix represents the population growth rate and the corresponding eigenvector is the stable size (or weight or stage) distribution (see Caswell 1989).

Structured matrix models including both stage, size, weight and age

The construction of models using both stage, size, weight, and age is possible (Caswell 1983, 1988, 1989) but such models are difficult to manipulate because of the large number of categories required. For zooplankton species, individuals proceed through a series of developmental stages with the probability of moving from one stage to the next dependent on the time already spent in the stage, but independent of the time spent in any previous stage. In such a model, individuals are classified by age within stages (or size/weight classes), as was first done by Parslow *et al.* (1979).

There are several variations of these matrix models, differing mainly in the expression of vital rates, which can vary with time depending on external (e.g. temperature, food concentration, competitors, predators) or internal (e.g. density-dependent) factors.

Examples of applications of matrix projection models for zooplankton population studies are given in Table 12.5. Two examples are described below.

Davis (1984a) presented a copepod model that simulates the movement of individuals through stages with a good representation of the duration of each stage. The model considers age-classes, one for each time step, separating new recruits in a stage from older individuals in the stage.

The transfer from one age class j to the next for non-molting individuals is given by:

$$N_{i,j+1} = \sigma_i(1 - \gamma_{i,j})N_{i,j} \tag{12.48}$$

where i is the stage, σ_i is the stage-specific survival rate and $\gamma_{i,j}$ the stage- and age-within-stage-specific probability of molting.

Table 12.5 Population dynamics models of zooplankton and ichthyoplankton. MM matrix model; ASMM age structured matrix model; SSM stage structured model; CASM continuous-age structured model; CASiSM continuous-age and size structured model; SASM stage and age structured model; WSM weight structured model; SiSM size structured model; IBM individual based model; LEM Lagrange ensemble model; CM cohort model.

Species	Authors	Type of model
Salps		
Thalia democratica	Ménard *et al.* (1994)	MM
Salpa fusiformis	Andersen and Nival (1986)	SSM
Medusa		
Pelagia noctiluca	Morand *et al.* (1992)	MM
Annelids (larval stages)		
Streblospio benedicti	Levin *et al.* (1987)	ASMM
Nephtys incisa	Zajac and Whitlatch (1989)	ASMM
Owenia fusiformis	Thiébault and Dauvin (1991)	ASMM
Pectinaria koreni	Carlotti (1996a)	SSM
Cladocera		
Daphnia sp.	Frank (1960)	ASMM
	Sinko and Streifer (1967)	CASM
	Streifer (1974)	CASiSM
	Argentesi *et al.* (1987)	CASM
	De Roos *et al.* (1992)	SSM
	Hogeweg and Richter (1982)	IBM
	Mooij and Boersma (1996)	IBM
Cirripeds (larval stages)		
Balanus glandula	Roughgarden and Iwasa (1986)	ASMM
Copepods		
Copepod sp.	Woods and Barkmann (1993)	LEM
	Caswell and Twombly (1989)	SSMM
	Hogeweg and Richter (1982)	IBM
Diaptomus sanguineus	Hairston and Munns (1984)	ASMM
Eurytemora affinis	Gaedke (1990)	SSM
Euterpina acutifrons	Sciandra (1986)	SASM
	Carlotti and Sciandra (1989)	SASM
	Carlotti and Nival (1992)	SASM
Acartia tonsa	Gaedke (1990)	SSM
Acartia clausi	Wroblewski (1980)	SSM
Paracalanus sp.	Hofmann and Ambler (1988)	SSM
Paracalanus parvus	Davis (1984b)	SASM
Pseudocalanus sp.	Davis (1984a, 1984b)	SASM
Calanus sp.	Steele and Mullin (1977)	CM
	Steele and Frost (1977)	CM
Calanus finmarchicus	Slagstad (1981)	WSM
	Davis (1984b)	SASM
	Miller and Tande (1993)	SASM
	Carlotti and Radach (1996)	SASM

(*continued*)

Table 12.5 *Continued*

Species	Authors	Type of model
Calanus finmarchicus	Carlotti and Wolf (1998)	IBM
	Bryant *et al.* (1997)	WSM
	Heath *et al.* (1997)	WSM
	Tande and Slagstad (1992)	WSM
Calanus marshallae	Wroblewski (1982)	SSM
Calanus glacialis	Slagstad and Tande (1990)	WSM
Calanoides acutus	Huntley *et al.* (1994b)	SSM
Calanus chilensis	Marin (1997)	SSM
Metridia pacifica	Batchelder and Miller (1989)	IBM
Metridia lucens	Batchelder and Williams (1995)	IBM
Decapoda		
Shrimps		
Penaeus astecus	George and Grant (1983)	SiSM
Penaeus sp.	Grant *et al.* (1988)	MM
Philocheras trispinosus	Labat (1991a, 1991b)	IBM
Krill		
Euphausia superba	Astheimer (1986)	SiSM
Euphausia superba	Hofmann *et al.* (1992)	IBM
Crabs (larval stages)		
Cancer magister	Moloney *et al.* (1994)	SiSM
Fish larvae		
Engraulix mordax	Lo *et al.* (1995)	SSMM
Sardinops sagax	Lo *et al.* (1995)	SSMM
Striped bass	Levin and Goodyear (1980)	ASMM
Gadus morhua	Werner *et al.* (1993)	IBM
Malanogrammus aeglefinus	Werner *et al.* (1993)	IBM
	Heath and Gallego (1998)	IBM
Theragra chalcogramma	Hinckley *et al.* (1996)	IBM
	Rose *et al.* (1996)	IBM
	Rose and Cowan (1993)	IBM
	van Winkle *et al.* (1993)	IBM

Transfer to the first age class of the next stage by molting individuals is given by:

$$\boldsymbol{N}_{i+1,1} = \sum_j \sigma_i \gamma_{i,j} \boldsymbol{N}_{i,j} \tag{12.49}$$

Egg production by adult females (stage 12), contributing to the first age-class (eggs, stage 1), is calculated as:

$$\boldsymbol{N}_{1,1} = \sum_j \sigma_{12} \boldsymbol{F}_{12,j} \boldsymbol{N}_{12,j} \tag{12.50}$$

In this model, the time step is 1 day. There is a matrix calculation with a finite-difference equation system. The transfer of animals from stage to stage and the mortality at each stage are expressed as simple linear functions. The transfer rate $\gamma_{i,j}$ (day^{-1}) (or probability of molting) from stage i to $i + 1$ depends on the stage but also on the age-

class. The transfer rate is determined from stage duration, using a polynomial approximation to the cumulative normal distribution function with mean equal to a calculated mean duration (D_i) and a standard deviation equal to 10% of the mean. The function is monotonic and is equal to zero for the first age and sharply increases at D_i. The mean duration D_i varies with temperature according to Belehrádek's equation (see page 590). The mortality rate σ_i (day^{-1}) is constant for each stage i, but among stages, mortality varies according to the susceptibility of that stage to physiological death, cannibalism, and predation.

The fecundity F is based on the temperature-dependent empirical equation of Corkett and McLaren (1978) (see section 12.3). Davis's (1984a) model gives a good representation of the duration of each stage and the model is a convenient basis for modeling species of animals with stage development.

Miller and Tande (1993) present a population model of *Calanus finmarchicus* with two matrices, one for the abundance in each age class of each stage and one for the associated molt cycle fraction. At each time step Δt, the stage duration D in the age class j of stage i is determined by temperature using Belehrádek's equation (equation 12.32):

$$\boldsymbol{D_{i,j+1} = D_{i,j} + \frac{\Delta t}{a_j(T + b_i)^{c_i}} \quad \text{and} \quad D_{i,1} = 0.} \tag{12.51}$$

Individuals are transferred to the next age class $j + 1$ of stage i if $D_{i,j} < 1$, and

$$\boldsymbol{N_{i,j+1} = \left(\sqrt[1/\Delta t]{1 - \mu_i} \right) N_{i,j}} \tag{12.52}$$

If $D_{i,j} \geqslant 1$, the molt cycle is completed, and the individuals molt to the first age class of stage $i + 1$, such that:

$$\boldsymbol{N_{i+1,1} = \sum_j \left(\sqrt[1/\Delta t]{1 - \mu_i} \right) N_{i,j}} \tag{12.53}$$

With this model, it is possible to simulate the movement of individuals through stages, with a realistic representation of the duration of each stage.

Hairston and Munns (1984) developed a model with interacting copepod and fish populations using matrix projections to study the effects of copepod diapause switching on system dynamics.

CONTINUOUS-TIME STRUCTURED POPULATION MODELS

McKendrick (1926) introduced an entirely different type of model for an age-structured population. It describes the dynamics of the age distribution on a continuous-time basis using partial differential equations, and is usually referred to as the McKendrick–Von Foerster equation. This type of model has been developed to the extent that it can be used to describe the dynamics of a population that is living in a fluctuating environment. In addition, it can also be applied to situations in which more than one physiological trait of the individuals (e.g. age, size, weight, and energy reserves) have strong influences on individual reproduction and mortality. The movement of individuals through the different structural classes is followed over time. Age and weight are continuous variables whereas stage is a discrete variable.

The general equation (Sinko and Streifer 1967) is:

$$\frac{\partial n(t, a, m)}{\partial t} + \frac{\partial n(t, a, m)}{\partial a} + \frac{\partial g(t, a, m) n(t, a, m)}{\partial m} = -\mu(t, a, m)\ n(t, a, m) \tag{12.54}$$

where n is abundances of individuals of age a and mass m at time t.

Continuous-time and age structured population models (CASM)

The formulation which best introduces classical demographic modeling is the Von Foerster equation (Von Foerster 1959). This equation describes population processes in terms of continuous age and time as follows:

$$\frac{\partial n(a, t)}{\partial t} + \frac{\partial n(a, t)}{\partial a} = -\mu(a, t)\ n(a, t). \tag{12.55}$$

The equation has both an initial age structure φ at $t = 0$:

$$n(a, 0) = \varphi_0(a) \tag{12.56}$$

and a boundary condition of egg production at $a = 0$:

$$n(0, t) = \int_0^\infty F(a, S_R)\ n(a, t)\ da \tag{12.57}$$

F is a fecundity function that depends on age (a) and the sex ratio of the population S_R. These kinds of equations are mathematically and computationally difficult to analyze, especially if the environment is not constant (Nisbet and Gurney 1982). Equations 12.55 and 12.57 together constitute a continuous version of the entire Leslie matrix . Equation 12.57 corresponds to the first row of the matrix, and the rest of the matrix corresponds to equation 12.55 (see Caswell 1989, 1997).

Thus, a system of ODEs can be obtained as an equivalent to Von Foerster's PDE.

Gurney *et al.* (1983) developed a combination of the Von Foerster description with simple time-delay models to describe insect life history. The classical continuous-time description of the age structured population is described by PDEs with the integral boundary condition transformed by a set of ordinary delay-differential equations corresponding to a functional or developmental class of the species life history.

Continuous time and weight structured population models (CWSM)

The same type of equation as equation 12.55 can be used where age is replaced by weight

$$\frac{\partial n(w, t)}{\partial t} + \frac{\partial g(w, T, P) n(w, t)}{\partial w} = -\mu(w, t)\ n(w, t). \tag{12.58}$$

The weight of the individual w and the growth g are influenced by the temperature T, the food P and by the weight itself through allometric metabolic relationships.

The equation has both an initial age structure φ at $t = 0$:

$$n(w, 0) = \varphi_0(w) \tag{12.59}$$

and a boundary condition of egg production at $w = w_0$:

$$N(0, t) = \int_0^\infty F(w, S_R)\ n(w, t)\ dw \tag{12.60}$$

F is the fecundity function which depends on weight (w) and the sex ratio of the population S_R.

Bryant *et al.* (1997) present in detail the numerical realization of this equation. This requires a representation of the continuous distribution $n(w, t)$ by a set of discrete values $n_i(t)$ that are spaced along the weight axis at intervals $\Delta w_i = w_{i+1} - w_i$. Using upwind difference discretization to solve the equations, and recasting the representation in terms of the number of individuals in the ith weight class, $N_i(t) \approx n_i(t)\,\Delta w_i$, the dynamic equation becomes:

$$\frac{dN_i}{dt} = \left[\frac{g_{i-1}}{\Delta w_{i-1}}\right] N_{i-1} - \left[\frac{g_i}{\Delta w_i}\right] N_i - \mu_i N_i \tag{12.61}$$

where $\mu_i(t)$ replaces $\mu(w_i,t)$. This describes the dynamics of all weight classes except the first ($i = 2$) and last ($i = Q$). If $R(t)$ represents the total rate of recruitment of newborns to the population, and all newborns are recruited with the same weight w_1, then the dynamics of the weight class covering the range Δw_1 are:

$$\frac{dN_1}{dt} = R - \left[\frac{g_1}{\Delta w_1}\right] N_1 - \mu_1 N_1 \tag{12.62}$$

If we assume that individuals in only the Qth weight class are adult, and that adult individuals expend all assimilated energy on reproduction rather than growth, the population dynamics of the adult population is given by:

$$\frac{dN_{Q-1}}{dt} = \left[\frac{g_{Q-1}}{\Delta w_{Q-1}}\right] N_{Q-1} - \mu_Q N_Q \tag{12.63}$$

and the rate of recruitment of newborns to the population is

$$R(t) = \beta(t) N_Q(t) \tag{12.64}$$

where $\beta(t)$ represents the per capita fecundity of an average adult at time t.

The weight intervals Δw_i increase with class number i as an allometric function. The growth rate $g(w, t)$ can be calculated by a physiological model (see section 12.3).

Slagstad and Tande (1990) and Tande and Slagstad (1992) present other applications of weight structured population models (WSM) to *Calanus* sp. populations (see Table 12.5).

To demonstrate how various factors affect the pattern of recruitment of crab larvae, Botsford *et al.* (1994) transformed equation 12.58 into a set of ODEs by the method of characteristics, and developed an analytical solution to these ODEs.

STAGE-STRUCTURED POPULATION MODELS BASED ON ODEs

Zooplankton populations often have continuous recruitment and are followed in the field by observing stage abundances over time. A large number of zooplankton population models deal with population structures in terms of developmental stage, using ODEs.

Simple stage structured population models

A single ODE can be used to model each development stage or group of stages. An example of such a model is given by Wroblewski (1980) to describe the dynamics of

Acartia clausi. He subdivided the population into four groups: eggs, nauplii, copepodites and adults. The equation system is:

$$\textbf{eggs} \qquad \frac{dN_1}{dt} = R - \alpha_1 N_1 - \mu_1 N_1 \tag{12.65}$$

$$\textbf{nauplii} \qquad \frac{dN_2}{dt} = \alpha_1 N_1 - \alpha_2 N_2 - \mu_2 N_2 \tag{12.66}$$

$$\textbf{copepodids} \qquad \frac{dN_3}{dt} = \alpha_2 N_2 - \alpha_3 N_3 - \mu_3 N_3 \tag{12.67}$$

$$\textbf{adults} \qquad \frac{dN_4}{dt} = \alpha_3 N_3 - \mu_4 N_4 \tag{12.68}$$

where R is recruitment, α is the molting rate to next stage, and μ is the mortality rate.

The system of ODEs is solved by Euler or Runge–Kutta numerical integration methods, usually with a short time step (approximately 1 h). This ODE system is quite similar to the Lefkovitch matrix. In this model, the transfer of animals from stage to stage and the mortality at each stage are expressed as simple linear functions. The transfer rate α_i day^{-1} is constant. The underlying assumption is that there is a continuum of ages of individuals in each stage. Thus some animals are always ready to mature to the next stage while others still require a full development time. Wroblewski takes $\alpha_i = 2/D_i$, where D_i refers to the development time of the ith stage. Because of the exponential formulation of copepod development, 87% of the individuals present at time zero will progress to stage $i + 1$ in the time interval D_i and the remaining 13.5% will take longer to complete their development. If we take α_i day^{-1} equal to $1/D_i$ the proportion of laggards becomes 36.8%. Generally, this second formulation is used because this model cannot mimic realistic development time within a stage.

The mortality rate μ_i day^{-1} is also constant in each stage, but among stages mortality can vary according to the susceptibility of that stage to physiological death, cannibalism and predation.

