Perumal Santhanam · Ajima Begum
Perumal Pachiappan *Editors*

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Preface

Zooplankton (In Greek: Zoon – animal; Planktos – wanderer/drifter) are minute, mostly microscopic drifting creatures mainly inhabiting the surface layers of aquatic ecosystems like seas, oceans, lakes, rivers, and ponds. The zooplankton along with phytoplankton forms the basic tiers of aquatic food chains and thus feeding the higher tropic organisms like fishes. The zooplankton comprises of wide range of organisms that are classified based on their size, depth, and geography of distribution and mainly in relation to their life cycle. While the holoplanktonic zooplankton lead their entire life as planktonic-mode of life (e.g., radiolarians), the meroplanktonic forms spend only their early stage of life as "plankton" and later stage as "nekton-swimmers" (e.g., eggs and larvae of fish). Their primary sources of food include bacterioplankton, phytoplankton, marine snow (detritus), and also some smaller zooplankton. Therefore, they are the primary consumers or secondary producers and hence form a vital link in the aquatic food web. Their distribution, abundance, and seasonal changes have a major impact on the annual fishery productivity/yield across the world's oceans. During the death and decay of these creatures, the carbon containing organic material sinks down onto deep oceans and thus they play an important role in carbon cycle.

The copepods, a major constituent of zooplankton population, are a potentially reliable source of protein to most of the economically important wild as well as aquacultured fishes. They are of a highly evolved and successful animal group in relation to the phylogenetic age, number of living species coupled with successful adaptive radiation. In view of their sheer abundance, diversity, and nutritional superiority, they are popularly called as "insects of the sea" and "living capsules." Millions of these tiny creatures produce countless fecal pellets that contribute to the snow formation and consequent nutrients and minerals flow to the surface waters. The study of the ecology of copepods/zooplankton would provide data – index of the fisheries potential of aquatic ecosystems. Some zooplankton, in view of their distribution in restricted areas, are considered to be "indicators of water masses." Moreover, these largely untapped microorganisms provide scope for prospecting for various medicines, cosmetics, and oils besides solution for sustainable fisheries and aquaculture.

This book documents the current state of the art of features of basic and applied aspects of zooplankton. The topics cover various aspects of zooplankton like ecology, economy, morphological, and molecular taxonomy of zooplankton. And also, culture and preservation of copepod eggs and the enzymatic/mosquitocidal property of copepods. Bioenvironment and pigments of copepods and their suitability as feed for larvae of fin fishes and shellfishes besides the biology of Artemia are the other aspects.

It is hoped that this book would be immensely useful to the postgraduate and research students of Marine Biology, Aquatic Biotechnology, Animal Science, and Fishery Science. The editors thank all the contributors and publishers.

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About the Editors

Dr. P. Santhanam is an assistant professor at the Department of Marine Science, School of Marine Sciences, Bharathidasan University, Tiruchirappalli, Tamil Nadu, India. He obtained his M.Sc, M. Phil and Ph.D Degrees in Marine Biology, from the C.A.S. in Marine Biology, Annamalai University. He has over 19 years of research and 11 years of teaching experience with over 130 publications in national and internationally respected journals, books and proceedings. He specializes in the areas of marine planktonology & aquaculture with reference to biodiversity, taxonomy, biology and culture of phyto and zooplankton for fish larviculture. He has successfully completed 5 major research projects funded by UGC, DST, DBT & SERB (Govt. of India) and is currently involved in 2 major research projects funded by the UGC & MoEF& CC (Govt. of India). He is a member in several scientific bodies and has served as an editorial board member for over 5 journals and as a reviewer for over 25 leading national and international journals. He received the DST Young Scientist Award in 2007, KTSTM Trust Best Researcher Award, and the Prof. Dr. M. Aruchami Award from Kongunadu Arts & Science College & Dr. M. Aruchami Research Foundation for his extraordinary contribution to Indian live-feed aquaculture, especially copepod. He has been expert-member of various committees.

Mrs. Ajima Begum is currently an assistant professor at the Department of Botany, Tihu College, Tihu, Nalbari, Assam, India. Before joining in Tihu College, she worked as an assistant professor at B. N. College, Dhubri, Assam. She also served as a research investigator at NIRD-NERC, in the project entitled "Training-cum-Documentation for Establishing Rural Industries of Medicinal and Aromatic plants in North Eastern Region". She has published over 10 research articles in leading national and international journals and participated in more than 6 national and international level trainings, conferences and seminars.

Dr. P. Pachiappan is a professor & head of the Department of Biotechnology, Periyar University, Salem, Tamil Nadu, India. Prior to his appointment at Periyar University, he was a faculty research associate, lecturer, reader and professor at the C.A.S. in Marine Biology, Annamalai University, where he received his Ph. D., Degree in 1989. He is also passed the UGC-National Educational Test in 1985. He has over 30 years of research experience with about 150 publications to his credit. His research interests include molecular taxonomy and live-feed aspects of plankton, fish larviculture and antibiotic principles/drugs from marine bioresources. He has successfully completed many major research projects funded by ICAR, MoES, DRDO, UGC (Govt. of India) and is currently co-ordinating the DST-FIST programme. He is a member of the Academic Council, Senate, Board of Research Studies, chairman of PG-Board of Studies in Biotechnology & Bioinformatics (of Periyar University) and a member of the Board of Studies at various colleges and universities. He was the chairman of the Periyar University Institutional Animals Ethical Committee & co-ordinator of the Periyar University Patent Facilitation Centre & Industrial MOU, an expert-member of the European Commission (EC) Projects Review committee, scientist member of Govt. Mohan Kumaramangalam Medical College Institutional Animal Ethics committee and Scientific Advisory committees. He is also a reviewer/editor for several national & international journals.

A Method of Collection, Preservation and Identification of Marine Zooplankton

P. Santhanam, P. Pachiappan, and A. Begum

Introduction

The name plankton originates from the Greek which means wandering or drifting. Victor Hensen coined the term 'plankton' in 1887. Plankton denotes a group of organisms—either animals or plants—which passively float and drift with the mercy of water currents, tides and waves. The animal component of the plankton is called zooplankton. It plays an important and probably the most significant role in aquatic productivity, determining the future commercial fishery of an area (Santhanam and Srinivasan 1994; Perumal et al. 1998; Perumal and Sampathkumar 2002). They form a vital link in any aquatic food web as primary consumers or secondary producers.

World total fishery production has been reported as 167.2 million tonnes in 2014 (FAO 2016), which including all aquatic organisms was used for human consumption or other commercial activity. This great and chief protein source for human beings has been practically supported and sustained by small food organisms present in the coastal and marine ecosystems, which cannot be neglected in the fisheries studies. Among these, 'zooplankton' deserves special attention, as all the aquatic higher organisms invariably require these smaller organisms at least during their early developmental stages (Kasturirangan 1963; Santhanam and Perumal 2008).

The zooplankton is multicellular animals, which have more species but fewer individuals. It is a diverse group of animals occurring both in marine and estuarine

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waters (Benfield et al. 2007). The distribution and abundance of the zooplankton are mainly influenced by the hydrographic conditions specific to the locality. Investigation on the species composition, population density and diversity of the zooplankton is necessary to assess the fishery potential of marine and estuarine ecosystems. Nowadays, the researchers are showing interests to work on biotechnological and other applied aspects of marine flora and fauna. The cumulative result is a lack for conventional taxonomist. The systematics and taxonomy of zooplankton for ecosystem health assessment and applied research are highly significant. In this context, this paper would be helpful for the researchers to learn more about systematics and taxonomy of marine and brackish-water zooplankton for ecological monitoring of any ecosystem.

Classification of Zooplankton

Based on protein content, the marine plankton can be classified as phytoplankton (plant plankton) and zooplankton (animal plankton). Zooplankton may be classified according to their habitat, depth-wise distribution, size and life cycle (Omori and Ikeda 1984). On the basis of habitat, the zooplankton is classified as marine plankton or haliplankton and freshwater plankton or limnoplankton. Based on habitat, the marine plankton is further divided as follows:

Oceanic plankton	Plankters inhabiting waters beyond continental shelves
Neritic plankton	Plankters inhabiting waters overlying continental shelves
Brackish-water plankton	Plankters inhabiting brackish-water areas such as estuaries, mangroves, lagoons, etc.

Based on the depth-wise distribution, zooplankton is classified as follows:

Pleuston	Living at surface of the sea
Neuston	Living in uppermost few to tens of mm of the surface
Epipelagic plankton	Living between 0 and 300 m
–Upper epiplankton	Living between 0 and 150 m
–Lower epiplankton	Living between 150 and 300 m
Mesopelagic plankton	Living between 300 and 1000 m
–Upper mesoplankton	Living between 300 and 700 m
–Lower mesoplankton	Living between 700 and 1000 m
Bathypelagic plankton	Living between 1000 and 3000 m
Abyssopelagic plankton	Living between 3000 and 4000 m
Epibenthic plankton	Living at the bottom

Based on size, different types of zooplankton have been recognized as follows:

Femtoplankton	0.02–0.2 μm (e.g. marine viruses)
Picoplankton	0.2–2.0 μm (e.g. Chrysophyta)

Nannoplankton	2–20 μm (e.g. diatoms, dinoflagellates)
Microzooplankton	20 μm−200 μm (e.g. rotifers, copepod nauplii)
Mesozooplankton	200 μm–2 mm (e.g. copepods, Cladocera, Chaetognatha)
Macrozooplankton	2–20 mm (e.g. ctenophores, salps, doliolids)
Megalozooplankton	>20 mm (e.g. jellyfish)

Based on life cycle, the zooplankton may be classified into two types, namely, holoplankton and meroplankton.

Holoplankton	The organisms which are planktonic throughout their life cycle that include tintinnids, copepods, chaetognaths and cladocerans
Meroplankton	The organisms which only for a part of their life act as plankton (e.g. fish larvae, veliger larvae)

Methods of Collection

There are three different types of plankton hauling such as horizontal hauling, vertical hauling and oblique hauling being followed for the collection of epipelagic, mesopelagic and bathypelagic plankton, respectively (Goswami 2004; Mitra et al. 2004; Santhanam and Perumal 2008).

Pump and Hose

Pump on Deck

An electrically operated rotary pump or centrifugal pump with attached flexible inlet and outlet rubber or a plastic hose pipe is suitable. The inlet hose is marked off in meters in order to read off the depth. A weight is attached near the open end of the inlet hose to make sure that the hose descends vertically when sent down. The pump may be used to constantly suck seawater with plankton into the vessel. The pumped water will be filtered through plankton mesh on deck.

Submersible Pump

The submersible pump can be attached near the open end of the inlet hose. The sample taken into the vessel from the specified depth is either transferred to a sedimentation chamber or poured through one or several hand nets of various sizes suspended one above the other. Several advantages encountered by using the plankton pump are collection of plankton from a desired depth, encountering of organisms at different depths in a particular stretch of the water column and continuous sampling from the moving ship itself which is not possible with water samplers.

Net Method

Closing Net

This net is provided with opening and closing mechanisms, and hence collection of plankton between the surface and bottom or from any desired depth in the vertical profile is possible by this method. The net is closed by means of a drop weight (messenger) which releases the throttle nose. The net must be drawn out immediately without stoppage. No more plankton can enter the closed net during take up.

The Clarke-Bumpus Horizontal Closing Net

The net consists of a solid front section, a brass tube 15 cm long and 12.7 cm in diameter attached to the filtering net portion by a bayonet lock. Propeller vanes are encased in the brass tube and a counter placed on the outside to register the volume of water that passes through the net. Brass tube is so mounted in a frame as to be movable up and down so that, regardless of the position of the frame, the net lies horizontally. A metal bar fixed between the brass tube at the front end and the net jar at the tail end acts as a stabilizer, and the two vanes located on the outer side of the brass tube help to keep the net in a horizontal position while in operation. The frame itself is so attached to a tow rope (draw line) that it can swing freely and that the opening of the brass tube is directed forwards by any movement of it. The brass tube can be opened and closed by a closing device.

Beyers Epibenthic Closing Net

This is a trawling-type net designed to be towed over the bottom and primarily intended for catching zooplankters which make vertical migrations. The net is housed in a circular framework which is also supported by a metal base. The net also has a closing band, above which a flow meter is attached outside. The net is towed using bridles which are asymmetrical. The asymmetry of the bridles and the circular framework make the net self-righting. The net is brought up by the wire attached to the back end of the frame. The net is closed by sending messenger.

Hardy's Continuous Plankton Recorder

It is torpedo-shaped plankton sampler. It consists of water tunnel, two rolls of the net silk, gear mechanism, propeller, stabilizing fin, horizontal stabilizing fin, diving plane, vibration damper, storage spool, formalin tank, etc. When the instrument is towed from a vessel, the propeller is turned by the passage of the water. The gears and adjoining spools containing the net silk strips are also simultaneously activated.

When water with plankton flows through the tunnel, one of the rolls of silk from the lower spool runs up through the water tunnel in the same way as a film in camera and filters the plankton which streams through the small square mouth of the instrument. Before this net strip containing the filtered plankton reaches the spool immersed in the formalin tank, another roll of silk from the upper spool spins out of cover the collecting strip and holds the plankton in place. Then the two strips are wound on the storage spool located in a container filled with formalin solution to preserve the plankton.

The Indian Ocean Expedition Standard Plankton Net

The plankton net consists of a cone-shaped gauze bag equipped with a metal ring at the wider mouth and closed at the narrow tail by a detachable plankton-collecting bucket. The non-filtering portion is generally made of coarse cloth attached with metal ring. The filtering portion is made up of different materials such as bolting silk, nylon or polyethylene. The net is towed from a boat for about 15 min using three suspension bridle lines at a speed of 2 nautical miles/h.

The Baleen Zooplankton Harvesting System

It consists of boat specifically designed for harvesting zooplankton. This vessel can filter the surface water at the rate of up to 400 l/s. Plankton is scooped onto a primary dewatering screen, and organisms are graded through a series of sieves. The graded and concentrated plankton is stored in wells and unloaded by pumping. The boat can be operated by one person.

Trawl Nets

A fishing boat equipped with a frame on which two to four plankton nets can be installed on both sides of the boat can be used for this purpose. When this moves at a speed of 1.5 km/h, average yields of 40 kg live zooplankton can be harvested in 1 h.

Flow-Through Harvesting

A propeller can be switched on from an anchored platform, bridge close to shore or on free-floating boat. The sucked water along with plankton can be filtered by using plankton net. The distance between the propeller and net should be maintained in a range from 0.3 to 1.5 m for proper filtration and to avoid the damage of nets.

Plankton Light Trapping

The standard light trap, equipped with a single high-output LED light, is powered by 2 C cells. They allow for deployment of up to 32 h. Upgrade to four batteries for improved system autonomy of up to 64 h. The zooplankton especially some species of copepods can be trapped by this method since the copepods have photoactive property.

Zooplankton Fixation and Preservation

For zooplankton, 5% formalin is the most widely used preservative. 40% formalin must be neutralized with agents like hexamethylamine ($C_6H_{12}N_{24}$) and borax. 80% of methyl alcohol is also an excellent preservative though causes shrinkage and decolouration. The formal alcohol is another important fluid for general preservation, which preserves colours.

Preparation of Formaldehyde Solutions

The concentrated formaldehyde (i.e. 40%) is usually used for fixing the zooplankters after the catch. However, for preservation, 2–4% solutions are commonly employed. In the preparation of a 4% solution, 10 ml of 40% formaldehyde is dissolved in 90 ml of seawater or distilled water. Similarly, 100 ml of 2% formaldehyde would contain 5 ml of 40% formaldehyde. The prepared solutions are normally kept in low temperatures, i.e. below 20 °C.

Neutralization of Formaldehyde

As the commercial formaldehyde has considerable acidity, it is always advisable to use the formaldehyde solution (whether concentrated or diluted) only after neutralizing it. To neutralize the acidity of the fixing (40%) and preserving (2–4%) formaldehyde solutions, excess $CaCO_3$ may be added when the pH of these solutions rises to 7. When formaldehyde solutions neutralized with $CaCO_3$ are stored in steel containers, the dissolved iron occasionally present may produce brown deposits. This can be prevented by using the following solutions:

1. Neutralization of 40% formaldehyde fixative: this is prepared by adding 10 g of Rochelle salt (potassium sodium tartrate—$KnaC_4H_4O_6.4.H_2O$) and 10 g of $CaCo_3$ to 1 liter of 40% commercial formaldehyde.
2. Preparation of 2–4% neutralized formaldehyde solution: 5 g of Rochelle salt is first dissolved in 1 liter of tap water. To 875 ml of this solution, 125 ml of 40% commercial formaldehyde and 10 g of $CaCo_3$ are added.

Formaldehyde Fixation of General Zooplankton Two methods are commonly employed:

1. To an empty container (say 500 ml capacity), neutralized 40% formaldehyde is added so as to make a final strength of 4% when the container is completely filled with seawater and zooplankton. In this case, 50 ml of neutralized 40% formaldehyde is added to the 300 ml of seawater earlier poured in to the container. The living, drained or concentrated zooplankters are then added and the jar dropped up with seawater. The jar is temporarily screened down and gently inverted a few minutes to mix the contents.
2. In the second method, 4% neutralized formaldehyde in seawater or in the distilled water is first added to half the level of an empty jar. The freshly collected and concentrated zooplankters are then added with a minimum of seawater. Subsequently, more of the 4% formaldehyde solution is added until the jar is full.

Such fixed zooplankton is left for a day or two and then filtered once again. The animals are finally preserved (stored) in small jars with plastic screw caps in the neutralized 5% formaldehyde solution or in a 75% ethanol. In replacing the fixative with the preservative, the fixative is carefully decanted. The sample jar is then filled to about three quarters with seawater or distilled water to which the correct volume of concentrated preservative (2.5 ml of buffered 40% formaldehyde or 7–5 ml preservatives with additives per 100 ml container volume) is added. Subsequently the jar is topped up with seawater or distilled water and sealed with a secure lid. It is also important if the jar is inverted several times to scatter the animals through the preservative. Such bottles should be checked monthly or quarterly for evaporation or animal condition. The plankton bottles should preferably be stored in a cool dark room.

Methods of Preservation of Individual Group of Zooplankton

Calcareous Plankton These include planktonic foraminiferans and certain gastropod molluscs which possess calcareous skeletons, namely, tests or shells made of calcium carbonate. These are fixed and preserved in 2% formaldehyde (pH 8–8.5) as follows: 5 ml of 40% formaldehyde buffered with borax is added to 90 ml of seawater containing living calcareous plankton. The buffered formaldehyde is prepared with 1000 ml of 40% formaldehyde to which are added 30 g sodium tetraborate ($Na_2B_4O_7$. H_2O-borax), 4.5 ml of propylene glycol and 0.1 ml of propylene phenoxetol. It is advisable to have the volume ratio of plankton biomass to preservative fluid at 1:9 and storage temperature at 15 °C.

Coelenterates (Cnidarians) The fixation of live cnidarians in a large glass jar may be done by gently adding 40% neutralized formaldehyde. Alternatively, the animals may be gently transferred to a 4% formaldehyde fixative using a spoon. These

coelenterates are preserved in 2% formaldehyde solution prepared with additives as follows: 10 ml stock solution (prepared using 500 ml of 40% formaldehyde to which the additives, viz. 50 ml of propylene phenoxetol and 450 ml of propylene glycol, are added) is added to 90 ml of seawater or distilled water. Hydromedusae may be preserved in 1% phenoxetol in distilled water and in the same fluid neutralized with 1% hexamine.

Ctenophores (Cnidarians) The animals are fixed in 1% trichloroacetic acid (1 g in 99 ml seawater) for 30 min. They are then temporarily preserved in 100 ml of seawater containing 1 ml of stock solution (50 ml of 4% formaldehyde containing 5 ml of propylene phenoxetol and 45 ml of propylene glycol). After a week, the animals are permanently transferred to 100 ml of seawater containing 5 ml of the above stock solution.

Chaetognaths (Arrow Worms) These are fixed with 5% neutralized formaldehyde which is prepared as follows: A solution containing 1 liter of tap water and 5 g of Rochelle salt is prepared. Then 125 ml of 40% commercial formaldehyde and 10 g of $CaCO_3$ are added to just 875 ml of the aforesaid solution. Fixatives containing osmic acid or Bouin's fluid can also be used for chaetognaths. The chaetognaths can be preserved in 2% formaldehyde in seawater at pH 6–7. 1% phenoxetol in distilled water and 50% ethylene glycol in distilled water are also added to preservatives.

Polychaetes The fixation of adult planktonic polychaetes is done by slowly adding neutralized formaldehyde until a concentration of about 2% is reached. They are preserved in either 2% neutralized formaldehyde or 70–80% ethanol. The fixation of polychaete larvae is done by pipetting Bouin's fluid heated to 60 °C into small volume of seawater containing the swimming larvae. The dead larvae are then transferred to cold Bouin's fluid for 1–3 h and subsequently washed and stored in ethanol.

Crustaceans For either fixation or preservation of crustacean zooplankton, the following solution is prepared and used. 5–10 ml of neutralized formaldehyde (prepared by adding 30–40 g of sodium tetraborate to 1000 ml of 40% formaldehyde) is added to 90–95 ml filtered seawater. The pH of this solution should be between 7 and 8. The ratio of plankton biomass to the preservative fluid should be approximately 1:4 by volume. Important species may be preserved in a 70% ethanol-distilled water solution. Specimens from a formaldehyde seawater solution may be transferred to an ethanol-distilled water solution as follows: The specimens, after thorough rinsing in distilled water, are first immersed in 30% ethanol for 10 min. Subsequently, the animals are immersed in 50% ethanol for 1 h and finally stored in 70% ethanol. A few drops of glycerol may be added to this solution, especially for the preservation of copepod and decapod plankton.

Planktonic Molluscs These include bivalve and gastropod veligers, shelled pteropods and heteropods and naked pteropods. The shell of these organisms is composed of aragonite, a most suitable form of calcium carbonate. Hence the pH of preservatives suitable to these organisms should drop to 6. Otherwise severe corrosion of the shells will take place. Further, the shells usually shiny, translucent, coloured and hard may lose their sculpture and become opaque-white and then chalky white, soft and brittle. Such shells may break easily when touched and eventually dissolve completely.

Veliger Larvae The veliger larvae of molluscs can be fixed with 90–95% aqueous ethanol which will prevent the dissolution of calcium carbonate. They may be preserved in a solution prepared as follows: 10 cc of 40% formaldehyde (buffered with sodium bicarbonate to 10) is added to 1 liter of filtered seawater containing 100 g of commercial sugar. The sugar besides preserving the colour of organisms act as a clearing agent and as a safety factor against evaporation. The veliger larvae can also be fixed in a 40% formaldehyde solution buffered to pH 8 with sodium glycerophosphate. After washing in freshwater, they can be stored in a solution of 1% propylene phenoxetol and 5–10% glycerol in freshwater.

Pteropods and Heteropods These animals can be fixed using 70% ethanol or 4% formaldehyde seawater buffered to pH 8. While the shelled forms are preserved in 70% ethanol, the naked forms are best preserved in 3–4% formaldehyde buffered to pH 8.

Thaliaceans The salps, doliolids and pyrosomes may be fixed in 4% formaldehyde. Subsequently, they are best preserved in 70% aqueous ethanol.

Appendicularians The appendicularians are fixed using 4% formaldehyde in seawater. While fixing, equal volumes of plankton and 4% formaldehyde are selected. Subsequently, they are preserved in 2% formaldehyde in seawater with a pH of 6.

Bottling and Labelling of Plankton

Storage of Zooplankton Generally glass containers with wide mouths and screw on plastic lids or good-quality polyethylene jars with polypropylene lids are suitable for fixation and storage.

Labelling Proper labelling of the collected zooplankton samples is more essential. All types of information regarding plankton collection should be written on the labels so that the plankton samples can be identified accurately. The label should contain enough information about the samples collected in order to assure proper

identification of the sample. The label is written with a light-coloured waterproof marker or wax pencil. It is also essential that apart from an external label, an internal label written on water-resistant paper be replaced in every jar containing a zooplankton sample. This internal label should contain the following information:

1. Station number
2. Date of sampling
3. Duration of sampling
4. Sampling depth
5. Type of net, mouth size and mesh size
6. Type of haul (horizontal, vertical, etc.)
7. Number of turns recorded by flow meter
8. Collector's name

Details on external label:

1. Bottle number
2. Station number
3. Date of sampling
4. Day/night
5. Sky-nature
6. Time
7. Depth of sampling site
8. Type of net
9. Mesh aperture
10. Flow meter reading
11. Collector's name

Zooplankton Mounting

Narcotisation

The initial reactions of zooplankters to any fixative and preservative are rapid and jerky movements, contraction of body and appendages. This can hinder species identification. This is controlled by temporarily anaesthetizing the specimens and allowing their recovery after necessary observations. The narcotizing solutions recommended are carbonated water, chloroform, methyl alcohol and magnesium chloride (about 7 g of magnesium chloride dissolved in 100 ml of distilled water). The carbonated water (1:20 by volume) is generally used as it is cheaper and easy to use in the field. The specimen should not be transferred directly to narcotizing solution. The narcotizing fluid is added drop by drop to the water containing the specimens. It should be remembered that the specimens are not kept there for a long period to

avoid any damage. As soon as the morphological characters are observed, the specimens are washed with distilled water and put back into the fixative.

Clearing

The fixed specimens must be cleared of any attached material such as detritus or precipitate. This can be done by removing the extraneous substances with fine forceps/needles without damaging the specimens. The specimens are immersed in clearing fluids such as lactic acid, glycerine and propylene glycol. The lactic acid is commonly used as a clearing agent, and care should be taken that specimens are not left in the lactic acid for a long period which would result in the disintegration of body tissues of zooplankton. Examination of external features becomes easier after clearing the specimens. To study the internal structures, staining of specimen is required.

Staining

Light staining of the specimens is carried out by adding a few drops of rose bengal, lignin pink, chlorazol black E and methylene blue added to the lactic acid. Borax carmine is used for staining small zooplankton, larval stages of crustaceans and ichthyoplankton (fish eggs and fish larvae). The lignin pink and chlorazol black E can penetrate the chitin and stain the internal tissues and facilitate dissection. Rose bengal is usually added to the internal tissues which facilitates dissection. Rose bengal is usually added to the zooplankton samples when the preservation is done.

Mounting

Permanent glass slides are made by using the natural or synthetic resins. Canada balsam, gum chloral, glycerine jelly and lactophenol are used as mounting agents. The Canada balsam dissolved in xylene or benzene is used for whole mounts. The disadvantage with balsam is that the mounts become dark with time. The lactophenol is widely used. This can be kept for a long time. Before mounting the whole specimen or the dissected parts, the slides and coverslips are thoroughly cleaned with ethanol and dried. A few drops of mountants are placed on the glass slide, and then the specimens or their dissected structures are transferred. The coverslip is supported by fragments of broken coverslip or wax. The slides should be completely dried and stored in a slide box for subsequent examination for species identification.

Micrometry of Plankton

By micrometry, while viewing through a microscope, the length, breadth and other details of an organism are measured. The size determination of the zooplankton forms an important aspect, especially, in preparing the report on the occurrence of new species or taxonomic studies for publication. In micrometry an ocular micrometer (graticule) plays an important role. The ocular micrometer is a circular glass piece which contains a scale of lines which are engraved or photographically reproduced (Fig. 12). This scale is of 10 mm in length divided into ten equal divisions. Thus, on the scale of the ocular micrometer, there are 100 divisions of 100 μm each.

Calibration: Ocular micrometer is mounted on the diaphragm inside the eyepiece of the chosen microscope at the focal of the eye lens. On the diaphragm inside the eyepiece, at one point, the image from the object is also focused, so that the two can be viewed simultaneously. Now, not only the object in focus, but superimposed on the object, the series of lines of the graticule is equally visible.

For the calibration of the graticule, a stage micrometer which is a microscopic slide of 7.5 × 2.5 cm has been engraved a scale of 1 mm long, divided into 100 divisions of 10 μm (0.01 mm) each. While calibrating, the stage micrometer is first placed on the stage of the microscope. Then it is focused and aligned with the ocular micrometer scale. The stage micrometer is then moved carefully until its zero line is in exact coincidence with that of the ocular meter, in order to find out how many divisions on the ocular micrometer scale correspond with a certain number of divisions on the stage micrometer scale. From this, the value (in μm) of one division of the ocular micrometer under the chosen microscope with fixed objective and eyepiece powers is calculated. If 30 divisions of the ocular micrometer correspond with 10 divisions of the stage micrometer scale, then these 30 divisions are equivalent to 100 μm. In other words, these 30 divisions occupy 100 μm space of the stage micrometer (as 1 division occupies 10 μm of the space in the stage micrometer and the total length of the scale is 1000 μm−1 mm). Thus one ocular micrometer division is equal to 100/30 = 3.3 μm. This calibrated value of the ocular micrometer is of a particular objective and eyepiece of a microscope. If size determination of an object is to be done in different objective or eye lenses, the ocular micrometer scale is calibrated for all the combinations of the different objectives and eyepieces, and all the values may be tabulated and can be used whenever it is required. The size of an individual plankton cell of a species may be determined using the calibrated ocular micrometer and micrometer as follows. For size determination, on the stage of the microscope, the specimen for which the size is to be measured is now placed instead of the stage micrometer. If the diameter of Cyclotella cell is to be determined, the zero of the ocular micrometer is focused against the edge of the cell, and the number of divisions of the ocular micrometer that occupy the diameter of the cell is found out. Number of calibrated ocular micrometer divisions multiplied by

the corresponding calibrated value would give a diameter of the said cell. For example, if the graticule divisions are 20, then the diameter of the cell is $20 \times 3.3\ \mu m = 66\ \mu m$.

Camera Lucida Drawings The zooplankton specimen must be accurately drawn in terms of proportions and magnifications. A camera lucida is invariably used to ensure accurate drawings of specimens.

Camera Lucida A small right-angled and reflecting prism is mounted above the eyepiece of a microscope. The reflecting surface of the prism is silvered except for one small patch in the centre. The prism mount carries a horizontal arm with an inclinable mirror to the end. The mirror should be set an angle of 45° to the vertical over the drawing paper (though small variations are permissible in the angle of the mirror, too much deviation results in distortion of the drawing).

Light from the drawing paper is reflected by the mirror onto the reflecting face of the prism, which reflects it into the eye of the observer. Light from the microscope eyepiece passes through the small unsilvered patch on the reflecting surface of the prism and enters the eye of the observer along with the light for the paper. As a result, the observer sees the object apparently superimposed on the paper. The observer concomitantly sees the pencil point if a pencil is used for the drawing.

Making a Camera Lucida Drawing The camera lucida should be attached to the microscope and properly positioned before undertaking the drawing of an organism that is mounted on a slide and placed to keep flat on the right-hand side of the microscope. Invariably, a fairly hard-leaded pencil should be used to obtain precision lines. In drawing with the right hand, the left eye should be at the eyepiece leaving the right eye free to look at what is being drawn without raising the head. By this method, only the main outline of the object is traced, which serves as the framework into which other details can be drawn later under the microscope but without the camera lucida.

Effective use of the camera lucida lies in the extract balancing of the intensity of light on the drawing paper and the object, respectively. For example, if the light from the object is too bright, the pencil point cannot be seen; on the other hand, if the paper is too bright, the object disappears altogether from view, and if the light to the specimen is too intense, it can be reduced by closing the iris diaphragm of the substage condenser.

Depicting Size of the Specimen in Drawing To determine the size of a zooplankton specimen, the stage micrometer is placed on the stage of the microscope immediately after removing the specimen slide but without altering the microscope set-up. Subsequently, a space equivalent to 100–200 μm of the millimeter scale is drawn alongside the object.

Taxonomic Identification of Zooplankton

Tintinnopsis tubulosa Levander

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. tubulosa*

Lorica possesses two regions-bowl and column. Oral flare absent. Bowl and column uniformly agglomerated.

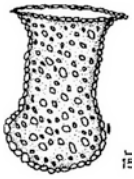
Tintinnopsis tocantinensis Kofoid and Campbell

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. tocantinensis*

The lorica is agglomerate and characterized by the presence of a cylindrical and elongated collar, ending with a bowl and one aboral horn. The agglomeration of sand particles is not intense as these particles are found scattered all over the body of the lorica. Length is 122–164 μm and oral diameter is 24–32 μm.

Tintinnopsis butschlii Daday

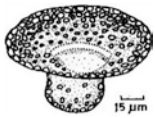
Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. butschlii*



The lorica is cylindrical, slightly flared orally and round aborally and forms a bowl here. Agglomeration is heavy in the bowl and moderate in the flare and collar. Length is 87.5–92.5 μm, and oral diameter is 62.1–64.8 μm.

Tintinnopsis mortensenii Schmidt

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. mortensenii*



This species is characterized by an agglomerate bowl which is enlarged anteriorly into a flare similar to that of a flower vase. The agglomeration is moderate on the bowl but dense on the rim of the flare. Length is 60–68 μm and oral diameter is 80–82 μm.

Tintinnopsis directa Hada

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. directa*

Characterized by the presence of a moderately erect lorica which is campanulate anteriorly and sub-spherical posteriorly. Agglomeration is light on the cylindrical part of the lorica and fairly pronounced on the bowl. Length is 84.5–92.5 μm and oral diameter is 51.5–52.8 μm.

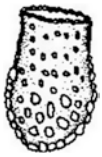
Tintinnopsis bermudensis Brandt

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. bermudensis*

The lorica of this species is divisible into a collar and a bowl portion. Agglomeration comprises on the collar and bigger particles on the bowl. Length is 90.8–94.0 µm and oral diameter is 31.0–31.8 µm.

Tintinnopsis lohmanni Laackmann

Phylum	Protozoa
Class	Polyhymenophora
Subclass	Spirotricha
Order	Oligotrichida
Suborder	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. lohmanni*



The lorica is small and consists of a short cylindrical collar portion and a bowl which is ovate. The bowl is more heavily agglomerate than the collar. Length is 57.0–57.8 µm and oral diameter is 26.2–26.6 µm.

Dictyocysta seshaiyai Krishnamurthy & Santhanam

Phylum	Ciliophora
Class	Ciliatea
Order	Oligotrichida
Family	Dictyocystidae
Genus	*Dictyocysta*
Species	*D. seshaiyai*

The lorica is divisible into bowl and collar portions. The collar possesses a single row of four rectangular windows, formed by four frames placed at equidistance from each other. The bowl is bulbous but slightly pointed aborally. Agglomeration with foreign particles is noticed both on the bowl and collar; however, on the collar the particles are large in size. Neither fenestrate nor reticulation is evident on the lorica. Total length is 72.0–82.8 µm and inner oral diameter is 48.5–54.0 µm.

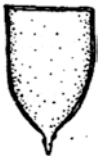
Tintinnopsis beroidea (Stein, 1867)

Kingdom	Protoctista
Phylum	Ciliophora
Class	Oligotrichea
Order	Tintinnida
Family	Codonellidae
Genus	*Tintinnopsis*
Species	*T. beroidea*

Usually bullet shaped with slightly pointed end; encrustations heavy; aboral part of lorica remains spherical, while oral portion sometimes starts narrowing.

Favella philippinensis Roxas

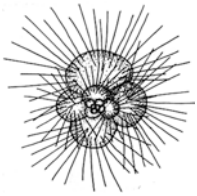
Phylum	Protozoa
Class	Polyhymenophorea
Subclass	Spirotrichia
Order	Oligotrichida
Family	Ptychocylididae
Genus	*Favella*
Species	*F. philippinensis*



The species characterized by the presence of a fairly long, clean and campanulated lorica. The posterior end of the lorica ends with a short pedicel-like structure called the aboral horn, which is provided with two small ears or winglike structures, one on either side of the aboral horn.

Globigerina bulloides d' Orbigny

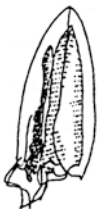
Phylum	Protozoa
Order	Foraminifera
Family	Orbulinidae
Subfamily	Globigerininae
Genus	*Globigerina*
Species	*G. bulloides*



Test consists of number of small chambers and all these are visible. The test is coarsely perforated.

Diphyes sp.

Phylum	Cnidaria
Class	Hydrozoa
Order	Siphonophorae
Suborder	Calycophorae
Genus	*Diphyes* sp.
Family	Diphyidae
Genus	*Diphyes*
Species	*Diphyes* sp.



Upper nectophore without many angles, oral margin without prominent teeth.

Pleurobrachia pileus Fabricius

Phylum	Ctenophora
Class	Tentaculata
Order	Cydippida
Family	Pleurobranchidae
Genus	*Pleurobrachia*
Species	*P. pileus*

A characteristic feature of the group is the rows of ciliary plates called comb plates, which are always eight in number. The body is egg shaped, biradially symmetrical, with long tentacles in a tentacle sheath and without oral lobe.

Obelia sp.

Phylum	Cnidaria
Subphylum	Medusozoa
Class	Hydrozoa
Subclass	Leptolinae
Order	Leptomedusae
Suborder	Proboscidoidea
Family	Campanulariidae
Genus	*Obelia*
Species	*Obelia* sp.

Medusae are saucer shaped with a very flat bell. There are 4 radial canals and 1 ring canal from which 24 solid marginal tentacles originate; body is transparent, and medusae luminescent with 8 abradial marginal vesicles.

Physalia physalis (Linnaeus, 1758)

Kingdom	Animalia
Phylum	Cnidaria
Superclass	Hydrozoa
Class	Siphonophorae
Order	Cystonectae
Family	Physaliidae
Genus	*Physalia*
Species	*P. physalis*

Presence of enormous coloured float formed simply as a hollow pocket by infolding of outer layer. At regular intervals, the whole body twists over to wet itself first on one side and then on the other. Presence of hydrophyllia or bracts which protect the gonophores and gastrozooids.

Porpita porpita (Linnaeus, 1758)

Phylum	Cnidaria
Subphylum	Medusozoa
Superclass	Hydrozoa
Class	Hydroidomedusae
Subclass	Anthomedusae
Order	Capitata
Suborder	Zancleida
Family	Porpitidae
Genus	*Porpita*
Species	*P. porpita*

It is a free-floating modified hydroid polyp up to 8 cm in diameter; numerous clavate tentacles bearing powerful stinging cells hang from the rim of horny central disc-shaped float which support the animal on the water surface; body tissues contain symbiotic algae.

Velella velella

Phylum	Cnidaria
Superclass	Hydrozoa
Class	Leptolida
Subclass	Anthoathecatae
Order	Capitata
Family	Porpitidae
Genus	*Velella*
Species	*V. velella*

It is a complex colony made up of a large number of polyps crowding the number under surface of an oval-shaped float; presence of central mouth with large number of smaller polyps; deep blue in colour with a translucent sail protruding above the water surface.

Aurelia aurita (Linnaeus, 1758)

Kingdom	Animalia
Phylum	Cnidaria
Superclass	Scyphozoa
Class	Scyphomedusae
Order	Semaeostomae
Family	Ulmaridae
Genus	*Aurelia*
Species	*A. aurita*

Bell is saucer shaped and about 50 cm in diameter; can be recognized by its bright purple to pale lilac-coloured ovaries or testes; presence of numerous fine short tentacles and eight sense organs around the edge of the umbrella.

Brachionus plicatilis (O. F. Muller)

Phylum	Aschelminthes
Class	Monogononta
Order	Ploima
Family	Brachionidae
Genus	*Brachionus*
Species	*B. plicatilis*

Lorica stippled in anterior margin and the posterior margin is broader than the anterior margin. Six anterior spines are present, which were equal in length and base is broad. The well-developed posterior spines are present.

Brachionus angularis Gosse, 1851

Kingdom	Animalia
Phylum	Rotifera
Class	Monogononta
Order	Ploima
Family	Brachionidae
Genus	*Brachionus*
Species	*B. angularis*

Lorica is circular in shape. Presence of four anterior spines, from which two marginal and two medium spines come out; presence of deep U-shaped sinus franked by dorsal median spines.

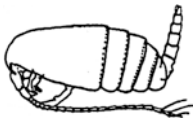
Brachionus calyciflorus Pallas, 1766

Kingdom	Animalia
Phylum	Rotifera
Class	Monogononta
Order	Ploima
Family	Brachionidae
Genus	*Brachionus*
Species	*B. calyciflorus*

Length and breadth of lorica, more or less the same; four occipital spines are present; V-shaped sinus with median spines longer than lateral spines.

Nannocalanus minor (Claus)

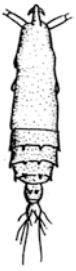
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Calanidae
Genus	*Nannocalanus*
Species	*N. minor*



The first antenna reaches up to caudal rami by about half of the body length. In males, the fifth legs contained few plumose setae on the right exopodite. Left leg distinctly longer than the right leg. The external marginal spines are greatly enlarged on the left exopodite.

Rhincalanus cornutus Dana

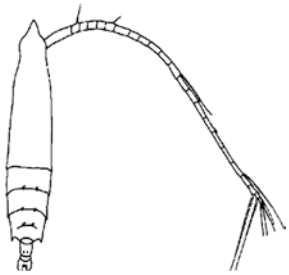
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Eucalanidae
Genus	*Rhincalanus*
Species	*R. cornutus*



It is a large-sized copepod with pointed head, and the anterior projection of the head is distinctly anchor shaped and the rostral filaments are visible. The antennules are much longer than the body. There are spines on the last three thoracic and abdominal segments. The sexes are distinguished by the form of the fifth legs with jointed exopod and endopods.

Rhincalanus nasutus Giesbrecht, 1888

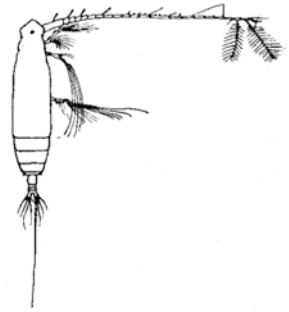
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Eucalanidae
Genus	*Rhincalanus*
Species	*R. nasutus*



The anterior projection of the head is conical, concealing the rostral filaments. The fifth leg of the female has one seta on the second segment and three setae on the third segment. The right fifth leg of the male is tipped with a curved claw.

Eucalanus elongatus (Dana, 1849)

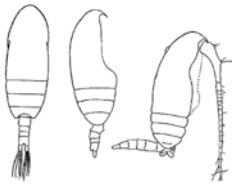
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Eucalanidae
Genus	*Eucalanus*
Species	*E. elongatus*



The head is triangular in shape. Lateral angles of the last thoracic segment pointed. There are two segments present in between the anal and genital segments. First and second segments of exopodite of A2 not fused; first segment of endopodite slightly longer than the second and over 3 times as long as it's broad. Inner margin of the second segment of basipodite of Md with three setae, divided into two nearly equal portions by the insertion of the endopodite; endopodite with two setae on the first segment and five setae on the second. Urosorne four segmented; an enlarged caudal seta present on the right side, posterior margin of metasome rounded in the Indo-Pacific forms but two blunt teeth present in the Atlantic form; length 4.4–8.25′ mm.

Paracalanus parvus (Claus)

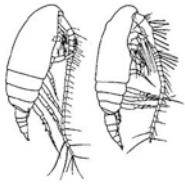
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Paracalanidae
Genus	*Paracalanus*
Species	*P. parvus*



First antennae not generally reaching beyond the caudal rami, surface of basipod 1 of legs 1 to 4 beset by hairs and bristles. In female, urosome is four segmented; fifth legs symmetrical, two segmented, genital opening oval, broader than long; length 0.8 to 10.00 mm. In male, urosome is five segmented; fifth legs asymmetrical, two segmented on the right and five segmented on the left, left foot much longer; bubble like eminence on cephalosome indistinct or absent in profile view; length 0.9–1.00 mm.

Paracalanus aculeatus Giesbrecht, 1888

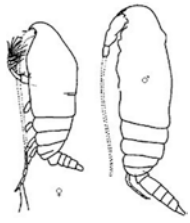
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Paracalanidae
Genus	*Paracalanus*
Species	*P. aculeatus*



Urosome four segmented in female and five segmented in male. Genital opening circular. Fifth leg asymmetrical and short. Length: female, 1.25 mm; male, 1.20 mm.

Acrocalanus gibber Giesbrecht, 1888

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Paracalanidae
Genus	*Acrocalanus*
Species	*A. gibber*

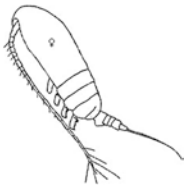


Female: deep body in lateral view, with a pronounced hump on the dorsal cephalosome; distinct though partial line of separation of the cephalosome from the first pedigerous segment is obvious; A1 reaches just to the furca or slightly beyond. Four

pairs of swimming legs, the distal toothed outer border of exopodite segment 3 of P4 is about 60% of the length of the proximal part of the toothed border; the distal teeth are strong compared to the fine teeth in *A. longicornis*. Genital segment longer than any of the others, with a prominent ventral swelling. Length is 0.93–1.28 mm. Male: distinct hump on the dorsal cephalosome; P5 is only present on the left and extends to the end of the urosome segment 3 or the middle of urosome segment 4 when this leg is fully extended; very simple limb, composed of four segments with two tiny spines on the last segment. Length is 0.94–1.24 mm.

Acrocalanus gracilis Giesbrecht, 1888

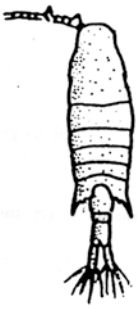
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Paracalanidae
Genus	*Acrocalanus*
Species	*A. gracilis*



Female: the anterior cephalosome is evenly round in lateral view and the line of separation of the cephalosome from the first pedigerous segment is very slight, if it can be seen at all; no obvious hump on cephalosome; A1 exceeds the end of the furca by two to four terminal segments; the distal toothed outer border of exopodite segment 3 of the P4 is about 80% of the length of the proximal part of the toothed border. The coxa of P4 in more convex than in *A. longicornis* and there are more spines distally. Length: 1.20–1.40 mm. Male: the anterior cephalosome is evenly round in lateral view and there is no hump. The P5 is asymmetrical and when fully extended reaches the end of urosome segment 2, the right foot is vestigial or absent, the left P5 is of five segments, the distal the largest. Length: 0.88–1.14 mm.

Centropages furcatus (Dana)

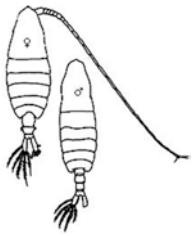
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Centropagidae
Genus	*Centropages*
Species	*C. furcatus*



In male, the posterior margin of the metasome provided with two smaller, more dorsally placed spines in addition to the two larger ones. The left exopodite of the fifth legs is two segmented and the right exopodite three segmented with a strong chela. The fifth legs are provided with a prominent inwardly pointed spine on the middle exposed segment in female. The right first antenna of the male is geniculated.

Centropages gracilis (Dana, 1849)

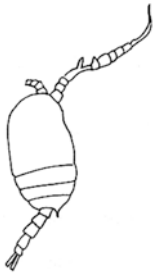
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Centropagidae
Genus	*Centropages*
Species	*C. gracilis*



The second urosome segment is small peglike projections laterally. First antennae extend beyond caudal rami by about five segments. Length: female, 2.0 mm; male, 1.0 mm.

Pseudodiaptomus aurivilli Cleve

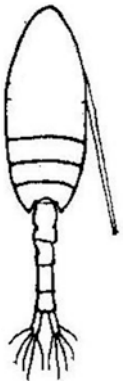
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pseudodiaptomidae
Genus	*Pseudodiaptomus*
Species	*P. aurivilli*



Urosome four segmented in female and five segmented in male. The first antennae alike on two sides, fifth legs as figured in male. In female first antennae geniculate on the right side, fifth legs highly complex, as figured.

Pseudodiaptomus serricaudatus (Scott T., 1894)

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pseudodiaptomidae
Genus	*Pseudodiaptomus*
Species	*P. serricaudatus*



Genital segment without spines and asymmetrical; urosome segment without regular row of teeth. The fifth leg of male is highly developed. The length of the female is 0.9–1.2 mm and male is 0.8–1.1 mm.

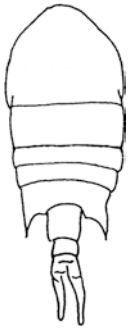
Pseudodiaptomus annandalei Sewell, 1919

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pseudodiaptomidae
Genus	*Pseudodiaptomus*
Species	*P. annandalei*

Taxonomic descriptions: genital segment with prominent spine on each side; the fifth leg is modified, and the right leg has claw on its terminal segment. The left fifth leg of male has two segments modified into forceps-like structure. Length of the female is 1.18 mm and male is 0.9 mm.

Temora discaudata Giesbrecht

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Temoridae
Genus	*Temora*
Species	*T. discaudata*



Body is bulky, short, compact and with massive head. It has long furcal rami over six times as long as broad and it is typically asymmetrical. The posterior margin of the metasome is drawn into spines which are not symmetrical, and the left spine is relatively larger.

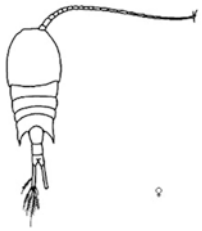
Temora turbinata Dana, 1849

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Temoridae
Genus	*Temora*
Species	*T. turbinata*

Posterior margin rounded the right leg of male is enlarged and modified. The distal segment has two spinulated setae. Length: female, 1.5 mm; male, 1.4 mm.

Temora stylifera Dana, 1849

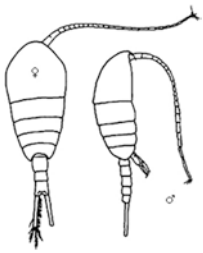
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Temoridae
Genus	*Temora*
Species	*T. stylifera*



Caudal rami asymmetrical, posterolateral corners of cephalosome expanded into laminae. Length: female, 1.35 mm; male, 1.3 mm.

Temora longicornis (Muller, 1792)

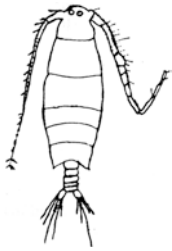
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Temoridae
Genus	*Temora*
Species	*T. longicornis*



Females: one caudal seta longer than others but not especially thickened; inner spine on terminal segment of P5 shorter than both terminal spines. Males: P5 left terminal segment (including stout terminal spine) shorter than penultimate segment, armed with one stout terminal spine, two small inner distal spines, and one terminal plumose seta.

Labidocera pavo Giesbrecht

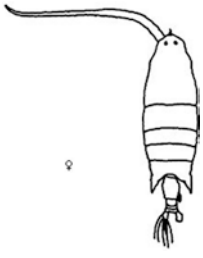
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Labidocera*
Species	*L. pavo*



The body with a hastate anterior end. Prominent eyes, eye lens on the dorsal side of the head region or cephalosome. The anterior margin of the head is with a pointed hook curved to the ventral side. The posterior end terminating in two spinelike processes. The metasome is three segmented, the first antennae are symmetrical, and the corners of the metasome ending in points are symmetrical.

Labidocera acuta (Dana, 1849)

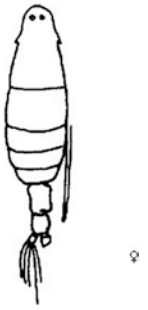
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Labidocera*
Species	*L. acuta*



Urosome five segmented in female and three segmented in male. Corners of metasome unlike on both sides. Eye lenses larger in male. Length: female, 3–3.4 mm; male, 2.8–3.4 mm.

Labidocera minuta Giesbrecht, 1889

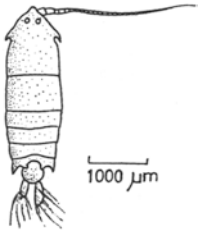
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Labidocera*
Species	*L. minuta*



In female posterior margin of metasome rounded with a peglike projection on the right side and in male metasome drawn out into spines. Length: female, 2.1 mm; male, 1.54 mm.

Pontella danae Giesbrecht

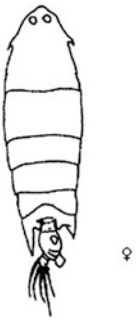
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Pontella*
Species	*P. danae*



In male, the urosome has five segments. The urosome and caudal rami are asymmetrical. The right antennae are geniculate. The lateral margins of the head have a hook on each side in female. One pair of dorsal eye lenses and one pair of rostral lenses are present. The urosome has two segments.

Pontella spinipes Giesbrecht, 1889

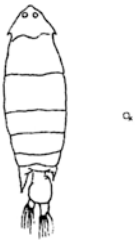
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Pontella*
Species	*P. spinipes*



Genital segment with transverse corrugations on dorsal surface right caudal ramus longer but setae shorter. Length: female, 4.5 mm; male, 4.3 mm.

Pontella securifer Brady, 1883

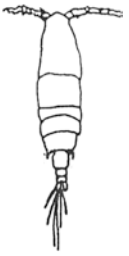
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Pontellidae
Genus	*Pontella*
Species	*P. securifer*



Genital segment with two or three fingerlike outgrowths on dorsal surface: exopods or fifth legs strongly curved with four outer spinules. Length: female, 4.4 mm; male, 4.3 mm.

Acartia spinicauda Giesbrecht

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Calanoida
Family	Acartiidae
Genus	*Acartia*
Species	*A. spinicauda*



The body is elongated with square anterior end with a typical pigmented eyespot. The second antennae with two jointed exopods in which the distal segment is as long as the proximal and the fifth leg always uniramous, slender and spinelike. The

posterior margin of the metasome is drawn into spines, and the spines are also present on the urosomal segments. The second urosomal segment almost as wide as long with two pairs of spines.

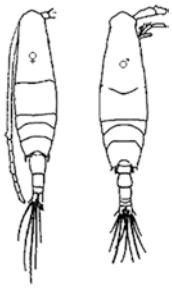
Acartia centrura Giesbrecht, 1889

Kingdom	Animalia
Phylum	Crustacea
Class	Maxillopoda
Subclass	Copepoda
Order	Calanoida
Family	Acartiidae
Genus	*Acartia*
Species	*A. centrura*

The spines of the metasome are as longer as *Acartia erythraea.* Terminal claw of fifth leg thickened little at the base. Straight and has notch. Length: female, 1.24 mm; male, 1.02 mm.

Acartia danae Giesbrecht, 1889

Kingdom	Animalia
Phylum	Crustacea
Class	Maxillopoda
Subclass	Copepoda
Order	Calanoida
Family	Acartiidae
Genus	*Acartia*
Species	*A. danae*



Fifth leg of female straight with a crowd of small teeth near the tip. Length: female, 1.0–1.22 mm.

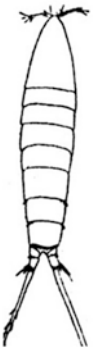
Microsetella norvegica (Boeck)

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Harpacticoida
Family	Ecnomidae
Genus	*Microsetella*
Species	*M. norvegica*

It is fusiform in shape. The body is gently compressed laterally. It has geniculated antennules. Third and fourth segments of the metasome and all the segments of the urosome carry transverse rows of spinules. The caudal rami are as long as broad and are indistinct. The caudal setae are also as long as the body nearly 11/2 times the length of the body.

Microsetella rosea (Dana, 1847)

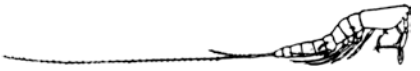
Kingdom	Animalia
Phylum	Crustacea
Class	Maxillopoda
Subclass	Copepoda
Order	Harpacticoida
Family	Miraciidae
Genus	*Microsetella*
Species	*M. rosea*



Slightly rosy in colouration. All segments of urosome with transverse rows of spinules. Caudal setae nearly twice the length of the body. Length: female, 0.4 mm; male, 0.3 mm.

Macrosetella gracilis (Dana)

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Harpacticoida
Family	Macrosetellidae
Genus	*Macrosetella*
Species	*M. gracilis*



Body is slender and laterally compressed. The antennule reaches as far back as the genital segment. Rostrum is prominent. Caudal setae cylindrical nearly one and half again as long as caudal rami, swimming legs long and narrow.

Euterpina acutifrons (Dana)

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Harpacticoida
Family	Tachinidae
Genus	*Euterpina*
Species	*E. acutifrons*

It is a monospecific genus as it has the only species. The body is subpyriform or attached, and the cephalosome is drawn out in front into a greatly prominent rostral projection which is acute at the tip. Fifth pair of legs formed by two undivided juxtaposed plates.

Dioithona rigida Giesbrecht

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Cyclopoida
Family	Oithonidae
Genus	*Dioithona*
Species	*D. rigida*

The anterior end is bluntly rounded. The first antennae reached up to end of third metasome only and it is twice geniculated, and the sheathing base appears to be present. The body is broader at the centre than at either end and the four segments are found behind the head. Body usually with much pigments. Paired egg sacs may sometimes be seen attached on the anterior end of the upper abdomen. Urosome nearly as long as metasome. Caudal setae coarsely plumose forming a fan.

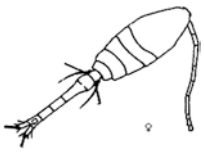
Oithona brevicornis Giesbrecht, 1891

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Cyclopoida
Family	Oithonidae
Genus	*Oithona*
Species	*O. brevicornis*

Head round shaped anteriorly. Prosome of adult is elongated and ellipsoid in shape. End of cephalasome is the widest part. Length: female, 600–700 μm.

Oithona similis Claus, 1866

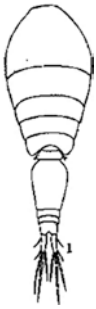
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Cyclopoida
Family	Oithonidae
Genus	*Oithona*
Species	*O. similis*



Prosome of adult is elongated and ellipsoid in shape. Head truncated anteriorly and as a sharply pointed rostrum in lateral view. Length and breadth of female measure 850 and 200 μm.

Oncaea venusta Philippi, 1843

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Family	Oncaeidae
Genus	*Oncaea*
Species	*O. venusta*



Second free metasome segment not raised into hump; anterior part of the body obovate in the female, less wide in the male; genital segment more conspicuously enlarged in the male than in female. Length: female, 1.1–1.27 mm; male, 0.7–1.0 mm.

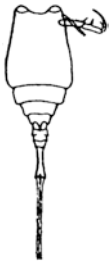
Corycaeus catus (F. Dahl, 1894)

Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Family	Corycaeidae
Genus	*Corycaeus*
Species	*C. catus*

Caudal rami very short, equal in length to the anal segment in the male, a little longer than the anal segment in the female, male genital segment with a ventral hook at the anterior body. Length: female, 1 mm; male, 0.8 mm.

Copilia vitrea (Haeckel)

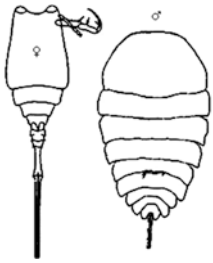
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Cyclopoida
Family	Corycaeidae
Genus	*Copilia*
Species	*C. vitrea*



Body is very transparent and slightly depressed. Head squarely truncated in front with corneal eye lenses near the corners. Second antennae are slender and prehensile with four segments. It has two prominent eyes each always some distance apart and a body tapering to a narrow apparent abdomen. The last segment of the abdomen has long, narrow furcal rami attached to it.

Copilia mirabilis Dana, 1849

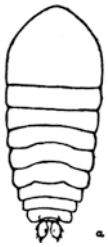
Kingdom	Animalia
Phylum	Crustacea
Class	Maxillopoda
Subclass	Copepoda
Order	Poecilostomatoida
Family	Sapphirinidae
Genus	*Copilia*
Species	*C. mirabilis*



Distance between eye lenses less than twice the lens diameter; length of anal segments five times its least width; endopod as long as first exopod segment in the fourth leg. Length: female, 2.22–4.1 mm.

Sapphirina ovatolanceolata Dana

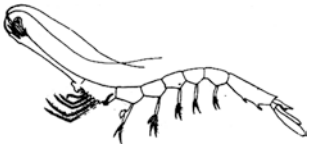
Phylum	Arthropoda
Class	Crustacea
Order	Copepoda
Suborder	Cyclopoida
Family	Sapphirinidae
Genus	*Sapphirina*
Species	*S. ovatolanceolata*



Body is generally long and narrow. It has prominent eyes set close together at the anterior end. Urosome is distinctly narrower than the metasome. Some of the metasome segments are as wide as cephalosome.

Lucifer hanseni Nobil

Phylum	Arthropoda
Class	Crustacea
Order	Decapoda
Genus	*Lucifer*
Species	*L. hanseni*



Body is elongated with long neck. Head, thorax and abdomen can be prominently distinguished. It has two groups of stalked eyes, one with short eyestalks and the other with long eyestalks.

Sagitta bipunctata Quoy and Gaimard

Minor Phylum	Chaetognatha
Class	Sagittoidea
Order	Aphragmophora
Suborder	Ctenodontina
Family	Sagittidae
Genus	*Sagitta*
Species	*S. bipunctata*

Body is elongated and comparatively slender and rigid. It is usually large and transparent. Body can be divided into head, trunk and tail. The posterior fin is slightly larger than the anterior fin and completely rayed. The head bears spines or jaws around the mouth with less numbers. The sides of the body are covered with numerous tufts of sensitive papillae.

Sagitta enflata Grassi, 1881

Phylum	Chaetognatha
Order	Aphragmophora
Family	Sagittidae
Genus	*Sagitta*
Species	*S. enflata*

Commonly known ‘arrow worm’ because of its long, straight, slender body; body is divisible into short head, long trunk and a short tailpiece; the mouth is with stout curved bristles and body with two pairs of horizontal side fins and tail fins.

Doliolum sp. Krohn

Phylum	Chordata
Subphylum	Urochordata
Class	Thaliacea
Order	Doliolida
Genus	*Doliolum* sp.

It has small and barrel-shaped body with an anterior inhalant branchial aperture and a posterior exhalent atrial aperture. The body is enriched by eight hoops of muscle, and the test is extremely thin and the underlying mantle forming a series of lobes around the inhalant and exhalent apertures.

Salpa fusiformis Cuvier

Phylum	Chordata
Subphylum	Urochordata
Class	Thaliacea
Order	Salpida
Genus	*Salpa*
Species	*S. fusiformis*

The mantle is thick and contains six muscle bands of which the first four and last two are fused together. It has solitary embryo. The musculature is very clearly seen and the muscle bands are peculiarly incomplete ventrally. The endostyle runs along the length of the pharynx.

Oikopleura parva Lohmann, 1896

Phylum	Chordata
Subphylum	Urochordata
Class	Copelata
Order	Appendicularia
Genus	*Oikopleura*
Species	*O. parva*

It is small larvacean plankton. It has an egg-shaped body. The well-developed ovary occupies the entire apical regions of the body mass. The tail is narrow, long supported by the central core of vacuolated cells comprising the notochord. It is hermaphrodite. More commonly found in coastal and shallow waters. The oceanic form is rare and it is large in size.

Podon sp.

Kingdom	Animalia
Phylum	Arthropoda
Class	Crustacea
Order	Cladocera
Family	Polyphenidae
Genus	*Podon*
Species	*Podon* sp.

Head demarcated from the body by a deep transverse groove; bivalved carapace forms a distinct dorsal chamber which acts as brood pouch; presence of biramous setose antennae behind the prominent compound eye.

Euphausia diomedeae Ortmann, 1894

Kingdom	Animalia
Phylum	Arthropoda
Class	Crustacea
Subclass	Brachiopoda
Order	Euphausiacea
Family	Euphausiidae
Genus	*Euphausia*
Species	*E. diomediae*

Possesses external gills attached to endopods of biramous thoracic limbs; presence of prominent pair of spines on each laterofrontal border and smaller one and each lateral margin of the carapace; they are of about 40 mm in length.

Mysis

Phylum	Arthropoda
Class	Crustacea
Order	Mysidacea
Group	Mysis

It looks like adult prawn. Well developed and eyes are stalked. Scales of antennae well developed. It has three walking legs chelate. Forked telson developed and forms into tail fin.

Alima larva

Phylum	Arthropoda
Class	Crustacea
Order	Stomatopoda
Group	Alima larva

It has pair of disembodied eyes. The large and triangle carapace covers much of the body of the animal. It has large rostrum and a pair of posterolateral spines is very needlelike. Two thoracic appendages are uniramous. Additionally, there are four or five pairs of biramous appendages present.

Crab zoea larva

Phylum	Arthropoda
Class	Crustacea
Order	Decapoda
Group	Crab zoea larva

The single dorsal and rostral spine can be elongated or very short. It has pair of lateral spines, one pair pointed downwards and the other one pointed forwards and upwards. The ends of most of the spines can be flattened like a spearhead.

Phyllosoma larva

Phylum	Arthropoda
Class	Crustacea
Order	Decapoda
Group	Phyllosoma larva

The larvae are very distinctive. Body is transparent, extremely broad, flat and with long thoracic legs.

Lingula larva

Minor Phylum	Brachiopoda
Class	Ecardines
Order	Atremata
Group	Lingula larva

It has a bivalved transparent shell with flat bottom enclosing the whole body. This is made up of coelomate unsegmented with a dorsal and ventral shell. The tentacles are distinct. The very young larva is half-moon shaped or slightly oval. The larva grows bigger; it becomes circular or oval again with the shorter axis from side to side.

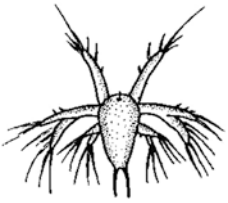
Trochophore larvae

Phylum	Annelida
Class	Polychaeta
Group	Trochophore larvae

Almost spherical body with little tuft of long cilia and sensory cells at the upper pole; the mouth is located equatorially; a main ciliated girdle runs around the sphere just above the equator.

Nauplii of *Penaeus indicus*

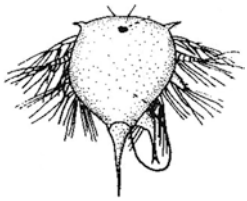
Phylum	Arthropoda
Class	Crustacea
Order	Decapod
Suborder	Macrura
Group	Shrimp nauplii



Ocellus present at the anterior median region of the body; presence of a pair of dorsally curved caudal setae at the posterior end of the body; first pair of appendage is uniramous, and second and third pair is biramous, with the third pair shorter than the other appendage.

Nauplii of *Balanus*

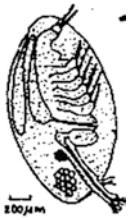
Phylum	Arthropoda
Class	Crustacea
Order	Decapod
Suborder	Macrura
Group	Barnacle nauplii



Body is triangular; presence of pair of posterior spines; tip of the rostrum is truncated.

Cypris of *Balanus*

Phylum	Arthropoda
Class	Crustacea
Order	Decapod
Suborder	Macrura



Presence of a bivalve shell; presence of a compound type and six pairs of thoracic appendages; consists of a short abdomen.

Veliger of Molluscs

Phylum	Mollusca
Class	Gastropoda/Bivalvia
Order	Thecosomata
Group	Veliger larva

Larvae are 1 ½ whorled shape; characterized by two dark pigmented particles; it is pale yellowish and has no sculpturing.

Bipinnaria larva of starfish

Phylum	Echinodermata
Class	Asteroidea
Group	Bipinnaria larva

Presence of a wavy band of cilia at the equatorial girdle; this band runs down on each side and loops around the front above and below where the mouth begins to form; before the mouth opens, the gut is developed with a tiny stomach and intestinal opening at the lower end of larva.

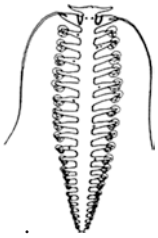
Brachiolaria larva

Phylum	Echinodermata
Class	Asteroidea
Group	Brachiolaria larva

Composed of three special appendages in the preoral region called brachiolarian arms; presence of adhesive papillae on the arms except for the medio-dorsal one; the arms are unciliated except the medio-dorsal one that bears circum-oral ciliary band.

Tomopteris sp.

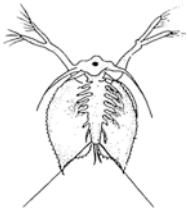
Phylum	Annelida
Order	Errantia
Genus	*Tomopteris*
Species	*Tomopteris* sp.



Eyes are small or lacking. Few setae were present or none. There is a pair of nuchal organs on each side of the head. The first pair of setiferous segments is often lost in the adult, and the second pair is often very elongated.

Penilia avirostris Dana

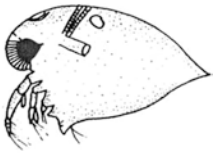
Order	Cladocera
Family	Polyphenidae
Genus	*Penilia*
Species	*P. avirostris*



Carapace forming a bivalve shell. The carapace does not cover the head and it has beaklike rostrum.

Evadne tergestina Claus

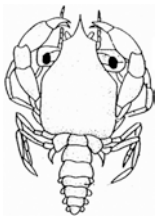
Order	Cladocera
Family	Polyphenidae
Genus	*Evadne*
Species	*E. tergestina*



The greatly reduced shell forming a brood sac and is cone shaped and pointed.

Larva of Brachyura megalopa

Phylum	Arthropoda
Class	Crustacea
Order	Cirripedia
Suborder	Brachiura
Genus	*Scylla*
Species	*S. serrata*



This stage appears in a period of 20 days. While all the appendages are well developed, the abdomen with five pairs of swimming pleopods is held in an extended position. The carapace, unlike in zoea, is depressed. A rostral spine long and slightly curved.

Creseis acicula Lane

Phylum	Mollusca
Class	Gastropoda
Order	Thecosomata
Genus	*Creseis*
Species	*C. acicula*

It is adult molluscan plankton. It is characterized by a pair of winglike extensions called parapodia with which they flap their way through the water. The wings are modified foot. The conical-shaped shell is either very thin or delicate. It is distinct and very often indicative of a seasonal cycle in hydrographic condition.

Fish egg and larva

Phylum	Chordata
Class	Pisces
Group	Fish egg and fish larva

Most of the fish eggs are generally spherical in shape with a clean yolk and oil globules. The fish larva has prominent eyes and mouthy, longer lower jaw, with an elongated tail fin. Pigments are often found on the body especially on the head and pectoral regions.

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Seasonal Composition and Diversity of Zooplankton from Muthupet Mangrove Wetland Ecosystem, Southeast Coast of India

P. Santhanam, N. Jeyaraj, J. Sivakumar, K. Jothiraj, S. Dinesh Kumar, and S. Ananth

Introduction

The mangrove environment has been considered as a plankton abundant area (Robertson and Blaber 1992), and it acts as feeding and nursery platform for a variety of secondary consumers like fish and invertebrates (Chong 2007). The carbon source of mangrove wetland is the main factor for their high productivity which has always been linked to detritus-based food web (Odum and Heald 1975). Zooplankters are tiny organism which was abundantly available in all depth of the ocean. The Indian Ocean plankton bibliography was composed by Prasad (2003). Subramanian (1987) has introduced the ecological survey of primary consumers in Indian Ocean. While going in to the mangrove-associated fishes, their main primary food is zooplankton, and also there is a need to study their community structure and abundance in relation with the environment (Chong 2007). Some of the researchers has made attempt on zooplankton ecology previously in the mangrove ecosystem worldwide (Robertson et al, 1988; McKinnon and Klumpp 1998; Krumme and Liang 2004). Studying about zooplankton ecology is an important feature of biological oceanography because of their major role in the marine food chain of the aquatic environment. Zooplankton dominantly occupied the intermediate level between the primary and tertiary producers. Zooplankton distribution and their life cycle have been determined by the physical and chemical characteristics of the marine environment.

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The present study dealt with the assessment of diversity and abundance of zooplankton from Muthupet mangrove wetland ecosystem, Southeast coast of India, in relation to the environmental factors.

Materials and Methods

Study Area

The Muthupet mangrove wetland is situated in the southern most end of the Cauvery Delta in the Thiruvarur district of Tamil Nadu (lat. 10° 20'N and long. 79° 35′E). It is part of a bulky coastal wetland complex called the Great Vedaranyam swamp. The lagoon was receiving huge amount of freshwater for the duration of northeast monsoon (October–January) which was containing soils from agriculture, mangrove leaf wastes, and excessive nutrients and wastes from shrimp- and fish-cultured ponds. The present study dealt with monthly variation of zooplankton in Muthupet mangrove area for 3 years from the period April 2008 to March 2011, and the sampling location was shown in Fig. 1. The samples were collected from three places. Station 1 is the starting point of the Muthupet lagoon, and this place was connected to the Palk Strait which was 1 km wide, and the depth ranges from 1.0 to 2.0 m; the bottom is characterized by fine silt. Sallimunai selected as station 2 which was located about 2 km away from the mouth of the lagoon. This place has 0.2–2 m depth with clayey silt as a bottom. For this study Sethukuda was chosen as station 3 which was in the western side of the Muthupet lagoon. Mostly this place has received bulk of freshwater with 0.5–1.0 m depth and silty clay as a bottom.

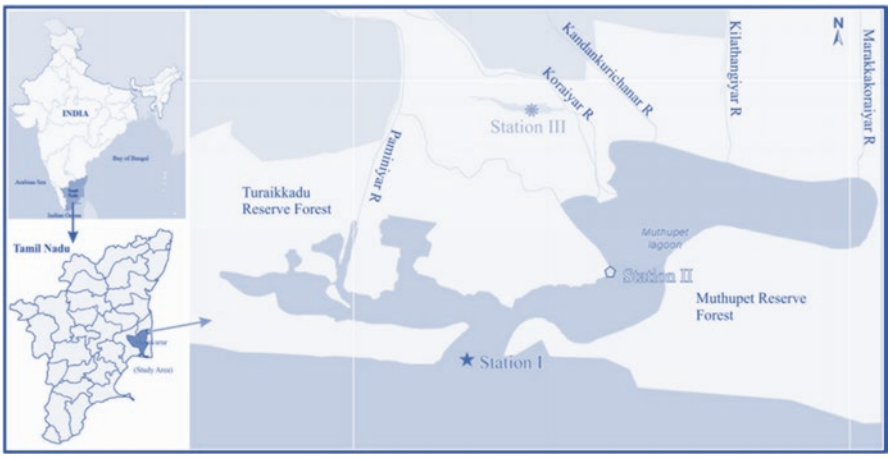


Fig. 1 Study area

Methodology

For estimation of physicochemical, biological parameters, the surface water samples were collected from the Muthupet mangrove at different stations by monthly intervals. Temperatures (atmospheric and surface water) were measured using a standard centigrade thermometer. Salinity was evaluated using refractometer (ERMA, Hand Refractometer, Japan). The pH was estimated using a standard ELICO grip pH meter. The dissolved oxygen was estimated by Winkler's method which was described by Strickland and Parsons (1972). After collection, the surface water samples was stored in polyethylene bottles and kept immediately in a temperature-controlled box and shifted immediately to the laboratory for further nutrient analysis. Before estimating nutrients, the water samples were filtered using a Millipore filtering systems with the help of 0.45 μM GFC filter paper, and then the inorganic nitrate, nitrite, phosphate, and reactive silicate were analyzed by standard procedures which were described by Strickland and Parsons (1972). Three-year rainfall data was obtained from the Indian Meteorological Unit, Govt. of India, located at Thiruvarur.

The collection of zooplankton was made by horizontal tows by using conical cylindrical plankton net with 2 m length and 0.5 m mouth diameter and equipped with a General Oceanics flow meter with 158 μm mesh. The collected zooplankton samples were fixed with 5% buffered formaldehyde for further analysis. Zooplankton samples were sorted under stereomicroscope, and their identification was made by the classical works of Kasturirangan (1963), Smith (1977), Todd and Lawerack (1991), Santhanam and Srinivasan (1994), Todd et al. (1996), Perumal et al. (1998), Ajmal Khan et al. (2001), Antony Fernando and Olivia Fernando (2002), and Young (2006). For quantitative analysis, zooplankton samples were placed on the counting chamber and counted under light microscope. Species diversity, richness, and evenness were calculated by using diversity software package (PAST – Paleontological Statistics, ver. 1.89). Simple correlation coefficient was used for interpretation.

Results

Physicochemical Parameters

The physicochemical characteristics of Muthupet water are mainly depending on the pattern of land runoff, and their seasonal variations were regulated by four different seasons, viz., post-monsoon (January–March), summer (April–June), pre-monsoon (July–September), and monsoon (October–December).Totally 5531 mm of rainfall was recorded for the period of 3 years from April 2008 to March 2011. The monthly rainfall was ranged between 5.8 and 986 mm during the period from April 2008 to March 2011. No rainfall was recorded during the month of February 2008, April–July 2009, February and March 2010, and April 2010 and March 2011.

The maximum rainfall was recorded during the month of November 2008 whereas the minimum during the month of January 2011 (Figs. 2a, 2b and 2c). The atmospheric temperature was recorded in the range between 25 and 34, 27 and 34, and 29 and 35 °C at stations 1, 2, and 3, respectively (Figs. 3a, 3b and 3c). The minimum temperature (25 °C) was recorded during the month of December 2008 at station 1, whereas the maximum (35 °C) was recorded during April 2009 at station 3. The surface water temperature was recorded in the range between 26 and 33.5 at station 1, 28 and 33.8 at station 2, and 28 and 34 at station 3. The minimum temperature (26 °C) was observed during the month of December 2008 at station 1, and maximum water temperature (34 °C) was noticed during the summer season at all the stations (Figs. 4a, 4b and 4c).