Gaedke (1990) presented similar stage structured population models for two interacting populations (*Acartia tonsa* and *Eurytemora affinis*) with nine groups of stages of nauplii (N) and copepodites (C): eggs, N1 to N3, N4 and N5, N6 and C1, C2 and C3, C4 and C5, and adults. In her paper, mortality rates are based on starvation, low salinity, predation on nauplii by *Acartia tonsa*, and predation by fish and carnivorous invertebrates. The formulation of the different components is fully described in the annexe of her paper. The model gives unrealistic stage abundance, beginning with a synchronous cohort, because minimum durations within each stage are not enforced. Thus, the generation time is artificially shortened due to a numerical diffusion of individuals through the stages. In the case of a stable environment where populations develop over several generations (small species), this effect would not be significant, and in such cases this model would be a simple and useful approach.

A similar model was built by Marin (1997) to describe the dynamics of *Calanus chilensis*. His model was built and run using STELLA-II version 3.07 (High Performance Systems, Inc), an interactive, iconographic software package. The basic equations and the diagram of the stage structured population model are presented in full detail in Marin's (1997) paper.

Andersen and Nival (1986) present a model of the population dynamics of salps considering five stages of oozooids and five stages of blastozooids. Assuming that each stage represents individuals of a given weight range, they computed a growth rate based on physiological functions (feeding and excretion) that is temperature- and food-

dependent, and related the demographic parameters (transfer, mortality and reproductive rates) to growth rate.

Hofmann and Ambler (1988) modeled the population dynamics of *Paracalanus* sp. within a model of a pelagic ecosystem. They used five variables for five groups of stages, with the model unit being the biomass of individuals in each stage. The change in biomass in each stage was caused by movements of individuals through the stage and also by the flux of matter linked to metabolism of the individuals.

Stage and age-within-stage structured population models

Sciandra (1986) used an intermediate model to those of Wroblewski (1980) and Davis (1984a) to model copepod population dynamics. His model had subdivisions within each stage and the movement of individuals through these subdivisions was simulated using ODEs. Because individuals stayed for a set time in each subdivision, these subdivisions cannot be termed 'age-classes' as in Davis's (1984a) matrix model.

Carlotti and Sciandra (1989) and Carlotti and Nival (1992) developed a model with two types of equation: finite-difference equations to transfer organisms from one age class to the next in each stage, and ODEs to represent the movement of organisms from any age class of one stage to the first class of the next stage. The time step of the transfer from one age class to the next was set at 6 h, whereas the time step of the movement from any age class of stage i to the first class of stage $i + 1$ was set at 1 h. The system of ODEs was solved using a fourth-order Runge–Kutta numerical integration. The model formulations are described below.

The first age-class of stage i:

$$\frac{dN_{i,1}}{dt} = R_i - \alpha_{i,1}N_{i,1} - \mu_{i,1}N_{i,1} \tag{12.69}$$

Other age-classes of stage i:

$$\frac{dN_{i,j}}{dt} = -\alpha_{i,j}N_{i,j} - \mu_{i,j}N_{i,j} \tag{12.70}$$

The rate of recruitment of newborns to the population in the first age class of eggs is the number of eggs spawned by mature females in the adult stage. The rate of recruitment of newly molted individuals in the first age class of a stage is:

$$R_i = \sum_{j=1}^{m} \alpha_{i-1,j}N_{i-1,j} \tag{12.71}$$

When the transfer from one age class to the next occurs, the individuals of the last two age classes ($m-1$ and m) are grouped in the last age class, the individuals of all age classes j are moved to those following, and the first age class is set to zero.

$$N_{i,m} = N_{i,m} + N_{i,m-1} \tag{12.72}$$

$$N_{i,j} = N_{i,j-1} \tag{12.73}$$

$$N_{i,1} = 0 \tag{12.74}$$

This process simulates the movement of cohorts of individuals through each stage. In a similar way to the matrix models that include stage and age, the structure of age-within-stage models allows the representation of different stage duration distributions to estimate molting probabilities. Mortality rates also can change with age within stage. Carlotti and his co-workers represented changes in the demographic parameters (molting rate,

mortality rate, egg production rate) as functions of individual properties of the organisms in the cohort (size, weight, growth rates), and the age distributions of the demographic parameters became a result of their simulations. Details of the different representations of the demographic processes in such structure are presented in the next sections.

Demographic parameters as functions of individual growth properties

In their model Carlotti and Sciandra (1989) represent the molting rate from stage i to stage $i + 1$ as a function of weight and growth rate. Molt determination is complex, but it is reasonable to believe that molting occurs only when a set of fundamental biological conditions are met (Carlotti 1996b).

The molting rate depends first on weight; animals should reach a critical molting weight. To represent a certain variability around the critical molting weight, S-shaped functions can be used. Carlotti and Sciandra (1989) used a Michaelis–Menten law with exponent. The increasing value of the exponent allows for reduced variability around the critical weight. Other S-shaped functions such as the hyperbolic tangent could be used. The molting rate depends also on the recent physiological condition of the organisms. Carlotti and Sciandra (1989) propose a linear function of the average specific growth rate (ASG) calculated over a given period Δt:

$$ASG_{i,j} = \frac{1}{\Delta t}\int_{t-\Delta t}^{t} \frac{G_{i,j}}{W_{i,j}}\,dt \tag{12.75}$$

where $G_{i,j}$ and $W_{i,j}$ are the growth of individuals in age-class j of stage i. Carlotti and Sciandra (1989) introduced a function whereby ingestion decreased when the weight of organisms in stage i was above the critical weight. As a consequence, individuals that reached the critical molting weight could molt, but those that remained in that stage diminished their probability of molting because of reduced ingestion and the decrease in the ASG value.

Mortality rate is usually considered constant in a stage. In fact, mortality rate varies as a function of food concentration, temperature and even age within stage (Carlotti and Nival 1992). Carlotti and Sciandra (1989) used an increasing hyperbolic function with mortality dependent on the specific growth rate. This function allowed for high mortality when energy budgets were unfavorable and low mortality when they were favorable. The average specific growth rate (ASG) could be used instead of the instantaneous specific growth rate to represent the effect of recent feeding history on the mortality rate. Egg production occurred when females reached a critical mature weight, following a sigmoidal function (similar to that used for the molting rate).

The links between physiological processes and demographic parameters suggested in this model resulted in stage duration distributions that were realistic (Carlotti and Nival 1992) as well as the frequently observed asymmetry of stage distributions in cultivated populations (Carlotti and Sciandra 1989).

Another type of continuous-time, physiologically structured population has been developed by De Roos *et al.* (1992) with the use of a numerical method called 'escalator boxcar train' (Goudriaan 1986). We do not know any examples where this has been applied to zooplankton species.

Demographic parameters as functions of age within stage

It is possible to simplify Carlotti and Sciandra's (1989) model, by developing age-dependent functions of molting rate, mortality rate and fecundity, and by removing all the physiological functions. The use of a gamma distribution to represent the age-

dependent molting rate, with its shape variations depending on food and temperature, appears to give a good parameterization (Souissi *et al*. 1997). In comparison with the simple model presented by Wroblewski (1980), the use of age-dependent molting rates allows for improved simulations of the time lags between stages in situations where cohorts are clearly identified. A similar model (called multi-transfer model) was used by Jellison *et al*. (1995) to identify stage durations and mortality rates.

DELAY DIFFERENTIAL EQUATION MODELS

Gurney *et al*. (1983), Nisbet and Gurney (1982), and Gurney *et al*. (1986) developed a modeling approach for species whose life history is made up of a number of well defined physiological stages, within which all individuals are assumed to be identical in feeding behavior and probability of death. The progress of an individual through a particular stage was quantified by a development index representing the state of development or the 'physiological age' of the individual within that stage at a particular time. The development index increased at the same instantaneous rate for all individuals in the stage at a given time. Maturation out of a stage occurred on achieving a fixed value of the development index.

Mathematically, this modeling approach is represented by a system of coupled ODEs for estimating population numbers, and delay-differential equations (DDEs) for estimating through-stage survival and stage duration. Crowley *et al*. (1987) give a complete description of such a model applied to a zygopteran with aquatic larval stages. No examples were found for copepods although the model structure is convenient for them. An extension of this model type was applied to the study of species with diapause stages (Gurney *et al*. 1992).

Several other models that include delay in stage recruitment have been used for the estimation of demographic parameters (for a review see Jellison *et al*. 1995)

STRUCTURED POPULATION MODELS TO ESTIMATE DEMOGRAPHIC PARAMETERS

A variety of cohort models have been developed for applying inverse methods; vital rates (e.g. mortality, stage duration) can be derived by fitting a population model to observed data (see Manly 1989 and 1990). All the structured models presented in the previous sections have been used for parameter identification: matrix models (Caswell and Twombly 1989), models with ODEs and models with DDEs (Rigler and Cooley 1974; Matthews *et al*. 1978; Parslow *et al*. 1979; Sonntag and Parslow 1981; Hairston and Twombly 1985; Saunders and Lewis 1987; Hay *et al*. 1988; Wood and Nisbet 1991; Jellison *et al*. 1995; Ohman and Wood 1996). The details of the techniques have been explained by Aksnes *et al*. (1997) and will not be repeated here.

STOCHASTICITY IN STRUCTURED POPULATION MODELS

Stochasticity can be included in structured population models either by influencing the environmental variable or the demographic process and vital rates. Stochastic events can be introduced into matrix models (Caswell 1989), as well as structured population models based on PDEs, ODEs and DDEs (Nisbet and Gurney 1982). However, no such examples have been found for marine zooplankton populations.

12.4.3 Individual-based models of a population

Individual-based models (IBMs), also called *i*-state configuration models (Metz and Diekmann 1986; Caswell and John 1992; Maley and Caswell 1993), describe population

dynamics by simulating the birth, development, and eventual death of a large number of individuals in the population. As powerful computers become more accessible, numerous IBMs of zooplankton populations have been developed, mainly to couple them with circulation models (see section 12.6.3). An increasing number of papers have appeared that have used this individual-based approach (De Angelis and Gross 1992; van Winkle *et al*. 1993).

BUILDING AN IBM

Maley and Caswell (1993) briefly presented the structure of IBMs, and the differences and the links between structured population models and individual-based models. Despite significant progress on model formulations for physiologically structured populations, several problems remain intractable. These problems include methods for dealing with the local character of many ecological interactions and the difficulty of jointly studying two complex ecological factors (i.e. investigating both the dynamic effects of age-structure and those of spatial variability). One solution to these problems is to focus on IBMs.

IBMs treat populations as collections of individuals, with explicit rules governing individual biology and interactions with the environment. Each biological component can change as a function of the others. Each individual is represented by a set of variables that store its *i*-state (e.g. age, size, weight, reserves, etc.). These variables may be grouped together in some data structure that represents a single individual, or they may be collected into arrays (e.g. an array of all the ages of the individuals, an array of all the sizes of the individuals, etc.), in which case an individual is an index number in the set of arrays. The *i*-state of an individual changes as a function of the current *i*-state, the interactions with other individuals, and the state of the local environment. The local environment can include prey and predator organisms that do not warrant explicit representation as individuals in the model. Population-level phenomena (e.g. temporal or spatial dynamics) or vital rates can then be inferred directly from the contributions of individuals in the ensemble.

The model starts with an initial population and the basic environment, then monitors the changes of each individual. At any time t, the *i*-state of individual j changes as:

$$\boldsymbol{X_{i,j}(t) = X_{i,j}(t - dt) + f(X_{1,j}(t - dt), \ldots, X_{i,j}(t - dt), \ldots, T, \ldots)} \qquad (12.76)$$

where $X_{i,j}(t)$ is the value of the *i*-state of individual j, and f is the process modifying $X_{i,j}$, as a function of the values of different *i*-states of the organism, and external parameters such as the temperature T. When the fate of all individuals during the time step dt has been calculated, the changes to the environment under the effects of individuals can be updated. Any stochastic process can be added to equation 12.76.

A simple example of a zooplankton IBM was presented by McLaren (1997) to study biases in estimating secondary production from copepod cohorts. This model needed three variables for each individual: the weight, the stage, and a variable specifying if the individual was dead or alive. The model was run with 50 000 individuals, each individual beginning with an initial weight drawn from a lognormal distribution. The weight of stage i at time t was

$$\boldsymbol{W_i(t) = (1 + G_i) W_i(t - 1)} \qquad (12.77)$$

where the growth rate G_i had been drawn from a random normal distribution. When the weight exceeded the critical weight for entering in a new stage, then

$$\boldsymbol{\mathrm{Stage}_i(t) = \mathrm{Stage}_i(t - 1) + 1} \qquad (12.78)$$

The probability of death M_i was taken to be constant for each stage, but could vary among the stages. For each individual for each time step, a random uniform variate $U(0, 1)$ was generated, and if $U(0, 1)$ was greater than M_i, the individual survived to the next time step. Weight increments of individuals that lived to the end of the simulation period were accumulated as 'growth' production, whereas the weight increments of dead individuals were accumulated as 'lost' production. Actual production was the sum of growth and lost productions.

Batchelder and Miller (1989) presented an IBM of *Metridia pacifica*, with the basic equations 12.77 and 12.78, but with greater detail in the representation of physiological functions. Individual growth was calculated as assimilation less respiration according to functions presented in section 12.3. The inter-individual variation in physiology was represented because the characteristics (growth parameters, mortality coefficient, and parameters connected with reproduction) of each individual were chosen at random. In addition to a constant stage-dependent mortality, individuals could also die by starvation, when their weights fell below the mean weight of the previous stage. Daily egg production by mature females was generated from a normal distribution of parameters of clutch size, clutch frequency, and total number of clutches. The model described the development of individuals and generated a population history over one year. To solve the problem of an increasing number of individuals (to the result of reproduction), which becomes too large for storage and increases the computational effort, Batchelder and Miller (1989) randomly selected a fractional sample (1/5) of the population when it was close to a maximum number (100 000 individuals in their simulation). Subsequent abundance reports were then multiplied by five. Similarly, Rose *et al.* (1993) studied different aspects of such resampling techniques with an IBM of fish.

In a refined version of their IBM, Batchelder and Williams (1995) represented the effects of vertical food distributions on individual growth and vertical distribution. In this version, the effect of temperature (the external driving variable) on biological functions was also taken into account. The results were presented in terms of individual weight trajectories and stage frequency distributions over time. A similar example of an IBM with a stochastic component was presented by Labat (1991a, 1991b), where the population dynamics and temporal changes in size structure of a shrimp species was simulated.

With the aim of coupling an IBM of *Calanus finmarchicus* with a circulation model of the Georges Bank region, Miller *et al.* (1998) developed a simple model that had six variables: three for position, one to register the individual as dead or alive, one for the stage, and one to define the relative age in the stage. A temperature-dependent fraction of the molt cycle was incremented at each time step, as in equations 12.77 and 12.78. Copepodite stage 5 had the possibility of entering a resting stage. A reproductive function with a temperature-dependent maturation time for clutches, was added when individuals became females.

To represent both the dynamics of a zooplankton population with an IBM and its interactions with the trophic environment, realistic numbers have to be simulated. The techniques used by Batchelder and Miller (1989) in fractionating the population are not always convenient. Another method is to assume that the basic unit of an IBM (usually a zooplankton individual) actually represents more than one individual. The individuals in the unit should be identical, as for a group of individuals that are born almost simultaneously and have similar mothers. Hogeweg and Richter (1982) used this approach to group eggs produced by females of similar size. Woods and Onken (1982)

termed a similar approach the 'Lagrangian-ensemble method', which was a modeling technique in which identical individuals born at the same time were grouped in one unit (also called a 'family' or 'particle'). They first applied this method to phytoplankton cells, and then to zooplankton (Woods and Barkmann 1993, 1994, 1995).

Carlotti and Wolf (1998) presented an application of the Lagrangian-ensemble method to the population dynamics of *Calanus finmarchicus* coupled with a 1-D ecosystem model, where new units were formed by grouping the eggs produced by females that occurred in the same depth layer. To simulate the annual ecosystem dynamics with realistic numbers of copepods ($>150\,000$ ind. m^{-2}), the number of units could not exceed 3000. Thus units could group up to 500 eggs at their time of creation. However, the number of units was large enough for producing realistic statistical distributions of the copepods in the water column. Carlotti and Wolf's (1998) model simulated the movements of organisms in relation to light (daily migration) and food concentration, similar to Batchelder and William's (1995) model for the physiology of organisms, but with a supplementary state of fatty reserves.