The recorded salinity of the Muthupet lagoon was varied from 7 to 39, 2 to 39, and 0.9 to 48‰ at stations 1, 2, and 3, respectively. The lowest salinity (0.9‰) was recorded during November 2010 at station 3, whereas the higher salinity (48‰) was observed during the month of July 2010 at station 3 (Figs. 5a, 5b and 5c). The hydrogen-ion concentration (pH) was recorded in the range between 7.5 and 8.49 at station 1, 7.23 and 8.5 at station 2, and 7.23 and 8.6 at station 3. The hydrogen-ion

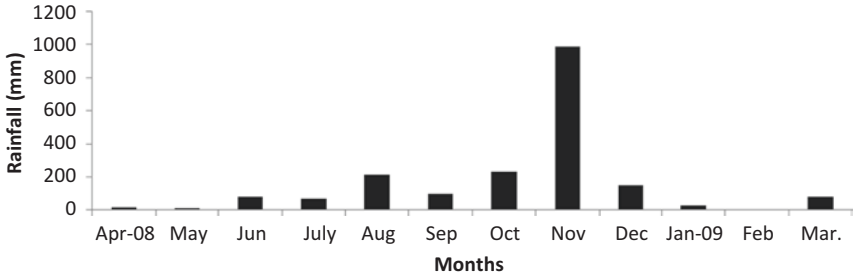


Fig. 2a Monthly variations in rainfall during April 2008–March 2009

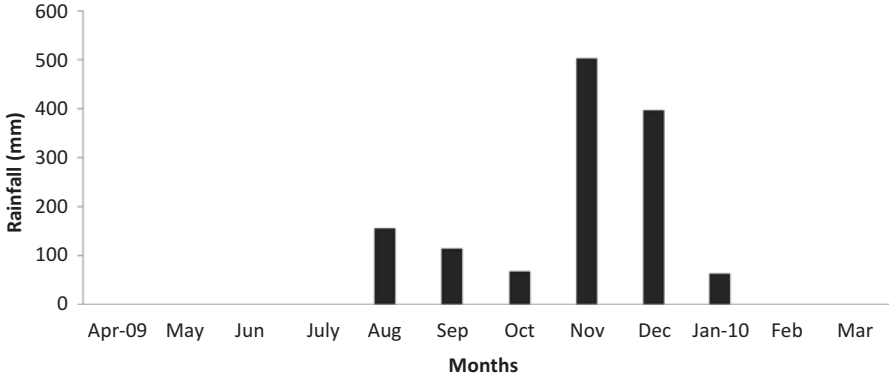


Fig. 2b Monthly variations in rainfall during April 2009–March 2010

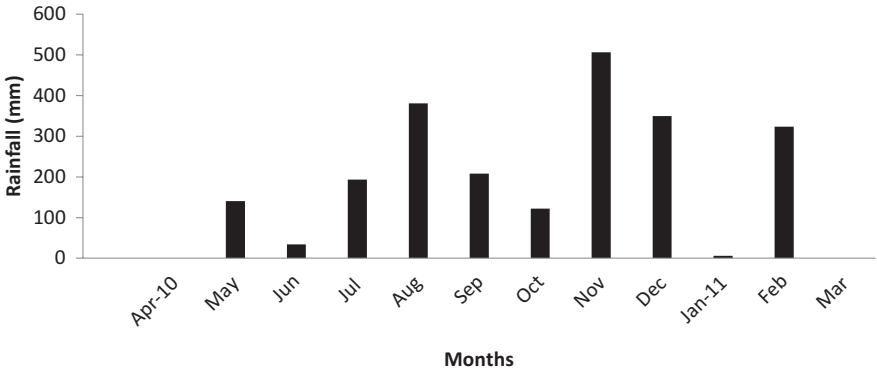


Fig. 2c Monthly variations in rainfall during April 2010–March 2011

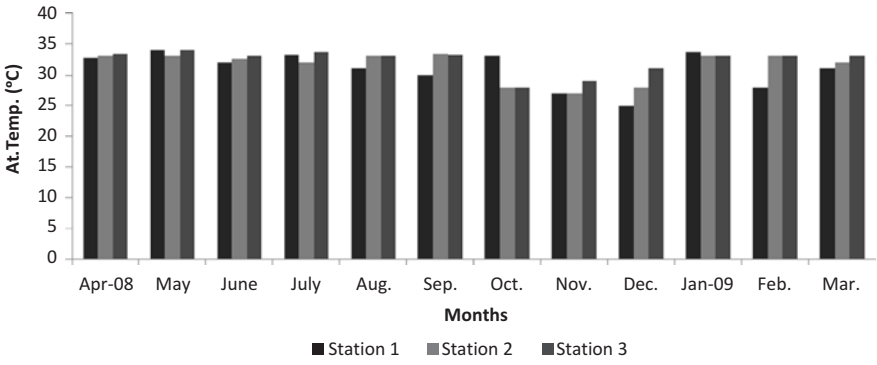


Fig. 3a Monthly variations in atmospheric temperature during April 2008–March 2009

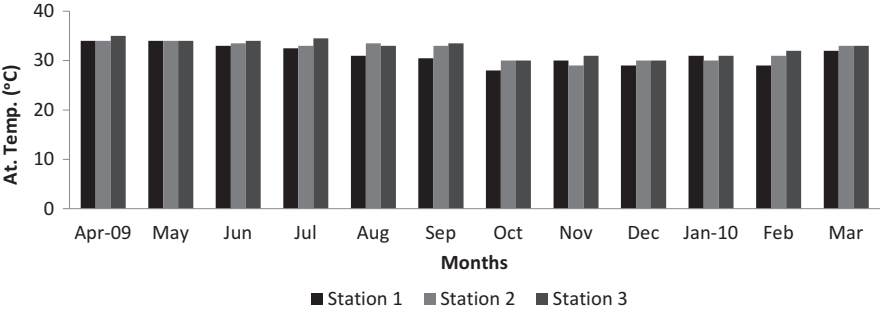


Fig. 3b Monthly variations in atmospheric temperature during April 2009–March 2010

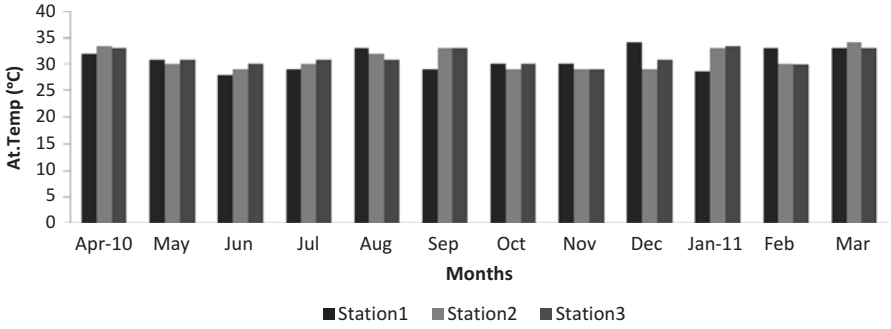


Fig. 3c Monthly variations in atmospheric temperature during April 2010–March 2011

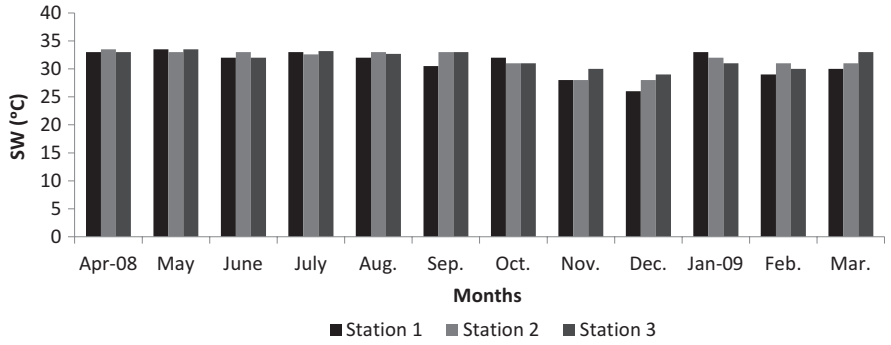


Fig. 4a Monthly variations in surface water temperature during April 2008–March 2009

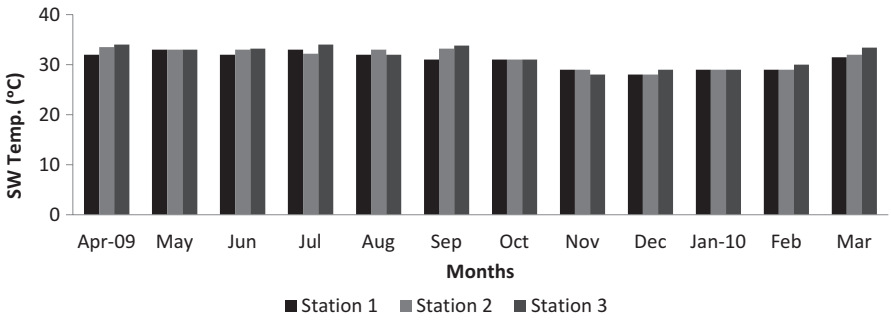


Fig. 4b Monthly variations in surface water temperature during April 2009–March 2010

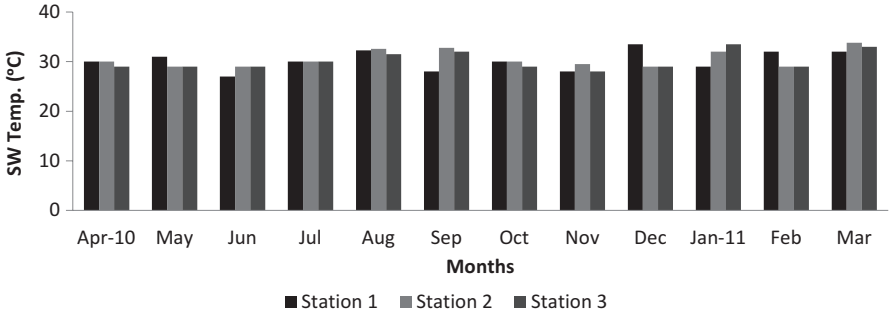


Fig. 4c Monthly variations in surface water temperature during April 2010–March 2011

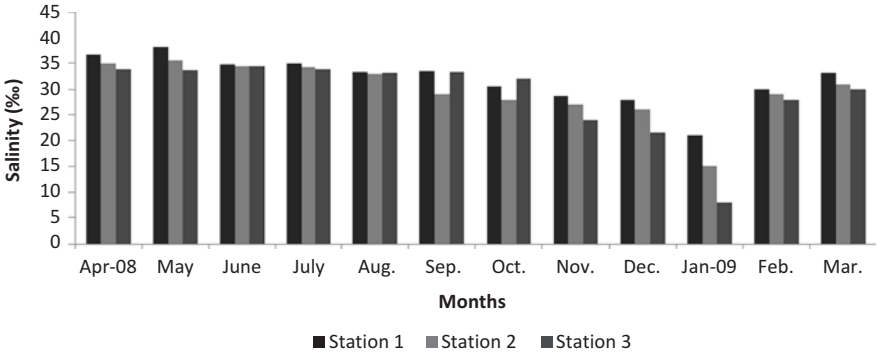


Fig. 5a Monthly variations in salinity during April 2008–March 2009

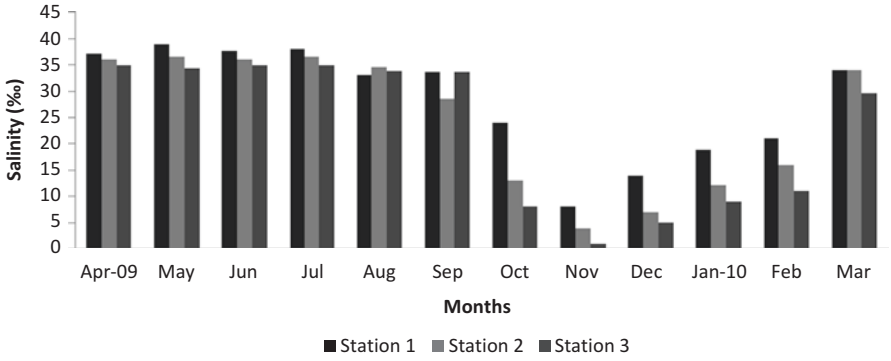


Fig. 5b Monthly variations in salinity during April 2009–March 2010

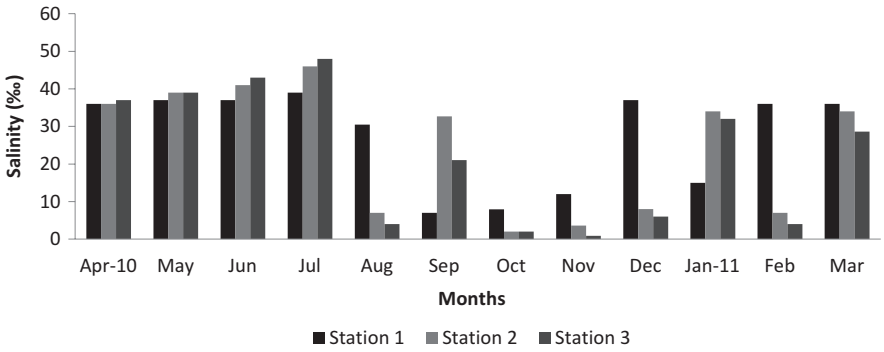


Fig. 5c Monthly variations in salinity during April 2010–March 2011

concentration was found too high (8.63) during the month of April 2010 at station 3 and low (7.23) during the month of February 2011 in station 2 (Figs. 6a, 6b and 6c). The dissolved oxygen concentration was recorded in the range between 2.71 and 4.72, 2.49 and 4.98, and 1.93 and 5.8 ml/l at stations 1, 2, and 3, respectively. The maximum dissolved oxygen concentration was noticed during the month of February 2011 at station 3, whereas the minimum was recorded during the month of July 2010 at station 3 (Figs. 7a, 7b and 7c).

The inorganic nitrate concentration (μM) was recorded in the range between 15.0 and 52.68 at station 1, 22 and 89 at station 2, and 14.23 and 89.2 at station 3. The maximum value (89.2μM) of nitrate was observed during the month of April 2010 at station 3 and the minimum (14.2μM) during the month of March 2010 at station 3 (Figs. 8a, 8b and 8c). The recorded inorganic nitrite concentration (μM) of stations 1, 2, and 3 was varied from 0.13 to 1.77, 0.21 to 1.63, and 0.15 to 1.96, respectively. The maximum nitrite was recorded during the month of November 2008 at station 3 whereas the minimum value during the month of April 2009 at station 1 (Figs. 9a, 9b and 9c). The concentration of phosphate (μM) was varied from 7.89 to 24.56, 9.67 to 43.1, and 11.23 to 35.7 at stations 1, 2, and 3, respectively. The minimum phosphate content was recorded during April 2009 at station 1, whereas the maximum phosphate content was recorded during April 2010 at station 2 (Figs. 10a, 10b and 10c). The reactive silicate concentration was always higher than other nutrients (NO_3, NO_2, and PO_4). The concentration (μM) of silicate was reported in the range between 21.35 and 209.45, 22 and 297.14, and 52.2 and 316.84 at stations 1, 2, and 3, respectively. The maximum silicate was noticed during monsoon season (November 2008) at station 3, whereas the low silicate content was reported during post-monsoon (January 2011) season at station 1 (Fig. 11a, 11b and 11c).

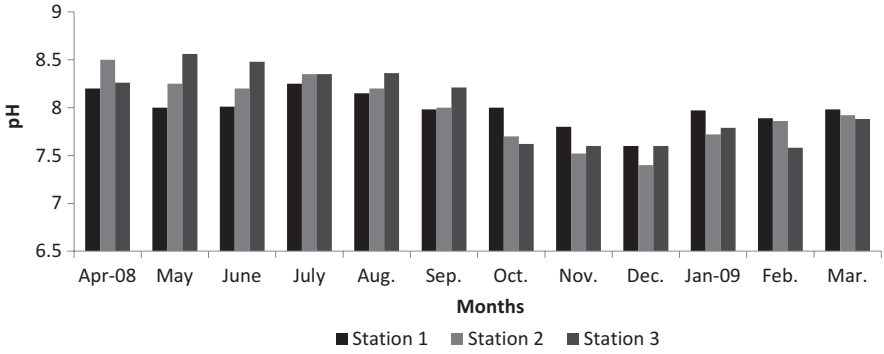


Fig. 6a Monthly variations in pH during April 2008–March 2009

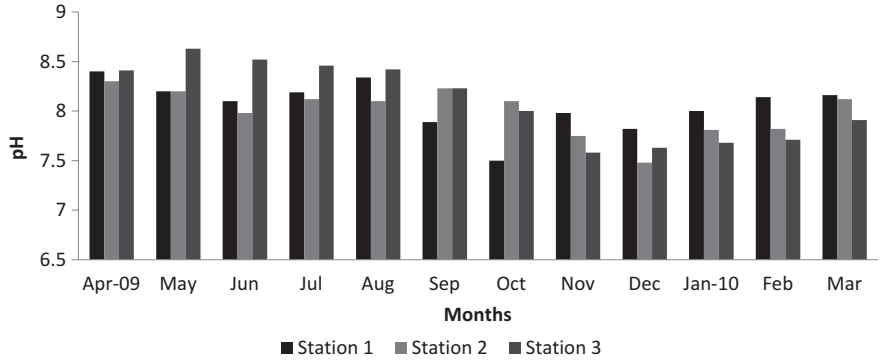


Fig. 6b Monthly variations in pH during April 2009–March 2010

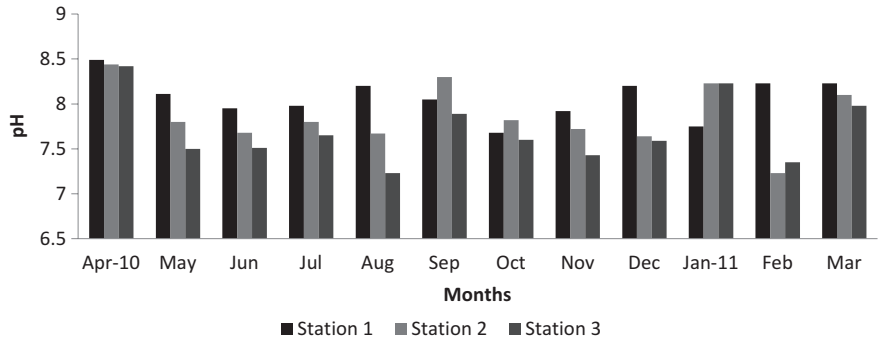


Fig. 6c Monthly variations in pH during April 2010–March 2011

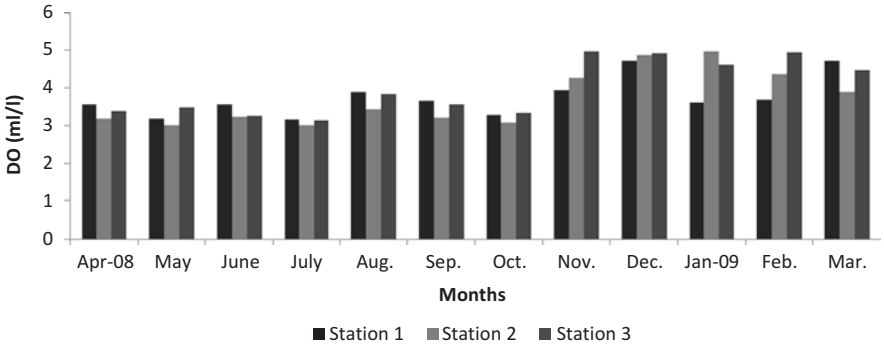


Fig. 7a Monthly variations in dissolved oxygen during April 2008–March 2009

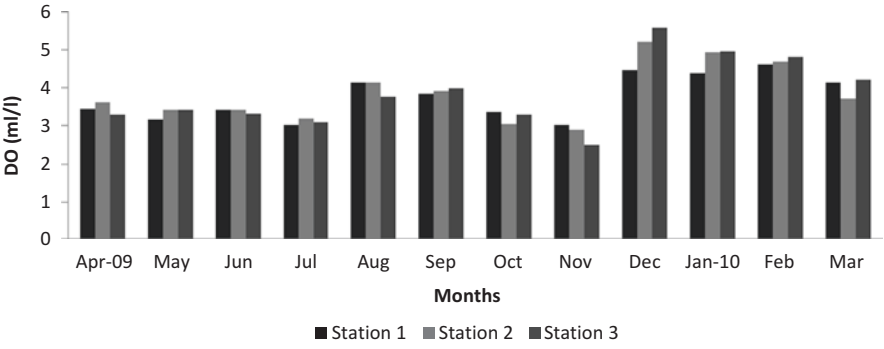


Fig. 7b Monthly variations in dissolved oxygen during April 2009–March 2010

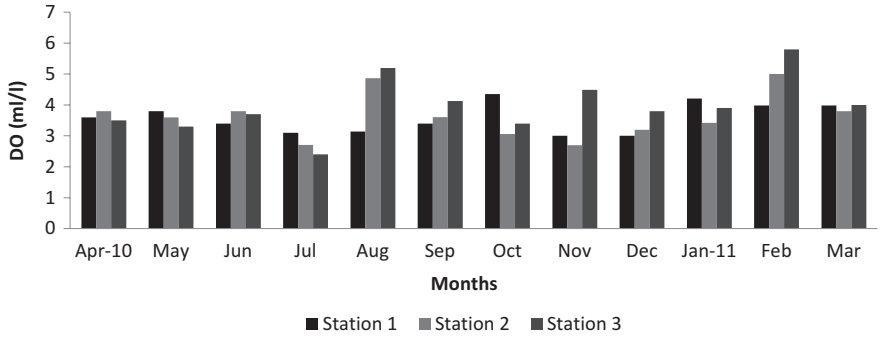


Fig. 7c Monthly variations in dissolved oxygen during April 2010–March 2011

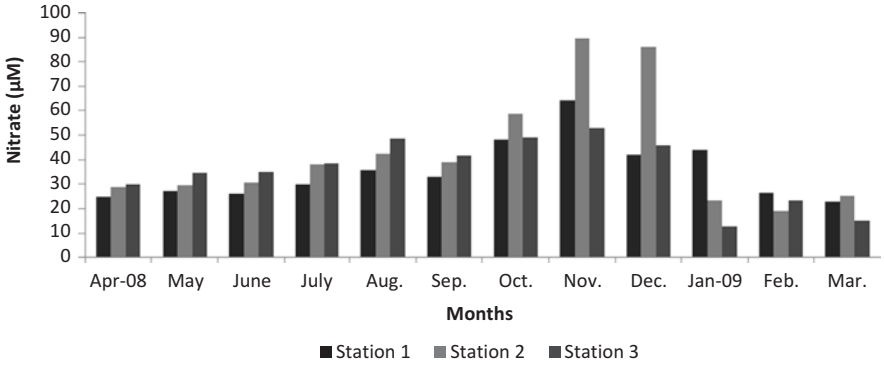


Fig. 8a Monthly variations in inorganic nitrate during April 2008–March 2009

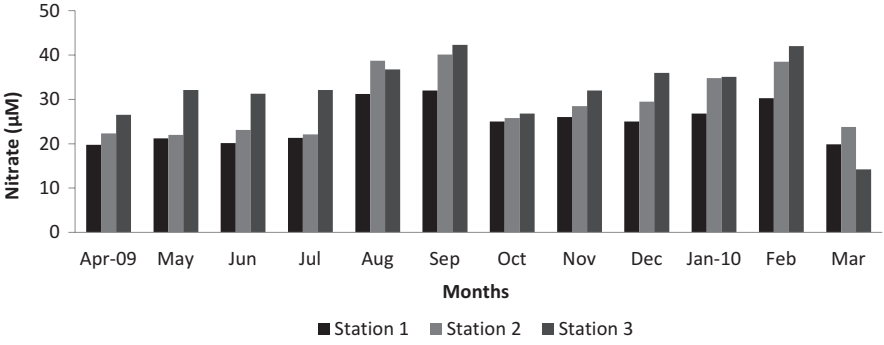


Fig. 8b Monthly variations in inorganic nitrate during April 2009–March 2010

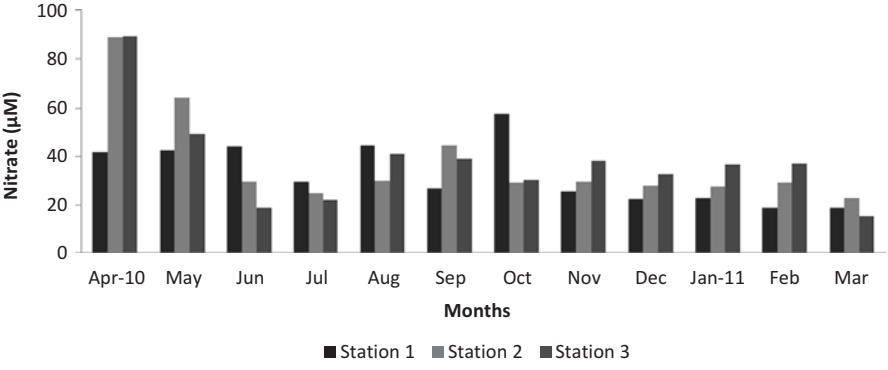


Fig. 8c Monthly variations in inorganic nitrate during April 2010–March 2011

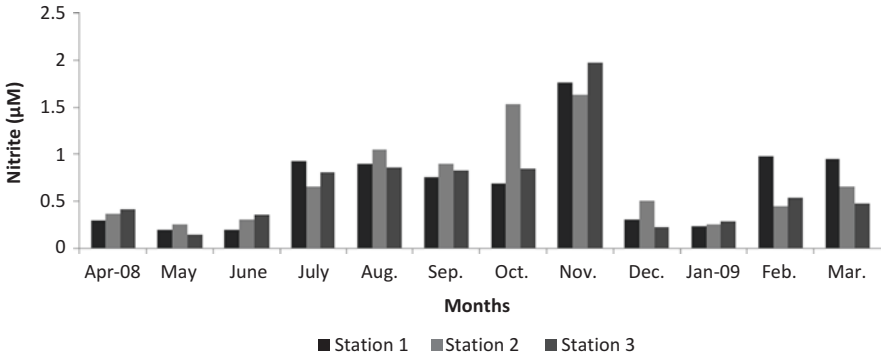


Fig. 9a Monthly variations in inorganic nitrite during April 2008–March 2009

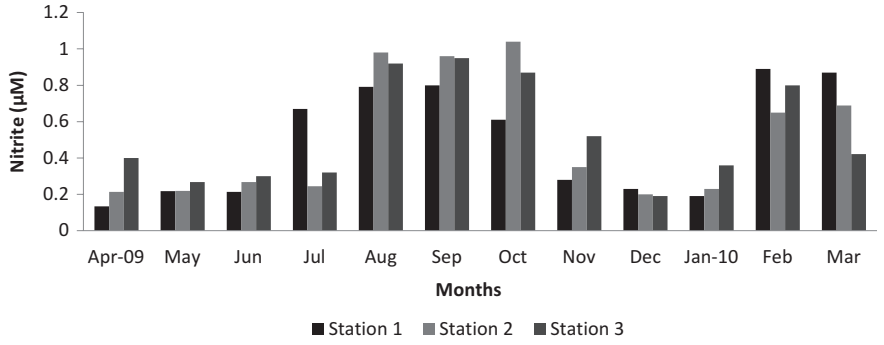


Fig. 9b Monthly variations in inorganic nitrite during April 2009–March 2010

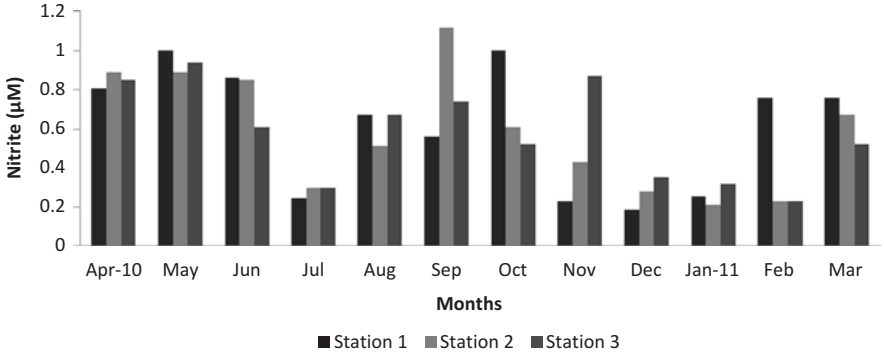


Fig. 9c Monthly variations in inorganic nitrite during April 2010–March 2011

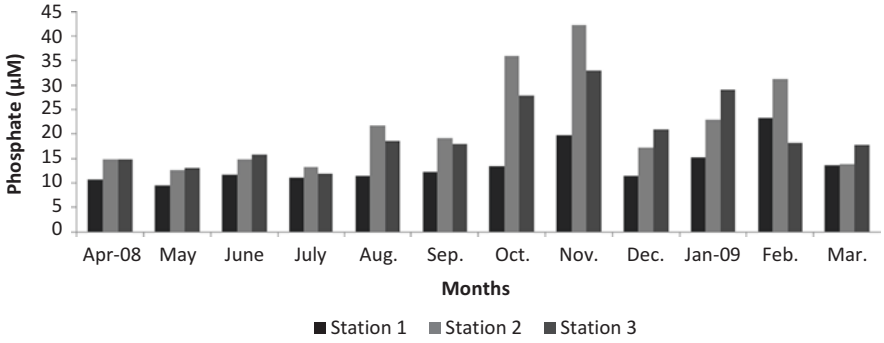


Fig. 10a Monthly variations in inorganic phosphate during April 2008–March 2009

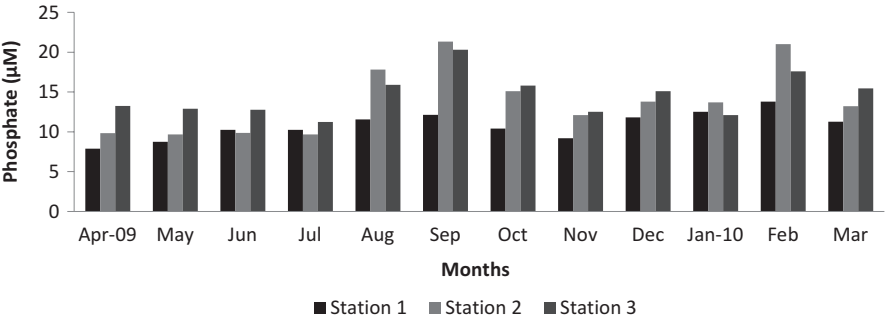


Fig. 10b Monthly variations in inorganic phosphate during April 2009–March 2010

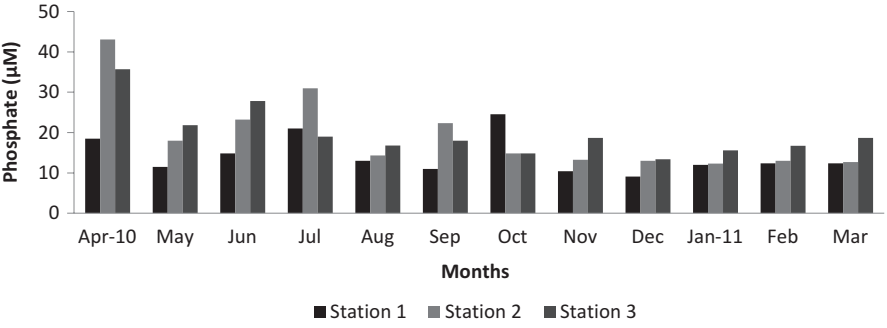


Fig. 10c Monthly variations in inorganic phosphate during April 2010–March 2011

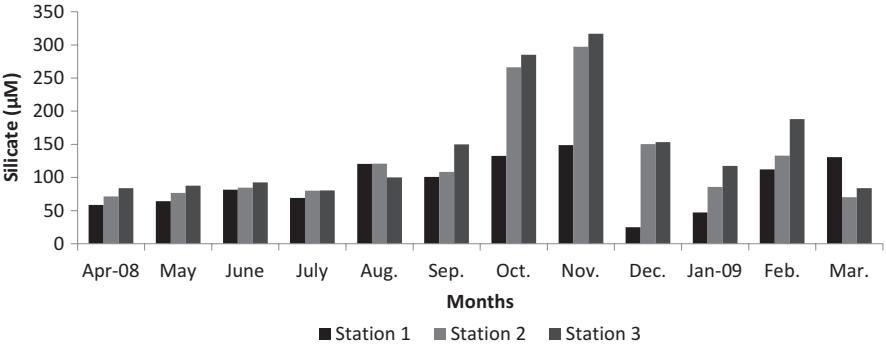


Fig. 11a Monthly variations in reactive silicate during April 2008–March 2009

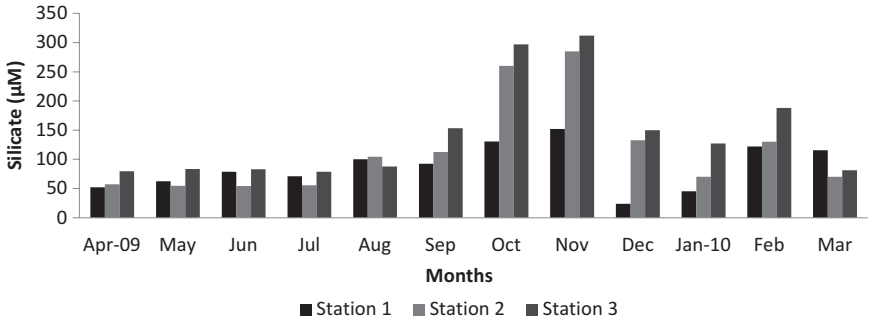


Fig. 11b Monthly variations in reactive silicate during April 2009–March 2010

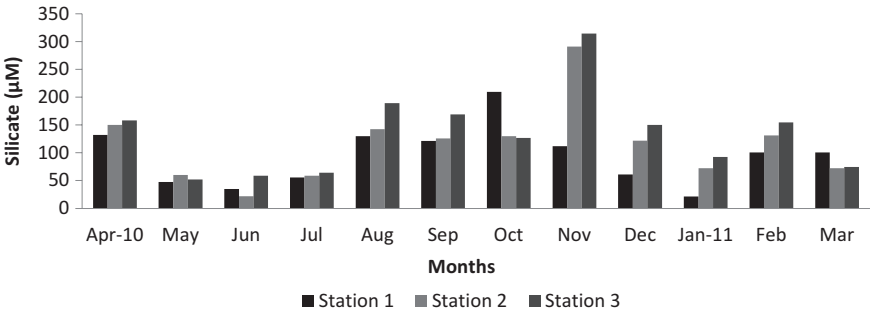


Fig. 11c Monthly variations in reactive silicate during April 2010–March 2011

Zooplankton

Species Composition

Totally 114 species of zooplankton belonging to 8 groups and 43 genera were identified from the Muthupet mangrove waters (Table 1) for the period of 3 years from April 2008 to April 2011. Among these, 74 species recorded belonged to Copepoda, 11 to Ciliata, 13 to Rotifera, 6 to foraminifera, 5 to Cladocera, 3 to Chaetognatha, and 1 each to decapoda and Polychaeta. Totally 96 species of zooplankton were reported at station 1, of these 68 species belonged to copepods; 11 to Ciliata; 6 to foraminifera; 3 each to Chaetognatha, Rotifera, and Cladocera; and 1 each to decapoda and Polychaeta. At station 2, totally 77 species of zooplankton were noticed. Of these, copepod constituted 53 species, foraminifera 4, Ciliata 5, Chaetognatha 3, Rotifera 7, Cladocera 3, and decapoda and Polychaeta each with 1. At station 3, totally 72 species of zooplankton were recorded which comprised of 41 species of copepods followed by rotifers (11), ciliates (6), Cladocera (5), foraminifera (4), Chaetognatha (3), and 1 each of Polychaeta and decapoda.

Percentage Composition

The copepods contributed for 71.87% followed by Ciliata with 15.66%, Chaetognatha with 3.19%, rotifer with 2.32%, decapoda with 1.37%, Cladocera with 1.32%, foraminifera with 1.64, and Polychaeta with 1.57 at station 1 (Figs. 12a, 13a, 14a). At station 2, copepods contributed with 68.89% followed by ciliate (8.76%), rotifer (7.62%), Chaetognatha (5.45%), decapoda (3.46%), Cladocera (2.69%), and foraminifera (3.36%) (Figs. 12b, 13b, 14b). At station 3, copepods comprised of 63.61%, followed by rotifer 14.17%, ciliate 9.59%, Cladocera 5.16%, Chaetognatha 2.45%, decapoda 2.52, and foraminifera 2.43% (Figs. 12c, 13c, 14c).

Population Density

The recorded population density of zooplankton was varied from 1,42,000 to 15,62,000 nos./m^3 at station 1. At station 2, it was encountered between 52,176 and 13,62,000 nos./m^3, and at station 3, the population density was ranged from 19,934 to 16,00,000 nos./m^3. The low zooplankton population was noticed during monsoon and post-monsoon seasons at stations 1, 2, and 3, respectively, and high population density was encountered during summer season at all the stations (Figs. 15a, 15b and 15c).

Table 1 List of zooplankton recorded from Muthupet mangrove waters during April 2008–March 2011

S. no	*Species Name*	Stn. *1*	Stn. *2*	Stn. *3*
	Copepoda-Calanoida			
1	*Acartia centrura*	+	+	+
2	*Acartia clausi*	+	+	+
3	*Acartia danae*	+	+	+
4	*Acartia erythraea*	+	+	+
5	*Acartia negligens*	+	+	+
6	*Acartia sewelli*	+	+	+
7	*Acartia southwelli*	+	+	+
8	*Acartia spinicauda*	+	+	+
9	*Acrocalanus gibber*	+	+	+
10	*Acrocalanus gracilis*	+	+	+
11	*Acrocalanus longicornis*	+	+	+
12	*Acrocalanus monachus*	+	+	–
13	*Candacia discaudata*	+	+	–
14	*Calanopia aurivilli*	+	+	+
15	*Canthacalanus pauper*	+	+	–
16	*Centropages calaninus*	–	+	+
17	*Centropages dorsispinalus*	+	–	–
18	*Centropages furcatus*	+	+	–
19	*Centropages orsinii*	+	–	–
20	*Centropages tenuiremis*	+	–	+
21	*Calanus tunicornis*	+	+	+
22	*Clytemnestra scutellata*	+	+	+
23	*Eucalanaus crassus*	+	+	+
24	*Eucalanaus elongatus*	+	+	+
25	*Eucalanus attenuatus*	+	–	–
26	*Eucalanus subcrassus*	–	–	+
27	*Euchaeta concinna*	–	+	–
28	*Lucicutia flavicornis*	+	–	–
29	*Labidocera acuta*	+	–	–
30	*Labidocera bengalensis*	–	+	–
31	*Labidocera minuta*	+	+	+
32	*Labidocera pavo*	+	+	+
33	*Labidocera pectinata*	+	+	+
34	*Nannocalanus minor*	+	+	+
35	*Paracalanus aculeatus*	+		+
36	*Paracalanus parvus*	+	+	+
37	*Pontella danae*	+	–	–
38	*Pontellopsis scotti*	+	+	+
39	*Pseudodiaptomus annandalei*	+	–	–

(continued)

Table 1 (continued)

S. no	*Species Name*	Stn. *1*	Stn. *2*	Stn. *3*
40	*Pseudodiaptomus aurivilli*	+	+	–
41	*Pseudodiaptomus serricaudatus*	+	+	–
42	*Rhincalanus cornutus*	+	–	–
43	*Rhincalanus nasutus*	+	–	–
44	*Scolecithrix danae*	+	+	–
45	*Temora discaudata*	+	+	–
46	*Temora stylifera*	+	–	–
47	*Temora turbinata*	+	+	+
48	*Tortanus barbatus*	+	–	–
49	*Tortanus gracilis*	–	+	–
50	*Undinula vulgaris*	+	–	–
	Harpacticoida			
51	*Bomolochus* sp.	+	–	+
52	*Euterpina acutifrons*	+	+	+
53	*Macrosetella gracilis*	+	+	+
54	*Macrosetella norvegica*	+	+	+
55	*Macrosetella oculata*	+	+	+
56	*Metacalanus aurivilli*	+	+	–
57	*Meteis jousseaumei*	+	+	–
58	*Microsetella gracilis*	+	+	+
59	*Microsetella norvegica*	+	+	+
60	*Microsetella rosea*	+	+	+
61	*Longipedia weberi*	+	+	+
62	*Longipedia coronata*	+	–	–
	Cyclopoida			
63	*Copilia mirabilis*	–	+	–
64	*Copilia vitrea*	+	+	–
65	*Corycaeus catus*	+	–	–
66	*Corycaeus danae*	+	–	–
67	*Corycaeus speciosus*	+	–	+
68	*Oithona brevicornis*	+	+	+
69	*Oithona linearis*	+	+	+
70	*Oithona plumifera*	+	+	+
71	*Dioithona rigida*	+	+	–
72	*Oithona similis*	+	+	–
73	*Oncaea venusta*	+	+	+
74	*Sapphirina ovatolanceolata*	+	–	–
	Foraminifera			
75	*Acanthometren* sp.	+	–	+
76	*Globigerina bulloides*	+	+	+
77	*Globigerina opima*	+	+	+

(continued)

Table 1 (continued)

S. no	*Species Name*	Stn. *1*	Stn. *2*	Stn. *3*
78	*Globigerina* sp.	+	+	+
79	*Chilostomella ovoidea*	+	+	–
80	*Pulvinulina menardii*	+	–	–
	Ciliata			
81	*Eutintinnus tenuis*	+	–	–
82	*Favella brevis*	+	–	+
83	*Favella philippinensis*	+	+	+
84	*Favella* sp.	+	+	+
85	*Tintinnopsis butscheii*	+	+	+
86	*Tintinnopsis cylindrica*	+	–	+
87	*Tintinnopsis directa*	+	–	–
88	*Tintinnopsis minuta*	+	–	–
89	*Tintinnopsis tocantinensis*	+	+	–
90	*Tintinopsis bermudensis*	+	–	–
91	*Tintinopsis tubulosa*	+	+	+
	Polychaeta larvae			
92	*Tomopteris* sp.	+	+	+
	Chaetognatha			
93	*Sagitta enflata*	+	+	+
94	*Sagitta bipunctata*	+	+	+
95	*Sagitta serratodentata*	+	+	+
	Rotifers			
96	*Brachionus calyciflorus*	+	+	+
97	*Brachionus angularis*	+	+	+
98	*Brachionus bidentata*	–	–	+
99	*Brachionus caudatus*	–	–	+
100	*Brachionus falcatus*	+	–	+
101	*Brachionus plicatilis*	–	+	+
102	*Brachionus quadricornis*	–	+	+
103	*Brachionus quadridentatus*	–	–	+
104	*Brachionus rubens*	–	+	+
105	*Brachionus urceolaris*	–	+	–
106	*Monostyla quadridentata*	–	–	+
107	*Platyias quadricornis*	–	–	+
108	*Filinia longiseta*	–	+	–
	Cladocera			
109	*Evadna* sp.	+	+	+
110	*Evadne tergestina*	–	–	+
111	*Moina* sp.	–	–	+
112	*Penilia avirostris*	+	+	+
113	*Bosmina* sp.	+	+	+
	Decapoda			
114	*Lucifer hansenii*	+	+	+

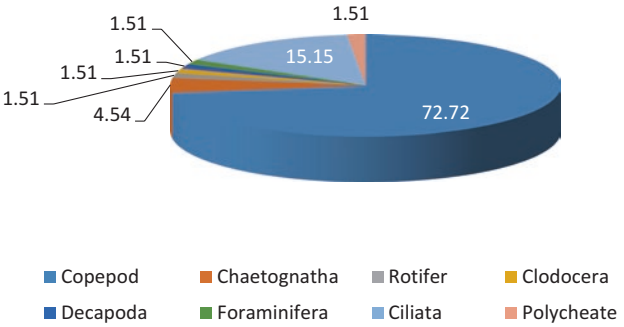


Fig. 12a Percentage composition of zooplankton at station 1 during April 2008–March 2009

Fig. 12b Percentage composition of zooplankton at station 2 during April 2008–March 2009

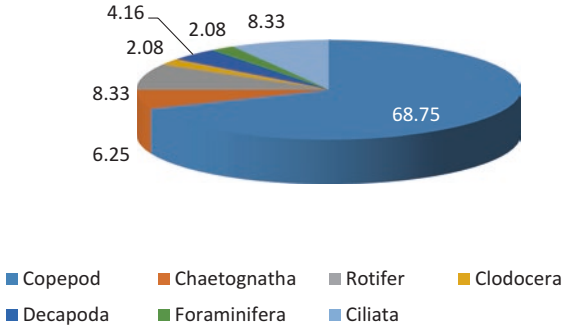
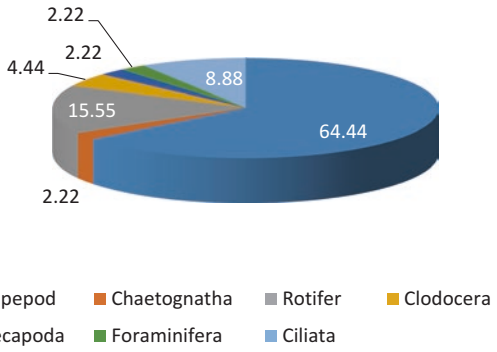


Fig. 12c Percentage composition of zooplankton at station 3 during April 2008–March 2009



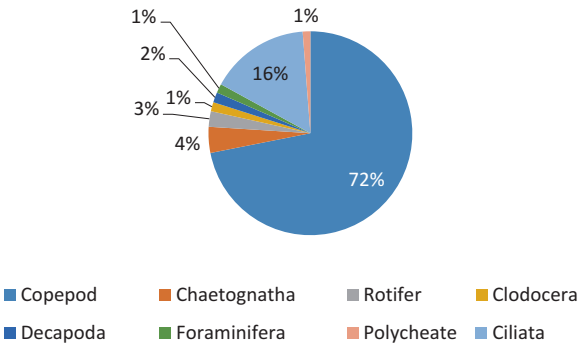


Fig. 13a Percentage composition of zooplankton at station 1 during April 2009–March 2010

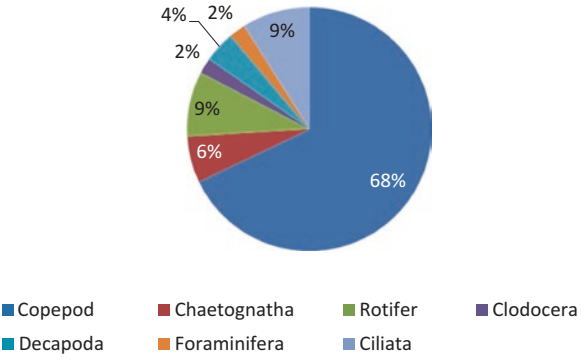


Fig. 13b Percentage composition of zooplankton at station 2 during April 2009–March 2010

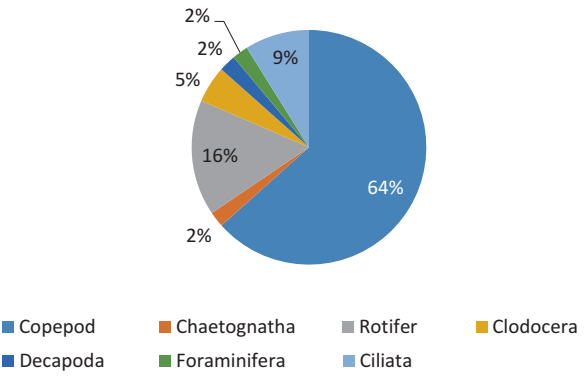


Fig. 13c Percentage composition of zooplankton at station 3 during April 2009–March 2010

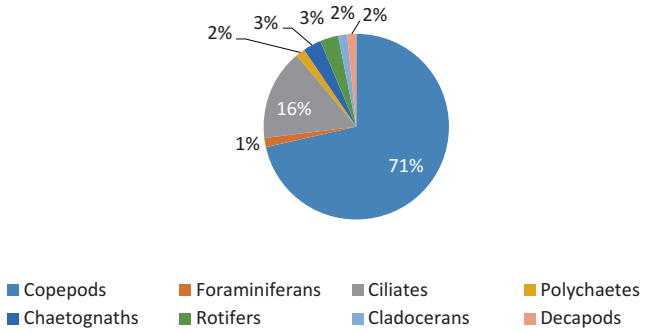


Fig. 14a Percentage composition of zooplankton at station 1 during April 2010–March 2011

Fig. 14b Percentage composition of zooplankton at station 2 during April 2010–March 2011

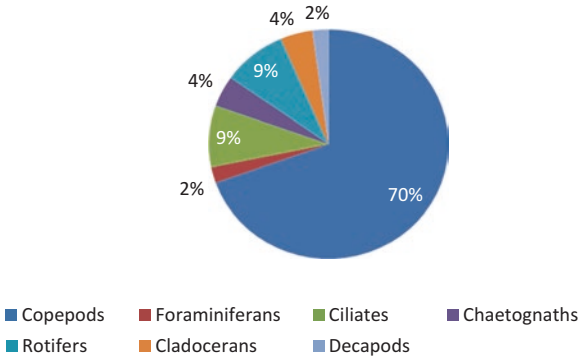
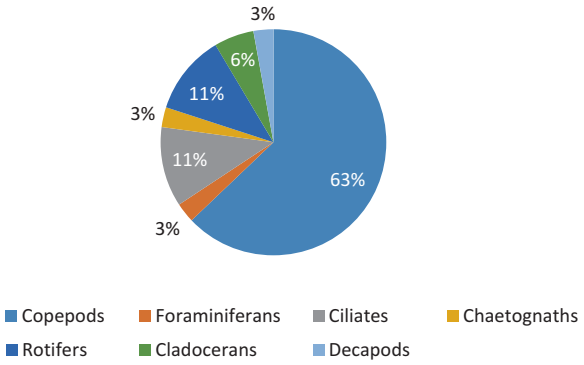


Fig. 14c Percentage composition of zooplankton at station 1 during April 2010–March 2011



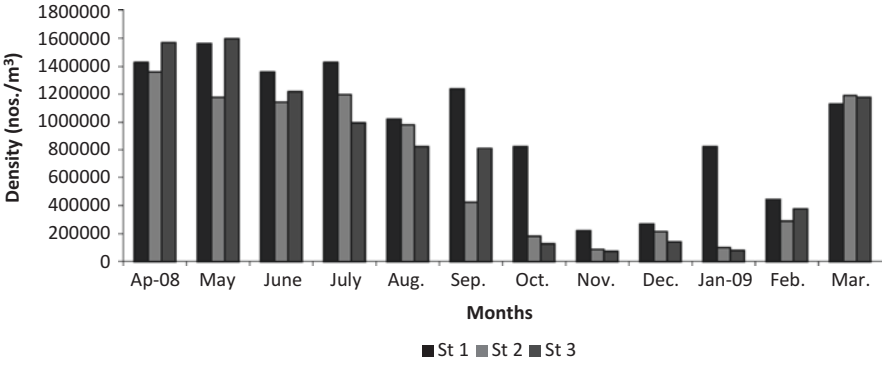


Fig. 15a Monthly variations in zooplankton density from April 2008–March 2009

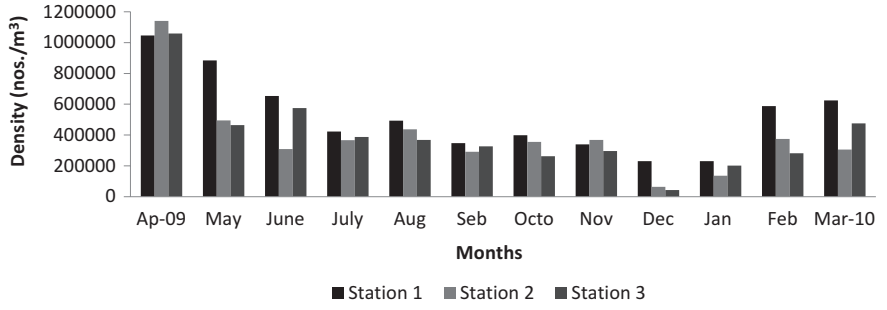


Fig. 15b Monthly variations in zooplankton density during April 2009–March 2010

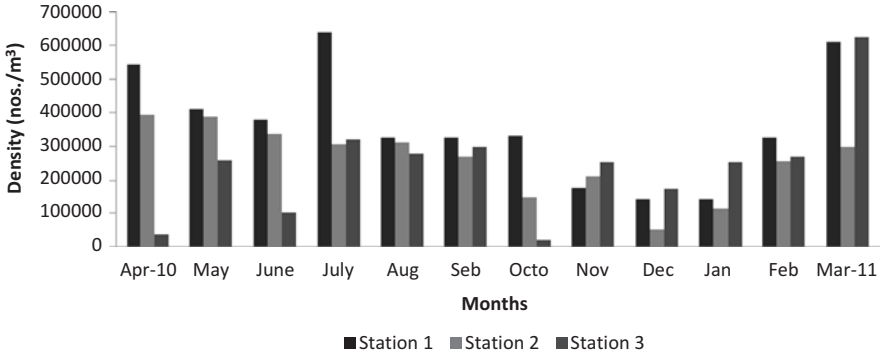


Fig. 15c Monthly variations in zooplankton density during April 2010–March 2011

Species Diversity

The zooplankton species diversity was reported in the range between 2.09 and 4.98, 3.02 and 4.80, and 2.42 and 4.24 at stations 1, 2, and 3, respectively. The least species diversity was encountered during the monsoon and post-monsoon seasons, and maximum species diversity was noticed during summer season (Figs. 16a, 16b and 16c).

Species Richness

The zooplankton species richness was reported in the range between 0.64 and 0.95, 0.15 and 0.98, and 0.5 and 0.94. The minimum species richness was recorded during the summer (station 1), post-monsoon (station 2), and monsoon (station 3) seasons, and the maximum value was noticed during the summer, post-monsoon and pre-monsoon (station 1), summer and pre-monsoon (station 2), summer, pre-monsoon, and post-monsoon seasons (Figs. 17a, 17b and 17c).

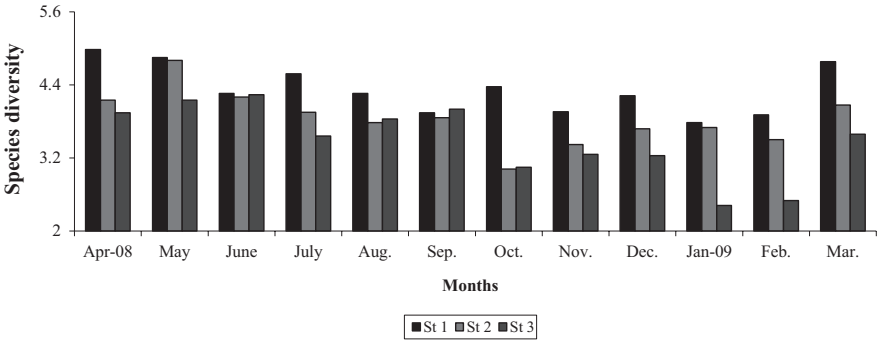


Fig. 16a Monthly variations in zooplankton species diversity during April 2008–March 2009

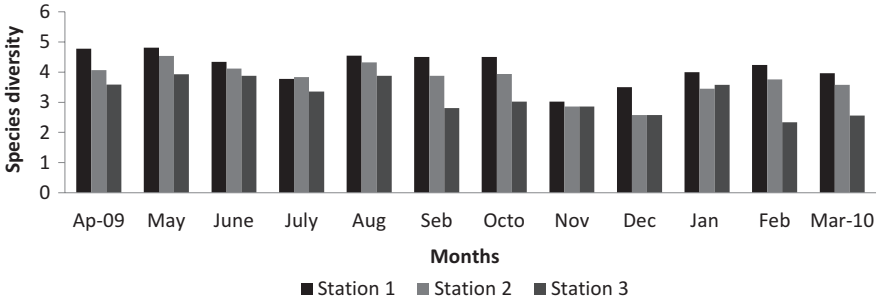


Fig. 16b Monthly variations in zooplankton species diversity during April 2009–March 2010

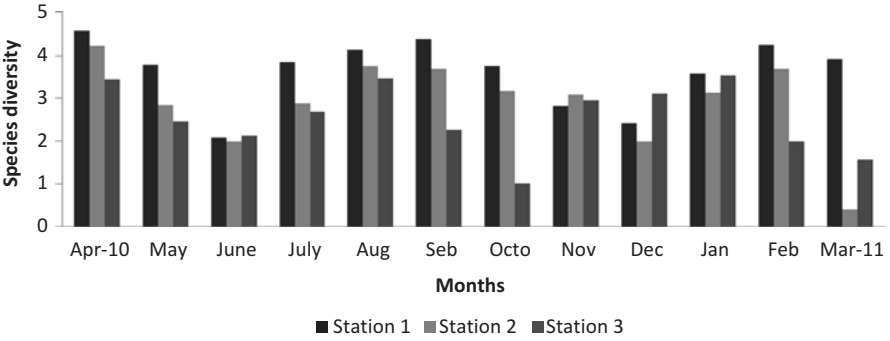


Fig. 16c Monthly variations in zooplankton species diversity during April 2010–March 2011

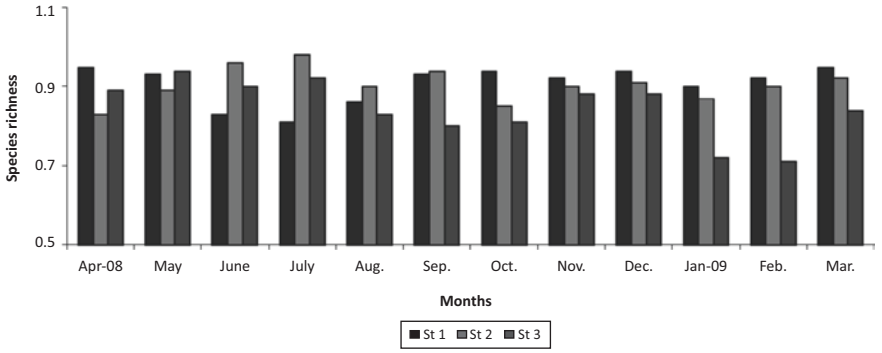


Fig. 17a Monthly variations in zooplankton species richness during April 2008–March 2009

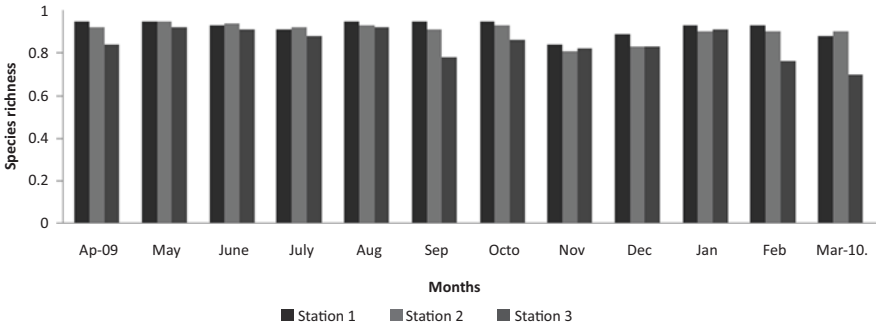


Fig. 17b Monthly variations in zooplankton species richness during April 2009–March 2010

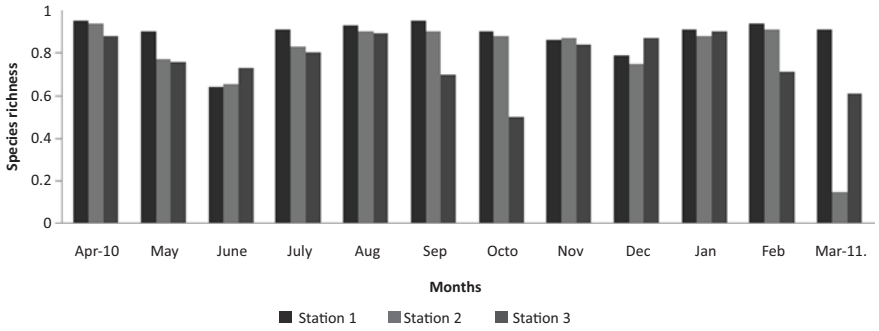


Fig. 17c Monthly variations in zooplankton species richness during April 2010–March 2011

Species Evenness

The recorded zooplankton species evenness was varied from 0.74 to 1.00, 0.41 to 1.00, and 0.73 to 1.00 at stations 1, 2, and 3, respectively. The low species evenness was observed during the month of March 2011 at station 2, whereas the maximum species evenness was noticed during monsoon and post-monsoon seasons (Figs. 18a, 18b and 18c). The correlation matrix results between the physicochemical and zooplankton were showed in Tables 2, 3, 4, 5, 6, 7, 8, 9, and 10.

(*$P < 0.01$; **$P < 0.001$)

Discussion

The present study inferred that the physicochemical parameters are responsible for the spatiotemporal variations of planktonic organisms. It is clear that the investigations on meteorological and hydrographical features are necessary for assessing the fertility and productivity of any ecosystems. An ecological interaction between organisms occupying an environment has been a matter of considerable study from brackish water and marine regions. Mangrove is a complex ecosystem that receives huge amount of dissolved inorganic inputs from a number of sources as runoffs through the course of river. Hence it is essential to investigate the fluctuations of various abiotic parameters in different seasons. A number of researchers have studied the physical and chemical characteristics of some Indian mangrove systems (Saravanakumar et al. 2008; Ashok Prabu et al. 2008; Rajkumar et al. 2009).

The Muthupet lagoon is a highly dynamic one on the east coast of India, and it has variable environment with considerable tidal influence. It has year round connection with Bay of Bengal and undergoes rhythmic fluctuations in marine and estuarine biotic community. Moreover a mangrove is a unique geographic area known for its high seasonally oscillating productive zone in plankton production. The present study was made to obtain the status of composition, density, and diversity of

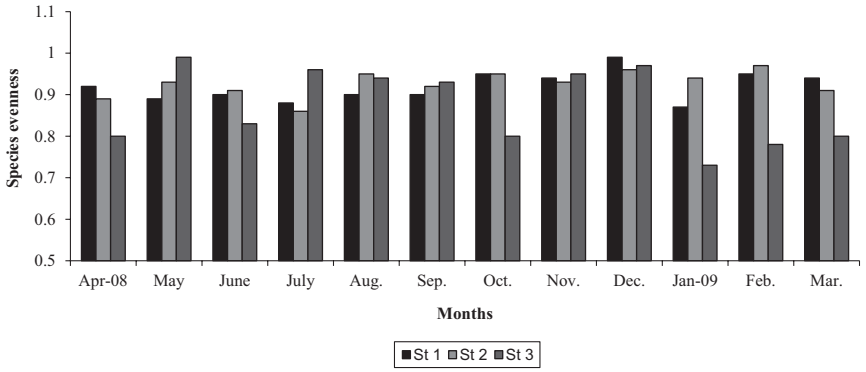


Fig. 18a Monthly variations in zooplankton species evenness during April 2008–March 2009

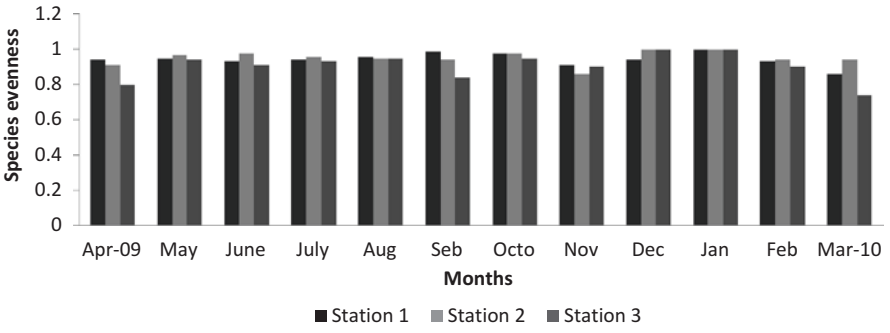


Fig. 18b Monthly variations in zooplankton species evenness during April 2009–March 2010

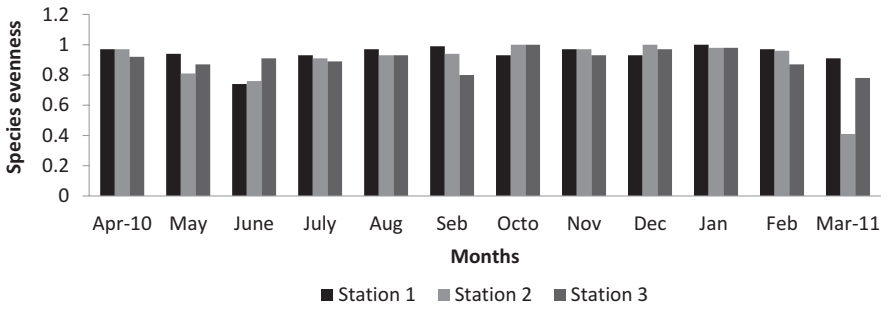


Fig. 18c Monthly variations in zooplankton species evenness during April 2010–March 2011

Table 2 Correlation coefficient between physicochemical parameters and plankton distribution at station 1 during April 2008–March 2009

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.45	1.00												
SWT	−0.44	0.97**	1.00											
S	−0.23	0.30	0.36	1.00										
pH	−0.35	0.78*	0.85*	0.52	1.00									
DO	0.17	−0.69	−0.76	−0.29	−0.61	1.00								
NO_3	0.83	−0.36	−0.38	−0.60	−0.46	0.07	1.00							
NO_2	0.75	−0.43	−0.41	−0.11	−0.09	0.18	0.47	1.00						
PO_3	0.38	−0.46	−0.46	−0.51	−0.38	0.13	0.33	0.60	1.00					
SiO_2	0.55	−0.11	−0.13	0.07	0.07	0.05	0.28	0.78*	0.48	1.00				
ZPD	−0.56	0.80*	0.82*	0.72*	0.79*	−0.50	−0.69	−0.46	−0.69	−0.21	1.00			
ZPDi	−0.31	0.43	0.40	0.74*	0.47	−0.08	−0.53	−0.28	−0.60	−0.15	0.64	1.00		
ZPR	0.07	−0.27	−0.36	−0.13	−0.46	0.38	0.13	−0.01	0.18	0.09	−0.30	0.12	1.00	
ZPE	0.27	−0.76	−0.82	−0.17	−0.69	0.63	0.24	0.22	0.33	0.16	−0.69	−0.03	0.56	1.00

*$P < 0.01$; **$P < 0.001$

Table 3 Correlation coefficient between physicochemical parameters and plankton distribution at station 2 during April 2008–March 2009

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.71	1.00												
SWT	−0.64	0.85*	1.00											
S	−0.15	0.24	0.43	1.00										
pH	−0.48	0.72*	0.88	0.72	1.00									
DO	0.19	−0.28	−0.67	−0.79	−0.75	1.00								
NO_3	0.75	−0.90	−0.79	−0.18	−0.65	0.27	1.00							
NO_2	0.77	−0.66	−0.43	−0.07	−0.40	−0.11	0.64	1.00						
PO_3	0.72	−0.61	−0.56	−0.42	−0.61	0.31	0.48	0.75	1.00					
SiO_2	0.81	−0.85	−0.68	−0.26	−0.66	0.17	0.76	0.86*	0.90**	1.00				
ZPD	−0.43	0.55	0.66	0.80*	0.88*	−0.65	−0.51	−0.43	−0.75	−0.68	1.00			
ZPDi	−0.44	0.60	0.54	0.53	0.65	−0.37	−0.47	−0.68	−0.81	−0.76	0.77	1.00		
ZPR	−0.04	0.15	0.05	0.26	0.12	−0.15	−0.02	−0.10	−0.34	−0.26	0.22	0.23	1.00	
ZPE	0.12	−0.27	−0.44	−0.46	−0.65	0.54	0.24	0.17	0.52	0.42	−0.68	−0.45	−0.38	1.00

*$P < 0.01$; **$P < 0.001$

Table 4 Correlation coefficient between physicochemical parameters and plankton distribution at station 3 during April 2008–March 2009

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.68	1.00												
SWT	−0.40	0.63	1.00											
S	−0.14	0.19	0.60	1.00										
pH	−0.38	0.68	0.84*	0.59	1.00									
DO	0.37	−0.27	−0.74	−0.69	−0.76	1.00								
NO_3	0.58	−0.56	−0.18	0.36	0.02	−0.19	1.00							
NO_2	0.90	−0.57	−0.20	0.04	−0.30	0.19	0.56	1.00						
PO_3	0.68	−0.78	−0.65	−0.67	−0.73	0.54	0.18	0.54	1.00					
SiO_2	0.74	−0.90	−0.65	−0.20	−0.71	0.38	0.50	0.71	0.79*	1.00				
ZPD	−0.46	0.69	0.84	0.68	0.84	−0.63	−0.22	−0.40	−0.83	−0.73	1.00			
ZPDi	−0.10	0.36	0.69	0.74	0.82	−0.66	0.30	−0.09	−0.61	−0.46	0.80	1.00		
ZPR	0.16	0.08	0.38	0.53	0.56	−0.46	0.42	0.03	−0.43	−0.25	0.57	0.75	1.00	
ZPE	0.32	0.01	0.13	0.33	0.34	−0.12	0.69	0.27	−0.24	0.00	0.14	0.49	0.65	1.00

*$P < 0.01$; **$P < 0.001$

Table 5 Correlation coefficient between physicochemical parameters and plankton distribution at station 1 during April 2009–March 2010

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1													
AT	−0.48	1.00												
SWT	−0.63	0.72	1.00											
S	−0.78	0.74	0.92**	1.00										
pH	−0.34	0.75	0.45	0.51	1.00									
DO	−0.01	−0.41	−0.58	−0.29	−0.01	1.00								
NO_3	0.30	−0.63	−0.44	−0.39	−0.22	0.48	1.00							
NO_2	−0.25	−0.36	0.12	0.16	−0.02	0.30	0.47	1.00						
PO_3	−0.02	−0.60	−0.53	−0.32	−0.24	0.82*	0.67	0.56	1.00					
SiO_2	0.14	−0.40	−0.03	−0.23	−0.20	−0.21	0.27	0.54	0.06	1.00				
ZPD	−0.55	0.72	0.61	0.64	0.63	−0.29	−0.53	−0.18	−0.58	−0.09	1.00			
ZPDi	−0.73	0.38	0.60	0.72	0.25	0.00	−0.01	0.09	−0.14	−0.18	0.63	1.00		
ZPR	−0.66	0.20	0.46	0.59	0.10	0.08	0.18	0.08	0.04	−0.29	0.37	0.93**	1.00	
ZPE	−0.07	−0.21	−0.06	−0.03	−0.34	0.06	0.50	−0.16	0.19	−0.31	−0.32	0.37	0.65	1.00

*$P < 0.01$; **$P < 0.001$

Table 6 Correlation coefficient between physicochemical parameters and plankton distribution at station 2 during April 2009–March 2010

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.64	1.00												
SWT	−0.58	0.91**	1.00											
S	−0.70	0.98**	0.91**	1.00										
pH	−0.66	0.77*	0.91**	0.79*	1.00									
DO	0.09	−0.24	−0.51	−0.33	−0.56	1.00								
NO_3	0.21	−0.25	−0.27	−0.33	−0.24	0.58	1.00							
NO_2	−0.08	0.00	0.20	0.01	0.31	−0.09	0.52	1.00						
PO_3	0.07	−0.21	−0.19	−0.27	−0.10	0.45	0.90**	0.74*	1.00					
SiO_2	0.66	−0.75	−0.49	−0.74	−0.35	−0.27	0.16	0.37	0.26	1.00				
ZPD	−0.32	0.51	0.55	0.47	0.63	−0.38	−0.34	−0.11	−0.29	−0.19	1.00			
ZPDi	−0.77	0.77	0.82	0.78	0.82	−0.35	−0.11	0.26	−0.04	−0.45	0.48	1.00		
ZPR	−0.88	0.73	0.76	0.76	0.76	−0.23	−0.16	0.22	−0.07	−0.55	0.33	0.94**	1.00	
ZPE	−0.33	0.02	−0.09	0.03	−0.18	0.48	0.00	−0.07	−0.02	−0.39	−0.49	0.11	0.37	1.00

Table 7 Correlation coefficient between physicochemical parameters and plankton distribution at station 3 during April 2009–March 2010

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.59	1.00												
SWT	−0.70	0.87*	1.00											
S	−0.63	0.93**	0.94**	1.00										
pH	−0.57	0.82	0.84	0.90	1.00									
DO	0.03	−0.42	−0.34	−0.32	−0.49	1.00								
NO_3	0.21	−0.12	−0.29	−0.16	−0.07	0.27	1.00							
NO_2	−0.03	−0.15	0.01	−0.04	−0.05	−0.09	0.35	1.00						
PO_3	−0.01	−0.15	0.08	0.00	−0.15	0.35	0.35	0.75	1.00					
SiO_2	0.57	−0.73	−0.66	−0.79	−0.62	−0.19	0.13	0.40	0.19	1.00				
ZPD	−0.48	0.77	0.65	0.64	0.59	−0.48	−0.41	−0.15	−0.23	−0.44	1.00			
ZPDi	−0.32	0.49	0.36	0.54	0.73	−0.40	−0.02	−0.23	−0.53	−0.49	0.44	1.00		
ZPR	−0.06	0.11	0.00	0.17	0.48	−0.24	0.25	−0.22	−0.55	−0.20	0.04	0.87*	1.00	
ZPE	0.30	−0.51	−0.56	−0.44	−0.15	0.26	0.53	−0.12	−0.27	0.21	−0.62	0.26	0.70	1.00

$P < 0.01$; $^{**}P < 0.001$

Table 8 Correlation coefficient between physicochemical parameters and plankton distribution at station 1 during April 2010–March 2011

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	0.31	1.00												
SWT	0.22	0.90	1.00											
S	−0.15	0.45	0.49	1.00										
pH	0.03	0.70*	0.50	0.64	1.00									
DO	−0.61	−0.08	0.02	−0.23	−0.28	1.00								
NO_3	−0.20	−0.23	−0.20	−0.11	−0.22	0.15	1.00							
NO_2	−0.43	0.08	0.02	0.14	0.16	0.54	0.63	1.00						
PO_3	−0.37	−0.29	−0.16	−0.06	−0.26	0.30	0.63	0.34	1.00					
SiO_2	0.17	0.23	0.09	−0.46	0.02	0.20	0.44	0.39	0.47	1.00				
ZPD	−0.43	0.19	0.04	0.53	0.61	0.17	0.36	0.70	0.19	0.05	1.00			
ZPDi	−0.51	−0.01	0.02	0.11	0.25	0.43	0.59	0.80	0.31	0.23	0.63	1.00		
ZPR	−0.42	−0.13	−0.01	0.00	0.06	0.39	0.57	0.59	0.36	0.17	0.37	0.93**	1.00	
ZPE	−0.05	−0.42	−0.25	−0.53	−0.44	0.18	0.34	−0.06	0.18	0.06	−0.32	0.37	0.65	1.00

*$P < 0.01$; **$P < 0.001$

Table 9 Correlation coefficient between physicochemical parameters and plankton distribution at station 2 during April 2010–March 2011

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.48	1.00												
SWT	−0.24	0.82*	1.00											
S	−0.70	0.39	0.15	1.00										
pH	−0.60	0.74*	0.54	0.52	1.00									
DO	0.03	0.31	0.20	−0.17	−0.29	1.00								
NO_3	−0.30	0.29	−0.19	0.32	0.50	0.08	1.00							
NO_2	−0.38	0.29	0.18	0.41	0.50	0.05	0.59	1.00						
PO_3	−0.39	0.21	−0.17	0.57	0.48	−0.13	0.70*	0.43	1.00					
SiO_2	0.70	−0.13	−0.08	−0.68	−0.08	−0.13	0.07	−0.15	−0.13	1.00				
ZPD	−0.25	0.29	−0.12	0.21	0.37	0.19	0.85	0.43	0.78	0.19	1.00			
ZPDi	−0.41	0.21	0.10	0.41	0.11	0.44	0.42	0.54	0.37	−0.46	0.48	1.00		
ZPR	−0.61	0.23	0.13	0.50	0.16	0.37	0.30	0.46	0.35	−0.68	0.33	0.94**	1.00	
ZPE	−0.40	−0.12	−0.04	0.19	−0.08	0.02	−0.26	−0.17	−0.22	−0.76	−0.49	0.11	0.37	1.00

*$P < 0.01$; **$P < 0.001$

Table 10 Correlation coefficient between physicochemical parameters and plankton distribution at station 3 during April 2010–March 2011

	RF	AT	SWT	S	pH	DO	NO_3	NO_2	PO_3	SiO_2	ZPD	ZPDi	ZPR	ZPE
RF	1.00													
AT	−0.62	1.00												
SWT	−0.39	0.77*	1.00											
S	−0.71	0.44	0.19	1.00										
pH	−0.71	0.83*	0.43	0.47	1.00									
DO	0.50	−0.21	0.07	−0.67	−0.38	1.00								
NO_3	−0.0**8**	0.28	−0.24	0.05	0.43	0.03	1.00							
NO_2	0.04	0.00	−0.25	0.08	0.04	−0.08	0.51	1.00						
PO_3	−0.47	0.19	−0.28	0.58	0.43	−0.26	0.61	0.52	1.00					
SiO_2	0.75	−0.33	−0.29	−0.72	−0.22	0.52	0.29	0.31	−0.13	1.00				
ZPD	−0.51	0.29	−0.15	0.53	0.47	−0.22	0.63	0.51	0.95	−0.14	1.00			
ZPDi	−0.33	0.09	0.02	0.54	0.05	−0.34	0.28	0.42	0.47	−0.33	0.44	1.00		
ZPR	−0.05	−0.17	−0.16	0.28	−0.18	−0.32	0.15	0.16	0.14	−0.21	0.04	0.87	1.00	
ZPE	0.34	−0.37	−0.18	−0.22	−0.41	−0.05	−0.16	−0.33	−0.49	−0.02	−0.62	0.26	0.70	1.00

*$P < 0.01$; **$P < 0.001$

zooplankton in Muthupet lagoon in relation to the prevailing hydrographical conditions. This information would be helpful in the ecological monitoring of this ecosystem in the future. The present study clearly indicated that the rainfall mainly inclined the changes on abiotic and biotic components of the Muthupet mangrove ecosystem. Monthly rainfall (mm) ranged between 5.8 and 986 mm during the period from April 2008 to March 2011. No rainfall was recorded during the month of February, April–July 2009, February–April 2010, and March 2011. The maximum rainfall was recorded during the month of November 2008 whereas the minimum during the month of January 2011. In the present study, more rainfall was reported during third year (April 2010–March 2011) than first year (April 2008–March 2009) and second year (April 2009–March 2010) of the study. It is well known that the presently noticed variations in salinity, dissolved oxygen concentration, and inorganic nutrients are mainly depending on the annual cycle of rainfall besides influences on zooplankton as agreed by Perumal (1993) and Santhanam and Perumal (2003).

The surface water temperature showed an increasing trend from December to April. The surface water temperature is influenced by the intensity of solar radiation, evaporation, freshwater influx, and cooling and mix up with ebb and flow from adjoining neritic waters. The surface water temperature was low during monsoon season owing to strong land sea breeze and precipitation, and it was high during summer due to high solar radiation as agreed by Santhanam and Perumal (2003), Ajithkumar et al. (2006), and Perumal et al. (2009). The salinity was found to be high during summer season and low during the monsoon at all the stations. The recorded maximum values could be attributed to the low rainfall, high rate of evaporation besides high temperature, and neritic water inundation toward the lagoon as agreed by earlier workers (Asha and Diwakar 2007; Perumal et al. 2008; Rajkumar et al. 2009; Sridhar et al. 2010). Hydrogen-ion concentration (pH) was remained alkaline throughout the study period at all the stations with maximum during summer season and the minimum during monsoon. The fluctuations in pH with respect to seasons are attributed to removal of CO_2 by photosynthesis through bicarbonate degradation, low primary productivity, reduction of salinity and temperature, and decomposition of organic matter as reported by Bragadeeswaran et al. (2007), Ashok Prabu et al. (2008), and Perumal et al. (2009). In the present study, the maximum dissolved oxygen content was reported during the month of February 2011 which might be due to cumulative effect of heavy freshwater discharges from upstream area after the monsoonal rainfall (Rajasekar 2003). However, very low dissolved oxygen concentration that was noticed during the month of December 2008 could be due to high rate of organic decomposition and the entry of agricultural runoff along with the sewage as reported by Bhatt et al. (1999) and Sachidanandamurthy and Yajurvedi (2006).

The nutrients are considered as one of the most important parameters in the mangrove environment influencing the distribution of zooplankton. It is well known that the distribution of nutrients is mainly based on the season, tidal conditions, and freshwater discharges from land sources and upstream dams. The nitrate content was found too low during post-monsoon season (January 2009), whereas the high

nitrate concentration was reported during monsoon season (November 2008). The recorded maximum nitrates in the Muthupet mangrove ecosystem during monsoon season could be due to the organic materials received from the catchment area during ebb tide (Ashok Prabu et al. 2008; Perumal et al. 2009). The recorded low value of post-monsoon nitrate level might be due to utilization by phytoplankton and low freshwater input (Bragadeeswaran et al. 2007; Soundarapandian et al. 2009).

In the present observation, the low nitrite value that was obtained during monsoon season may be due to less freshwater inflow and high salinity as reported by Angadi et al. (2005) and Saravanakumar et al. (2008). However, the high nitrite value that was noticed during monsoon season could be due to the increased phytoplankton excretion, oxidation of ammonia, and reduction of nitrate and by recycling of nitrogen and also due to bacterial decomposition of planktonic detritus present in the environment as supported by Govindasamy et al. (2000). The denitrification and air-sea interaction exchange of chemicals are also responsible for the presently observed increased nitrite value (Perumal et al. 2008). The inorganic phosphate content that was recorded to be more in monsoon season might be due to the regeneration and release of total phosphorous from bottom mud into the water column by turbulence and mixing and the weathering of rock-soluble alkali metal phosphates carried into Muthupet lagoon as agreed by earlier workers (Santhanam and Perumal 2003; Damotharan et al. 2010). The phosphate content was also increased due to land drainage which carried super phosphates applied in the agricultural fields as fertilizers and alkyl phosphates used in households as detergents can be other sources of inorganic phosphates during the monsoon season as agreed by Bragadeeswaran et al. (2007) and Perumal et al. (2009). The summer low phosphate content reported could be attributed to the limited flow of freshwater, high salinity, and utilization of phosphate by phytoplankton (Rajasegar 2003). The variation in phosphate content was also might be due to the processes like adsorption and desorption of phosphates and buffering action of sediment under varying environmental conditions as opined by Santhanam and Perumal (2003).

Generally, the silicate content was found to be higher than other nutrients. The recorded higher monsoonal silicate content may be due to heavy freshwater inflow derived from land drainage and silicate reached out from rocks. Further, the exchange of silicate from the bottom sediment might be due to turbulent nature of water that is also responsible for the presently recorded higher silicate content in the study area as agreed by Rajasegar (2003) and Perumal et al. (2009). However, the silicate content that was found to be low during post-monsoon and summer season could be attributed to uptake of silicates by diatoms for their frustule formation (Thillai Rajasekar et al. 2005; Ashok Prabu et al. 2008 and Santhanam et al. 2012). In the present investigation, the concentration of inorganic nutrients was recorded too high at station 3 followed by stations 2 and 1, which could be attributed to more organic matter input from catchment area to the lagoon. It is also believed that more leaf litter fall at station 3 which has dense mangrove vegetation might be a possible reason for the increased nutrients level than the rest of the stations studied presently. The 3-year investigation clearly indicated that the inorganic nutrient concentration was comparatively higher during the period of first year (April 2008–March 2009)

than second (April 2009–March 2010) and third years (April 2010–March 2011). The higher nutrient concentrations noticed during the first year could be attributed to maximum rainfall besides land drainage.

In the present study, totally 84 species of zooplankton belonging to 8 groups and 43 genera were recorded for 3-year periods from April 2008 to March 2011. Of these, 59 species belong to Copepoda, 10 species to Ciliata, 7 species to Rotifera, 2 species to Cladocera, and 1 each to decapoda, foraminifera, and Polychaeta. Among the zooplankton, the copepods were found to be dominant throughout the year in all the stations with appreciable numbers and species, which could be due to availability of plenty of food organisms as evidenced by high phytoplankton population during the same season and also due to continuous breeding and reproductive capacity of copepods besides survival to widely changing environmental conditions prevailed as agreed by Santhanam and Perumal (2003), Mwaluma et al. (2003), Perumal et al. (2008), Abowei et al. (2008), and Davies and Otene (2009). The zooplankton species composition was found to be more in station 1 (mouth) than stations 2 and 3 and could be attributed to availability of more neritic zooplankton forms as reported by Santhanam and Perumal (2003), Perumal et al. (2008), and Santhanam et al. (2012).

The zooplankton population showed considerable spatial and temporal variations depending upon the environmental conditions prevailed and attendance of phytoplankton. The recorded low zooplankton density during monsoon season could be ascribed to heavy input of freshwater during which only a few species of zooplankton have been reported. Similar monsoonal minimum population was reported in other waters (Mwaluma et al. 2003; Lo et al. 2004; Sterza and Fernandes 2006; Saravanakumar et al. 2007; Perumal et al. 2008; Santhanam et al. 2012). The population density of zooplankton was attenuated with salinity changes as evidenced by the high zooplankton density that was encountered during summer season due to increased salinity and phytoplankton population as reported by Subbaraju and Krishnamurthy (1972), Madhupratap (1978), Shanmugam et al. (1986), McKinnon and Thorrold (1993), Santhanam and Perumal (2003), and Santhanam et al. (2012). The zooplankton population density was comparatively rich in station 3 (Sethukuda) than stations 1 and 3 because station 3 is receiving more organic matter from the catchment areas as opined by Santhanam and Perumal (2003), and Saravanakumar et al. (2007).

In the present study, the minimum species diversity was obtained during monsoon season owing to the presence of least number of species. The minimum number of species recorded during the monsoon season might be due to the heavy monsoonal flood, which washed the allochthonous species, and even autochthonous species, which could not survive in very low salinity. The turbidity during this season may also be responsible for lower values. Similar observations were also made earlier by Maruthanayagam and Subramanian (1999), Eswari and Ramanibai (2004), Melo junior et al. (2007), and Perumal et al. (2008). The maximum diversity was recorded during summer which corresponds to the high population density, the high salinity,

and the more number of species observed during the same season. Similar findings were also reported by Damodara Naidu (1980) from Vellar estuary, Madhupradap et al. (1981) from Andaman Sea, Karuppasamy and Perumal (2000) from Pichavaram mangrove waters, and Saravanakumar et al. (2007) from mangrove waters of Gulf of Kachchh. In the present investigation, the species diversity was higher at station 1 (mouth) than in stations 2 and 3 which might be due to the presence of more neritic forms and the stable hydrological conditions prevailed. Especially the salinity plays an important role in higher diversity recorded at station 1. The minimum species diversity that was observed in station 2 (Sallimunai) could be attributed to the constant changes owing to high freshwater sources from adjacent channels, and agriculture lands were making the estuary more dynamic in turn fluctuations in diversity values. In the present study a reverse trend was observed in evenness values. Very low species evenness was obtained in summer season due to unequal distribution of the species in these months, and the high evenness values were obtained during monsoon season in both stations which indicated that the species were equally distributed and thus not allowing a single species to be dominant over others as reported earlier by Kumar (1993) from Vellar estuary, Ambikadevi (1993) and Saraswathy (1993) from Arasalar and Cauvery estuary, Karuppasamy and Perumal (2000) from Pichavaram mangrove waters, and Saravanakumar et al. (2007) from Gulf of Kachchh waters.

Conclusion

The loads and population structure of copepods in the Muthupet mangrove estuary showed the physical and chemical parameters related to the spatiotemporal variations with the current rainfall pattern. The huge amount of inorganic nutrients that were recorded in sampling places is expressed that all the sources comes from the rainfall, land drainage, influx of freshwater, and mangrove litter fall. The overall population of copepod showed a bimodal pattern of distribution. Among the copepod family, calanoid copepods were found to be dominant followed by Cyclopoida and Harpacticoida. The population density of the copepods showed the positive correlation with temperature and salinity and negative correlation with rainfall, pH, and dissolved oxygen. Finally, this study concluded that the Muthupet mangrove ecosystem and closest neritic waters can be well thought out as one of the major productive habitats in Southern India with more biodiversity of zooplankton which can be helpful for fish potential of this ecosystem.

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DNA Barcoding of Copepods

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Introduction

Copepods are the most studied zooplankton focussing on diversity, morphology, taxonomy, phylogeny, distribution, life-cycle strategies, feeding behaviour and adaptation to various environmental conditions (Bradford-Grieve et al. 2010; Blanco-Bercial et al. 2011; Saiz and Calbet 2011). They are the most diverse taxon which extend over vast geographic ranges and are planktonic almost throughout their life cycle. Precise identification is based on our knowledge of morphological traits found among copepods. The common occurrence of spectacular ontogenetic changes and also higher levels of morphological plasticity persisted among copepods have remained a challenge for the species identification that can be readily addressed through the molecular level (McManus and Katz 2009). More recently, biodiversity assessments have been increasingly concentrated on molecular-based genetic methods (Bucklin et al. 2010a; Grant et al. 2011). The molecular taxonomical analysis has heralded a new era in solving the confusion caused by cryptic species (Goetze 2003, 2010; Miyamoto et al. 2010).

Copepod populations showed higher levels of genetic divergence (Goetze 2003). The remarkable evolutionary conservation of divergence found among copepods species has resulted in genuine difficulties in the identification of the numerous sibling species as many of which could be discriminated only by subtle morphological

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and morphometrical characters. To distinguish between sibling and cryptic species morphologically is time-consuming, and the procedure requires detailed microscopy. Therefore, various methods have to be developed and followed for the accurate identification of species. One accurate method for identification of sibling species is to apply a molecular method, such as species-specific polymerase chain reaction (PCR) (Bucklin et al. 1999).

Burton (1996) underlined the importance of adopting the molecular methods for species identification. Despite the rapid recent growth of molecular techniques, molecular biologists have often been less thorough in precise identification of species. As a result, the large number of sequences submitted in the public databases is linked to be mis- or unidentified species. To overcome the problem, "total evidence" approach in identifying copepod, by using both molecular and morphological evidences, has to be followed, whenever possible (Mcmanus and Katz 2009).

Copepod represents a species complex with more restricted distributional patterns. The available morphological data for subspecies of copepods has been differentiated based on the morphological variability. But this is not suitable for the species that comes under convergent evolution which could hinder this task (Fuentes-Reinés and Suárez-Morales 2014). For example, recent gene-sequencing studies in copepods revealed the evidences of cryptic speciation (Schizas et al. 1999; Rocha-Olivares et al. 2001), incipient speciation (Schizas et al. 1999, 2002) and pseudo-sibling species (Schizas et al. 1999, 2002; Rocha-Olivares et al. 2001; Staton et al. 2005). Therefore, DNA barcoding has been proved to be a useful tool for species delimitation among copepods (Rocha-Olivares et al. 2001). Recently molecular markers in species discrimination allowed genetic studies in phylogeography (Avise 2009) and which yielded better understanding of the marine ecosystem (Selkoe et al. 2008).

Nowadays, different types of molecular techniques are being used for identification and discrimination of a particular species at any stage of development (eggs to adults) for closely related species of copepods, viz. restriction fragment length polymorphism (RFLP), random amplified polymorphic DNA (RAPD) and barcoding through DNA marker sequences. Among them, DNA barcoding is more advanced in which different kinds of nuclear and mitochondrial gene markers are being used (Bucklin 2000). DNA barcoding could be based upon sequence diversity in small segment of DNA (Tautz et al. 2003).

Currently, only a few nuclear and mitochondrial markers have been applied successfully to resolve phylogenetic relationships among copepods. Cytochrome oxidase I (COI) and the small and large subunits of mitochondrial rRNA (12S and 16S) have been used as molecular markers for identifying or determining the phylogenetic relationships of copepods (Bucklin et al. 1995, 2003; Bottger-Schnack and Machida 2010). 18S and 28S are highly conserved genes used to resolve phylogenetic relationship among families or genera of copepods in which 28S rDNA region has been used for species-level identification (Hassouna et al. 1984; Sonnenberg et al. 2007). ITS gene refers to a non-coding DNA situated between the structural rRNA used for species identification and DNA array technologies (Engelmann et al. 2009; Landis and Gargas 2007).

In India, barcoding studies in copepods are found to be scarce. To date, there is minimum sequence data available in GenBank particularly for copepods in India. The present identification through morphological alone results in false identification of species particularly invasive, cryptic and sibling species. To overcome the problem, barcoding studies for harpacticoid copepod population of different geographical regions are essential to know the genetic divergence that occurred between inter- and intraspecies level. This would pave the way for clearing the confusion pertaining to the species identification of copepods.

DNA Barcoding

DNA barcoding is a taxonomic method using genetic markers that enables species identification and recognition and to identify unknown species whether species should be combined or separated. It can help to detect overlooked species with subtle or complex morphological traits and stimulate collection of additional genetic, morphological, ecological, geographical and behavioural data (Bucklin and Frost 2009; Hebert et al. 2004; Smith et al. 2008; Steinke et al. 2009). Barcode study can be applied for identification of marine invasive species. In some instances, mitochondrial gene sequence variation can reveal the geographic source region and pathway of invasion. Two studies have been reported about the usefulness of mitochondrial gene to study invasive pathways of a bryozoan (Mackie et al. 2006) and comparative analysis of COI diversity of native and introduced populations of an amphipod (Ashton et al. 2008).

Genetic Markers Commonly Used for DNA Barcoding in Copepods

Mitochondrial Genome

In metazoans, the mitochondrial genome is circular and has double-stranded DNA molecule (mtDNA), length usually 16 kb but can vary from 14 to 48 kb conserved with 37 genes, 13 protein-encoding genes, 22 transfer RNA (tRNA) genes and one or more non-coding region(s) containing signals for transcription and replication of the mt DNA (Wolstenholme 1992). Mitochondrial genome can be widely used for population studies (Park et al. 2004; Nuwer et al. 2008), phylogeography (Park et al. 2004; Burton et al. 2007) and phylogenetic relationships at various taxons, particularly in arthropods (Simon et al. 2006; Hassanin 2006; Place et al. 2005). Hebert et al. (2003a) proposed DNA barcoding system for animal life could be based upon sequence diversity in cytochrome c oxidase subunit 1 (COI). Diversity in the amino acid sequences coded by 59 sections has constantly placed species into higher

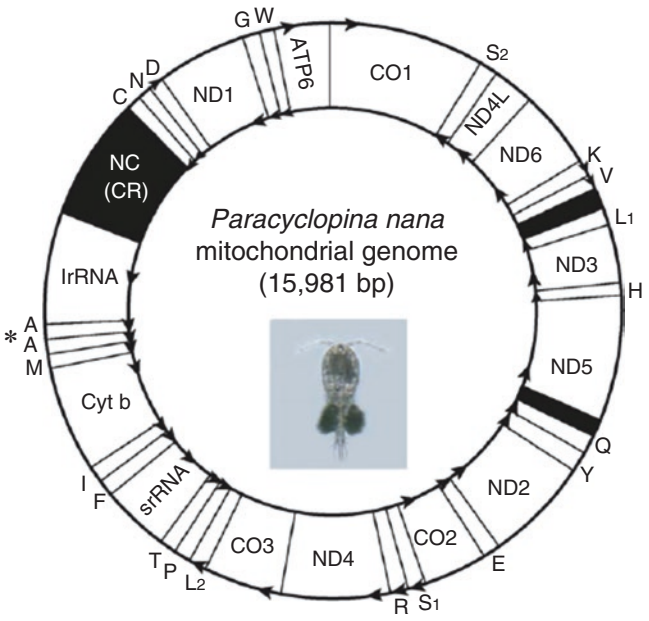


Fig. 1 Gene organization of the mitochondrial genome of the cyclopoid copepod *Paracyclopina nana* (Ki et al. 2009)

taxonomic categories (from phyla to orders). Mitochondrial COI gene is an appropriate marker for species-level identification because it showed intra- and interspecific phylogenetic relationship in invertebrates (Soh et al. 2012). It is the most conserved protein-coding gene found in the mitochondrial genomes of animals (Brown 1985) and one of the widely used markers to study population genetics and evolution (Shao and Barker 2007).The barcode region showed a marked divergence of genetic distance within metazoan species (typically <3%) and between species (typically 10–25%; Hebert et al. 2003b) (Fig. 1).

Nuclear Genome

The 18S rRNA is the small ribosomal subunit used to study the relationships from generic or species to superfamily levels in copepods (Bucklin et al. 2003; Figueroa 2011). Sequence data from these genes is widely used in molecular analysis to reconstruct the evolutionary history of organisms, especially in invertebrates, due to slow evolutionary rate that makes it suitable to reconstruct ancient divergences. The 28S rRNA gene is the large ribosomal subunit that has been used to examine phylogenetic relationship among invertebrate species. The gene is used for taxonomic identification of species with subtle or an ambiguous morphological characteristic which makes it a good marker for the identification of cyclopoid copepod *Oithona* (Cepeda et al. 2012).The internal transcribed spacers (ITS) of nuclear rRNA genes

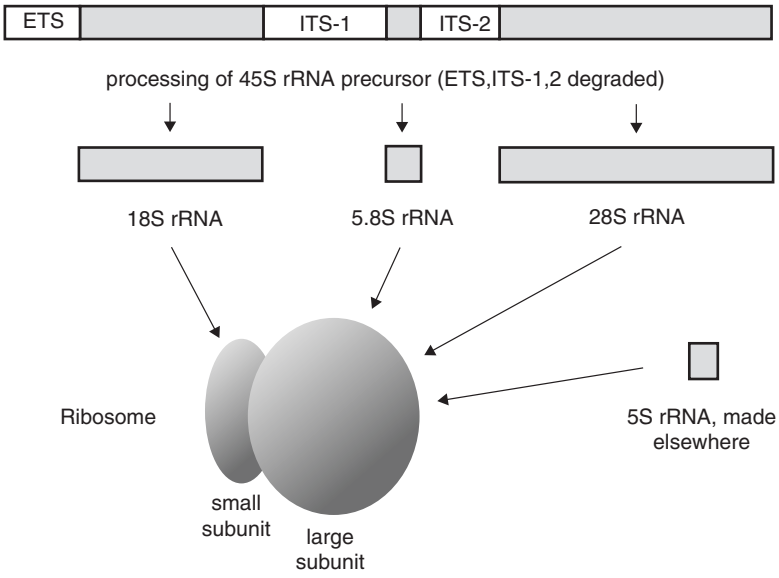


Fig. 2 Ribosomal DNA gene cluster (www.rzuser.uni-heidelberg.de/~bu6/Introduction11.html)

have been applied for species identification for many marine groups, especially those for which COI has proved problematical. The combined sequences of ITS2 and 28S would show genetic variability with species-level resolution. Phylogenetic relationships among copepods at higher systematic levels (ordinal, familial and generic) have been resolved using the 18S and 28S nuclear rRNA genes and at the lower taxonomic levels (species and populations) have been resolved using the ITS2 of the nuclear rDNA gene cluster (Bucklin and Frost 2009; Goetze 2005; Wyngaard et al. 2010; Thum and Harrison 2009; Ki et al. 2009) (Fig. 2, Tables 1, 2, 3 and 4).

DNA Barcoding of *Nitokra affinis* Gurney: 1927

Copepods Collection and Preservation

Zooplankton samples were collected from Vellar estuary (Fig. 3), Parangipettai, Tamil Nadu, Southeast coast of India using horizontal towing of a zooplankton net (158 μm). The collected samples were immediately transported to the laboratory, vigorously aerated and thoroughly rinsed to reduce contamination by the other zooplankter. Then, the zooplankters were screened to isolate the size fractions containing predominantly adult copepods and later-stage copepodites. This was accomplished by first crude screening through a 500-μm mesh to eliminate particularly fish and prawn larvae. Then, the rotifers, nauplii of copepods and barnacles from the collected samples were removed by rinsing the samples for 2 hr in a zooplankton washer fitted with a 190 μm mesh size, and the remaining adult copepods were immediately preserved in 5% formalin and 95% ethanol for taxonomic and molecular analysis, respectively.

Table 1 Different primers for various marine invertebrates

Taxon	Marker	Primer name	Direction	Sequence (5′–3′)	References
COI	LCO-1490	Forward	GGTCAACAAATCATAAAGATATTGG	Folmer et al. (1994)	HCO2198 Nancy
COI	HCO-2198	Reverse	TAAACTTCAGGGTGACCAAAAAATCA	Folmer et al. (1994)	LCO1490
COI	jgLCO1490	Forward	TITCIACIAAYCAYAARGAYATTGG	Geller et al. (2013)	jgHCO2198
COI	jgHCO2198	Reverse	TAIACYTCIGGRTGICCRAARAAYCA	Geller et al. (2013)	gLCO1490
COI	Nancy	Reverse	CCCGGTAAAATTAAAATATAAACTTC	Simon et al. (1994)	LCO1490
cyt b	UCYTB151F	Forward	TGTGGRGCNACYGTWATYACTAA	Merritt et al. (1998)	UCYTB270R
cyt b	UCYTB270R	Reverse	AANAGGAARTAYCAYTCNGGYTG	Merritt et al. (1998)	UCYTB151F

Table 2 Different primers for various marine invertebrates

Taxon	Marker	Primer name	Direction	Sequence (5′–3′)	References
28S	28S-F1a	Forward	GCGGAGGAAAAGAAACTAAC	Ortman (2008)	28S-R1a
28S	28S-R1a	Reverse	GCATAGTTTCACCATCTTTCGGG	Ortman (2008)	28S-F1a
28S	D9/10 forward	Forward	CGGCGGGAGTAACTATGACTCTCTTAAGGT	Zardoya et al. (1995)	D9/10 reverse
28S	D9/10 reverse	Reverse	CCGCCCCAGCCAAACTCCCCA	Zardoya et al. (1995)	D9/10 forward
18S*	18A1 mod*	Forward	CTGGTTGATCCTGCCAGTCATATGC	Raupach et al. (2009)	1800
18S*	1800 mod*	Reverse	GATCCTTCCGCAGGTTCACCTACG	Raupach et al. (2009)	18A1
*Internal sequencing primers	F1	Forward	AGCAGCCGCGGTAATTCCAGCT	Laakmann et al. (2013)	
*Internal sequencing primers	CF2	Forward	GAAACTTAAAGGAATTGACGGAA	Laakmann et al. (2013)	
*Internal sequencing primers	CR1	Reverse	CCTTCCGTCAATTCCTTTAAGT	Laakmann et al. (2013)	
*Internal sequencing primers	R2	Reverse	AGCTGGAATTACCGCGGCTGCT	Laakmann et al.(2013)	
18S	18SE	Forward	CTGGTTGATCCTGCCAGT	Hillis and Dixon (1991)	18SL

Table 3 Different primers for various copepods

Taxon	Marker	Primer name	Direction	Sequence (5′–3′)	References	Primer pairings
Copepoda	COI	Cop-COI-2189R	Reverse	GGGTGACCAAAAAATCARAA	Bucklin et al. (2010b)	LCO1490
Copepoda	COI	Cop-COI-1498F	Forward	AAYCATAAAGAYATYGGDAC	Bucklin et al. (2010b)	HCO-2198
Copepoda	COI	Cop-COI-2105R	Reverse	CGRTCHGTHARNARYATDGTAATDGC	Bucklin et al. (2010b)	LCO1490
Copepoda	COI	Crus-COI-2198R	Reverse	CCHACDGTAAAYATRTGRTG	Bucklin et al. (2010b)	LCO1490
Copepoda	COI	Crus-COI-2428R	Reverse	TTAATHCCHGTDGGNACVGCAAT	Bucklin et al. (2010b)	LCO1490
Copepoda	COI	HCO-Co-2358	Reverse	CCHACDGTAAAYATRTGRTG	Bucklin et al. (2010b)	LCO1490
Copepoda: Eucalanidae	COI	COI_RNI	Forward	GTAGT(AGCT)GTAAC(AT)GCTCATGC	Goetze and Bradford-Grieve (2005)	COI_VH
Copepoda: Clausocalanus	COI		Forward	GAGCCTGGTCAGGAATAATCG	Blanco-Bercial and Alvarez-Marques (2007)	
Copepoda: Clausocalanus	COI		Reverse	GGTCTCCTCCTCCTCCAACAT	Blanco-Bercial and Alvarez-Marques (2007)	
Copepoda: Calanoida	COI	LCO-1703	Forward	CTATTTGATTGGAGGATTTGG	Hill et al. (2001)	Internal primer

Copepoda: Calanoida	COI	LCO-1719	Forward	GGATTTGGTAACTGATTAGTGCC	Hill et al. (2001)	Internal primer
Copepoda: Calanoida	COI	H2612-COI	Reverse	AGGCCTAGGAAATGTATAGGGAAA	Figueroa (2011)	L592-RCOI
Copepoda: Calanoida	COI	L592-RCOI	Forward	AACCTTAATACATCTTTTTATGATG	Figueroa (2011)	H2612-COI
Copepoda: Pleuromamma	COI	PLXI_VH		CCAAACGTTTCTTTCTTCCC	Goetze (2011)	PLXI_VL
Copepoda: Pleuromamma	COI	PLXI_VL		TCAGCCAGGGTCTTTAATTGG	Goetze (2011)	PLXI_VH
Copepoda: *Calanus helgolandicus*	COI	ChelgCOI-F	Forward	GGCCAAAACAGGGAGAGATA	Papadopoulos et al. (2005)	ChelgCOI-R
Copepoda: *Calanus helgolandicus*	COI	ChelgCOI-R	Reverse	CGGGACTCAGTATAATTATTCGTCTA	Papadopoulos et al. (2005)	ChelgCOI-F
Copepoda: Eucalanidae	16S	16SAR	Forward	CGCCTGTTTATCAAAAACAT	Braga et al. (1999)	16SCB
Copepoda: Eucalanidae	16S	16SCB	Reverse	ATTCAACATCGAGGTCACAA	Braga et al. (1999)	16SAR
Copepoda: Acartia	16S	16S-167	Forward	GACGAGAAGACCCTATGAAG	Bucklin et al. (1998)	16sbr-H
Copepoda: Acartia	16S	16sbr-H	Reverse	CCGGTTTGAACTCAGATCATGT	Palumbi (1996)	16S-167
Copepoda: Pareucalanus	16S	16S_PAR1		GCTAAGGTAGCATAATAATTAGTT	Goetze (2003)	
Copepoda: Subeucalanus	16S	16S_SUB2		AAGTGCTAAGGTAGCATAAT	Goetze (2003)	
Copepoda: Eurytemora	16S	16SA2	Forward	CCGGGT(CT)TCGCTAAGGTAG	Lee (2000)	16SB2
Copepoda: Eurytemora	16S	16SB2	Reverse	CAACATCGAGGTCGCAGTAA	Lee (2000)	16SA2

Table 4 Different primers for various copepods

Taxon	Marker	Primer name	Direction	Sequence (5′–3′)	References	Primer pairings
Copepoda: Skistodiaptomus	16S	Skisto-1	Forward	TGGTAAGGTAGCATAATAAT	Thum and Harrison (2009)	Skisto-2
Copepoda: Skistodiaptomus	16S	Skisto-2	Reverse	CCGGTTTGAACTCAGATCATGT	Thum and Harrison (2009)	Skisto-1
Copepoda: Calanidae and Eucalanidae	cyt b	L10319-CYB	Forward	CCTTGGGGKCAGATGTCTTTTTGGG	Machida et al. (2004)	H10648-CYB
Copepoda: Calanidae and Eucalanidae	cyt b	H10648-CYB	Reverse	GATAAAATTTTCWGGGTC	Machida et al. (2004)	L10319-CYB
Copepoda: *Neocalanus cristatus*	cyt b	Necr- CYB-L1	Forward	TTGGTGGTGACTTGGTACAGTGG	Machida et al. (2004)	
Copepoda: Oncaeids and *Tigriopus*	12S	L13337-12S	Forward	YCTACTWTGYTACGACTTATCTC	Machida et al. (2002)	H13842-12S
Copepoda: Oncaeids, Eucalanidae, Calanidae	12S	H13842-12S	Reverse	TGTGCCAGCASCTGCGGTTAKAC	Machida et al. (2004)	L13337-12S
Copepoda: Disseta	28S	F352-28S	Forward	AGACCGATAGTMAACAAGTACCGT	Machida and Tsuda (2010)	
Copepoda: Disseta	28S	R768-28S	Reverse	TAGACTCCTTSGTCCGTGTTTCA	Machida and Tsuda (2010)	
Copepoda: Calanoida	18S	18S-693R	Reverse	AAACCTCTGGCAAAACTACG	Bucklin et al. (2003)	18SE
Copepoda: Calanoida	18S	F1665-18S	Forward	CCGTCGCTACTACCGATTGAACG	Machida (unpubl) in Figueroa (2011)	R73-5.8S

Copepoda: Calanoida	18S	R73 - 5.8S	Reverse	GTGTCGATGTTCATGTGTCCTGC	Machida (unpubl) in Figueroa (2011)	F1665-18S
Copepoda: Diaptomidae	18S	18S-1F	Forward	AACCTGGTTGATCCTGCCAGT	Thum (2004)	18S-1R
Copepoda: Diaptomidae	18S	18S-1R	Reverse	TGGTGCCCTTCCGTCAATTCCT	Thum (2004)	18S-1F
Copepoda: Diaptomidae	18S	18S-2F	Forward	CTGGTGCCAGCAGCCGCGG	Thum (2004)	18S-2R
Copepoda: Diaptomidae	18S	18S-2R	Reverse	TTGATCCTTCTGCAGGTTCACCTAC	Thum (2004)	18S-2F
Copepoda: Mesocyclops	18S	18 s329	Forward	TAATGATCCTTCCGCAGGTT	Spears et al. (1992)	18sI-
Copepoda: Mesocyclops	18S	18sI-	Reverse	AACT(CT)AAAGGAATTGACGG	Spears et al. (1992)	18 s329
Copepoda: Eucalanidae	ITS2	ITS3F	Forward	GCATCGATGAAGAACGCAGC	White et al. (1990)	ITS10R
Copepoda: Eucalanidae	ITS2	ITS10R	Reverse	TACGGGCCTATCACCCTCTACG	Geerken and Wyngaardunpubl. Data in Goetze (2003)	ITS3F
Copepoda: Acartia	ITS2	ITS-4	Reverse	TCCTCCGCTTATTGATATGC	White et al. (1990)	IST-5
Copepoda: Acartia	ITS2	IST-5	Forward	GGAAGTAAAAGTCGTAACAAGG	White et al. (1990)	ITS-4
Copepoda: Paracalanidae	Histone H3	H3aF	Forward	ATGGCTCGTACCAAGCAGACVGC	Colgan et al. (1998)	H3aR
Copepoda: Paracalanidae	Histone H3	H3aR	Reverse	ATATCCTTRGGCATRATRGTGAC	Colgan et al. (1998)	H3aF

After morphological identification, the copepods collected from Vellar estuary were immediately preserved in 95% ethanol to facilitate DNA isolation. In addition, copepods (sample 2) were also collected from aquaculture farm located at Mimisal, Pudukkottai district, Tamil Nadu, Southern India and preserved in 95% ethanol to facilitate DNA isolation (Fig. 3).

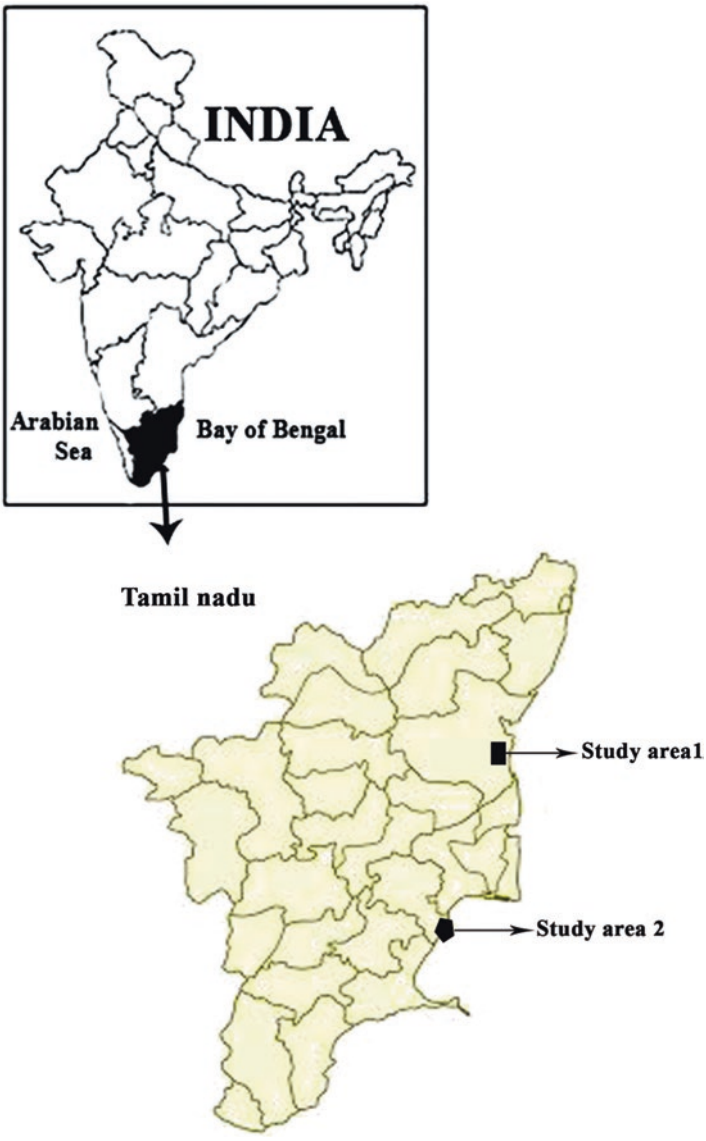


Fig. 3 Map showing the study area where the copepods were collected. Study area 1 (Vellar estuary, Parangipettai, sample 1); study area 2 (aquaculture farm, Mimisal, sample 2)

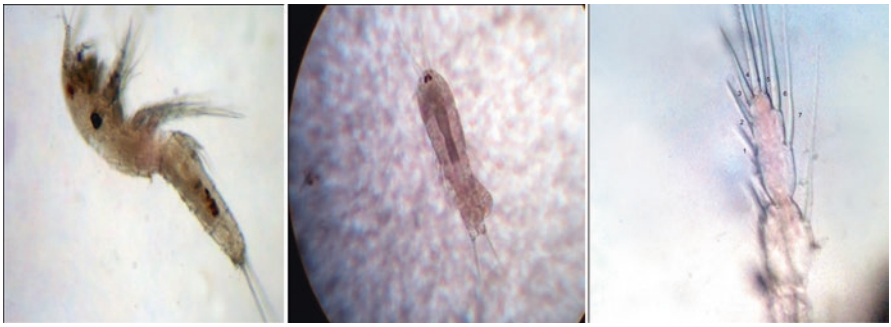


Fig. 4 Some morphological photos of *Nitokra affinis* (Rajthilak et al. 2015)

Morphological Description of Copepods

The copepods belonging to different families were identified by referring the standard keys. Specimens of copepods were isolated and separated, and their morphological features were evaluated to detect the family, genus and species of the copepod based on standard keys provided. Copepods were observed under a dissecting binocular and compound microscope, and their appendages were photographed with a well-equipped camera (Fig. 4). For clear information, SEM, TEM and drawings are also preferable. After confirmation of the species, the specimens were deposited according to the ICZN (International Code of Zoological Nomenclature).

Copepod Genomic DNA Isolation

Sample Preparation for DNA Isolation
The copepods were prepared for molecular analysis (both PCR and sequencing) by rehydrating individual copepods in 0.5 ml distilled water in a microcentrifuge tube for 24 h (Bucklin et al. 1995). In some cases, individual copepods were boiled in distilled water for 10 min to evaporate the alcohol before molecular analysis.

Isolation of DNA from Copepods (Spin-Column Protocol)

- Individual copepods were cut into two halves and placed in 1.5 ml microcentrifuge tube, and to this, 180 µl ATL buffer and 20 µl of proteinase K were added.
- The microcentrifuge tube was vortexed and incubated at 56 °C for 2 h. After incubation, 200 µl of AL Buffer and 200 µl 100% ethanol were added. The mixer was vortexed again. After vortexing, the mixture was placed in DNeasy Mini spin column with 2 ml collection tube and then centrifuged at 6000 × g (8000 rpm) for 1 min, and the flow-through and the collection tube were discarded.
- Then centrifuged at 6000 × g (8000 rpm) for 1 min. and discarded the flow-through and the collection tube.

- DNeasy Mini spin column was placed in new 2 ml collection tube, and 500 µl buffer AW1 was added. The column was centrifuged at 8000 × g rpm. Flow-through and collection tube were discarded.
- DNeasy Mini spin column was placed in new 2 ml collection tube, and 500 µl buffer AW2 was added and centrifuged at 20,000 × g (14,000 rpm) for 3 min. Flow-through and the collection tube were discarded out.
- The excess flow was allowed through the collection tube, DNeasy Mini spin column was once again placed in old collection tube (step 5) and column was centrifuged at 14,000 rpm for 1 min. Flow-through and collection tube were discarded out. Then, the DNeasy membrane was allowed to dry for 1 min at room temperature.
- The spin column was placed in a clean 2 ml microcentrifuge tube, and to this, 200 µl AE buffer was added. The spin column was again incubated at room temperature for 1 min and then centrifuged at 6000 × g (8000 rpm) for 1 min.
- The flow-through tube contained the DNA. Collected DNA was stored in 4°C until further analysis.

PCR reaction mix

Autoclaved deionized water	13.5 µl
*10X *Taq* buffer	1.5 µl
Forward primer 1 µm/µl	1.0 µl
Reverse primer 1 µm/µl	1.0 µl
dNTPs 10 mM/µl	1.0 µl
Taq polymerase 1.5 U/µl	1.0 µl
DNA template	1.0 µl
Total volume for one reaction	**20 µl**

Primers

18S rRNA gene (Takenaka et al. 2012)	
21F	5′-ATCTGGTTGATCCTGCCAGT-3′
1778R	5′-AATGATCCTTCCGCAGGTTC-3′

Cytochrome c oxidase subunit I (Folmer et al. 1994)	
LCO1490:	5′-GGTCAACAAATCATAAAGATATTGG-3′
HC02198:	5′-TAAACTTCAGGGTGACCAAAAAATCA-3′

PCR Program

Steps	Action	Time (min)
Initial denaturation	94 °C	5
Denaturation	94 °C	1

Steps	Action	Time (min)
Annealing	52 °C	1
Gone to	Step2	30 times
Final extension	72 °C	3
Held	4 °C	

Agarose Gel Electrophoresis

- 1 g of agarose was dissolved in 100 ml of 1X Tris acetate ethylenediaminetetraacetic acid (TAB) buffer.
- Agarose mixture was heated in the microwave oven for 60–90 sec interval with constant shaking.
- The mixture was swirled and was made sure that agarose has melted without any formation of lumps or particles.
- Agarose was allowed to cool for several minutes, and add 2 µl of ethidium bromide. Agarose solution was poured into a sealed gel tray without any air bubbles.
- After the gel had completely hardened, the comb was carefully removed, and the gel was immersed in 1X TAB running buffer.
- PCR-amplified products were loaded (1:1 ratio with gel loading dye) into wells; 100 bp DNA ladder was added in the first well.
- Electrical leads were connected to the electrophoresis chamber, and the gel was run with a current of 90 V for 30 min.
- The gel was carefully removed and viewed with Kodak Gel Logic 1500 documentation system with UV filter, and the amplified products of the PCR were visualized, and the images were captured.

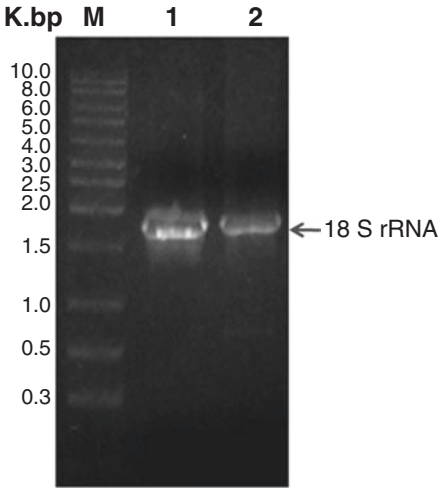
DNA Sequencing

DNA sequencing was performed at BioServe Biotechnologies Pvt. Ltd., Hyderabad, by using the specific primers of 18S rRNA and cytochrome C oxidase subunit I gene (COI).

Phylogenetic Analysis

The sequences were initially edited in the Gene Tool and Bio-edit software packages. Then, the edited sequences were submitted to NCBI database. DNA homology searches were performed using BLASTN 2.2.24 programs at NCBI, and similarity sequences were retrieved for phylogenetic analysis. A multiple alignment of all similarity sequences was done by Clustal W 2.1, and it was used to determine levels of differentiation between genera and species. Phylogenetic analysis was carried out by neighbour-joining (NJ) search with Kimura two-parameter model which was conducted using MEGA version 4.0.2. The tree was bootstrapped using 1000 sub-replicates. Similarly, pairwise nucleotide distances were estimated with the Kimura two-parameter model among the obtained partial 18S rRNA sequences, and out-groups were calculated using MEGA Ver. 4.0.2 (Plate 1).

Plate 1 Electrophoresis profile of amplified PCR product of 1800 bp filament of the 18S rRNA sequences



Lane M: Marker DNA
Lane 1: Vellar Estuary Sample
Lane 2: Aquafarm Sample

Results

The partial 18S rRNA sequences of samples 1 and 2 collected from different locations (Vellar estuary at Parangipettai, Cuddalore Dist., Tamil Nadu, India, and aquaculture farm at Mimisal, Pudukkottai Dist., Tamil Nadu, India) were compared with the already available homologous sequences pertaining to the family Ameiridae (NCBI-BLAST database).

Totally five sequences for the family Ameiridae were retrieved from NCBI database to compare the BLAST similarity homologous sequences between the genus (intergeneric) and species (interspecies) and within the genus and species (intrageneric or intraspecific) with our sequence. Among the five retrieved sequences, two belonged to the same genus *Nitokra* (*Nitokra hibernica* and *Nitokra spinipes*), and three belonged to the different genera: *Argestigens* sp., *Sarsameira* sp. and *Ameira scotti*.

The present sequences, partial 18S rRNA of sample 1 (*Nitokra affinis*) and sample 2 (*Nitokra* sp.), have been deposited in the NCBI (GenBank accession numbers: JX438707.1 and KF657669.1) (Figs. 5 and 6) (Table 5).

Sequence Similarity of Sample 1

The 18S rRNA gene sequence of *Nitokra hibernica* has been retrieved as the most homologous sequence with 95% similarity of 0.00 E value based on 99% of query coverage. The existing DNA sequences in NCBI for copepod species such as *Argestigens* sp., *Sarsameira* sp., *Ameira scotti* and *Nitokra spinipes* were found

```
          10         20         30         40         50         60         70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAGCCATGCATGTCCAAGTACAAGCCTCATTAAGGTGAAACCGCGAATGGCTCATTAAATCACATCTAAT

          80         90         100        110        120        130        140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATACCGGACAGTGCCAGTTACTTGGATAACTGCGGTAATTCTGGAGCTAATACATGCTGACGAGCCCCGA

          150        160        170        180        190        200        210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTCACGTGAAGGGCGCTTTTATTAGACCAAAACCAAACGTCCTCGGACGTCCCCCTTGGTGACTCTGAA

          220        230        240        250        260        270        280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAACTTTTTGCTGATCGCACGGCTCCACGCCGGCGACGCGTCCGTCTAAGGTGTGCCCTATCAACTGTCG

          290        300        310        320        330        340        350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGTGGCATAGACGCCCACAGTGGTTTTGACGGGTAACGGGGAATCAGGGTTCGATTCCGGAGAGGGAG

          360        370        380        390        400        410        420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCTGAGAAACGGCTACCACTTCTACGGAAGGCAGCAGGCACGCAAATTACCCACTGGCCGAAGGCCGAGG

          430        440        450        460        470        480        490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TAGTGACGAAAAATAACGATACCGGACTCATCCGAGGCCCGGTAATCGGAATGAGTACACTCTAAACCCT

          500        510        520        530        540        550        560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTAACGAGGAACAATTGGAGGGCAAGTCTGGTGCCAGCAGCCGCGGTAATTCCAGCTCCAATAGCGTATG

          570        580        590        600        610        620        630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTAAAGTTGTTGCGGTTAAAAAGCTCGTAGTTGGATCTCACCACCCCTGCGGCGGTCGCCTGTTCGGGCG

          640        650        660        670        680        690        700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GACTGCCGTCTCTGGGGTGTGACGCTTCGTCGGAGCCTGGGGGTGCTCTTAACCGAGTGTCCCCTGGCGC

          710        720        730        740        750        760
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....
CGTCGGGTTTACTTTGAAAAAATTAGAGTGCTCAACGCAGCCTTACACGCCTGGATATCCGTGCATGGA
```

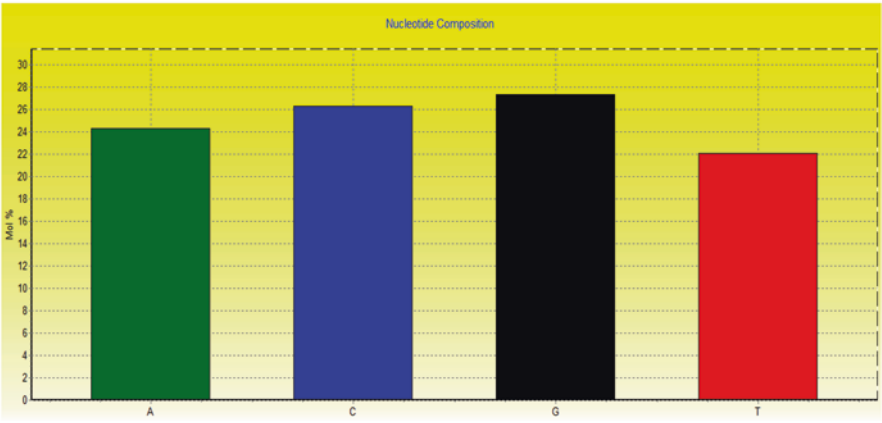


Fig. 5 *Nitokra affinis* (Source: Vellar estuary) (GenBank accession number: JX438707.1) and nucleotide composition

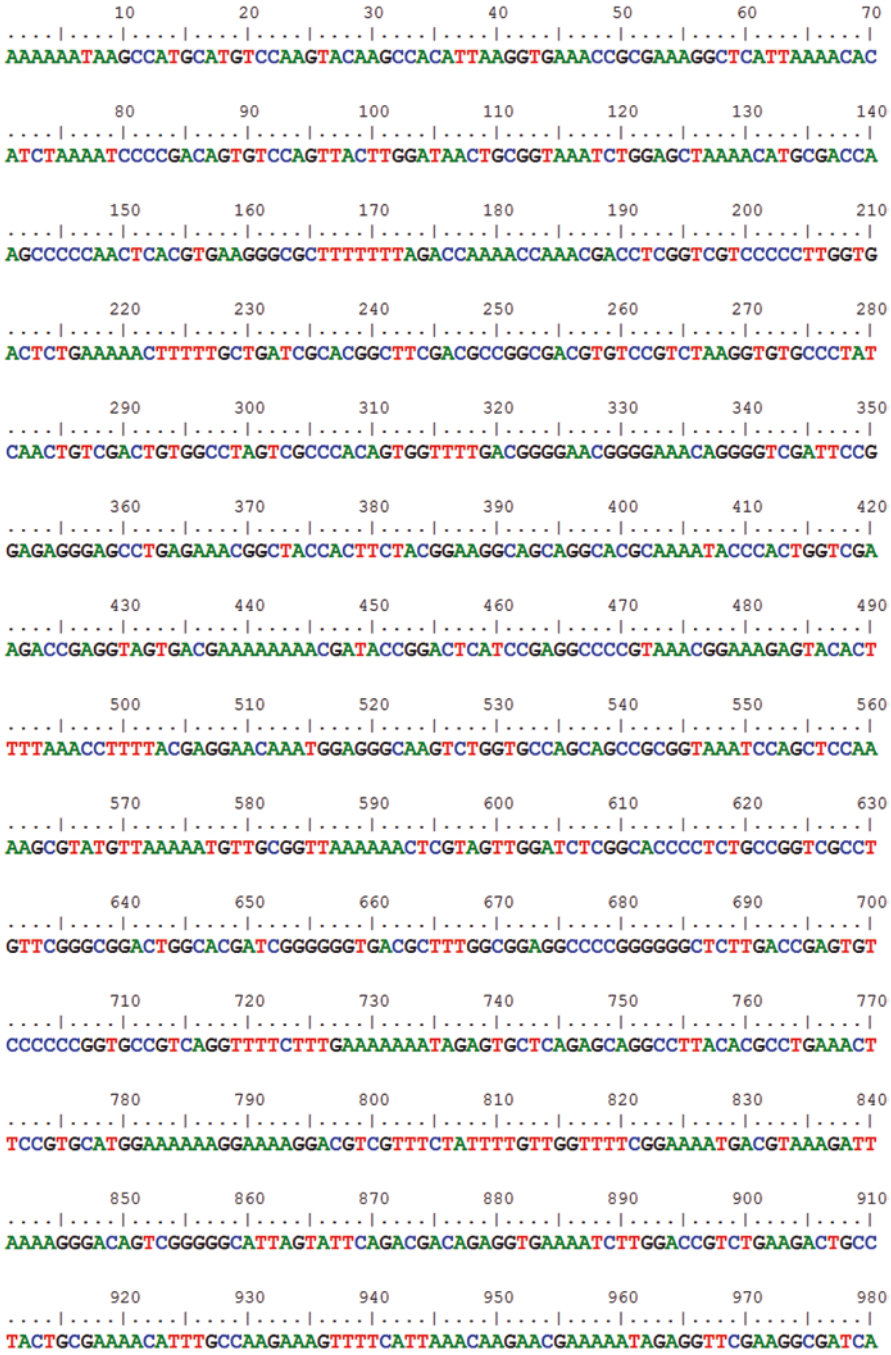


Fig. 6 *Nitokra* sp. (Source: aquaculture farm) (GenBank accession number: KF657669.1) and nucleotide composition

```
          990       1000       1010       1020       1030       1040       1050
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GATACCGCCCTAGTTCTAACCATAAACGATGCCAGCTAGCGATCCGAGGCTGTTTTTCTTGTAGGCTCCT

          1060       1070       1080       1090       1100       1110       1120
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGGGCAGCTTTCCGGGAAACCAAAACTCTTGGGTTCCGGGGGAAGTATGGTTGCAAAACTGAAACTTGAA

          1130       1140       1150       1160       1170       1180       1190
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGGAAATGACGGAAGGGCACCACCAGGCGTGGAGCCTGCGGCTTAAATTGACTCAACACGGGAAAACTCA

          1200       1210       1220       1230       1240       1250       1260
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCAGGCCCCGACACCGGCAGGATTGACAGATTGAGAGCTCTTTCTCGATTCGGTGGGGGGTGGTGCATGG

          1270       1280       1290       1300       1310       1320       1330
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CCGTTCTTAGTTGGTGGAGTGATTTGTCTGGTTAAATCCGATAACGAACGAGACTCTGTCCTGCTAACTA

          1340       1350       1360       1370       1380       1390       1400
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GTTCCGGGGGCCCCTTTTCCGCCCCCGAAAAAACTTCTTAGAGGGACTGGCGGCATCTAGTCGCACGAGA

          1410       1420       1430       1440       1450       1460       1470
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGGAGCAAAAACAGGTCTGTGATGCCCTTAGATGTTCTGGGCCGCACGCGCGCTACACTGAAAAGCTCAG

          1480       1490       1500       1510       1520       1530       1540
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CGTGTCCTCCCTTTCCGAGAGGAACGGGGAACCCCCTGAACCCCTTTCGTGGTAGGGATCGGGGCTTGCA

          1550       1560       1570       1580       1590       1600       1610
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AATCTTCCCCCTGAACCAGGAAAGCCCAGTAAGCGCAAGTCATAAGCTTGCGTTGATTACGTCCCTGCCC

          1620       1630       1640       1650       1660       1670       1680
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTGTACACACCGCCCCTCGCTACTACCGATTGAACGTTTTTGTGAGGTATTTGGACTGGACCTGGGGGG

          1690       1700       1710       1720       1730       1740
....|....|....|....|....|....|....|....|....|....|....|....|....|...
CTTCACGGCCGCCTTGTTTGTCCGGGAAGACTCCCCAACTTGAGCGTTTTGAGGAAGTAAAAA
```

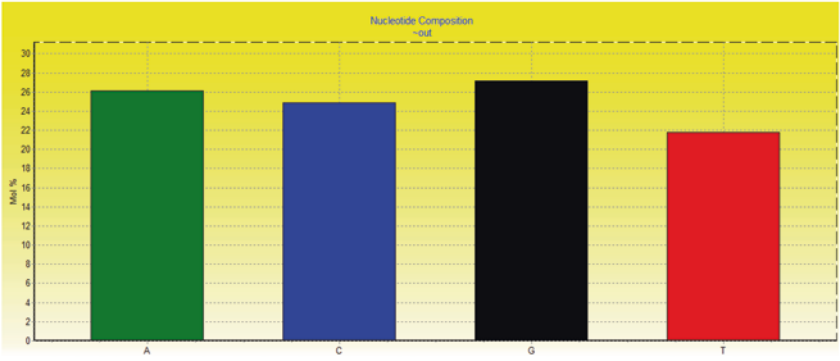


Fig. 6 (continued)

Table 5 Comparison of 18S rRNA sequences of *Nitokra* copepods collected from Vellar estuary and aquaculture farm

	Nitokra affinis JX438707.1	*Nitokra* sp. KF657669.1
Length in base pairs	769	1743
Molecular weight in Daltons		
Single stranded	234042.00	530275.00
Double stranded	467979.00	1060210.00
Base pair composition		
G + C content (%)	53.58	52.04
A + T content (%)	46.42	47.96
A	187 (24.32%)	456 (26.16%)
T	202 (26.27%)	434 (24.90%)
G	210 (27.31%)	473 (27.14%)
C	170 (22.11%)	380 (21.80%)

Table 6 BLAST similarity hits obtained for 18S rRNA sequence isolated from sample 1 (*Nitokra affinis*) collected from Vellar estuary, Parangipettai

Description	Max score	Total score	Query cover	E value	Ident	Accession
Nitokra sp. RC-2012 18S ribosomal RNA gene, partial sequence	1421	1421	100%	0.0	100%	JX438707.1
Nitokra hibernica 18S ribosomal RNA gene, partial sequence	1192	1192	99%	0.0	95%	EU380305.1
Argestigens sp. Greenland-RJH-2007 18S ribosomal RNA gene, partial sequence	1048	1048	99%	0.0	91%	EU380306.1
Sarsameira sp. Greenland-RJH-2007 18S ribosomal RNA gene, partial sequence	961	961	99%	0.0	89%	EU380304.
Ameira scotti 18S ribosomal RNA gene, partial sequence	953	953	99%	0.0	89%	EU380303.1
Nitokra spinipes strain CCUMP 40 18S ribosomal RNA gene, partial sequence	645	645	65%	0.0	90%	JQ315748.1

to be 91%, 89% and 90% similarity with 0.0 E value, respectively. The detailed BLAST results for *Nitokra* affinis are shown in Table 6.

Sequence Similarity of Sample 2

The 18S rRNA gene sequence of *Nitokra hibernica* has been retrieved as the most homologous sequence with 96% similarity of 0.00 E value based on 99% of query coverage. The sequences of *Argestigens* sp., *Ameira scotti*, *Sarsameira* sp. and *Nitokra spinipes* were found to be 90%, 88% and 85% similar with 0.0 E value, respectively. The detailed BLAST result for *Nitokra* sp. is shown in Table 7.

Table 7 BLAST similarity hits obtained for 18S rRNA sequence isolated from sample 2 (*Nitokra* sp.) collected from an aquaculture farm in Mimisal

Description	Max score	Total score	Query cover	E value	Ident	Accession
Nitokra sp. CR-2012 isolate PU 3 18S ribosomal RNA gene, partial sequence	3219	3219	100%	0.0	100%	KF657669.1
Nitokra hibernica 18S ribosomal RNA gene, partial sequence	2819	2819	99%	0.0	96%	EU380305.1
Argestigens sp. Greenland-RJH-2007 18S ribosomal RNA gene, partial sequence	2220	2220	99%	0.0	90%	EU380306.1
Ameira scotti 18S ribosomal RNA gene, partial sequence	2078	2078	99%	0.0	88%	EU380303.1
Sarsameira sp. Greenland-RJH-2007 18S ribosomal RNA gene, partial sequence	2063	2063	99%	0.0	88%	EU380304.1
Nitokra spinipes strain CCUMP 40 18S ribosomal RNA gene, partial sequence	662	1521	84%	0.0	85%	JQ315748.1

Table 8 Clustal W 2.1 similarity score value for 18S rRNA gene sequences of the *Nitokra affinis* and *Nitokra* sp.

Seq A	Name	Length	Seq B	Name	Length	Score
1	*Nitokra affinis* JX438707.1	769	2	*Nitokra* sp. KF657669.1	1743	91.55

Clustal W 2.1 Multiple Sequence Alignment of *Nitokra affinis* and *Nitokra* sp. Collected from Different Locations

The multiple sequence alignment was done by Clustal W 2.1 for *Nitokra affinis* and *Nitokra* sp. collected from different locations. Multiple sequence alignment similarity score value between *Nitokra affinis* and *Nitokra* sp. was 91.55% (Table 8).

Nucleotide Pairwise Distance for Nitokra affinis

The nucleotide pairwise distances for *Nitokra affinis* within family Ameiridae ranged between 0.058 and 0.195. *Nitokra affinis* had lesser 0.058 pairwise distances with the intra-generic *Nitokra hibernica*, and huge pairwise distance of 0.153 was observed in the intra-generic *Nitokra spinipes*. Among the inter-generic, *Nitokra affinis* had lesser 0.101 pair wise distance with the *Argestigens* sp. and higher 0.132 distance with *Sarsameira* strain. The detailed result for nucleotide pairwise distance for *Nitokra affinis* is shown in Table 9.

Table 9 Pairwise distance of nucleotide sequences of partial 18S rRNA of *Nitokra affinis* and selected family Ameiridae out-groups. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra affinis	*Nitocra hibernica*	*Argestigens* sp.	*Sarsameira* sp.	*Ameira scotti*	*Nitokra spinipes*
JX438707.1 *Nitokra affinis*		0.010	0.013	0.015	0.014	0.016
EU380305.1| *Nitocra hibernica*	0.058		0.012	0.015	0.013	0.016
EU380306.1 *Argestigens* sp.	0.101	0.090		0.010	0.012	0.016
EU380304.1. *Sarsameira* sp.	0.132	0.125	0.069		0.015	0.017
EU380303.1 *Ameira scotti*	0.124	0.115	0.089	0.138		0.019
JQ315748.1 *Nitokra spinipes*	0.153	0.161	0.148	0.165	0.195	

Nucleotide Pairwise Distance for Nitokra *sp.*

The pairwise nucleotide distances for *Nitokra* sp. within family Ameiridae varied from 0.042 to 0.188. *Nitokra* sp. had lesser 0.042 pairwise distance with the intra-generic *Nitokra hibernica*, and a huge pairwise distance of 0.188 was observed in the intra-generic *Nitokra spinipes*. Similarly, among intergeneric members, *Nitokra* sp. had lesser 0.105 pairwise distance with the *Argestigens* sp. and higher 0.127 distance with *Sarsameira* strain. The detailed result for nucleotide pairwise distance for *Nitokra* sp. is shown in Table 10.

Nucleotide Pairwise Distance Between Nitokra *spp.*

Nitokra affinis had 0.102 nucleotide pairwise distance with the intra-generic *Nitokra* sp. collected from different locations, viz. Vellar estuary, Parangipettai, and an aquaculture farm in Mimisal) (Table 11).

Table 10 Pairwise distances of nucleotide sequences of partial 18S rRNA of *Nitokra* sp. and selected family Ameiridae out-groups. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra sp.	*Nitocra hibernica*	*Argestigens* sp.	*Ameira scotti*	*Sarsameira* sp.	*Nitokra spinipes*
KF657669.1 *Nitokra sp.*		0.005	0.008	0.009	0.009	0.012
EU380305.1 *Nitocra hibernica*	0.042		0.006	0.007	0.007	0.010
EU380306.1 *Argestigens* sp.	0.105	0.067		0.006	0.005	0.010
EU380303.1 *Ameira scotti*	0.124	0.086	0.061		0.008	0.011
EU380304.1. *Sarsameira* sp.	0.127	0.085	0.052	0.087		0.010
JQ315748.1 *Nitokra spinipes*	0.188	0.147	0.131	0.150	0.125	

Phylogenetic Tree for Nitokra affinis

The partial 18S rRNA sequence of 769 bp of *Nitokra affinis* and selected Ameiridae out-groups were used to construct a gene tree using neighbour-joining method (Fig. 7). The tree branched out into two main clusters. In the first hierarchical level, the dendrogram tree was divided into two main clusters; *Nitokra spinipes* occurred separately, while the rest of the genus of family Ameiridae occupied on another cluster.

In the second hierarchical level, the members within family of Ameiridae was divided into two groups: the intra-generic strains of the *Nitokra* were paired on one clade (*Nitokra affinis* and *Nitokra hibernica*) with bootstrap value of 97, and the members of the intergeneric strains (*Argestigens* sp., *Sarsameira* sp. and *Ameira scotti*) are located on the other group in which *Argestigens* sp. and *Sarsameira* sp. again paired to another clade separating *Ameira scotti*.

Table 11 Pairwise distances of nucleotide sequences of partial 18S rRNA of *Nitokra* spp. and selected family Ameiridae out-groups. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra affinis	*Nitocra hibernica*	*Argestigens* sp.	*Nitokra* sp.	*Sarsameira* sp.	*Ameira scotti*	*Nitokra spinipes*
JX438707 *Nitokra affinis*		0.010	0.013	0.012	0.015	0.014	0.016
EU380305.1 *Nitocra hibernica*	0.059		0.012	0.008	0.015	0.013	0.016
EU380306.1 *Argestigens* sp.	0.101	0.092		0.015	0.010	0.012	0.016
KF657669.1 *Nitokra* sp	0.102	0.042	0.132		0.017	0.015	0.019
EU380304.1. *Sarsameira* sp.	0.132	0.127	0.069	0.165		0.015	0.017
EU380303.1 *Ameira scotti*	0.124	0.116	0.089	0.156	0.138		0.019
JQ315748.1 *Nitokra spinipes*	0.153	0.163	0.148	0.206	0.165	0.195	

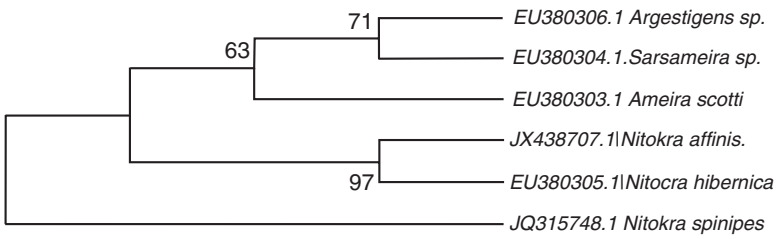


Fig. 7 Phenogram of partial 18S rRNA gene of *Nitokra affinis* and selected Ameiridae out-groups. Phylogenetic tree reconstruction was carried out by neighbour-joining method using Kimura two-parameter model, tree was bootstrapped at 1000× and values were shown at the branch point

Phylogenetic Tree for Nitokra *sp.*

The partial 18S rRNA sequence of 1743 bp of *Nitokra* sp. and selected Ameiridae out-groups were used to construct a gene tree using neighbour-joining method (Fig. 8). The tree branched out into two main clusters according to different genera as expected, with *Nitokra spinipes* diverged from the rest of the genus.

In the first hierarchical level, the dendrogram tree was divided into two main clusters, in which *Nitokra spinipes* was entirely diverged from the members of inter-generic strains assembled on another cluster. The second hierarchical level showed the intergeneric strain of *Sarsameira* sp. occurred separately from the rest of the inter generic members. The members within intergeneric strains were again separating *Argestigens* sp. from the rest of the members which was observed in the third hierarchical level. Whereas in the fourth hierarchical level, the tree was again separating intergeneric strain *Ameira scotti* from the intra-generic members (*Nitokra* sp. and *Nitokra hibernica*), and the intra-generic members assembled to a separate clade with high bootstrap value of 100.

Phylogenetic Tree for Nitokra *spp.*

Combining *Nitokra* spp. (*Nitokra affinis* and *Nitokra* sp.) with selected Ameiridae out-groups was used to construct a gene tree using neighbour-joining method (Fig. 9). The tree branched out into two main clusters according to different genera as expected, with *Nitokra spinipes* on one cluster delineating the rest of the genus on another main cluster.

In the first hierarchical level, the dendrogram tree was divided into two main clusters, with *Nitokra spinipes* diverged from the rest of the genus on another main cluster. In the second hierarchical level, the members of the family Ameiridae is divided into two groups: the intergeneric strains of family Ameiridae occurred in one group, and the members of the intra-generic strain occurred in another clade with high bootstrap value of 96. In the third hierarchical level, the intra-generic strains (*Nitokra* sp. and *Nitokra hibernica*) were occurred on one clade with high bootstrap value of 100 which was clearly separate *Nitokra affinis* occurred on the another group.

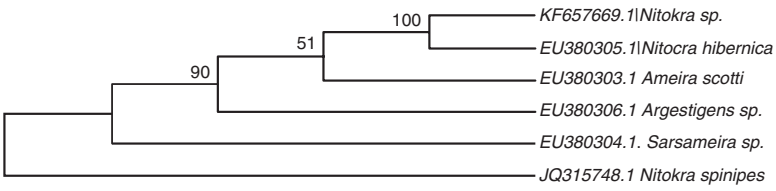


Fig. 8 Phenogram of partial 18S rRNA gene of *Nitokra* sp. and selected Ameiridae out-groups. Phylogenetic tree reconstruction was by neighbour joining using Kimura two-parameter model; tree was bootstrapped 1000×. Bootstrap value is given at the branch point

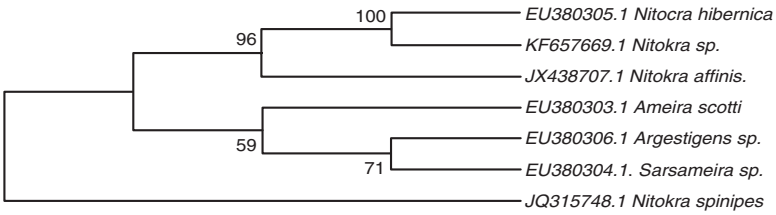
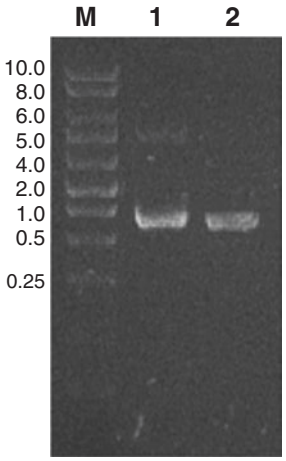


Fig. 9 Phenogram of partial 18S rRNA gene of *Nitokra* spp. and selected Ameiridae out-groups. Phylogenetic tree reconstruction was by neighbour joining using Kimura two-parameter model; tree was bootstrapped 1000×. Bootstrap value is given at the branch point

Plate 2 Electrophoresis profile of amplified PCR product of 650 bp filament of the COI gene; ladder shows 100–1000 bp band obtained



Lane M: Marker DNA
Lane 1: Vellar Estuary Sample
Lane 2: Aquafarm Sample

Cytochrome Oxidase Subunit I

The present mitochondrial cytochrome oxidase subunit I sequences (mtCOI) of *Nitokra affinis* and *Nitokra* sp. collected from different locations were sequenced and deposited in the NCBI (GenBank accession numbers: KC678692.1 and KF673354) (Plate 2).

There were no sequences belonging to family Ameiridae available in the GenBank for mtCOI. Therefore, we have retrieved four sequences of suborder harpacticoids belonging to different families from NCBI database in order to construct phylogenetic tree and nucleotide pairwise distances for *Nitokra* spp. (collected from different locations) and with the selected out-groups.

Among the four retrieved sequences, *Canthocamptus* sp. belonged to the family Canthocamptidae, two belonged to the same family Miraciidae (*Miraciidae* gen and *Stenhelia* sp.) and one belonged to the family Euterpinidae (*Euterpina acutifrons*) (Figs. 10 and 11) (Table 12).

```
          10         20         30         40         50         60         70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TCCCAACCAAATCATAAAGATATTGGAACTTTACAGGTTTTCTAGCGGGGACATGAGCTGGATAGTAGGA

          80         90        100        110        120        130        140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ACTGGATTAAGAATATTGATTCGTTTAGAGTTAGGTCAACCTGGTTCATTAATAGGTGATGACCAATCTT

         150        160        170        180        190        200        210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATAATGTCGTTGTCACAGCACATGCTTTTATTATAATTTTTTTTATAGTTATGCCTGTTTTAATTGGAGG

         220        230        240        250        260        270        280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATTTGGAAACTGATTAGTGCCTTTAATACTTGGTGCTCCTGATATAGCTTTTCCTCGATTAAACAATATG

         290        300        310        320        330        340        350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
AGATTTTGATTTCTTCTGCCCGCTTTAATTTTATTATTAAGAAGTAGAATAGTTGAAAGAGGAGCAGGAA

         360        370        380        390        400        410        420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTGGGTGAACAGTGTACCCGCCATTAAGGTCTAATATTTCCCATGCTGGGGGCTCAGTTGATTTAGCAAT

         430        440        450        460        470        480        490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTTTTCTTTGCATTTGGCAGGAATTTCGTCCTTGTTAGGAGCTGTAAATTTTATTAGGACTTTAGGAAAT

         500        510        520        530        540        550        560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTGCGGACTTTTGGCATATTTTATGATCGTATACCATTATTTTGTTGAGCGGTATTAGTGACAGCTGTTT

         570        580        590        600        610        620        630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTACTGTTAAGATTACCTGTTTTAGCAGGAGCCATTACAATGCTTCTGACAGATCGTAATTTAAATAC

         640        650        660        670        680        690        700
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TACTTTCTATGATGTAGCGGGAGGAGAGGGACCCTGTTTTATATCAACACTTATTTTGATTTTTTGGTCA

         710
....|....|....
CCCGGAAAATAAAA
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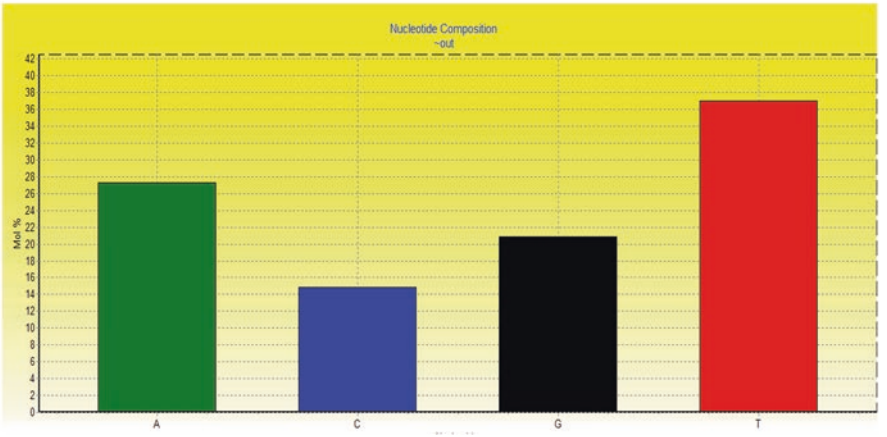

Fig. 10 *Nitokra affinis* (Source: Vellar estuary) (GenBank accession number: KC678692) and nucleotide composition

```
          10         20         30         40         50         60         70
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTACAGGTTTTCTAGCGGGGACATGAGCTGGATAGTAGGAACTGGATTAAGAATATTGATTCGTTTGGAG

          80         90        100        110        120        130        140
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTAGGTCAACCTGGTTCATTAATAGGTGATGACCAATCTTATAATGTCGTTGTAACAGCACATGCTTTTA

         150        160        170        180        190        200        210
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTATAATTTTTTTTTATAGTTATGCCTGTTTTAATTGGGGGTTTTGGAAACTGATTAGTGCCTTTAATACT

         220        230        240        250        260        270        280
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TGGTGCTCCTGATATAGCTTTTCCTCGATTAAACAATATGAGATTTTGATTTCTTCTGCCCGCTTTAATT

         290        300        310        320        330        340        350
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
TTATTATTAAGAAGTAGAATGGTTGAAAGAGGAGCAGGAACTGGGTGAACAGTGTACCCGCCATTAAGGT

         360        370        380        390        400        410        420
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTAATATTTCCCATGCTGGGGGCTCAGTTGATTTAGCAATTTTTTCTTTGCATTTGGCAGGAATTTCGTC

         430        440        450        460        470        480        490
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
CTTGTTAGGAGCTGTAAATTTTATTAGGACTTTAGGAAATTTGCGGACTTTTGGAATATTTTATGACCGA

         500        510        520        530        540        550        560
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
ATACCTTTATTTTGTTGAGCGGTATTAGTGACAGCTGTTTTACTACTGTTAAGATTACCTGTTTTAGCAG

         570        580        590        600        610        620        630
....|....|....|....|....|....|....|....|....|....|....|....|....|....|....|
GAGCCATTACAATGCTTCTGACAGATCGTAATTTAAATACAACTTTTTATGATGTGGCGGGAGGAGAGGG

         640
....|....|....|....
ACCCTGTTTTATATCAACA
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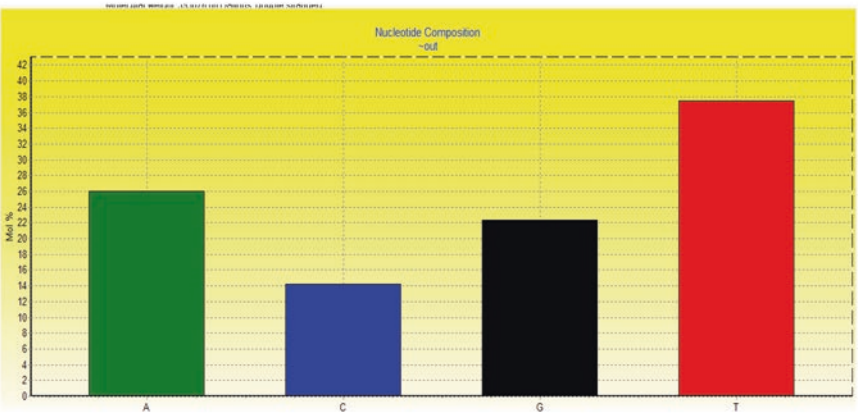

Fig. 11 *Nitokra* sp. (Source: Aquafarm) (GenBank accession number: KF673354) and nucleotide composition

Table 12 Comparison of COI gene sequences collected from Vellar estuary and aquaculture farm

	Nitokra affinis KC678692	*Nitokra* sp. KF673354
Length in base pairs	714	649
Molecular weight in Daltons		
Single stranded	214449.00	194553.00
Double stranded	432343.00	393076.00
Base pair composition		
G + C content (%)	35.71%	36.52%.
A + T content (%)	64.29%	63.48%.
A	195 (27.31%)	169 (26.04%)
T	106 (14.85%)	92 (14.18%)
G	149 (20.87%)	145 (22.34%)
C	264 (36.97%)	243 (37.44%)

BLAST Result for Nitokra affinis

The BLAST analysis was conducted for *Nitokra affinis* by comparing the partial mitochondrial COI gene sequences of *Nitokra affinis* with the available online database of GenBank. The BLAST analysis showed that the 81% high similarity with the partial mitochondrial COI gene region of calanoid copepod *Euchirella messinensis* (GenBank accession number: GU171308).