Several IBMs have been developed for the early life history of fish populations (see review by Tyler and Rose 1994). Techniques of resampling (Rose *et al.* 1993) or grouping of individuals (Scheffer *et al.* 1995) have also been presented for fish models. In recent studies by Hermann *et al.* (1996), Werner *et al.* (1996), Hinckley *et al.* (1996), Gallego and Heath (1997) and Heath and Gallego (1997), detailed physiological processes were introduced into IBMs of fish coupled with circulation models. These approaches attempted to derive conclusions about the population based on the distinct and unique life histories of the individuals. The coupling of IBMs to spatially explicit physical models adds the space dimension that is necessary to include environmental constraints that affect individuals as they move in an environment. These constraints include regions of poor growth, increased mortality, dispersion, etc. The approach integrates the unique temporal and spatial history of the individual larvae, each of which is exposed to different prey concentrations and physical parameters. In this manner, the growth of individual larvae can be understood in terms of a detailed time history of the food available to the larva, which itself is a function of the unique trajectory of each larva through the prey field, and the ability to encounter (and capture) the prey (see page 578).

OBJECT-ORIENTED PROGRAMING (OOP)

Object-oriented programing (OOP) is a technique that has been applied to IBMs recently. In OOP, the individuals, interaction structure, and environment are all defined as objects. Papers by Baveco and Lingeman (1992), Silvert (1993) and Maley and Caswell (1993) give good introductions to OOP illustrated by simple examples. There are very few examples of models using OOP for zooplankton populations. Laval (1995, 1996, 1997) presented such a model to simulate the development of a tunicate bloom, taking account of the physiology of salps and the colonization of space by its members with their spatial interactions. Population dynamics models based on OOP have been developed for cladocerans and copepods (Hogeweg and Richter 1982; Mooij and Boersma 1996).

CONSTRAINTS IN BEHAVIOR

IBMs are focused at resolving physiological and behavioral differences within populations. In this way, IBMs can describe population effects caused by individual variability more precisely than can SPMs. However, in standard IBMs this is done by specifying

how organisms respond to their environment, either by a fixed rule or by an elaborate IF-ELSE IF table. This may not always help the modeled individuals to behave in an evolutionarily optimal way. There are many things that can happen to a copepod, and it is not possible to combine all events in a predescribed decision matrix. Neither would the modeler know the appropriate response in many of these situations. Classical IBMs with defined behavioral rules are therefore best suited for simulations in rather simple and stable environments.

Alternatively, IBMs can derive their trade-off rules from life history theory, as suggested by Tyler and Rose (1994). A different approach would be to model the optimal decisions, and then let the population act accordingly on an individual basis, as in Stochastic Dynamic Programing (SDP; Clark and Mangel 1999). This type of forcing has been used by Fiksen and Giske (1995) and Fiksen and Carlotti (1998). However, SDP also has inherent weaknesses. The method can easily solve state-dependent or density-dependent optimal behavior in a changing environment, but cannot combine state- and density-dependencies in one model. The ING method (Huse and Giske 1998), described on page 627, can overcome many of these obstacles, but at a price of high CPU demand.

12.4.4 Models of interactions between zooplankton populations

In this section we look at models of direct interactions between species. Indirect interactions such as competition for food are treated in section 12.5. Direct interactions can be of different types: predation by one species on another, crossed predation of adults of several species on juveniles of other species, and cannibalism by adults on juveniles.

INTERACTION MODEL WITH TWO VARIABLES

Simple models of two-species interactions take the form:

$$\frac{dN_1}{dt} = r_1 N_1 - k_1 N_1 N_2 \tag{12.79}$$

$$\frac{dN_2}{dt} = r_2 N_2 - k_2 N_1 N_2. \tag{12.80}$$

These population models represent some special experimental situations or typical field situations. As an example, Legovic (1987) studied the dynamic properties and the steady state of a simple predator–prey model to represent the predation of the jellyfish *Pelagia noctiluca* (N_2 in mg C m^{-3}) on fish eggs and zooplankton (N_1 in mg C m^{-3}):

$$\frac{dN_1}{dt} = rN_1\left(1 - \frac{N_1}{K}\right) - k_1 N_2\left(\frac{N_1}{k_2 + N_1}\right) - k_3 N_1 \tag{12.81}$$

$$\frac{dN_2}{dt} = k_4 k_1 N_2\left(\frac{N_1}{k_2 + N_1}\right) - k_5 N_2 \tag{12.82}$$

where r is the intrinsic growth rate of zooplankton (day^{-1}), K the carrying capacity of the prey population, k_1 the maximum specific predation rate of jellyfish (day^{-1}), k_2 the half-saturation constant (mg C m^{-3}), k_3 the specific mortality by other predators (day^{-1}), k_4 the conversion of prey biomass into jellyfish biomass (no units), and k_5 the specific mortality of jellyfish (day^{-1}). The results indicate different causes of an increase

in numbers of jellyfish, which include an increase in prey, the decrease of carnivorous competitors, and the decrease of jellyfish predators.

Gaedke and Ebenhöh (1991) presented an interaction model between two estuarine species of copepods *Acartia tonsa* and *Eurytemora affinis*, with equations similar to equations 12.79 and 12.80, but with (1) predation by *Acartia* on the two species (N_1 is replaced by N_2 in the second term on the right hand side of equation 12.80), (2) a term of biomass gain of *Acartia* by this predation, and (3) a density-dependent loss term caused by predation by invertebrates or starvation of the two species. This simple model did not result in stable coexistence between the two species with a reasonable parameter range under steady-state conditions. A more complex structured model was also built for comparison (see below).

Note that simple deterministic models of a number of ecological interactions can induce chaotic behavior, comparable with many field observations (Scheffer 1991).

POPULATION INTERACTIONS USING STRUCTURED POPULATION MODELS

Direct and indirect interactions between two estuarine copepods *Eurytemora affinis* and *Acartia tonsa* were studied by Gaedke (1990) and Gaedke and Ebenhöh (1991) using two stage-structured population models with stage-specific interactions (with similar equations to equations 12.65–12.68) and abiotic and biotic forcing variables: temperature, salinity, primary production, phytoplankton species composition, and seasonal abundance of fish, carnivorous zooplankton (mysids, chaetognaths and coelenterates) and *Noctiluca miliaris*. The stage-structured population models allowed the predation of large individuals of *A. tonsa* (copepodites 4 to adults) on nauplii of both species to be represented. Predation on nauplii depended on the combined abundances of predator (C4 to adults) and prey (eggs and nauplii) stages and was calculated for each stage. The results of this detailed numerical model were compared with results obtained using a simpler model with two variables. Greve (1995) presented a model of mutual predation between *Calanus helgolandicus* and *Pleurobrachia pileus*.

Carlotti and Slagstad (1997) developed an ecosystem model of the Greenland Sea in which zooplankton was represented by two copepod populations of *Calanus hyperboreus* and *Oithona similis*. The simulations indicated that the predation of *Calanus* on *Oithona* was necessary to sustain the *Calanus* population. *C. hyperboreus* is assumed to feed preferentially on phytoplankton, but supplements its diet with *Oithona*. A first grazing value (G1) with phytoplankton as the only food item was calculated with a type I feeding function (see Table 12.2). Then a second grazing value (G2) was calculated on the food constituted by phytoplankton plus the biomass of *Oithona* above a threshold biomass. The real predation was calculated as the difference between the two calculated grazing values (G2 − G1).

Cannibalism has been shown to occur in zooplankton (Daan *et al.* 1989), and a few theoretical models have investigated the consequences for the population dynamics (Gabriel 1985; Van den Bosch *et al.* 1988).

12.4.5 Further reading

Cushing, J.M., 1977. *Integrodifferential equations and delay models in population dynamics. Lecture Notes in biomathematics 20*. Springer-Verlag, Berlin, 196 pp.

McDonald, N., 1978. *Time lags in biological models. Lecture notes in biomathematics 27*. Springer-Verlag, Berlin, 112 pp.

Metz, J.A.J. and Diekmann, O., 1986. *The dynamics of physiologically structured populations. Lecture notes in biomathematics 68*. Springer-Verlag, Berlin, 511 pp.

Wood, S.N. and Nisbet, R.M., 1991. *Estimation of mortality rates in stage-structured populations. Lecture notes in biomathematics 90*. Springer-Verlag, Berlin, 101 pp.

12.5 MODELS OF ZOOPLANKTON COMMUNITIES

12.5.1 Zooplankton bulk models in ecosystem models

THE REPRESENTATION OF HERBIVOROUS ZOOPLANKTON IN NPZ-TYPE ECOSYSTEM MODELS

Modeling of ocean biogeochemical processes developed rapidly in the last decades and a number of text books on marine ecosystem modeling and related techniques (e.g. data assimilation) have been published. The book by Evans and Fasham (1993) presents a synthesis of the model-building process at the ecosystem level and linked aspects: level of resolution, linkage between physical, chemical and biological components, representation of trophic functional units and associated processes. A chapter devoted to zooplankton modeling describes the difficulty of representing very diverse groups of organisms with one or two variables. Previous monographs by Steele (1974), Kremer and Nixon (1978), Platt *et al.* (1981), Nisbet and Gurney (1982), Walsh (1988) and Fransz *et al.* (1991) also provide useful introductions for the student.

In the last two decades, ecosystem models have been developed to simulate more site-specific situations (Fasham *et al.* 1990; Hofmann *et al.* 1980; Hofmann 1988; Hofmann and Ambler 1988). These models have mainly used deterministic differential equations to describe ecosystem dynamics, although some papers have explored stochastic approaches to modeling (Fasham 1977; Kremer 1983). Models of pelagic ecosystems have been reviewed by Totterdell (1993) and the modeling of the zooplankton compartment in ecosystem models has been treated by Anderson *et al.* (1993).

The representation of zooplankton

In ecosystem models, the zooplankton compartment corresponds to a highly aggregated entity with organisms covering a large size range (Table 12.6). In Fasham *et al.*'s (1990) model, a one-compartment zooplankton model integrated organisms from bacterivorous flagellates through ciliates, copepods, and euphausiids (all of which may be partly herbivorous or carnivorous), to wholly carnivorous chaetognaths. To take into account the functional diversity of zooplankton, some modelers have divided zooplankton into two or more size classes (e.g. Frost 1987; Moloney and Field 1991), and such models will be described further in sections 12.5.2 and 12.5.3.

The ultimate goal of many ecosystem models is to embed the ecosystem model in a 3-D general circulation model from the mesoscale (e.g. Flierl and Davis 1993; Dadou *et al.* 1996) to basin scale (Wroblewski *et al.* 1988). The models have to be as detailed as necessary but as simple as possible (see Evans and Fasham 1993).

The simplest model has three components: nutrient (N), phytoplankton (P) and herbivorous zooplankton (Z). Such models are termed 'NPZ' models. They are driven by physical processes such as mixing or upwelling, which introduce nutrients into the euphotic zone and are closed at the upper level by some 'mortality' of herbivores (e.g.

Table 12.6 Groups of organisms, taxon or species represented in bulk zooplankton variables of ecosystem models.
N nutrients; P phytoplankton; Z zooplankton; D detritus; M microzooplankton and/or bacteria; F fish; DON dissolved organic nitrogen.

Species or groups	Authors	Type of ecosystem model	Process studied
Herbivores			
copepods	Evans and Parslow (1985)	NPZ	Annual plankton cycle
copepods	Klein and Steele (1985)	NPZ	Spatial simulation of the pelagic ecosystem
copepods	Evans (1988)	NP(2)Z	Annual plankton cycle
copepods	Aksnes and Lie (1990)	N(2)P(2)ZD	Annual plankton cycle in a fjord
copepods	Aksnes and Wasmann (1993)	PZD	Role of zooplankton grazing in export production
copepods	Walsh (1975)	N(4)PZDF	Spatial simulation of the Peru upwelling ecosystem
copepods	Wroblewski (1977)	N(2)PZD	Spatial simulation of the Oregon upwelling ecosystem
copepods	Slagstad (1985)	NPZD	Plankton dynamics in the marginal sea ice zone
copepods	Fransz and Verhagen (1985)	N(3)P(2)ZD	Annual plankton cycle in the southern North Sea
copepods	Franks *et al.* (1986)	NPZ	Effect of a food-level acclimatation by copepods
copepods	Andersen *et al.* (1987)	N(2)P(2)Z(2)C	Plankton dynamics in mesocosms
copepods	Wroblewski and Richman (1987)	NPZF	Plankton dynamics during wind mixing events
copepods	Andersen and Nival (1988)	NPZ(2)D(5)	Sedimentation of biogenic particles
copepods	Wroblewski (1989)	NPZ	Plankton dynamics in the North Atlantic basin
copepods	Fasham *et al.* (1990)	N(2)PZDM and DON	Plankton dynamics at station India
copepods	Steele and Henderson (1992)	NPZ	Role of predation in plankton models
copepods	Kawamiya *et al.* (1995)	N(2)PZD and DON	Plankton dynamics at station Papa
copepods	Ross *et al.* (1993)	NPZC and DON	Annual plankton cycle in a fjord
copepods	Robinson *et al.* (1993)	NPZ(2)	Annual plankton cycle
Calanus sp.	Wasmann and Slagstad (1993)	N(3)P(2)MZD(2)	Annual dynamics in the Barents Sea
salps	Andersen and Nival (1988)	NPZ(2)D(5)	Sedimentation of biogenic particles
appendicularians	Andersen *et al.* (1987)	N(2)P(2)Z(2)C	Plankton dynamics in mesocosms
Carnivores			
ctenophores	Parson and Kessler (1987)	NPZ(3)DM(2)F	Plankton and fish production
ctenophores	Andersen *et al.* (1987)	N(2)P(2)Z(2)C	Plankton dynamics in mesocosms
ctenophores	Ross *et al.* (1993)	NPZC and DON	Annual plankton cycle in a fjord
euphausiids	Robinson *et al.* (1993)	NPZ(2)	Annual plankton cycle
chaetognaths	Andersen *et al.* (1987)	N(2)P(2)Z(2)C	Plankton dynamics in mesocosms
anchovy	Walsh (1975)	N(4)PZDF	Spatial simulation of the Peru upwelling ecosystem
anchovy	Wroblewski and Richman (1987)	NPZF	Plankton dynamics during wind mixing events
salmon	Parson and Kessler (1987)	NPZ(3)DM(2)F	Plankton and fish production

Steele and Henderson 1981; Evans and Parslow 1985). The interactions between the three components can be expressed in mass units per m^3:

$$dN/dt = \textbf{input} - \textbf{phytoplankton uptake} + \textbf{zooplankton metabolic losses} \quad (12.83)$$

$$dP/dt = \textbf{phytoplankton uptake} - \textbf{zooplankton grazing} \quad (12.84)$$

$$dZ/dt = \textbf{assimilated food} - \textbf{zooplankton metabolic losses} - \textbf{predation} \quad (12.85)$$

where:

- input = $p(No - N)$, p being the mixing rate from a deep high-nutrient source of constant concentration No
- phytoplankton uptake = $n(N)f(P)$, where $n(N)$ is some nutrient uptake function and $f(P)$ is a function representing self-shading
- zooplankton grazing = $g(P)Z$, where $g(P)$ is the grazing function
- zooplankton egestion = $a(P)Z$, where $a(P)$ is the egestion rate and $a(P) = (1 - \alpha)g(P)$
- assimilated food = grazing − egestion = $\alpha g(P)Z$, where α is the assimilation rate
- zooplankton metabolic losses = $e(P)Z$, with $e(P) = \beta\alpha g(P)$, where β is the excretion rate
- predation = $\alpha(1 - \beta)g(P)h(Z)Z$, where $h(Z)$ parameterizes predation by higher trophic levels.

Explanations for the formulations of processes and the values of parameters concerning nutrients and phytoplankton can be found in Steele and Henderson (1981). Here, we will focus on the processes relating to zooplankton. Some formulations for $g(P)$, $a(P)$, $e(P)$ and $h(Z)$ are presented in Table 12.7.

Steele (1974) represented zooplankton as a single growing cohort (age class) of mesozooplankton. The cohort was represented by two equations, one equation for the rate of change of individual weight from W_1 to W (in mass unit per individual), and one equation to represent the decrease of individual numbers in the cohort from N_1 to N (in numbers of individual per m^3) as a result of density dependent mortality and predation.

$$dW/dt = \textbf{assimilated food} - \textbf{metabolic losses} \quad (12.86)$$

$$dN/dt = \textbf{density dependent mortality} + \textbf{predation} \quad (12.87)$$

where:

- assimilated food = $\beta g(P)W^{0.7}$, where β is the assimilation rate and $0 < \beta < 1$
- metabolic losses = $\lambda g(P)W^{0.7} + \gamma W^{0.7}$, with the first term proportional to the assimilated food and the second term independent of food intake
- density dependent mortality = $-v(N - N_1)(W - W_1)/(H + NW)$
- predation = $h'(N)N$, and $h'(N)$ parameterize the predation by higher trophic levels.