BLAST Result for Nitokra *sp.*

The BLAST analysis was conducted for *Nitokra* sp. by comparing the partial mitochondrial COI gene sequences of *Nitokra* sp. with the available online database of GenBank. BLAST results showed 80% closest similarity with *Mesocyclops edax* (GenBank accession number: JQ284449.1).

Nucleotide Pairwise Distance for Nitokra *spp.*

The pairwise nucleotide distances for *Nitokra* spp. within order harpacticoid varied from 0.020 to 0.451 (Table 18). *Nitokra affinis* (collected from Vellar estuary) had lesser 0.020 pairwise distances with the intra-generic *Nitokra* sp. (collected from an aquaculture farm at Mimisal).

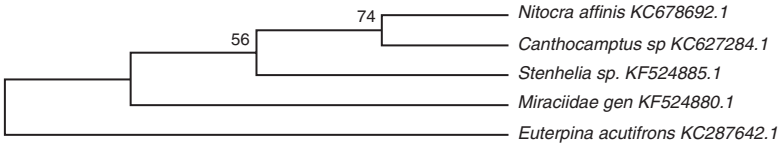

Fig. 12 Phenogram of partial mitochondrial COI gene region of *Nitokra affinis* and selected harpacticoid out-groups. Phylogenetic tree reconstruction was by neighbour joining using Kimura two-parameter model; tree was bootstrapped 1000×. Bootstrap value is given at the branch point

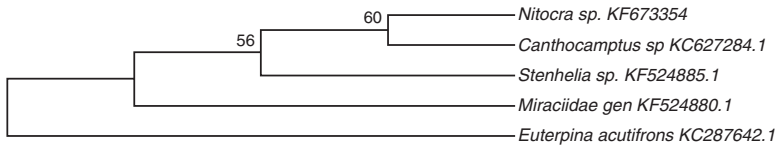

Fig. 13 Phenogram of partial mitochondrial COI gene region of *Nitokra* sp. and selected harpacticoid out-groups. Phylogenetic tree reconstruction was by neighbour joining using Kimura two-parameter model; tree was bootstrapped 1000×. Bootstrap value is given at the branch point

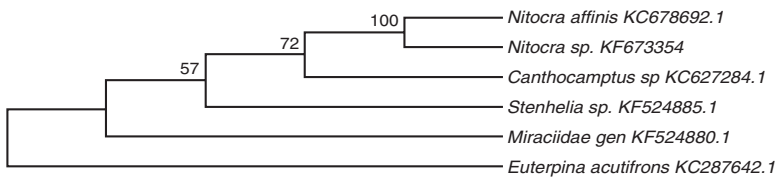

Fig. 14 Phenogram of Partial mitochondrial COI gene region of *Nitokra affinis* and *Nitokra* sp. and selected harpacticoid out-groups. Phylogenetic tree reconstruction was by neighbour joining using Kimura two-parameter model; tree was bootstrapped 1000×. Bootstrap value is given at the branch point

Phylogenetic Tree for *Nitokra affinis* and *Nitokra* sp. Partial Mitochondrial COI Sequences for Harpacticoid

The partial mitochondrial COI sequences of *Nitokra affinis* (collected from Vellar estuary) and *Nitokra* sp. (collected from an aquaculture farm at Mimisal) from different locations and selected harpacticoid out-groups obtained from NCBI were used to construct a gene tree using neighbour-joining method (Fig. 1).

In the trees (Figs. 12 and 13), *Nitokra affinis* and *Nitokra* sp. are clearly distinct from the rest of the harpacticoid copepods. In the tree (Fig. 14), the harpacticoid strain of *Euterpina acutifrons* that occurred in the separate cluster diverged from the rest of harpacticoid copepods. Then, the tree formed two groups separating *Miraciidae* from the rest of the harpacticoid out-groups followed by *Stenhelia* sp. (Miraciidae family).The tree again assembled to another clade constituting *Canthocamptus* sp. and *Nitokra* strain. The intra-generic strains of *Nitokra* (*Nitokra*

affinis and *Nitokra* sp.) collected from different locations assembled on a separate clade with high bootstrap value of 100 are clearly distinct from the closely related *Canthocamptus* sp. (family Canthocamptidae) and harpacticoid out-groups.

Clustal W 2.1 Multiple Sequence Alignment of Intra-generic *Nitokra affinis* and *Nitokra* sp. Collected from Different Locations

The multiple sequence alignment was done by Clustal W 2.1 for *Nitokra affinis* and *Nitokra* sp. to compare the intra-generic variation collected from different locations. The *Nitokra affinis* and *Nitokra* sp. differed by 12 bases which was highlighted (Fig. 15). Multiple sequence alignment similarity score value between *Nitokra affinis* and *Nitokra* sp. was 98.15 (Table 13).

Within the sample base pair differences were obtained based on substitution; Transition events (substitution of a pyrimidine by a pyrimidine or a purine by a purine) and Transversion events (substitution of a purine by a pyrimidine or a pyrimidine by a purine). The substitution events are illustrated in Table 14.

Clustal W 2.1 Multiple Sequence Alignment of Amino Acids of Intra-generic *Nitokra affinis* and *Nitokra* sp. Collected from Different Locations

The multiple sequence alignment was done by Clustal W 2.1 for *Nitokra affinis* and *Nitokra* sp. for amino acids to compare the intra-generic variation from different location. Since no changes were noticed in the protein level the score value recorded as 100 (Fig. 16 and Tables 15, 16, 17, and 18).

Conclusion

In our findings, the 18S rRNA and mtCOI markers showed different kinds of genetic divergence within the related species. The 18S rRNA for *Nitokra affinis* and *Nitokra* sp. showed genetic divergence that ranged between 4.2% and 20.6%, whereas the mtCOI showed 2% to 44.5% variations. The 18S rRNA which is a slowly evolving nuclear small subunit gene has been used to resolve deep phylogenetic relationship among the species within the family levels including invertebrates (Bucklin et al. 2003; Halanych et al. 1995, 1998). Thus, 18S rRNA for *Nitokra affinis* and *Nitokra* sp. showed closest similarity from the available homologous sequence in the NCBI database and which showed deep phylogenetic relationship within family level.

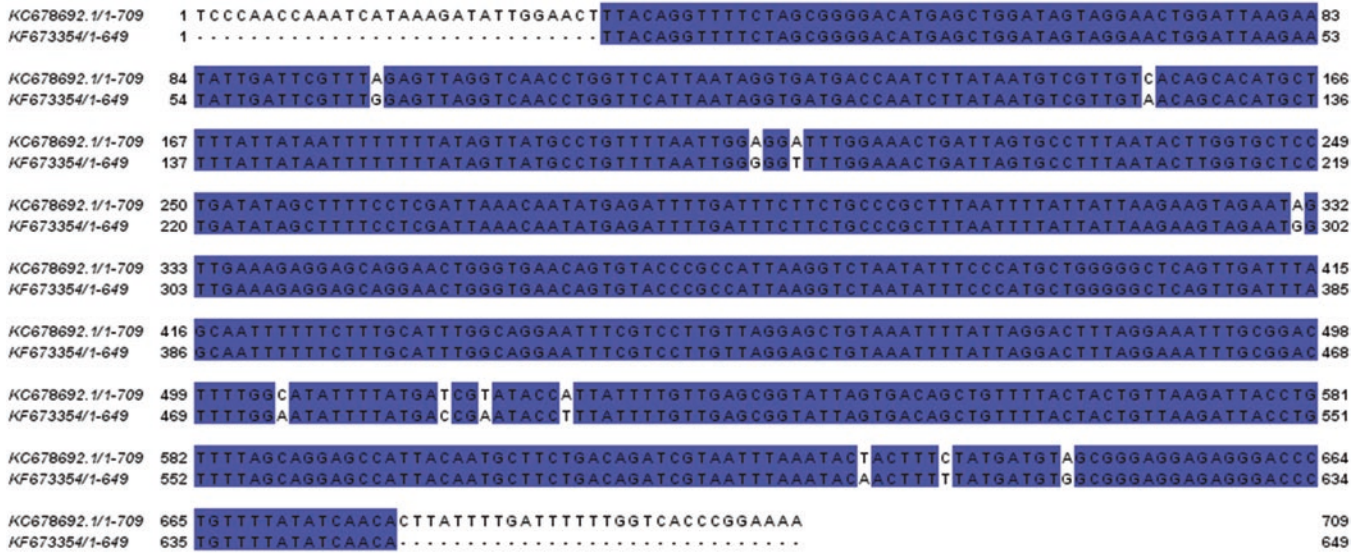

Fig. 15 Similarity of the COI gene sequences between the *Nitokra affinis* (KC678692.1) and *Nitokra* sp. (KF673354.1) collected from Vellar estuary and aquaculture farm (Mimisal)

Table 13 Similarity between COI gene sequences of the samples collected from Vellar estuary and aquaculture farm

Seq A	Name	Length	SeqB	Name	Length	Score
1	*Nitokra affinis* KC678692	714	2	*Nitokra* sp. KF673354.1	649	98.15

Table 14 Number of substitution events in comparisons between the mtCOI gene sequence for *Nitokra affinis* and *Nitokra* sp. Substitutions are separated into transitions and transversions

S. No.	Base pairs		Type of mutations
	Nitokra affinis	*Nitokra* sp.	
1	A	G	Transition
2	C	A	Transversion
3	A	G	Transition
4	A	T	Transversion
5	A	G	Transition
6	C	A	Transversion
7	T	C	Transition
8	T	A	Transversion
9	A	T	Transversion
10	T	A	Transversion
11	C	T	Transition
12	A	G	Transition

Fig. 16 Similarity of the amino acid sequences between the samples *Nitokra affinis* and *Nitokra* sp. collected from Vellar estuary and aquaculture farm (Mimisal)

Further, the mtCOI showed clear intraspecific variation among *Nitokra affinis* and *Nitokra* sp. since it is the most conservative protein-coding gene found in the mitochondrial genomes of animals (Brown 1985). It is also a good marker to study the population genetics and evolution. However, in our result, the genetic divergence between *Nitokra affinis* and *Nitokra* sp. collected from different locations exhibited a less variation of 2% but showed a huge genetic divergence with different selected harpacticoid copepods. This emphasizes that mtCOI is a very useful marker for comparison of sequence divergence within or between populations of the species.

Table 15 Similarity between COI amino acid sequences of the samples collected from Vellar estuary and aquaculture farm

Seq A	Name	Length	SeqB	Name	Length	Score
1	*Nitokra affinis* KC678692	236	2	*Nitokra* sp. KF673354.1	214	100

Table 16 Partial mitochondrial COI gene region of nucleotide pairwise distance (Kimura two-parameter model) of *Nitokra affinis* and selected harpacticoid out-groups. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra affinis	*Canthocamptus* sp.	*Miraciidae gen*	*Euterpina acutifrons*	*Stenhelia* sp.
Nitokra affinis KC678692.1		0.024	0.031	0.033	0.025
Canthocamptus sp. KC627284.1	0.305		0.029	0.029	0.027
Miraciidae gen KF524880.1	0.420	0.411		0.033	0.026
Euterpina acutifrons KC287642.1	0.442	0.420	0.425		0.032
Stenhelia sp. KF524885.1	0.312	0.377	0.347	0.438	

Table 17 Partial mitochondrial COI gene region of nucleotide pairwise distance (Kimura two-parameter model) of *Nitokra* sp. and selected harpacticoid out-groups. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra sp.	*Canthocamptus* sp.	*Miraciidae gen*	*Euterpina acutifrons*	*Stenhelia* sp.
Nitokra sp. KF673354		0.028	0.034	0.036	0.028
Canthocamptus sp KC627284.1	0.309		0.034	0.035	0.035
Miraciidae gen KF524880.1	0.402	0.412		0.036	0.031
Euterpina acutifrons KC287642.1	0.439	0.421	0.423		0.037
Stenhelia sp. KF524885.1	0.301	0.383	0.347	0.439	

Table 18 Partial mitochondrial COI gene region of nucleotide pairwise distance (Kimura two-parameter model) of *Nitokra* spp. and selected harpacticoid out-group. Pairwise nucleotide distances were shown in the lower left column, while standard error estimates were shown on the upper right column

	Nitokra affinis	*Nitocra* sp.	*Canthocamptus* sp.	*Miraciidae gen*	*Euterpina acutifrons*	*Stenhelia* sp.
Nitokra affinis KC678692.1		0.005	0.027	0.035	0.037	0.029
Nitokra sp. KF673354	0.020		0.027	0.034	0.037	0.029
Canthocamptus sp KC627284.1	0.307	0.312		0.035	0.035	0.033
Miraciidae gen KF524880.1	0.418	0.409	0.412		0.036	0.030
Euterpina acutifrons KC287642.1	0.445	0.451	0.421	0.423		0.037
Stenhelia sp. KF524885.1	0.314	0.311	0.383	0.347	0.439	

Therefore in the future, there is a need for the population studies on copepods from different geographical locations of Indian waters to estimate their genetic divergence. For that purpose, our study would frame a key factor to determine molecular phylogeny in copepods.

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A Method of Bio-efficacy Potential of Zooplankton (Copepod) for the Control of Vector Mosquitoes

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Introduction

Mosquitoes (Diptera: Culicidae) are one of the main threats for many people throughout the world; subsequently they act as vectors for indispensable pathogens for the following infections, malaria, dengue, yellow fever, West Nile, and parasites, such as filariasis (Murugan et al. 2015). Mosquitoes are the most critical group of insects in the context of public health, because they transmit numerous diseases, causing millions of deaths annually. An annual estimation of 390 million cases worldwide, growing incidence and more frequent epidemics, dengue is an increasingly important public health challenge (WHO 2012; Tran et al. 2015). As there is no vaccine or treatment for dengue, prevention and control of this disease depend on vector control to reduce viral transmission (Guzman and Kouri 2002). The key flight path of dengue is *Aedes aegypti*, a domestic mosquito that rears mostly in artificial water ampoules (Focks et al. 1981). In this situation, mosquito flight path rheostat is a main anticipation thing. In recent times, eco-friendly resistor tackles have been instigated to increase mosquito control. Substantial hard work has been conceded out investigating the efficacy of botanical products, and many plant-borne compounds have been reported as excellent toxins against mosquitoes, acting as

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adulticidal, larvicidal, ovicidal, oviposition deterrent, growth and/or reproduction inhibitors, and/or adult repellents (Conti et al. 2014; Murugan et al. 2015; Benelli et al. 2015a, b). Marine creatures are a gorgeous home of fundamentally new and biologically active metabolites, and cyclopoid copepods are noticeable predators in lots of aquatic ecosystems and have been acting as biological substitutes in efficacious programs to control mosquito larvae.

Copepods are tiny aquatic organisms. Many of compounds are used in the biological inhibition of mosquitoes, as well as copepods. Many species of cyclopoid copepods are victims of early mosquito larvae and have been successfully used in programs for controlling mosquito-transferred contagions, such as dengue (Nam et al. 1998, 2005). Cyclopoid copepods have been shown to be active predators of *Aedes aegypti* larvae in both laboratory investigation and environs trials (Kay et al. 1992). *Mesocyclops thermocyclopoides* is an incredibly familiar species in tropical and subtropical zones, and it was evaluated as a biological control agent against *Aedes* species. This copepod nurses on the first and second instars of the mosquito larvae, seriously off ending around seven individuals per day (Schaper and Hernández 1998). More than a few species of copepods, comprising *Mesocyclops aspericornis*, *M. thermocyclopoides*, *M. guangxiensis*, and *M. longisetus*, have been described as prospective biological regulator agents of *Ae. aegypti* (Rawlins et al. 1997; Jekow et al. 1998; Manrique-Saide et al. 1998; Schaper 1999; Locantoni et al. 2006). However, to evaluate new expected insecticides, more than a few factors need to be assessed. Among these factors, it is imperative to distinguish the time the harmfulness of the agent begins and in what way extended they sustain poisonous dosages for mosquito larvae. Moreover, it is essential to distinguish the lowest concentrations of sublethality in affecting mosquito enhancement. Thus, the objective of this study was to increase the predictive capability, larvicidal activity, predation by the copepod *Megacyclops formosanus*, and the combined effect of the copepod with different concentrations of the microalgae against vector mosquito larvae (Table 1).

Collection of Copepods

Copepods for aquaculture startup can be composed from ponds ditches and other eminence water bases. They can be both by promptly plummeting an appropriate bottle in the water, predominantly near inundated undergrowth. After dipping, slowly pour out 2/3 of the water in the container, and look at the remaining water for signs of the copepods. Even though they are small, they can be seen swimming in the collection vessel. If copepods are present, quickly pour the contents into a clean container, label, and cap securely.

The other technique of copepods collection is filtering water through two in-line sieves, either by forcing or by raising water with a bucket and bucketing over the sieves. The top sieve can be a usual kitchen filter; its purpose is to capture large debris that may be present in the water. Copepods will be caught in the subsequent

Table 1 Copepod species found to be promising biocontrol agents for mosquito control in different habitats

Species	Habitat	Mosquito species	Country	References
Mesocyclops aspericornis	Crab holes, tree holes,artificial containers	*Aedes* and *Culex* sp.	French Polynesia	Riviere et al. (1987)
M. albidus	Tire piles	*Ae. albopictus*	(New Orleans) USA	Marten (1990)
M. longisetus	Water storage containers	*Anopheles*	Honduras (Louisiana)	Marten et al. (1994)
M. venezolanus		*Ae. aegypti*	USA (operational)	
M. thermocyclopoides	Water storage urns,vases, and bromeliad	*Ae. aegypti*	Brazil	Vasconcelos et al. (1992)
M. albidus				
M. thermocyclopoides	200–500 ml bowls in the laboratory	*Culex quinquefasciatus*, *An. stephensi*, and *Ae. aegypti*	India	Mittal et al. (1997)
M. longisetus	Laboratory glass bowls and small artificial pools	*Ae. aegypti*	Brazil	Santos and Andrade (1997)
M. aspericornis				
Macrocyclops spp.	Domestic containers	*Ae. aegypti*	Caribbean Islands	Rawlins et al. (1997)
Mesocyclops spp.				
Acanthocyclops vernalis	Laboratory	*Ae. canadensis*		Andrealis and Gere (1992)
Diacyclops bicuspidatus	Bowls	*Ae. stimulans*		
Mesocyclops spp.	Natural mosquito breeding habitat	*Ae. aegypti*	Vietnam (operational)	Nam et al. (1998)
Mac. Albidus	Residential ditches	*C. quinquefasciatus*		Marten et al. (2000)
Mesocyclops spp.	Manholes and pits	*Ae. aegypti*	Queensland, Australia	Kay et al. (2002)
		Ae. albopictus		Dieng et al. (2002)
M. aspericornis		*Ae. aegypti*	Thailand	Kosiyachinda et al. (2003)
M. thermocyclopoides	Bowls	*C. quinquefasciatus*, *An. stephensi*	India	Kumar and Rao (2003)
M. albidus	Plastic pools	*Ae. aegypti* and *Ae. albopictus*	USA	Rey et al. (2004)
M. thermocyclopoides with *Bacillus thuringiensis*	Earthen jars	*Ae. aegypti*	Thailand	Chansang et al. (2004)

(continued)

Table 1 (continued)

Species	Habitat	Mosquito species	Country	References
M. annulatus	250 ml plastic containers	*Ae. aegypti* and *C. pipiens*	Argentina	Micieli et al. (2002)
M. longisetus and *M. albidus*	Rainwater, marsh, rice fields, and residential area	*Anopheles* spp. and *C. quinquefasciatus*	New Orleans (USA)	Marten et al. (1994)
M. thermocyclopoides	Laboratory	*An. stephensi* and *C. quinquefasciatus*	India	Ram Kumar and Ramakrishna Rao (2003)
M. thermocyclopoides	Laboratory	*Ae. aegypti*	India	Mahesh Kumar et al. (2012)
M. formosanus	Laboratory	*Ae. aegypti*	Taiwan	Kalimuthu et al. (2014)
M. annulatus	Freshwater ponds and laboratory	*Ae. aegypti* and *C. pipiens*	Argentina	Micieli et al. (2002)
M. albidus	Laboratory and field studies	*Ae. aegypti* and *Ae. albopictus*	Vero Beach (USA)	Rey et al. (2004)
Acanthocyclops vernalis	Laboratory conditions	*Ae. aegypti*	Saudi Arabia	Alshammari et al. (2015)
M. aspericornis	Breeding sites (water tanks and muddy areas)	*An. stephensi* and *Ae. aegypti*	India	Murugan et al. (2015)

sieve which should be set up as follows: get hold of a part of 200 μm mesh outsized adequate to shelter a subsequent sieve, push in the middle to roughly the shape of the strainer bowl and secure the edges to the strainer with a rubber band. Behind filtering through this setup, either remove the netting and invert in a container of clean dechlorinated water or turn the subsequent sieve setup upside down over a sanitary container and bathe with sanitary dechlorinated water from a squirt bottle. Add water to the collection bottles as required, shield and sticky label.

Isolation of Copepods

After collection, the copepods are screened to isolate the size fraction containing predominantly adult and late-stage copepods. This is achieved by first removing fish and prawn larvae. Grading is accomplished by using a set of superimposed sieves with varying mesh sizes and with decreasing mesh size from upstream to downstream. Copepod samples are screened coarsely through a 500 μm mesh to remove fish and prawn larvae. Then the samples were screened through a 190 μm mesh to remove rotifers, nauplii of copepods, and barnacles. After grading, copepods are

identified using standard manuals, monographs, and textbooks (Sewell 1947; Edmondson 1959; Kasturirangan 1963; Williamson 1991; Battish 1992; Reddy 1994; Dussart and Defaye 1995; Perumal et al. 2000; Altaff 2004; Santhanam and Perumal 2008).

Indoor Stock Culture of Copepods

Subsequent to the species authentication, identified amounts of gravid females were isolated by the use of capillary tubes, fine brush, and needle and are stocked initially in a low volume of glass beakers and conical flasks in case with microalgae without aeration. Later, the copepods are sub-cultured into average volume of plastic containers filled with filtered water and are provided with vigorous aeration. The most favorable water quality circumstances like temperature, salinity, pH, and dissolved oxygen are sustained. Copepods are fed with a daily ration of microalgae diet in the constant concentration. Copepods are, in general, cultured in freshwater. The stock is sustained regularly at a temperature of 29 °C. Copepod stock cultures are maintained in cylindrical, flat-bottomed, polyethylene 5 or 7 L plastic cans at air-conditioning room. Primarily the stock is conserved with 50–80 adult copepods. The microalgae cultures grown non-axenically in 5 L flasks are provided as groceries.

Stock Culture of Copepod

The stock cultures of copepod is taken from copepod culture collections (Marine Planktonology & Aquaculture Laboratory, Department of Marine Science, Bharathidasan University, Tiruchirappalli – 608 502, Tamil Nadu, India). The culture is started by inoculating 10 gravid female copepods into a quadrilateral crystal aquarium overflowing with 3 L of a culture medium containing microalga *Chlorella vulgaris* as a victim for the copepods. The copepods are reared at 27 ± 2 °C temperature, pH 7, and a photoperiod of 12 h (light):12 h (dark) regime in a culture rack using fluorescent lights. They are fed mosquito larvae for 3rd instar, and are then starved for 24 h prior to the experiment.

Collection of Eggs and Maintenance of Larvae

The eggs of *Aedes*, *Anopheles*, and *Culex* species are obtained from Vector Control Research Center (VCRC), Pondicherry, India, and Centre for Research in Medical Entomology (CRME), Madurai Field station of Tamil Nadu, India, using an “O”

type brush. These eggs are brought to the laboratory and transferred to 18 × 13 × 4 cm lacquer trays comprising 500 ml of water for hatching. The mosquito larvae are fed with pedigree dog biscuits and yeast at 3:1 ratio. The feeding is sustained until the larvae renovated into the pupal phase (Kumar et al. 2012).

Maintenance of Pupae and Adults

The pupae were harvested from the culture trays and transferred to malleable bottles (12 × 12 cm) encompassing 500 ml of water with the help of a dipper. The plastic jars are kept in a 90 × 90 × 90 cm mosquito cage for adult emergence. Mosquito larvae are kept at 27 ± 2 °C, 75–85% RH under a photoperiod of 14 L:10 D. A 10% sugar solution is provided for a period of 3 days before blood feeding (Kumar et al. 2012).

Blood Feeding of Adult

The adult female mosquitoes are permitted to feed on the blood of a rabbit (one rabbit per day, exposed on the dorsal side) for 2 days to ensure adequate blood feeding for 5 days. After blood feeding, enamel trays with water from the culture trays are placed in the cage as oviposition substrates (Kumar et al. 2012).

Larval and Pupal Toxicity Test

Laboratory colonies of mosquito larvae/pupae are used to test the larvicidal/pupicidal activity. Twenty-five first to fourth instar larvae (I, II, III, and IV) and pupae are introduced into a 500 mL glass beaker containing 249 mL dechlorinated water. Larval food is given to the test larvae during the experimental period. At each tested concentration, two to five trials are performed, and each trial consisted of five replicates (Kalimuthu et al. 2013). The control group's mortalities were corrected using Abbott's formula (Abbott 1925).

$$\text{Corrected mortality} = \frac{\text{observed mortality in treatment} - \text{Observed mortality in control}}{100 - \text{Controlmortality}} \times 100$$

$$\text{Percentage mortality} = \frac{\text{No.of dead larvae / Pupae}}{\text{Number of larvae / pupae introduced}} \times 100$$

The LC_{50} and LC_{90} are intended for toxicity data using probit analysis (Finney 1971).

Predatory Efficiency Test

Adult copepods are used to measure the predatory activity toward the four instars (I, II, III, and IV) and pupae of the mosquito larvae. One hundred individuals' mosquito larvae of each instar and 10 adult copepods are introduced into split 500 ml glass beakers containing 250 ml of dechlorinated water. The mosquito larvae are replaced daily with new ones. Each mosquito instar copepod treatment is replicated five times. The control group consisted of 250 ml of dechlorinated water without copepods. The glass beakers are inspected after 24, 48, 72, 96, and 120 h, and the number of prey consumed by the predators is recorded (Kalimuthu et al. 2013).

Predation of Copepods Against Malaria and Dengue Mosquitoes

Predation efficiency of copepod adults is evaluated contrary to *Anopheles stephensi* and *Aedes aegypti* larvae. For each instar, 100 nos. of mosquitoes are introduced, with 10 nos. of copepods, in a 500 mL glass beaker containing 250 mL of dechlorinated water. Mosquito larvae are replaced every day with fresh ones. For each mosquito instar, five replicates are conducted. The control is 250 mL of dechlorinated water exclusive of copepods. All beakers are tartan after 1, 2, 3, 4, and 5 days, and the number of prey enthusiastic by copepods is recorded. Predatory efficiency is calculated using the following formula:

$$\text{Predatory efficiency} = \left[\frac{\left(\text{number of consumed mosquitoes} / \text{number of predators}\right)}{\text{total number of mosquitoes}}\right] * 100$$

Conclusion

The present method demonstrates ecological importance of copepod in the control of mosquito *Anopheles*, *Aedes*, and *Culex* larvae. The research chapter reveals that some species may serve as a nutritious food for mosquito larvae, whereas others are harmful. Mosquito larvae are incapable to succeed their growth if dense copepods are abundant in the aquatic environment to inhibit the larvae intake of sufficient supplementary nourishment to fulfill their nutritional requirements. This occasionally happens in environments as shown in Fig. 1.

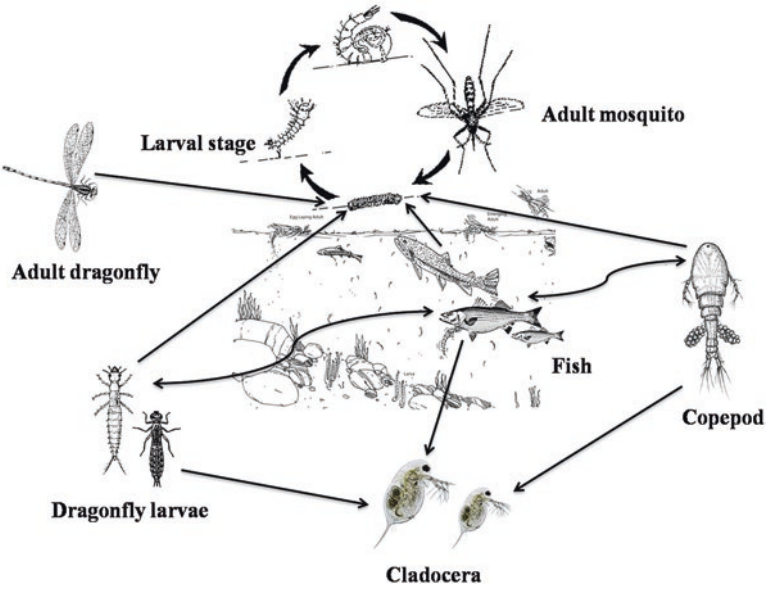

Fig. 1 Diagrammatic representation of the interactions between mosquito larvae and their predators (Ram Kumar and Jiang-Shiou Hwang 2006)

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Techniques in the Collection, Preservation and Morphological Identification of Freshwater Zooplankton

N. Manickam, P. Santhanam, and P. Saravana Bhavan

Introduction

The word plankton (Greek – "wanderers" or "drifters") was coined by German Marine Biologist Victor Hensen (1887). The terminology "plankton" is plural (singular – plankter). Plankton community is a heterogeneous group of tiny plants (phytoplankton) and animals (zooplankton) adapted to suspension in the sea and fresh water. Their intrinsic movements, if any, are so feeble that they remain essentially at the mercy of every water current. It is a potentially functional community of similar organizational rank implicit in the terms forest or grassland communities. The terminology "plankton" included all organic particles, which float freely and involuntarily in the open water independent of shore and bottom. The dependence of plankton upon water movement for maintenance and transport is accurately implied in this definition (Greek word – planktos, meaning wandering). The term plankton refers to any small biota (from microns to centimeters) living in the water and drifting at the mercy of currents, ranging from bacteria to sea jelly.

The definition of plankton is rather loose, as we often include sea jelly and krill (euphausiids and their larval forms) as plankton, yet they are active swimmers and, therefore, technically referred to as "nekton". Sometimes, even good swimmers

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such as late-stage fish larvae are incorrectly termed "planktonic", as they are often caught in the plankton nets, particularly at night. Another definition of plankton is simply "material which is caught in a fine mesh net". Plankton refers to those minute aquatic forms which are non-motile or insufficiently motile to overcome water currents, and transport and live suspended in the open or pelagic water. Hensen stated that plankton did not specifically exclude non-living particles. Therefore, plankton is regarded as being synonymous with "seston" in Kolkwitz's (1912) terminology. The term "seston" (ceston) denotes particulate dead or living matter drifting passively in water. Ceston is divided into bioceston, the floating organisms in water, and abioceston, the non-living particulate matter, such as clay, sand etc. The term "tripton" coined by Wilhelmi (1917) for non-living particulate matter is still valid. Investigation of the origin of tripton identifies two major groups: eutripton, the term used for all material of autochthonous origin (the particles originating in the lake or pond itself) and pseudotripton, the particulate matter of allochthonous origin (originating outside the lake or pond). Depending on their ability to carry out photosynthetic activity, plankton are divided into phytoplankton (plant plankters) and zooplankton (animal plankters).

Plankton can also be classified on the basis of size, using capture methods. The net plankton are those retained by a tow net, while nanoplankton will normally pass through these nets. The plankton can be further divided as follows: megaloplankton ➔ more than 8 cm (*Physalia*, *Velella*, *Porpita*, all marine), macroplankton ➔1 mm to 1 cm, mesoplankton ➔ 0.3 mm to 1 mm, microplankton ➔ 0.06 mm to 0.5 mm, nanoplankton ➔ 0.005 mm to 0.06 mm and ultraceston ➔ 0.0005 mm to 0.005 mm in size. The term "picoplankton" has been introduced recently for exceedingly small organisms of less than 0.0005 mm in size.

Plankton are also distinguished as holoplankton and meroplankton: the former remain planktonic throughout their lives and the latter only for a short period, e.g. glochidium larvae of freshwater mussels. Plankton are sometimes referred to by their habitat. The plankton species from ponds are called heleoplankton, of lakes limnoplankton, of running water potamoplankton (rheoplankton) and of salt water haliplankton. The pseudoplankton are those which occupy the upper layer of water accidentally. Examples of these include creeping organisms which rise up through the aquatic vegetation and are accidentally caught in the plankton collection.

Zooplankton

The term zooplankton is derived from the Greek words zoo, meaning animals, and plankton, meaning wanderers. The members of zooplankton include the marine and freshwater planktonic community that drifts according to the water currents. The planktonic plants are called phytoplankton and the planktonic animals are called zooplankton (APHA 1985).The zooplanktonic assemblage inhabiting freshwater comprises Protozoa, Coelenterata, Rotifera, Gastrotricha, Bryozoa and Arthropoda. Zooplankton are microscopic animals which do not have the power of locomotion and move against the water currents. They occupy an intermediate position of the

food web and zooplankton mediate the transfer of energy from lower to higher trophic levels (Waters 1977); thus, zooplankton represent an important link in aquatic food chains and contribute significantly to secondary production in a freshwater ecosystem (Sharma 1998; Manickam 2015).They range in size from microscopic protozoans to the largest sea jelly of over 10 μm in length. Most zooplankton occupy the second or third tropic levels of the aquatic food webs, such as the herbivores, carnivores and omnivores, and play a very important key role in the food webs. The minute size of phytoplankton denotes that aquatic grazers are very small and, therefore, there exist many steps or links in aquatic food webs which are necessary to support large carnivores, such as finfish and shellfish.

Laboratory studies of zooplankton samples offer an opportunity to study the tremendous diversity of organisms found in plankton communities. Many of the zooplankton, though important, require specific and more elaborate study techniques that go beyond the frame of the present course. The metazoan zooplankton can be distinguished into two major groups; the holoplankton (or permanent plankton), that spend their entire life cycle as plankton (for example, Copepoda, salps and some sea jellies), and the meroplankton (or temporary plankton), which are only planktonic for part of their life cycle as plankton (usually at the larval stage forms of benthic or nektonic adults).

The macroplankton are generally more abundant in coastal areas because of the vicinity of the benthic realm. Some of the most important metazoan holoplankton in the marine system are the copepods, a group of small crustaceans. In marine systems, Copepoda replace the water fleas (cladoceran) of freshwater in terms of abundance, diversity and importance. Almost any zooplankton tow net from all marine planktonic communities will contain numerous copepods. Although meroplankton is often also termed “larval plankton”, the nauplii and copepodids of copepods do not belong to the meroplankton but to the holoplankton. Despite their abundance and diversity in freshwater systems, much smaller numbers of species of water fleas (cladoceran) occur in marine waters. At times, they can occur in considerable abundance.

Freshwater zooplankton is dominated by protozoans, rotifers and three subclasses of Crustacea, i.e. Cladocera, Copepoda and Ostracoda. The planktonic Protozoa have limited locomotion, but the rotifers, Cladocera and Copepoda microcrustaceans and certain immature insect larvae often move extensively in quiescent water. Many pelagic Protozoa (5–300 μm) are meroplanktonic, in that only a portion, usually in the summer, of their life cycle is planktonic. These forms spend the rest of their life cycle in the sediments, often encysted throughout the winter period. Many protozoans feed on bacteria-sized particles (most cells <2 μm) and, thereby, consume a size class of bacteria and detritus generally not utilised by large zooplankton. Although most rotifers (150 μm–1 mm) are sessile and are associated with the littoral zone, some are completely planktonic. These species can form major components of the zooplankton. Most rotifers are non-predatory and omnivorously feed on bacteria, small algae and detrital particulate organic matter. Most food particles eaten are small (<12 μm in diameter). Most cladoceran zooplankton are small (0.2–3.0 mm), have a distinct head and the body is covered by a bivalve carapace. Locomotion is accomplished mainly by means of the large second antennae. The

productivity of suspension feeding zooplankton is higher than that of predaceous zooplankton.

Much of the wild zooplankton are an important source of live food organisms and they can play a vital role in the hatchery production of seeds. The live food provides highly essential nutrition to achieve good growth and survival rates of commercially important finfish and shellfish larvae. The diversity of zooplankton in freshwater and marine water habitats is very high, and many of these species have great potential for mass culture and use in aqua hatcheries. Recently, mass culture technology has been established for the culture of Rotifera (*Brachionus* sp.), Cladocera (*Daphnia*, *Ceriodaphnia* and *Moina*) and Copepoda (Calanoida and Cyclopoida), as these organisms have nutritive value and a wide spectrum of size suitability for freshwater and marine water fish, prawn and shrimp larvae. Zooplankton are ideal organisms for toxicological studies and environment monitoring. Due to their short life span and wide distributions, many zooplankton act as ecological indicators.

Methodology

In the study on freshwater zooplankton, depending on the study target, different types of observations may be required for the correct identification of plankton up to the species level, population counts to determine their population density and seasonal abundance, determination of the biomass or wet weight or volume, dry weight etc. This chapter deals with the systematic methods for the sample collection, concentration, fixation, preservation, morphological identification, dissection, measurement and determination of taxonomically important characteristics, and life-cycle strategies and species development stages are discussed.

The methods pertaining to other aspects are omitted here but detailed information can be found in the works by Welch (1948), Edmondson (1959), Schwoerbel (1970), Ruttner (1975), Battish (1992), Murugan et al. (1998) and Altaff (2004). Zooplankton research is a very important new strand for students, researchers and those involved in the environmental management of governments and non-governmental organisations (NGOs). The fundamental information essential for any studies of biologists (botany and zoology) include an animal's morphology, physiology and body development stages. This chapter reveals the methods of collection, preservation and taxonomic identification of freshwater zooplankton.

Collection of Zooplankton Samples

The zooplankton samples are collected by using plankton nets. In fact, there are many methods for concentrating the zooplankton and a vast amount of literature is now available describing the various equipment and methods for planktonic study.

A critical review of this literature can be found by Downing and Rigler (1984). Here, we mention some easy methods and equipment which can be made by the worker herself/himself, which have been tested by the authors with good results. The plankton samplings should be made in freshwater or seawater during the early morning hours (5.00 am to 7.00 am), which is the ideal time for plankton to distribute themselves at the pelagic and epipelagic layers of the water system.

Plankton Nets

Except for nanoplankton, all other categories of plankton can be easily collected by nets. There are many types of nets available (Welch 1948), such as tow nets, Birge core nets and Juday plankton trap nets, yet their shapes remain by and large conical, with a metallic ring forming the mouth of the net, around which bolting cloth is stitched in a manner so as to narrow at the cone end. The net cloth is available in a variety of materials, such as silk, organdie, muslin, terylene, nylon and assorted synthetic fibres. Naturally, the mesh size will vary from cloth to cloth. Generally, a synthetic cloth of uniform mesh is preferred over silk, as it is tougher and the fibres do not swell in water. Synthetic cloths of 35 to 335 micron mesh size are used for zooplanktonic collection. The plankton nets (Fig. 1) are available in various shape and sizes, and can be purchased from most scientific instruments suppliers. Generally, nets according to Apstein's design are used in limnological pursuits, but the filtering surface chosen nowadays constitutes synthetic fibre of variable mesh size.

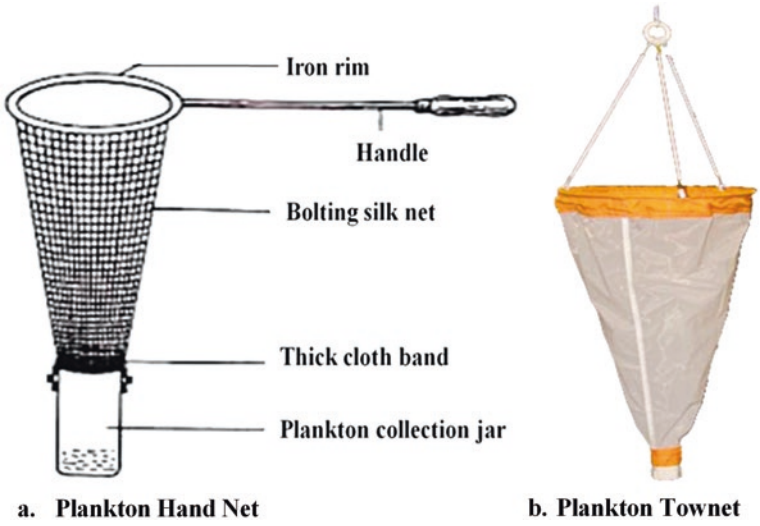


Fig. 1 Two types of plankton nets: (**a**) plankton hand net; (**b**) plankton tow net

Preparation of Zooplankton Nets

The plankton nets can be prepared easily in different sizes. Take one square metre of synthetic cloth of uniform mesh. For the Protozoa and Rotifera, cloth of 55 micron mesh size is suitable, but for Cladocera, Copepoda and Ostracoda, a 335 micron mesh size serves the purpose better. Polysynthetic gauze is recommended, as it does not swell on being wetted. For taxonomic studies, a net of muslin, organdie, nylon or terylene can also be used, but these cloths get spoiled easily.

Concentration of Plankton Samples

A number of methods are available by which plankton can be concentrated. The method chose should keep in mind the planktonic group of interest. There are three methods that are commonly used in plankton concentration: filtration, centrifugation and photoconcentration.

Filtration Method

The plankton samples can be further concentrated by sieving through a fine mesh or sieving through a membrane. Application of the vacuum technique can hasten the filtration process but causes considerable damage to the zooplankters.

Centrifugation Method

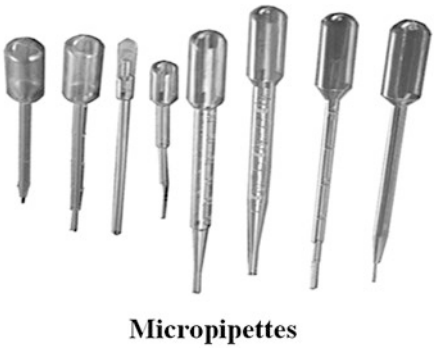
This is a useful method and, perhaps, only suitable for nanoplanktonic collection. The sample is poured into graduated centrifuge tubes of 10–30 mL capacity and rotated in a manual or electric centrifuge (Fig. 2) for 10–20 min at different rates of revolution (1500–2000 rpm), after which the supernatant water is removed. A hand-operated centrifuge is also useful during staining when the reagents have to be changed frequently.

Photoconcentration Methods

The various plankters have a differential affinity towards light. A suitable source of light can be used in aggregating both photopositive species, which move towards the light source, and photonegative organisms, which move away from it. The

Fig. 2 Types of centrifuge: (**a**) hand centrifuge; (**b**) electric centrifuge

Fig. 3 Different types of micropipettes



experienced worker uses different sources and intensities of light, keeping in mind the plankters under study. Thus, the different plankters can be pipetted using micropipettes (Fig. 3) from the culture near the light source or from darkness and then preserved.

Fixation and Preservation of Plankton Sample

Once the sample has been collected, it must be fixed and preserved at the earliest opportunity. Many killing and preserving reagents have been tested by scientists. Some are used for cytological, anatomical and morphological studies, but only the methods useful for taxonomic studies are discussed here. Formalin is one of the best (hence, it is widely used) fixing and killing agents of organisms. It is used in different concentrations, from 2 to 10%, and it is used to preserve stages from microorganism to early larvae (prepared from 40% formaldehyde solution available

commercially). The prepared solutions are normally kept at low temperature, i.e. below 24 °C. One of the simplest methods of formalin solution preparation is 40% formaldehyde added with 60% distilled water and mixed slowly for the preparation of 100% formalin (40% formaldehyde solution). Delicate forms such as Protozoa and Rotifera are best preserved in 2% and 4% formalin, respectively. For the medium-sized plankters such as Cladocera, Copepoda and Ostracoda, 5–6% formalin solution is suitable, but for the larger forms, such as fairy shrimps (Anostraca), preservation in 10% formalin is recommended. For these larger forms, an injection formalin solution can be given for better results. The collected plankton samples are filtered to remove the water and then the samples are transferred to polyethylene bottles filled with formalin (different groups of species require different concentrations or percentages of formalin).

Transfer and Isolation of Zooplankters

Zooplankton is very small and very difficult to handle. For any type of study, their isolation, separation and transfer from one slide/reagent to another is required. These steps require special tools and skills. Various workers have recommended many types of needles, droppers and brushes mounted on a stick. However, the animals can be isolated and transferred very easily by specially designed microloops and micropipettes, which can be prepared simply without requiring any special skills.

Separation and Staining of Plankton Sample

The preserved zooplankton is segregated groupwise, including Rotifera, Cladocera, Copepoda and Ostracoda. They are separated under a high-power stereo binocular zoom dissection microscope using a fine needle and brush. From the planktonic concentrate preserved in formalin, transfer the material to a vial for staining. Individual species of plankton are mounted on microscopic slides on a drop of 20% glycerin after staining with eosin and Rose Bengal. First, the material should be washed with water twice or thrice and then a few drops of 20% glycerin added after staining with aqueous Eosin or Rose Bengal. The staining solution is easily prepared using 1 g of Eosin or Rose Bengal powder added to 100 mL of distilled water, followed by proper mixing. One drop of stain will be added to zooplankton samples. Then, after 5–10 min, the stain will be removed and the specimen washed with distilled water twice. The specimens are now ready. One specimen is transferred to a glass slide and a drop of Farrant's medium or glycerin is added to it. Water will be mixed in an equal volume to cover a slide. Farrant's medium is best for this purpose because it is a clearing agent and the specimen can be transferred into it directly

from the water. If covered with a cover slip, the slide will become permanent after sealing with wax. Study the specimen under a stereo binocular dissecting microscope. The specimen can be orientated in a dorsal, lateral or ventral view. If the specimens are sparse in the collection, a diagram of the whole specimen can be drawn with the help of a camera lucida before dissection. Next, using fine needles, separate the parts of taxonomic importance and make a diagram of them uncompressed (without the cover slip). Before drawing a figure, ascertain that the organism or its part is orientated in the view designated in the key for ready comparison.

Identification of Zooplankton

The accurate identification of plankton requires examination in detail under high magnification. The preserved samples should be identified as fresh as possible because long preservation periods distort the specimens and their pigment fades partially or completely. The identification methods for each zooplanktonic group are described in their respective sections in this chapter. The identification of zooplankton is made referring to the standard manuals, textbooks and monographs (Dujardin 1841; Edmondson 1959; Michael 1973; Victor and Fernando 1979; Sharma and Michael 1980, 1987; Battish 1992; Reddy 1994; Dhanapathi 2003; Altaff 2004). Specific manuals are used for the identification of different groups of freshwater zooplankton, e.g. for rotifers (Battish 1992; Dhanapathi 2003), cladoceran (Michael and Sharma 1988; Murugan et al. 1998), copepods (Sewell 1947; Reddy 1994; Dussart and Defaye 1995; Huys and Boxshall 1991; Williamson 1991) and ostracods (Victor and Fernando 1979). The schematic outline of the zooplankton is shown in Fig. 4.

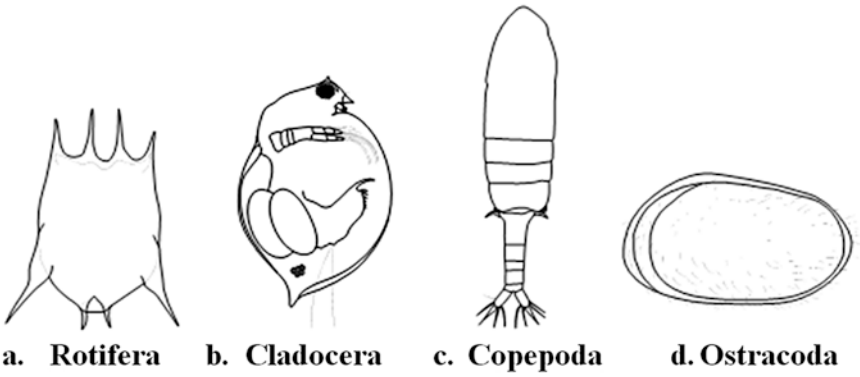


Fig. 4 Schematic outline of zooplankton: (**a**) morphology of the Rotifera; (**b**) side view of Cladocera; (**c**) dorsal view of Copepoda (Calanoida); (**d**) side view of Ostracoda. (Source: MRC Environment Programme Report 2015)

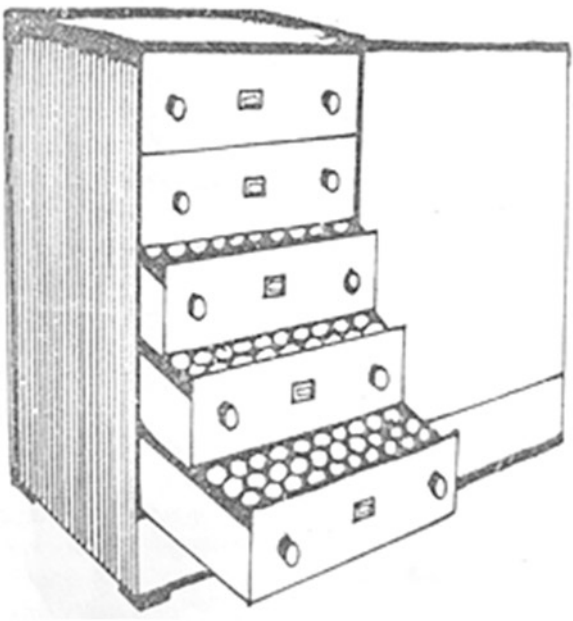


Fig. 5 Zooplankton sample storage box. (Source: Battish 1992)

Storage of Plankton

The collection of plankton samples, like any other scientific endeavour, is a difficult and time-consuming task, especially when the material is to be collected from a vast area involving remote and hard–to-reach sites. Moreover, it may not always be possible to collect the same taxa again, even from the same site of earlier collection, due to the seasonal and erratic occurrence of these forms. Therefore, it is highly imperative to preserve and store the sample properly for future study and to make them available to other workers. The samples can be stored in any good quality glass and plastic wares, but specimen tubes of 8×3 cm of good quality plastic are more convenient. These tubes can be placed serially into specially designed boxes (Fig. 5) and stored. The solution in these tubes should be checked every 1 or 2 months as a safeguard against desiccation.

Labelling of Plankton Sample

The collection of samples without a proper label is of no use for study and research purposes. Detailed information such as location of sample collection, date of collection and name of collection should be properly written on the data sheet maintained in the laboratory. Therefore, it is highly desirable that a suitable label be affixed to each tube before storage. The label should indicate all the details required by future researchers to enable them to locate and identify specimens exactly. The label

should be made from high quality paper and entries legibly written in good quality ink to safeguard against damage and fading of the notes.

Measurement and Magnification Lines of Plankton Species

The dimensions of organisms are important and zooplankter are, likewise, always compared and judged based on their size. There are two methods by which plankters can be measured, by an ocular micrometer or a stage micrometer. Further, a rough estimate can always be made if the field of vision of the microscope is measured by keeping a simple scale under the objective. The magnification lines can be drawn on a card by placing a stage micrometer under various combinations of lenses with the help of a camera lucida. Then, by means of a fine divider, the length of the plankters can be measured by taking the dimension from the magnification lines as the unit if the figure has been drawn with the camera lucida (Fig. 6). The diagram as well as the magnification lines for each plankter may be needed for recording and publication purposes.

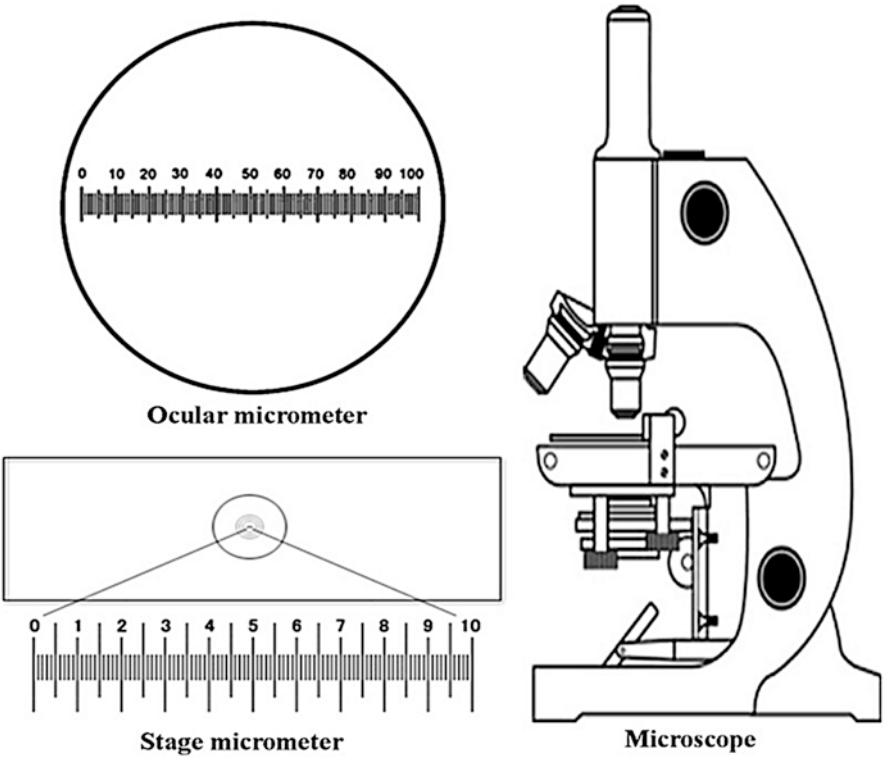


Fig. 6 Ocular micrometer, stage micrometer and microscope

Ocular and Stage Micrometer Method

The ocular micrometer is put in the eyepiece of a microscope and a stage micrometer on the mechanical stage (Fig. 7). Now, turn the stage micrometer in such a way that the micrometers coincide. The number of lines in the stage micrometer which coincide with the line of the ocular micrometer will depend upon the magnifying lenses used. The stage micrometer has a scale of 1 mm in length, carrying ten marks at equal intervals, each at 0.1 mm. These are further divided into ten marks each. By the coinciding of the ocular micrometer and stage micrometer, the actual difference in microns between the lines of the ocular micrometer can be calibrated by using the following formula: 10 spaces on the ocular micrometer = (X) space on the stage micrometer. Since the smallest spaces on the stage micrometer are known to be 0.01 mm apart, then the ocular micrometer space = (S) times of 0.01 mm/10. Once the ocular micrometer is calibrated, the objects can be directly measured by placing them under the same magnifying system of lenses.

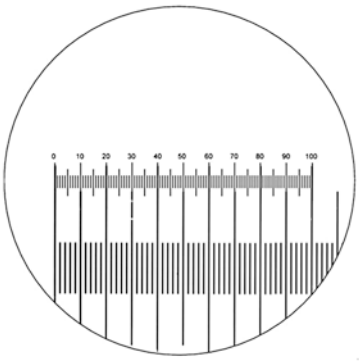
Preparation of Illustrations

The illustration of plankton can be done with the help of a camera lucida. Preference should be given to live and uncompressed specimens without a cover slip, which are mandatory for such delicate organisms as Protozoa and Rotifera. Movements of these plankters can be impeded by narcotization and so forth. The species of animals should be figured in the same view for easy comparison with those in the literature. The photomicrographs taken with a good camera at a chosen magnification are also of great help in taxonomic work.

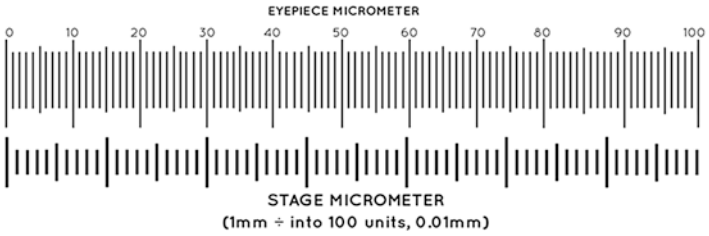
Histological Studies

Histological studies of specimens have to be fixed in aqueous Bouin's fluid for 12 h and after washing in running tap water for 1 h. They are dehydrated in 50%, 70% and 90% absolute ethanol to clear in xylene, embedded in paraffin wax and serial sections (L.S., V.S. and T.S.) are taken. The sections are deparaffinised in xylene and hydrated in descending series of ethanol up to 70%. The counterstaining is done with alcoholic eosin and then passed through 90% absolute ethanol. After complete dehydration, xylene is used for clearing and then mounting is done in DPX (Pantin 1964). The muscular, digestive and reproductive system can be studied under a compound microscope and photomicrographed.

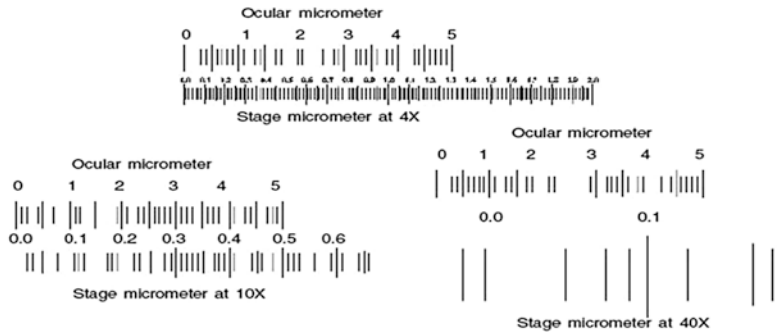
2. Microscope



1. Coinciding ocular micrometer and stage micrometer



3. Two micrometer coincided



4. Two micrometer coincided of different magnification on 4X, 10X and 40X

Fig. 7 Calibration of the micrometer

Scanning Electron Microscope (SEM) Studies

The zooplankton species are fixed in 40% glutaraldehyde for 12 h at 3.0–5.0 °C and washed in 0.1 M phosphate buffer solution. A post-fixation is carried out in 1% osmium tetroxide for 1 h at room temperature and further washed in 0.1 M phosphate (PO_4) buffer solution, dehydrated in ascending series of ethyl alcohol and the specimens are then treated with propylene oxide and air-dried below room temperature. The specimens are intact and undamaged for gluing on self-sticking carbon tape at different orientations with the help of a fine brush. Specimens are then coated with a palladium gold ion sputtering device at a thickness of 200 A°. The specimens are scanned in an SEM at 15 kV. The photomicrographs are taken at different angles and magnifications.

Transmission Electron Microscope (TEM) Studies

The zooplankton species are fixed in 2.5% glutaraldehyde for 6 h at 4 °C and washed in 0.1 M phosphate buffer (pH 7.4). This is followed by post-fixation in 1% osmium tetroxide for 2 h at 4 °C, further washing with 0.1 M phosphate buffer and dehydration through an ascending series of acetone, cleared in toluene and embedded in araldite. Ultra-thin sections of 60–90 nm have to be a taken using an ultramicrotome. These ultra-thin sections are stained with uranyl acetate and lead citrate, washed, dried and, finally, observed under a TEM.

Rotifera

Rotifers are the “wheel animalcules”, microscopic aquatic animals of the phylum Rotifera. The name “rotifer” is derived from the Latin word meaning “wheel-bearer” and they have a length of 0.4–2.5 mm; this refers to the crown of cilia around the mouth of the Rotifera. Rotifera is the most important soft-bodied metazoans (invertebrates) among the plankton. The rotifers are numerically abundant in freshwater, brackish water, marine and interstitial habitats. Among the zooplankton, rotifers are next in abundance to copepods. They can be easily identified by their anterior ciliated wheel organ, called the corona, used for locomotion and sweeping food particles towards the mouth. Their name comes from this apparent rotating wheels motion of the cilia. The mouth is generally anterior and the digestive tract contains a set of jaws (trophi) to grasp the food particles and crush them. Relatively few (about 100) ubiquitous Rotifera species are planktonic and a much larger number (about 300) are sessile and associated with sediments and vegetation of the littoral zones.

Planktonic rotifers have a very short life cycle (<14 days) under favourable conditions of temperature, food and photoperiod. Females are more common than

males and most of the species' males are unknown, as they only live for a few hours to 3 days. Since the Rotifera have short reproductive stages, they increase in abundance rapidly under favourable environmental conditions (Dhanapathi 2000). About 1700 species of Rotifera have been described from different parts of the world and 500 species (only 330 species belonging to 63 genera and 25 families have been authenticated so far) were described from Indian water bodies (Arora and Mehra 2003; Kiran et al. 2007). Rotifers inhabit the thin films in water that form around soil particles. The habitats of Rotifera may include still water environments, such as ponds and lake bottoms, as well as flowing water environments, such as rivers or streams. Rotifers are commonly found in mosses and rocks, in soil or leaf litter on mushrooms growing near dead trees, in tanks of sewage treatment plants and even on freshwater crustaceans and aquatic insect larvae (Orstan 1999).

Rotifera are multi-cellular animals with body cavities that are partially lined by mesoderm. These organisms have specialised organ systems and a complete digestive tract that includes both a mouth and an anus. Since these characteristics are all unique animal characteristics, Rotifera are recognised as animals, even though they are microscopic. Most of the Rotifera species range in length from about 200 to 500 micrometers. However, a few species, such as *Rotaria neptunia*, may be longer than a millimetre (Orstan 1999). The freshwater rotifers are microscopic animals and their diet must consist of matter small enough to fit through their tiny mouths during filter feeding. Rotifera are primarily omnivorous, but some species have been known to be cannibalistic. The diet of Rotifera most commonly consists of dead or decomposing organic materials, as well as unicellular algae and other phytoplankton that are primary producers in aquatic communities. Such feeding habits make some rotifers primary consumers. Rotifers are, in turn, prey to carnivorous secondary consumers, including shrimp and crabs. Reproduction in Rotifera is rather unusual. Several types of reproduction have been observed in Rotifera. Some species consist only of females that produce their daughters from unfertilised eggs, a type of reproduction called parthenogenesis.

Morphology of Rotifera

Rotifera has a transparent cylindrical-shaped body, lined by a thin cuticle. In the majority of rotifers, the cuticle thickens to form a lorica. The lorica has an arched dorsal plate and a flat ventral plate, connected by a flexible cuticular membrane, the sulci. The body can be divided into the head, trunk and foot. The head bears the corona, mouth and sensory organs. The corona is a ciliary organ, which has an anterior and a posterior line of cilia. The trunk forms the major part of the body and encloses the organs concerned with digestion, excretion and reproduction. The foot and toes are located in the posterior region of the body and they are useful for locomotion and attachment. The foot and toes can be withdrawn into the body. The pharynx contains a masticating apparatus called mastax, which contains the trophi (Fig. 8).

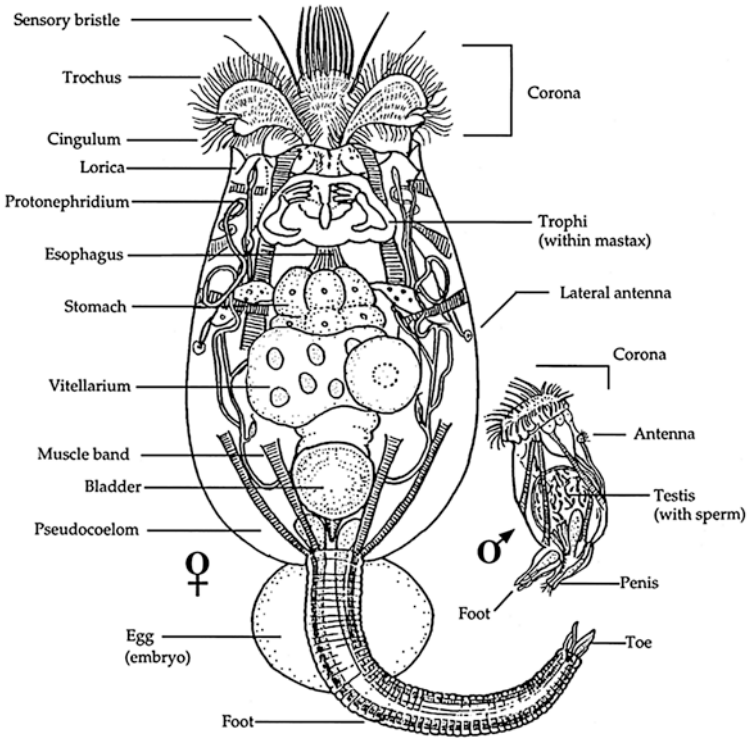


Fig. 8 The typical structure of rotifers (female and male *Brachionus plicatilis*). (Source: modified from Koste 1980)

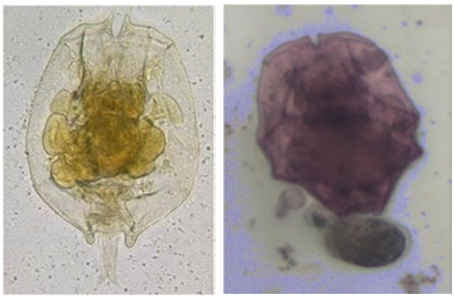
Taxonomically Important Characters of Rotifera

The lorica is present or absent. Its shape, sculpture, presence or absence of spines (movable or fixed), size, sulci or ridges, cuticular tubercles, skipping blades and presence of antennae or antenna are important to note. In the corona, its shape, structure, ciliation, rostrum and rostral lamellae, presence or absence of cement glands and location of the mouth on apical field of the corona are important characters. The foot is present or absent. Its shape, annulations or segmentation, presence of pedal glands and their number and shape, presence of other structure on the foot, i.e. styles, spurs etc. and their number are useful characters. A mastax structure and its type is an important taxonomic character to preserve in specimens. The eyes, retrocerebral sac and digestive tract are also helpful in the identification of some rotifers.

Taxonomy and Systematic of Rotifera

***Brachionus angularis* (Gosse, 1851)**

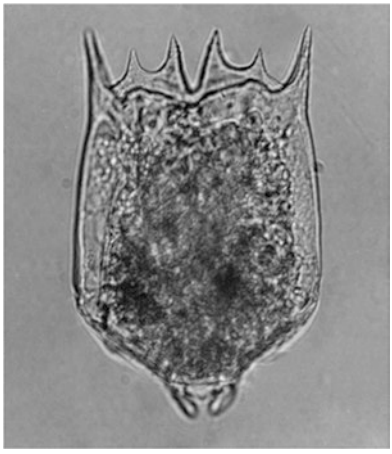
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. angularis* (Gosse, 1851)



Lorica firm, lightly or heavily stippled, divide into dorsal and ventral plates; dorsal plate with pattern of cuticular ridges, moderately compressed dorsoventrally; anterodorsal margin with two median spines flanking a V-shaped notch; lateral and intermediate spines usually obliterated, intermediate spine may be present in some; mental margin rigid, somewhat elevated with a shallow median notch; foot opening rather large, somewhat variable in shape; large foot aperture in ventral plate flanked by cuticular protuberances; posterior spines wanting. Measurements: Length of lorica 80–89 µm, maximum width of lorica 76–83 µm.

***Brachionus bidentata* (Anderson, 1889)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. bidentata* (Anderson, 1889)

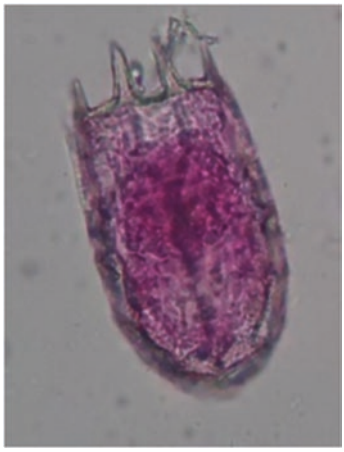


The lorica firm, stippled, with definite pattern of plaques, lorica divided into dorsal, ventral and basal plates. The dorsal and ventral plates soldered together for three-fifths of the lorica and, thereafter, united to a third basal plate, dorsal margin with six spines, lateral always longer than medians. In medians longer than intermediates,

mental margin flexible, elevated in the middle, posterior spines may vary in length and position of origin, but may be absent; foot opening with footsheath. Measurements: Length of lorica 121–155 µm, maximum width of lorica 120–129 µm, length of anterior intermediate spine 11–15 µm, length of anteromedian spine 27–32 µm, length of anterolateral spine 28–31 µm, length of posterior spine 14–19 µm.

***Brachionus budapestinesis* (Daday, 1885)**

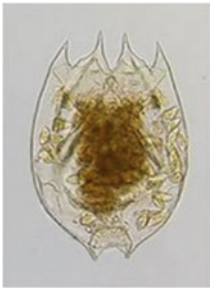
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. budapestinesis* (Daday, 1885)

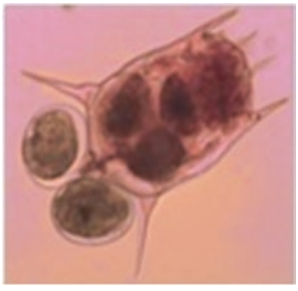


The lorica firm, oval, divided into dorsal and ventral plates, ornamented with pattern of cuticular ridges on both dorsal and ventral plates, dorsoventral depth about two-thirds the width. The anterodorsal margin with four spines, median pair longer than laterals, their distal end curved ventrally, posterior spines wanting, mental edge nearly straight, with small median unflanked notch, foot with V-shaped aperture dorsally and larger oval opening ventrally. Measurements: Length of lorica 86–111 µm, maximum width of lorica 70–120 µm, length of median spines 31–38 µm, length of lateral spines 18–24 µm.

***Brachionus calyciflorus* (Pallas, 1776)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. calyciflorus* (Pallas, 1776)

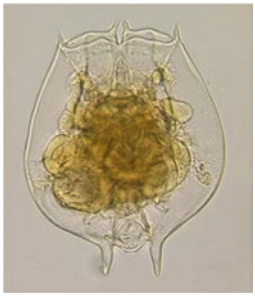


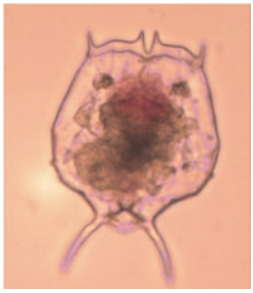


Lorica flexible, oval, not separated into dorsal and ventral plates; body slightly compressed dorsoventrally, anterior dorsal margin with four broad-based spines of variable length. The medians longer than laterals; mental margin flexible, usually somewhat elevated, with shallow V- or U-shaped notch, unflanked. The posterior spines present or absent; posterolateral spines usually absent, lorica smooth or lightly stippled. Measurements: Length of lorica 218–225 µm, maximum width of lorica 180–200 µm, length of anteriomedian spine 65–90 µm, length of lateral spine 46–71 µm, length of posterior spine 0–162 µm. *Brachionus calyciflorus* is an exceedingly variable species, especially true of its size, length of occipital spines and presence and length of posterior spines.

***Brachionus caudatus personatus* (Ahlstrom, 1940)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. caudatus personatus*

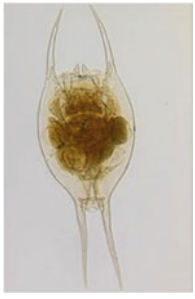


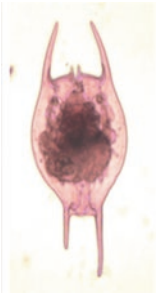


The characters of the main species, lorica heavily stippled, with a pattern of cuticular ridges more or less distinct. The lorica moderately compressed dorsoventrally; occipital spines six, lateral occipital spines larger than median spines. At times twice as long as medians, intermediate spines reduced, mental margin wavy, posterior spines not developed in the same plane as the axis of the body. Measurements: Length of lorica 145 µm, length of anterolateral spine 25 µm, length of anteriomedian spine 14 µm, length of posterolateral spine 45 µm.

***Brachionus diversicornis* (Daday, 1883)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. diversicornis* (Daday, 1883)

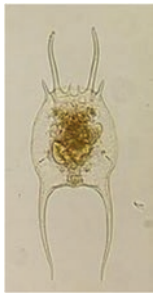


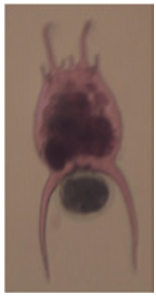


Lorica firm, lightly stippled, divided into dorsal and ventral plates, quite compressed dorsoventrally, occipital margin with four spines, medians short, laterals long, intermediates completely obliterated. The mental margin more or less rigid, somewhat elevated with a median V-shaped notch. Posteriorly, lorica becomes narrower and carries two diverging spines, the right spine usually larger, the left short or nearly equal in length to the right. The foot opening between bases of posterior spines; rounded tongue-like projection of dorsal plate overhangs foot opening. Measurements: Length of lorica 220–228 µm, maximum width of lorica 186–190 µm, width of lorica near posterior end 75–80 µm, length of anterolateral spine 110–115 µm, length of median spines 19–22 µm, length of posterior right spine 117–120 µm.

***Brachionus falcatus* (Zacharias, 1898)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. falcatus* (Zacharias, 1898)

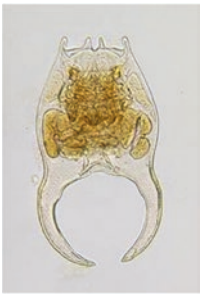


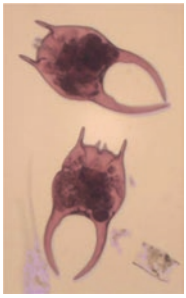


Lorica firm, lightly stippled, greatly compressed dorsoventrally and composed of dorsal and ventral plates. Anterodorsal margin with six spines, intermediate spines considerably larger than laterals and medians, curve laterally outwards or ventrally towards head of the animal. The median spines mostly equal to laterals but sometimes smaller, mental edge firm and wavy without spine and without elevation towards the centre. Posterior spines widely separated basally, long, their width much greater than anterior spines, parallel or bow outwards, converge, then twist towards their apices, thus completing full arch; foot opening between bases of posterior spines, sub-square hole in ventral plate. Foot opening unflanked. Occasionally, two, sometimes only one, eggs present at the bases of posterior spines. Measurements: Length of lorica 122–155 µm, maximum width of lorica 126–175 µm, length of anterior intermediate spine 90–120 µm, length of anteromedian spine 11–22 µm, length of anterolateral spine 10–031 µm, length of posterior spine 148–170 µm.

***Brachionus forficula* (Wierzejski, 1891)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. forficula* (Wierzejski, 1891)

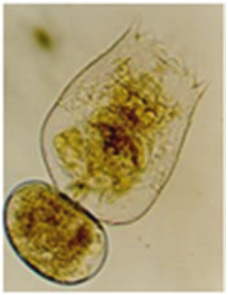




Lorica, firm, stippled, divided into dorsal and ventral plates, moderately compressed dorsoventrally, anterior margin with four occipital spines, anterolateral longer than anteromedian spines. The intermediate spines wanting, all occipital spines rounded at tips, rarely pointed, mental margin rigid with two well-marked lobes, lorica terminates posteriolaterally two stout, long and sub-square spines, basally widely separated and tapering to blunt points, geniculate swellings present at bases of posterior spines. Foot opening between bases of posterior spines. Usually, one egg attached in between bases of posterior spines. Measurements: Length of lorica 90–120 µm, maximum width of lorica 91–130 µm, length of anterolateral spine 31–41 µm, length of anteromedian spine10–12 µm, length of posterior spine 60–155 µm.

***Brachionus plicatilis* (Muller, 1786)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. plicatilis* (Muller, 1786)

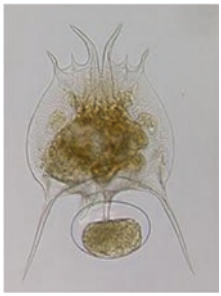
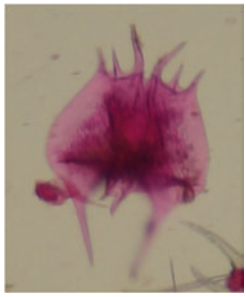


Lorica flexible, lightly stippled, more or less oval, greater width about two-thirds the length of lorica from the anterior end. It narrows anteriorly and not sharply separated into dorsal and ventral plates, slightly compressed dorsoventrally. Anterodorsal margin with six broad-based acutely pointed saw-toothed spines, nearly equal in length, posterior spines wanting, mental margin separated into four lobes. The foot

opening with a small sub-square aperture dorsally and longer V-shaped aperture ventrally. Measurements: Length of lorica 234–240 µm, maximum width of lorica 213–220 µm, length of anteromedian spine 16–18 µm, length of anterolateral spine 20–24 µm.

***Brachionus quadridentatus* (Hermann, 1783)**

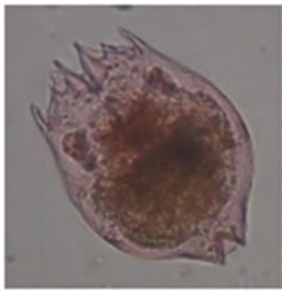
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. quadridentatus* (Hermann, 1783)

Lorica firm, moderately compressed dorsoventrally and divided into dorsal and ventral plates, occipital margin with six spines, medians longest, curved outwards and, when extra-long, bent downwards over the head. The laterals longer than the intermediates. Mental margin rigid, wavy, elevated, with median notch flanked on either side by a small tooth-like papilla. Posterolateral spines usually present but their length varies, ventroposterior portion of lorica prolonged in the form of lobular footsheath around the base of retractile foot. In the sheath on dorsal side with well-defined sub-square piece. Measurements: Length of lorica 115–118 µm, maximum width of lorica 120–230 µm, length of anterior median spine 40–92 µm, length of intermediate spine 8–15 µm, length of lateral spine 20–45 µm, length of posterior spine 0–148 µm.

***Brachionus rubens* (Ehrenberg, 1838)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Brachionus* (Pallas, 1776)
Species: *B. rubens* (Ehrenberg, 1838)

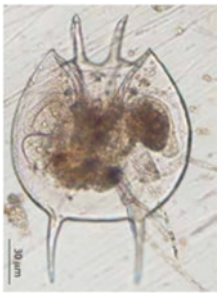



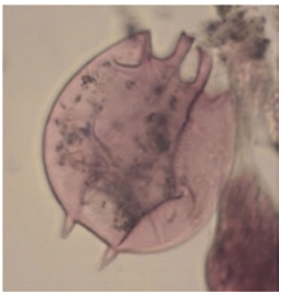
Lorica firm, oval, smooth, compressed dorsoventrally and composed of dorsal and ventral plates; anterior dorsal margin with six spines, medians longest, intermediates somewhat longer than laterals. The medians and intermediates with peculiar

asymmetric shape (saw-toothed), each spine with a narrow anterior part, then rounding outwards and forming a broad base. All these spines provided with strengthening ridges, mental margin serrated and markedly elevated towards the centre with a central notch. Posterior spines absent, foot opening sub-square and small. Measurements: Length of lorica 146–150 µm, maximum width of lorica 100–180 µm, length of median spine 30–32 µm, length of intermediate spine 2–5 µm, length of lateral spine 0–20 µm, length of posterior spine 148–170 µm.

***Platyias quadricornis* (Ehrenberg, 1882)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Platyias* (Harring, 1914)
Species: *P. quadricornis* (Ehrenberg, 1882)

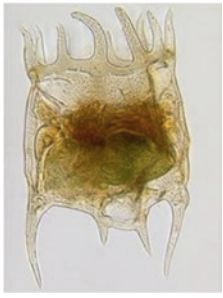


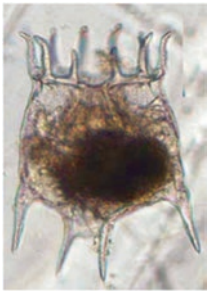


Lorica firm, more or less circular, moderately compressed dorsoventrally, tuberculated or stippled, composed of dorsal and ventral plates. The dorsal plate slightly convex, shield-like and with a regular pattern of facets consisting of three central pentagons surrounded by eight marginal areas, the ventral plate comparatively flat. The anterodorsal margin with two stout median spines that taper very slightly and bluntly rounded to nearly truncated at their tips, usually bending ventrally somewhat, mental margin rigid and depresses towards the centre, posteriorly, lorica terminates in short or long stout parallel spines, one-third to one-half the length of lorica from posterior end. The foot is in two segments, with two toes. Measurements: Length of lorica 286–290 µm, maximum width of lorica 247–250 µm, length of anterior spines 97–100 µm, length of posterior spine 128–130 µm.

***Plationus patulus* (Muller, 1786)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Plationus* (Segers, Murugan & Dumont, 1993)
Species: *P. patulus* (Muller, 1786)





Lorica is sub-rectangular, wider than long, slightly compressed dorsal-ventral. The anterior lorica has ten large spines, unequal. Anterior dorsal margin with six spines, unequal, pair of central spines longer than others and curved inwards. The dorsal plate with a pentagonal ornamentation. Anterior ventral margin with four spines, unequal, pair of central spines shorter than laterals. Posterior of lorica unsymmetrical, with two spines, stout, short. Foot opening in ventral plate, somewhat deflected to the right, bounded by two short unequal spines. Measurements: Length of lorica 60–90 µm, maximum width of lorica 247–250 µm, length of anterior spines 97–100 µm, length of body 110–130 µm.

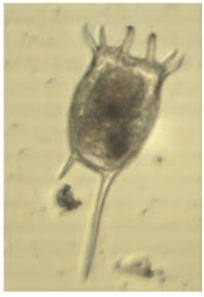
***Keratella cochlearis* (Gosse, 1851)**

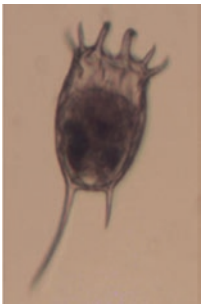
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Keratella* (Bory de St. Vincent, 1822)
Species: *K. cochlearis* (Gosse, 1851)

Lorica is spoon-shaped, dorsal plate convex, ventral plate flat. Posterior lorica margin with a long and stout spine. Anterior dorsal margin with six spines and median spines longer than others and curved outwards. The dorsal plate with a characteristic median longitudinal line, with symmetrically arranged plaques on either side. Measurements: Total length 200 µm, length of lorica 100 µm.

***Keratella tropica* (Apstein, 1907)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Keratella* (Bory de St. Vincent, 1822)
Species: *K. tropica* (Apstein, 1907)



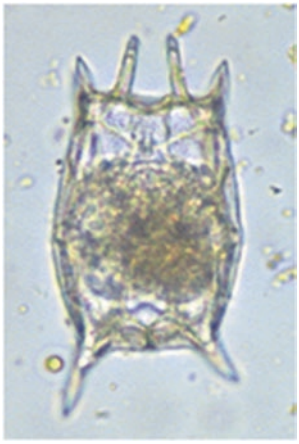


The genus with characters of anterodorsal margin with six spines, medians longest, intermediates. The mental edge bifurcate, posterior spines unequal, width of lorica at bases of posterior spines smaller than width at occipital margin. Three hexagonal plaques on dorsal plate of lorica and a small, four-sided plaque between

the posterior border of the lorica and the last hexagonal plaque, lorica pustulated. Measurements: Length of lorica 110–115 µm, maximum width of lorica 91–95 µm, length of anterolateral spine 42–45 µm, length of anteromedian spine 27–29 µm, length of median spine 50–55 µm, length of posterior right spine 90–95 µm, length of the posterior left spine 18–20 µm.

***Keratella procurva* (Thorpe, 1891)**

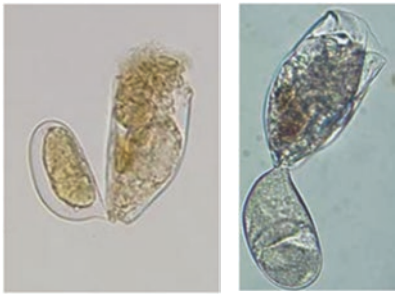
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Keratella* (Bory de St. Vincent, 1822)
Species: *K. procurva* (Thorpe, 1891)



The anterodorsal margin with six spines, medians longest and curved ventrally, laterals shortest, posterior spines almost equal. The posterior margin, dorsal plate of lorica with three median plaques and one pentagon, terminating in short median line. Measurements: Length of lorica 82–85 µm, maximum width of lorica 63–65 µm, length of lateral spines 13–16 µm, length of intermediate spines 17–20 µm, length of median spine 23–25 µm.

***Anuraeopsis fissa* (Gosse, 1851)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Anuraeopsis* (Lauterborn, 1900)
Species: *A. fissa* (Gosse, 1851)

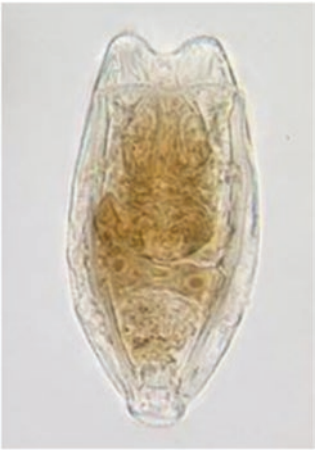


Lorica more or less cylindrical, stipple, obtusely pointed posteriorly, dorsal plate without sculpture, anterodorsal margin with a shallow notch in the middle, lateral elevations hardly to be regarded as spines, mental margin almost straight, with a

depression in the middle. Some individuals with single egg carried posteriorly or ventrally. Measurements: Length of the body 71–75 µm, maximum width of lorica 46–48 µm. Species is very well represented in clean water during the major part of the year.

***Anuraeopsis navicula* (Rousselet, 1892)**

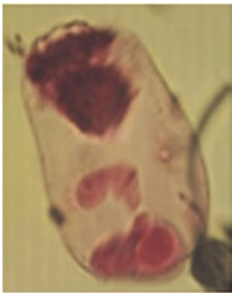
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Anuraeopsis* (Lauterborn, 1900)
Species: *A. navicula* (Rousselet, 1892)



Lorica oblong, stipple, dorsal plate with definite sculpture, anterodorsal margin with a shallow notch in the middle, mental edge divided into two lobes, with a depression in the middle. Measurements: Length of the body 92–95 µm, maximum width of lorica 48–50 µm.

***Asplanchna intermedia* (Hudson, 1886)**

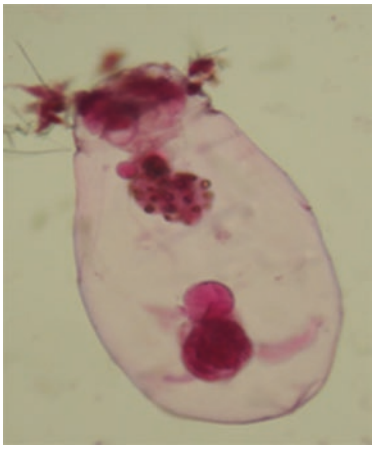
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Asplanchna* (Gosse, 1850)
Species: *A. intermedia* (Hudson, 1886)



Large, transparent with sacciform body, greatly contractile, without lorica; foot or any other extensions of cuticle wanting, corona reduced to a thin course of cilia around the head. They enclose a large apical area; with large body cavity, stomach, lying well away from body wall, intestine absent; trophi inundate; with single spherical, band- or horseshoe-shaped ovary. Frequently in viviparous, with one or several embryos.

***Asplanchna brightwellii* (Gosse, 1850)**

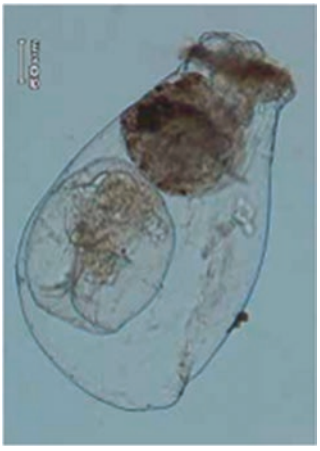
Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Brachionidae (Ehrenberg, 1838)
Genus: *Asplanchna* (Gosse, 1850)
Species: *A. brightwellii* (Gosse, 1850)



Body large, sacciform shape and transparent. Intestine, foot and toes are absent. Trophi incudate with rami possessing horn-like projections at outer margins of the base and inner spines at the middle and flame bulbs 18. Germovitellarium with 30–32 nuclei. Feeds on other Rotifera species. Measurements: Length of the body 350 µm, width 196 µm, trophi 70 µm.

***Asplanchna priodonta* (Gosse, 1850)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Asplanchnidae (Eckstein, 1883)
Genus: *Asplanchna* (Gosse, 1850)
Species: *A. priodonta* (Gosse, 1850)



Body very transparent, sacciform shape, with head, trunk and foot. Anterior lorica margin occipital with a circling of ciliate. Trophi are characteristic, inner edge of the ramus with 4–6 teeth at the anterior end. Ovary rounded. Measurements: Length of the body 300–700 µm (female).

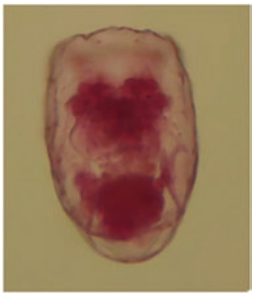
***Asplanchna sieboldii* (Leydig, 1854)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Asplanchnidae (Eckstein, 1883)
Genus: *Asplanchna* (Gosse, 1850)
Species: *A. sieboldii* (Leydig, 1854)

Females with horseshoe or bell shape, transparent, polymorphic. Foot absent. Trophi with a departure from the type is an auxiliary tooth on the outside of each ramus. The gastric is lobe shaped. Measurements: Length of the body 400 µm (female).

***Polyarthra multiappendiculata* (Arora, 1962)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Synchaetidae (Hudson & Gosse, 1886)
Genus: *Polyarthra* (Ehrenberg, 1834)
Species: *P. multiappendiculata* (Hudson, 1886)

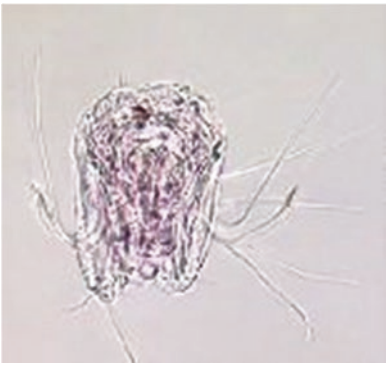


The body more or less oval or sub-square, with flattened cuticular appendages (paddles) attached in four groups to dorsolateral and ventrolateral surface near anterior end; in addition, setiform projections may be present in some trophi virgate. Speciation of the genus based on shape, length and structure of paddles, number of nuclei in vitellarium, location of lateral antennae and presence of an extra pair of small, cuticular appendages on the ventral side.

Polyarthra multiappendiculata of 'paddles' 12 in number, six on each side arranged into four groups, reaching slightly beyond posterior end of the body; two antennae carrying long cilia at their tip present on the anterior side, surrounded by ciliary wreath; lateral antennae small and hardly traceable; pair of setiform projections present on ventral side. Measurements: Length of the body 106–108 µm, width 64–68 µm.

***Polyarthra indica* (Segers & Babu, 1999)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Synchaetidae (Hudson & Gosse, 1886)
Genus: *Polyarthra* (Ehrenberg, 1834)
Species: *P. indica* (Segers & Babu, 1999)



The body illoricate and a little squarish. In four groups of lateral paddles inserted dorsally and ventrally in the neck region. Each group with three paddles of equal length extending beyond the posterior end of the body. Accessory pair of ventral paddles present between ventral bundles. Vitellarium with 4–6 nuclei. Trophi modified virgate type.

***Polyarthra vulgaris* (Segers &Babu, 1999)**

Phylum: Rotifera
Class: Monogonata (Remane, 1933)
Order: Ploima (Remane, 1933)
Family: Synchaetidae (Hudson & Gosse, 1886)
Genus: *Polyarthra* (Ehrenberg, 1834)
Species: *P. vulgaris* (Carlin, 1943)

Polyarthra vulgaris is an aloricate rotifer, with four sets of blade-like projections or paddles (Gilbert 1985). A ciliated corona surrounds the mouth, leading to the gullet, trophi (jaws), oesophagus, stomach and intestine, and the anus on the ventral surface. The cilia surrounding the corona are used in feeding and locomotion. Females (most common in collections) possess a large ovary consisting of a germarium and vitellarium. The oviduct empties into the cloaca. The male reproductive system contains a testis and sperm duct. The tactile sense organs consist of the two lateral antennae and dorsal antenna and a cephalic retrocerebral organ (Edmondson 1959). The paddles are attached to strong, striated muscles within the body by integumentary hooks. When the muscles contract, they pull the hooks downwards and

cause the paddles to elevate. As the rotifer lacks any protective outer covering, it is the rapid movement of the paddles that allows it to escape predation (Gilbert 1985).

***Lecane luna* (Muller, 1776)**

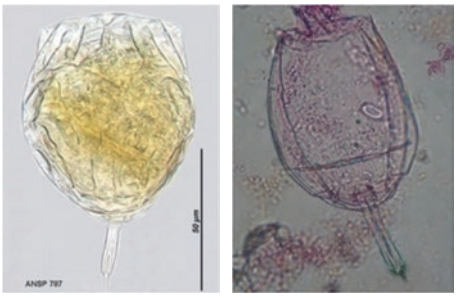
Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Ploima (Hudson & Gosse, 1886)
Family: Lecanidae (Remane, 1933)
Genus: *Lecane* (Nitzsch, 1827)
Species: *L. luna* (Muller, 1776)

The genus of lorica broadly ovate in outline composed of dorsal and ventral plates; foot projects through hole in ventral plate near posterior end, with toe rudimentary segments, of which only the posterior is movable, bearing two toes, separated, rarely fused at the base.

Lorica broadly pyriform or sub-circular, ventral plate ovate, both dorsal and ventral plates with lunate anterior notch, anterior margin of dorsal plate considerably narrower and projects beyond ventral plate in the centre, second foot joint large and broad, toes parallel sides but bear a claw at the distal end. Measurements: Length of lorica 130–135 µm, maximum width of lorica 46–48 µm.

***Lecane inopinata* (Harring & Myers, 1926)**

Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Ploima (Hudson & Gosse, 1886)
Family: Lecanidae (Remane, 1933)
Genus: *Lecane* (Nitzsch, 1827)
Species: *L. inopinata* (Harring & Myers, 1926)

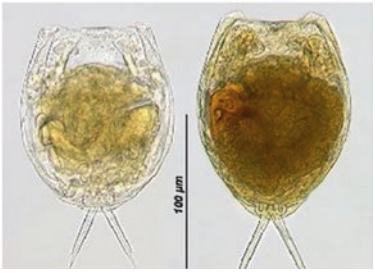


The body oval in outline, dorsal plate oval, ventral plate sub-oval, triangular cusps present at the anterior external angles, ventral plate with two or three transverse ridges, toes fused for one-third of their length, long and slender, slightly

enlarged at their junction with foot joint and end in acute claws. Measurements: Length of lorica 138–140 µm, maximum width of lorica 110–112 µm, length of toe 52–53 µm.

***Lecane papuana* (Murray, 1913)**

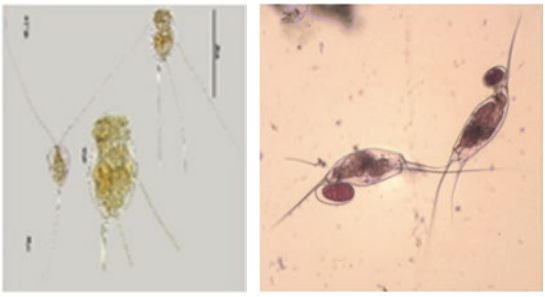
Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Ploima (Hudson & Gosse, 1886)
Family: Lecanidae (Remane, 1933)
Genus: *Lecane* (Nitzsch, 1827)
Species: *L. papuana* (Murray, 1913)



The lorica sub-circular, anterior, dorsal margin straight and ventral with V-shaped sinus. Ventral plate slightly narrower than the dorsal plate. Second foot joint robust, toes two, slender, parallel sides. ending in claws with basal spicule.

***Filinia terminalis* (Plate, 1886)**

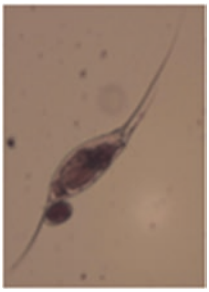
Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Floscullariacea (Harring, 1913)
Family: Trochosphaeridae (Harring, 1913)
Genus: *Filinia* (Bory de St. Vincent, 1824)
Species: *F. terminalis* (Plate, 1886)



The *Filinia* lorica is thin, flexible, fusiform, barrel- or cup-shaped; appendages (spines) long setiform extensions of cuticle, movable; two long anterolateral spines and one lone posterior spine, may be terminal or lateral and additional posterior small spine present in some; dorsal antenna absent; lateral antennae rarely present; corona in the form of circumapical girdle, differentiated into trochal and singular circlets; foot wanting; trophi malleoramate. It is a common plankton form. The lorica barrel-shaped when contracted; anterior lateral spine equal in length; with one terminal posterior spine. Females of the species carry the egg sac near the posterior end. The mastax is non-ramate and there may or may not be the presence of a lorica. Males are small and degenerate. Measurements: Length of retracted lorica 100–102 µm, maximum width of retracted lorica 48–50 µm, length of anterior spine 345–350 µm, length of posterior spine 257–260 µm.

***Filinia opoliensis* (Zacharias, 1898)**

Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Floscullariacea (Harring, 1913)
Family: Trochosphaeridae (Harring, 1913)
Genus: *Filinia* (Bory de St. Vincent, 1824)
Species: *F. opoliensis* (Zacharias, 1898)



Lorica fusiform in preserved specimens, four setiform spines present, two anterior and two posterior, unequal. Measurements: Length of retracted lorica 98–100 µm, maximum width of retracted lorica 36–38 µm, length of small anterior spine 89–90 µm, length of long anterior spine 208–210 µm, length of small posterior spine 26–28 µm, length of long posterior spine 107–110 µm.

***Filinia longiseta* (Ehrenberg, 1834)**

Phylum: Rotifera
Class: Eurotatoria (De Ridder, 1957)
Order: Floscullariacea (Harring, 1913)
Family: Trochosphaeridae (Harring, 1913)
Genus: *Filinia* (Bory de St. Vincent, 1824)
Species: *F. longiseta* (Ehrenberg, 1834)

The genus has two equal anterior setiform appendages and one posterior, ventral or sub-terminal in position. Spine has not bulged, foot absent. Measurements: Length of retracted body 178–180 µm, maximum width of retracted body 118–120 µm, length of anterior spine 242–250 µm, length of posterior spine 380–400 µm.

Crustacean Zooplankton

This group comprises members belonging to the well-known phylum Arthropoda. The sub-phylum Crustacean has two divisions, Entomostraca and Malacostraca. It has three classes, Branchiopoda (order: Cladocera), Copepoda (orders: Calanoida, Cyclopoida and Harpacticoida) and Ostracoda (order: Podocopa). This is the largest phylum in terms of the number of species and, among zooplankton, holds the highest position in terms of both systematic and secondary consumers in the food chain. Animals belonging to this class are free-living crustaceans with compound eyes, usually a carapace, at least four pairs of trunk limbs which are, in most cases, broad lobed and fringed on the inner edges with bristles.

Cladocera

Cladocera is a primary freshwater monophyletic group, an important component of the micro-crustacean zooplankton. Cladocera has an important role in the benthic fauna. Most of the Cladocera species are found to be primary consumers, where they feed on microscopic algae and fine particulate matter in the detritus, influencing the cycling of energy. About 600 species of freshwater Cladocera have been reported throughout the world, whereas only110 species have been discovered in India (Korovchinsky, 1996). They inhabit most types of continental fresh and saline water habitats, occurring most abundantly in both temporary and permanent stagnant waters. Cladocera is an ancient group of Palaeozoic origin.

Cladocera are commonly known as “water fleas”. This group mostly inhabits the freshwater, except the three marine genera, namely, *Podon*, *Penilia* and *Evadne.* Among the freshwater forms, a few genera are planktonic, while the majority are littoral, living among the weeds, and some of them live on the bottom mud. The planktonic forms are usually transparent and colourless, while the littoral forms are pigmented and coloured. Cladocera is a term loosely applied to a variety of small freshwater crustaceans which swim in a rapid jerky manner by flipping their antennae or other appendages. More specifically, the term is usually applied to the Cladocera crustaceans. They range in size from <250 μm in the chydorid genus *Alonella* to 4–6 mm in the daphniid genera *Daphnia* and *Simocephalus*, the two most common genera. During the summer season, the majority of them reproduce parthenogenetically, producing only female offspring from eggs which require no fertilisation. The young mature to a well-developed stage within the brood chamber of the parent, sustained initially by their egg yolk and then by a secretion from the inside of the brood chamber. Towards autumn, a combination of conditions not entirely predictable sees the production of males, which fertilise special eggs, capable of surviving winter and hatching the following spring into females, which will continue the parthenogenetic cycle.

Morphology of Cladocera

The body of a typical Cladocera is divided into the head, thorax, abdomen and post-abdomen, and is covered by a bivalve cuticular carapace. The outline of the carapace may look either oblong or oval or quadrangular. It may also be oblong and oval, oval and quadrangular or semicircular in shape. Most of the Cladocera have a carapace covering both sides of the body but opening ventrally. The head bears antennules and antenna. The antennules, in most cases, are a simple structure bearing olfactory setae laterally or terminally. The antennules may be situated on the ventral side of the head or anterior margin.

The antennae are attached close to the posterior margin of the head and are usually biramous. The posterior part of the body ends in a broad structure known as the post-abdomen that usually bears the anus. The post-abdomen is a single structure mostly employed in Cladocera taxonomy, especially in certain families. Its shape, the number and arrangement of anal spines, and the number and location of the lateral setae are of taxonomic value. The post-abdomen terminates in a pair of claws and bears two long abdominal setae. The following section describes some species of Cladocera that were taxonomically clarified for identification purposes (Fig. 9).

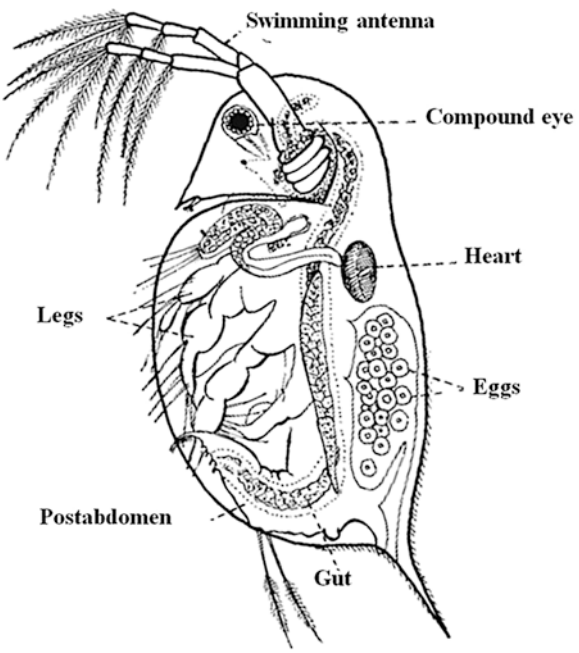
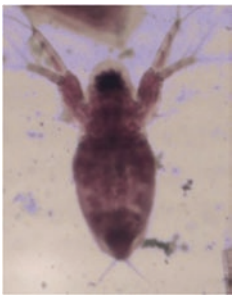


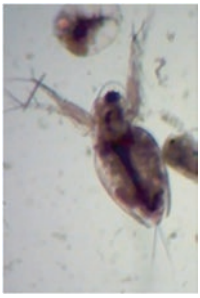
Fig. 9 General structure of a cladoceran

Taxonomy and Systematics of Cladocera

***Diaphanosoma sarsi* (Richard, 1895)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Sididae (Baird, 1850)
Genus: *Diaphanosoma* (Fischer, 1850)
Species: *D. sarsi* (Richard, 1895)

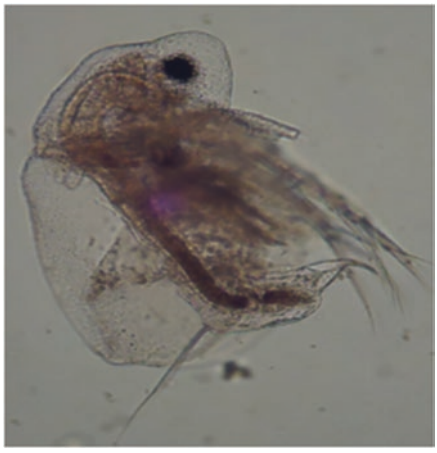




In females, eye large and situated near the anterior margin of the head; antennules small, two-jointed, with terminal olfactory setae and slender flagellum; reflexed antenna reaches posterior end of carapace; ventroposterior end of carapace provided with small teeth, numbering about 6–20; post-abdomen narrow, without anal spines; claws with three basal spines. Males; smaller than female, with long antennules; olfactory setae laterally placed. Measurements: Females length 0.8–0.94 mm, males length 0.77 mm, length of antennule of male 0.33 mm.

***Diaphanosoma excisum* (Sars, 1885)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Sididae (Baird, 1850)
Genus: *Diaphanosoma* (Fischer, 1850)
Species: *D. excisum* (Sars, 1885)



Carapace is almost oblong in outline and posterior end is abruptly truncate. Head is large, oblong-quadrate and obtusely truncate anterior. Eye is relatively large with numerous crystalline lenses. Antennae are not reaching the posterior margin of valves. Post-abdomen narrow with fine setules and claw with three basal spines and decreasing in size proximally.

***Daphnia carinata* (King, 1853)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Daphniidae (Straus, 1850)
Genus: *Daphnia* (Muller, 1785)
Species: *D. carinata* (King, 1853)

Head is large with roughly semicircular anterior margin. Body is oblong in shape. Rostrum is recurved and usually in contact with ventrolateral present. Ocellus is small. Dorsal margin of carapace is arched. The caudal spine is long and upturned, three abdominal processes are present. Post-abdomen is tapering distally with 10 to13 anal spines, claw is short and curved. Basal spine is absent. The claw is provided with minute setae.

***Daphnia magna* (Straus, 1820)**

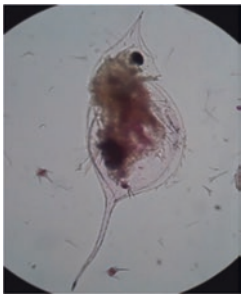
Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Daphniidae (Straus, 1850)
Genus: *Daphnia* (Muller, 1785)
Species: *D. magna* (Straus, 1820)

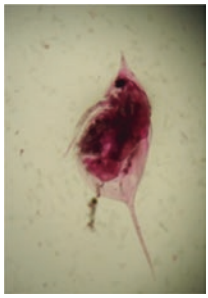
Body thick, oval, translucent; head compressed, rostrum present; prominent eye and small ocellus present; antennules small, olfactory setae not extending beyond rostrum; fornix well developed, carapace produced into posterior spine; dorsal and ventral spinules present on carapace, beginning before its middle; dorsal margin of

post-abdomen deeply sinuate, bears proximal set of seven and distal set of four spines; three abdominal processes help to retain eggs in brood pouch; alimentary canal straight, with well-developed hepatic caeca anteriorly. Measurements: Length of female 3.52–4.00 mm. *Daphnia magna* has been reported to exceptionally reach a length of 6 mm (Johnson 1952). It is the largest of all the known species of the genus *Daphnia.*

***Daphnia lumholtzi* (Sars, 1885)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Daphniidae (Straus, 1850)
Genus: *Daphnia* (Muller, 1785)
Species: *D. lumholtzi* (Sars, 1885)

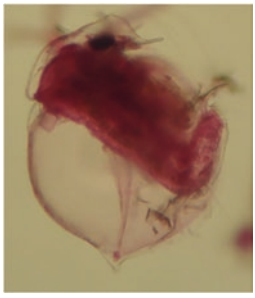


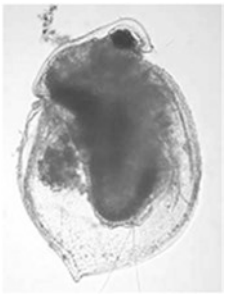


The female head moderate in size and prolonged into a head spine of variable length anteriorly (helmet), eyes moderate in size, small ocellus present, cervical sinus present, antennules small but olfactory setae extend slightly beyond rostrum, fornix very well developed, prolonged into triangular spines laterally (view dorsally). The carapace broad anteriorly but tapers posteriorly (view dorsally), dorsal edge of carapace almost straight in young specimens, while ventral curved. Shell spinules present only on posterior half of ventral edge of carapace and continue on both sides of prominent shell spine, spinules situated at a greater length of spine, post-abdominal with sinuate dorsal margin having 12–14 anal spins ending in well-developed claws. Two or three abdominal processes, prominent in live specimens, without setae. Measurements: Female length 2.23–3.33 mm, maximum length of helmet observed 0.27 mm, length of shell spine 0.80 mm.

***Ceriodaphnia cornuta* (Sars, 1888)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Daphniidae (Straus, 1850)
Genus: *Ceriodaphnia* (Dana, 1853)
Species: *C. cornuta* (Sars, 1888)

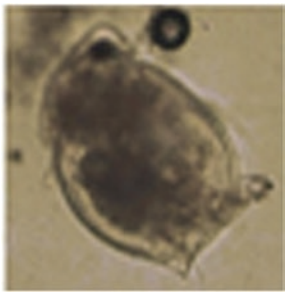




Head produced in front antennules into a short, cervical, acutely pointed horn-link process; eye quite large; ocellus not prominent; valves reticulated, more or less rounded in outline with the upper and lower margins arched, posteriorly produced into short and blunt shell spine; dorsal and ventral shell margins without spinules; post-abdomen with 5–7 anal spines, claws short and smooth, stout and with fine setules. Measurements: Length of body 0.55 mm, width of body 0.31 mm.

***Ceriodaphnia reticulata* (Jurine, 1820)**

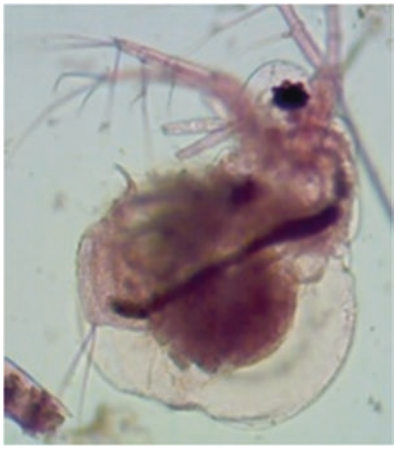
Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Daphniidae (Straus, 1850)
Genus: *Ceriodaphnia* (Dana, 1853)
Species: *C. reticulata* (Jurine, 1820)



Head depressed, rounded anteriorly, without spine or horn; eye and ocellus quite prominent; antennules small with terminal setae and a single lateral seta situated after proximal half of length of antennule; carapace rounded, with a deeply arched ventral margin, whereas dorsal margin less arched, more or less straight in specimens without eggs, posteriorly ending in a small shell spine (superaposteal angle), shell reticulations not distinct; valves with smooth edges, appear postulated; post-abdomen with seven anal spines, the distal being smaller than the others; claws with a proximal comb of five teeth at some distance from the base. Measurements: Length of body 0.86 mm, width of body 0.61 mm.

***Moina brachiata* (Jurine, 1820)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Moinidae (Goulden, 1968)
Genus: *Moina* (Baird, 1850)
Species: *M. brachiata* (Jurine, 1820)



Female: Head depressed to the extent that vertex lies almost at the level of ventral margin of valves; antennules attached to ventral convex part of head, supra-ocular depression well defined; valves more or less oblong, ending bluntly posteriorly, slightly flattened laterally and faintly marked; dorsal margin of valves edge; post-abdomen well defined with post-anal part beset with 7–11 spines besides a bident. Measurements: Length of body 1.8 mm, width of carapace 0.73 mm, length of antennules 0.16 mm. Male: Smaller than female, with long ciliated antennules, three hooks present at its distal end, while sense hair situated in mid-proximal part; first leg with hook.

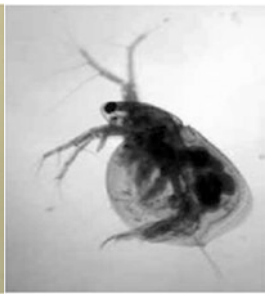
***Moina micrura* (Kurz, 1874)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Moinidae (Goulden, 1968)
Genus: *Moina* (Baird, 1850)
Species: *M. micrura* (Kurz, 1874)

Female: Body elliptical, small in size (0.31 mm); head rounded anteriorly, small supra-ocular depression present, eye large with numerous lenses, ocellus wanting; antennules small, movable, more or less spindle shape, bearing five sensory hairs distally; antenna well developed, valves oval with rounded posterior margin, dorsal margin without spinules, ventral margin bearing spinules; terminal portion of post-abdomen small, bearing five thick and ciliated anal denticles and a non-ciliated bident spine; claws small, pectinate, hearing spinules on dorsal margin. Summer eggs present. Colour: Light yellow. Male: Not found. Measurements: Length of body 0.31–0.34 mm, height of body 0.17–0.19 mm.

***Moinodaphnia macleayi* (King, 1853)**

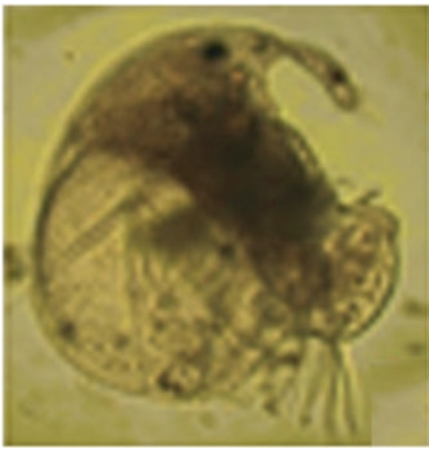
Phylum: Arthropoda
Class: Crustacea
Subclass: Branchiopoda (Latreille, 1817)
Order: Cladocera (Latreille, 1829)
Family: Moinidae (Goulden, 1968)
Genus: *Moinodaphnia* (Herrick, 1887)
Species: *M. macleayi* (King, 1853)



Female: Compressed and crested dorsally with elliptical valves completely covering body except head; head triangular, with flat ventral side carrying long and thin antennules, large eye present near tip of the head, ocellus present; fornices well developed; antenna long, basal segment bearing two sensory setae, endopod three segmented and bearing normal setae, in exopod distal segment with four setae; post-abdomen with tapering distal end, ten feathered spines and a bident spine; claws without pectin but with fine hair. Ephippial female not found. Measurements: Length of body 0.9–1.0 mm. Male: Not found.

***Macrothrix goeldii* (Richard, 1897)**

Phylum:	Arthropoda
Class:	Crustacea
Subclass:	Branchiopoda (Latreille, 1817)
Order:	Cladocera (Latreille, 1829)
Family:	Macrothricidae (Norman & Brady, 1867)
Genus:	*Macrothrix* (Baird, 1843)
Species:	*M. goeldii* (Richard, 1897)



Body ovate; dorsal edge of shell serrate, especially in posterior half, ventral margin of valves provided with short and long setae; antennules with slight notches along anterior margin with cluster of hair in distal notch, broaden distally and carry 3–4 long and several short sensory setae at the end; post-abdomen broad, not bilobed and bears strong denticles along its dorsal margin. Measurements: Female, length of body 0.471 mm, width of body 0.033 mm, length of antennule 0.011 mm.

Copepoda

The class Copepoda constitutes the dominant zooplankton groups of both freshwater and marine habitats. It is the largest class of Entomostracan in Crustacea. It includes ten orders, viz. Platycopodia, Calanoida, Misophroida, Cyclopoida, Geleylloida, Mormonilloida, Harpacticoida, Poecilostomatoida, Siphonostomatoida and Monstrilloida. They include three free-living groups, viz. Calanoida, Cyclopoida and Harpacticoida (Fig. 10). They play a vital role as primary consumers in the aquatic ecosystem. Free-living Copepoda show variation in their morphology; however, they are sexually reproducing organisms and show similar patterns of their life

cycle. The freshwater calanoid copepod *Sinodiaptomus* (*Rhinediaptomus*) *indicus* is taken as a typical Copepoda and its life cycle is described. Worldwide, there are over 5500 species of free-living copepods. The calanoids include about 2300 species, the cyclopoids include about 450 species and the harpacticoids include approximately 2800 species (Bowman and Abele 1982).

Sinodiaptomus (*Rhinediaptomus*) *indicus* is one of the most common calanoid copepods of freshwater bodies in South India. Copepoda frequently comprise a major portion of the consumer biomass in aquatic habitats and play a significant role in food webs, as both primary and secondary consumers, and as a major source of food for many larger invertebrates and vertebrates (Williamson 1991). Copepods are used as biological indicators for certain ecosystems (Dussart and Defaye 1995). They can be used as agents of biological control (Rivieeret al. 1987). Copepods are an excellent food with high nutritional value for zooplanktivorous fishes and prawn (Bulkowskiet al. 1985; Altaff and Chandran 1995). The higher rate of survival is obtained in fish larvae with live food than with refrigerated plankton food (Sharma and Chakarabarathi 2000).

Morphology of Copepoda

In freshwater calanoid copepods, the male is obviously larger than the female; the mean lengths of the males and females of this group are 1.2 and 1.4 mm, respectively, including the caudal rami and excluding the caudal setae. A major articulation between segments six and seven divides the body into an anterior prosome and

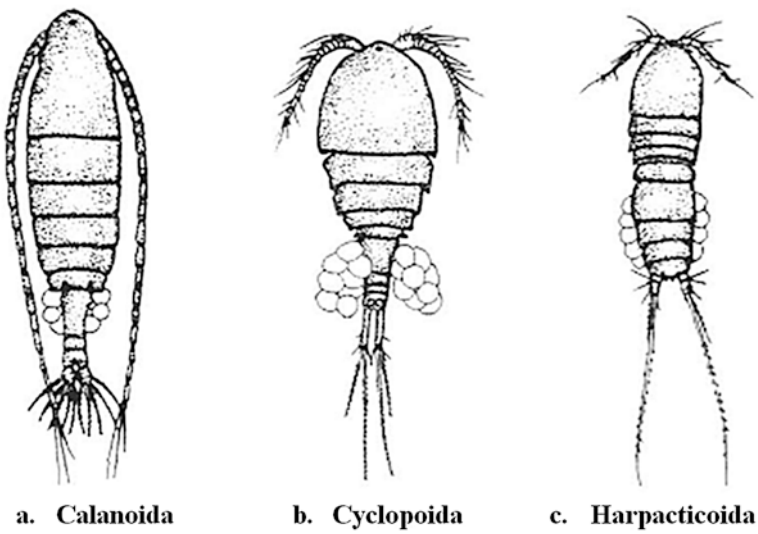


Fig. 10 The major groups of Copepoda. (Source: Smith and Fernando 1978)

posterior urosome. The prosome consists of five cephalic and six thoracic segments. All five cephalic segments and the first thoracic segment are fused together to form a compound cephalic somite. The anterior end of the prosome has a characteristic median naupliar eye and a ventrally curved rostrum with two rostral filaments. The sixth thoracic segment of the female extends posteriorly to form the metasomal wings. The urosome comprises the last thoracic segment and four abdominal segments in males and two abdominal segments in females. The last urosomal segment is bifurcated and ends in two caudal rami, and each ramus bears six setae. There are 11 pairs of appendages in the prosome; they include five pairs of cephalic (antennules, antenna, mandible, maxillula and maxilla) and six pairs of thoracic appendages (maxilliped and five pairs of swimming legs). The urosome is devoid of any appendages.

Calanoid Copepods

Calanoid copepods are divided into two groups based on the major articulation of the body, namely Gymnoplea and Podoplea. In Gymnoplea, there are no appendages at the body segments posterior to the major articulation (Platycopida and Calanoida). In Podoplea, there are reduced appendages on the body segment posterior to the major articulation (another eight orders).The superfamily Centropagiden consists of 11 families. Freshwater calanoids are included in only one family, Diaptomidae. More than 40 genera of diaptomids are known in the world. The following genera are found to be dominant in Indian waters: *Heliodiaptomus*, *Sinodiaptomus*, *Phyllodiaptomus*, *Paradiaptomus*, *Allodiaptomus*, *Neodiaptomus* and *Spicodiaptomus*.

Morphology of Calanoids

All diaptomids are very much homogenous in their structure and habits. Their identification is based on differences in minute morphological details, especially the appendages. The diaptomids body is elongated, cylindrical, spindle shaped and clearly segmented. The total length of the body including the caudal setae ranges between 1.00 and 2.75 mm. The body has two divisions; an anterior part consisting of six-segmented metasomes and a posterior narrow five-segmented urosome in males and two- or three-segmented urosome in females (Fig. 11).

Cyclopoid Copepods

Cyclopoid copepods are physically diminutive, yet their presence in aquatic systems can be great. Cyclopoids usually range in length from 0.5 to 2.0 mm, and are primarily benthic, although a few species thrive in the pelagic zones of lakes, seas and oceans. Within these larger bodies of water, cyclopoid biodiversity tends to be highest in the littoral zone. Their diversity and abundance can also be great in shallower bodies of water, such as wetlands and temporary ponds. There are a number of species that are quite common in interstitial and subterranean systems, in water that collects in the various parts of plants (phytotelmata) and that are associated with the thin, laminar flow of seepage areas (hygropetric). Some species are euryhaline and occur in brackish water and saltwater, as well as freshwater. Cyclopoid copepods play an important role in aquatic food webs as either primary consumers or predators. They can also be an important source of food for larval, juvenile and adult fish of many species. Cyclopoids are intermediate hosts of many parasitic worms (tapeworms, cestodes, roundworms) that infect vertebrates, including humans (Fig. 12).

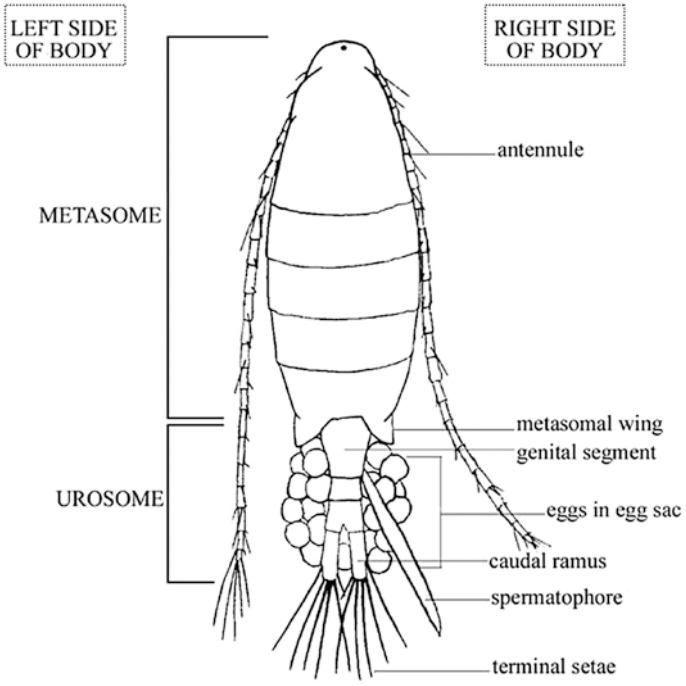


Fig. 11 Typical structure of female calanoid copepods. (Source: Balcer et al. 1984)

Morphology of Cyclopoids

The order Cyclopoida comprises species in which the last thoracic somite is different. Antennules of the females consist of 9 to 17 segments. Antennae are uniramous. Maxilliped is simple, without prehensile claw. P1–P4 biramous, with exopodite and endopodite consisting of a varying number of segments, most often two or three. P5 reduced. Two genital pores, situated sub-dorsally on each side of the seminal receptacle (Fig. 13).

Harpacticoid Copepods

According to Dussart and Defaye (1995), the superorder Harpacticoida includes two orders, namely Longipediformes and Harpactiformes. The former contains almost exclusively marine species. The latter comprises families which have representatives in fresh as well as brackish water. The harpacticoids contain over 300 species and subspecies belonging to 463 genera and 54 families; however, the great majority of species is assumed to be still unknown, mainly because large regions of the Earth have not yet been extensively sampled (Willen 1999). Harpacticoids are

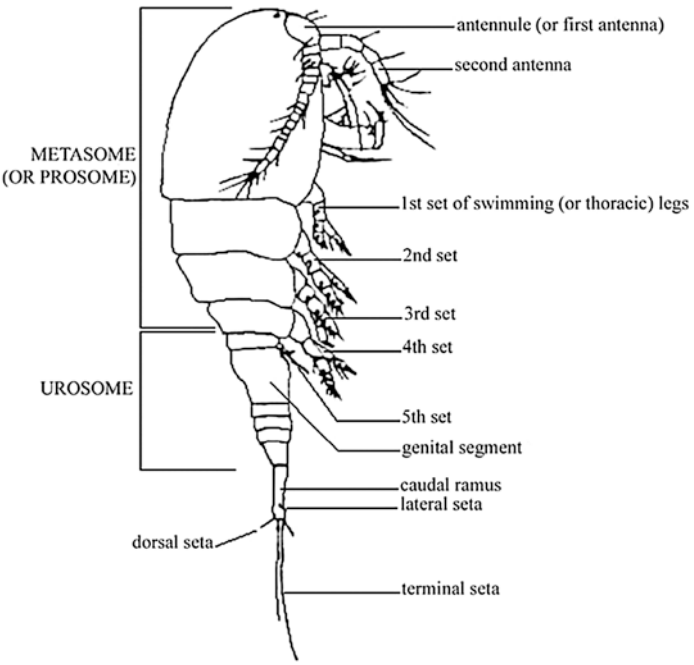


Fig. 12 Lateral view of a cyclopoid copepod. (Source: Smith and Fernando 1978)

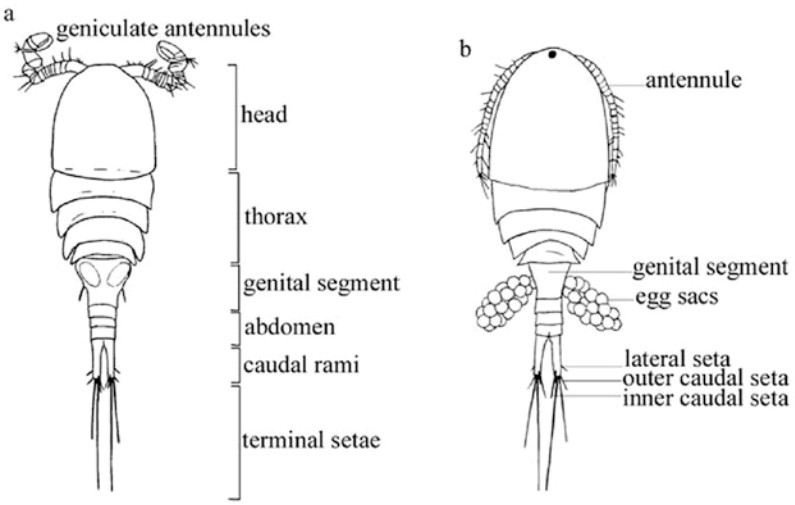


Fig. 13 Typical structure of a cyclopoid copepod: (**a**) males; (**b**) females. (Source: Balcer et al. 1984)

primarily benthic organisms. They are adapted to several kinds of freshwater and marine environments. A few are planktonic or live in association with other organisms. In marine sediments, they represent the most abundant meiofaunal group after the nematodes. Harpacticoids may also be found in semi-terrestrial habitats, such as damp moss and leaf litter. Harpacticoids are widespread across the world. Some genera have a distribution restricted to one continent, but most of them are cosmopolitan. Many have distributions related more to environmental factors than geographic location. The temperature and pH seem to influence their distribution and abundance (Williamson 1991; Dussart and Defaye 1995).

Morphology of Harpacticoids

Harpacticoids are linear in shape, with the prosome slightly wider than the urosome and the entire body gradually tapering posteriorly. The harpacticoid body is divisible into an anterior prosome and posterior urosome. The prosome is further divided into a cephalosome, with all the head appendages, the first appendages and a metasome including somites with legs 1 to 4. The urosome starts with the somite that bears the fifth leg, continues with the remaining posterior somites and terminates with the caudal rami bearing caudal setae. The anus opens on the last urosomal somite (Fig. 14).

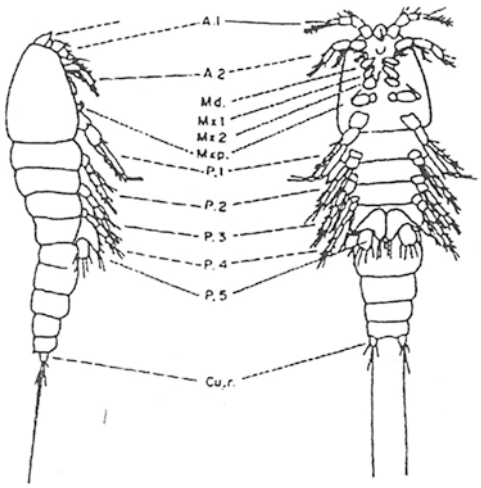
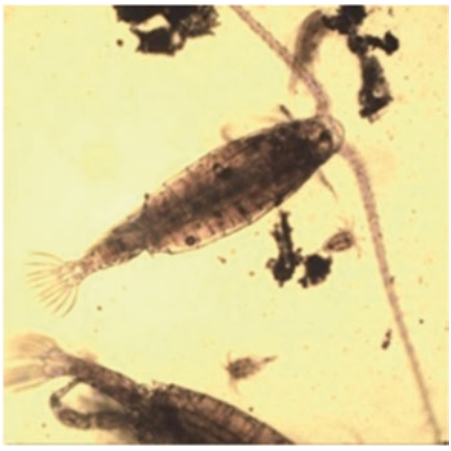


Fig. 14 General structure of a harpacticoid copepod. (Source: Altaff 2004)

Taxonomy and Systematics of Copepoda

Heliodiaptomus viduus (Gurney, 1916)

Phylum:	Arthropoda
Class:	Maxillopoda (Dahl, 1956)
Subclass:	Copepoda (Milne-Edwards, 1840)
Order:	Calanoida (Sars, 1903)
Family:	Diaptomidae (Baird, 1850)
Genus:	*Heliodiaptomus* (Kiefer, 1932)
Species:	*H. viduus* (Gurney, 1916)



The male cephalothorax broad posteriorly, tapering anteriorly; right metasomal wing with two hyaline spines. Penultimate segments of metasoma with spinular

girdle in some specimens; furcal rami and setae of normal shape; fourth abdominal segmented, asymmetrical, slightly swollen on right side. Antennules 25 segmented, reaching the end of the fourth abdominal segment, antepenultimate segment of right antennules having a hyaline process and a short outwardly turned hook. Right fifth leg with two small hyaline processes in the second basal joint; endopodite longer than the first joint of expedite, second joint of exopodite laterodistally with spine, first joint of the exopodite produced laterally into a pointed process and bears a hyaline process on its posterior face. Measurements: Length of body 1.74–1.80 mm, colour whitish. Female: Like male, both antennules similar to left antennules of male. Metasomal wing almost symmetrical, urosome three segmented. Claws of fifth leg asymmetrical, left claw denticulate on both margins, right claw bears teeth only on inner margin; endopodite long and cylindrical with two curved spinules and fine bristles at the tip. Measurements: Length of body 1.82–2.17 mm, colour whitish.

***Sinodiaptomus* (*Rhinediaptomus*) *indicus* (Sewell, 1934)**

Phylum:	Arthropoda
Class:	Maxillopoda (Dahl, 1956)
Subclass:	Copepoda (Milne-Edwards, 1840)
Order:	Calanoida (Sars, 1903)
Family:	Diaptomidae (Baird, 1850)
Genus:	*Sinodiaptomus* (Kiefer, 1937)
Species:	*S. indicus* (Sewell, 1934)

The female in P5, coxal spine is strong. The first exopodite segment is nearly twice as long as wide. The third segment is small but well defined and its inner spine slightly dilated proximally and reaching middle of end claw. In the male, the urosome is bent to the right side. Right P5 coxa is large. Basis is wider than long, with massive chitinous outgrowth. The second expedites segment is two to three times longer than wide. End claw is strong and slightly variable in shape. Endopodite is one segmented and somewhat longer than the first exopodite segment. Left P5 endopodite is two segments and reaches the distal margin of the first exopodite segment.

***Eucyclops speratus* (Lilljeborg, 1901)**

Phylum: Arthropoda
Class: Maxillopoda (Dahl, 1956)
Subclass: Copepoda (Milne-Edwards, 1840)
Order: Cyclopoida (Burmeister, 1834)
Family: Cyclopoidae (Dana, 1853)
Genus: *Eucyclops* (Claus, 1893)
Species: *E. speratus* (Lilljeborg, 1901)

As in other species under the genus *Eucyclops*, spinules are present (reduced in *Eucyclops macrurus* Sars) on the other margin, as its caudal rami is comparatively longer (by more than five times) but the lateral spinules are very small. The antennules are 12 segmented and reach beyond the cephalothorax. Measurements: Female length of body 0.96–1.2 mm.

***Mesocyclops hyalinus* (Rehberg, 1880)**

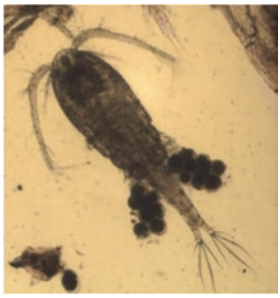
Phylum: Arthropoda
Class: Maxillopoda (Dahl, 1956)
Subclass: Copepoda (Milne-Edwards, 1840)
Order: Cyclopoida (Burmeister, 1834)
Family: Cyclopoidae (Dana, 1853)
Genus: *Mesocyclops* (Claus, 1893)
Species: *M. hyalinus* (Rehberg, 1880)

Female: Body slender, cephalothorax oval, much broader than abdomen. Furcal rami as long as last two abdominal segments, inner margin of caudal rami bare; terminal plumose setae of furcae long, outermost shortest. Antennules 17 seg-

mented, reaching slightly beyond the metasoma, last two segments longer than the previous three, last segment with smooth hyaline membrane. The inner spine of terminal segments of the fifth leg are apical or subapical in position; inner terminal spine but shorter than terminal segments. Colour white, opaque with dark spots. Measurements: Female length of body 0.86–0.90 mm. *Mesocyclops hyalinus* can be very easily distinguished from *Mesocyclops leuckarti* (described below) due to the presence of a smooth hyaline membrane on the terminal segment of the antennules and smooth outer margin basis of the maxilla.

***Mesocyclops leuckarti* (Claus, 1857)**

Phylum:	Arthropoda
Class:	Maxillopoda (Dahl, 1956)
Subclass:	Copepoda (Milne-Edwards, 1840)
Order:	Cyclopoida (Burmeister, 1834)
Family:	Cyclopoidae (Dana, 1853)
Genus:	*Mesocyclops* (Claus, 1893)
Species:	*M. leuckarti* (Claus, 1857)



Female: Body slender, cephalothorax oval, much broader than abdomen. The furcal rami is as long as the last two abdominal segments; inner margin of caudal rami bare; terminal setae of furcae plumose, unequal, outermost shortest. Antennules 17 segmented, reaching slightly beyond the metasoma, last two segments together longer than previous three, last segment with a serrated or notched hyaline plate antenna four jointed; basis of maxilla with conspicuously ribbed outer margin and bears a long seta on a distal outer angle, second joint longer and bears two long setae, one at its distal end, the other on its inner side. Measurements: Female length of body 1.0–1.38 mm, male length of body 0.75–0.92 mm. Colour is whitish.

***Thermocyclops hyalinus* (Rehberg, 1880)**

Phylum: Arthropoda
Class: Maxillopoda (Dahl, 1956)
Subclass: Copepoda (Milne-Edwards, 1840)
Order: Cyclopoida (Burmeister, 1834)
Family: Cyclopoidae (Dana, 1853)
Genus: *Thermocyclops* (Kiefer, 1927)
Species: *T. hyalinus* (Rehberg, 1880)

Antennules reaching slightly beyond the metasome and consisting of 17 segments. Last two segments together longer than the previous three; last segment with a smooth hyaline membrane without any notches. The inner spine is P5 apical or subapical in position in the second segment. Inner terminal of spine is P4, endopod distinctly longer than the outer terminal spine and shorter than the terminal segment. The inner margin of the caudal ramus was bare.

Ostracoda

Ostracoda taxonomy has been of great interest because of their possible use as indicator species of climate and ecosystem changes. Ostracoda are bivalve crustaceans found numerously in both freshwater and marine environments. There are, overall, 1700 known living species of Ostracoda, of which about one-third are freshwater forms. They inhabit a wide variety of aquatic habits, like freshwater, lakes, pools, swamps, streams and heavily polluted areas (Edmondson 1959). Ostracoda are microscopic, bivalve crustaceans, usually 0.4–3 mm long, with valves of low-Mg calcite. The two valves comprise the carapace and protect the soft body parts. The valves are closed by the adductor muscles attached to the carapace. These muscles leave scars on the valves and can be used to identify individuals in the family and superfamily levels. Other important taxonomic characters used for identification are the structure of the marginal zone of the valve, the external valve surface, pigmentation of the carapace and differences in appendages and other soft parts (Dole-Olivier et al. 2000; Meisch 2000; Horne et al. 2002). The abundance of these provide a good food for aquatic organisms. Approximately 110 species are known from the inland subcontinent (Patil and Gouder 1989).

Ostracoda, commonly known as “seed shrimps” or “mussel shrimps”, are small crustaceans, but the freshwater Ostracoda are usually smaller than a millimetre. Ostracoda live in fresh, brackish and marine waters, including streams, springs, ponds, lakes, estuaries and oceans. Some of the taxa are found in groundwaters, semi-terrestrial environments or even in terrestrial plants that accumulate water, such as bromeliads. Non-marine Ostracoda species display diverse lifestyles. Some are benthic, living on or within the bottom substrate, while others are nektobenthic, with swimming abilities, but also live on the bottom substrate or plants. Some species can live in interstitial environments (Mezquita et al. 2005). In freshwater ponds, they are usually found scuttling around among the submerged plants and debris at the shallow edges and are less common in the open waters. Ostracoda display different tolerances to water column physico-chemical variables. Eurytopic species are adapted to a wide range of environmental conditions, while stenotopic species are adapted to a narrow range of environmental conditions. Like other crustaceans, Ostracoda moult, generally passing through eight stages to reach adulthood. An Ostracoda life cycle may last a few months or >2 years (Horne et al. 2002; Smith and Horne 2002). Ostracoda constitute a model group in ecological and evolutionary studies, and are used as indicator species in climate and ecosystem change studies (Butlin and Menozzi 2000; Smith and Horne 2002; Viehberg 2004; Martens et al. 2008).

Morphology of Ostracoda

Organisation of the Ostracoda body can be seen after the removal of one valve. There are seven pairs of modified biramous appendages. The first four pairs are cephalic appendages. They are first antennae, second antennules, mandible and maxillae three pairs of thoracic legs of the thoracic appendages. As well as these, there is a pair of furcal rami attached to the posterior end of the body. These are not normally regarded as true appendages. The second antennules, first thoracic legs and the furcal rami exhibit sexual dimorphism. Their bodies are flattened from side to side and protected by a bivalve-like, chitinous or calcareous valve or shell. The hinge of the two valves is in the upper (dorsal) region of the body. Ostracoda are grouped together based on gross morphology, but the group may not be monophyletic; their molecular phylogeny remains ambiguous. They have a laterally compressed body which is not distinctly segmented. A bivalve carapace is present enclosing the head and the trunk with the limbs. There are three pairs of thoracic appendages which are stout and cylindrical and not phyllopodus. The abdomen is sedimentary; the internal organs like the gonads extend into the valves of the carapace, a feature by which this order can be distinguished from other bivalve crustaceans. The following are some species of Ostracoda that have been taxonomically clarified for identification purposes (Fig. 15).

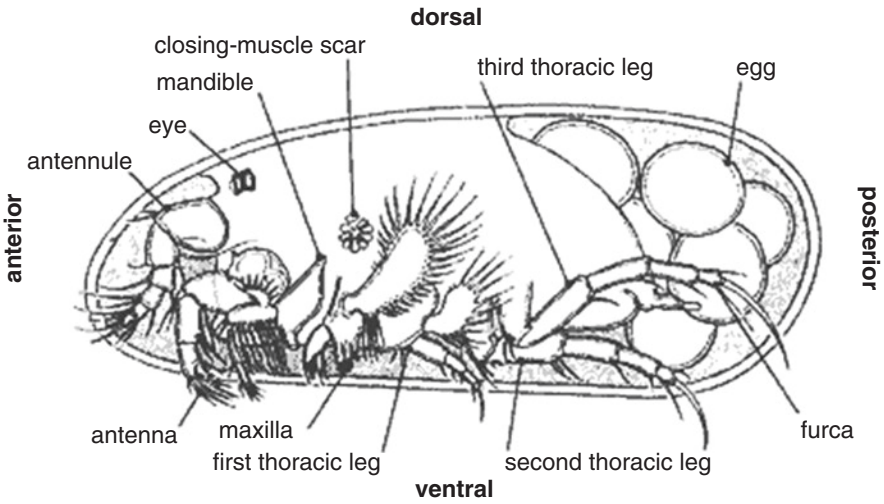
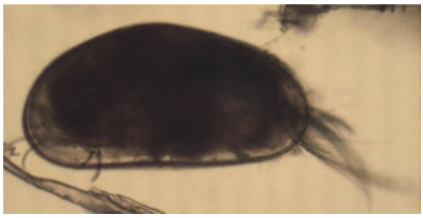


Fig. 15 Typical structure of Ostracoda. (Source: Google in BGS)

Taxonomy and Systematics of Ostracoda

***Cypris protubera* (Muller, 1776)**

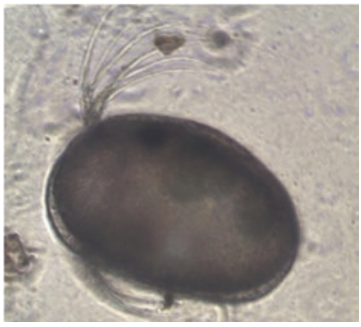
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Cypris* (Muller, 1776)
Species: *C. protubera* (Muller, 1776)



Valves are tumid and there are two prominent tubercles at the postero-ventral margin of valves. Legs 1–4 segmented, third and fourth segments united. Furca is nearly straight and symmetrical.

***Eucypris bispinosa* (Victor & Michael, 1975)**

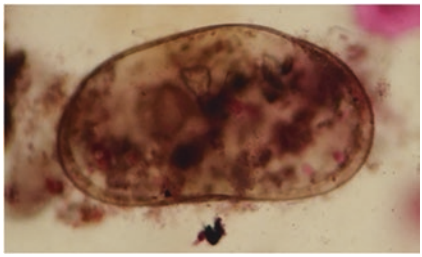
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Eucypris* (Vavra, 1891)
Species: *E. bispinosa* (Victor & Michael, 1975)



Shells of normal shape, elongated, valves are moderately compressed and sub-equal. The penultimate segment of the second thoracic leg divided. Symmetrical furca, usually short, less than half the length of the valves, and it is strongly built with a long, slender terminal claw.

***Strandesia elongata* (Stuhlmann, 1888)**

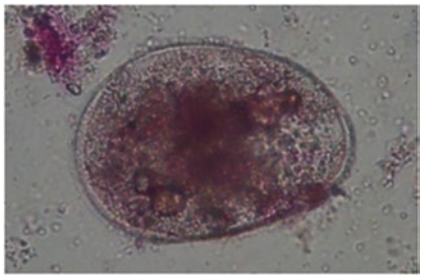
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Strandesia* (Stuhlmann, 1888)
Species: *S. elongata* (Stuhlmann, 1888)



The shell is broadly arched dorsally, height considerably greater than half the length. Maxillary spines moderately toothed. Valves sub-elliptical, elongate. Furcal rami are symmetrical.

***Cyprinotus nudus* (Brady, 1885)**

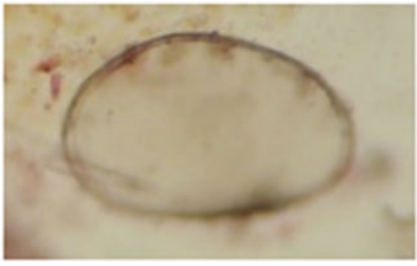
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Cyprinotus* (Brady, 1886)
Species: *C. nudus* (Brady, 1885)



A species with smooth valve surface, furca 20 times longer than its narrowest width and with fine hairs on its ventral margin. Margin of one valve is tuberculated. Terminal seta of furcal rami nearly as long as the terminal claw. Surface of valves is smooth, subovate. The eye is large and prominent. Furcal rami are almost straight. Male is smaller than the female species.

***Heterocypris dentatomarginatus* (Baird, 1859)**

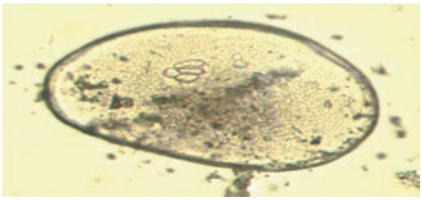
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Heterocypris* (Claus, 1892)
Species: *H. dentatomarginatus* (Baird, 1859)



Valves usually subovate, not tumid and unequal, left valve overlaps the right valve and tuberculate. Furcal rami well developed, symmetrical and short.

***Candonocypris dentatus* (Victor & Michael, 1975)**

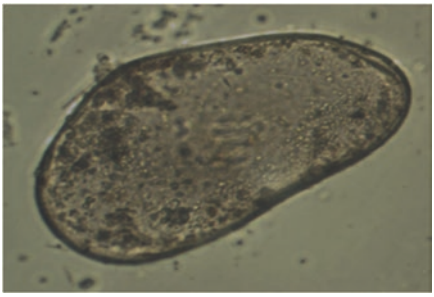
Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Candonocypris* (Vavra, 1891)
Species: *C. dentatus* (Victor & Michael, 1975)



Elongate and sub-oblong valves, distal segment of the maxillary palp broadened, spines smooth, furcal rami symmetrical. Transparently whitish colour. Equivalve, not tumid, pars incisive with large molar-shaped teeth. In males, hemipenis is finely granulated and ejaculatory duct with 40 or 41 crowns.

***Cypretta fontinalis* (Hartmann, 1964)**

Phylum: Arthropoda
Class: Crustacea
Subclass: Ostracoda (Latreille, 1806)
Order: Podocopida (Sars, 1866)
Family: Cyprididae (Baird, 1845)
Genus: *Cypretta* (Vavra, 1895)
Species: *C. fontinalis* (Hartmann, 1964)



At the anterior margin of each valve was a row of radiating septa. The terminal seta of the furca is present. A valve was not uniformly dark pigmented. The surface of valves was sparsely hairy, maxillary spines with slender, delicate teeth. Furca are almost straight, symmetrical and sub-terminal claws slender.

Conclusion

The collection, preservation and morphological identification of freshwater zooplankton for understanding their taxonomical features to fulfil the needs of the scientific community and adjudging the health and wealth of any ecosystem is essential to maintaining a pristine aquatic environment. In this context, the taxonomic key presented in this chapter is considered to be highly essential.

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A Technique on the Culture and Preservation of Marine Copepod Eggs

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Introduction

The search for the ideal copepod for marine fish larvae that can be cultured intensively is ongoing. Copepods are nutritionally suitable for marine fish larvae (Sargent et al. 1997; Stottrup 2000) and constitute a large percentage of the diet in the natural environment (Hunter 1981; Munk and Nielson 1994). Moreover copepods are way too higher in nutritional composition when compared to the traditional live feeds such as *Artemia* nauplii and rotifers. But it is difficult to culture copepod at sufficient densities to be economically efficient on a commercial scale, because they require high water volumes for cultivation in captivity (Esmaeili and Amiri 2011). Even though more than 12,000 species of copepods have been identified and classified (Humes 1994), a few species only are being cultured for the purpose of rearing fish larvae. Out of ten orders of copepods, only three orders, viz., Calanoida, Harpacticoida, and Cyclopoida, are being cultured widely around the world. Among these, the calanoid species receives much attention due to their abundance in pelagic waters and ease of culture in controlled environments. Apart from this, many calanoid copepods are capable of releasing free eggs unlike the cyclopoid and harpacticoid copepods which release their nauplii from the egg sacs itself. The production of diapause eggs has been recorded in many calanoid species during abnormal environmental conditions. These diapause eggs can undergo a long period of metabolic arrest until the environment turns to favorable conditions. This fact aids in the storage of diapause eggs for a longer period and could be used for culture when needed.

In order to meet the demand of hatcheries for large quantities of copepods at one time, mass-scale production techniques and viable methods for long-term cold storage of copepod eggs must be defined. Both subitaneous and diapause eggs have been investigated for storage and use in aquaculture. Diapause eggs are produced

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when environmental conditions signal the possibility of long-term changes; thus they have hardened shells. However, dormancy can be induced in subitaneous eggs if they are exposed to unfavorable environmental conditions, viz., salinity changes or low temperatures, etc. (Uye and Fleminger 1976). Such eggs can be preserved using different preservation techniques for many months and used as an inoculums for copepod culture. This chapter deals with some of the effective and proven methods of preservation of copepod eggs (Table 1).

Methods

The following are the criteria for selection of copepod species for egg storage:

- Dominant and cultivable species in lab scale
- Type of spawning (free spawner/egg carrying)
- High fecundity (eggs per female)
- Registered production of resting eggs
- Short generation time

Copepod Culture

Culture of calanoid copepods is quite simple, once proper environmental and food conditions are met. To start a culture, advanced copepodites and adult copepods are to be stocked at a ratio of 50 individuals per liter of filtered seawater with salinity of 30 psu and temperature around 25 ± 1 °C with a photoperiod of 12:12 h LD. The copepods should be fed with suitable microalgae at a rate of 25,000 cells/ml daily. Care should be taken in siphoning to remove detritus and sufficient water volume to allow addition of the new feed volume every day. Male calanoid copepods make use of their antennae to grasp the female for mating. So avoid rough handling (mostly vigorous aeration) which may result in damaging the antennae and similarly causes decline in the population. Adults will begin producing eggs in 9–12 days and release the eggs freely in the water column.