The combination of the two equations gave the changes in zooplankton biomass. When the weight reached the maturity weight, the growth rate became storage for reproduction, and reproduction occurred after a given delay. The adults were then lost to predation and a new cohort was born. Landry (1976) presented a slightly extended version of Steele's (1974) model with the creation of a cohort each day and some modification in the processes. Note that the mortality function (h') in Steele's (1974)

Table 12.7 Mathematical formulation of processes linking zooplankton to other variables in ecosystem models.
P phytoplankton or food; Z zooplankton; $g(P)$ grazing rate; $a(P)$ assimilation rate; $e(Z)$ excretion or respiration rate; $h(Z)$ predation rate. (See quoted references for the meaning of parameters.)

Formulation	Authors	Zooplanktonic organisms
Zooplankton grazing		
$g(P) = I_{max}P$	Andersen and Nival (1988)	salps
$g(P) = I_{max}(1 - e^{-\alpha P})$	O'Brien and Wroblewski (1973)	copepods
	Wroblewski and O'Brien (1976)	copepods
$g(P) = I_{max}\alpha(1 - e^{-\alpha P})$	Franks *et al.* (1986)	copepods
$g(P) = I_{max}(1 - e^{-\alpha(P-P')})$	Wroblewski (1977)	copepods
	Andersen *et al.* (1987)	copepods and appendicularians
$g(P) = \dfrac{I_{max}P}{\alpha + P}$	Scheffer (1991)	zooplankton
	Doveri *et al.* (1993)	zooplankton
$g(P) = \dfrac{I_{max}P^2}{\alpha + P^2}$	Steele and Henderson (1981)	zooplankton
$g(P) = \dfrac{I_{max}(P - P')}{\alpha + (P - P')}$	Steele (1974)	copepods
	Walsh (1975)	copepods
	Evans and Parslow (1985)	copepods
	Frost (1987)	microzooplankton and copepods
	Robinson *et al.* (1993)	copepods and euphausiids
Zooplankton assimilation		
$a(P) = \alpha g(P)$	Steele and Henderson (1981)	copepods
$a(P) = \dfrac{E_{max}\epsilon\ e^{\alpha(P-P')}}{E_{max} + \epsilon(e^{\alpha(P-P')} - 1)}$	Wroblewski (1977)	copepods
Zooplankton excretion and/or respiration		
$e(Z) = \theta$	Fasham *et al.* (1990)	copepods
$e(Z) = \theta\gamma^T$	Andersen *et al.* (1987)	copepods
$e(Z) = \delta g(P)$	Walsh (1975)	copepods
	Evans and Parslow (1985)	copepods
	Wroblewski and O'Brien (1976)	copepods
$e(Z) = \delta g(P) + \theta$	Steele (1974)	copepods
	O'Brien and Wroblewski (1973)	copepods
Food-dependent mortality		
$h(Z) = a$ if $P \leq P'$	Andersen and Nival (1988)	mortality of copepods and salps
$h(Z) = \dfrac{b}{P} + c$ if $P > P$		
$h(Z) = a\exp\left(-b\dfrac{P}{Z}\right)$	Andersen *et al.* (1987)	appendicularian mortality

(*Continued*)

Table 12.7 *Continued*

Predation rate		
$h(Z) = a$	Wroblewski *et al.* (1988)	copepods' predators
	Evans and Parslow (1985)	copepods' predators
	Fasham *et al.* (1990)	copepod's predators
$h(Z) = aZ$	Steele and Henderson (1981, 1992)	copepods' predators
$h(Z) = aZ - b$ if $c < Z < d$		
$h(Z) = 0$ if $Z \leq c$	Aksnes and Lie (1990)	copepod's predators
$h(Z) = ad - b$ if $d \leq Z$		
$h(Z) = aZe^{-\eta T}$	Kawamiya *et al.* (1995)	copepod's predators
$h(Z) = \frac{aZ}{b+Z}$	Frost (1987)	copepods on microzooplankton
	Hofmann and Ambler (1988)	*Paracalanus* sp. predators
	Landry (1976)	copepods
$h(Z) = \frac{a(Z-Z')}{b+(Z-Z')}$	Robinson *et al.* (1993)	euphausiid's predation
$h(Z) = a(1 - e^{-bZ})$	Wroblewski and Richman (1987)	anchovy's predation
$h(Z) = a(1 - e^{-b(Z-Z')})$	Andersen *et al.* (1987)	ctenophores' predation on copepods

model was related to individuals, whereas the similar function in equation 12.85 is related to biomass.

A description of the pelagic ecosystem with three variables is not sufficient in many situations, but the possible range of additional variables is large and could be of many different types. Ohuchi *et al.* (1986) present a review of models with three variables (NPZ), four variables (NPZD), five variables of which two are nutrients (N(2)PZD), five variables with fish (NPZDF), and seven or eight variables of which three or four are nutrients (N(3)PZDF and N(4)PZDF). Models with a more detailed representation of phytoplankton grazers will be presented on page 618.

When a detritus compartment is added, zooplankton can feed on detritus, and produce detritus as fecal pellets or carcasses. In Fasham *et al.*'s (1990) model with seven variables, zooplankton can feed on bacteria, phytoplankton and detritus.

Grazing rate

The feeding function, $g(P)$, of the zooplankton compartment is the individual functional response per unit mass. Different types of grazing functions are found in ecosystem models. The simplest one is the Michaelis–Menten expression but other expressions also have been used (see Table 12.2 in section 12.3.1 and Table 1 in Ohuchi *et al.* 1986). Steele (1974) used a Michaelis–Menten equation with a food threshold, and observed that the value of the food threshold could change the stability of the system (but see Landry 1976, and Steele 1976).

Extending Evans and Parslow's (1985) modeling approach for the subarctic North Pacific, Frost (1987) used two categories of grazers, microzooplankton feeding on phytoplankton and mesozooplankton feeding both on phytoplankton and microzooplankton.

Evans (1988) added another phytoplankton group (Q) to the Evans and Parslow (1985) model. The grazing of herbivores was modeled by a Michaelis–Menten hyperbola above a threshold concentration of food, where food was total phytoplankton $pP + qQ$, as perceived by herbivores.

Fasham *et al.* (1990) developed a more complex formulation to represent the switching between different food resources, presented in section 12.3.1. Fasham *et al.* (1990) mainly discuss the choice of food preference parameters and not the form of the feeding function and its related parameters.

Assimilation and metabolic losses

The assimilation rate, $a(P)$, is usually considered to be constant. However, Franks *et al.* (1986) used a mathematical formulation of the grazing response that was derived from Mayzaud and Poulet (1978), and which simulated the change of feeding rate, and indeed assimilation, with food concentration. Wroblewski (1977) presented a formulation of egestion rate as a function of food availability (see Table 12.7).

Excretion or respiration rates, $e(P)$, are expressed either as a fixed proportion of the zooplankton biomass, or as a fixed proportion of the ingestion rate, or both (see Table 1 in Ohuchi *et al.* 1986). Density dependent excretion or respiration are not really justified, although they could represent reduced respiratory loss in winter.

Mortality

Zooplankton mortality represents a model closure term. Steele (1976) and Steele and Henderson (1981, 1992, 1995) have shown that the mathematical form of this closure term can have a large influence on the dynamics of simple NPZ plankton models and on zooplankton demographics if it is represented as in Steele's (1974) model.

Steele and Henderson (1981, 1992) studied a three-component system consisting of phytoplankton, zooplankton and a nutrient. They examined three forms for the zooplankton specific mortality rate: constant (the closure is density-independent and named 'linear'), a linear function of zooplankton biomass (the closure is density-dependent and is named 'quadratic'), and a Michaelis–Menten function of zooplankton biomass (see Table 12.7). They concluded that the choice of the form of the closure term can determine overall patterns for all variables and be particularly relevant to the question of nutrient limitation versus grazing control.

The use of a Michaelis–Menten mortality has some biological foundation: zooplankton mortality consists of natural mortality, which may be caused by disease or starvation, and mortality due to higher predators. Both disease and predation are likely to be density-dependent processes. However, Steele and Henderson (1992) pointed out that there are no suitable observational data that will allow the parameters of a Michaelis–Menten function to be estimated. Steele and Henderson (1995) presented simulation results showing the importance of the correct representation of predator abundance cycles for the demographics of herbivorous copepods.

Andersen *et al.* (1987) considered two types of mortality for herbivorous zooplankton: mortality caused by starvation and mortality caused by predation. Mortality by starvation is an inverse function of the food concentration.

Vertical distribution of zooplankton

An important feature of mesozooplankton behavior is diel vertical migration. Many species migrate from daytime depths below 200 m up to the surface at night where they feed on the phytoplankton. There has been little modeling of this migration (Wroblewski 1982; Andersen and Nival 1991; Steele and Henderson 1998). However,

the migration patterns depend on species-specific behavior, and are difficult to represent in bulk-mass models of zooplankton.

Simulation results

It should be stressed that, in order to fully understand the dynamics of a model, it is important to analyze inter-compartment flows. If observational data of flow rates are available then they provide strong constraints on the model. Without such observations, modeled flows can be used to calculate bacterial or zooplankton growth efficiencies to check that they lie within the known range of experimental observations. The technique of 'flow analysis' provides a powerful tool for the analysis of either observed or modeled flow networks (Fasham 1985; Wulff *et al.* 1989). The mathematical analysis of simple food chain systems with three components (PZF) by Scheffer (1991) and five components (NPZF(2)) by Doveri *et al.* (1993) indicates that the dynamics of the model can be very complex.

The main biogeochemical functions of herbivorous (omnivorous) zooplankton, identified through field and modeling studies (Totterdell *et al.* 1993), are:

- control of lower trophic levels (phytoplankton, microzooplankton, bacteria) and the transfer of material to higher trophic levels
- the transfer of material from upper ocean layers to depth with the production of fecal pellets and carcasses
- a downward flux of matter linked to diel vertical migrations.

Aksnes and Wasmann (1993) showed the significance of zooplankton grazing for export production in a theoretical PZD model.

Other functions have been identified and depend on the organisms, such as the production of shells by pteropods, or detritus consumption by copepods.

FROM A SINGLE GRAZER TO SEVERAL GRAZERS

Functional groups of zooplankton

The NPZ-type models generally treat one copepod species as the herbivore in a simple food chain, whereas there is usually a succession of species (Davis 1987). Moreover, other herbivorous organisms, like salps or appendicularians can have a shorter and stronger impact on the ecosystem than do copepods. Copepods themselves develop through developmental stages and the ratio between the adult weight and the egg weight can be three orders of magnitude. However, Totterdell *et al.* (1993) recommended that life-history strategies should only be modeled explicitly if their effects are indispensable for the results and predictions sought, and they cannot be reproduced by some implicit formulation. The subdivision of zooplankton into several functional groups is treated in this subsection because the representation of processes is similar to that presented in section 12.5.1. The subdivision of zooplankton based on size or stage-development is presented in sections 12.5.2 and 12.5.3.

General formulation of a biological component

If we group several functional groups into one compartment, organisms with very different turnover times should not be combined (turnover time is defined as the organism pool size divided by the flux of biomass through that pool). Fasham (1993)

presented a general equation for change in bulk-biomass X of a zooplankton group k, based on an equation formalized by Wiegert (1979):

$$\frac{dX_k}{dt} = \sum_{i=1}^{m} e_{ik}\tau_k p_{ik} f_{ik} X_k - (\mu_k + \phi_k + \rho_k)X_k - \sum_{l=1}^{n} \tau_l p_{kl} f_{kl} X_l \qquad (12.88)$$

The first term on the right side of equation 12.88 represents the assimilated ingestion or uptake by species k from all the other modeled species or abiotic sources. The second term represents physiological losses, and the third term represents predation on species j by other species. The parameters are defined as follows:

- e_{ik} assimilation efficiency of species k using the resource i
- τ_k the maximum specific ingestion uptake rate of species k
- p_{ik} the preference of species k for the resource i (if predators are dynamically switching between resources then p_{ik} will be a function of the other resources as well (see section 12.3.1)
- f_{ik} the limitation of ingestion of species k by resource i, which is usually a function of X_i
- μ_k specific loss rate due to excretion
- ϕ_k specific loss rate due to natural mortality
- ρ_k specific loss rate due to respiration.

The form of the different processes is similar to those presented in Table 12.7 and section 12.5.1.

The equations governing the biological processes can be linked to physical models that provide environmental forcing.

Examples

Several models studying the role of lower trophic levels in material cycles have changed the zooplankton bulk compartment into several compartments to represent the diversity of microorganisms (e.g. Pace *et al.* 1984). Moloney *et al.* (1986) and Moloney and Field (1991) subdivide the group of heterotrophic organisms into the components mesozooplankton, microzooplankton, heterotrophic flagellates, and bacteria. Mesozooplankton feed on large phytoplankton and on microzooplankton following a Michaelis–Menten hyperbola above a threshold concentration of food. Food in their models is the sum of phytoplankton and microzooplankton. Metabolic losses are composed of two terms, the first proportional to ingestion and the second proportional to biomass. The second term also includes zooplankton mortality which is not explicitly represented.

In some models, different categories of large mesozooplankton are considered. Andersen *et al.* (1987) modeled plankton dynamics of an enclosed water column, based on the CEPEX project. A simple NPZC model with one herbivore compartment (copepods) and one carnivore compartment (chaetognaths and ctenophores) did not adequately represent the development of plankton populations in the enclosure, and a second version taking into account separate categories of nutrients, of phytoplankton and of herbivores was developed. Herbivores were divided into copepods and appendicularians. Ingestion rates of both herbivore groups followed an Ivlev relationship (see Table 12.7), and the food consisted of the two phytoplankton categories with different capture efficiencies for prey and predators. Excretion rates of the two herbivore groups were temperature dependent. Mortality of copepods was caused by carnivorous predation whereas the mortality of appendicularians was a function of the ratio of phyto-

plankton biomass over appendicularian biomass (see the formulations in Table 12.7). The second model gave a better fit of the observed dynamics.

In a pelagic ecosystem model of the Ligurian Sea, Andersen and Nival (1988) took into account two important groups of grazers: copepods and salps. Whereas copepod grazing is described by an Ivlev function, the grazing rate of salps was proportional to the phytoplankton concentration. Excretion by the two groups was temperature-dependent and food-independent, and mortality was an inverse function of food concentration (see Table 12.7).

A few attempts have been made to model estuarine/marine and shelf ecosystems as a whole, taking into account benthic and pelagic processes as well as advection and dispersion (Kremer and Nixon 1978; Radford and Joint 1980; Baretta and Ruardij 1988; Baretta *et al.* 1995). In these models, the biological components have been aggregated into functional groups. Each functional group is represented by a sub-model. Broekhuizen *et al.* (1995) developed a zooplankton submodel taking into account omnivorous zooplankton (copepods) feeding on phytoplankton and microzooplankton, and carnivorous zooplankton (copepods and gelatinous plankton). Planktivorous fish ate both omnivorous and carnivorous zooplankton. Each prey taxon of any zooplankton group was consumed in proportion to its instantaneous relative abundance, and the grazing function was given by a type II functional response to the total food concentration. The assimilation rate and active metabolism were proportional to ingestion, and the basal metabolism of a group was proportional to its biomass. Mortality other than predation by fish was proportional to biomass.

The ECOPATH II model (Christensen and Pauly 1992) is an example of a modeling technique in which higher trophic levels that feed on zooplankton, especially commercially exploited fish, are well represented.

12.5.2 Size-structured zooplankton community

SIZE-STRUCTURED ECOSYSTEM MODELS

An alternative approach to using functional groups in models, is to subdivide zooplankton into groups that are based on organism size. There is a theoretical basis for defining size-related compartments, because growth and metabolic rates are often found to be dependent on organism size (Peters 1983). Several models represent size-structured zooplankton communities. Because some models defined functional groups on the basis of size (Pace *et al.* 1984; Moloney *et al.* 1986), they can also be considered as size-structured models.

One of the first size-structured models was developed by Vinogradov *et al.* (1972) to simulate the time evolution of a community in the Pacific equatorial upwelling zone. This model is built on the basis of eight functional groups. Zooplankton are subdivided in small-sized herbivores, large-sized herbivores, omnivores, small-sized carnivores and large-sized carnivores.

Steele and Frost (1977) simulated the size structure of herbivorous zooplankton and their prey, and their interactions, in a nutrient–phytoplankton–herbivore–carnivore dynamics model. Their study focused mainly on the population structure of filter-feeding copepods (*Calanus* and *Pseudocalanus* sp.) and the relative abundance of species which, at any developmental stage, differ markedly in body size.