Egg Collection

The eggs can be collected by siphoning from the bottom of the container and rinsed through sieves with mesh size of 100 and 70 μm. After collecting the new eggs, they should be filtrated and washed thoroughly to remove all sorts of waste particles such as decomposed algae, feces, ciliates, nauplii, and exoskeletons of copepods. Before going for any further experiments on preservation of copepod eggs, the collected

Table 1 List of calanoid copepod species known to produce resting eggs (Marcus Engel 2005)

Species	Location	References
Acartia adriatica	Porto Cesareo, Lonic Sea, Italy	Belmonte (1997)
A. bifilosa	Southampton, Southampton Water, UK	Castro-Longoria (1999)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
A. californiensis	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
A. clausi	Aquaculture enclosures, W-Norway	Naess (1996)
	Aquaculture enclosures, N-Norway	Naess (1996)
	Pacific Ocean, CA, USA	Marcus (1990)
	Inland Sea of Japan, Japan	Uye et al. (1979)
	Mission Bay, Pacific Ocean, USA	Uye and Fleminger (1976)
A. erythraea	Inland Sea of Japan, Japan	Uye et al. (1979)
A. grani	Malaga Harbour, Mediterranean, Spain	Guerrero and Rodriguez (1998)
A. hudsonica	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
	Narragansett Bay, Atlantic Ocean, USA	Sullivan and McManus (1986)
A. italica	Porto Cesareo, Lonic Sea, Italy	Belmonte (1997)
A. josephinae	Porto Cesareo, Lonic Sea, Italy	Belmonte and Puce (1994)
	Otranto, Adriatic Sea, Italy	Belmonte and Puce (1994)
A. latisetosa	Adriatic Sea, Italy	Belmonte (1992)
A. pacifica	Inland Sea of Japan, Japan	Uye (1985)
A. plumosa	Inland Sea of Japan, Japan	Uye (1985)
A. spinicauda	Xiamen, Taiwan Strait, China	Chen and Li, unpublished in: Marcus (1996)
A. steueri	Onagawa Bay, Pacific Ocean, Japan	Uye (1980)
A. teclae	Aquaculture enclosures, W-Norway	Naess (1996)

(continued)

Table 1 (continued)

Species	Location	References
A. tonsa	Tampa Bay, Gulf of Mexico, USA	Suderman and Marcus (2002)
	Southampton, Southampton Water, UK	Castro-Longoria (2001)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Schlei Fjord, Baltic Sea, Germany	Madhupratap et al. (1996)
	Pacific Ocean, CA, USA	Marcus (1990)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	Narragansett Bay, Atlantic Ocean, USA	Sullivan and McManus (1986)
	La Jolla, Pacific Ocean, USA	Uyeancl Fleminger (1976)
A. tsuensis	Inland Sea of Japan, Japan	Uye (1985)
Anomalacera pafersoni	Gulf of Naples, Mediterranean, Italy	Lanora and Santella (1991)
A. ornata	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
Boeckella hamata	Lake Waihola, South Island, NZ	Hall and Burns (2001)
Calanopia americana	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
C. thompsoni	Inland Sea of Japan, Japan	Uye et al. (1979)
Centropages abdominalis	Inland Sea of Japan, Japan	Uye et al. (1979)
C. furcatus	Alligator Harbor, Gulf of Mexico, USA	Marcus, 1989
C. hamatus	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Kiel Bay, Baltic Sea, Germany	Madhupratap et al. (1996)
	Aquaculture enclosures, W-Norway	Naess (1996)
	S-North Sea	Lindley (1990)
	Drogheda, Irish Sea, Ireland	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	White Sea, Russia	Perzova (1974)
C. *ponticus*	Black Sea	Sazhina (1968)

(continued)

Table 1 (continued)

Species	Location	References
C. *typicus*	S-North Sea	Lindley (1990)
C. *yamadai*	Inland Sea of Japan, Japan	Uye et al. (1979)
C. velificatus	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
Epilabidocera longipedata *E. amphitrites*	Pacific Ocean, CA, USA	Marcus (1990)
	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
Eurytemora americana	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
E. affinis	Norrbyn, Baltic Sea, Sweden	Albertsson arid Leonardsson (2000)
	StorfjÃ¤rden Baltic Sea, Finland	Katajisto et al. (1998)
	Schlei Fjord, Baltic Sea, Germany	Madhupratap et al. (1996)
	Aquaculture enclosures, W-Norway	Naess (1996)
	Aquaculture enclosures, N-Norway	Naess (1996)
	Pettaquamscutt Estuary, USA	Marcus et al. (1994)
	Yaquina Bay, Pacific Ocean, USA	Johnson (1980)
E. pacifica	Onagawa Bay, Pacific Ocean, Japan	Uye (1985)
E. velox	Brackish water lake, SE-France	Champeau (1970)
Gippslandia estuarina	Hopkins River Estuary, Victoria, AUS	Newton and Mitchell (1999)
Gladioferens pectinatus	Lake Waihola, South Island, NZ	Hall and Burns (2001)
Labidocera aestiva	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
	Woods Hole, Atlantic Ocean, USA	Lawson and Grice (1976)
L. bipinnata	Inland Sea of Japan, Japan	Uye et al. (1979)
L. trispinosa	La Jolla, Pacific Ocean, USA	Uye (1985)
L. scotti	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Alligator Harbor, Gulf of Mexico, USA	Marcus (1989)
L. wollastoni	S-North Sea	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)

(continued)

Table 1 (continued)

Species	Location	References
Pontella media	Turkey Point, Gulf of Mexico, USA	Chen and Marcus (1997)
	Gulf of Mexico, LA, USA	Chen and Marcus (1997)
	Woods Hole, Atlantic Ocean, USA	Grice and Gibson (1977)
P. mediterranea	Gulf of Naples, Mediterranean, Italy	Santella and Ianora (1990)
	Cap Ferrat, Mediterranean, France	Grice and Gibson (1981)
	Black Sea	Sazhina (1968)
Sinocalanus tenellus	Fukuyama, Japan	Uye (1985)
Sulcanus conflictus	Hopkins River Estuary, Victoria, AUS	Newton and Mitchell (1999)
Temora longicornis	Menai Bridge, Irish Sea, UK	Castellani and Lucas (2003)
	Aquaculture enclosures, S-Norway	Naess (1996)
	S-North Sea	Lindley (1990)
	Margate, English Channel, England	Lindley (1990)
Tortanus derjugunii	Xiamen, Taiwan Strait, China	Chen and Li (1991)
T. discaudatus	Pacific Ocean, CA, USA	Marcus (1990)
T. forcipatus	Inland Sea of Japan, Japan	Kasahara et al. (1974)

eggs can be stored in normal saline water (30 psu) at 2 °C temperature for few days as *Acartia* non-diapause eggs can be stored for 2–6 weeks (Marcus and Wilcox 2007).

Counting of Eggs

A subsampling system is developed as following the method of Drillet (2010). After cleaning the eggs, they should be transferred into 500 ml seawater in a 500 ml plastic container. The container should be shaken for 15 s, and then after waiting for a few seconds to allow air bubbles to travel to the top of the container, the container should be reversed five times and a 10 ml subsample should be removed from the container by using a pipette. This should be repeated for five times, and the number of eggs in the subsamples can be counted. The average of the number of eggs in the subsamples can be calculated, and thereby the total number of eggs in the stock solution in the container could be estimated with a 10% deviation.

Cold Storage of Copepod Eggs

After collecting the eggs from the copepod culture vessels, the eggs should be cleaned and transferred into vials containing 1 μm filtered seawater adjusted to a salinity of 30 ppt. These vials should be stored in refrigerator at a constant temperature of 2–3 °C for predetermined time period (Hagemann 2011a). Before using the eggs for hatching experiments, the vials stored in refrigerator should be transferred to incubator to be heated to room temperature. Before the onset of incubation, a water exchange should be conducted in the vials to ensure that oxygen is present in the vials during the incubation, in case the oxygen had been depleted during the storage period. After the incubation, the eggs can be transferred from the vials to test tubes to carry out hatching experiments.

Cryopreservation of Copepod Eggs

Many experiments have shown that the copepod resting eggs can tolerate freezing down to −25 °C and that they are able to resist low temperatures (3–5 °C) for a longer period. The copepod resting eggs are generally obtained from sediments. They need to be cleaned properly prior to their use as inoculums. The copepod eggs collected from the culture vessels should be sieved through 150 μm and 70 μm sieves. For further cleaning of fraction-sized particles from copepod eggs, the eggs should be added to centrifuge tubes containing a 1:1 solution of sucrose and distilled water and centrifuged at 300 rpm for 5 min, and the supernatants should be washed through a double sieve of 100 μm and 70 μm. Then the copepod eggs should be immersed in the antibiotics such as kanamycin sulfate and oxytetracycline HCl (Drillet et al. 2007). The addition of antibiotics is to eliminate the microbial growth. Then the eggs should be washed with 1 μm filtered sterile seawater and transferred to storage vials and stored at determined temperature. In cryopreservation of copepod eggs, some of the most commonly used cryopreservants are methanol, glycerine, DMSO, ethylene glycol, and propylene glycol which are not toxic to the embryos. After preserving the eggs at determined temperature and time period, the eggs should be made ready for hatching by exposing the tubes containing eggs at room temperature for 10–20 min. Then the eggs should be carefully transferred to vials containing 30 ppt of filtered seawater. The eggs should be then allowed to hatch at room temperature (27 °C) with 12:12 h light-dark cycle with ambient light source.

Optimum Conditions for Preservation of Copepod Eggs

Salinity

The hypersaline water up to a concentration of 100 g/L has potential for short- and long-term storage of subitaneous *A. tonsa* eggs (Ohs et al. 2009). Andreas Hagemann (2011a, b) has also stated that hypersaline waters up to 50, 75, and 100 PSU resulted in 83, 85, and 76% of hatching success, respectively, when stored for up to 7.5 months. Holmstrup et al. (2006) observed that seawater with a salinity of 50‰ proved as a good cold storage media for periods up to 3 months. For short-term storage of copepod eggs, it is preferable to use 30‰ salinity, which could be effective for higher hatching rate.

Temperature

A decrease in the ambient temperature decreases the metabolic activities of an embryo. A study done by Drillet et al. (2005) showed that subitaneous *A. tonsa* eggs remained viable with a high hatching success for up to a period of 12 months of cold storage (2–3 °C) and that cold storage does not affect the reproductive capacity of the following generations. The developmental time from nauplii to adult copepods could increase slightly with the duration of cold storage (10 and 14 days for fresh and 12 months for cold stored eggs, respectively), most likely because the nauplii use up their energy reserves during cold storage and need to rebuild these after hatching before they can molt into the following naupliar stage.

Antibiotics

The application of antibiotics in copepod egg preservation has an important role as a disinfectant. Such antibiotics significantly increase the shelf life of stored eggs. However, exposure for a longer period or high concentrations of antibiotics would be lethal to the copepod eggs. The concentration of antibiotic should be adjusted depending on the period of cold storage planned. Drillet et al. (2007) have observed that antibiotics such as kanamycin sulfate and oxytetracycline HCl with glucose increased the shelf life of preserved eggs when compared to untreated eggs. He also stated that long storage periods with the use of antibiotics are likely to decrease the viability of the eggs and subsequent nauplii, which tend to become smaller after extended periods of cold storage.

Time Period

The duration of the storage period plays a significant role in copepod egg preservation as it directly affects the hatching rate of eggs when stored for a prolonged time (Drillet et al. 2011) in a study observed that the hatching success of copepod eggs increased during the first 5 months, from 0% to 83%, whereupon it decreased until no more hatching occurred after 12 months and also stated that eggs stored for more than 14 months showed no hatching. Many studies have suggested that for longer storage, fresh eggs could be more efficient than the older ones. After long storage periods, the eggs may not be able to hatch due to the lack of exploitable energy.

Cryopreservants

The recommended methods for cryopreservation of shrimp eggs may be similar for copepod eggs. The use of cryopreservants in cold storage of copepod eggs is very scarce. But cryopreservation of penaeid shrimp, *Penaeus japonicus*, embryos, naupliі, and zoea has been attempted by Gwo and Lin (1998) and Vuthiphandchai et al. (2005) and reported that methanol, glycerine, DMSO, ethylene glycol, and propylene glycol are shown not to be toxic to embryos, and the embryos remained viable following exposure to 0 °C. Ohs et al. (2009) observed that cryopreservants such as methanol, ethylene glycol, and glycerine at 5 M produced high viability of hatching in subitaneous eggs of *Acartia tonsa* at an exposure temperature of 4 °C. Propylene glycol and dimethyl sulfoxide produced high viability at 2 M at an exposure temperature of 4 °C.

Conclusion

Aquaculture of finfish and shellfish species has increased dramatically over the past few decades. However, many species generate larvae that are too small to be cultured using rotifer and *Artemia*-based hatchery technologies. After several decades of research, copepods are generally accepted to be of the appropriate size and nutritional value needed to support many of the challenging finfish and shellfish species through the critical first-feeding period. However, still the technology for copepods is at an early stage of development and requires significant advancement prior to widespread commercial implementation. In this state, an intensive culture and preservation of copepod resting eggs adopting suitable preservation techniques to use copepod resting eggs as an inoculum in hatcheries are greatly essential which ensure the sustainable aquaculture practices.

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Introduction to *Artemia* Culture

T. Veeramani, P. Santhanam, N. Manickam, and C. Rajthilak

Introduction

Artemia, the brine shrimp, is an excellent live food for cultivable aquatic species. It is in great demand for use in shrimp hatcheries, fish hatcheries and ornamental fish culture farms. The genus *Artemia* is a cosmopolitan taxon that has typically adapted to live in the stressful environmental conditions of higher saline habitats, such as salt lakes, lagoons and solar salt works all over the world (Vanhaecke et al. 1987; Triantaphylidis et al. 1998). *Artemia* populations have been identified in about 600 natural salt lakes and salt works along the coastlines of tropical, subtropical and temperate regions, and further survey efforts are being conducted to identify more *Artemia* biotopes (Bossier et al. 2004). *Artemia franciscana* is native to North, Central and South America (Bowen et al. 1985), while other *Artemia* species include *Artemia persimilis* in South America (Gajardo et al. 1999), *Artemia salina* in the Mediterranean Sea (Gajardo et al. 1999), *Artemia urmiana* in Lake Urmia, Iran (Triantaphyllidis et al. 1997; Manaffar 2012), *Artemia sinica* in China (Naihong et al. 2000), *Artemia tibetiana* in Tibet (Naihong et al. 2000) and *Artemia* sp. in Kazakhstan (Pilla and Beardmore 1994). *Artemia* has been introduced in Brazil, Australia (Geddes 1980), the Philippines, Thailand (Tarnchalanukit and Wongrat 1987), India, Sri Lanka (Hoa et al. 2007) and Vietnam (Baert et al. 1997). *Artemia* can withstand habitats whose salinity levels range from 10 to 340 g L^{-1}, with fluctuating ionic compositions and temperatures (Van Stappen 2002). *Artemia franciscana* is a native stain from America, originating in the San Francisco Bay, California and the Great Salt Lake, Utah (Persoone and Sorgeloos 1980).

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To date, there is no artificial feed that can be a complete substitute for *Artemia*; thus, *Artemia* nauplii remain essential in many hatchery operations (Sorgeloos et al. 2001). *Artemia* is rich in highly unsaturated fatty acids (HUFA; docosahexaenoic acid [DHA] and eicosapentaenoic acid [EPA]), which are considered essential fatty acids for larval fish development (Dhont and Van Stappen 2003). *Artemia* are non-selective filter feeders, feeding on protozoa, bacteria, microalgae, organic detritus etc. (Anh et al. 2009). They graze on very small food particles ranging in size from 1 to 50 μm (D'Agostino 1980; Stappen 1996; Dhont and Sorgeloos 2002).

The aquaculture of pond natural habitats due to the availability of food from natural microalgae can be used for extensive culture. Other by-products from agriculture, the food industry and some organic manures like rice bran and corn bran are used for small-scale aquaculture. These foods are useful to increase the reproduction and growth of *Artemia* (Wear and Haslett 1987; Wurtsbaugh and Gliwicz 2001). Organic foods and industrial waste are decomposed and converted into fertiliser, which can produce feed for *Artemia*, such as yeast, bacteria, algae and some kinds of microorganisms (Brands et al. 1995; Zmora and Shpigel 2006). The traditional use of chicken waste stimulates algal growth in the algal pond; hence, rice bran and soybean meal are helpful for *Artemia* growth via supplements. Consequently, organic waste manure has been considered as a food (Brands et al. 1995; Baert et al. 1997).

Large quantities of pig manure are not utilised and are, instead, dumped into the public sewage systems, such as ponds, rivers, lakes etc. Pig manure has been used in fish ponds to fertilise the pond water; hence, there is no need to apply supplementary feed (Lan et al. 2004).The various supplementary feed ratios are green water (GW), pig manure (PM), rice bran (RB) and soybean meal (SB). PM + RB and PM + SB stimulate better biomass production of *Artemia* (Anh et al. 2009). Therefore, agricultural and veterinary waste is a potential source of organic fertiliser for *Artemia* culture and it can reduce the pressure on the environment. Worldwide aquaculture concentrates on the culture of aquatic species like fish, mud crabs, bivalves and marine snails, polychaetes etc. However, recently, aquaculturists have begun to concentrate on culturing *Artemia* for producing *Artemia* cysts, biomass and frozen items. Therefore, there is an increasing demand for *Artemia* in the aquaculture industry, which encourages the economic development of any country.

The culture of *Artemia* can improve the economic value of local populations. *Artemia* culture is practiced to educate local communities for economic gain (Ogello et al. 2014). Approximately 200 tons of *Artemia* cysts are used as a live feed in aquaculture hatcheries in India (CMFRI 2006). No attempt has been made so far to develop a proper methodology to produce *Artemia* on a commercial scale. Despite the above mentioned reason, worldwide aquaculture industries are now focusing on the setting up of *Artemia* cultures in saline-based environments.

Need for *Artemia* Culture

The use of *Artemia* nauplii as a live fish food began in the 1930s, when scientists discovered their unrivalled nutritional value (Bengtson et al. 1991). During the 1970s and 1980s, the Great Salt Lake in Utah supplied around 90% of the world's demand for *Artemia* cysts. However, some poor harvests from the Great Salt Lake in the mid-1990s and increasing aquaculture demands for cysts forced the development of alternative sources of cysts for world utilization. To meet the demand for *Artemia* cysts, *Artemia* aquaculture should be carried out on a large scale along with the salt works, wherever possible.

Indeed, many authors agree that *Artemia* could be the solution to the mass mortalities experienced in many global fish and shellfish hatcheries (Bengtson et al. 1991; Merchie 1996; Sorgeloos 1998). *Artemia* nauplii can stimulate the spawning in brood stocks of shrimps (Burton et al.1998). *Artemia* nauplii are carriers of probiotics in marine fish (King 2002). Over 2000 tons of *Artemia* dry cysts, costing about 65 USD/1 kg, are used annually to feed fish and shellfish (De Los et al. 1980). Salt production companies now agree that salt works operations require substantial populations of *Artemia* to control the problems associated with algal blooms and to improve salt quality. Scientists consider *Artemia* as a model organism for research fields such environmental ecotoxicology (Nunes et al. 2006). *Artemia* filter and feed on microalgae, halophilic bacteria and other suspended organic matters, clear water and help to increase the evaporation speed in salt production. This clear water is conveyed from evaporation ponds to crystallisation ponds to produce a final product of good quality salt.

Classification of *Artemia*

Kingdom:	Animalia
Phylum:	Arthropoda
Subphylum:	Crustacea
Class:	Branchiopoda
Order:	Anostraca
Family:	*Artemia*
Species:	*Artemia franciscana* Kellogg, 1906

There are seven species and 50 strains of *Artemia* recorded so far in the world's salt lakes and ponds, commonly known as *Artemia franciscana*, *Artemia sinica*, *Artemia parthenogenetica*, *Artemia* sp., *Artemia tunisiana*, *Artemia urmiana* and *Artemia persimilis* (Lavens and Sorgeloos, FAO 1996) (Fig. 1).

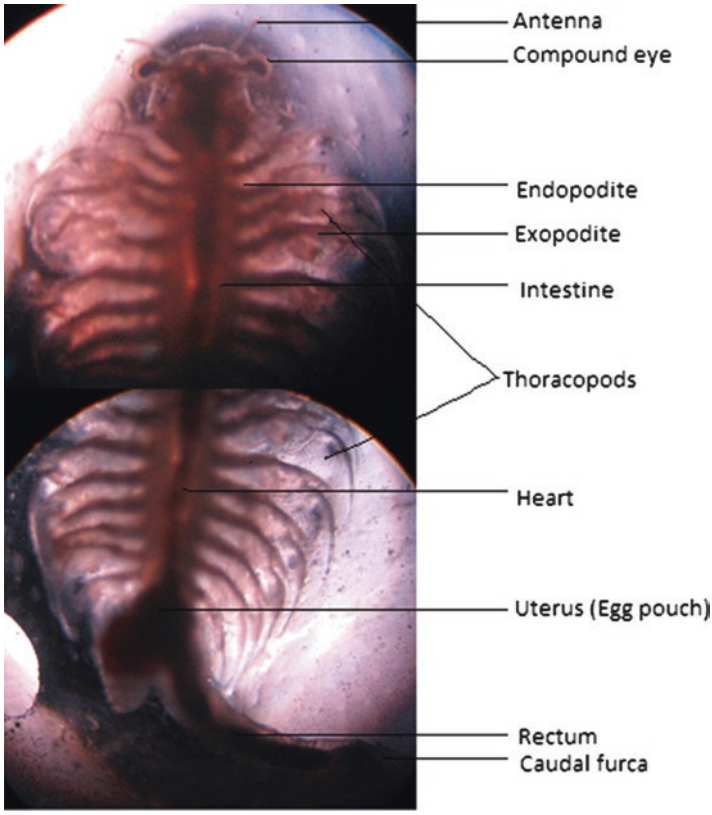

Fig. 1 Microscopic view of *Artemia*

Adult Males and Females of *Artemia*

Artemia have a monosex (*Artemia parthenogenetica*) and bisexual nature. Males and females are separate individuals. *Artemia* have oviparous and ovoviviparous modes of reproduction. The uterus of a single female *Artemia* can hold around 200 eggs. *Artemia* contain three eyes; one simple eye developed during the fingerlings phase of development and, during adult species development, two additional compound eyes. *Artemia* never sleep, as they must be constantly awake in order to breathe, and are continuous filter feeders. The male *Artemia* has two reproductive organs (two big antennas) called graspers, and the female has one small antenna and a large uterus.

The male and female *Artemia* have two compound eyes, antenna intestine, thoracopods, intestine, rectum, telson and caudal furca etc. They also have a single simple eye, which can sense light. *Artemia* nauplii swim towards the light, while the adult *Artemia* swim away (Emslie 2003) (Fig. 2).

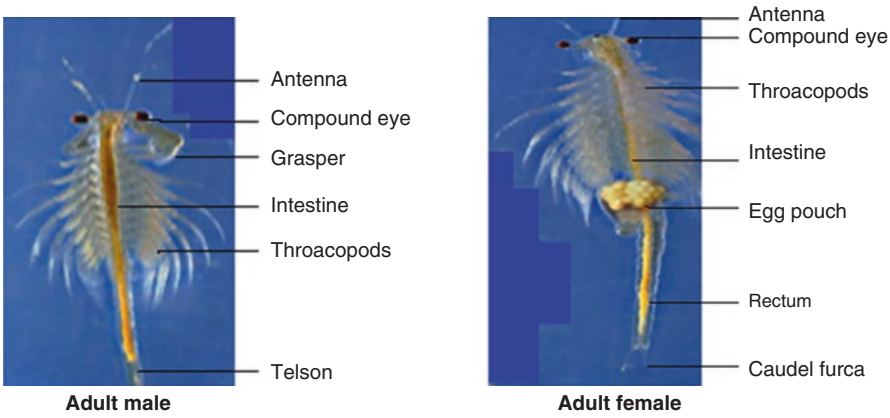

Fig. 2 Adult male and adult female of *Artemia*

General Biology and Ecology of *Artemia*

Brine shrimp (*Artemia*) date from 100 million years ago, according to their osmoregulatory function. They are found in a wide range of salt environments, ranging in salinity from 10 to 350 ppt, which characterises various chemical compositions. According to radio carbon analysis, *Artemia* cysts are more than 10,000 years old. Hence, in optimum conditions, *Artemia* can live for several months. The development of *Artemia* will take 8 days from the nauplius to the adult stages, and they can release 300 cysts or nauplii every 4 days. The brine shrimp development stage takes approximately 15 moults, its lifespan being 3–6 months. *Artemia* can live in environments of huge O_2 deficiency and the minimum required O_2 level is in the range 0.3–0.5 mg/L. *Artemia* are non-selective filter feeders, feeding on microalgae, various bacteria, detritus matters, yeast and other organic food items. *Artemia* can reproduce both sexually (e.g. *A. franciscana*) and asexually (e.g. *A. parthenogenetica*). *Artemia* lay eggs, or cysts, a process called oviparous reproduction. They have a hard shell and an exceptional ability to survive.

Embryonic Development

The developing stage of the egg is self-contained and the yolk is evenly distributed during cleavage, along with the blastomeres. The egg development formations rapidly grow through the cleavage and blastula stages within the female brood sac. After formation, the eggs are deposited in a particular environment and they can remain encysted. The embryonic development of cysts is arrested during the early gastrula stages. Throughout this process, there are around 4000 cells that are activated in the embryo of the egg, but no organs are discernible during these stages.

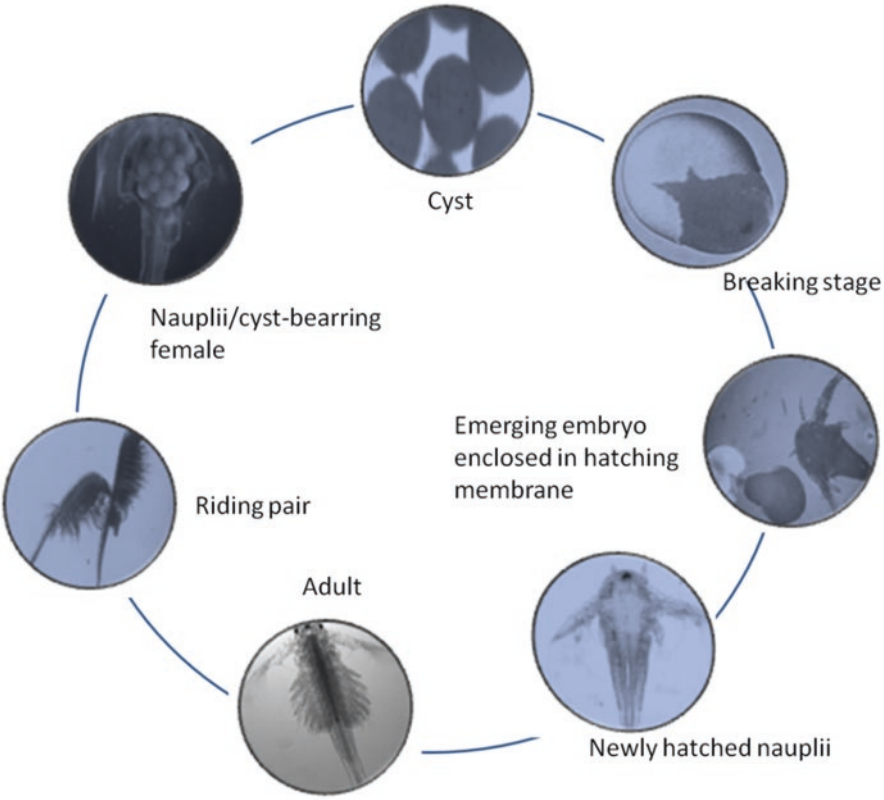

Fig. 3 Embryonic development stages of *Artemia*

When the encysted eggs are exposed to good conditions (rehydration), they swell and rapid development of the embryo occurs. Hatching occurs between 24 and 48 h. Due to the temperature, during the first few hours, the nauplius stays within a hatching membrane that hangs beneath the cyst shell. After 12 h, the nauplius is completely developed and is called the umbrella stage.

Post-embryonic Development of *Artemia*

After hatching, the nauplii develop into different stages (Fig. 3). Stage 1 (instar 1): Free-swimming and about 0.4–0.5 mm in length and brownish-orange in colour based on yolk materials. In a sense, the body consists of various organs. On the head are three pairs of appendages and one pair of small first antennules (antennae), a

pair of developed second antennae, a pair of mandibles and a large labrum, which covers a ventral mouth of the organisms, and the nauplii eye is poorly developed. The nauplii do not have a complete digestive system, are weak swimmers for about 12 to 20 h and then moult into metanauplius larvae. Stage 2 (instar II): Commonly called metanauplius larvae, they are translucent in colour and about 0.6 mm in length. The trunk region is noticeably longer and this metanauplius swims strongly using its second antennae, which are well developed. This stage starts filter feeding, the main foods of which are microalgae, bacteria and detritus sized 1 to 50 μm, which are filtered out by the second antennae and ingested into the functional digestive tract.

After the instar II development stage, classified as metanauplius, the next stage is to develop the mouthparts appendage. Post-naupliar stages: There are seven stages, and paired thoracic appendages are formed. Fully developed compound eyes, reduced labrum and defined abdominal segment. Post-larval stages: Reduction of antennae and length of the eyestalks, formation of sexual organs. Juvenile stages: The larva grows and differentiates through about 15 moults, and has well-developed organs. Important morphological as well as functional changes take place.

Pre-adult stage: In this stage, locomotor functions are lost and sexual differentiation occurs between males and females. The male species develop hooked graspers, whereas the female antennae are reduced to sensory appendages and thoracopods are now separated into three functional parts; telopodites and endopodites change their functions into locomotory organs and filter feeding, and expedites are formed as a gills. These stages cover 14 to 15 moults and, sometimes, 17 moults.

Adult stage: Well-developed animals (males and females) are called adults. This adult stage is reached around 3 weeks after hatching. The animals range from 0.7 to 1.2 mm in length and the adults may live for up to about 4 months.

Morphology and Life Cycle of *Artemia*

Brine shrimp have three eyes; a simple eye developed during the fingerling stage of development and two additional eyes developed by the adult organisms. A male *Artemia* has two large antennae as reproductive organs on its head and a female has one small antenna with a large uterus, containing approximately 200–300 eggs. In the natural environment at certain times of the year, *Artemia* produce cysts that can float on the water surface and are thrown ashore by the wind and waves. These cysts are metabolically inactive and do not develop further, as long as they are kept dry. Upon immersion in the seawater, the biconcave-shaped cysts will hydrate and become a spherical shape and, within the shell embryo, resume their interrupted metabolism. After 20 h, the outer membrane of the cyst bursts (“breaking”) and the embryo will appear, surrounded by the hatching membrane.

Pond Production of *Artemia*

Solar salt works ponds providing suitable habitats for *Artemia* can be useful for healthy and biological salt production processes. *Artemia* can control algal blooms and prevent salt contamination during salt production. Brine shrimp a play major role in releasing metabolites, which can enrich nutrients and function to develop red halobacterium in the crystallization salt pond. This halobacterium will encourage the heat obstruction process to increase the rate of salt production. Hence, the efficient management of *Artemia* in salt pan environments is essential to produce large quantities of good quality salt. Based on this benefit, several countries have integrated the production of *Artemia* in salt works with the aquaculture industry.

Site Selection

The first and foremost requirement for embarking on the venture of *Artemia* culture is the acquisition of suitable land. A suitable site is one that can support optimum conditions for the growth of *Artemia* at the targeted production level. Various parameters are considered when choosing the site for *Artemia* pond preparation.

Climatology/Meteorology

The area should have the characteristics of low rainfall, low humidity, long duration of sunshine and high atmospheric temperature. This will ensure the availability of high-salinity water for culture, via a high evaporation rate.

Topography

The *Artemia* culture pond should be chosen to be as flat as possible for ease of use, while the location should be a naturally sloped region to give a proper drainage system. The pond site should be near the seashore, with high tide inflow to fill the pond with seawater via gravity and tidal current, so as to reduce the fuel energy requirements of pumping the water.

Soil Condition

The *Artemia* pond should contain rich organic matter and natural fertiliser for enriching the soil, as well as suitable heavy clay soil to reduce underground water percolation and pond water leakage. Acid and sulphate soils are the major problems causing reduction of the soil and water pH levels. Based on this, mangrove and swamp areas should be avoided.

Others

While selecting the site for *Artemia* culture, the entrepreneur should ensure the following:

1. The area should be accessible preferably by a road.
2. The site should have access to seawater as well as high-saline groundwater.
3. The area should be flood-free.
4. Social problems due to the drainage of waste water should be properly taken care of.
5. Availability of necessary infrastructure, namely, electricity, communication facilities etc., are needed for successful management.

Types of Pond

Artemia culture can be carried out in three types of ponds, viz. concrete ponds, earthen ponds and plastic-lined ponds.

Pond Preparation

Artemia culture ponds are prepared following the pre-stocking procedure, including the drainage system. At the end of the culture, the water should be drained through a canal and the unwanted materials removed, the pond bottom scraped, all dykes elevated and the pond levelled up to 40 to 50 cm from the bottom. The pond should be allowed to dry for up to 2 weeks. Derris roots have to be applied to kill the

predators in the pond. The ponds will be filled with saline water, filtered through 500-μm nylon screens to eliminate fish eggs and larvae (Baert et al. 1996; Anh 2009). At the start of the culture period, the water level should be maintained in the ponds at about 4–5 cm above the platform and then gradually increased up to 40 cm during the culture period.

Hatching and Inoculation of Artemia

High quality 'A' grade *Artemia* cysts (OSI brand) were purchased from a registered dealer and kept stored in a cool place. The hatching conditions according to Stappen (1996) were followed. Seawater of salinity 35 ppt gives constant illumination and aeration; while maintaining a temperature of 25–28 °C, freshly hatched nauplii are inoculated during the late afternoon. Ponds are inoculated when the water salinity does not exceed 80 ppt at a stocking density of about 100 per litre (Anh et al. 2009).

Stocking in Large Salt Ponds

The average *Artemia* nauplii stocking density of 5 to 10 per litre should be maintained. The operation of a large pond depends on the water and area coverage. The nauplii should be stocked in a hypersaline water circuit to prevent contamination and predators should be controlled by using bleached water. The stocking of *Artemia* nauplii should be at optimum salinity and involve the use of several stocking ponds to help prevent algal bloom problems.

Stocking in Small Ponds

The starting stocking density level should be high in number, at around 100 nauplii per litre, and the water turbidity should be maintained in the 15–25 cm range. At high stocking densities, the optimum oxygen level should be maintained. When the turbidity is below 25 cm, the stocking density should be reduced to 45 to 65 nauplii per litre. High stocking density will stimulate oviparous reproduction. If the stocking density is very low, the animals can produce a greater number of female *Artemia* through ovoviviparous reproduction. The maintenance of essential food in the stocking pond is important, as low food stocks will affect the growth of the animal. During the culture period, the optimum levels of temperature, oxygen, pH and feed should be maintained.

Application of Fertilisers to Artemia *Ponds*

Manure application is important for *Artemia* culture to increase algal growth, especially in high-salinity water. During algal growth, various factors influenced by the natural composition include ions in seawater and pH maintenance by the calcium composition. Salinity, temperature and pH are essential parameters for improving the algal density in the culture pond. When applying chicken waste, cow dung, organic waste and pig manure etc., inorganic nutrients like the nitrogen phosphorous proportion are maintained in the culture pond.

Feeding Procedure

Algae, Organic and Supplementary Feeds

Feeding will start from day 5 after inoculation. Algae feed will be supplied twice per day (*Artemia* is a continuous filter feeder), by organic wastes stored in a plastic bag, which will be perforated and submerged underwater for 2–3 days and then moved daily around the *Artemia* pond to allow its gradual spreading over the pond bottom. Rice bran and ragi flour (about 10–20% of which are particle sizes ≤50 μm) will be incubated (fermentation with yeast) in freshwater for 24 h before application (Anh et al. 2009). The fermented matter will be diluted with water and filtered by using a 20–50-μm mesh net and the filtered milky water applied to the *Artemia* culture pond.

Pond Management

Adequate management of *Artemia* ponds is highly essential. The pond bottom should be raked on a daily basis to prevent contamination of the water and soil and to remove unwanted gas from the bottom of the pond. Dumper organic matters are dissolved and used for *Artemia* feed. The particular amount of water is adjusted depending on turbidity, salinity of culture ponds and algae ponds. After 7 weeks of culture, about 10–20% of the pond water is exchanged weekly to minimise the increase in salinity and to remove weak *Artemia* nauplii that have accumulated in the corners of the pond (Anh et al. 2009). The water is discharged from the *Artemia* ponds and filtered over a 500-μm screen to prevent the escape of adult and juvenile *Artemia*.

Sample Collection

The water temperature is measured at 7 am and 12 pm, the salinity at 7 am and the turbidity (using a Secchi disc) at 12 pm on a daily basis. Survival is determined at days 5 and 11 (evaluating only the original population inoculated). To collect nine points of samples per pond, a square plankton net (mesh size 100 μm, surface area 0.25 m^2) is used, which is towed vertically through the water column at each sampling in the early morning (Anh et al. 2009; Baert et al. 2002).The temperature and salinity are measured using a Celsius thermometer and hand refractometer. The water pH and dissolved oxygen are analysed using a pH pen and DO meter, respectively. Nutrients (C, N, P and Si) and major ions (Ca, Mg and K) are analysed by adopting the standard methods described by Strickland and Parsons (1972). *Artemia* samples are collected by using a square plankton net with mesh size 100 μm and surface area 0.25 m^2 in the early morning and the biological study is as described by Baert et al. (2002). The culture management of *Artemia* is performed according to Kellogg (1906).

Monitoring of the Artemia *Population*

The *Artemia* population is monitored visually every day in the morning before pond raking and parameters such as animal gut fullness, faecal pellets, swimming behaviour, animal colour (normal/red/green), colour of the brood pouch (white, brown), animal appearance (appendages for any bacterial or fungal diseases or abnormalities), predators (mysids, crustacean larvae), competitors (rotifers, ciliates) and prey (microalgae, cyanobacteria) are observed microscopically.

Artemia *Cyst Collection and Processing*

The harvesting of *Artemia* cysts and biomass is carried out based on the procedure of Anh and Hoa (2004). The *Artemia* biomass has to be collected every 21 days by using a scoop net and the excess water from the biomass collection is removed with the help of a cloth. *Artemia* cysts are collected by using a scoop net shaped as an isosceles triangle (100 μm mesh size). The collected cysts are segregated and stored in brine water for diphase. After 3 months, the cysts are dried by using a cyst dryer or solar dried.

Cyst Evaluation, Sieving and Packing

Before packing into tins or containers, the quality of the cysts (moisture content, number of cysts per gram, hatching percentage, hatching efficiency etc.) should be evaluated. The dried cysts should be passed through a shaker sieve (230–250 μm) to avoid other non-cyst impurities. The dried cysts are very hygroscopic in nature. In open air, they quickly absorb atmospheric humidity and become soaked, which results in loss of energy and poor hatching. Hence, cysts should be packed in air-tight vacuum packs or nitrogen-flushed containers.

Artemia Biomass Collection and Processing

Biomass Harvest

Artemia biomass is harvested partially when the population density exceeds 200 adults per litre. A dip net or dragnet with a cod end of around 200 microns mesh size can be used to harvest the *Artemia* biomass. The net should be emptied at an interval of 15 min to avoid damage of the accumulated *Artemia* in the cod end. To harvest the biomass, 800-micron-sized sieves are also used. Biomass harvesting can be continued until the density of the *Artemia* population drops below 100 adults per litre.

Cleaning, Packing and Freezing

The harvested biomass should be cleaned and rinsed with adequate clean filtered seawater. Then, they should be packed in a 0.5–1-kg polythene cover and thermally sealed. The sealed packs should be kept over crushed ice until completion of the packing process or loading into the freezer. A strict cold chain should be maintained to avoid quality deterioration and autolysis by its own proteolytic enzymes. Then, the frozen biomass packs are stored at <−18 °C. The shelf life of the product is 3–6 months.

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Optimisation of the Culture Conditions of *Nannocalanus minor* (Copepoda: Calanoida)

K. Jothiraj and P. Santhanam

Introduction

By studying the biology of copepods, including their growth, survival and reproductive behaviour, we can obtain baseline information on the culture of particular species at the laboratory- and pilot-scale levels. This information can be used in the development of feed for the commercial production of fish seeds. Most of the research conducted to date has focused on copepod taxonomy and ecology. However, studies on the biology of copepods are limited. Amongst the marine copepods, experimental work has been conducted with different species of the genus *Oithona* by Murphy (1923), Gibbons and Ogilvie (1933), Rao (1958), Haq (1965), Goswami (1975), Lampitt (1978), Perumal et al. (2000) and Santhanam and Perumal (2002). Very limited reports are available on the biology of copepods in Indian waters that investigate feeding behaviour, developmental biology and reproductive biology (Rao 1958; Krishnaswamy 1950; Goswami 1975; Saraswathy and Santhakumari 1982; Shrivastava et al. 1999; Perumal et al. 2000; Santhanam and Perumal 2002).

Despite the ability to sample meso- and microzooplankton and successfully conduct experimental studies with them (Mullin and Brooks 1967; Paffenhofer 1984), the oceanographic community has focused its research on larger representatives; thus, the importance of small species has generally been neglected or underestimated (Mazzocchi and Paffenhoper 1998). Studies on the fecundity and egg production of copepods can indirectly estimate population dynamics and secondary production in the aquatic ecosystem (Hirche and Niehoff 1996). Considerable information is available on the copepod species to provide a clear understanding of their biology, including their larval development (Yoon et al. 1998; Merrell and Stoecker 1998), variations in body length (Schnack et al. 1989), feeding and survival biology (Metz 1998; Shrivastava et al. 1999; Perumal et al. 2000), sex ratio (Moore and

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Sander 1983; Fredrika Norrbin 1993) and fecundity or egg production (Nival et al. 1990; Hirche 1992; Rodriguez et al. 1995; Peterson and Dam 1996; Hirche and Kwasniewski 1997; Uye and Murase 1997; Richardson and Verheye 1998; Koski et al. 1999; Koski and Kuosa 1999; Irigoien et al. 2000; Ku Kang et al. 2000; Hirche and Halsband 2001).

Several experimental studies have been conducted on the developmental biology and reproductive biology of copepods (Lopez 1996; Caramujo and Boavida 1999; Cervetto et al. 1999). A more complete understanding of the mortality of early developmental stages is important. Their life cycle and the effects of temperature and food quality and quantity on post-embryonic development and reproduction have, indeed, been the subject of many studies (Andersen and Nielsen 1997; Devreker et al. 2004; Halsband-Lenk et al. 2002; Santhanam and Perumal 2002; Cook et al. 2007; Almeda et al. 2010). This chapter provides a number of biological observations on the calanoid copepod *Nannocalanus minor.* For selected species, data are now reported on the survival in low and high food concentrations. The growth of *N. minor* in different microalgal feeds was examined in order to compare variations in body length, larval development, survival rate and reproductive parameters such as egg production and egg hatching rate, with reference to temperature and algal food concentration.

Materials and Methods

Sample Collection

The copepod samples were collected from the coastal waters of Nagapattinam, Muthupet, Parangipettai and Pichavaram by using plankton net (158 μm). The collected samples were taken to the laboratory and provided with aeration using a battery aerator. In the laboratory, the calanoid copepod *N. minor* was identified using the classical key (Kasturirangan 1963) as follows: *Female*: First antennae reaches caudal rami; length is 1.9–2.0 mm. *Male*: Five legs, with a few plumose setae present on the right exopodite; the left leg distinctly longer than the right leg; external marginal spines greatly enlarged on the left exopodite; length is 1.70 mm. After the confirmation of species, the healthy adults of *N. minor* were removed from the collected samples by using a fine capillary tube.

Growth and Survival of Copepods

The isolated copepods were kept overnight in 250-mL glass bowls containing filtered seawater (1 μm) of ambient salinity (32‰), with aeration for starving prior to the experiment. The monocultures of algae, viz. *Chlorella marina*, *Dunaliella* sp., *Isochrysis galbana*, *Nannochloropsis* sp., *Coscinodiscus centralis*, *Chaetoceros*

affinis and *Skeletonema costatum*, were cultured separately in the laboratory by using F2 medium (Guillard 1972). The individuals of *N. minor* were stocked in 100-mL glass bowls containing filtered seawater with 32‰ salinity. Seven types of live phytoplankton, viz. *C. marina*, *Dunaliella* sp., *I. galbana*, *Nannochloropsis* sp., *C. centralis*, *C. affinis* and *S. costatum*, and different concentrations of algal feed were used for the survival study. Ten individuals of copepods were maintained separately in each bowl for each algal concentration. They were transferred daily to a new bowl with freshly filtered seawater and feed. The daily mortality was recorded carefully. The experimental sets were maintained at 28 ± 1 °C till the death of all animals. For the experiment on the effect of food concentration, the concentrations of unicellular algae were quantified by cell counts using a Sedgewick counting chamber under a compound microscope. Cell concentrations of 10,000 and 20,000 cells/mL were used for the present experiment. For the growth estimation, the nauplii, copepodite and adult copepods fed with different types of algae were collected from the respective tanks. The total length of the different stages of copepods were measured under a microscope at a magnification of 10× by using ocular and stage micrometers, from the tip of the prosome to the end of the caudal rami, excluding the caudal setae.

Egg Production and Hatching Rate

For fecundity measurement under different experimental conditions, male and female copepods were placed in Pyrex test tubes at the ratio of 1:1. The test tubes were provided with mixed microalgae of *C. marina*, *Dunaliella* sp., *I. galbana*, *Nannochloropsis* sp., *C. centralis*, *C. affinis* and *S. costatum*.

Effect of Temperature

To determine the appropriate experimental temperature range for *N. minor*, males and females were maintained under food-saturated conditions, with excess food consisting of *C. marina*, *Dunaliella* sp., *I. galbana*, *Nannochloropsis* sp., *C. centralis*, *C. affinis* and *S. costatum* for studying the temperature effect on fecundity. Adult males and females of *N. minor* were placed in Pyrex test tubes (2.5 cm mouth diameter, 15 cm depth) containing 20 mL of the culture water. Temperature fluctuations were controlled by using air conditioning. Temperatures above 25 °C were maintained by using a water bath. An immersion heater in the water bath can be used to control the ambient temperature. The water was stirred using an air stone so that a layer of heated water does not form at the surface and overheat the animals. The water level within the animal containers was maintained slightly higher than that of the heated water bath. This causes gentle stirring of the water in the animal containers as the surface loses heat to the atmosphere and convection currents are established. Eggs produced were placed on a watch glass and counted under a binocular microscope.

The effect of temperature on the egg production rate of copepods was determined by incubating eggs in a test tube placed in an air-conditioned room by maintaining temperatures of 18 ± 1, 20 ± 3, 25 ± 3 and 30 ± 1 °C. Each experiment was carried out in triplicate.

Effect of Food Concentration

To investigate the effects of different food types and concentrations on the egg production of *N. minor*, the male and female copepods were isolated and kept in Pyrex test tubes containing filtered seawater and starved for 24 h. Food concentrations were diluted from stock cultures of known concentrations determined by microscopic counts. The food was kept constant at 1000, 5000, 10,000 and 20,000 cells/mL, with one control containing filtered water without algae. During the experiment, a stirrer agitated the water in the beakers at 2–6-h intervals. Cell counts were measured before and at the end of the experiment. The temperature was kept constant at 25 ± 3 °C. The test tubes were covered with black cloth to reduce illumination. After the experiment, the eggs were collected from the bottom of the tubes and counted under a light microscope.

Hatching Succession

To estimate the hatching succession, laid eggs were incubated in test tubes along with filtered seawater for 96 h. After that, the hatched nauplii were counted using a binocular microscope. The effect of temperature on the hatching rate of copepods was determined by incubating eggs in a test tube placed in an air-conditioned room by maintaining temperatures of 18 ± 1, 20 ± 3, 25 ± 3 and 30 ± 1 °C. Each experiment was carried out in triplicate. The hatched nauplii from individual test tubes were placed in Petri plates and counted under a binocular microscope. For examining the effect of algal food concentration, the eggs were placed in test tubes with filtered seawater. The food was given at the following concentrations: 1000, 5000, 10,000 and 20,000 cells/mL, with one control containing eggs without algae. The nauplii were counted under a binocular microscope. Each experiment was performed in triplicate.

Results

Survival Rate of N. minor *in Low Food Concentrations*

The survival of *N. minor* recorded in the present study depended on the food type and concentration. 100% survival was noted for about 2–7 days at low food concentrations. In *C. marina*, 100% survival occurred up to the 7th day, 49% survival was

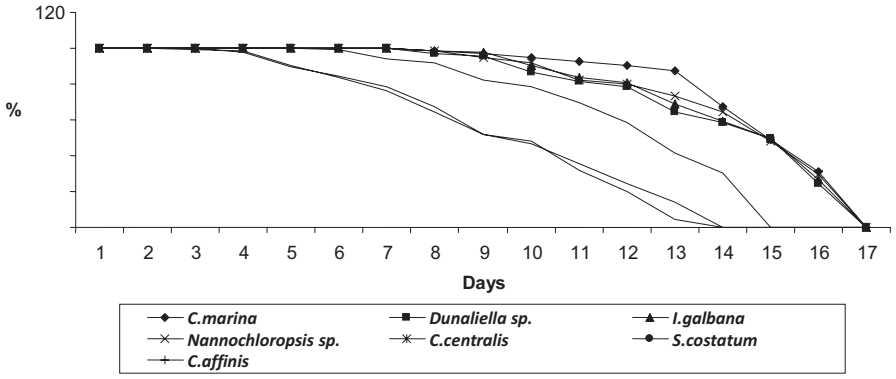

Fig. 1 Survival of *N. minor* fed with low food concentrations and different food types

observed on the 15th day and total mortality occurred only from the 17th day onwards. However, *S. costatum* has poorer survival than the rest of the feed types used, where 100% survival was observed only up to the 2nd day. After that, survival declined to 48% on the 10th day and complete mortality was noted from the 14th day onwards (Fig. 1).

Survival Rate of N. minor *in High Food Concentrations*

In high food concentrations, 100% survival was noticed for about 3–9 days. The maximum days of survival was reported in *C. marina*, where 100% survival occurred up to the 9th day, 48.6% survival was observed on the 17th day and total mortality from the 19th day onwards. However, *S. costatum* showed poorer survival than the rest of the feed types used in this experiment, where 100% survival was observed up to the 3rd day only. After that, survival declined to 48.5% on the 11th day and complete mortality from the 15th day onwards (Fig. 2).

Growth of N. minor

The effects of microalgal diets on the growth of copepod *N. minor* are shown in Tables 1, 2, 3, 4, 5, 6 and 7. The growth experiment of *N. minor* showed that those fed *S. costatum* recorded the least growth, where the length of nauplii I was 0.094 mm and the length of nauplii VI was 0.188 mm. The body length of copepodite I and adult females and males were 0.39, 1.906 and 1.743 mm, respectively. The growth was comparatively higher in *C. marina* feed, where nauplii I hatched after 24 h with a length of 0.107 mm and the body length of nauplii VI was 0.275 mm. The length of CI and CV were 0.427, 1.563 (females) and 1.266 mm (males),

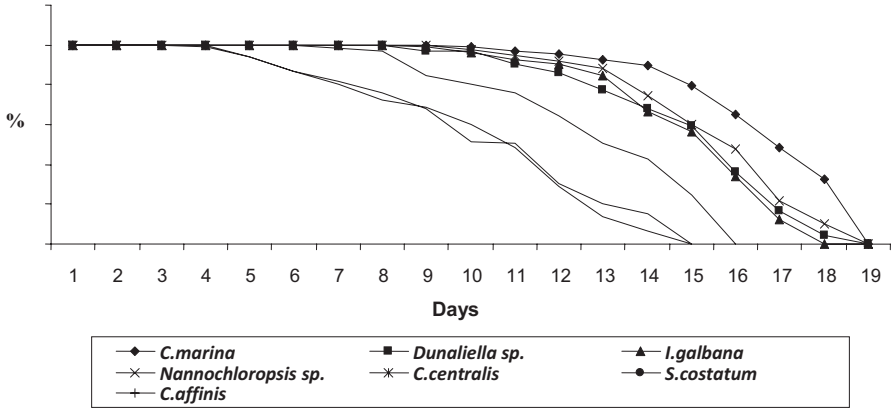

Fig. 2 Survival of *N. minor* fed with high food concentrations and different food types

Table 1 Daily mean growth of *C. marina* fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.107 ± 0.002	–
0.4	0.127 ± 0.001	–
0.42	0.136 ± 0.002	–
0.34	0.155 ± 0.004	–
0.39	0.179 ± 0.005	–
25	0.275 ± 0.021	–
24.67	–	0.427 ± 0.009
30.19	–	0.55 ± 0.026
36.34	–	0.789 ± 0.005
38.46	–	0.944 ± 0.004 (female)
	–	0.931 ± 0.006 (male)
60	–	1.563 ± 0.020 (female)
	–	1.266 ± 0.075 (male)
60	–	1.963 ± 0.037 (female)
	–	1.756 ± 0.030 (male)

respectively. The lengths of adult female and male copepods were 1.963 and 1.756 mm, respectively. The growth of *Dunaliella* sp. fed copepods showed a length of 0.096 mm (nauplii I) and the nauplii VI body length was 0.208 mm. The body length of CI and CV were 0.394, 1.42 (females) and 1.046 mm (males), respectively, and adult females and males were 1.936 and 1.74 mm, respectively. The length of *I. galbana* fed nauplii I was 0.104 mm and that of nauplii VI was 0.201 mm. The body lengths of CI and CV were 0.414, 1.493 (females) and 1.15 mm (males), and the lengths of adult females and males were 1.933 and 1.75, respectively.

The growth of *Nannochloropsis* sp. fed copepods is shown in Table 4. Here, the length of nauplii I was 0.107 mm and that of nauplii VI was 0.24 mm. The body lengths of CI and CV were 0.456, 1.526 (females) and 1.176 mm (males), while the

Table 2 Daily mean growth of *Dunaliella* sp. fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.096 ± 0.005	–
0.4	0.107 ± 0.001	–
0.42	0.118 ± 0.003	–
0.34	0.139 ± 0.001	–
0.39	0.167 ± 0.003	–
25	0.208 ± 0.012	–
24.67	–	0.394 ± 0.003
30.19	–	0.445 ± 0.006
36.34	–	0.652 ± 0.016
38.46	–	0.894 ± 0.001 (female)
	–	0.820 ± 0.005 (male)
60	–	1.42 ± 0.045 (female)
	–	1.046 ± 0.041 (male)
60	–	1.936 ± 0.015 (female)
	–	1.74 ± 0.02 (male)

Table 3 Daily mean growth of *I. galbana* fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.104 ± 0.004	–
0.4	0.113 ± 0.003	–
0.42	0.125 ± 0.004	–
0.34	0.145 ± 0.003	–
0.39	0.168 ± 0.001	–
25	0.201 ± 0.013	–
24.67	–	0.414 ± 0.005
30.19	–	0.481 ± 0.025
36.34	–	0.743 ± 0.044
38.46	–	0.94 ± 0.03 (female)
	–	0.906 ± 0.005 (male)
60	–	1.493 ± 0.085 (female)
	–	1.15 ± 0.026 (male)
60	–	1.933 ± 0.005 (female)
	–	1.75 ± 0.01 (male)

lengths of adult females and males were 1.946 and 1.873 mm, respectively. The lengths of *N. minor* nauplii I and nauplii VI which were fed on *C. centralis* were 0.094 and 0.188 mm, respectively. The body lengths of CI and CV were 0.384, 1.366 (females) and 0.988 mm (males), and adult females and males were recorded as 1.963 and 1.743 mm, respectively. The growths of *C. affinis* fed *N. minor* nauplii I and nauplii II were 0.096 and 0.191 mm, respectively. The body lengths of CI and CV were 0.383, 1.4 (females) and 0.987 (males), and adult females and males were 1.953 and 1.74 mm, respectively.

Table 4 Daily mean growth of *Nannochloropsis* sp. fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.107 ± 0.001	–
0.4	0.118 ± 0.001	–
0.42	0.127 ± 0.003	–
0.34	0.155 ± 0.004	–
0.39	0.181 ± 0.007	–
25	0.24 ± 0.036	–
24.67	–	0.456 ± 0.041
30.19	–	0.527 ± 0.010
36.34	–	0.768 ± 0.018
38.46	–	0.946 ± 0.020 (female)
	–	0.921 ± 0.006 (male)
60	–	1.526 ± 0.035 (female)
	–	1.176 ± 0.025 (male)
60	–	1.946 ± 0.055 (female)
	–	1.873 ± 0.100 (male)

Table 5 Daily mean growth of *C. centralis* fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.094 ± 0.003	–
0.4	0.106 ± 0.002	–
0.42	0.115 ± 0.004	–
0.34	0.129 ± 0.004	–
0.39	0.149 ± 0.001	–
25	0.188 ± 0.001	–
24.67	–	0.384 ± 0.013
30.19	–	0.449 ± 0.013
36.34	–	0.671 ± 0.016
38.46	–	0.916 ± 0.025 (female)
	–	0.813 ± 0.005 (male)
60	–	1.366 ± 0.050 (female)
	–	0.988 ± 0.002 (male)
60	–	1.936 ± 0.035 (female)
	–	1.743 ± 0.040 (male)

Effect of Temperature on Egg Production and Hatching Succession

Egg Production of *N. minor*

The minimum mean egg production of 5.66 ± 0.57 eggs/female/day was observed at 18 ± 1 °C, whereas the maximum mean egg production of 25.5 ± 0.57 eggs/female/day was recorded at 25 ± 3 °C. At 30 ± 1 °C, the mean egg production was slightly decreased (16 ± 1 eggs/female/day) compared to that at 25 ± 3 °C (Table 8).

Table 6 Daily mean growth of *S. costatum* fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.094 ± 0.002	–
0.4	0.108 ± 0.013	–
0.42	0.115 ± 0.003	–
0.34	0.124 ± 0.004	–
0.39	0.148 ± 0.003	–
25	0.188 ± 0.005	–
24.67	–	0.39 ± 0.003
30.19	–	0.440 ± 0.015
36.34	–	0.66 ± 0.015
38.46	–	0.888 ± 0.002 (female)
	–	0.833 ± 0.020 (male)
60	–	1.28 ± 0.01 (female)
	–	0.983 ± 0.003 (male)
60	–	1.906 ± 0.015 (female)
	–	1.743 ± 0.04 (male)

Table 7 Daily mean growth of *C. affinis* fed *N. minor*

Rearing period (h)	Nauplii length (mm)	Copepodite length (mm)
24	0.096 ± 0.002	–
0.4	0.106 ± 0.002	–
0.42	0.116 ± 0.003	–
0.34	0.126 ± 0.003	–
0.39	0.155 ± 0.005	–
25	0.191 ± 0.004	–
24.67	–	0.383 ± 0.012
30.19	–	0.472 ± 0.032
36.34	–	0.668 ± 0.017
38.46	–	0.906 ± 0.012 (female)
	–	0.847 ± 0.034 (male)
60	–	1.4 ± 0.07 (female)
	–	0.987 ± 0.003 (male)
60	–	1.953 ± 0.020 (female)
	–	1.74 ± 0.036 (male)

Hatching Succession of *N. minor*

The lowest percentage of 41.16% hatching was observed at 18 ± 1 °C, whereas a high hatching percentage (96.07%) was obtained at 25 ± 3 °C (Table 8). The hatching of *N. minor* at 30 ± 1 °C was reported to be lower (72.87%) than that at 25 ± 3 °C (Table 8).

Table 8 Mean egg production and hatching rate of *N. minor* at different temperatures

Temperature (°C)	Egg production (eggs/female/day)	Hatching rate	Hatching (%)
18 ± 1 °C	5.66 ± 0.57	2.33 ± 0.57	41.16
20 ± 3 °C	11.66 ± 1.52	9 ± 1	77.18
25 ± 3 °C	25.5 ± 0.57	24.5 ± 0.57	96.07
30 ± 1 °C	16 ± 1	11.66 ± 0.57	72.87

Effect of Algal Concentration on Egg Production and Hatching Rate

Egg Production of *N. minor*

The egg production rate was found to be increased with increasing food concentration (Table 9). The highest mean egg production (32 ± 1.52 eggs/female/day) was achieved with the highest algal food concentration of 20,000 cells/mL, while the lowest egg production (3 ± 1 eggs/female/day) was obtained at the lowest food concentration of 1000 cells/mL (Table 9).

Hatching Succession of *N. minor*

During low algal food concentration (1000 cells/mL), the hatching percentage was 44.33%, whereas at high algal cell concentration (20,000 cells/mL), it was reported to be 93.75% (Table 9). The correlation between hatching rate and food concentration was highly significant (*P*) at an 'r' value of 0.98776 (Table 10).

Discussion

The survival rate of *N. minor* was found to increase with increased food concentration and decrease with decreased food concentration. In low food concentrations, the survival was low due to the lack of food. As could be easily understood, because of the insufficient food supply, the copepods cannot show further metabolism and survival, so the species becomes stressed, followed by mortality. However, in high algal food concentrations, the survival was high owing to the presence of the required amount of food, as agreed earlier by Davis (1984) in the calanoid copepod *P. parvus*. The copepod survival normally relying on a combination of microalgae species fed to copepods might be a reason for the high survival observed in this study, which could be due to the smaller cell size and high concentration, as opined by Stottrup (2003) and Luis et al. (2010).

The variations observed in the survival of *N. minor* with different algal feeds could be related to the morphology of algae used in this investigation. In the present experiment, high survival was observed in *C. marina*, which might be due to its

Table 9 Mean egg production and hatching rate of *N. minor* in different algal concentrations

Algal concentration (cells/mL)	Egg production (eggs/female/day)	Hatching rate	Hatching (%)
1000	3 ± 1	1.33 ± 0.57	44.33
5000	14 ± 1.41	8 ± 1.41	57.14
10,000	22 ± 1.41	19 ± 0	86.36
20,000	32 ± 1.52	30 ± 0.577	93.75

Table 10 Correlation coefficient (r) values between temperature, food concentration and reproductive performance of *N. minor*

S. no.	Parameters	Egg production	Hatching rate
1	Temperature	0.63093	0.53455
2	Food concentration	0.97217*	0.98776**

*$P < 0.01$; **$P < 0.001$

smallest size compared to the other algal feeds used here. The present findings also supported observations by previous workers (Shrivastava et al. 1999; Perumal et al. 2000; Rauquirio and Fernande 2002; Cook et al. 2007; Luis et al. 2010). Similarly, the assimilation efficiency of *N. minor* was found to be higher in high food concentrations and, also, *C. marina* feed because of its efficiency to feed on smaller prey, agreeing with the findings of Lalli and Parsons (1997). However, *N. minor* showed a lower survival in *S. costatum*, owing to the lower preying capabilities of copepods on chain-forming diatoms and, also, the mouthparts of copepods do not facilitate the capture of larger food organisms and, therefore, in this experiment, low survival was noticed in *S. costatum*, as supported earlier by Ganf and Russell (1985) and Perumal et al. (2000). The increase in copepod survival at a water temperature of 25 ± 3 °C reflects its preference for an optimum temperature, which compares favourably with previous reports of Chinnery and Williams (2004) and Cook et al. (2007). It is also known that the variations in salinity also affect the growth and survival of copepods. The presently maintained salinity was found to be suitable for obtaining high survival for copepod *N. minor*. This is also supported by Chinnery and Williams (2004), who reported the maximum growth and survival in copepods such as *Acartia bifilosa*, *A. clausi*, *A. discaudata* and *A. tonsa* when the salinity was maintained above 33‰. Similar results were also reported by Cervetto et al. (2006), Oliveira (2006) and Danilo et al. (2008). The present observation on the survival of copepods provides realistic information for culturing *N. minor* in hatchery conditions. The experiment also indicated that the level of food concentration and suitable algal food are very much necessary for copepods at different trophic levels.

In the present study, the growth of copepods achieved depended mainly on algal feeds. The maximum growth in *N. minor* was achieved in those which were fed *C. marina*, while the lowest growth was obtained for *S. costatum* fed copepods. It is clear that the food size limitation might effectively act as a filter for small copepods (Ulrika et al. 2009). The size and structure of algal food might influence the slow growth of *S. costatum*. The chain-forming nature and larger size of *S. costatum* may

not be a suitable for *N. minor* to feed on them. Due to the unfavourable size of *S. costatum*, it has resulted in the low growth in copepods, agreeing with the observations of Hirst and Bunker (2003). Furthermore, the temperature and salinity may also have direct influences on the growth of copepods. The temperature and salinity maintained in this study resulted in good growth in copepod *N. minor*. The present findings also coincided with the earlier reports of McKinnon et al. (2003) and Milione and Zeng (2008).

The effects of various dietary microalgae on growth parameters have been evaluated with many calanoid copepods, including *Pseudodiaptomus pelagicus* (Ohs et al. 2010), *Pseudodiaptomus euryhalinus* (Puello-Cruz et al. 2009), *Gladioferens imparipes* (Payne and Rippingale 2000), *Acartia sinjiensis* (Knuckey et al. 2005), *Acartia tonsa* (Stottrup and Jensen 1990; Stottrup et al. 1999; Kleppel and Burkart 1995), *Acartia omorii* (Shin et al. 2003) and *Temora stylifera* (Buttino et al. 2009). The importance of food quality for copepod production is a subject that has attracted researchers' attention in recent years (Kleppel et al. 1998; Koski et al. 1998; Laabir et al. 1999; Jones et al. 2002; Koski and Klein Breteler 2003; Irigoien et al. 2005). Different algal diets are known to affect the development of both juveniles and adults (Knuckey et al. 2005).

External factors such as food quantity and quality have also been shown to strongly affect the growth and reproduction of these small pelagic crustaceans (Nejstgaard et al. 2001; Rey et al. 2001; Hassett 2004). Experiments conducted in the laboratory indicated that a diet based on specific diatoms negatively impacts egg hatching success, which might be due to the larger size (Ianora and Poulet 1993). The effects of various micro algal diets and combinations on *Bestiolina similis* daily egg production, egg hatching success and population growth have been studied (Camus et al. 2009). Food quality has been shown to strongly affect the copepod egg production, embryonic and post-embryonic development, and hatching success of *Pseudocyclops xiphophorus*, which has been demonstrated with some diets that are poorer than others in inducing maximum fecundity (Brugnano et al. 2008).

Food condition is also an important factor affecting copepod growth and reproduction. The clutch size and hatching success in copepod *Pseudocalanus newmani* were not related to the biomass of any food items in the field and the body size in wild females was within the range of well-fed females reared in the laboratory (Ban et al. 2000). The effects of temperature and salinity on the productivity of calanoid copepods have also shown their clear effects on egg production, egg hatching rates and survival (Hall and Burns 2002; Peck and Holste 2006; Holste and Peck 2006; Milione and Zeng 2008).

Temperature has a major influence on the physiology and life-cycle process of copepods. Studies on the effects of different temperatures on various aspects of the life cycle and physiology of the copepod development times, egg production and hatching were determined for organisms reared individually in the laboratory under saturated food conditions (Bonnet et al. 2008; Brugnano et al. 2008). Numerous studies have shown that the nutritional condition of zooplankton changes with diet quantity and quality, which affects their secondary productivity

in terms of growth rates and egg production (Checkley 1980; Durbin et al. 1983; Kiorboe et al. 1985; Verity and Smayda 1989; Kleppel and Burkart 1995; Hazzard and Kleppel 2003).

Temperature is a key abiotic factor regulating the growth and reproductive potential of copepods and a variable in the development of production regimes (Santos et al. 1999; Peterson 2001; Isla and Perissinotto 2004; Holste and Peck 2006; Milione and Zeng 2008; Sun et al. 2008; Sullivan et al. 2007). Up to now, many experimental studies have shown that water temperature and food conditions are the most important factors affecting the life history parameters of copepods (Ban 1994). The effects of six different temperatures (24, 26, 28, 30, 32 and 34 °C) on survival, development time, reproductive output and population growth were investigated by Rhyne et al. (2009) in order to define the optimal temperature for culture. Hassett and Crockett (2009) investigated the growth response of one copepod, *Eurytemora affinis*, to the dietary cholesterol level at two temperatures.

The present investigation clearly indicated that the egg production of *N. minor* was affected by temperature, which is supported by the ANOVA value (F = 2.959719) (Table 11). Presently, the maximum numbers of eggs were noticed when the temperature was maintained at 25 ± 3 °C, while low numbers of eggs were obtained at low temperature (18 ± 1 °C). In contrast, the egg production decreased at an unstable temperature of 30 ± 1 °C, as supported by Hopcroft and Roff (1998). In this way, the availability of food (Ban 1994) and temperature (White and Roman 1992) are considered the main factors in determining egg production, which are also governed by the quality and quantity of food, as agreed by Kleppel (1992). The egg production of the copepod *N. minor* studied here was found to be relatively higher in comparison with that for *A. tonsa* and *A. clausi* (Cervetto et al. 1993; Rodriguez et al. 1995). Lee et al. (2003) stated that the copepod *Pseudocalanus newmani* did not spawn at 20 °C. Such an inhibitory effect of many species explains this well. They concluded that the temperature-dependent growth might influence the egg production in copepods.

Koski and Kuosa (1999) described that the increase in temperature above 13 ± 1 °C did not significantly increase the egg production in *A. bifilosa*. In contrast, the increased egg production due to temperature is relatively low. In the present experiment, a temperature increase up to 25 ± 3 °C resulted in high egg production, indicating its adaptation to optimum temperatures. The observation of Koski and

Table 11 One-way analysis of variance (ANOVA) between the temperature, algal concentration and egg production of *N. minor*

Parameters	Source of variation Group	Df	SS	MS	F
Temperature and egg production	Between	1	146.0341	146.0341	2.959719*
	Within	6	296.043	49.34052	
Food concentration and egg production	Between	1	8.61125	8.61125	0.056318
	Within	6	917.4175	152.9029	

*$P < 0.025$

Kuosa (1999) varied from the present study and another study on different species (Durbin et al. 1992). Hirche and Halsband (2001) noticed the peak egg production in *Centropages typicus* and *A. clausi* from the North Sea of Germany during the summer months. Kiorboe and Nielsen (1994) reported on the low egg production in *Temora* sp. and *Pseudocalanus* sp. during the winter season in Kattegat. Similar results were also described by several workers, including Rob and Elaine (2009) in *Tigriopus brevicornis*, Milione and Zeng (2008) in *A. sinjensis*, Devreker et al. (2009) in *Eurytemora affinis* and Linda et al. (2009) in *Temora longicornis.*

In the present observation, egg production was found to be positively correlated with algal concentration (r = 0.97217) (Table 11). The maximum mean egg production was observed with the maximum cell concentration. These results strongly support the hypothesis that the rates of egg production of *N. minor* are limited immediately by the availability of phytoplankton (Hirche 1990). Hirche and Kwasniewski (1997) described that the feeding had a strong influence on the egg production rate, which was much higher in females of *C. finmarchicus* exposed to different feeding conditions. Runge (1984) and Kimoto et al. (1986) noticed significantly larger clutches when the species were reared at high algal food concentrations of *C. finmarchicus* and *Sinocalanus tenellus*, respectively. The rate of egg production was significantly better related to feeding on greater food availability (Checkley 1980). From the present study, it could be inferred that the egg production increased with increasing food concentration, while increasing temperature did not significantly increase the egg production. However, *N. minor* seemed to give good results in egg production with high food concentration. Irigoien et al. (2005) described that the daily egg production and hatching success rates of the calanoid copepods *Calanoides carinatus* and *Rhincalanus nasutus* can increase depending on the algal food concentration and, also, composition. A similar observation was also made by Koski and Kuosa (1999) in the calanoid copepod *A. bifilosa*, Ceballos and Álvarez-Marqués (2006) in *Calanus helgolandicus* and *Calanoides carinatus*, Ianora et al. (2007) in *Centropages typicus* and Murray and Marcus (2002) in *Centropages hamatus*. Nival et al. (1990) found no egg production in calanoid copepod *Centropages typicus* when reared at a food concentration of 500 cells/mL. It is easily understood that all of the available energy is being used for survival. However, a food concentration of 5000 cells/mL was enough for metabolic and egg production, which is reported in *Centropages* sp. In our species, the egg laying started at 1000 cells/mL, but in the case of *Centropages* sp., the egg laying started at 5000 cells/mL. From these results, it could be inferred that *N. minor* are the most suitable for culturing and other physiological monitoring with minimum food availability.

Apart from food concentration, the food quality is also considered to be important for the reproductive success of copepods, as reported by Runge and Plourde (1996). Factors such as particle size and species composition of food also influence egg production (Koski and Kuosa 1999). Runge and Plourde (1996) and Arnaud et al. (2001) suggested that the nutritive value of food also controlled the egg production of copepods. Whitehouse and Lewis (1973) noticed that the number of eggs increased in the freshwater cyclopoid copepod *Cyclops abyssorum* when the food

was given in unlimited amounts. There are many observations published from investigations conducted in natural water and laboratory conditions which described that food limited the copepod egg production (Bautista et al. 1994; Ianora et al. 2007).

The reproductive success of copepods is not only dependent on the egg production rate, but also on the egg hatching rate, which may not be affected by food quality in the same manner as egg production (Miralto et al. 1998). It is widely reported that the hatching rate also increased with increased temperature up to a certain limit and increased food concentration. In our experiments, a low hatching rate was observed at low temperatures and low food concentrations, whereas a high hatching rate was observed at optimum temperature and high food concentration, respectively. An insufficient food supply and inadequate temperature might the reasons for a low hatching rate, as agreed by Burkhart and Kleppel (1998), Koski et al. (1999) and Belmontle and Pati (2007). Some workers also stated that low egg viability may also be dependent on the biochemical composition (polyunsaturated fatty acids) of the algal food used (Jonasdottir and Kiorboe 1996).

Inference

The effects of temperature and food concentration on the survival, egg production and population density of calanoid copepod *N. minor* were studied in this chapter. Four different temperatures (18 ± 1, 20 ± 3, 25 ± 3 and 30 ± 1 °C) and seven different algal diets (*Chlorella marina*, *Dunaliella* sp., *Isochrysis galbana*, *Nannochloropsis* sp., *Coscinodiscus centralis*, *Chaetoceros affinis* and *Skeletonema costatum*) were analysed in this study. The food was given at concentrations of 1000, 5000, 10,000 and 20,000 cells/mL. The survival rate of *N. minor* at low food concentrations of *C. marina* was 100% up to the 7th day and 49% survival was observed on the 15th day. At high food concentration of *C. marina*, the maximum days of survival of *N. minor* was reported, where 100% survival occurred up to the 9th day and 48.6% survival was observed on the 17th day. The growth was higher in *C. marina* feed compared to the other feeds, where the nauplii I (NI) hatched after 24 h with length 0.107 mm and the body length of NVI was 0.275 mm. The lengths of CI and CV were 0.427, 1.563 (females) and 1.266 mm (males). In this study, the maximum growth in *N. minor* was achieved in copepods fed *C. marina*, while the lowest growth obtained was among those fed *S. costatum*.

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A Method of Estimation of Enzymatic Activity of Copepods

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Introduction

Nutrition is extremely important to any sector of commercial animal production, especially fish and crustacean larviculture. Different types of diets are available, and live feed plays a key role in the growth of many aquatic animals. The main problem in most marine fish farming is the high mortality rate associated with the initial feeding of larvae (Garcia-Ortega et al. 2000). For many fish species, live feed yields better results in terms of growth and survival when compared to fish fed with artificial diets (Dabrowski 1984). In fact, the commercial production of fish and crustacean larvae, postlarvae, and juveniles depends on the supply of live prey, mainly the rotifers *Brachionus* sp. and nauplii of *Artemia* (Kolkoviski 2001; Koven et al. 2001; Sorgeloos et al. 2001). Although these animals offer low nutritional value, their production is relatively easy, and this ensures their predominance as live prey (Støttrup 2000). Copepods could be an alternative to this problem. They are recognized as an important source of nutrients, and they are underused in the aquaculture because brine shrimp offers the advantage of storage in their cysts of resistance (Drillet et al. 2006). Copepods are promising candidates for large-scale aquaculture as live feed for fish and crustacean larvae (Lima and Souza-Santos 2007; Olivotto et al. 2008a, b) because the digestion of food in the early stages of fish and shrimp growth can be improved by a set of enzymes that are synthesized in the midgut region of copepods (Brunet et al.1994).

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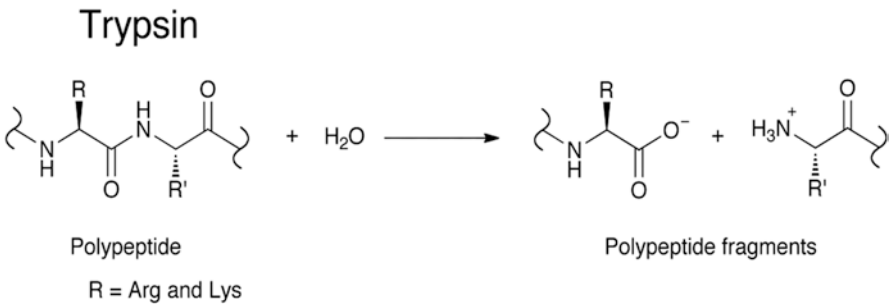

Fig. 1 Trypsin enzymatic reaction

Digestive enzymes have been extensively studied in copepods mainly to investigate how enzyme levels reflect their feeding activities (Hirche 1981; Mayzaud and Mayzaud 1981; Baars and Oosterhuis 1984; Head et al. 1984; Mayzaud et al. 1984). For some enzymes, correlations of their activity with the concentration and quality of food were found in calanoid copepods. Amylase activity in copepodite stage V of *Calanus finmarchicus* (Tande and Slagstad 1982) and carbohydrase activities in total zooplankton population (Mayzaud et al. 1984) showed a die1 cycle, whereas it took several days to induce laminarinase in *Euphausia pacifica* and *Calanus pacificus*, when the proper substrate was available (Cox 1981). Hassett and Landry (1988) found no short-term changes in digestive enzymes, assuming a long-term acclimation of the enzyme level. The use of the highly sensitive fluorescence technique developed by Ueberschär (1988) allowed us to measure enzymatic activities in individual specimens of small copepod species.

Enzymatic Reaction

Trypsin is a pancreatic serine protease with substrate specificity based upon positively charged lysine and arginine side chains (Fig. 1). The enzyme is excreted by the pancreas and takes part in the digestion of food proteins and other biological processes. Trypsin is a medium-sized globular protein and is produced as an inactive proenzyme, trypsinogen (Chen et al. 2009).

Materials and Methods

Sample Preparation

Usually enzyme samples are prepared either as wet homogenates by grinding in buffer or distilled water, by sonic or thermal homogenization, or as dry homogenates after acetone dehydration or freeze-drying. Whenever the enzyme analysis

cannot be run immediately, deep freezing (−60 °C) the tissue samples or homogenates and storing at the same temperature is usually a method of choice for both phytoplankton and zooplankton. However, when several enzymatic activities must be determined on the same sample, dry homogenates provide a safer storage of the material between assays than the freezing procedure. Before adopting such procedures, thorough examination of their applicability to the zooplankton and their sources of variability is necessary. One of the best advantages of live feed is the presence of a high content of digestive enzymes (Kuzmina and Golovanova 2004). Thus, studies on the characterization of digestive enzymes from these organisms are essential to evaluate the similarity between the properties of enzymes from live feed organisms.

Homogenate Preparation

In order to estimate enzyme activities in copepods, 100 freshly caught specimens are collected with fine forceps and put in to the 0.89% of sodium chloride solution. Grind in a potter blender (homogenization). After homogenization the sample is sonified for 30 s. And the sample is centrifuged at 12,000 g for 10 min. to remove cell debris and nuclei.

Substrate Stock Solution 10^{-3}

43.5 mg DL-BAPA is dissolved in 1 ml of dimethyl sulfoxide (DMSO). This solution make up to 100 ml with 0.05 M tris buffer pH −8.2 containing 0.02 M $CaCl_2$. Care must be taken to dissolve all of the DL-BAPA in the dimethyl sulfoxide. Presence of any crystals causes precipitation to occur on standing. The temperature of the substrate stock solution is never allowed to fall below 25 °C.

Enzyme Stock

Dissolve concentration of 90–450 μg trypsin/ml of 0.001 M HCl.

Measurement of Enzyme Activity in Copepod (Uberschar 1985)

For all enzyme activity measurements, proper blanks and controls are run simultaneously, including a mixture of substrate and inactivated enzyme (boiled 10 min). 0.9 ml of water is added to 5 ml of substrate stock solution. The substrate and

enzyme mixture is allowed to equilibrate in a thermostatically controlled both at 25 °C for 5 min. At zero time, 0.1 ml of enzyme stock solution (9–45 μg trypsin) is added. And the reaction is allowed to run for 600 s. Addition of 1.0 ml of 30% acetic acid is needed to terminate the reaction. The quantity of p-nitroaniline is estimated spectrophotometrically at 410 nm. The control tube shows no self hydrolysis.

The relationship between in situ feeding activity and digestive enzyme activity is confounded by the interactions of past feeding history, present food conditions, and the metabolic needs of the copepods (Samain et al. 1985; Roche Mayzaud et al. 1991). In order to interpret digestive enzyme activities of field populations, it is necessary to understand the factors that can lead to different enzyme distributions. Laminarin-like compounds are the primary storage carbohydrates of diatoms; hence, activity levels of laminarinase are indicative of the copepod's ability to utilize (digest and assimilate) diatoms as a food source. β-Glucosidase is involved in the secondary digestion of laminarin, while a-glucosidase fulfills a similar role for the substrate amylose.

Marine fish larvae feed primarily on copepods of which calanoids are the dominant food source in open marine waters (Turner 2004), whereas harpacticoids are an important link between primary producers and fish larvae in coastal areas. Feed will have a significant role in the larval growth process if the feed nutrients can be absorbed by the larval body cells. Digestive enzyme activity is a biological indicator to determine the ability of larvae to digest the feed (Gawlicka et al. 2000). Therefore, protease, amylase, trypsin, and lipase are the biological indicators of larval ability to digest protein, carbohydrate, and fat contained in the feed. Digestive enzyme activity is influenced by several factors, such as age of larvae (Wang et al. 2006), substrate (Cara et al. 2003), and kind of feeds and feed composition (Navarro-Guillén et al. 2015).

In general copepods have high protein content (44–52%) and a good amino acid profile, with the exception of methionine and histidine. They (copepodites and adults) are believed to contain higher levels of digestive enzymes which may play an important role during larval nutrition. The early stages of many marine fish larvae do not have a well-developed digestive system and may benefit from the exogenous supply of enzymes from live food organisms. They passed more quickly through the gut and were better digested than other live feeds.

Trypsin-like enzymes are widely occurring proteolytic enzymes in vertebrates and invertebrates such as copepods. Copepods maximize protein ingestion through selective feeding. Hence, trypsin activity can serve as a sensitive index of digestive activity and may elucidate life-cycle strategies. Trypsin was chosen since it is the major pancreatic enzyme to degrade protein in the early stages of fish larvae and plays a key role in activating other proteolytic enzymes (Rønnestad et al. 2013). This enzyme is a member of a large family of serine proteases that cleaves proteins and peptides on the carboxyl side of arginine and lysine. It is the most important digestive enzyme in animals and humans due to its role in activating other enzymes (Klomklao et al. 2007; Kishimura et al. 2007).

Tryptic enzyme activity has been suggested as an appropriate indicator of the fitness and survival potential of organism. Tryptic-like enzymes are universally

abundant among marine organism and are present in a measurable amount even in the youngest stages of fish larvae and in their potential food organisms. Hallberg and Hirche (1980) showed that the developmental stage, season, feeding condition, sex, and perhaps reproductive state affected enzyme levels in calanoid copepods (*Calanus finmarchicus*, *C. helgolandicus*) from the Gullmar fjord (West Sweden). Higher trypsin activities were generally found in shallow than deep waters in summer and autumn. These depth-related patterns are consistent with enzyme activities (trypsin, amylase) and distribution patterns of *Calanus finmarchicus* and *C. hyperboreus* in Fram Strait/Greenland Sea (Hirche 1989).

Hence, trypsin activity can serve as a sensitive index of digestive activity and may elucidate life-cycle strategies. Moreover, alterations in enzyme activity have also been observed in decapods as a response to ontogenetic changes, i.e., increased activities associated with higher energy turnover in later developmental stages or as a result of a change in the function and relative size of the digestive tract (Lovett and Felder 1990; Lemos et al. 2009). However, the digestive metabolism in general is complex, and the measurement of activities of a specific enzyme does not differentiate between its synthesis and secretion and an enzyme pool stored in cells. Moreover, it is presumed that only secretion reflects the instantaneous digestive activity (Mayzaud 1986). Consequently, only the "digestive potential" of a given enzyme during a particular time can be determined in homogenates of whole individuals (Head and Harris 1985).

Studies involving digestive enzymes have been carried out in recent decades in order to fill the gaps of knowledge concerning the physiology and biology of aquatic animals. Lauff (1983) described a specific trypsin activity of 9.3 U.mg21 of protein for *Artemia salina* (Linnaeus 1758), which is the main live feed used in aquaculture. *Daphnia carinata* King, 1853, have a trypsin activity of 0.21 6 0.02 U.mg21 of protein (Kumar et al. 2005). In *Monia* sp. (an important member of pond cladocerans), trypsin and chymotrypsin activity was 47.3 and 3.9 U.mg21 of protein, respectively (Lauff and Hofer 1984). A trypsin activity of 2.27 6 0.12 U.mg21 of protein is described for the crude extract of *T. biminiensis*. A range of values of trypsin activity have been observed in the literature from 0.21 to 47.3 U.mg21 of protein). In fact, in this case, it is very difficult to compare these values, mainly because there is no stand between the experimental conditions employed by the authors.

The presence of both proteinases and peptidases can be related to efficient digestion and incorporation of key nutrients (amino acids and peptides). Adequate nutrition is one of the principal factors influencing larval survival and depends on an effective ingestion, digestion, and assimilation of essential nutrients (Lazo et al. 2007). The knowledge of these physiological and metabolic peculiarities, as well as how they can be affected by diverse factors, provides a good tool with which to optimize fish and crustacean cultures (Furne et al. 2008). To elucidate variation in physiological processes under changing nutritional conditions, the determination of various types of digestive enzyme activities has proven successful in pelagic copepod organisms (Hassett and Landry 1990; Mayzaud et al. 1992; Ueberschär 1995; Meyer et al. 2002). The production of digestive enzymes is regulated by ingestion and by metabolic requirements of the consumer (Bamstedt et al. 2000). The former

is the theoretical basis for the use of enzyme activities as proxies of feeding activity levels. While changes in the food environment are usually poorly reflected in enzyme activity, extremely high or low feeding activities are clearly resolved (Bamstedt et al. 2000).

The intra- and interspecific differences in levels of trypsin activity study reflect different digestive activities in relation to the species life-cycle strategies rather than temperature effects related to water stratification. If enzyme activity is measured at optimum laboratory conditions, the data reflect the digestive potential of trypsin, irrespective of the influence of in situ water temperature in situ activity.

The higher level of trypsin activity of the smaller copepod *Oithona similis* is consistent with the principle of increasing specific metabolic rates with decreasing organism size, e.g., respiration rates of differently sized copepod species (Mayzaud et al. 2002a, b). However, recent findings indicate extremely low respiration rates in *O. similis* (Castellani et al. 2005). As Lampitt and Gamble (1982) suggested for *Oithona nana*, *O. similis* might save energy due to its usually motionless behavior (Drits 1984; Paffenhöfer 1993; Hwang and Turner 1995) resulting in low respiration rates despite relatively high trypsin activities and nutritional needs. Differences in trypsin activities in males confirmed previous assumptions that male *Paracalanus minutus* most likely do not feed (Norrbin 1994), while *Oithona similis* males may feed (Eaton 1971) and hence may have a longer life span after fertilization of the females.

Trypsin was retained in the intestine during two consecutive pulses of feeding and defecation of copepods. The content of trypsin is related to the digestional status of the animal. As has been previously shown (Pedersen et al. 1987), the content of intestinal trypsin was low in starving fish, and a significant rise in trypsin was observed following ingestion of a meal. This rise in trypsin level can only be attributed to secretion of proform from the pancreas into the intestine. The post-ingestion trypsin content was substantially higher than basal level even after the meal had been digested and defecated (starvation and secretory response tests; trypsin-turnover tests). The persistently high content of trypsin post-digestion most likely relates to retention of the enzyme, as known mechanical and chemical stimuli for pancreatic secretion are absent, and a secretion inhibitory stimulus (i.e., trypsin, Schneeman and Lyman 1975) is present.

Conclusion

Understanding enzymatic activity in aquatic animals is important for the choice of specific ingredients for artificial diets and can lead to a better food conversion ratio, as well as a reduction in the dependence on live feed during early life stages. Enzyme activity measurements sometimes used to ascertain the distribution and quantification of physiological activity of marine copepods are sensitive to a number of sources of variability of both methodological and environmental origins. The

relationship between the feeding rate of copepods and the concentration of food available is central to a general understanding of the marine food chain. The evidence supports the idea that feeding rate is proportional to concentration of food up to a certain level, above which it tends toward a constant or saturation level.

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A Study on Assessing the Feeding, Survival, Fecundity, and Postembryonic Development of Zooplankton *Nitocra affinis* (Copepoda: Harpacticoida)

R. Nandakumar and P. Santhanam

Introduction

Copepods are the most copious metazoan subclass on the planet and generally dominate the mesozooplankton, constituting more than 80% of its biomass (Verity and Smetacek 1996). Copepods play a primary role in the marine fish larval diets (Santhanam and Perumal 2012a, b), and on average copepods make up more than 50% of the fish larval gut contents (Stottrup 2000). By forming a vital link between primary and tertiary production, copepods play an important role in the transferring of nutrients and energy in marine ecosystem (Kiorboe 1997; Santhanam et al. 2013). It is well recognized that many marine fish larvae cannot thrive on the traditionally used live feeds like rotifers (*Brachionus* sp.) and *Artemia* sp., and this represents a major challenge to the aquaculture industry (Chesney 2007; McKinnon et al. 2003; O'Bryen and Lee 2005), as these species include several high-valued food fishes such as tropical snappers (Lutjanidae.) and groupers (Serranidae and Epinephelinae) and also several marine ornamental species, such as marine Angel fishes (Pomacanthidae) and the seahorse *Hippocampus subelongatus* (Payne and Rippingale 2001; Vander Lugt and Lenz 2008). On the other hand, copepods have been proven as ultimate food for many cultured marine larvae (Matias-Peralta et al. 2012; Hernandez Molejon and Alvarez-Lajonchere 2003), showing considerable advantages while comparing with rotifers and *Artemia* (Chen et al. 2006). The advantages of copepods over commonly used hatchery live feeds comprise of their many naupliar and copepodite stages that provide a wide variety of prey sizes for cultured larvae (Chen et al. 2006). Additionally, the nutritional profile of copepods generally matches the needs of marine fish larvae (Stottrup 2000; Evjemo et al. 2003; McKinnon et al. 2003). These advantages make copepods as the potential live feed for the successful larval culture of species that is difficult to be cultured using

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traditional live feeds (O'Bryen and Lee 2005). Moreover, addition of copepods in diets of previously cultured species could promote their survival, development, and pigmentation (Stottrup 2000; Knuckey et al. 2005; Vander Lugt and Lenz 2008). In spite of these promising features, the use of copepods in aquaculture remains inconsistent (Marcus et al. 2004; Camus and Zeng 2008; Santhanam and Perumal 2012a). This underutilization is mostly accredited to their relative low productivity in intensive culture (O'Bryen and Lee 2005), which could in turn be partly attributed to the lack of research on this field.

The quality and quantity of the feed are the most important factors regulating the production of copepod culture. As such, the effects of different microalgal diets on egg production (Kleppel et al. 1998; Koski and Kuosa 1999; Calbet and Alcaraz 1996; Turner et al. 2001; Santhanam and Perumal 2012b; Payne and Rippingale 2000), egg hatching success, mortality, and development (Leandro et al. 2006; Knuckey et al. 2005) have been reported for many calanoid species, such as *Acartia omorii* (Shin et al. 2003), *A. sinjiensis* (McKinnon et al. 2003; Knuckey et al. 2005), *A. tonsa* (Holste and Peck 2006), *Calanus helgolandicus* (Lacoste et al. 2001), and *Gladioferens imparipes* (Payne and Rippingale 2000, Santhanam and Perumal 2012a). Some studies have reported the response of paracalanid copepods to different food sources (McKinnon et al. 2003; Vander Lugt and Lenz 2008), and none appears to have considered the effects of various microalgal combinations. The productive capacity of any copepod species in intensive culture is largely related to female egg production as it is a direct indicator of population recruitment, as well as a measure to determine the total production rate of adult females (Shin et al. 2003). Though, subsequent egg hatching rate, nauplius and copepodite survival, and development rates, all compose the production rate of copepods (Santhanam et al. 2013; Milione and Zeng 2007). A broad study, including assessment of the population increase after a period of culture, will therefore provide a better understanding of the effects of algal diets on copepod culture productivity. *Nitocra affinis* is a tiny benthic copepod and is a much preferred food for several tropical fish larvae (Sampey et al. 2007). Harpacticoid copepods are broadly dispersed in most of the temperate and tropical waters and often dominate the copepod communities of surface waters.

Small size and natural abundance of *N. affinis* make it a preferred prey for an immense group of marine larvae which make them excellent candidates as live feed for fish larvae. McKinnon et al. (2003) suggested that *N. affinis* is among tropical copepods with high potential for aquaculture hatcheries and better for tropical larval fish culture. Therefore an adequate amount of research is needed to standardize the culture techniques to fully comprehend their potential. In the present study, a series of experiments were carried out to standardize suitable feed, salinity and temperature on survival, nauplius production, feeding rate, population growth, and development of *N. Affinis* for their cultivation.

Materials and Methods

Microalgae Culture

Marine microalgal strains were obtained from the Central Institute of Brackishwater Aquaculture, Chennai, Tamil Nadu, India. Microalgal cultures (*Chlorella marina* (CHL), *Isochrysis galbana* (ISO), *Dunaliella salina* (DUN), *Nannochloropsis oculata* (NAN), and *Tetraselmis suecica* (TET)) were cultured in 1 L container at temperature between 23 and 25 °C, 30 ppt salinity, and light intensity of 45–60 mmol photons/m^2/sec. using Walne medium (1974). The seawater was filtered using a filter bag (1 μm) and sterilized in autoclave. The vessels used for algal culture were completely sterilized and dried before use. Microalgae in exponential phase were harvested and fed to copepods.

Experiments

For survival experiment, ten adults of *N. Affinis* were placed into new 50 ml beakers filled with corresponding water salinity and algae, at 28–30 °C and on a 12L:12D light/dark cycle. The culture medium was changed once in every 2 days. The copepods were fed with algae at a rate of 25,000 cells ml-1. The dead organisms and fecal pellets were removed with a pipette. Survival of the animals was determined under a stereomicroscope and recorded from the start of the experiment. For fecundity experiment, a gravid female of *N. affinis* was placed in 20 ml vial filled with filtered seawater (Plate 4). The experimental vials were kept in dark throughout the study period but were exposed to light for 3–4 h every day during which period they were examined. The copepods were monitored daily to find out the time of the nauplius release, and the culture media were changed once in every 2 days. After releasing the nauplii, the copepod was transferred to a new 20 ml vial filled with a fresh culture medium. The newly hatched nauplii were fixed by adding a drop of 5% formalin and counted under a dissecting microscope. To estimate the population density of *N. affinis*, 15 adult copepods were stocked into each culture bottle (1 L). Adult copepods were separated from the stock culture by draining the culture water through a 158 μm sieve. The copepods were immediately placed in a petri dish with a small amount of seawater. Individuals were randomly captured using a fine-tipped pipette and transferred to the experimental bottles. During the experimental period (17 days), the copepods were fed with designated diets daily. After 17 days, all the contents from the triplicates were drained through a 58 μm sieve, and the total number of nauplii, copepodites, and adults of *N. affinis* retained was fixed in 5% formalin solution. Population counts were made using a Sedgewick Rafter counter under a high-powered microscope (Optika).

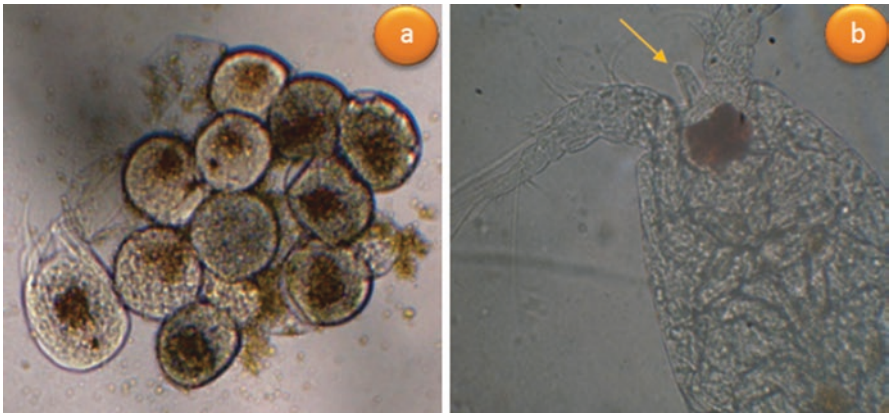

Plate 1 Egg sac (**a**) and prominent rostrum (**b**) of *N. affinis*

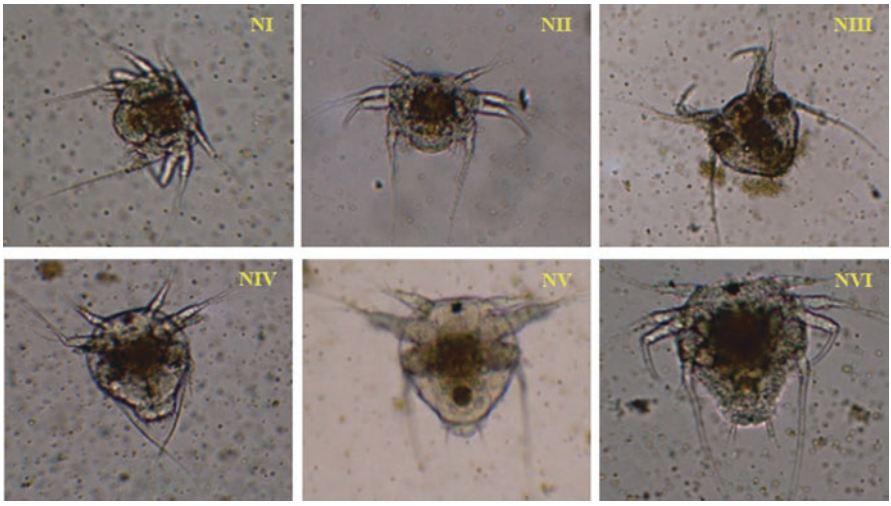

Plate 2 Naupliar stages of *N. affinis* (NI-NVI)

For development experiment, the containers were checked at regular intervals (four to eight times daily) to find out the time of nauplius release. Once the female completed the release of its nauplii, it was transferred to a new plate with fresh culture medium. The following parameters were measured and observed daily: (a) time between the appearance of the egg sac and its hatching (maturation time of egg sac), (b) time between hatching of the first egg sac and the appearance of the next (interval time between egg sacs), (c) development time from nauplii I (NI) to copepodid I (CI) and from CI to adult (including gravid females; Plates 1, 2, 3, and 4), (d) time interval between hatching of nauplii to the time this nauplii hatch its own eggs (generation time; hatch to hatch), and (e) the life span (longevity) of female *N. affinis*.

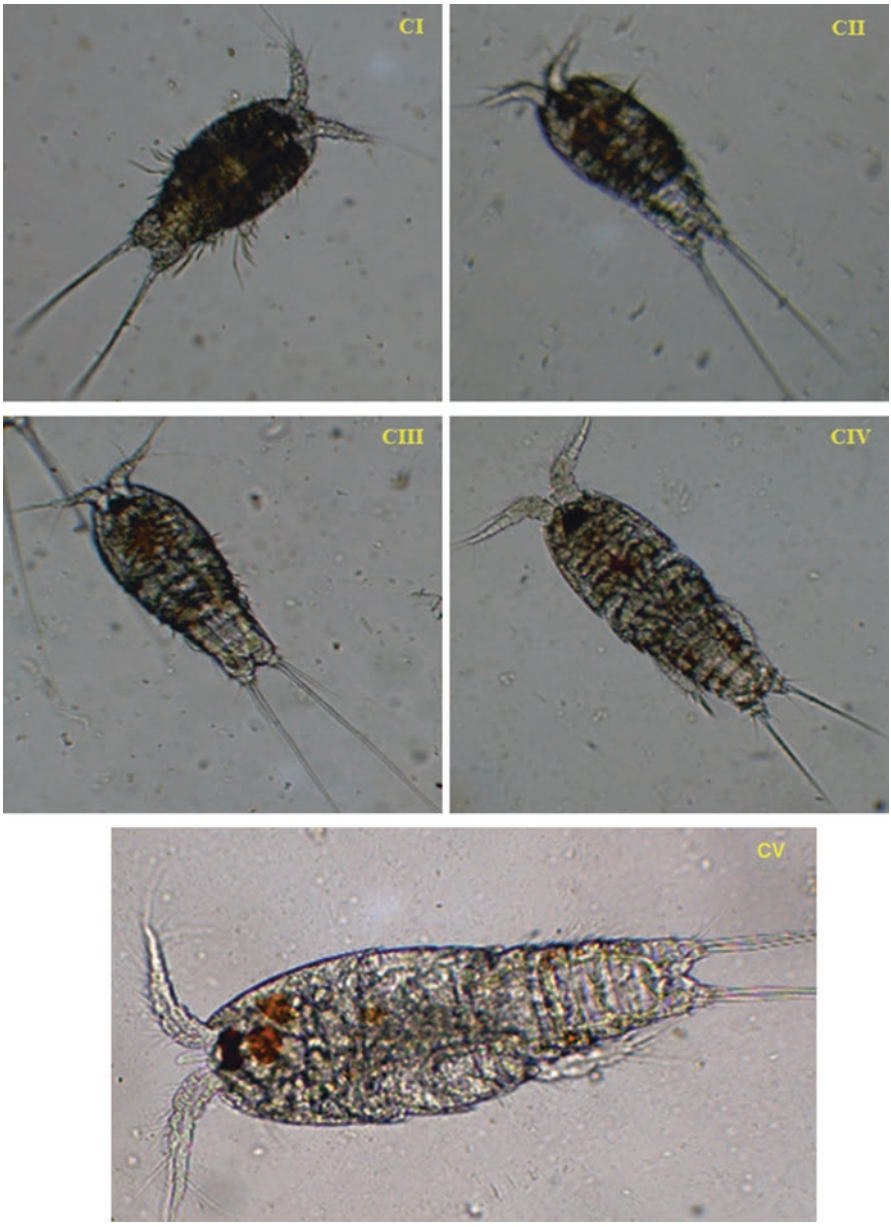

Plate 3 Copepodite stages of *N. affinis* (CI-CV)

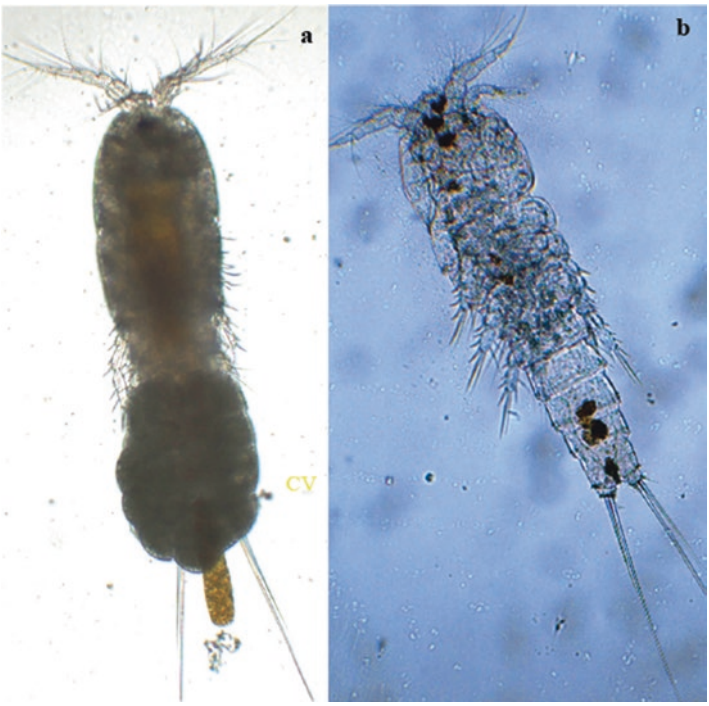

Plate 4 Adult stages of *N. affinis* (a) female and (b) male

The nauplii from the first three egg sacs (from each treatment) were reared in the culture media until the female hatched the first egg sac to determine the generation time, while the original females were maintained until they die to determine longevity.

Statistical Analysis

The data were analyzed using one-way ANOVA. Tukey's test was used to rank the groups if significant ($P < 0.05$) difference was found in the ANOVA test. Data are presented as mean ± S.E. All statistical analyses were performed using GraphPad Prism version 5.

Results

Diet Experiments

Survival

Mostly every tested feeds showed survival rate of more than 50%. The copepod survival was significantly low in NAN and DUN compared to other diets. Among the individual feeds tested, ISO induced best survival (89%) followed by CHL and TET. The copepod survival had a sharp decline from 80% on 3rd day to 40% on 6th day with NAN. NAN was the only treatment which resulted in total mortality by the end of 10th day (Fig. 1a).

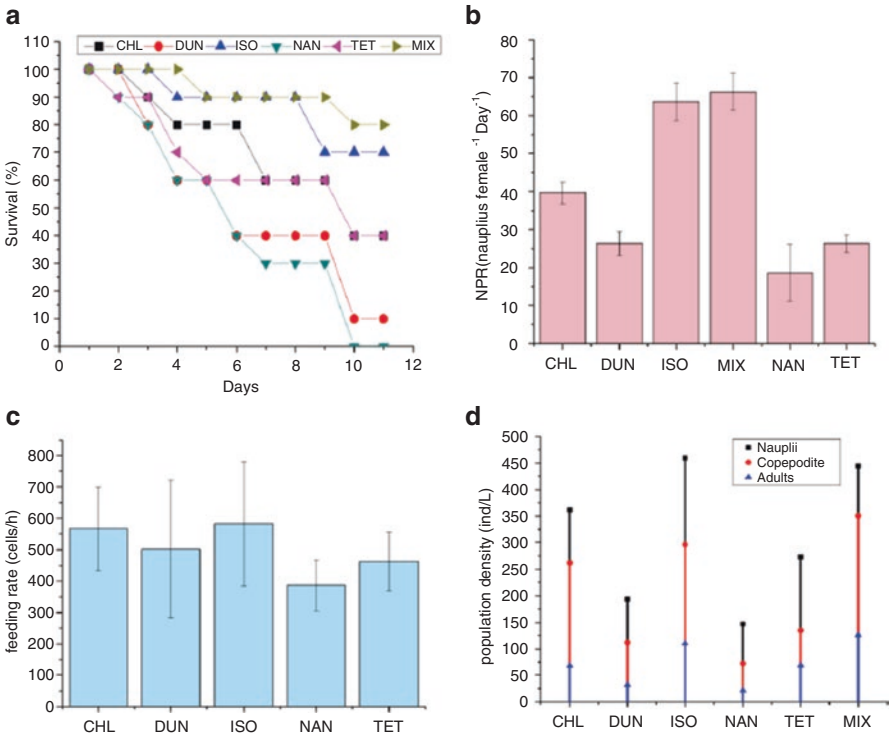

Fig. 1 (**a**) Survival; (**b**) nauplius production rate; (**c**) feeding rate; (**d**) population density of copepods fed on different diets. Copepods were cultured under identical condition of 28 ± 2 °C and 30 ± 2 ppt and values expressed as mean ± S.E

Nauplius Production Rate (NPR)

The influence of five different microalgal diets on NPR (nauplius female^{-1} day^{-1}) of *N. affinis* is shown in Fig. 1b. The results showed that maternal food significantly influenced *N. affinis* nauplius output ($p < 0.05$). The highest NPR (66.3 ± 4.9 nauplius female^{-1} day^{-1}) was produced by the mixed diet, which was over three times higher than that of the lowest NPR (18.6 ± 7.4 nauplius female^{-1} day^{-1}), recorded for NAN. Among the individual feeds tested, ISO showed better NPR of 63.6 ± 4.9 nauplius female^{-1} day^{-1}. CHL, DUN, and TET were the next best feeds with NPR of 39 ± 2.8, 26.3 ± 3.14, and 26.3 ± 2.25 nauplius female^{-1} day^{-1}, respectively.

Feeding Rate

The feeding rate of copepods under different algal diets is shown in Fig. 1c. Feeding rate was maximum of 582.6 ± 198.2 cells/h in ISO. NAN feed did not elicit a good feeding response as evidenced by lower feeding by copepods (386.6667 ± 81.7 cells/h). Better feeding rates were also observed in CHL and DUN diets with values of around 566.6 ± 132.09 and 501 ± 219.1 cells/hr.

Population Growth

The average total number of population density obtained for a trial of 17 days is shown in Fig. 1d. The type of feed used was also shown to have considerable effect on population density of copepods ($P < 0.05$). The highest total population density of *N. affinis* was observed in mixed algal diet (920 ± 98.27). In the case of individual diets, ISO yielded high result followed by CHL with population densities of 866 ± 67.8 and 658 ± 69.5, respectively. NAN feed yielded the lowest density among the feeds tested with mean population being 241 ± 51.15. The results showed considerable differences in the numbers of the life-stage categories, i.e., nauplii, copepodites, and adults in different diet treatments. The total number of nauplii was remarkably higher in the MIX diet treatment. The ISO diet remarkably differed from the other diets in that it had substantially higher numbers of both nauplii and copepodites. Both MIX and ISO also had noticeably higher numbers of adults when compared to the rest of the diet.

Development Time

The fastest ($p < 0.05$) embryonic development time for copepod was found in the mixed algal diet, which hatched within 19 ± 1.54 h, followed by ISO and TET which hatched within 25 ± 2.36 h and 25.33 ± 2.73 h, respectively. The longest ($p < 0.05$) maturation time which was 29 ± 1.54 h resulted from feeding with

DUN. The shortest time interval between egg sac occurred at 20 ± 1.54 h for the copepod fed with mix diet, while the longest interval between egg sac was obtained from copepod fed with CHL, which took 33.3 ± 2.25 h. Copepods fed with NAN failed to produce a second egg sac. The development time from first stage of nauplii to first stage of copepodid (N1-C1) was found longest 5.75 ± 0.22 ($p < 0.05$) when fed with TET feed. On the other hand, the shortest ($p < 0.05$) development time (3.13 ± 0.15 days) was achieved by copepods fed with MIX diet. The shortest ($p < 0.05$) time taken to develop from CI to adult was 3.52 ± 0.42 days which was achieved by copepod fed with MIX diet, while the longest development time was 5.97 ± 0.18 days which was found in copepod fed with NAN diet (Table 1). Similarly, the shortest ($p < 0.05$) generation time (10.68 ± 0.73 days) was achieved by copepod fed MIX diet, whereas *N. affinis* fed with *N. oculata* had the longest ($p < 0.05$) generation time (16.59 ± 0.53 days) among all the treatments (Table 1). The longevity of *N. affinis* fed with mixed algal food and CHL was found to be significantly longer ($p < 0.05$) than those fed with other food types, while copepods fed with NAN were found to have the shortest ($p < 0.05$) longevity compared with the rest of the treatment (Table 1).

Salinity Experiments

Survival

The mean survival was above 50% at all tested salinities. Although survival rate was found maximum at the salinity ranges between 30 and 35 ppt, mortality was slower at 30 ppt with final survival of 80%. Even though salinity had significant impact on survival, maximum effects were noticed at higher salinities. The minimum survival rate of 20% was obtained for copepods cultured at 45 ppt (Fig. 2a).

Nauplius Production Rate

Salinity significantly ($P < 0.05$) affected the nauplius production rate of *N. affinis* (Fig. 2b). The salinity tolerance of *N. affinis* seemed to be in the range of 25–35 ppt. The results showed that *N. affinis* was adaptive under varying salinity conditions, although the nauplius production decreased beyond the tolerance range. NPR was found to be highest (73 ± 4.09) when the salinity was maintained at 30 ppt followed by 35 ppt with an NPR of 69 ± 6.28. The lowest nauplius production (33.3 ± 5.75) was recorded at 45 ppt which was about two times lower than NPR at 30 and 35 ppt. The higher salinities appeared to have adverse effect on nauplius production of *N. affinis* as evidenced by the present results.

Table 1 The mean ± SE maturation time of egg sac, time interval between egg sac, development time from nauplii I to copepodid I and from copepodid I to adult, generation time, and female longevity of *N. affinis* grown under different diets

Parameters	CHL	DUN	ISO	MIX	NAN	TET
EDT (hrs)	27.3 ± 1.4^{a}	29 ± 1.54^{ab}	25 ± 2.36^{ab}	19 ± 1.54^{b}	28.66 ± 1.36^{c}	25.33 ± 2.73^{a}
IES (hrs)	33.3 ± 2.25	26.7 ± 4.5	26 ± 2.68	20 ± 1.54	na	31 ± 3.57
N1-C1 (days)	5.02 ± 0.18^{ad}	5.24 ± 0.3^{ade}	4.01 ± 0.16^{b}	3.13 ± 0.15^{c}	5.47 ± 0.18^{de}	5.75 ± 0.22^{e}
C1-A (days)	5.61 ± 0.54^{a}	4.74 ± 0.65^{abc}	4.01 ± 0.16^{b}	3.52 ± 0.42^{bc}	5.97 ± 0.18^{a}	5.41 ± 0.39^{a}
GT (days)	12.91 ± 1.39^{a}	13.64 ± 1.19^{ab}	12.37 ± 0.20^{a}	10.68 ± 0.73^{a}	16.59 ± 0.53^{b}	14.54 ± 0.72^{ac}
FL (days)	27.3 ± 1.36^{ade}	12.3 ± 3.14^{a}	23.6 ± 2.58^{cbe}	28.33 ± 6.71^{bd}	11.33 ± 3.61^{a}	14 ± 1.54^{ac}

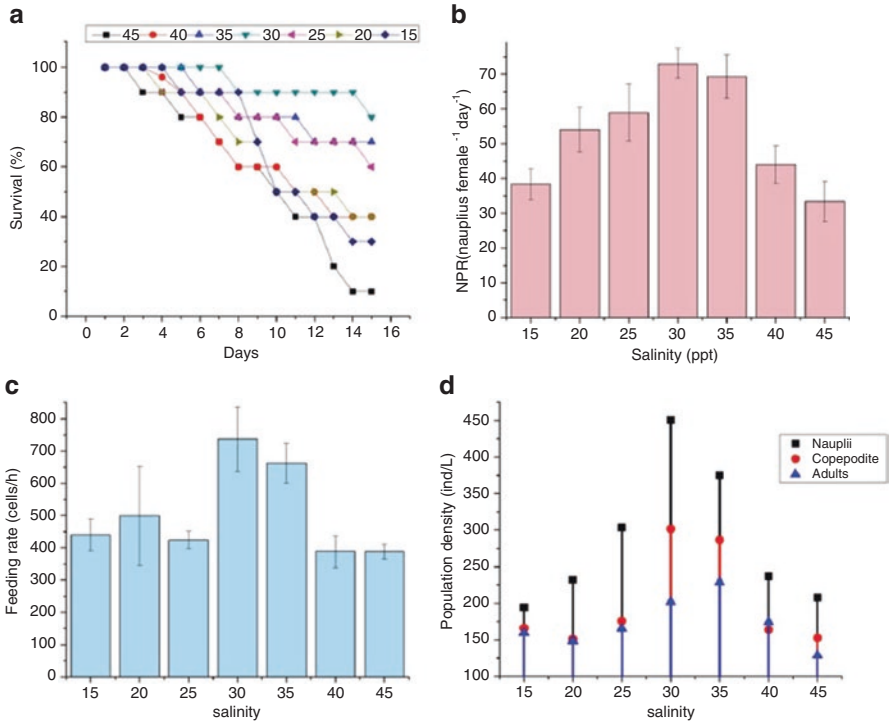

Fig. 2 (**a**) Survival; (**b**) nauplius production rate; (**c**) feeding rate; (**d**) population density of copepods exposed to different salinities. Copepods were incubated under identical condition of 28 ± 2 °C and 25,000 cells/ml of mixed algal feed

Feeding Rate

Feeding rate was maximum around 737 ± 100.2 cells/h when the salinity was maintained at 30 ppt. No clear trend was observed in the feeding rate experiment with copepods exposed to lower salinity (15 and 20 ppt) exhibiting better feeding rate than copepods exposed to 25 ppt. However 30 ppt seemed to have better feeding rate on the whole, and either a decrease or increase in salinity beyond 30 ppt, a drop in feeding rate was observed (Fig. 2c).

Population Growth

Salinity also had considerable effect on population density of copepods ($p < 0.05$). Mean population density of *N. affinis* was found highest at 30 ppt, reaching 953 ± 93.53 from an initial of 15 adults. Lowest population mean (489 ± 72.16) was obtained when copepods were maintained at 45 ppt. Population density was dropped almost by 50% compared to highest mean population when salinity increased to

45 ppt. Copepods survived for a longer period at salinities between 30 and 35 ppt compared to other levels. Salinity had no significant effect on nauplius production. An average nauplius production of 24 nauplius female^{-1} day^{-1} was recorded for all salinity treatments (Fig. 2d). The high tolerance capacity of the *N. affinis* can be added to the fact that the copepods have been cultured in the laboratory for more than 6 months. Hence they could have adapted to laboratory conditions and are able to survive better after being cultured.

Development Time

The observed results showed that *N. affinis* could survive in salinity ranging from 10 to 35 ppt, although under lower salinities (10–35 ppt) the overall reproduction seemed to be decreased ($p < 0.05$), development time and generation time were significantly lengthened ($p < 0.05$), and the longevity of female was significantly shortened ($p < 0.05$) compared to higher salinities.

The embryonic development time was shortest at 35 ppt, which hatched within 18 ± 2.25 h, followed by 30 ppt with an EDT of 18.3 ± 2.68 h. The embryonic development time was longest in 40 ppt, which was around 28 ± 0 h. The interval between egg sac formations was longest at 15 ppt (24.6 ± 2.73) and shortest at 35 ppt (20 ± 2.87). Copepods exposed to 45 ppt failed to produce a second egg sac. The nauplius to copepodite transformation was shortest at 30 ppt which took place with 3.13 ± 0.32 days, while the longest period was 5.59 ± 0.14 and 5.47 ± 0.38 days which occurred at 40 and 45 ppt. The copepodite to adult transformation was longest in 40 ppt which took place at around 6.03 ± 0.09, while it was shortest (3.52 ± 0.14) in 30 ppt. The shortest generation time of 10.68 ± 0.47 was observed at 30 ppt, while it was longer (14.52 ± 0.30) at 45 ppt. The female longevity was longer for about 30.3 ± 4.41 and 28.3 ± 6.71 in 30 and 35 ppt, respectively (Table 2).

Effects of Temperature

Survival

The mean survival was above 50% at all tested temperatures until 6th day. Total mortality was earlier at 34 °C by the end of 11th day. However survival was higher (73%) at 25 °C by the end of 6th day, followed by 28 and 30 °C with 70% survival. The final survival was higher at 28 °C with a survival of 40% followed by 30% at 30 °C (Fig. 3a).

Table 2 The mean ± SE maturation time of egg sac, time interval between egg sac, development time from nauplii I to copepodid I and from copepodid I to adult, generation time, and longevity of *N. affinis* grown under different salinities

Parameters	15	20	25	30	35	40	45
EDT (hrs)	28.6 ± 2.25^{a}	22 ± 3.22^{ab}	20.6 ± 1.37^{ab}	18.3 ± 2.68^{b}	18 ± 2.25^{b}	25.3 ± 2.73^{ab}	27.3 ± 4.22^{a}
IES (hrs)	24.6 ± 2.73	23.3 ± 2.25	25.3 ± 3.61	22.3 ± 1.54	20 ± 2.87	28 ± 0	0
N1-C1 (days)	4.9 ± 0.46^{ad}	4.85 ± 0.23^{a}	4.94 ± 0.09^{a}	3.13 ± 0.32^{bc}	4.01 ± 0.15^{bd}	5.59 ± 0.14^{a}	5.47 ± 0.38^{a}
C1-A (days)	4.76 ± 0.20^{a}	5.4 ± 0.28^{ade}	5.19 ± 0.21^{ade}	3.52 ± 0.14^{bc}	4.73 ± 0.42^{c}	6.03 ± 0.09^{de}	5.8 ± 0.11^{e}
GT (days)	13.73 ± 0.52^{a}	14.16 ± 0.63^{ade}	13.7 ± 1.23^{ac}	10.68 ± 0.47^{b}	12.08 ± 0.73^{c}	13.65 ± 0.17^{ad}	14.52 ± 0.30^{de}
FL (days)	15 ± 2.36^{a}	14.3 ± 2.73^{ade}	16.3 ± 2.06^{ade}	30.3 ± 4.41^{bc}	28.3 ± 6.71^{c}	13.6 ± 3.38^{d}	12 ± 3.22^{e}

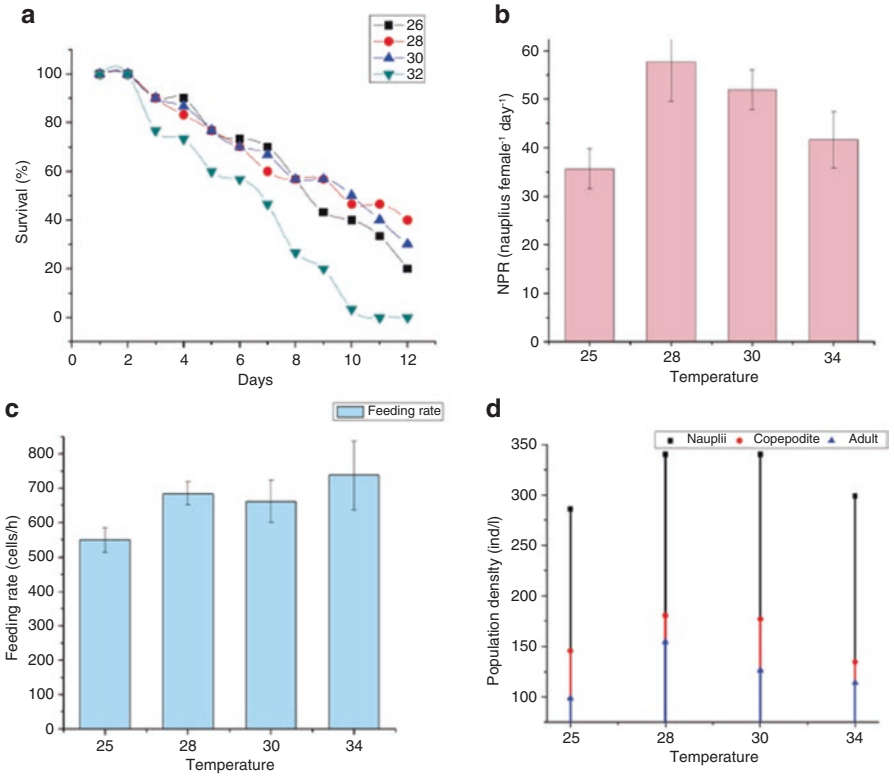

Fig. 3 (**a**) Survival; (**b**) nauplius production rate; (**c**) feeding rate; (**d**) population density of copepods exposed to different temperature. Copepods were provided identical condition of 25,000 cells/ml of algae and maintained at 30 ± 2 ppt

Nauplius Production Rate (NPR)

Temperature significantly ($P < 0.05$) affects the nauplius production rate of *N. affinis*. The copepod tolerated temperature variations between 28 and 30 °C (Fig. 3b). NPR was found to be highest (57 ± 8.06 nauplius female^{-1} day^{-1}) when the temperature was maintained at 28 °C followed by 30 °C with an NPR of 52 ± 4.09. The lowest nauplius production (35 ± 4.22) was recorded at 25 °C. In the present study lowest temperature employed had more effect on the NPR of copepods.

Feeding Rate

Feeding rate was maximum of around 737 ± 100.2 cells/h when the temperature increased to 34 °C. No clear trend was observed in the feeding rate experiment with copepods exposed to different temperature conditions. However, lower feeding rate of 549.6 ± 35.44 cells/h was noticed when maintained at lower temperature. On the whole a gradual increase in feeding rate along with the temperature was observed (Fig. 3c).

Table 3 The mean ± SE maturation time of egg sac, time interval between egg sac, development time from nauplii I to copepodid I and from copepodid I to adult, generation time, and longevity of *N. affinis* grown under different temperature

Parameters	25 °C	28 °C	30 °C	34 °C
EDT (hrs)	33.56 ± 1.39^{a}	19.6 ± 1.36^{bcd}	21.6 ± 2.25^{cd}	22.3 ± 1.36^{d}
IES (hrs)	30.6 ± 2.25^{a}	20.6 ± 2.25^{bcd}	21.33 ± 1.86^{cd}	24.6 ± 1.36^{d}
N1-C1 (days)	7.6 ± 0.41^{d}	3.96 ± 0.5^{cd}	4.58 ± 0.23^{bcd}	4.27 ± 0.21^{a}
C1-A (days)	4.48 ± 0.28^{a}	3.52 ± 0.42^{a}	6.1 ± 0.15^{bc}	5.19 ± 0.69^{c}
GT (days)	13.7 ± 0.39^{acd}	10.68 ± 0.73^{b}	12.72 ± 0.68^{cd}	13.45 ± 0.49^{d}
FL (days)	19 ± 2.68^{a}	26.6 ± 4.92^{a}	24.3 ± 5.08^{a}	23 ± 3.89^{a}

Population Density

Temperature also had significant impact on population growth of copepods ($p < 0.05$). Mean population density of *N. affinis* was high at 28 °C, reaching 675 ± 43.65 ind L-1 from an initial of 15 adults (Fig. 3d). Lowest population mean (530 ± 33.7) was obtained when copepods were maintained at 25 °C. Copepods survived longer, and development time from nauplii to adults was also earlier at 28 °C compared to other temperature. Salinity had no significant effect on nauplius production. An average nauplius production of 20 nauplius female^{-1} day^{-1} was recorded for both 28 and 30 °C. The higher tolerance of the *N. affinis* at 28–30 °C can be attributed to the fact that they are copepods adapted to our laboratory conditions.

Development Time

The embryonic development time was shortest at 28 °C, which hatched within 19.6 ± 1.36 h, followed by 30 °C with an EDT of 21.6 ± 2.25 h. The embryonic development time was longest in 25 °C, which was around 33.5 ± 1.39 h. The interval between egg sac formations was longest at 25 °C (30.6 ± 2.25) and shortest at 28 °C (20.6 ± 2.25). The nauplius to copepodite transformation was shortest at 28 °C which took place with 3.96 ± 0.50 days, while the longest period was 7.63 ± 0.41 days which occurred at 25 °C. The copepodite to adult transformation was longest in 34 and 30 °C which took place at around 6.19 ± 0.69 and 6.1 ± 0.15, while it was shortest (3.52 ± 0.42) in 28 °C. The shortest generation time of 10.68 ± 0.73 was observed at 28 °C, while it was longer (13.74 ± 0.39) at 25 °C. Female longevity was longest for about 26.6 ± 4.9 at 28 °C (Table 3).

Discussion

Copepods are well adapted to deal with sudden changes in feeds, temperature, and salinity under natural conditions (Santhanam and Perumal 2012a; Milione and Zeng 2007). Copepods in estuarine have tolerance to a wide range of salinities (Chinnery

and Williams 2004; Castro-Longoria 2003). This is a valuable feature for aquaculture, as copepods used as live feed for fish larvae remain alive in different temperature and salinity ranges that are required for the culturing fish larvae (Santhanam and Perumal 2012a). The outcome of this study showed that survival of *N. affinis* was higher at salinities between 30 and 35 ppt. High survival of over 80% was achieved under these salinities. Sun and Fleeger (1995) stated that harpacticoid copepods grew best in the salinity regime of 25–35 ppt, although it can continue to survive in the salinity range of 10 to 60 ppt. In general harpacticoid copepods are better known to have an ample tolerance to salinity differences due to their diverse natural habitats with periodic exposure to salinity fluctuations. Rhodes (2003) also stated that the salinity tolerance of *Nitocra lacustris* ranged from 10 to 40 ppt. Recently, Zaleha and Jamaludin (2010) observed the optimum condition for the maximum production of a tropical harpacticoid copepod *Pararobertsonia* sp. to be around 35 ppt. Though *N. affinis* was capable of surviving in a broad range of salinity levels, the female reproduction has been observed to decline under high salinities as agreed by many reports (Hall and Burns 2002, Calliari et al. 2008 and Santhanam and Perumal 2012b, Chen et al. 2006). In this study, the maximum growth rate peaked at 30–35 ppt and decreased at both higher and lower salinities. Under these salinities, copepods matured and were able to form eggs earlier. Hastening of the biological cycle in terms of minimum and mean generation time is maximal for salinities 30 and 35 ppt, a range corresponding that of the original habitat of the species, a coastal marine environment where lower salinities may occur locally or temporally. In the present study, the highest growth rate was observed at 30–35 ppt and declined at lower salinities (25–10 ppt).This may be related to the fact that these copepods are well adapted to the salinity range of 30–35 ppt in our laboratory for longer than a year. Moreover, these are salinities experienced by the copepods in the wild. However, it is hard to term a salinity threshold for estuarine species (Uye and Fleminger 1976; Santhanam and Perumal 2012a), as different species may acclimatize to variable salinity regimes at different stages of their life cycle due to their changing habitats.

Apart from salinity, feed is also an important factor that can impact growth, egg hatching success, and nauplius survival in copepods (Santhanam et al. 2013; Santhanam and Perumal 2012b). With a total of six diets tested, MIX and ISO were among the best promoting high nauplius production and population density. In case of survival, highest values were obtained in the mixed diet trials, followed by ISO. Though MIX feed yielded the best result, ISO showed results almost close to MIX. Many reports have stressed the use of binary algal diets for higher fecundity in copepods (Stottrup 2000; Payne and Rippingale 2001; Knuckey et al. 2005 and Lee et al. 2006). Even though, a few studies (Lee et al. 2006; Milione and Zeng 2007; Zhang et al. 2013) have reported better results using *I. galbana* as a mono diet. In the present study, the better results obtained in ISO may be because of the higher amounts of polyunsaturated fatty acids (PUFA) specifically docosahexaenoic

acid present in ISO. Still very little is studied about the requirements of fatty acid in copepods; the importance of diet rich in (n-3) PUFA, especially DHA and EPA, for the egg production and egg development has been emphasized in the works of Lacoste et al. (2001). Klein-Breteler et al. (2004) also stated that the production of highly unsaturated fatty acids improved copepod egg production. Similar observations were made by Matias-Peralta et al. (2012), Zhang et al. (2013), and Santhanam et al. (2013). The least effective diet was NAN which was unable to sustain survival and population density. From the obtained results, NAN was not a suitable monoalgal diet for enhancing the development rate of *N. affinis*. Though DUN and TET showed better results than NAN, they cannot be considered as finest feeds. Inferior efficacy of these diets is likely to be a result of a lack of necessary nutritional elements that could not be fully compensated individually. Morehead et al. (2005) also found that *N. oculata* did not support population growth of copepods and attributed lack of DHA and other highly unsaturated fatty acids despite its high EPA for ineffectiveness of NAN diet. Payne and Rippingale (2000) also indicated the indigestibility of the cell wall for poor results associated with *N. oculata*.

Though several tropical organisms have continuous breeding seasons, there were indications that the majority of these species spawn either exclusively or most actively in the warmer months, so as to make the best use of time, space, and most specifically abundant food availability (Matias-Peralta et al. 2012; Kinne 1963). In this experimental study, the feeding condition (except feed experiments) was constantly optimal for *N. affinis*, and the growth was not affected by shortage of food. The ambient temperature at which *N. affinis* resulted the maximum overall reproductive activities, shortest development time, and longest life span was observed at temperature ranging from 30 to 35 °C. This water temperature range match to that of the temperature variations in the natural habitat of *N. affinis*. Although in the natural habitat, water temperature could go down to 20 °C during early morning, extended exposure to low temperature as described in this study could considerably affect the overall reproductive capacity, development time, and longevity.

Santhanam and Perumal (2013) also stated that only a handful of laboratory studies have unveiled the relationship between temperatures and developmental rates for harpacticoid copepods. Data on the duration of the larval stages of marine harpacticoid copepods and the influence of environmental factors (specifically temperature) and their potential interaction on postembryonic development are relatively limited. Hicks and Coull (1983) also mentioned that development rates in harpacticoids depend on temperature and development time tends to decrease with increasing temperature.

In short, survival rate and nauplius production of *N. affinis* were influenced by feed and salinity. Salinity of around 30–35 ppt can be considered optimal for growth and survival of this copepod. Diet experiment with MIX and ISO resulted in excellent survival and population growth. Hence the study suggests using of ISO feed, salinity of 30 ppt, and temperature of around 28 °C for effective mass culture of *N. affinis*.

Conclusion

This study describes the survival, nauplius production rate, and developmental biology of marine harpacticoid copepod, *Nitocra affinis*, at different salinity, diet, and temperature. Copepods used in the experiments were reared as batch culture in laboratory. In diet experiments, the survival was higher with 89% in *Isochrysis galbana*; the highest NPR (66.3 ± 4.9 nauplius female^{-1} day^{-1}) was obtained in the mixed diet, the feeding rate was maximum with 582.6 ± 198.2 cells/h in ISO, and the highest population density was obtained in mixed algal diet (920 ± 98.27). The shortest development time was found in the mixed algal diet, which hatched within 19 ± 1.54 h. In salinity experiments, the highest survival (80%) was observed in 30 ppt; NPR was found to be highest (73 ± 4.09) at 30 ppt; feeding rate was maximum of around 737 ± 100.2 cells/h at 30 ppt; the highest population density was recorded in 30 ppt, with 953 ± 93.53 ind L^{-1}; and the embryonic development time was shortest at 35 ppt (18 ± 2.25 h). In temperature experiments, survival was higher (73%) at 25 °C; NPR was highest (57 ± 8.06 nauplius female^{-1} day^{-1}) at 28 °C; feeding rate was maximum of around 737 ± 100.2 cells/h at 34 °C; population density was high at 28 °C, reaching 675 ± 43.65 ind L^{-1}; and the shortest embryonic development time was observed at 28 °C, which hatched within 19.6 ± 1.36 h.

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Evaluation of the Suitability of Marine Copepods as an Alternative Live Feed in High-Health Fish Larval Production

P. Santhanam, N. Jeyaraj, K. Jothiraj, S. Ananth, S. Dinesh Kumar, and P. Pachiappan

Introduction

Copepods comprise the first vital link in the marine food chain between primary producers and fish. The fast-growing hatchery production of larvae for mariculture, as well as the increased attention on new species and ornamental fish cultures for replacing wild fisheries, requires the development of suitable larval feeds; this need cannot be met by conventional species of live feeds, such as rotifers and *Artemia*. Thus, interest in copepods has increased and the use of copepods as live feed in aquaculture has gained momentum. Copepods may be the only prey of acceptable size for small larvae of some fish species or the only type of live feed that is suitable for the rearing of fishes with altricial larvae. Marine copepods are the major diet for most fish larvae in the ocean. They have high amounts of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and other polyunsaturated fatty acids (PUFAs), either from their phytoplankton diet or accumulated despite low PUFA levels in their diet. Furthermore, copepods play an important role in fish larval digestion by

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producing exogenous digestive enzymes. The newly hatched marine fish larvae are very small; hence, they cannot be fed rotifers as live feed for the first feeding. This chapter discusses the methods for rearing newly hatched marine fish Asian seabass *Lates calcarifer* larva fed with marine copepods as live feed for the first feeding.

Materials and Methods

Copepod Culture

For the culturing of copepods, the copepod samples were collected from the Nagappattinam coastal waters early in the morning by using a plankton net with a 158-μm mesh. Vigorous aeration was provided to the collected copepod samples by using battery aerators and the samples were transported immediately to the laboratory and thoroughly rinsed to reduce the contamination from other zooplankters. From the collected samples, individual copepod species like *Nannocalanus minor* and *Paracalanus parvus* were isolated and identified under the microscope using the standard keys of Kasturirangan (1963). After confirmation of the indigenous species name of the copepod, 200 individuals of them, including both males and females, were isolated and stocked into an oval-shaped, flat-bottomed fibreglass tank (0.54 m diameter, 0.81 m length), containing 100 L of filtered seawater, with vigorous aeration provided by using an aquarium aerator. The seawater was filtered through a membrane filter (1 μm) before use. Maintenance of optimised water quality parameters such as temperature, salinity, pH and dissolved oxygen in copepod culture water, are important to obtain high yield. The mixed microalgae were given as feed for copepods at the cell concentration of 30,000 cells/mL. The copepod cultures were harvested every 14 (for *N. minor*) and 12 (for *P. parvus*) days by gentle siphoning. Finally, the adult male and female copepods were used to restart the stock culture. Water quality parameters such as temperature, salinity, pH and dissolved oxygen, and the population density, growth and survival of nauplii, copepodites and adults were observed daily to maintain healthy stock culture.

Algal Culture

For providing feed for copepods, we need to maintain the microalgal pure culture as a primary feed for the primary consumer. The common microalgal species, such as *Chlorella marina*, *Dunaliella salina, Isochrysis galbana, Nannochloropsis salina* and *Tetraselmis suecica*, were maintained in temperature-controlled room separately in 250-mL, 500-mL, 1-L and 2-L conical flasks, 5-L plastic jars and 15-L transparent plastic buckets containing filtered seawater at 20–23 °C temperature, 30‰ salinity, 12:12 L:D photoperiod and 150 μmol m^{-2} s^{-1} light intensity and fertilised with Walne's medium (Walne 1970).

Rotifer and Artemia *Culture*

The rotifer *Brachionus plicatilis* and *Artemia* nauplii cultured separately in a 100-L tank at 32‰ salinity, 29 °C temperature and with vigorous aeration and illumination for estimating the comparative efficacy of the live feed. The rotifer and *Artemia* feeds have equal compositions of mixed microalgae. Samples of *B. plicatilis* and *Artemia* nauplii were harvested from the culture tank and used as feed for fish larvae. To find out the initial biochemical composition of the rotifer and *Artemia*, after collection from the tank, they were washed with distilled water and kept frozen under nitrogen at −80 °C for further analyses.

Evaluation of the Live Feed Suitability of Copepods

To estimate the effects of rotifer, *Artemia* nauplii and copepod feeding on the growth, survival and biochemical composition of seabass *Lates calcarifer*, larvae was examined according to Santhanam and Perumal (2012). Fourteen-day-old seabass *L. calcarifer* larvae were obtained from ICAR-Central Institute of Brackishwater Aquaculture, Chennai, India. The fish larvae were acclimatised in the laboratory before the experiment. The fish larval rearing experiment was conducted in triplicate and live feed such as rotifers, *Artemia* and copepods used in the density ranges of 5000–10,000, 4000–6000 and 2000–3000 individuals L^{-1}, respectively. One hundred larvae were stocked in the 100-L FRP tanks filled with filtered seawater with vigorous aeration using an aquarium aerator. Daily, 50% of the water was exchanged with fresh filtered seawater to avoid ammonia formation from the faecal matter. Every 7 days, the fish larvae were harvested from the experimental tank and their length and weight was measured, and samples handled as per the procedures and stored in the desiccators for the biochemical composition analyses. Water quality parameters such as temperature, dissolved oxygen and salinity were estimated daily and maintained at the optimised levels. The rearing experiment lasted for 21 days, with a 12:12 h L:D cycle being maintained. The daily ration was given three times per day at 06:00, 13:00 and 19:30 h. The total length (TL) and wet weight (WW) of the fish larvae were measured and recorded at the beginning and end of the experiment for estimating the effects of different live feeds (rotifers, *Artemia* and copepods). The biochemical composition of both feeds and fish larvae were estimated at the initial and final stages of the experiment. Growth of larvae was determined by seine sampling from various points of each tank using a 158-μm scoop net. The excess moisture was removed using tissue paper and the length and weight measurements of the larvae was estimated within a few seconds, to avoid larval mortality. If any mortality is observed, the dead larvae were collected and stored at 40 °C.

Analysis of Samples

Water Quality Parameters

The water temperature and salinity were measured using a standard centigrade thermometer and hand refractometer (ERMA, Japan), respectively. The dissolved oxygen concentration of water was estimated by Winkler's method (Strickland and Parsons 1972).

Morphometric Parameters

Fish sampling was carried out for every 7 days for morphometric analyses (length, weight and survival). The fish growth rate (dry weight) was calculated from the body weight (mg) based on the formula derived by Ricker (1979): $G = (W_2 - W_1)/(T_2 - T_1)$, where W_2 and W_1 represent the final and initial weights of the fish larvae, respectively, and $(T_2 - T_1)$ is the duration of the experimental period. For length (L) analyses, the initial length (I_L) of fish larvae was subtracted from the final length (F_L) with the help of the following formula ($L = F_L - I_L$). The survival of the larvae was determined at each sampling. Survival was calculated as the percentage of fish larvae remaining in each tank from the estimated number stocked initially.

Nutritional Composition

Rotifer, *Artemia* and copepod fed fish larvae were harvested from the experimental tank and then washed with distilled water and stored in the desiccators for further analyses. The initial and final concentrations of nutritional compounds of fish larvae were analysed. The moisture, protein, carbohydrate, lipid and ash contents in live feeds and fish larvae fed on various live feeds were estimated following the standard methods (Rajendran 1973; Raymont et al. 1964; Dubois et al. 1956; Folchet al. 1956; AOAC 1995). The amino acids composition of live feed and fish larvae were analysed according to Yamamoto et al. (1994) using High Performance Liquid Chromatography (HPLC). For analyses of fatty acid composition in live feeds and fish larvae, 0.4 g of dried samples were homogenised with 2:1 (v/v) combination of chloroform and methanol mixture, and they were extracted using the modified method of Bligh and Dyer (1959). After the extraction of samples, esterification with 1% H_2SO_4 and fatty acid methyl esters (FAME) were estimated as per the AOAC (1995) using Gas Chromatography Mass Spectrometry (GCMS).

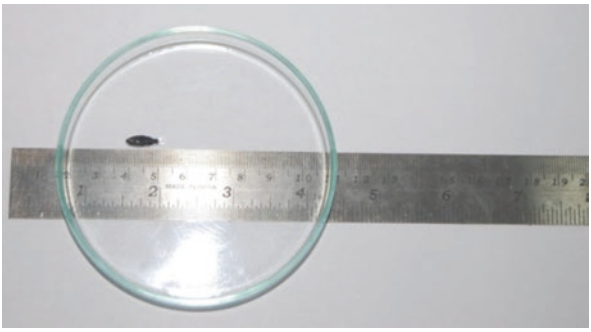

Plate 1a *Brachionus plicatilis* fed *L. calcarifer* larva (21st day)

Plate 1b *Artemia* nauplii fed *L. calcarifer* larva (21st day)

Results

Growth and Survival of Larvae of Seabass, Lates calcarifer

The length and weight of copepod *N. minor* fed seabass *L. calcarifer* larvae (Plate 1c) was comparatively higher than rotifer (Plate 1a) and *Artemia* nauplii fed larvae (Plate 1b). The total mean length of *N. minor* fed larvae was reported as 21.33 ± 1.52 mm, 30.6 ± 1.15 mm and 43.3 ± 1.52 mm on the 7th, 14th and 21st days, respectively, and the weight was 111.6 ± 7.05 mg, 254.3 ± 4.04 mg and 333.6 ± 11.01 mg on the 7th, 14th and 21st days, respectively. The *N. minor* fed seabass larvae showed a remarkable survival of 98%, 94 ± 4% and 92.6 ± 0.57% on the 7th, 14th and 21st days, respectively. The detailed results are given in Table 1. The analyses of variance (ANOVA) on the growth and survival of *L. calcarifer* larvae fed with *B. plicatilis* and *N. minor* showed significant results. Among the parameters analysed, the 14th day weight was higher, with $P < 0.005$. The remaining parameters showed significance, with $P < 0.01$, whereas the initial length and weight were not significant. *Artemia* nauplii and *N. minor* fed larvae also reached significance, with $P < 0.01$, whereas the initial length and weight were not significant (Plate 1c).

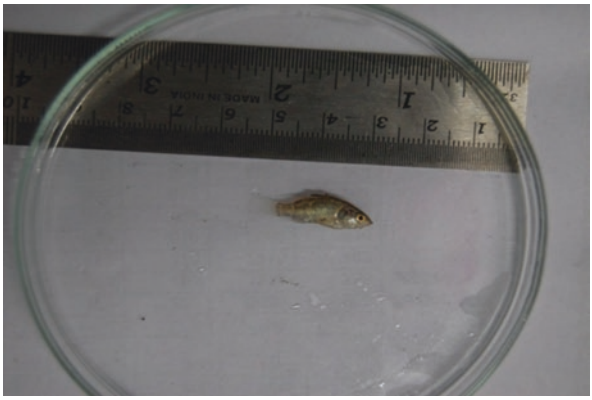

Plate 1c *Nannocalanus minor* fed *L. calcarifer* larva (21st day)

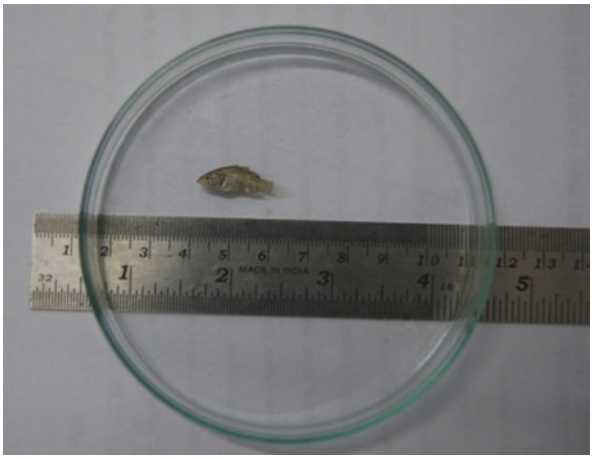

Plate 1d *Paracalanus parvus* fed *L. calcarifer* larva (21st day)

Sea bass *L. calcarifer* larvae fed with the copepod *P. parvus* grew faster (Plate 1d) compared to larvae fed with rotifers and *Artemia* nauplii. The total mean length of copepod *P. parvus* fed seabass larvae was reported as 22.6 ± 2.51 mm, 31.66 ± 0.57 mm and 44.33 ± 2.08 mm on the 7th, 14th and 21st days, respectively, and the weight was 116.3 ± 9.07 mg, 257.3 ± 4.93 mg and 338 ± 8.185 mg on the 7th, 14th and 21st days, respectively. The *P. parvus* fed seabass larvae showed a remarkable survival of 98 ± 1%, 95 ± 2% and 93 ± 1% on 7th, 14th and 21st days, respectively, whereas the seabass larvae reared on *Artemia* nauplii had the lowest survival rate, being 94.33 ± 0.57%, 88 ± 1% and 82 ± 2% on the 7th, 14th and 21st days, respectively. However, the survival of rotifers fed seabass larvae was 91 ± 1%, 84 ± 2% and 77 ± 2.6% on the 7th, 14th and 21st days, respectively (Table 1). The ANOVA results indicated that the 14th day weight of *B. plicatilis* and *P. parvus* fed seabass larvae showed a highly significant result, with $P < 0.005$. The other parameters reached significance at $P < 0.01$, whereas the initial length and weight were not significant. Similarly, the 14th day weight of *Artemia* nauplii and *P. parvus* fed

Table 1 Growth and survival of seabass *L. calcarifer* fed with different live feeds

Feeding regimes	Initial		7th day			14th day			21st day		
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Survival (%)	Length (mm)	Weight (mg)	Survival (%)	Length (mm)	Weight (mg)	Survival (%)
B. plicatilis	9.23 ± 1.0	12.6 ± 1.5	11.76 ± 0.2	23.66 ± 2.5	91 ± 1	18.6 ± 1.1	89.3 ± 0.5	84 ± 2.0	25.66 ± 0.5	132 ± 3.7	77 ± 2.6
Artemia nauplii	12.33 ± 1.1	24 ± 3.0	13.83 ± 1.2	29.33 ± 1.5	94 ± 0.5	21.33 ± 1.5	94 ± 1.0	88 ± 1.0	26.66 ± 1.5	170.66 ± 6.6	82 ± 2.0
N. minor	10.66 ± 2.0	23.33 ± 9.4	22.6 ± 2.5	116.3 ± 9.0	98 ± 0	31.66 ± 0.5	257.3 ± 4.9	95 ± 2.0	44.33 ± 2.0	338 ± 8.1	93 ± 1.0
P. parvus	10.66 ± 2.0	23.33 ± 9.4	21.33 ± 1.5	111.6 ± 7.5	98 ± 0	30.6 ± 1.1	254.3 ± 4.0	94 ± 4.0	43.33 ± 1.5	333.6 ± 11.0	92.6 ± 0.5

seabass larvae showed a highly significant ANOVA, with $P < 0.005$, and the other parameters were significant at $P < 0.01$. However, the initial length and weight were not significant.

Biochemical Composition of Seabass Larvae

The biochemical composition analyses clearly indicated the nutritional strength of copepods. The proximate compounds such as moisture, protein, lipid, carbohydrate and ash contents of copepod *N. minor* fed seabass larvae were reported as 81.5%, 65.04%, 15.23%, 11.34% and 8.29%, respectively. The moisture, protein, lipid, carbohydrate and ash contents of *P. parvus* fed seabass larvae were 81.5%, 65.35%, 15.33%, 11.23% and 8.09%, respectively (Table 2). The protein and lipid contents of rotifer and *Artemia* nauplii fed larvae were comparatively lower than that of copepod fed larvae. The recorded percentages of moisture, protein, lipid, carbohydrate and ash contents of rotifer fed larvae were 81.39%, 60.51%, 13.66%, 11.19% and 14.64%, respectively. However, the percentage of moisture, protein, lipid, carbohydrate and ash contents of *Artemia* nauplii fed fish larvae were 81.12%, 63.31%, 12.55%, 13.29% and 10.84%, respectively (Table 2).

The total amino acid content of 100% was recorded in copepod *N. minor* fed larvae, with dominance of glutamic acid (14.56%), arginine (10.12%), alanine (9.87%), lysine (9.44%), aspartic acid (7.86%) glycine (6.34%) and leucine (5.18%). The highest amino acid content of 99.9% was recorded in *P. parvus* fed larvae, with dominance of glutamic acid (11.78%), serine (11.12%), arginine (10.23%), lysine (10.12%), alanine (8.97%), aspartic acid (6.87%) and glycine (6.23%) (Table 3). The *Artemia* nauplii fed larvae contained 69.44% of total amino acids. Among the total amino acids, arginine, glutamic acid, lysine and alanine were reported with percentages of 9.44%, 8.14%, 7.87% and 7.36%, respectively. The total amino acid level of *B. plicatilis* fed larvae was 66.65%. The amino acids glutamic acid, alanine, tyrosine, aspartic acid and lysine were observed at high percentage levels of 11.61%, 7.63%, 7.58%, 7.47% and 7.39%, respectively. Detailed results of the amino acids composition of different live feeds fed larvae are given in Table 3.

The total fatty acid content of copepod *N. minor* fed seabass larvae was 100%. Among these, fatty acids such as 14:01, 16:00, 18:1-n9, 18:01 and 17:00 were recorded with maximum percentages of 22.8%, 14.87%, 8.97%, 8.67% and 5.56%,

Table 2 Biochemical composition of *L. calcarifer* fed with different live feeds

Feeding regimes	Moisture (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)
Initial	80.26 ± 0.12	62.54 ± 0.21	11.33 ± 2.51	12.91 ± 0.38	13.22 ± 0.04
B. plicatilis	81.39 ± 0.04	60.51 ± 0.52	13.66 ± 1.52	11.19 ± 0.24	14.64 ± 0.30
Artemia nauplii	81.12 ± 0.30	63.31 ± 0.53	12.55 ± 0.50	13.29 ± 0.13	10.84 ± 0.17
N. minor	81.5 ± 0.17	65.04 ± 0.06	15.3 ± 1.06	11.34 ± 0.11	8.29 ± 0.04
P. parvus	81.5 ± 0.12	65.35 ± 0.12	15.33 ± 2.51	11.23 ± 0.17	8.09 ± 0.04

Table 3 Amino acid composition of *L. calcarifer* fed with different live feeds

Amino acids	Initial larvae	Rotifer fed larvae	*Artemia* fed larvae	*N. minor* fed larvae	*P. parvus* fed larvae
Arginine	3.25	3.07	9.44	10.12	10.23
Histidine	2.41	2.04	3.7	4.35	4.89
Isoleucine	2.73	2.72	1.92	3.46	4.45
Leucine	5.71	5.42	4.89	5.18	6
Lysine	5.76	7.39	7.87	9.44	10.12
Methionine	1.77	1.85	3.45	2.34	2.89
Phenylalanine	2.35	0.33	3.65	3.55	4.56
Threonine	3.16	3.11	3.22	3.89	4.56
Cystine	0.36	0.01	0.01	1.12	1.89
Valine	2.9	2.85	3.09	3.45	3.35
Alanine	7.21	7.63	7.36	9.87	8.97
Aspartic acid	7.23	7.47	6	7.86	6.87
Glutamic acid	11.6	11.61	8.14	14.56	11.78
Glycine	5.28	0.48	3.17	6.34	6.23
Serine	3.1	3.09	2.21	12.34	11.12
Tyrosine	2.03	7.58	1.32	2.13	2.08
Total	66.85	66.65	69.44	100	99.99

respectively. The eicosapentaenoic acid (EPA) and DHA contents of *N. minor* fed fish larvae were 6.7% and 8.96%, respectively. The fatty acid level of *L. calcarifer* larvae fed with *P. parvus* was 100%. Among the total fatty acids, components like 14:00 (15.74%), 18:1-n9 (11.54%), 16:00 (9.78%), 18:01 (8.67%) and 18:00 (6.51%) were recorded in higher amounts (Table 4). The fatty acid level of *L. calcarifer* larvae fed with rotifers was 69.35%. Among the total fatty acids, the components 16:00 (18.91%), 18:1n-9 (9.08%), 18:3 (8.97%), 17:00 (6.91%) and 16:01 (6.78%) were found to be high in rotifer fed sea bass larvae. The *Artemia* nauplii fed larvae contained more fatty acids like 16:00 (21.3%), 18:1-n9 (12.62%), 18:3-n6 (12.15%), 17:00 (9.92%) and 22:01 (5.98%) (Table 4). The HUFAs such as EPA and DHA were higher in *P. parvus* fed larvae than the *B. plicatilis* and *Artemia* fed larvae. The larvae fed with the copepod *P. parvus* contained higher EPA (7.2%) and DHA (10.23%) compared to rotifer fed larvae (4.89% and 0.37%, respectively) and *Artemia* nauplii fed larvae (5.25% and 1.76%, respectively) (Table 4).