Moloney and Field (1991) and Moloney *et al.* (1991) presented a size-based plankton model of the Benguela ecosystem, but general enough to simulate interactions within plankton communities of any ecosystem. Community structure and transfer processes

were all size-dependent, and all model parameters were determined by body size, using empirically determined relationships calculated from published data (Peters 1983; Moloney and Field 1989). In the original model (Moloney and Field 1991), autotrophic and heterotrophic groups comprised organisms in size ranges from 0.2 to 200 μm ESD (equivalent spherical diameter) and 0.2 to 2000 μm ESD respectively, and were divided into size classes using a logarithmic scale. A logarithmic scale of 10 was used because the resulting size classes were similar to traditional categories described by Sieburth *et al.* (1978). In the size range 0.2–2 μm ESD, picophytoplankton and bacterioplankton were considered, in the size range 2–20 μm ESD nanophytoplankton and heterotrophic flagellates, in the range 20–200 μm ESD net phytoplankton and microzooplankton, and in the range 200–2000 μm mesozooplankton. Moloney and Field's model simulated flows of carbon and nitrogen.

In the model, ingestion rate of heterotrophs was a function of prey concentration between a range of size classes, and following a Michaelis–Menten relationship:

$$g(P_i) = \frac{I_{max}(P_i - P_i')}{\alpha + \sum_{i=\min}^{\max} (P_i - P_i')} \tag{12.89}$$

where the specific ingestion rate of size class i by size class j was determined by the maximum mass-specific size-dependent ingestion rate of size class j (I_{max}). P_i is the standing stock of size class i, and P_i' is the threshold. Assimilation was taken to be constant, and respiration and excretion were proportional to biomass.

SIZE SPECTRUM THEORY

The biomass size spectrum model initially proposed by Sheldon and Parsons (1967) and Sheldon *et al.* (1972, 1973) has been developed in successive steps by Platt and Denman (1978) and Silvert and Platt (1978, 1980). Heath (1995) presented a synthesis of biomass size spectrum theory (see also Gaedke 1992, 1993; Gaedke and Straile 1994; Blanco *et al.* 1994, 1998).

The size distribution of organisms in the pelagic food chain can be described by the following time-dependent equation (Silvert and Platt 1980):

$$\frac{\partial N}{\partial t} + w\frac{\partial}{\partial w}(bG) + bM = 0 \tag{12.90}$$

where $\beta(w, t)$ is a biomass density function such that

$$b(w, t) = \beta(w, t)dw \tag{12.91}$$

$b(w, t)$ is the mass of particles per unit volume in the size interval from w to $w + dw$ at time t. Silvert and Platt (1980) assumed predation scales in a perfectly isometric fashion, i.e. the range of particle sizes acceptable as prey scales as w. Thus $dw \sim w$.

The function $G(w)$ is the specific growth rate of particles:

$$G(w) = \frac{1}{w}\frac{\partial w}{\partial t} \tag{12.92}$$

and the function $M(w)$ is the specific rate of change in numbers of particles due to mortality and reproduction:

$$M(w) = \frac{1}{N}\frac{\partial N}{\partial t} \tag{12.93}$$

and $N = \beta/w$.

The basic assumptions of the size spectrum theory (Silvert and Platt 1980) are that the system is in steady state, with no immigration and emigration of biomass, predation is the only source of mortality, large organisms eat only smaller ones that are a constant fraction of their own size, there is a constant input of energy to the smallest size classes in the spectrum, and the flow of energy (biomass) is in one direction only, from small particles to large ones.

Silvert and Platt (1980) showed that one possible solution to equation 12.90 is

$$\beta(w)G(w) \sim w^{-c} \tag{12.94}$$

where the exponent c is such that:

$$K_1 c = q^{c-1} \tag{12.95}$$

and parameter K_1 is the growth efficiency, i.e. growth divided by ingestion. Specific growth rate can be expressed as:

$$G(w) = kw^{-x} \tag{12.96}$$

where k and x are constants.

Combining equations 12.93 and 12.95,

$$\beta(w) \sim w^{x-c} \tag{12.97}$$

Silvert and Platt (1980) showed that

$$M(w) = \frac{G(w/q)}{K_1 q}\frac{\beta(w/q)}{\beta(w)} \tag{12.98}$$

and from equations 12.94 and 12.95

$$M(w) = \frac{k}{k_1} q^{c-1} w^{-x} = ckw^{-x} \tag{12.99}$$

Somatic growth represents the difference between the assimilated ration and losses due to metabolism and reproduction (reproductive loss is small relative to metabolic losses and is therefore ignored). Metabolism is found to take the form αw^{γ} and $\gamma = 1 - x$. Thus

$$\frac{dw}{dt} = pI - \alpha w^{1-x} = G(w)w = kw^{1-x} \tag{12.100}$$

for all w.

The ingestion rate becomes:

$$I = \frac{k + \alpha}{p} kw^{1-x} \tag{12.101}$$

and growth efficiency:

$$K_1 = \frac{p(k+1)}{\alpha} \tag{12.102}$$

Peterson and Wroblewski (1984) estimated the four parameters k, x, K_1, and q from the literature on a range of pelagic species and concluded that:

- $x \sim 0.25$
- $k = 4.23\ 10^{-3}\ \mathrm{d}^{-1}$
- $c \sim 1.22$ with $K_1 = 0.14$ and $q = 3.45\ 10^{-4}$

The steady-state constraint on this basic model, with constant energy input to the smallest size classes, dictates that it can only be considered to represent some long-term average distribution of biomass across the full range of organisms in an ecosystem, such that temporal trends are eliminated. The shortest such time period over which the model could apply would be 1 year, thereby removing the dominant seasonal signal in the energy input (at high latitude), and several data sets supported this conclusion (see references in Heath 1995). However, Thiebaux and Dickie (1993a, 1993b) have suggested that within any trophic group in the ecosystem other factors are involved which cause the biomass spectrum to be non-linear. Considering variations in the biomass spectrum on some time scale shorter than one year, Silvert and Platt (1978, 1980) showed that perturbations in the energy input to the ecosystem must propagate up the spectrum as a damped wave. Feedback mechanisms, such as reproduction, will lead to smearing of the wave, but the mathematics of this process are complicated.

New technological developments such as the Optical Plankton Counter will probably encourage further development of the size-based approach to data analysis (Heath 1995; Zhou and Huntley 1997). Applications of biomass spectrum theory to fish larvae are presented by Beyer (1989).

12.5.3 Size- and stage-structured zooplankton populations in ecosystem models

The life cycle of dominant zooplankton species in ecosystems can be represented in detail in ecosystem models, when the representation of an average individual is not sufficient.

Steele (1974), Steele and Mullin (1977) and Steele and Frost (1977) have developed an ecosystem model in which the dynamics of *Calanus* were represented as successive cohorts. Hofmann (1988) used a stage structured model of *Paracalanus* sp. in five groups of stages which were feeding on two size categories of phytoplankton. Stage and age-structured population models (see page 602) have been coupled with ecosystem models (Fransz 1981; Davis 1984a, 1984b; Carlotti and Radach 1996).

Koslow (1983) studied the role of predatory interactions in the regulation of the size structure of marine zooplankton communities. The model contained size-structured populations of large (*Calanus* sp.) and small (*Paracalanus parvus* and *Acartia clausii*) herbivorous zooplankton, and invertebrate carnivores (chaetognaths). Planktivorous fish were represented by one compartment.

12.5.4 Further reading

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Ulanowicz, R.E. and Platt, T., 1985. Ecosystem theory for biological oceanography. *Can. Bull. Fish Aquat. Sci.*, **213**: 260 pp.

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12.6 MODELING SPATIAL DYNAMICS OF ZOOPLANKTON

Spatial distribution, dispersion and patchiness of zooplankton are important features of the ocean (Mann and Lazier 1991). They are influenced by a set of biological and physical processes of which the relative contributions are difficult to extract. Mathematical models that include biological as well as circulation processes provide an approach for investigating and separating environmental and biological factors that control plankton distribution. Many of the examples found in the literature study the effect of some selected factors, assuming that other factors have negligible effects. Simulated plankton distributions obtained with models treating plankton as simple drifters and for which dispersion is determined solely by circulation, show strong differences from observed distributions. The primary conclusion from these models is the need to include biological effects, and a thorough effort has been made in this direction during the past decade.

12.6.1 Modeling active behavior and counter-gradient search

An organism's sensory systems are evolutionary adaptations that enable the organism to react to environmental variations. Zooplankton will benefit from organs that allow them to locate food and mates and to avoid predators and other harmful situations. The evolution of such organs is therefore under strong selection pressure. The simplest formulation of predator–prey contact rates is obtained by assuming that either the prey or the predator is stationary while the other is moving about with an average speed v and that the predator has a reaction volume with a surface A outside which it cannot detect prey (Gerritsen and Strickler 1977). Then the total number of encounters between predators and prey during a time period T is given by

$$E = TNPAv \tag{12.103}$$

where N and P are concentrations of predators and prey respectively (Giske *et al.* 1994). This model can be easily reformulated to express feeding rates of predators and mortality rates of prey. It has been elaborated in two directions: models of contact rates and models of perception ranges (Table 12.8). Contact rate models can incorporate swimming of both predator and prey, pause swimming, and turbulent water motions.

The effect of small-scale physics on plankton ecosystems is a recent research topic (Yamazaki and Osborn 1988; Granata and Dickey 1991; Denman and Gargett 1995). Recent theoretical models suggest that small-scale turbulence is a significant component in the encounter rate between a larval fish and their planktonic prey (Rothschild and Osborn 1988; MacKenzie and Leggett 1991; McKenzie *et al.* 1994). Few studies of this effect have been found to date. Using numerical simulation methods, Yamazaki *et al.* (1991) demonstrated that the contact rate model of Rothschild and Osborn (1988) is valid. Lagrangian models which mimic the aggregation of planktonic organisms in turbulent flow allow one to take into account small-scale non-linear processes, and to study the behavior of planktonic organisms in conjunction with such structures (Yamazaki 1993).

Models of perception ranges focus on the sensory organs of predators and prey, and mathematical formulations are available for the lateral line, vision, hearing and olfaction. Although zooplankton use all sensory systems simultaneously (Bollens *et al.* 1994), no single model has yet incorporated all of these.

The sensory systems of fish take up information at several spatial and temporal scales.

Table 12.8 Models of sensory systems of fish and mortality risk of zooplankton.

Authors	Modeled processes
Passive and tactile encounter	
Gerritsen and Strickler (1977)	contact rate between a predator and a prey
Rothschild and Osborn (1988)	effect of turbulent motion on contact rates
MacKenzie and Leggett (1991)	effect of wind and tide on encounter rates between fish larvae and zooplankton
MacKenzie and Kiørboe (1995)	encounter rates for cruising and pause travel predators in calm and turbulent conditons
Kiørboe and Saiz (1995)	copepod feeding and predation risk in turbulence
Caparroy and Carlotti (1996)	energy budget for copepod in turbulent regime
Eiane *et al.* (1997)	comparison of tactile and visual predation in the vertical field
Fiksen *et al.* (1998)	combination of light and turbulence for predation by fish larvae
Olfaction	
Jumper and Baird (1991)	detection range of odor plume
Baird and Jumper (1995)	mate location by olfaction
Baird *et al.* (1996)	odor spread by diffusion, turbulence and advection
Moore *et al.* (1994)	odor spread in benthic boundary layer flow
Light and vision	
Aksnes and Giske (1993)	model of visual range of planktivorous fish
Aksnes and Utne (1997)	
Giske *et al.* (1994)	vision-based (light-dependent) mortality risk for zooplankton
Hearing, lateral line and pressure fields	
Rogers and Cox (1988)	underwater sound propagation and biological responses
Kalmijn (1988)	detection range of hydrodynamic and acoustic fields
Bleckmann (1993)	model of detection by lateral line system

The lateral line system can detect zooplankton movement at very small distances, and therefore can be the first tool available for prey detection by fish larvae (Blaxter and Batty 1985). Vision is also a near-field instrument, because the decay rate of images underwater is fast (Aksnes and Utne 1997). Sound propagates better in water than in air (Rogers and Cox 1988), and hearing is therefore a good long-distance tool. Olfaction can be used to trace prey or a mate along a concentration gradient (Baird and Jumper 1995) and it is the only sense to measure 'past experience'. It can also be used to indicate concentrations of predators and hence predation risk for prey (Larsson and Dodson 1993). The lateral line and vision systems give more precise information than hearing or olfaction, and final prey capture will most often rely on one of these. Hearing or olfaction is probably more important for the early phase of prey location and for predator avoidance.

12.6.2 Modeling behavioral mechanisms, aggregation, and schooling patches

MODELING ZOOPLANKTON BEHAVIOR AT THE 'MICRO-SCALE'

The simplest model of dispersion is a random walk model, where individuals move along a line from the same starting position (see Okubo 1980; Possingham and Rough-

garden 1990; Renshaw 1991; Brown and Rothery 1993). Below we describe models that are used to simulate movement of zooplankton individuals.

Random walk models (RWMs) have been used for the last 50 years (Yamazaki 1993). They involve a succession of movements along a line segment where the direction and the length of each move is randomly determined (Yamazaki and Okubo 1995). The RWM and its extensions, as well as numerous applications can be found in Okubo (1980) and Berg (1983). In plankton ecology, RWMs have been used mainly for the study of phytoplankton trajectories in mixing circulation or Langmuir cells (see Yamazaki 1993), and for behavior of fish larvae (Okubo 1986). There are few RWMs for zooplankton studies. Davis *et al*. (1991) presented a series of models exploring the role of swimming and patchy food supply on the growth of a predator (fish larvae preying on copepods, or copepods preying on phytoplankton cells). From a RWM, they derived an appropriate form for a diffusion term and combined this with a growth model (similar to those presented in section 12.3). A general analysis of the growth/swimming model showed the dependence upon the parameters and the patch structure. The authors used this model to study the effect of turbulence, which induces more frequent encounters but also dissipates prey patches. Tiselius *et al*. (1993) presented a model of individual copepods, taking into account the feeding process in a patchy prey environment, as well as growth and reproduction. In their model, copepod motility followed a RWM.

Yamazaki made initial attempts to simulate a zooplankter in a flow field, first as a passive particle, then with behavior based on a RWM (Yamazaki *et al*. 1991). These models showed that organism behavior must be integrated into studies of small-scale physics. The only way to properly account for the effects of small-scale physics (e.g. turbulence) is to develop Lagrangian models and implement these into realistic simulated flow fields, for example turbulent flow from direct numerical simulations based on the Navier–Stokes equations.

A different method for modeling the movements of organisms is by utilizing evolutionary theory (section 12.3.3). Tools for this approach will be discussed in Evolutionary modeling approaches for optimal spatial distributions, section 12.6.2.

From animal aggregation to patch dynamics models

Here we denote aggregation as a grouping of conspecific individuals without any implication of mutual attraction (Ritz 1994). Passive aggregations are caused by physical factors (e.g. currents, light). Active aggregations can be permanent or temporary, as a response to food concentration or predation, or for mating. Different types of models have been built, some of them focusing on the structure and shape of aggregations depending on internal and external physical forces, others dealing with the benefits for individuals of living in groups with regard to feeding (foraging models) and to predation. There are many models dealing with living in groups (see for review Pulliam and Caraco 1984), but few have been applied to marine zooplankton.

Models for studying aggregation size, structure and shape need the development of physical models to which can be added the swimming behavior of organisms. The focus of much modeling work has been the study of the maintenance of animal aggregations (swarms, patches, schools, etc.) in the face of dispersive forces acting on them from the environment (Okubo 1980).

The Lagrangian approach can take into account the behavior of individual organisms, and the effects of the physical environment upon them. The Lagrangian approach is straightforward, but the nature of the mathematics, i.e. 'n-particle motion dynamics', makes any analysis computer intensive with an increasing degree of non-linear aspects.

Yamazaki (1993) reviewed available Lagrangian simulations for animal groupings (presented with others in Table 12.8). Yamazaki (1993) introduced numerical Lagrangian models that incorporate an attractive force component in organism aggregations (see also Yamazaki and Okubo 1995). Yamazaki and Haury (1993) used such a model to find a generalized relationship between an organism's locomotion ability (e.g. swimming speed and 'motivation' to maintain a swarm), perception distance (measuring the ability to sense and orient toward neighbors), and the strength of diffusing forces (e.g. turbulence) in a zooplankton swarm.

Although Eulerian approaches are mathematically tractable, the methods do not explicitly address the density dependence of aggregating individual behavior within a patch. Okubo (1980, 1986) has reviewed much of the work in mathematical modeling of animal aggregations that use a Eulerian approach, incorporating diffusion and advection terms to represent random walk processes and attractive forces. Several models have emphasized the forces that internally maintain schools (Okubo 1980; Anderson 1981). Anderson (1981) represented a school size change by Fokker–Planck stochastic differential equations, having linear terms for school size increase and decrease.

Group living is an important feature for some zooplankton species and fish larvae. Group membership is supposed to be helpful for the individual in feeding and avoiding predators (Pulliam and Caraco 1984; Clark and Mangel 1986). Foraging and feeding in groups may give benefits for locating new or richer food patches, but also increases competition for resources within a prey patch. Clark and Mangel (1986) present a set of simple mathematical models to investigate the relationship between evolutionary fitness of individual foragers and the size of the foraging group.

Another important function of aggregation is to confer protection from predators upon its members. Swartzman (1991) approaches the formation and maintenance of fish groups under predation. He described a birth–death model that is expressed as a system of differential-difference equations describing the probability of having different numbers of schools in a $1\,km^2$ study region. The equilibrium solution was obtained analytically, and results were presented for different combinations of prey and predator characteristics. These results were interpreted to indicate under what conditions schooling is likely.

EVOLUTIONARY MODELING APPROACHES FOR OPTIMAL SPATIAL DISTRIBUTIONS

The theory of evolution by natural selection predicts that the gene pools of populations will become adapted to their physical and biological environment (section 12.3.3 and Figure 12.1). Optimal spatial distributions have been investigated by several modeling approaches (see review by Giske *et al.* 1998a). The simple equation for population growth has branched into four bodies of theory (Figure 12.2), of which two have been utilized to predict individual behavior.

Optimization

The earliest theory for optimal spatial distributions of individuals within a population was the Optimal Foraging Theory (OFT) (Emlen 1966; MacArthur and Pianka 1966). OFT is not derived from any specific equation related to Darwinian fitness, but assumes that increased efficiency during feeding will benefit the organism by providing more time for other fitness-related tasks (such as mating or hiding). OFT was the major theory in use by behavioral ecologists in the 1970s, and diversified into theories of optimal diet, optimal habitat and several more (see review by Schoener 1987). In contrast, Zaret

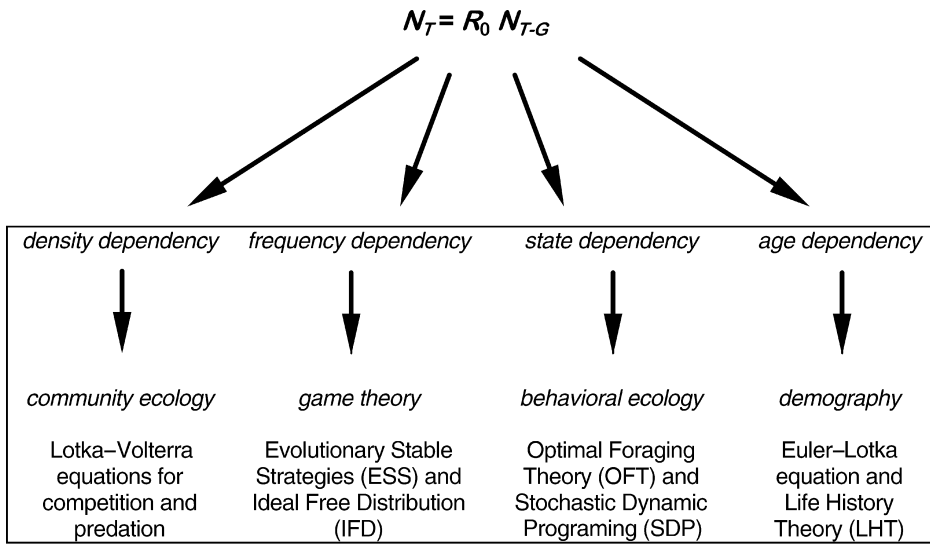

Fig. 12.2. The simple equation for population growth has spawned several theories in quantitative ecology (Stearns 1992), each using a partial description of the organisms and their environment. Within community ecology, mortality and fecundity are assumed depending on density-dependent factors as resources, competitors, diseases and predators. Within game theory, the rate of spread of a strategy is assumed to depend on the frequency of alternative strategies. In models in behavioral ecology, animals will act according to some measure of their state, while organisms, according to classical life history theory, behave, reproduce, and survive as a function of their age or stage. N = population size, R_0 = (adult) offspring per parent, G = generation time and T = temperature.

(1980) expressed frustration about the predominantly feeding-based approach to aquatic ecology; he showed many examples of predation-structured aquatic communities.

Also in 1966, the first papers appeared that used demography as a tool for predicting life histories of individuals (Murdoch 1966, Williams 1966). Demography can be linked directly to fitness by the Euler–Lotka equation (Figure 12.2) and may therefore solve conflicts between the importance of feeding versus predation. Individual motivations for behavior in Life History Theory (LHT) account for factors influencing fecundity and survivorship, and generate predictions of optimal trade-offs between environmental forces over a long period (Werner and Gilliam 1984; Aksnes and Giske 1990; Salvanes *et al.* 1995). Typical for LHT models is that individuals are characterized by their age and not individual characteristics such as competitive ability or hunger. McLaren (1963, 1974) proposed a model for vertical distribution of copepods based on maximization of the reproductive rate. McLaren's (1963, 1974) work, being earlier than the papers leading to LHT, was largely met with scepticism. We take this opportunity to congratulate him on his pioneering and original work.

The main drawback of Euler–Lotka-based LHT (the reliance on age as the factor characterizing individuals) may be solved by SDP (Stochastic Dynamic Programing). Dynamic optimization with SDP (Houston *et al.* 1988; Mangel and Clark 1988; Clark and Mangel 1999) allows descriptions of the internal state of individuals, which may lead

to both variable and fluctuating motivations among individuals over short time periods (see Box 12.1). SDP has yielded considerable attention lately with a few applications in zooplankton and ichthyoplankton ecology. SDP has been used to study diel (Clark and Levy 1988; Rosland and Giske 1994; Fiksen and Carlotti 1998) and ontogenic (Fiksen and Giske 1995; Fiksen 1997; Rosland and Giske 1997) vertical distribution of zooplankton and fish, large scale horizontal distribution (Mangel 1994; Fiksen *et al.* 1995) and optimal group size (Mangel 1990). A disadvantage of SDP for ecological modeling is that optimal solutions in dynamic programing depend on the future of the individual and the environment. The optimal behavior of an individual at time t thus depends on expected density of predators and food at time $t + x$. Hence, the behavior found for time t is truly optimal only as long as it does not change the expected state of the environment (food and predators) at $t + x$. Trophodynamics can therefore not be modeled by the backwards-calculating SDP.

Box 12.1 Dynamic Programing

Finding optimal distributions by backward iteration proceeds as follows: relative fitness is first determined for each possible energetic state at the end of the simulated period, for example by a life-history related function. Generally, these fitness values are termed $F(x, t, T)$, where x is the value of the state variable, t is the current time step and T is total number of time steps.

Consider an SDP model for optimal vertical positioning of a pelagic predator. During a time step, an animal may choose among several habitats. Predation risk (p), probability of finding food (e), the energetic value of the food (f) and metabolic costs (c) may differ among habitats. (For simplicity of presentation, we assume here that all prey in a habitat are equal and that one or no items can be caught in a period.) In a period (from t to $t + 1$) an animal of state x (x, t) either finds food and changes its state to ($x - c + f, t + 1$), or does not find food, and changes to ($x - c, t + 1$). The average new state (x'_{t+1}) for an animal of state x_t in a habitat is therefore

$$x'_{t+1} = e(x_t - c + f) + (1 - e)(x_t - c) \tag{1}$$

and the probability of surviving the time step is $(1 - p)$. The fitness value of the states $x - c + f$ and $x - c$ at final time ($t = T$) is now already found, and fitness of state x in habitat h at $t = T - 1$ is

$$F(x, h, t = T - 1) = (1 - p)[eF(x - c + f, T) + (1 - e)F(x - c, T)] \tag{2}$$

$F(x, h, t = T - 1)$ is then calculated for all habitats, and the optimal habitat is where $F(x, h, t)$ is maximal. In this optimal location $[h(x, T - 1)]$ the fitness value of animals of state $(x, T - 1)$ is

$$F(x, T - 1) = (1 - p^*)[e^*F(x - c^* + f^*, T) + (1 - e^*)F(x - c^*, T)] \tag{3}$$

where * refers to environmental values in the optimal habitat. Having found $F(x, T - 1)$ and $h(x, T - 1)$ for all x, calculation of fitness values and the corresponding optimal locations for all states at $t = T - 2$ proceeds according to the general equation

$$F(x, t) = (1 - p^*)[e^*F(x - c^* + f^*, t + 1) + (1 - e^*)F(x - c^*, t + 1)] \tag{4}$$

(From Giske *et al.* 1998b).General textbooks discussing SDP are Mangel and Clark (1988) and Clark and Mangel (1999). The latter book also shows how a population may be modeled when all individuals follow the optimal trajectory of the SDP equation.

Game theory

Game theory addresses optimal solutions to frequency-dependent problems, i.e. situations where the optimal decision depends on what other individuals do. Two game theoretic approaches have been utilized in ecological modeling, the evolutionary stable strategy (ESS) and the ideal free distribution (IFD). Game theory, like optimality theory in OFT, was introduced to ecology from economics (Maynard Smith 1982; Parker 1984). Commonly, the goal of a game is to find a strategy (pure or mixed) that can persist in a population, and the Evolutionary Stable Strategy (ESS) is a well-known example (Maynard Smith and Price 1973; Maynard Smith 1974). A strategy is an ESS if, once established in the population, a new mutant or immigrant cannot invade the population (see Box 12.2).

Iwasa (1982) constructed the first aquatic ESS model. He modeled the diel vertical distribution of zooplankton (that ate phytoplankton and were eaten by fish) and fish. The game between predator and prey led to light-dependent behavior at both trophic levels. Gabriel and Thomas (1988a, 1988b) developed this game further, but included negative density-dependent effects of competitors within each trophic level. Their model thus was both frequency- and density-dependent. Hugie and Dill (1994) also constructed a habitat-selection game between zooplankton and a planktivorous fish population. Their model links ESS and the other game theoretical approach, the IFD (see below).

Ideal Free Distribution

The Ideal Free Distribution (IFD) is a theoretical model for studying density-dependent effects on the spatial distribution of optimal individuals in a group (Fretwell and Lucas 1970). 'Ideal' means that each individual animal is able to choose the habitat that maximizes its fitness rewards, and 'free' means that there are no costs associated with entering this habitat. For animals that forage in a patchy environment, for convenience the rewards are often supposed to be equivalent to food intake rate. Generally, in classical IFD, all individuals are alike, but this constraint has been relaxed in several approaches (see review by Tregenza 1995). The relaxation of the original assumptions

Box 12.2 The evolutionary stable strategy (ESS)

A strategy is an ESS if, once established in the population, a new mutant or immigrant cannot invade the population. This can be stated as

$$S_e(1-\epsilon) > S_m(\epsilon) \tag{1}$$

where S_e and S_m are the pay-offs of the established and mutant strategies when their frequencies of occurrence are $(1-\epsilon)$ and ϵ, respectively $(\epsilon \ll 1)$. However, a mixed ESS, or an evolutionary stable polymorphism, can be established if the pay-offs of the two (or more) strategies are the same at some frequency of occurrence

$$S_e(x_s) = S_m(x_s) \tag{2}$$

and when a change in frequency of occurrence from this balance leads to reduced fitness of the strategy:

$$S_e(x) < S_e(x_s) \tag{3}$$

for x close to x_s.

usually leads to very computer-intensive models (e.g. Giske *et al*. 1997). A limitation of IFD is that it cannot explain the fluctuating motivation of individuals according to time and state. Combined effects of density and internal state may be studied by SDP. Giske *et al*. (1997) present in detail an IFD model of similar copepods in the water column which includes both density-dependent predation risk and food supply (See Box 12.3).

Box 12.3 The Ideal Free Distribution (IFD)

Under Ideal Free Distribution models with equal competitors, the total number of individuals distribute themselves among habitats so that the gain of an individual is equal in all habitats. In the classical version of the model, food was the resource, and the individual gain (S_i) in a habitat (i) declined from a density-independent maximum B_i by a habitat-specific function of competitor density d_i

$$S_i = B_i - f_i(d_i), i = 1, 2, \ldots, L(\text{habitats}) \quad (1)$$

The IFD is achieved when individual gain is equal among habitats

$$S_1 = S_2 = \ldots = S_i \quad (2)$$

and total numbers in the $K \leq L$ occupied habitats match population size N_T

$$N_1 + N_2 + + + N_K = N_T \quad (3)$$

For zooplankton in a natural environment we would not expect that food gradients alone could cause an IFD for three reasons: (1) there are combinations of low competitor density and high food production where individual feeding rate will not be influenced by a (minor) increase in competitor density, and more important, (2) one of the main resources for development and fitness of aquatic organisms may be environmental temperature and (3) predation risk will affect fitness in a density-dependent manner. Habitat profitability may then be expressed directly in terms of the habitats' expected contribution to the fitness (Φ) of the animal, and ideal free individuals will distribute so that

$$\Phi_1 = \Phi_2 = \ldots = \Phi_i \leftrightarrow \rho_1 = \rho_2 = \ldots = \rho_i \quad (4)$$

as r is a population parameter constant for all habitats and is the average of all ρ_i' (see equation 12.37). In a situation where feeding will be sufficient for growth and where reproduction may occur unhindered by, for example, seasonal constraints, fitness is proportional to the life-history trade-off g/M, as shown above. Then we may write

$$\Phi_1 = \Phi_2 = \ldots = \Phi_i \leftrightarrow g_1/M_1 = g_2/M_2 = \ldots = g_i/M_i \quad (5)$$

(Giske *et al.* 1997). In IFD terms we will find the relation between the optimum number in each habitat, so that gain is equalized by competition and predation risk dilution. Fecundity, growth and survival will be derived from mechanistical equations of the environment and density-dependent responses. The ideal free distribution – where no individual could increase its fitness by moving – is obtained when competitor density in each available habitat is adjusted so that the ratio g/M is equalized.

According to equation (1) habitat profitability is a decreasing function of concentrations of foragers, while in equation (5) the fitness may be maximal at some intermediate zooplankton concentration with low resource competition but with substantial dilution of predation risk. In the latter case, with a bell-shaped habitat profitability curve, there are two zooplankton concentrations that give the same fitness in each habitat. This makes it far more complicated to calculate the expected spatial distribution of a population. Methods for this calculation are discussed in Giske *et al.* (1997).

Adaptation tools

Over the past few decades, evolutionary modeling philosophies have emerged that do not rely on maximizing any explicit fitness measure, as R_0 or r, but rather mimic the process of natural selection. Hence these methods are labeled 'adaptation tools' as compared to the 'optimization tools' described on page 627. Curiously, this tradition also may be traced back to 1966, when Fogel *et al.* (1966) presented Evolutionary Programing. Later, other techniques such as Evolutionary Strategies (Rechenberg 1973), Genetic Algorithms (Holland 1975) and Genetic Programing (Koza 1992) appeared. We will briefly describe the genetic algorithm and show how it may be linked to a tool in neurobiological modeling, the Artificial Neural Network (ANN) to produce a new class of models which may be useful for individual-based modeling.

The genetic algorithm (GA) is a powerful search technique that mimics evolution by natural selection – the principles of Charles Darwin (1859) are used to evolve optimal solutions to a given problem. The method was developed in the late 1960s and early 1970s by John Holland and co-workers at the University of Michigan (Holland 1975). Although the GA was created based on an evolutionary approach (Holland 1975), this method has had very few applications in biology and especially in ecology as pointed out by several authors (Goldberg 1989; Davis 1991; Saila 1996; Toquenaga and Wade 1996). GA may be used as a regular search technique finding globally or near-globally optimal solutions to a given problem. The GA may have some advantages over conventional methods in ecology, specifically in cases of many decision variables, important trophic couplings and where density dependence is important. In such cases SDP, ESS and IFD models are intractable whereas the GA may provide solutions.

The GA may be considered as a wheel where each turn represents a generation. Some general basic features of the GA are:

1) code with solutions to the problem, where alternative parameter values are given as alleles of genes
2) evaluation and ranking of solutions
3) selection of solutions – thus reproducing good alleles more than poor alleles
4) 'crossing over' and 'mutation' providing variability to solutions.

The major asset of the GA is its intrinsic forcing (through natural selection) as opposed to optimization models that are driven by extrinsic forcing (through a fitness or goal function). The intrinsic forcing may help the GA find its goal and adapt dynamically to changing environments and population characteristics.

The GA is seldom encountered in the ecological literature in general, and most biological applications seem to be in molecular biology (Goldberg 1989). Applications of GA in aquatic biology are, however, mostly related to spatial distribution of fish. D'Angelo *et al.* (1995) used a GA to examine relationships between physical characteristics of streams and trout distribution data. They found that the GA was a more efficient predictor of trout distribution than was proportional trout distribution and multiple linear regression equations. Dagorn *et al.* (1995, 1997) applied a GA to control a neural network (e.g. Saila 1996) to investigate spatial distribution of tuna in the Indian Ocean. The neural network was used to search for high concentrations of food which are normally associated with thermal fronts. They applied two different models to solve the problem, and the GA model where the tuna were given learning abilities proved to be a much better predictor of tuna movement than the method using search without learning.

Thus, this type of model is situated between the ING method (see below) and conventional GA models.

In Artificial Neural Network (ANN) models the human brain is used as a model for decision-making based on sensory inputs (Hopfield 1982; Kohonen 1984). The ANN is organized as a neuroconnector net, which connects sensory input data with behavioral actions. Information from the sensory neurons is passed by adjustable 'synapses' to a series of releaser 'neurons'. Between these and a corresponding series of 'behavior' neurons there is a series of fixed weight synapses. Finally, there may be inhibition synapses between behavior neurons. A neuron will 'fire' a signal through a synapse if the inputs to this neuron are above an 'input threshold'.

In the first series of adjustable synapses the neuroconnector net may learn to find the appropriate weights of the signal as well as the optimal thresholds for each input register. Learning (i.e. reinforcement) occurs because connections between the sensory input and the corresponding behavioral action are strengthened if the behavior is adequate, and weakened if not (Rummelhart *et al.* 1986). By training, the neural network may learn to respond to signals. This can be done in a known environment (where the correct output = behavior is known) by 'back propagation' (Rummelhart *et al.* 1986) or (for complex problems) by evolving the best network by a genetic algorithm (van Rooij *et al.* 1996). Links to evolutionary fitness can be made by evolving the connection strength by a genetic algorithm (GA) linked to reproduction, rather than training the ANN on obvious situations (van Rooij *et al.* 1996).

Huse and Giske (1998) and Huse (1998) proposed a new method for individual-based modeling of organisms. In their method, behavioral responses to sensory information was governed by one or more ANNs that were evolutionary adapted to the physical and biological environment by a GA. They called this method Individual-based Neural-network Genetic-algorithm (ING, Figure 12.3), and have used it to model capelin and capelin–cod interactions in the Barents Sea. The ING concept can handle both state and density dependencies, and may allow for trophic couplings by several populations of adaptive agents. The ING approach thus seems to be able to unify all three major branches of evolutionary theory in Figure 12.2. The major disadvantages are the intractability of the associated ANNs (so that the decision rules are not easily interpreted by the scientist) and the high demands on computer performance to evolve the appropriate ANNs by the GA.

MODELS OF PLANKTON PATCHINESS GENERATED BY POPULATION DYNAMICS INTERACTIONS

It is well recognized that spatial and temporal variability – patches and population blooms – are not merely noise but essential features of food web dynamics. This variability ensures adequate feeding rates and reproduction, and is important in determining the fate of natural populations of zooplankton and ichthyoplankton in a dynamic physical environment. In the sea, continuous spatial records are obtained from ships, and spectral analysis is used to define the biological patterns and compare them with physical observations. Patchiness in plankton occurs in the presence or absence of marked physical patterns.

Steele and Henderson (1992) simulated phytoplankton–zooplankton patchiness in the horizontal dimension, generated by non-linearities in a simple phytoplankton–zooplankton interaction model. The variable parameters were the mortality of herbivores with a white-noise stochastic forcing in the spatial domain and the eddy diffusion

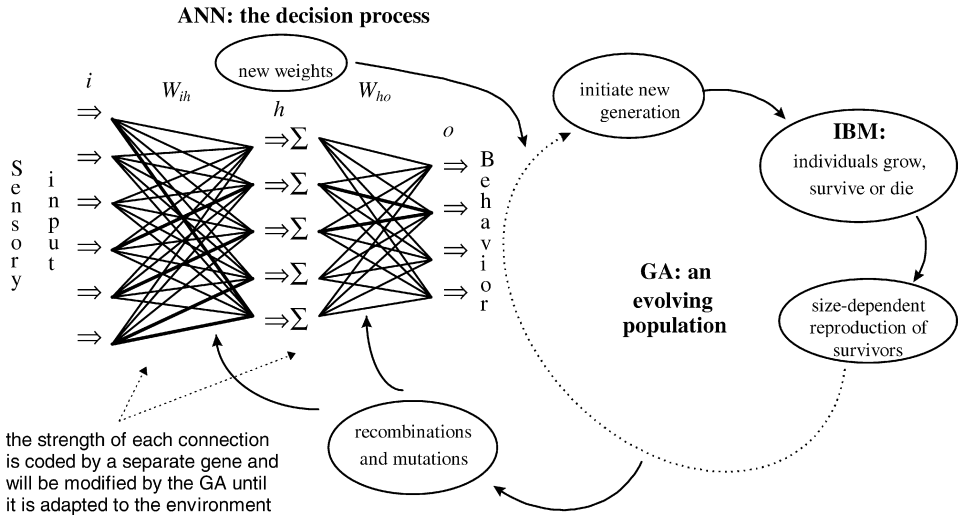

Fig 12.3. The ING concept: one or more artificial neural networks (ANN) code for the decision processes utilized in an individual-based model (IBM). A cohort of individuals with randomly set individual strings coding for the weighting of the ANN are initiated in the first time step. The strings are then adapted using the genetic algorithm (GA) through the principle of evolution by natural selection. Differences among the strings are expressed through differences in weights of the ANN, which leads to behavioral differences. The *i*, *h*, and *o* refer to input, hidden, and output layers respectively. The input layer consists of internal or environmental input. The output nodes fire if their values are above certain threshold values. The lines indicate the relationships among the different layers. Each of the nodes in the *i*-layer are connected to all the nodes in the *h*-layer which again are connected to all the nodes in the *o*-layer. W_{ih} and W_{ho} are the weight matrices of connection strengths between the nodes of the layers. Potential weight differences are indicated by the variation in line thickness of the connections. The GA will evolve the 'gene pool' to contain the best possible response to the combinations of sensory inputs received throughout the lives of the individuals, as measured by the fecundity that results from the IBM given a particular ANN. Modified from Giske *et al.* (1998a).

coefficient. Other examples are given by Nisbet *et al.* (1993) and Rothschild and Ault (1996).

GRID-BASED MODELS

Tischendorf (1997) gave a short review of modeling methods for spatially explicit simulations in heterogeneous landscapes. Grid-based models are starting to be used in spatial zooplankton dynamics, and are mostly associated with new modeling approaches of individual movements. Grids divide a continuous two-dimensional space into discrete units of equal size and shape, i.e. cells. Each cell can easily be selected by indices because of its defined position in a matrix. By this arrangement, cells relate descriptive information (e.g. state variables and transition rules) both to each other (by fixed neighborhood relationships) and to the area they cover. Movements of individuals are commonly expressed by rules that either assign individuals to other cells or change

cumulative cell state variables that describe a spatial class of individuals. Such movement rules can be influenced by landscape features associated with cells. Object-oriented modeling of individual movements uses grids to represent space (e.g. Laval 1997). Cellular automata represent such a class of models for population interactions in space (Phipps 1992; Caswell and Etter 1993), but there are very few examples in zooplankton ecology (Delgado and Marin 1998).

12.6.3 Coupling IBMs and spatially explicit models

PASSIVE PARTICLE TRAJECTORIES FROM LAGRANGIAN TRANSPORT IN MODEL CIRCULATION FIELDS

This approach uses simulated currents from sophisticated 3-D hydrodynamic models driving Lagrangian models of particle trajectories to examine dispersion processes.

The approach is relatively straightforward and is a first step in formulating spatially explicit individual based models (IBMs). Given a 'properly resolved' flow field, particle (larval fish/zooplankton/meroplanktonic larvae) trajectories are computed (generally with standard Runge–Kutta integration methods of the velocity field). Specifically, hydrodynamic models (Box 12.4) provide the velocity vector $\boldsymbol{v} = (u, v, w)$ as a function of location $\boldsymbol{x} = (x, y, z)$ and time t and the particle trajectories are obtained from the integration of

$$d\boldsymbol{x}/dt = \boldsymbol{v}(\boldsymbol{x}, \boldsymbol{y}, \boldsymbol{z}, \boldsymbol{t}). \tag{12.104}$$

These trajectories could be modified by turbulent dispersion as described below. Once the larval/particle position is known, additional local physical variables can be estimated along the particle's path, for example temperature, turbulence, light, etc., and input to the IBM (see section 12.4.3). The physical quantities are then included in biological, for example physiological or behavioral, formulations of IBMs (see section 12.4.3).

In general, flows can be three-dimensional, baroclinic and time-dependent. Driving forces are the winds, tides, atmospheric heating and cooling and prescribed inflows. Estimates of local turbulence levels are derived from advanced turbulence closure models. The simulations can become quite intensive computationally and the model output of the physical model can be large if 3-D physical data is saved on the order of every hour.

Simulations considering trajectories of plankton as passive particles are a necessary step before considering any active swimming capability of planktonic organisms. They show the importance of physical features in the aggregation or dispersion of the particles (see as examples Ishizaka and Hofmann 1988; Oliver *et al*. 1992; Werner *et al*. 1993).

Effect of turbulence on dispersion

Small scale turbulence can be represented in various ways. Some Lagrangian models include a stochastic term to mimic diffusive processes (e.g. Walsh *et al*. 1981). Others use a diffusion coefficient using a random walk procedure as in Black and Gay (1990).

Werner *et al*. (1996) and Hannah *et al*. (1998) present a more complex representation. The dispersal of particles in heterogeneous turbulent fields (e.g. near or within turbulent boundary layers) can lead to aggregations that are not realistic if the dispersal process is not treated carefully (e.g. Thompson 1987; and Holloway 1994). Legg and Raupach (1982) proposed a Langevin equation to derive a Markov equation for the vertical velocity of a particle (or zooplankton organism) in a flow where the turbulence is heterogeneous.

Box 12.4 The hydrodynamical model equations

Acceleration in x direction:

$$\frac{\partial u}{\partial t} = fv - u\frac{\partial u}{\partial x} - v\frac{\partial u}{\partial y} - w\frac{\partial u}{\partial z} - \frac{1}{\rho}\frac{\partial p}{\partial x} + A_v\nabla^2 u + \frac{\partial}{\partial z}A_v\frac{\partial u}{\partial x}. \quad (1)$$

Acceleration in y direction:

$$\frac{\partial v}{\partial t} = fuv - u\frac{\partial v}{\partial x} - v\frac{\partial v}{\partial y} - w\frac{\partial v}{\partial z} - \frac{1}{\rho}\frac{\partial p}{\partial y} + A_h\nabla^2 v + \frac{\partial}{\partial z}A_v\frac{\partial v}{\partial x}. \quad (2)$$

Vertical velocity is found from the equation of continuity:

$$0 = \frac{\partial w}{\partial z} + \frac{\partial u}{\partial x} + \frac{\partial v}{\partial y} \quad (3)$$

and surface elevation

$$h = \int w_1 dt \quad (4)$$

where

u, v horizontal velocity components in x and y direction, respectively
w vertical velocity component
w_1 vertical velocity of surface elevation, i.e. upper layer
h height of the free surface from the undisturbed mean
f Coriolis parameter
ρ density
A_h horizontal eddy diffusion of momentum
A_v vertical eddy diffusion of momentum
p pressure found by the hydrostatic equation

$$p = \int_z^h rgdz + P_a \quad (5)$$

where P_a is the atmospheric pressure.

The space–time variations of water density ρ are found by the functional relationship

$$\rho = \rho(S, T) \quad (6)$$

where S and T are the salinity and temperature of the water, respectively. These scalar fields can be modeled by a balance equation of the form

$$\frac{\partial c}{\partial t} = \frac{\partial}{\partial x}(uc) - \frac{\partial}{\partial y}(vc) - \frac{\partial}{\partial z}(wc) + K_h\nabla^2 c + \frac{\partial}{\partial z}\left(K_v\frac{\partial c}{\partial z}\right) + \delta_c \quad (7)$$

where

c S or T
K_h horizontal eddy diffusion of salinity and temperature
K_v vertical eddy diffusion of salinity and temperature
δ_c thermodynamic interaction between the upper layer and the atmosphere ($c = T$) or supply of salt and fresh water during freezing or melting respectively ($c = S$) and the operator ∇^{-2} means

$$\nabla^2\Theta = \frac{\partial^2\Theta}{\partial x^2} + \frac{\partial^2\Theta}{\partial y^2} \quad (8)$$

where Θ is any function of x and y.

Vertical mixing is calculated as a function of the Richardsons number, wave height and parametrization of tidal mixing in shallow areas. (From Giske *et al.* 1998b.)

The Langevin equation for the dispersion of particles is:

$$dw/dt = -\alpha w + \lambda\xi(t) + F \tag{12.105}$$

where $\alpha = 1/\tau_1$ and τ_1 is the Lagrangian integral time scale (or auto-correlation time scale) estimated from $N_q = \sigma_w^2 \tau_l$, where N_q is the turbulent exchange coefficient (see Galperin *et al.* 1988), σ_w is the Lagrangian velocity variance ($\sigma_w^2 = 0.3q^2/2$); $\lambda = \sigma_w^2\sqrt{2/\tau_1}$; $\xi(t)$ is Gaussian noise of zero mean and unit variance; and $F = \partial(\sigma_w^2)/\partial z$ is a term involving the gradient in the turbulent velocity variance.

The Markov chain for w_{n+1}, the turbulent vertical velocity at time step $n + 1$, becomes:

$$w_{n+1} = a_n w_n + b_n \sigma_{wn} \xi_n + C_n \tag{12.106}$$

where

$$a_n = \exp(-a_n/\tau_{1n}),\ b_n = [1 - \exp(-2\Delta t/\tau_{1n})]^{1/2} \tag{12.107}$$

$$C_n = (F/\alpha)[1 - \exp(-\Delta t/\tau_{1n})] \tag{12.108}$$

and Δt is the time step.

This approach was used in the studies of Hannah *et al.* (1998) wherein potential upper-ocean pathways for the supply of *Calanus finmarchicus* from the Gulf of Maine to Georges Bank were investigated by numerically tracking particles in realistic 3-D seasonal-mean and tidal flow fields. Hannah *et al.* (1998) found that upper-ocean drift pathways for biota in the southern Gulf of Maine are strongly sensitive to biological and/or physical processes (including turbulent dispersion) that affect vertical position (in relation to the surface Ekman layer) and horizontal position (in relation to topographic gyres).

TRAJECTORIES OF ACTIVELY SWIMMING PARTICLES FROM LAGRANGIAN TRANSPORT IN MODEL CIRCULATION FIELDS

Plankton transport models that include biological components typically use a prescribed vertical migration strategy for all or part of an animal's life history or a vertical motion (sinking or swimming) that is determined by animal development and growth (Hofmann *et al.* 1992; Werner *et al.* 1993; Verdier *et al.* 1997). The simulated plankton distributions from these models tend to compare better with observed distributions than models that use passive particles. Sensitivity studies show that behavior is an important factor in determining larval transport and/or retention.

Biological and physical models can be coupled in the following way. First, the physical model can be run with a particular physical scenario (winds, boundary conditions, etc.), then the biological model can be used. Organisms, considered as particles, can be tracked in the 3-D velocity field calculated by the physical model using a standard Euler or Runge–Kutta integration:

$$dx/dt = v_a(x, y, z, t) + v_b \tag{12.109}$$

where v_a is the velocity vector from the circulation model as a function of location $x = (x, y, z)$, t is time and v_b is the swimming speed of the organism depending on prescribed or dynamical biological properties. Hofmann *et al.* (1992) and Capella *et al.* (1992) present a time- and temperature-dependent model combining temperature effects on development times, physiology and density of krill embryos and larval sinking and ascending rates. Verdier *et al.* (1997) consider a prescribed age-dependent vertical swimming velocity of annelid larvae.

The coupling of IBMs of zooplankton and 3-D circulation models is a recent field of study, even for fish models (Tyler and Rose 1994). Generally, models that describe the spatial heterogeneity of the habitat have been designed to answer questions about the spatial distribution of a population rather than questions about the numbers and characteristics of surviving individuals. The simplest biological representation concerns the swimming ability of the planktonic organism. As questions concerning biological aspects of dispersion receive increasing attention through programs like GLOBEC, the number of models with biological detail (see section 12.4.3) is likely to increase.

The dispersion of several species on the Georges Bank has been studied recently (US-GLOBEC Georges Bank program): a *Calanus finmarchicus* model (Miller *et al*. 1998), a larval fish model applied to haddock and cod (Werner *et al*. 1996), and a model of scallop larvae (Tremblay *et al*. 1994). They all use the three-dimensional hydrodynamic model developed by Lynch and collaborators (Lynch *et al*. 1992, 1996). Other spatially explicit IBMs for fish larvae have been developed (see Tyler and Rose 1994 for a review; Hinckley *et al*. 1996). Verdier *et al*. (1997) presented a spatially explicit IBM for meroplanktonic larvae in the Bay of Banyuls.

Tyler and Rose (1994) emphasized that models that incorporate individual-based formulations and physical habitat modeling techniques not only increase our understanding of the link between spatial and temporal dynamics of zooplankton and fish populations, but also allow us to explore the potential effects of habitat alteration on these populations. Using this approach, biological mechanisms that are strongly dependent on habitat and that are not fully understood could be studied by examining different scenarios. For example, Moloney and Gibbons (1996) used an IBM of herbivorous zooplankton coupled with simplified 1-D profiles of food and temperature to study the effects of different scenarios of diel vertical migration on daily ingestion.

12.6.4 Spatial zooplanton dynamics with advection-diffusion-reaction equations (ADRE)

The general biological–physical model equation used to describe the interaction between physical mixing and biology is:

$$\frac{\partial \boldsymbol{C}}{\partial t} + \nabla \cdot (\boldsymbol{v_a C}) - \nabla \cdot (\boldsymbol{K} \nabla \boldsymbol{C}) = \textbf{‘biological terms’} \tag{12.110}$$

where $C(x, y, z, t)$ is the concentration of the biological variable which is either a functional group (zooplankton), a species, or a developmental stage (in which case the number of equations would equal the number of stages) at position x, y, z at time t. The concentration can be expressed as numbers of organisms or biomass of organisms per unit volume, and

- $v_a(u_a, v_a, w_a)$ represents the advective fluid velocities in x, y, z directions;
- K_x, K_y, K_z are diffusivities in x, y, z directions;
- $\nabla = (\partial/\partial x, \partial/\partial y, \partial/\partial z)$ is the Laplacian operator.

On the left hand side of equation 12.110, the first term is the local change of C, the second term is advection caused by water currents, and the third term is the diffusion or redistribution term. The right hand side of equation 12.110 has the biological terms that

represent the sources and sinks of the biological variable at position x, y, z as a function of time.

The biological terms may or may not include a velocity component (swimming of organisms, migrations, sinking, ...) and the complexity of the biological representation can vary from the dispersion of one variable (the zooplankton biomass, or the concentration of a cohort) in NPZ ecosystem models (see section 12.5) to detailed population dynamics with stage-structured populations. The following sections give examples of passive and active dispersion of zooplankton biomass or targeted populations.

The numerical methods used to solve ADREs are finite difference techniques (see for instance Sewell 1988). Examples given by Okubo (1980, chapter 6) include solution methods for ADREs.

MODELING PASSIVE DISPERSION WITH ADREs

There are several examples of models of passive dispersion of planktonic organisms. The spatial dependence is usually reduced from three to two or one dimension depending on the question being asked, and the equations are solved analytically or numerically. However, the simultaneous partial differential equations that model such systems can be solved analytically only under very special circumstances, depending on the assumptions regarding the boundary conditions and on the functional forms used in the model. Results of analytical solutions are of interest because they can delimit parameter space for simple situations, and also because they can serve as a useful check to the solutions obtained by numerical methods.

Sundby (1983, 1991) presented a simple model of vertical egg distribution as a function of the properties of the water (density, current and turbulent diffusion) and physical properties of the eggs (buoyancy and dimension). The basic equation 12.110 is then reduced to the vertical component of the diffusion equation:

$$\frac{\partial C(z,t)}{\partial t} - \frac{\partial}{\partial z}\left[K(z,t)\frac{\partial C(z,t)}{\partial z}\right] = \textbf{mortality} + \textbf{spawning} - \frac{\partial[w_b(z,t)C(z,t)]}{\partial z} \tag{12.111}$$

where $C(z,t)$ is the concentration of eggs in numbers per unit volume, $K(z,t)$ is the vertical eddy diffusivity coefficient, and z is the vertical coordinate.

The biological terms are reduced to the mortality of eggs, the input from spawning, and the sinking of eggs with $w_b(z,t)$ as the vertical velocity of the eggs.

To solve equation 12.111, $w_b(z,t)$ and $K(z,t)$ must be known. The vertical velocity is expressed as $w_b = f(d, \Delta\rho, v)$, where d is the diameter of the egg, $\Delta\rho$ is the difference in density (buoyancy) between the egg, ρ_e , and the ambient water, ρ_w, and v is the viscosity of the water. Sundby presented the solutions in steady-state situations (first term equal to zero) and with an equal value for mortality and spawning.

To study the passive settlement of planktonic larvae onto bottoms of different roughness, Eckman (1990) and Gross *et al.* (1992) developed 1-D advection-diffusion models with particular attention to the effect of the turbulent boundary layers. The equations were solved with a finite-difference technique.

Hill (1990) presented a model of pelagic dispersion of lobster larvae in the horizontal plane. The model is two-dimensional in space and the concentration of larvae is depth-averaged. Current and turbulence fields are also considered as depth-averaged values.

The concentration $C(x, y, t)$ of larvae at a position x, y at time t, is governed by the following advection-diffusion equation:

$$\frac{\partial C(x,y,t)}{\partial t} + \frac{\partial [u_a(x,y,t)C(x,y,t)]}{\partial x} + \frac{\partial [v_a(x,y,t)C(x,y,t)]}{\partial y} - \frac{\partial}{\partial x}\left[K(x,t)\frac{\partial C(z,t)}{\partial x}\right] - \frac{\partial}{\partial y}\left[K(y,t)\frac{\partial C(y,t)}{\partial y}\right] = mC(x,y,t) \tag{12.112}$$

where $u_a(x, y, t)$ and $v_a(x, y, t)$ are depth averaged water velocities in the x and y directions respectively, and $K(x, t)$ and $K(y, t)$ are the turbulent diffusion coefficients in the x and y directions. In his model, Hill (1990) assumed a constant current speed u_a in the x direction, no current in the y direction ($v_a = 0$), and constant and equal turbulent diffusion in both x and y directions. The biological term represented reduction of larval concentration because of mortality of organisms at an instantaneous mortality rate, m.

With the same assumptions, Hill (1990, 1991) studied the relative contributions of turbulent diffusion and advection, as well as mortality and dispersal, to the dispersion of the larvae. He used analytical solutions of simplified versions of equation 12.112, corresponding to idealized configurations.

Similar models have been used to investigate the advection, diffusion, and mortality of Pacific herring larvae (McGurk 1989), the movements of larvae released from a well-defined region into a tidal current (Richards *et al.* 1995), and the dispersal and recruitment of a larval population of barnacles in a coastal habitat (Possingham and Roughgarden 1990). Possingham and Roughgarden (1990) presented, in detail, the use of finite difference methods to solve numerically the differential equations describing the dynamics of the distribution and abundance of adult and larval barnacles.

Hofmann (1988) presented a two-dimensional spatially-dependent advection-diffusion model of biological variables of the pelagic ecosystem on the outer southeastern US continental shelf. The dynamics of the copepod *Paracalanus* sp. was taken into account in this model (Hofmann and Ambler 1988).

MODELING ACTIVE VERTICAL SWIMMING WITH ADREs

The swimming behavior and/or buoyancy effects of organisms are included in equation 12.110 by the addition of a Laplacian term $\nabla(v_b C)$ for the swimming velocities, with $v_b(u_b, v_b, w_b)$ representing the swimming velocities in x, y, z directions. However for zooplankton organisms (not for fish larvae) the horizontal effect is negligible ($u_b = v_b = 0$).

Dekshenieks *et al.* (1996) presented a vertically structured and time-dependent model to investigate the effects of changes in the physical environment on the vertical distribution of oyster larvae, as determined by vertical stratification in temperature and salinity. The vertical distribution of a given size class, C_i, was assumed to be governed by:

$$\frac{\partial C_i(z,t)}{\partial t} + \frac{\partial [w_a(z,t)C_i(z,t)]}{\partial z} - \frac{\partial}{\partial z}\left[K(z,t)\frac{\partial C_i(z,t)}{\partial z}\right] = \mathbf{growth}_{i-1} - \mathbf{loss}_i - \frac{\partial [w_b(z,t)C_i(z,t)]}{\partial z}. \tag{12.113}$$

The first term on the left hand side represents the rate of change of the number of larvae in a particular size class i in a size-structured population model. The second term

represents the effect of advective vertical transport by the fluid flow. The value of w_a is specified as a constant velocity or as a function that varies in space and time. The coefficient K determines the rate of vertical diffusion and may be specified as either a constant or a variable value. The first two terms on the right hand side represent the biological processes that determine the rate of transfer to the next largest size. The number of larvae in a particular size class changes by growth of new individuals from the previous size class (growth_{i-1}), and the loss of individuals to the next largest size (loss_i) (see weight-structured models on page 600). Larval growth was modeled for the whole life cycle as a function of ambient temperature, salinity, turbidity and food, and the size range was partitioned into 271 size classes.

Vertical migration is represented by the last term on the right hand side of equation 12.113. Larval swimming ability w_b is parameterized using observed dependencies on temperature, salinity and larval size. Therefore, the total advective velocity, w, is composed of contributions from the vertical circulation w_a, and the size-dependent biologically produced vertical movement (sinking or swimming) w_b, with $w = w_a + w_b$.

The addition of biological terms makes equation 12.113 complex, and analytical solutions are difficult to extract without simplifications. As a consequence, such equations are solved numerically. Dekshenieks *et al*. (1996) used a Crank–Nicholson implicit finite difference scheme (see Sewell 1988) with a time step of 12 min, which was adequate to resolve the transfer of larvae between size classes.

A similar approach was used by Andersen and Nival (1991) and Richards *et al*. (1996) to simulate the vertical distribution of zooplankton (euphausiids and copepods respectively) that vary in space and time as a result of diel vertical migration. They studied the role of light intensity, the rate of change in light intensity, and the relative rate of change in light intensity. The equation they used is the same as equation 12.113, except that there are no biological processes affecting the biomass or the number of organisms; the vertical speed of the organisms, w_b, only depends on light. These authors present in detail in their papers the numerical methods that they used.

Studies of the distribution of organisms in regions where zooplankton may be aggregated (e.g. upwelling and downwelling regions, Langmuir circulations, internal waves) are usually undertaken with 2-D models. Wroblewski (1980, 1982) modeled population dynamics (with a stage-structured population model) of copepod species embedded in a circulation system simulating the upwelling off the Oregon coast. Simulations of the dynamics of *Calanus marshallae* (Wroblewski 1982) focused on the interaction between diel vertical migration and offshore surface transport.

The zonal distribution of the life-stage categories C_i of *C. marshallae* over the Oregon continental shelf was modeled by the two-dimensional (x, z, t) equation:

$$\begin{aligned}\frac{\partial C_i(x,z,t)}{\partial t} &- \frac{\partial[u_a(x,z,t)C_i(x,z,t)}{\partial x} - \frac{\partial[w_a(x,z,t)C_i(x,z,t)]}{\partial z} \\ &- \frac{\partial}{\partial x}\left[K(x,t)\frac{\partial C_i(x,z,t)}{\partial x}\right] - \frac{\partial}{\partial z}\left[K(z,t)\frac{\partial C_i(x,z,t)}{\partial z}\right] \\ &= \textbf{population dynamics} + \frac{\partial[w_{bi}(x,z,t)C_i(x,z,t)]}{\partial z}\end{aligned} \tag{12.114}$$

where w_{bi} is the vertical swimming speed of the ith stage, assumed to be a sinusoidal function of time:

$$w_{bi} = w_{si}\sin(2\pi t) \tag{12.115}$$

with w_{si} the maximum vertical migration speed of the ith stage. The population dynamics model was presented on page 602.

The upwelling zone extended 50 km from the coast down to a depth of 50 m, and was divided into a grid with spacing 2.5 m in depth and 1 km in the horizontal. The author used a finite difference scheme with a time step of 1 h, which fell within the bounds for computational stability.

MODELING THE DISPERSION OF A POPULATION IN CIRCULATION MODELS WITH ADREs

An important development in zooplankton modeling is to make full use of the increased power of computers to simulate the dynamics of zooplankton (communities or populations) in site-specific situations by coupling biological and transport models, giving a high degree of realism. Structured population models and individual-based models allow detailed simulations of zooplankton populations in different environmental conditions.

In the last decade, some efforts have been made to develop such physical–biological models. The current development of advanced circulation models will strongly increase the number of such studies.

Davis (1984b) developed a species-specific model for *Pseudocalanus* sp. incorporating 13 stages in an idealized steady gyre around the Georges Bank. Equation 12.110 was written as:

$$\frac{\partial N_{i,j}}{\partial t} + \frac{2\pi}{T_r}\frac{\partial N_{i,j}}{\partial \theta} - \frac{K}{r^2}\frac{\partial^2 N_{i,j}}{\partial \theta^2} = \textbf{population dynamics} \qquad (12.115)$$

where $i = 1$ to 13 for all developmental stages, K is the horizontal coefficient of eddy diffusivity, r is the radial distance from the center of the bank, and θ is the angular coordinate.

The equation can be solved analytically without biological terms, but the addition of the biological term (see Continuous-time structured population methods, section 12.4.2) makes the analytical solution intractable, and a numerical solution of the complete biological–physical model was required. Advection and diffusion were approximated as centered difference terms and were solved numerically using the improved Euler method.

Recent progress has been made in the representation of the physical part of Davis's (1984b) model. Lewis *et al*. (1994) investigated the role of wind variability on the dispersion of a copepod population, using an advanced 3-D physical model of Georges Bank without tides. Currently the simulations presented by Lynch *et al*. (1998) are probably the most sophisticated example of the dispersion and dynamics of a zooplankton population in a site-specific study (Georges Bank). Equation 12.110 was reduced to the horizontal dimension making assumptions about the vertical distribution of organisms. Fluid velocities in the horizontal plane were derived from a hydrodynamical model (Lynch *et al*. 1996). The transport equations were solved using a Galerkin method (Sewell 1988). The population dynamics were represented by a stage- and age-structured population dynamics model similar to the model presented by Davis (1984a; see page 600 for details) with biological parameters relevant for *Calanus finmarchicus*. Lynch *et al*.'s (1998) model results gave a credible scenario for the initiation of the spring *Calanus* bloom by simulating the emergence of copepodites at the right time and place in comparison to observations.

12.6.5 Spatial distribution of zooplankton in ecosystem models coupled with ADREs

Physical–biological models of various levels of sophistication have been developed recently for different regions of the ocean. An overview of many marine interdisciplinary models is given in Wroblewski and Hofmann (1989), Hofmann (1993) and Hofmann and Lascara (1998).

Biological models were configured first as compartmental ecosystem models in an upper ocean mixed-layer (e.g. Fasham *et al*. 1990). Zooplankton can be represented by one variable (as in NPZ models) or more (e.g. by distinguishing mesozooplankton and microzooplankton). In this second case, the model generally takes into account several size classes of phytoplankton. This class of ecosystem model has been coupled to one-dimensional physical models (McGillicuddy *et al*. 1995a; Prunet *et al*. 1996a, 1996b; Oguz *et al*. 1996), and embedded into two-dimensional (Klein and Steele 1985) and three-dimensional circulation models (Fasham *et al*. 1993; Sarmiento *et al*. 1993; McGillicuddy *et al*. 1995b; Moisan *et al*. 1996; Levy *et al*. 1998).

As emphasized by Spitz *et al*. (1998), one of the difficulties with these models is obtaining an estimate of parameters. A systematic and non-subjective technique of adjusting the model parameters consists of using observations in conjunction with a data assimilation technique (Armstrong *et al*. 1995; Matear 1995; Lawson *et al*. 1995, 1996; Prunet *et al*. 1996b). A second difficulty is the presentation and interpretation of results. Ecosystem models are complex, and when biological effects are combined with physical effects in a 3-D environment, a large amount of information is produced. This information results from non-trivial interactions and dynamics, and there is much scope for developing innovative ways to summarize or integrate this information into a meaningful form.

12.6.6 Further reading

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