Discussion

The present study examined the growth and survival of larvae of Asian seabass *L. calcarifer* vis-à-vis live food organisms, viz. rotifer, *Artemia* and copepods. The growth and survival were relatively high when copepods *N. minor* and *P. parvus* were used as food. Similar results were reported for the grouper *Epinephelus*

Table 4 Fatty acid composition of *L. calcarifer* fed with different live feeds

Fatty acids	Initial larvae	Rotifer fed larvae	*Artemia* fed larvae	*N. minor* fed larvae	*P. parvus* fed larvae
12:00	–	–	–	0.98	1.21
14:00	0.14	–	–	8.9	3.9
15:00	–	–	–	0.97	1.34
16:00	9.76	18.91	21.3	14.87	9.78
17:00	6.89	9.08	9.92	5.56	4.76
18:00	2.1	5.48	–	5.8	6.51
20:00	–	–	–	0.8	1.56
21:00	0.64	3.94	–	0.92	1.54
22:00	–	0.14	–	0.23	2.18
24:00	0.001	0.28	0.02	0.1	1.65
14:01	–	–	–	22.78	15.74
16:01	4.03	6.78	–	1	2.43
18:01	0.21	–	2.88	8.67	8.67
18:1-n9	4.51	8.97	12.62	8.97	11.54
18:3-n3	–	–	–	3.1	4.23
18:3-n6	0.26	0.18	12.15	0.5	1
18:2-n6	6.64	6.91	–	0	0.56
20:2-n6	–	–	–	0	0.32
20:4-n6	1.24	2.56	–	0.05	1.87
20:4-n5	–	0.86	–	0.02	1
20:5-n3	2.01	4.89	5.2	6.7	7.2
22:6-n3	1.52	0.37	1.76	8.96	10.23
22:01	1.04	–	5.98	0.12	0.78
Total	40.99	69.35	71.83	100	100

coioides larvae fed with mixed zooplankton (Toledo et al. 1996, 1997; Doi et al. 1997). The influence of zooplankton abundance in the feeding periodicity of *L. calcarifer* was observed earlier by Barlow et al. (1993), who stated that the feeding rate of seabass larvae increased when the zooplankton density was increased. The Bojonegara Research Station for Coastal Aquaculture in West Java was used to collect marine fish larvae of seabass *L. calcarifer* and larvae of the grouper *E. fuscoguttatus* fed with copepods. Substitution of *Artemia* nauplii with wild copepods has often improved the growth and survival of grouper (*E. fuscoguttatus*) larvae and it has also been proved by Slamet and Diani (1993). Similarly, Drillet et al. (2006) confirmed that the mixed strains of copepods can replace or supplement rotifers and *Artemia*. The length and weight were all significantly lower in those seabass larvae reared on *Artemia* nauplii, and the survival of seabass reared with *Artemia* nauplii showed larger variation when compared with those reared on copepods (Table 1). Initially, there were no changes in the length and weight of *Artemia* fed animals up to the 4th day. In rotifer fed larvae, there was no change in the length and weight up to the 3rd day from stocking. The lower growth was noticed in rotifers fed animals

Table 5 Biochemical composition of wild collected *N. minor* and *P. parvus*

Composition (%)	*N. minor*	*P. parvus*
Moisture	71.41 ± 0.168	73.72 ± 0.34
Protein	63.16 ± 1.27	57.92 ± 0.435
Carbohydrate	20.18 ± 0.746	23.33 ± 0.746
Lipid	12.53 ± 0.393	13.82 ± 0.31
Ash	4.196 ± 0.195	4.84 ± 0.157

Table 6 Amino acid composition of wild copepods

Amino acids	*N. minor*	*P. parvus*
Arginine	4.23	7.65
Histidine	2.21	2.68
Isoleucine	2.1	3.4
Leucine	9.86	9.23
Lysine	5.49	4.34
Methionine	4.78	3.98
Phenylalanine	2.45	3
Threonine	14.1	10.98
Cystine	0.061	0
Valine	6.56	7.89
Alanine	9.8	9.87
Aspartic acid	8	8.76
Glutamic acid	17	16.78
Glycine	3.019	3.26
Serine	5.89	4.98
Tyrosine	4.45	3.2
Total	100	100

compared to copepods fed animals, and this might be due to the larvae fed with unscreened *B. plicatilis* and, so, the larvae have to spend more energy searching for smaller prey. A similar reason was suggested earlier by Laurence (1977). The growth and survival of *L. calcarifer* larvae were comparatively higher with a copepods diet than with rotifers and *Artemia* nauplii diets. Hence, initially, the length and weight increased faster than the other feeds. The survival rate was also comparatively double that of rotifers and *Artemia* fed animals. The present result was further confirmed by earlier works (Santhanam 2002; Evjemo et al. 2003; Rajkumar and Vasagam 2006; Olivotto et al. 2008). The growth of the fish larvae was extensive and contradictory, apparently due to the variety of physical and biological conditions of water used in larval rearing. The maintenance of water quality parameters such as temperature, salinity and dissolved oxygen was helpful for good results in the larval rearing of *L. calcarifer*. The nutritional value of food (Tables 5, 6 and 7), particularly the quantity and quality of n-3 highly unsaturated fatty acids (n-3 HUFA), are known to affect the growth and survival of seabass larvae. In the present study, the length and weight of *L. calcarifer* larvae were higher with the copepods

Table 7 Fatty acid composition of wild copepods

Fatty acids	*N. minor*	*P. parvus*
14:00	12.47	16.51
16:00	27.58	32.89
16:01	7.36	4.2
17:00	5.52	–
18:00	11.36	12.39
18:01	8.78	11.35
18:2n-6	2.15	1.14
18;3n-6	0.42	–
18:3n-3	0.64	2.16
18:4n-3	1.24	1.12
20:01	1.22	1
20:2n-6	0.58	–
20:4n-6	2.07	1.21
20:4n-3	0.21	–
20:5n-3	6.78	6.17
22:00	0.89	–
22:01	0.45	–
22:5n-6	0.34	–
22:5n-3	0.67	1.76
22:6n-3	8.43	7.1
24:00:00	0.84	1
Total	100	100

diet (*N. minor* and *P. parvus*), which might be due to the higher amount of n-3 HUFA (EPA and DHA) present in *N. minor* and *P. parvus* compared to that in *B. plicatilis* and *Artemia* nauplii. This is in agreement with the findings of Watanabe et al. (1983), Watanabe (1993), Evjemo and Olsen (1997) and Olivotto et al. (2010). Fukunaga et al. (1990) observed nutritional deficiency in the grouper *E. suillus*. Payne and Rippingale (2000) reported the low length and weight in the seahorse *Hippocampus subelongatus* fed with *Artemia* nauplii, might be due to its low content of EPA and DHA. In the present experiment, the low growth and survival in seabass larvae fed with rotifers and *Artemia* nauplii could only plausibly be a reflection of the inadequate nutritional value of rotifers and *Artemia* nauplii. Toledo et al. (1999) recorded similar results in the grouper *E. coioides* larvae. Prey density also has a large effect on feeding behaviour and growth of fish larvae, and this was proved by Hunter (1981). In the same way, the better growth was previously observed in African catfish fed with *Moina dubia* than *Artemia* nauplii by Adeyemo et al. (1994). In fact, copepods as feed clearly outperforms others in terms of meeting fish larval HUFA requirements (Santhanam 2002; Bell et al. 2003; Rajkumar and Vasagam 2006; Van der Meeren et al. 2008). The size of the prey and the mouth size of the larvae could also influence the growth of the *L. calcarifer* larvae during the initial feeding stage. In the present study, the length and weight of *L. calcarifer*

was observed in rotifers and *Artemia* fed animals after 4 days of growth. In fact, slow growth was observed might be due to small mouth size of the larvae at the initial stage. Similar observations were also made by Maneewong et al. (1986) and Kayano (1988) in grouper fishes. Seabass *L. calcarifer* larvae can be reared successfully with nauplii of *N. minor* and *P. parvus* with normal pigmentation compared with those fed on *Artemia* nauplii and rotifers. Naess and Lie (1998) carried out an experiment on rearing halibut larvae using rotifers and *Artemia* with abnormal pigmentation. Many of the advantages are available by using copepods as feed in fish larviculture. The superior nutritive value of copepods can give rise to good results on pigmentation and development (Nellen 1981; Witt et al. 1984; Norsker and Stottrup 1994; Heath and Moore 1997). Kraul et al. (1993) observed that the copepod *Euterpina acutifrons* improved stress resistance in the larval mahi-mahi. The copepod nauplii served as the first food for fish larvae (Stottrup 2000) and had an important role in larval digestion due to its source of exogenous digestive enzymes. This was put forth by Munilla-Moran et al. (1990) in *Eurytemora hirundoides*. Similar results were also found by Santhanam (2002), Santhanam and Perumal (2005) and Rajkumar and Vasagam (2006). Several workers successfully carried out experiments on the larval rearing of fish fed with copepods. Doi et al. (1997) observed the feeding performance and growth of larvae of red snapper *Lutjanus argentimaculatus* fed with nauplii of *Acartia sinjiensis*. They observed good growth and feeding intake in copepod fed larvae compared to *Brachionus* sp. Nanton and Castell (1999) observed significantly higher growth in haddock larvae fed with copepods compared to those fed with rotifers. The use of copepod *Tisbe* sp. nauplii as a supplement to a basic rotifers diet also enhanced the feeding rates and increased the growth and survival of the first feeding turbot larvae (Stottrup and Norsker 1997). Payne et al. (1998) studied the larval rearing of pipefish and they observed good growth and 99% survival with copepods diet. Barlow et al. (1993) observed the maximum growth and survival of larvae of barramundi (*L. calcarifer*) reared in ponds and, thus, it is necessary to have abundant zooplankton while the fish grow from 10 to 40 mm TL. More recently, Stottrup (2000) studied the nutritive value and live food suitability of marine copepods for fish larvae and also gave an account on the advantages of copepods used in larviculture.

Conclusion

The present results obviously direct that the marine copepods *Nannocalanus minor* and *Paracalanus parvus* could be considered as a promising and nutritionally superior alternative live feeds to potentially replace the existing nutritionally doubtful traditional live feeds such as rotifer *B. plicatilis* and brine shrimp *Artemia nauplii*. Furthermore, it was evident that Asian seabass larvae could actively feed on the copepods than traditional feeds. The present study established that the copepods *N. minor* and *P. parvu*s both can be considered as a potential live feed for rearing of Asian seabass *L. calcarifer* larvae.

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Assessing the Efficacy of Marine Copepods as an Alternative First Feed for Larval Production of Tiger Shrimp *Penaeus monodon*

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Introduction

Intensive production of marine shrimp is mainly depending on live prey in rearing of the first feeding shrimp larval stages. Commonly rotifers and brine shrimps are the primary live feed for the shrimp, and commercial shrimp production has conventionally acclimatized with this. Because, commercial scale need fast growing and high reproductive rates live pray and they mainly depend on rotifers which fulfil all the requirements. At the same time, brine shrimp *Artemia* can be collected in nature and stored as cysts until needed. Regrettably, still marine larviculture faces an unbalanced live feed which contains low nutritional compositions, and it reflects in shrimp larval survival and their disease resistance capability. While there is a mass production of shrimp larvae, the high and fluctuating costs of *Artemia* push to find an alternative live prey such as copepods (Abate et al. 2015; Drillet et al. 2008).

Copepods are dominant metazoans inhabiting coastal and oceanic environment. Polyunsaturated fatty acids (PUFAs) were found high in copepods (Støttrup et al. 1999). The polyunsaturated fatty acids such as docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) were dominant in the copepods and also for reproduction and growth of copepods; DHA and EPA are

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considered as essential (Klein Breteler et al. 1999). HUFAs are necessary for copepods for their ontogenetic development (Tang et al. 2001) and positively influence egg production rates, and these constituents are therefore regarded as key for shrimp and fish larval performance (Kattner et al. 2007). In the present chapter, the effects of two copepods (*N. minor* and *P. parvus*) as live food on survival, growth and pigmentation of black tiger shrimp, *Penaeus monodon*, post larvae were compared with *Artemia* nauplii and rotifer.

Materials and Methods

Microalgae Culture

Several species of microalgae were cultured according to the procedure explained in Chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production" to feed copepods.

Rotifer and Artemia *Culture*

For comparative study of shrimp larval growth and survival with reference to copepod, rotifer and *Artemia* were cultured separately according to the procedure explained in Chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Isolation and Culture of Copepods

Collection, isolation and intensive culture of marine copepods species such as *Nannocalanus minor* and *Paracalanus parvus* were cultured in the laboratory adopting the standard procedure as explained in Chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Biochemical Analysis of Live Feed and Shrimp Larvae

The estimation of biochemical composition such as moisture, protein, carbohydrate, lipid, ash, amino acids and fatty acids in live feed and shrimp larvae was estimated according to the standard procedure as explained in Chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Larval Rearing of Tiger Shrimp Penaeus monodon

The Post Larvae (PL 10) *P. monodon* were obtained from the Rank Marine Hatchery (Marakkanam, Pondicherry, India) and stocked at a density of 50 PL L-1. An air compressor was used to provide constant aeration to each FRP tanks. The FRP tanks were maintained under a12:12 L: D cycle. Shrimp PL were fed at 8 h interval daily with rotifer *B. pilicatilis and Artemia nauplii* and copepods *N. minor* and *P. parvus* in the density of 8–10 individuals ml-1. Salinity was maintained at 28‰. The experiment lasted for 21 days. The shrimp larval sampling was made every 7 days once for morphometric analyses (Length, Weight, and Survival). Shrimp growth rate was calculated from the body weight (mg) based on the formula derived from Ricker (1979): G = (W2–W1)/(T2–T1), where W2 and W1 represent the final and initial weight of the shrimp, respectively, and (T2–T1), the duration of the experimental period. Survival was calculated as the percentage of shrimp remaining in each tank from the estimated number stocked initially. For length (L) analyses, initial length (IL) of shrimp was subtracted from final length (FL) with help of following formula (L = FL–IL).

Statistical Analysis

The results obtained were statistically analysed using analysis of variance (ANOVA) test with species length, wet weight and survival rate.

Results

Growth and Survival of Tiger Shrimp, P. monodon *Larvae*

The length and weight of copepod *N. minor*-fed shrimp *P. monodon* larvae (Plate 1c) were comparatively higher than rotifer- (Plate 1a) and *Artemia* nauplii-fed larvae (Plate 1b). The total mean length of *N. minor*-fed larvae was reported as 21.0 ± 0 mm, 28.5 ± 0.5 mm and 34.86 ± 1.09 mm on 7^{th}, 14^{th} and 21^{st} day, respectively, and weight was 24.83 ± 0.28 mg, 29.5 ± 0.5 mg and 36.16 ± 3.32 mg on 7^{th}, 14^{th} and 21^{st} day, respectively. The *N. minor*-fed shrimp larvae showed a remarkable survival of 100 ± 0%, 100 ± 0% and 98.5 ± 0.51% on 7th, 14th and 21st day, respectively (Table 1). The length, weight and survival of rotifer- and *N. minor*-fed shrimp larvae showed significant result at $P < 0.01$ except initial length, weight, 7^{th} day survival and final day survival which are not significant. However *Artemia* nauplii- and *N. minor*-fed shrimp larvae showed significance at $P < 0.01$, whereas the initial length, weight and 7^{th} day and final day survival were not significant (Plate 1c).

Plate 1a Rotifer *B. plicatilis*-fed *Penaeus monodon* larva (21st day)

Plate 1b *Artemia* nauplii-fed *Penaeus monodon* larva (21st day)

Plate 1c Copepod *N. minor*-fed *Penaeus monodon* larva (21st day)

Plate 1d Copepod *P. parvus*-fed *Penaeus monodon* larva (21st day)

The tiger shrimp, *P. monodon*, grew faster at *P. parvus* (Plate 1d) compared to rotifer and *Artemia* nauplii. The mean total length of copepod *P. parvus*-fed post larvae of *P. monodon* was 21.66 ± 0.57 mm, 28.66 ± 0.57 mm and 35.16 ± 1.25 mm on 7th, 14th and 21st day, respectively. Similarly the mean weight of *P. parvus*-fed larvae were 25.23 ± 1.07 mg, 30 ± 1 mg and 36 ± 3 mg on 7th, 14th and 21st day(s), respectively. The survival of copepod *P. parvus*-fed shrimp larvae showed good survival. Here the 7th, 14th and 21st day survival of *P. parvus*-fed shrimp larvae were 100%, 100% and 98.93 ± 0.9%, respectively (Table 1). The final average length and weight of rotifer- and *Artemia* nauplii-fed shrimp were 24.33 ± 0.57 mm and

Table 1 Growth and survival of tiger shrimp *P. monodon* fed with different live feeds

Feeding regimes	Initial		7th day			14th day			21st day		
	Length (mm)	Weight (mg)	Length (mm)	Weight (mg)	Survival (%)	Length (mm)	Weight (mg)	Survival (%)	Length (mm)	Weight (mg)	Survival (%)
B. plicatilis	12.3 ± 0.2	11.3 ± 1.5	14.0 ± 2.0	12.1 ± 0.7	99.3 ± 1.1	16.4 ± 0.4	15.8 ± 1.0	98.0 ± 0.1	24.3 ± 0.5	18.8 ± 0.2	97.93 ± 0.30
Artemia	12.3 ± 0.2	11.3 ± 1.5	16.0 ± 1.0	13 ± 1.7	99 ± 1.0	19.0 ± 1.0	18.0 ± 2.6	98.3 ± 0.5	28.9 ± 0.0	21.3 ± 1.5	98.2 ± 0.34
N. minor	12.66 ± 0.5	11.46 ± 0.4	21.6 ± 0.5	25.2 ± 1.0	100 ± 0.0	28.6 ± 0.5	30.0 ± 1.0	100 ± 0.0	35.1 ± 1.2	36 ± 3.0.0	98.9 ± 0.90
P. parvus	12.66 ± 0.5	11.46 ± 0.4	21.0 ± 0.0	24.8 ± 0.2	100 ± 0.0	28.5 ± 0.5	29.5 ± 0.5	100 ± 0.0	34.8 ± 1.0	36.1 ± 3.3	98.56 ± 0.51

18.83 ± 0.28 mg and 28.93 ± 0.05 mm and 21.33 ± 1.52 mg, respectively. The final day survival of *P. monodon* post larvae fed with rotifer and *Artemia* nauplii were 97.93 ± 0.30% and 98.2 ± 0.34%, respectively. The 21st day survival of copepod-fed shrimp larvae was higher (98.93 ± 0.90%) than rotifer- and *Artemia* nauplii-fed one (Table 1). ANOVA results showed that the rotifer- and *P. parvus*-fed shrimp larval growth and survival were significant at $P < 0.01$ excluding initial length, weight and 7th and final day survival which are not significant. Similarly *Artemia* nauplii- and *P. parvus*-fed shrimp larvae growth and survival were significant at $P < 0.01$ except initial length, weight and 7th and final day survival which are not significant.

*Biochemical Composition of Shrimp (***P. monodon***) Larvae*

The moisture, protein, carbohydrate, lipid and ash contents of copepod *N. minor*-fed *P. monodon* larvae were 82.45%, 65.85%, 16.5%, 11.18% and 6.51%, respectively. The results on biochemical composition analysis can clearly indicate that high level of protein (65.99 ± 0.01%) and lipid (16.33 ± 0.57%) was obtained in *P. parvus*-fed larvae than those fed with rotifer and *Artemia* nauplii. The rotifer-fed larvae were found to have 63.73 ± 0.25% of protein and 10.4 ± 0.62% of lipid. The protein (64.06 ± 0.29) and lipid (11.46 ± 0.5) content of *Artemia* nauplii was higher than that of rotifer (Table 2).

The highest amino acid content of 52.81% was recorded in *N. minor*-fed larvae of *P. monodon*. In *B. plicatilis*-fed larvae, the amino acid content was 30.63%, and the *Artemia*-fed larvae contain 33.67%. The amino acids such as isoleucine (6.2%), glutamic acid (5.67%), lysine (5.65%), aspartic acid (4.98%), argentine (4.84%) and alanine (4.68%) were reported to be high in copepod *N. minor*-fed *P. monodon* larvae. The amino acid level of *P. monodon* larvae fed with *P. parvus* was 61.36%. Among these total amino acids, the components such as glutamic acid, aspartic acid, isoleucine, alanine, argentine and lysine were reported as predominant with the percentages of 6.78, 6.12, 5.65, 5.18, 5.14 and 5.12, respectively (Table 3).

Total amino acid content of rotifer-fed *P. monodon* larvae was 30.63%. The amino acids such as glutamic acid, glycine, cystine and valine were reported as major in the percentages of 3.6, 3.04, 2.98 and 2.24, respectively. Similarly *Artemia* nauplii-fed larvae contained the components like alanine, glutamic acid, threonine, histidine and glycine in the percentages of 4.34, 3.42, 3.42, 2.34 and 2.34, respectively (Table 3).

Table 2 Biochemical composition of *P. monodon* fed with different live feeds

Feeding	Moisture (%)	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)
Initial	80.26 ± 0.42	63.48 ± 0.42	10.15 ± 0.25	13.5 ± 0.44	12.87 ± 0.07
B. plicatilis	81.39 ± 0.29	63.73 ± 0.25	10.4 ± 0.62	12.41 ± 0.44	13.46 ± 0.14
Artemia nauplii	81.12 ± 0.24	64.06 ± 0.29	11.46 ± 0.5	14.07 ± 0.24	10.37 ± 0.27
N. minor	82.0 ± 0.21	65.85 ± 0.16	16.53 ± 0.15	11.18 ± 0.05	6.51 ± 0.27
P. parvus	81.5 ± 0.62	65.99 ± 0.01	16.33 ± 0.57	11.13 ± 0.21	6.55 ± 0.41

Table 3 Amino acid composition of *P. monodon* fed with different live feeds

Amino acids	Initial larvae	Rotifer-fed larvae	*Artemia*-fed larvae	*N. minor*-fed larvae	*P. parvus*-fed larvae
Arginine	2.87	2.18	2.04	4.84	5.14
Histidine	0.69	0.42	2.34	2.54	3.32
Isoleucine	0.18	2.34	1.56	6.2	5.65
Leucine	2.94	1.87	2.12	3.24	3.78
Lysine	2.54	2.98	2.41	5.65	5.12
Methionine	1.37	2.22	1.23	2.78	3.14
Phenylalanine	1.34	1.94	1	2.98	2.98
Threonine	0.96	0.82	3.42	1.36	2.13
Cystine	0.01	0.11	1.3	1	1.98
Valine	2.13	2.24	2.56	1.9	3.1
Alanine	2.14	2.61	4.34	4.68	5.18
Aspartic acid	1.96	2.71	1.43	4.98	6.12
Glutamic acid	3.24	3.16	3.42	5.67	6.78
Glycine	2.41	3.04	2.34	2.9	3.4
Serine	1.78	0.81	0.62	0.25	1.2
Tyrosine	1.44	1.18	1.54	1.84	2.34
Total	28	30.63	33.67	52.81	61.36

Totally 18 fatty acids were reported in copepod *N. minor*- and *P. parvus*-fed larvae followed by *Artemia* nauplii-fed larvae with 15 fatty acids and rotifer-fed larvae with 13 fatty acids. The results indicated that the fatty acids such as 16:00, 16:01 and 18:00 were found to be dominant in the percentages of 29.31, 8.84 and 6.51 at rotifer-fed larvae. Similarly 16:00, 18:1-n9 and 18:01 were higher in the levels of 28.91%, 22.61% and 10.88%, respectively, in *Artemia*-fed larvae. The fatty acid profile of *P. monodon* larvae fed with *N. minor* was comparatively higher. Eighteen fatty acids were recorded in the present study. Among these, fatty acids like 16:00 (8.56%), 18:01 (8.53%) and 22:00 (7.14%) were recorded more when compared with other components. The high amount of EPA and DHA was recorded in copepod, *N. minor*-fed larvae. The EPA and DHA concentrations were 8.13% and 9.71%, respectively. It is several folds higher than rotifer- and *Artemia* nauplii-fed larvae. The total fatty acid content of 100% was reported in *P. parvus*-fed larvae with dominance of 18:1-n9 (11.57%), 22:6-n3 (11.34%), 20:5-n3 (9.89%), 22:00 (6.53%) and 20:01 (6.1%). The highly unsaturated fatty acids (HUFAs) such as EPA and DHA were higher in *P. parvus*-fed shrimp larvae than rotifer- and *Artemia* nauplii-fed animals. The copepod, *P. parvus*-fed larvae showed high level of EPA (9.89%) and DHA (11.34%) than those of rotifer-fed larvae (2.47% and 0.26%) and *Artemia* nauplii-fed larvae (3.68% and 1.41%). The detailed result on fatty acid composition of tiger shrimp *P. monodon* was given in Table 4.

Table 4 Fatty acid composition of *P. monodon* fed with different live feeds

Fatty acids	Initial larvae	Rotifer-fed larvae	*Artemia*-fed larvae	*N. minor*-fed larvae	*P. parvus*-fed larvae
12:00	0.618	2.98	0.44	31	27
14:00	11.25	29.31	28.91	8.56	4.35
15:00	–	–	1.29	1	0
16:00	2.72	6.51	–	1.89	1.8
17:00	–	–	0.007	–	0
18:00	0.647	2.98	–	4.12	6.1
20:00	–	–	0.099	1.4	1.89
21:00	0.002	–	0.005	7.14	6.53
22:00	–	0.29	0.003	0.65	1.54
24:00	1.01	–	–	0.14	2.12
14:01	3.43	8.84	–	5.46	4.67
16:01	4.48	–	10.88	8.53	3.89
18:01	4.21	12	22.61	9	11.57
18:1-n 9	–	–	3.38	1.25	1.62
18:3-n 3	1.02	0.65	–	–	0.5
18:3-n 6	2.86	4.34	0.11	0.2	0
18:2-n 6	–	–	–	0	0.23
20:2-n 6	0.01	–	–	0.66	1.17
20:4-n 6	–	0.61	0.003	0	1.47
20:4-n 5	–	2.47	3.68	8.13	9.89
20:5-n 3	0.21	0.26	1.41	9.71	11.34
22:6-n 3	0.09	0.3	2.47	1.16	2.32
22:01	0.618	2.98	0.44	31	27
Total	32.557	71.54	75.297	100	100

Discussion

Feeding experiments of *P. monodon* larvae fed with *N. minor* and *P. parvus* showed better survival and growth than with rotifer *B. plicatilis* or even with *Artemia* nauplii (Table 1). This may be due to the higher levels of required essential fatty acids (EPA and DHA) of copepod, *N. minor* and *P. parvus*. Similar statements were documented by earlier workers like Watanabe et al. (1989), Kitajima et al. (1979), Read (1981) and Reigh and Stickney (1989) in *Macrobrachium rosenbergii* with freshwater copepods. In larval rearing experiments on the larvae of *P. monodon* with four types of live-food organisms, *B. plicatilis*- and *Artemia* nauplii-fed animal showed lower growth than those of *N. minor* and *P. parvus* feed types. The lower growth and survival of larvae fed with *Artemia* nauplii might be due to the low nutritional value of *Artemia* nauplii. The results also suggested that the calanoid copepods, *N. minor* and *P. parvus*, are a better source of nutrients, as they supply all required essential nutrients (as evidenced by their biochemical profile amino acid and fatty acid content).

The low survival rate of larvae of *P. monodon* was noticed with *Artemia* nauplii diet. But the higher survival rate of *P. monodon* was observed with copepod *N.*

minor and *P. parvus* diet. Earlier investigations also noticed higher survival and weight gain in the larvae of *P. monodon* when fed with zooplankton. They also reported that laboratory-cultured *Acartia plumosa* were successfully used as a supplementary feed in the place of *Artemia* diet in hatchery. *P. monodon* larvae fed with *Artemia* nauplii showed slightly higher survival rate than those of rotifer, *B. plicatilis*, and low survival rate than copepod-fed larvae which might be due to the nutrient deficiency. Lovett and Felder (1988) and Santhanam et al. (2004) also expressed the similar trend in *P. monodon*.

The recorded higher amino acid content in *N. minor*- and *P. parvus*-fed larvae might be due to the higher concentration of amino acids available in copepods than those in *B. plicatilis* and *Artemia* nauplii. The deficiency of histidine, phenylalanine, methionine and threonine in *Artemia* nauplii may be the reason for lower growth and survival obtained in shrimp larvae fed with *Artemia* nauplii. The amino acid content was low in *B. plicatilis*- and *Artemia* nauplii-fed animal than those fed on *N. minor*- and *P. parvus*-fed larvae, and this may be the reason for decreased growth and survival recorded in *P. monodon* larvae fed on *B. plicatilis* and *Artemia* nauplii. Barlow et al. (1995) have reported similarly. The glutamic acid level was always higher in *B. plicatilis*, *Artemia* nauplii and *N. minor* and *P. parvus*. The cystine concentration is very much low in all the live-food organisms than other amino acid compounds.

The amino acid content of the *N. minor*- and *P. parvus*-fed larvae of the *P. monodon* was comparatively higher than the *B. plicatilis*- and *Artemia* nauplii-fed animals. The amino acid content was higher in initial samples of the *P. monodon* than the final samples of the *Artemia* nauplii-fed larvae which may be due to the higher metabolic rate of early development of *P. monodon*. *P. monodon* larvae fed with *N. minor* and *P. parvus* showed the higher growth and survival than the *B. plicatilis*- and *Artemia* nauplii-fed larvae, and it might be due to the amino acid superiority. Similar observations were reported earlier by Lovett and Felder (1988). Safiullah (2001) reported similar result for freshwater prawn, *Macrobrachium rosenbergii*, fed with freshwater copepods, *Mesocyclops aspericornis* and *Sinodiaptomus indicus*. The low content of arginine, methionine and phenylalanine may be the reason for low growth and survival noticed in *P. monodon* larvae fed with *Artemia* nauplii, and this is in line with the findings of Cowey and Forster (1971) and Watanabe et al. (1989). The present experiments suggest that copepods can form good live feed for the larvae of *P. monodon*. When the larvae are stocked in the tank, they can actively feed on the copepods by improving their growth and survival.

Conclusion

The commercial production of shrimp in nowadays has been highly hampered by vulnerable diseases and be short of suitable feed. Shrimps appearing bright are generally considered to be of good quality. So developing a feed that has high pigmentation value is equally essential as a nutritionally good feed. The present chapter

dealt with the assessing the efficacy of marine copepods *N. minor* and *P. parvus* as first feed on shrimp larvae *Penaeus monodon*, and the results were compared with other live feeds like *Artemia* and rotifer. The results revealed that the shrimp PLs fed with copepod had high growth rate and higher survival from PL fed on *Artemia* and rotifer, which indicate that copepod has potential to be used as live feed for the hatchery rearing of *P. monodon* PLs in replacing *Artemia* and rotifer. Further study is needed on optimization of wastewater-cultured copepod as a live feed to yield maximum growth and survival. The present study concluded that using *N. minor* and *P. parvus* served as potential live feed for successful larval production of tiger shrimp *P. monodon* in indoor level and aqua hatcheries. Further, the present study gives proof to convert the tiger shrimp *P. monodon* for ornamental purpose since it grew with maximum pigmentation when fed with *N. minor* and *P. parvus*.

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Biofloc-Copefloc: A Novel Technology for Sustainable Shrimp Farming

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Introduction

Since the 1990s, aquaculture has become the fastest growing animal food-producing industry in the world (FAO 2014). It has experienced a tremendous growth in productivity during this period (Asche et al. 2008, 2013). Thus, aquaculture has been one of the most promising animal food-producing sectors, with the lowest feed conversion ratio (Smil 2011), while providing sufficient and highly nutritional food for a growing world population. Particularly, the industry will play a crucial role in meeting increasing demands for healthier animal proteins and lipids. Thus, the contribution of aquaculture in alleviating obesity and its resultant health and social benefits is clear and should not be underestimated.

However, despite these positive attributes and success stories, the aquaculture industry has also faced many challenges, including environmental issues (Asche et al. 1999), fish meal (feed) problems (Naylor et al. 2000), disease outbreaks (Asche et al. 2009) and price volatility (Oglend 2013). If aquaculture is to live up to its promises, it must follow a sustainable production line. Among other things, sustainability should be achieved in an economically efficient and cost-effective way.

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To this end, potential barriers should be identified throughout the entire production cycle, and better practices and innovations have to be implemented accordingly.

One of the sub-sectors in the aquaculture industry that requires substantial advances is nutrition and feeds for farmed fish and shrimp. These nutrition-related issues must be considered in order to achieve a balance in safe and nutritious food production and sustainability in aquaculture. Better practices and innovations in larval rearing are particularly necessary for major upscaling in the production of existing species and for efficacious farming of "new" species (Dhert et al. 2001; Mahjoub et al. 2013). Larval rearing is the backbone of the aquaculture industry because it is the stage at which the process begins. The success of larval rearing is, in turn, significantly dependent on larval feeding, which needs substantial improvements in itself (Dhert et al. 2001; Turingan et al. 2005). As formulated feed has not yet been effectively developed, the role of live feeds for the success of larval rearing is absolutely vital (Dhert et al. 2001). Live feeds are crucial given the fact that the lion's share of commercially farmed species (such as turbot, shrimp and grouper) needs live feeds for the first feeding (Mahjoub et al. 2013). Hence, to sustain productivity growth and introduce "new" farmed species in the aquaculture industry, the larval rearing stage has to be enhanced and better practices should be adopted. To this end, the focus ought to be on live feeds, as the success of larval feeding relies directly on improvements in live feeds.

As the human population continues to grow, food production industries such as aquaculture will need to expand as well. Shrimp farming has become competitive and, as such, the technology utilised needs to be efficient in all aspects, viz. productivity, quality, sustainability, biosecurity and commercial surety. In order to preserve the environment and its natural resources, this expansion will need to take place in a sustainable way. The prime goals of aquaculture expansion must be to produce more aquaculture products without significantly increasing the usage of natural resources, such as land and water; to develop sustainable aquaculture systems that will not damage the environment; to establish systems providing an equitable cost/benefit ratio to support economic and social sustainability. All these prerequisites for sustainable aquaculture development can be met by biofloc-copefloc technology.

Global aquaculture production has increased in recent years and it is predicted that aquaculture will provide the most reliable supply of seafood in the future. However, there are many controversial issues in aquaculture regarding food safety, nutrition and sustainability, many of which are directly related to nutrition and feeds for farmed shrimp. These nutrition-related issues must be considered in order to achieve a balance in safe and nutritious food production and sustainability in aquaculture. This chapter highlights recent studies and discusses new and innovative aspects of shrimp farming. Some issues in the arena of shrimp nutrition require consideration and improvement, such as feed and nutrient efficiency, overfeeding and waste, fish meal and fish oil replacements, fish health, biotechnology and human health concerns. The findings reviewed in this chapter show promise towards improvement of the aquaculture industry through innovative farming technology. This review is an update in shrimp nutrition research and provides insight on the progression and evolution of this field in order to meet the needs of the industry, with the purpose of achieving a balance in seafood production and environmental

sustainability. The outcome of this review encourages the use of biotechnology as a tool to meet seafood production and environmental sustainability, in order to ensure global food security in the future and to improve our use of resources.

What Is Biofloc-Copefloc?

Biofloc-copefloc is a new shrimp farming technique introduced in Thailand, developed on the basis of biofloc technology; an advantage of floc particles is the use of natural food as the main food for shrimps, which is a strictly non-food industrial use. Biofloc-copefloc technology of the recirculation aquaculture system is the most advanced technology in the shrimp farming industry that provides natural live diets of "copepods" for post-larvae prior to stocking, aids pond water stability, enhances the survival rate, has the fastest growth rate, is highly profitable, totally sustainable and does not cause any destruction to our environment.

Why is Biofloc-Copefloc Technology Needed?

It is well accepted that the Biofloc-Copefloc technology is highly essential in the current scenario for various reasons includes: To produce more aquaculture products without significantly increasing the usage of the basic natural resources of water and land. To develop sustainable aquaculture systems that will not damage the environment. To build up systems providing an equitable cost/benefit ratio to support economic and social sustainability.

Advantages of Biofloc-Copefloc

Biofloc-copefloc technology is considered as an efficient alternative system since nutrients could be continuously recycled and reused. Free ammonia in water is converted into biomass protein in heterotrophic microorganisms, gathered into biofloc particles suspended in water. They improve the level of biosecurity, reduce the risk of infection due to water exchanges, do not pollute the environment, reduce the cost of food, drugs and chemicals, and shrimp grow faster. A simple but strictly non-food industrial use can put shrimp PL10–12 directly into the pond without acclimatisation. There is no need for periodic water exchange, which increases energy costs and also increases the risk of infectious pathogens from water supplies. There is also no need for any kind of filter system, which requires complex equipment, operating costs and high technical capabilities. The copefloc system is able to improve the environment as it creates natural food biomass and contributes reusable waste nutrients from aquatic animals, a fairly simple operation. Copefloc technology completely obviates the use of chemicals or antibiotics, thus improving the quality of products and goods.

Food Source for Shrimp

Feeds used in this system are mainly grain and natural food items like microalgae, copepods and amphipods. These foods are nutritious and very good for the growth of shrimp. Copepods are a very important food for fish and shrimp, especially in the childhood stage, and microbes can transform organic waste into higher protein content.

Mapping Process of Food for Shrimp with Copefloc

Copepods will be inoculated in ponds with depths in the range 1.2–1.5 m. Oxygen enrichment will be done by strong aeration for 24–48 h. Rice bran at 300 kg/ha will be fermented with probiotic placed in a tubular cheese cloth bag at the bottom of the pond. Continuous aeration will be carried out for 7–10 days to develop a favourable environment for copepods in the pond. Biofloc primer solution will be prepared by fermenting the mixture at a ratio of 1 L of water, 10 g cereal (fishmeal, soybean meal etc.) and 10 mL of cultured pure strain of *Bacillus subtilis* with bacterial density 10^6/mL. Fermentation will be performed under conditions of strong aeration for 48 h, maintaining the pH at 6.0–7.2 by the addition of a buffer pH, at temperatures in the range 25–28 °C. When microorganisms thrive, large bubbles floating on the surface will be formed and biofloc bait is added to the pond.

Culture of Probiotic Bacteria *Bacillus subtilis*

The probiotic bacteria *Bacillus subtilis* is cultured according to Zokaeifar et al. (2012). *Bacillus subtilis* will be grown in Lysogeny broth using a shaking incubator at 30°C for 48 h. The cultures will then be centrifuged at 3000 × g for 10 min at 4 °C and, after discarding the supernatant, the pelletted bacteria will be re-suspended and washed three times in sterile normal saline solution (NSS, 0.9% NaCl). The cell densities of the suspensions will be calculated using a spectrophotometer at 600 nm and also correlated to the colony-forming units (CFUs) using the spread-plate technique. These suspensions will be used as the inoculum for mass culture in the shrimp pond.

Culture of Microalgae

The microalgae culture is done according to Perumal et al. (2015). In brief, the algal stock culture will be maintained in a special air-conditioned room. Stock cultures will be kept in 1- and 2-L culture flasks, and 5- and 15-L plastic containers.

The water will be filtered by using a filter bag (1 micron), sterilised by using an autoclave and, after cooling, transferred to the culture flask. Culture flasks will be plugged with cotton or covered by aluminium foil. All vessels used for algal culture will be sterilised properly and dried in an oven before use. The culture medium used for indoor stock culture of microalgae will be selected depending on the species. About 10 mL of the inoculum in the growing phase will be transferred to the culture flasks and the culture will be provided with 1000 lux light and 12:12 h light and dark cycle. After 8–10 days, the maximum exponential phase will be obtained. The temperature and salinity will be maintained between 23 and 25 °C and 28 and 30 ppt, respectively, for the entire culture period. Continuous aeration will be provided for culture. After 8–10 days, when the maximum exponential phase has been reached, light will be reduced to 500 lux for further growth. The time required for the maximum cell densities varies depending on the species. Under controlled conditions of light and temperature, with or without aeration, the algae will grow. At the time of the maximum exponential phase of growth, the colour of the culture turns dark green. A maximum of five culture flasks will be kept for each species as stock culture.

For large-scale production of microalgae, fibreglass tanks will be used. For efficient growth of algae, commercial fertilisers, namely ammonium sulphate, superphosphate and urea at a ratio of 10:1:1, will be added. For 100 L of water, 2 L of inoculum will be added to the culture tank. Continuous and vigorous aeration will be provided to the culture and the culture will always be kept in suspension.

Culture of Copepods

The copepods are cultured according to Santhanam et al. (2015). Copepods will be collected from freshwater, brackish water and the marine environment using a plankton net with mesh size 158 μm. After collection, the mixed zooplankton samples will be screened using a set of superimposed sieves of varying mesh sizes, with decreasing mesh size from upstream to downstream. Zooplankton samples will be screened coarsely through a 500-μm mesh to remove fish and prawn larvae. Then, the samples will be screened through a 190-μm mesh to remove rotifers and nauplii of copepods. Finally, samples containing predominantly adult, late-stage copepodids and egg-bearing female copepods will be collected using a fine brush, needles and a Stempel pipette under a microscope. Females will be selected for the purpose of making the culture monotonous and free of contamination from other copepods.

A known number of copepods, including males and females or gravid females, will be isolated using a fine brush, needles and a Stempel pipette. Isolated copepods will be stocked initially in 250-mL glass beakers and conical flasks provided with microalgae without aeration. Later, copepods will be sub-cultured into 7-L plastic containers filled with filtered water and vigorous aeration will be instigated. Then, the copepods will be transferred to an oval-shaped, flat-bottomed fibreglass tank filled with 100 L of filtered water and vigorous aeration invoked for mass culture. The water quality parameters, such as temperature, salinity, pH and dissolved oxygen,

will be maintained in the ranges 26–30 °C, 28–32‰, 7.5–8.5 and 5.0–7.5 mL/L, respectively. Copepods will be fed with a daily ration of mixed microalgae, viz. *Chlorella marina*, *Dunaliella salina*, *Isochrysis galbana* and *Nannochloropsis* sp., at a concentration of 25,000 cells/mL.

Pilot-scale Culture of Copepods

The large scale culture of copepods will be done by using different capacity FRP tanks. The FRP tanks will be washed with a low-residue laboratory detergent (e.g. Alconox or Sparkleen) and water, followed by thorough rinsing. Then, the tanks will be treated with 100% muriatic acid (HCl) solution, followed by thorough rinsing with filtered water. The tanks will be leached three times (24 h each time) to remove all water-soluble remnants of the manufacturing process. The tanks will be filled to the rim with filtered, UV-treated water and the salinity adjusted as needed, and water will be chlorinated with 60 mL (0.2 mL/L) commercial 10% hypochlorite solution per litre. The treated system will be allowed to stand for 24 h. Thereafter, the system will be de-chlorinated with 60 mL of stock thiosulfate solution. Vigorous aeration will then be started. After an hour, a "free chlorine" test strip will be dipped and zero "free chlorine" remaining will be verified. The treated water will be aerated to at least 6 mg/L dissolved oxygen (DO).

The water conditions, such as salinity, pH, DO, colour and scent (particularly the "rotten egg" smell of H_2S), will be checked prior to collection and treatment. The water will be serially filtered through 50, 10 and 1-µm mesh bags followed by sand filter, carbon filter, biological filter and then passed through an ultraviolet steriliser. The filter bags will be cleaned and then sanitised overnight in hypochlorite solution once a week under normal usage. Filtered, UV-treated water will be used directly to culture copepods. The filtered water will be treated with 10% commercial hypochlorite solution at 0.2 mL/L and left to stand overnight without aeration. After that, the water will be dechlorinated with thiosulfate solution volume for volume (v/v) at 0.2 mL/L. The dechlorinated water will be used for filling all wash bottles, stacked-sieve holders, harvesting samples, population counts etc.

Pilot-scale copepods culture will start with a clean FRP tank, algae and filtered, UV-treated water. Tanks will be stocked with a known number of gravid females. Gravid females will release the nauplii within 36–42 h. The detritus will be removed daily using graded sieves connected with a siphon hose, and then adults and nauplii will be returned to the tank. The sequential batch cultures will start at 5–7-day intervals for continuous copepods production. Cool, filtered, interior air will be used for aeration. Disposable inline 0.2–µm-pore antibacterial air filters will be used for all aeration. Aeration will be used to maintain algae culture, CO_2 saturation, pH stability and uniform mixing. The stacked-sieve holder and wash bottles will be washed with treated seawater at the culture tank temperature. The siphon hose will be con-

nected to the siphon head and the stacked sieves. The copepods will be harvested and filtered onto a wet free-standing sieve. As the tank's water level drops, frequent rinsing will be done to remove the copepods stuck to the sidewalls. Thirty percent of the tank volume will be exchanged weekly with new treated water.

Culture of Amphipods

Amphipods culture is performed according to Baeza-Rojano et al. (2013). Cylindrical tanks 100 L in capacity will be used for amphipod culture. The tanks will be supplied with running seawater, with a complete water replacement of the tank every hour. Salinity is maintained in the range 37–39 g L − 1 and DO in the range 5–9 ppm. The water temperature fluctuated naturally (25 ± 3 °C) and a natural photoperiod, 14 h light 10 h dark, is used. During the experimental period, amphipods will be fed daily with *Artemia* nauplii (500 mL of seawater with a density of 1400 nauplii/mL) and a mixture of microalgae, such as *I. galbana* and *Tetraselmis suecica* (2 L of seawater with mean densities of 11.9×10^6 cells L^{-1} and 2.9×10^6 cells L^{-1}, respectively). *Artemia* nauplii is previously enriched with a synthetic enrichment market product and, when the *Artemia* and microalgae are added to the tanks, the water inflow is reduced to increase their residence time (Nakajima and Takeuchi 2008). Amphipods are collected from the estuary, coastal intertidal area, mangrove area and backwater. The individuals are isolated from the sediment and are transported with aeration, together with some sediment or oyster shells, to the experimental station. Inside the culture tank, three different plastic meshes of dimensions 30 × 50 cm are used as artificial substratum for the amphipods. The culture is started with a known number of brooders added to the tank (e.g. 125 females and 125 males). Three months after the introduction of the brooders to the tank, the plastic meshes containing amphipods are recovered individually. From these stock cultures, amphipods are mass cultured in larger cement or FRP tanks and further introduced to the shrimp culture pond.

Operations

The biofloc-copefloc system (Fig. 1) does not create any waste, so there is no need to pump the system for waste collection convergence. Instead, the aeration system layout needs to provide more oxygen to the shrimp, microorganisms and natural food in the pond. The copefloc farming technology using a bottom aeration system is designed with small holes drilled on the flank of the PVC pipe, the distance between the holes being in the range 25–30 cm, forming a network with an area covering about 40% of the total area of ponds, instead of the water fan system which

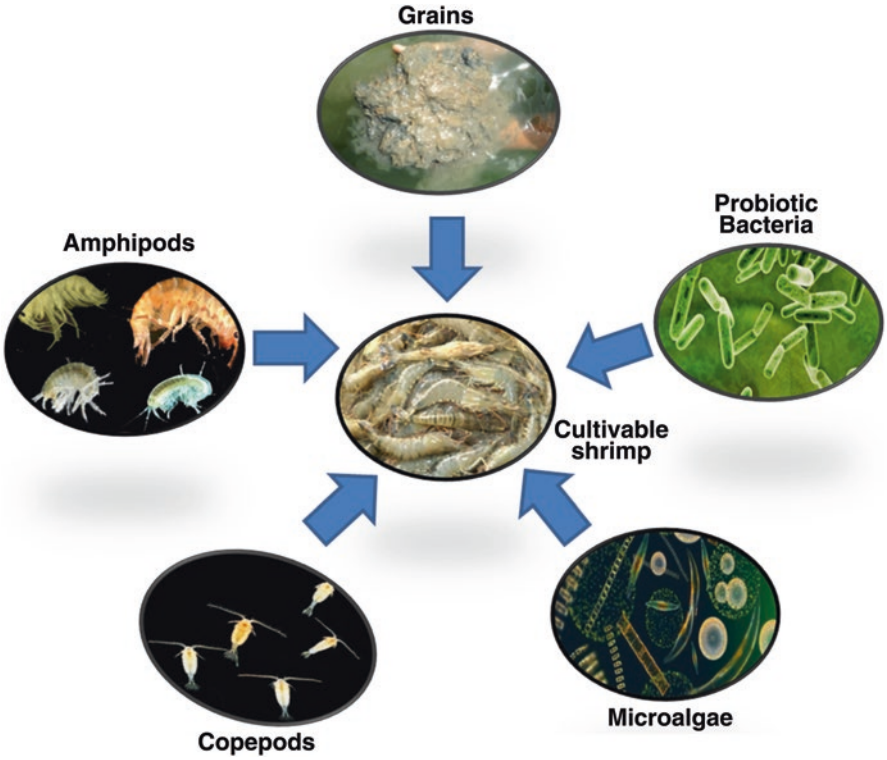

Fig. 1 A typical biofloc-copefloc shrimp farming system

is not or very seldom used. The stocking density is less than 50 individuals/m^2. The shrimp density grows well and is a completely natural feed. During the breeding process, the shrimp do not need to eat. Instead, farmers have to manage and maintain the population and density of natural food, the biofloc volume in the pond. For sampling and calculating the daily copepod density using buckets, 50–100 L of water at different positions in the pond are taken and then filtered through plankton nets with mesh size in the range 50–70 microns, added into 600-mL vials and formalin fixed at 2–4%. One millilitre is pipetted and counted under a microscope at magnifications of 10× and 40× by moving the chamber counting method by coordinates. Then, the density of copepods is computed and the amount of probiotics applied to the pond for feeding copepods and creatures adjusted. To stimulate the formation and maintenance of biofloc stability, a biofloc carbon source primer should supplement and complement the culture system. There are many sources of carbon: cereals, molasses, bagasse pulp, straw, grass. The concentration of biofloc should be maintained at<1 mL/L during the production cycle.

Conclusion

The concept of biofloc-copefloc is gaining increased attention nowadays as a sustainable intensive shrimp farming technology. High stocking densities are now possible with this technique, which combines biofloc-copefloc technology with natural live feed production in ponds, which we call copefloc technology. The copefloc farm will provide some of the rarest insights into such farm design and management concepts as sustainable alternatives for shrimp farming, which are equally applicable to intensive farming for fish.

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Methodologies for the Bioenrichment of Plankton

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Introduction

Aquaculture is expanding worldwide to meet the protein requirements of humans. The basic requirement in culture practice is seed production, while the major constraint is larval nutrition (Imelda 2003). Larviculture—specifically, the initiation of feeding in early larval stages—is a major bottleneck for the industrial scale-up of fish and shellfish cultures. Larval survival also varies with the type of organism, with a rate of <10% in finfish, <1% in mud crabs, <20–40% in shrimp and <20% in molluscs. Evolutionarily, most fish and crustacean larvae are motile prey organisms and encounter problems with the initiation of inert/dry diets. Even if they accept the diets, their poor enzymatic activity and non-functional stomachs will not allow them to digest the existing formulated diets (Pedersen et al. 1987; Pedersen and

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Hjelmeland 1988; Agh and Sorgeloos 2005). Thus, improving the acceptance of dry diets for fish larvae and formulating more digestible and less polluting diets are important tasks for aquaculturists. The challenge in larval nutrition lies in the fact that live feeds are not completely replaced in hatchery operations. Therefore, once this is achieved, live food (phytoplankton and zooplankton) will remain an important food source for the starting of feeding in the early larval stages. Among the important starter feeds used in larviculture are newly hatched nauplii of *Artemia* and rotifer *Brachionus plicatilis*. The successful development of commercial hatcheries and farms has been made possible by several improvements in the production techniques of this live food (Candreva et al. 1996; Dehasque et al. 1998; Agh and Sorgeloos 2005). When compared to rotifers and *Artemia* nauplii, the traditional live feeds provided to marine fish larvae, copepods can improve larval growth and survival and the ratio of normally pigmented juveniles when fed either alone or as a supplement (Kraul 1983; McEvoy et al. 1998; Nanton and Castell 1999). Thus, the ability to culture these organisms at a scale adequate for marine larviculture would present a major step forward for the production of many marine species that require a better suited diet nutritionally than that provided by the traditional live prey (Josianna and Stottup 2006). It is believed that the optimal formulations for the first feeding of larvae should simulate the yolk composition and, to some extent, reflect the nutrient requirements and metabolic capacities of pre-feeding finfish and shellfish of other organisms (Imelda 2003).

Bioenrichment

Bioenrichment, or bioencapsulation, is the process involved in improving the nutritional status of live food organisms, either by feeding or incorporating within them various kinds of nutrients (Imelda 2003). The practical and experimental examples for enrichment diets are unicellular algae (Watanabe et al. 1980), emulsion (Leger et al. 1987), liposomes (Ozkizilick and Chu 1994) and microencapsulated diets (Southgate and Lou 1995; Han et al. 2000). The marine larvae, in general, require omega-3 polyunsaturated fatty acids (PUFA) such as eicosapentaenoic acid (EPA, 20:5n-3) and, even more importantly, docosahexaenoic acid (DHA, 22:6n-3) for their normal development and survival. Apart from EPA and DHA, arachidonic acid (ARA, 20:4n-6) has also been recognised as essential for marine fishes. ARA is a precursor of eicosanoids responsible for osmoregulation, cardiovascular functions, neural control and reproduction. In contrast to freshwater species, most marine organisms do not have the capacity to biosynthesise these essential fatty acids (EFAs) from the lower chain of unsaturated fatty acids, such as linolenic acid (18:3n-3).

In view of the fatty acid deficiency of *Artemia*, research has been conducted to improve its lipid composition by pre-feeding with n-3 highly unsaturated fatty acid (HUFA)-rich diets. It is fortunate, in this respect, that *Artemia*, because of its primitive feeding characteristics, allows a very convenient way to manipulate its biochemical composition. Thus, since *Artemia*, on moulting to the second larval stage

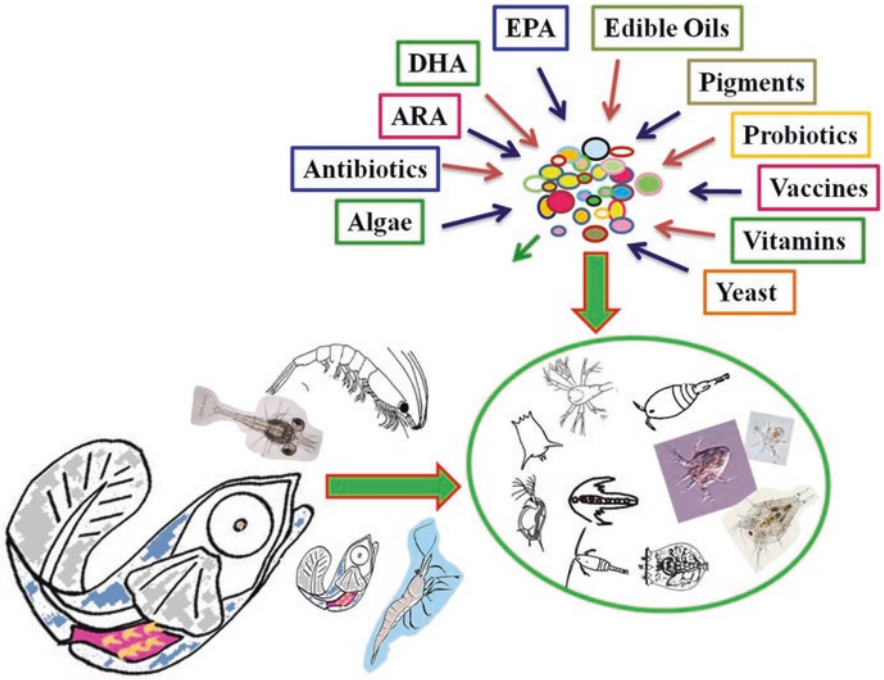

Fig. 1 Bioenrichment of rotifers, copepods, cladocerans and *Artemia* is a vector for the transfer of specific components into the finfish and shellfish cultured larvae

(i.e. about 8 h following hatching), is non-selective in taking up particulate matter, simple methods have been developed to incorporate lipid products into the brine shrimp nauplii prior to offering them as a prey to the predator larvae. This method of bioencapsulation, also called *Artemia* enrichment or boosting, is widely applied in marine fish and crustacean hatcheries all over the world for enhancing the nutritional value of *Artemia* with essential fatty acids (Van Stappen 1996; Agh and Sorgeloos 2005). The bioenrichment or bioencapsulation of rotifers, copepods, cladocerans and *Artemia* is a vector for the transfer of specific components into the finfish and shellfish cultured larvae (Fig. 1).

The rotifer *B. plicatilis* and anostracan *Artemia* vary in lipid class composition, n-3 HUFA content and DHA is practically absent. For this reason, the nauplii need to be enriched before they can be used for feeding freshwater and marine larvae. Providing suitable live food at an appropriate time plays a major role in achieving maximum growth and survival of the young of finfish and shellfish. The appropriate diet is an essential element of success in larval rearing (Watanabe and Kiron 1994; Olivotto et al. 2006). Rotifers and *Artemia* nauplii are regularly used as live food, but contain low concentrations of omega-3 fatty acids and carotenoids. To overcome

these shortfalls, rotifers and brine shrimp for use in feeding larval fishes are often enriched with lipid-containing algae and/or artificial enriching agents (Olivotto et al. 2006; Yamasaki et al. 2007; Seixas et al. 2010). The most important factor governing the nutritional quality of live feeds for aquaculture practices is the essential fatty acid content, particularly EPA and DHA, commonly called HUFA.

The filter feeders (rotifers, *Artemia* nauplii and copepods) have been used as biological carriers for transferring essential nutrients to predator larvae using the bioencapsulation technique (Leger et al. 1986). The nutritional status of live food organisms can be improved through various techniques of enrichment and bioencapsulation. Bioencapsulation in aquaculture can be defined as a process where a live organism incorporates a certain product orally and it becomes a live capsule. The nature of the product given can vary depending on the desires of the aquaculturist. The process is generally directed to the incorporation of some essential elements for the diet (Lovell 1990; Chen 1997).

The enrichment is commonly achieved by placing the nauplii in a medium, generally an emulsion, containing EPA and DHA. Nauplii are passive filter feeders and, thus, incorporate in their digestive tract the emulsions, acting as live vehicles. The degree of success in modifying the fatty acid profile of the nauplii has been shown to be influenced by the type of the enrichment diet, the enrichment conditions and the live food strain itself. The differences in enrichment conditions are related to the salinity of the culture medium, the concentration of experimental emulsion, enrichment duration and temperature following starvation. Also, the species and geographical origin of the *Artemia*, Cladocera and copepods affect the success of the enrichment procedure.

Essential Factors to Be Considered Prior to Bioencapsulation

Several factors are considered to be paramount in the bioenrichment of live feeds: 1. Selection of live feed taking into account the acceptability of the organism and size for common use as: microalgae (2–20 μm), rotifers (5–200 μm), *Artemia* (200–400 μm), *Moina* (400–1500 μm), *Daphnia* (200–400 μm), calanoid copepods (*Acartia* sp., *Paracalanus* sp. and *Calanus* sp.) and cyclopoid copepods (*Oithona* sp., *Paracyclopina* sp. and *Apocyclops* sp.); 2. The nutritional quality, digestibility and acceptability before and after encapsulation; 3. Increasing the level of enrichment media to be incorporated into the carrier organism, which depends on the nutritional quality of the carrier organism before incorporation and is based on feeding trials conducted in the laboratory; 4. A cost-effective likelihood of enrichment; 5. The purity of the culture of the carrier organism; 6. The easy procurement of carrier organisms, possible culture techniques and catchability of the carrier organism by target species, and its easy reproduction. Once all of the above factors are fulfilled, one can obtain a viable and effective enrichment.

Methods of Bioenrichment

Bioenrichment of Artemia *Nauplii with HUFA*

Artemia cysts are hatched under the high light intensity of a plastic tank or cylindroconical containers filled with filtered seawater, with vigorous aeration. Before inoculating the cysts (4 gL^{-1}) in seawater, they are disinfected with hypochlorite solution of 200 $gL^{-1}$and hydrated for 0.30 min in 1000 mL of seawater. Then, the cysts are washed with tap water to remove (disinfect) the hypochlorite and decapsulate them. Half of the cysts per litre of disinfected natural or artificial seawater are incubated for 24 h at a temperature of 28 °C and provided with 2000 lux light and strong aeration. After hatching, the nauplii (<90% instar I) are separated from the empty cyst shells and transferred to 2-L glass tubes (cylindroconical) in a water bath at 28 °C, with continuous aeration supplied from the bottom of the cone using an additional air stone to enable regular monitoring of the oxygen levels and pH. The nauplii are harvested and subjected to counting to determine the survival, i.e. counting of dead nauplii (no Lugol) and total nauplii (+Lugol) from 3 × 250-μL samples per cone. The aeration is stopped and nauplii are concentrated using light. The nauplii are sieved and rinsed well with tap water. The sieved nauplii are transferred into vials and frozen at −30 °C until further processing. Then, the *Artemia* nauplii are enriched using an emulsion containing 30% and 50% of total omega-3 (n-3) HUFA (% of dry matter) with an EPA/DHA ratio of 0.73 and 0.84, respectively, and an emulsion devoid of n-3 HUFA. *Artemia* nauplii enrichment is performed at a temperature of 28 °C and 34 ppt salinity for a period of 24 h. The larvae are then cleaned in water and 28.9 mg g^{-1} enrichment (DHA) could be obtained with 50% n-3 HUFA emulsions at 0.39 L^{-1} (Imelda 2003).

Method of Enrichment of Artemia *Nauplii with Free Amino Acids (FAA)*

The amino acids are the major substrates of aerobic metabolism during the development of the embryo and yolk sac in the larvae of marine and freshwater species, which have pelagic eggs. Due to rapid larval growth and development, there is a large amino acid requirement to both maintain the appropriate concentration in the tissues necessary to obtain an optimal growth rate and amino acid utilisation, and to supply the fuel for energy metabolism. The need to add dietary FAA for marine/freshwater fish larvae is also suggested by the large pool of free amino acids found in marine/freshwater invertebrates, such as copepods, which are natural food (live food) for the larvae in the sea. *Artemia* contain lower levels of FAA, especially methionine. This suggests that methionine is a limiting amino acid for fish larvae when fed *Artemia* nauplii.

One approach to enrich *Artemia* nauplii with water-soluble substances, such as FAA, is to use liposomes. The liposomes are spherical vesicles (10–20 μm) in which an aqueous volume is entrapped by a membrane composed of lipid molecules, usually in the form of phospholipids. These are potent delivery vectors for hydrophilic as well as hydrophobic nutrients and are potential carriers for FAA. The phospholipid from concentric bilayers is dispersed in an aqueous medium enclosing the aqueous material in the core, as well as within the bilayered lamellae. The compatible size range and complete digestibility of liposome make them a good vehicle for the study of nutritional requirements of aquatic filter feeders.

Preparation of Liposome

The egg yolk phosphatidylcholine (PC), cholesterol (10:4 w/w), 100 mg of egg yolk and 40 mg of cholesterol are dissolved in 5 mL of chloroform:methanol (2:1 v/v) in a round-bottomed flask. The solvents are evaporated at 30 °C under nitrogen in a rotary evaporator and the resulting lipid film is further dried for 30 min in vacuum desiccators. 5 mL of 530 mv NaCl solution is added into the flask, along with 5 mL of 300 mm methionine, and rotated slowly for 30 min at 35 °C. The resulting liposome suspension is extruded through a polycarbonate filter (0.6 μm). Liposomes can provide protection against degradation, as well as easier incorporation of the lipids contained in them.

Method for the Enrichment of **Artemia** *Nauplii with Vitamin C*

Standard Enrichment Procedure

Enrichment with vitamin C is a secondary step to enrichment with HUFAs. Therefore, in order to enrich the live food with vitamins, fatty acid emulsion is used as a carrier of this component.

The fatty acid emulsion is prepared as explained earlier; ➔ Add the 10–20% ascorbyl palmitate (AP) w/w to the fatty acid emulsion; ➔ Mix properly using an electric mixer until vitamin C is dissolved [dilute with double distilled filtered water (DDFW) if necessary]; ➔ Preserve the emulsion containing vitamin C in a refrigerator before use; ➔ Nauplii are enriched with two doses of the above mixture at 0.0 h and at 10–12 h during the process of enrichment; ➔ The enrichment process is the same as explained for HUFA; ➔ Stop aeration after 24 h and siphon the nauplii into a clean beaker containing filtered seawater; ➔ Preserve the enriched *Artemia* nauplii in a cold incubator with gentle aeration.

Sample Preparation

➔ The samples should be stored in a deep freezer at −80 °C for vitamin C analysis before the procedure is started or should be processed immediately after

enrichment; ➔ Take a live sample (e.g. enriched *Artemia* nauplii; copepods and cladocerans) and place over a sieve; ➔ Rinse the sample very well with tap water; ➔ Dry the bottom side of the sieve using paper; ➔ Cut the sample into small pieces; ➔ Transfer 0.5–1 g of the sample into a plastic test tube; ➔ Add 100 μL of internal standard (isoascorbic acid) to the test tube; ➔ Add 2 mL of standard solution (1 mm EDTA +2 mm hemocystein) to the sample (in 500 mL of DDFW for fish sample or 1 g MPA + 1 mL acetic acid + 0.3774 g EDTA + 500 mL DDFW for *Artemia* sample); ➔ Homogenise the sample for 1–2 min at 4 °C; ➔ Transfer the supernatant to a clean test tube; ➔ Again, add 2 mL of the above solution to the sample and repeat earlier steps; ➔ Once again, add 1 mL of standard solution and repeat the earlier steps in order to have about 5 mL of supernatant; ➔ Centrifuge the homogenised sample for 5–10 min at 10,000 rpm at 4 °C; ➔ Filter the sample through cartridge powder already conditioned with methanol, DDFW and standard solution; ➔ Now the sample is ready for injection; ➔ Preferentially, three samples should be made available for each analysis.

Method of Enrichment of Artemia *Nauplii with Antibiotics*

Antibiotics are also incorporated into HUFA emulsion for the enrichment of *Artemia* nauplii. Prepare fatty acid (FA) emulsion according to standard procedure; ➔ Calculate the amount of antibiotic required for enrichment of the fish/shrimp larvae (it may differ according to the weight, size or species); ➔ Add the calculated amount of antibiotic (e.g. 10% w/w) to the fatty acid emulsion; ➔ Mix the content properly using an electric mixer until the antibiotic is dissolved in the fatty acid emulsion; ➔ Preserve the emulsion containing antibiotic in a refrigerator before use; ➔Nauplii are enriched with two doses of this mixture at 0.0 h and for 10–12 h during the process of enrichment; ➔ The enrichment process is the same as explained for HUFA; ➔ Stop aeration after 24 h and siphon the nauplii into a clean beaker containing filtered seawater; ➔ Preserve the enriched *Artemia* in a cold incubator with gentle aeration.

Method of Enrichment of Artemia *Nauplii or Copepods with Probiotics and Prebiotics*

Standard Procedure for Enrichment with Probiotics

Take newly hatched *Artemia* nauplii or copepods in 500-mL bottles; ➔ Prepare a solution with 150 mg L^{-1} docosahexaenoic acid (DHA) and 50 mg L^{-1} of probiotic bacteria (commercial preparation) or bacterial suspension at a concentration of 10^7–10^8 CFU L^{-1} (if you have the probiotic bacteria isolated by your own methods); ➔ Add this solution to the bottle containing *Artemia* nauplii or copepods; ➔ Incubate

for 14–20 h (first step of enrichment); ➔ Count the bacteria associated to 1 day post-hatching (dph) *Artemia* on selected media; ➔ If larvae have to be fed with *Artemia*, a second step of enrichment is needed; ➔ To this end, 1 dph *Artemia* are further enriched with DHA (50 mg L^{-1}) and probiotic preparation (50 mg L^{-1}); ➔ Distribute enriched *Artemia* with a peristaltic pump.

Standard Procedure for Enrichment with Prebiotics
Take newly hatched nauplii in 500-mL bottles; ➔ Prepare a solution with 150 mg L^{-1} DHA and 10, 30 or 60 mgL^{-1} of prebiotic powder; ➔ Add this solution to the bottle containing nauplii; ➔ Incubate for 14–20 h (first step of enrichment); ➔ Count bacteria associated to 1 dph *Artemia* on selected media; ➔ If larvae have to be fed with *Artemia*, a second step of enrichment is needed; ➔ 1 dph *Artemia* are further enriched with DHA (50 mg L^{-1}) and probiotic powder (50 mg L^{-1}); ➔ Distribute enriched *Artemia* with a peristaltic pump.

Standard Encapsulation Protocol
Artemia cysts are hydrated, decapsulated and incubated at 28 °C under continuous aeration and illumination. After 24 h, hatched nauplii are scooped, washed and stored at 20 °C for 5 h before being transferred to 50-mL plastic centrifuge tubes for enrichment. Add the 0.625 mL of 300 mm methionine and an equal amount of methionine in their respective liposome suspensions. After 16 h, the nauplii are washed with tap water and transferred to seawater. The direct enrichment of nauplii is enriched with methionine directly dissolved in the culture water and the nauplii are analysed for FAA after 16 h.

Preparation of Enrichment Medium

The preparation of enrichment medium is as follows: ➔ Take 5 mL or 5 g of fish oil and prepare separately with the addition of poultry egg yolk (5 ml/L), mixed and emulsified in 100 mL of double distilled water (D.H_2O), homogenise the content for 2–3 min in a homogeniser or mix the content manually by vigorous shaking; ➔ Observe the content under a microscope to ensure proper emulsion; ➔ Store the enrichment medium in a refrigeration until use; ➔ The emulsifiers may be added to maintain the emulsion or strong shaking prior to use reforms the emulsion; ➔ The mixture is homogenised and stirred well until an emulsion is formed; ➔ The stability of the oil emulsion is checked; ➔ Enrichment media may be supplemented with water and lipid-soluble vitamins like vitamin A, D, E and K during homogenisation.

One more potential risk of using fish oil for HUFA enrichment is that, in the n-3 HUFA, particularly DHA, very small triacylglycerol (TAG) micelles generated in enrichment procedures are prone to autoxidation, especially under continuous aeration employed for extended periods. The addition of natural antioxidants vitamins E and C (generally added as oil-soluble α-tocopheryl acetate and ascorbyl palmitate), which are not effective until they are hydrolysed in the intestinal tract and absorbed,

and/or synthetic antioxidants like ethoxyquin or butylated hydroxyanisole (BHA), minimises peroxidation.

Bioenrichment of Nutrition

Bioenrichment with Algae

As good quality algae is available in large quantities, they are may be used as an excellent live food diet for boosting the fatty acid content in rotifer, Copepoda and Cladocera. The specific content of the EFA, such as EPA (20:5n-3) and DHA (22:6n-3) in some microalgae (e.g. 20:5n-3 in *Nannochloropsis oculata*; Watanabe 1979; Sukenik et al. 1993 and 22:6n-3 in *Isochrysis galbana* and *Rhodomonas* sp.; Lubzens et al. 1985; Mourente et al. 1993), and their relatively easy mass culture make them very attractive in commercial hatcheries. Rotifers incubated in these algae cultures (at approximately 5–25 × 10^6 algae.mL^{-1}) are incorporated in the EFA in a few hours and attain a DHA/EPA level above 2 in *Isochrysis* and below 0.5 for *Tetraselmis*. However, most of the time, algae of good quality are not available in large enough quantities, are too labour-intensive to be produced or too expensive for rotifer enrichment (Coutteau et al. 1997; Agh and Sorgeloos 2005). For this reason, rotifers are generally boosted in oil emulsions before they are fed to the predators. The latter may be kept in clear water or in "green water". This "green water" consists of ±0.2 × 10^6 algal cells.mL^{-1} (*Tetraselmis*, *Nannochloropsis* or *Isochrysis*) and is used as a "water conditioner" and as a nutritional factor to maintain an appropriate HUFA content in the live prey before they are eventually ingested by the predator (Dhert et al. 1998; Agh and Sorgeloos 2005).

The use of storable algal products (algal pastes and frozen algae) has found some new interest for rotifer cultures (Hamada et al. 1993; Lubzens et al. 1995; Agh and Sorgeloos 2005). Lubzens et al. (1995) and Agh and Sorgeloos (2005) accredit this new interest in the new products to the facts that: (1) the algal products can be transported and stored for longer periods (2 weeks for pastes), relieving the hatcheries from their direct dependence; (2) algae can be cultured under conditions that ensure the highest quality; (3) the chemical composition and quality can be determined in advance; (4) high-density rotifer cultures can be obtained.

Nutritional Bioenrichment of Zooplankton

The enhancement of rotifer *Brachionus* sp., copepods and cladocerans as food for marine fish and shrimp larvae has been achieved through enrichment with various diets containing different levels of n-3 HUFA. These diets include microalgae, lipid emulsions, fish oils, microparticles and microcapsules containing lipids. The HUFA content of the enriched rotifers is, to a great extent, a reflection of the content of

these fatty acids in the diets. Enriched rotifers, copepods and cladocerans have been shown to be able to maintain their improved nutritional value for several hours at 10 °C during their application as live food for marine fish and shrimp larvae.

The theory of long- and short-term methods of enrichment has also been explored for live feed enrichment. In the long term, it combines growth and n-3 HUFA enrichment during the production phase. Short-term enrichment involves short (<24 h) exposure to a high concentration of specific HUFA-rich feed following culture. The latter is important for coldwater fishes.

Short-term Enrichment of Rotifers with Microalgae

The microalgae are used for short-term enrichment of rotifers before they are given to the fish larvae to improve the content of n-3 fatty acids in the rotifers. Short-term feeding with algae affects the protein content and dry weight of the rotifers.

Enrichment with Oil Emulsions

For the enrichment or boosting of rotifers, several approaches can be used: (1) Adjustment of the lipid and vitamin content of the rotifers just before feeding them to other organisms, referred to as short-term enrichment (generally less than 8 h of exposure); (2) Feeding of rotifers on a complete diet or long-term enrichment (rearing of the rotifers on the enrichment diet for more than 24 h). Much of the research has elaborated on both techniques and each of them has its benefits and disadvantages. The short-term enrichment technique has the advantage of being fast and flexible, but, very often, produces lower quality rotifers with a too high lipid content (Dhert et al. 1990; Stottrup and Attramadal 1992; Agh and Sorgeloos 2005) and poor hygienic quality. The biggest problem with this enrichment technique resides in the fact that many rotifers are lost when they are concentrated (sticking of the rotifers) at high density. Also, the transfer of oil to larval rearing tanks with consequent loss of water quality and associated problems of larval viability have been reported (Foscarini 1988; Agh and Sorgeloos 2005). On top of that, the retention time of the nutrients, which are mainly accumulated in the digestive tract of the rotifers, is very short and can create problems when the rotifers are not eaten immediately.

Since rotifers are not selective for the uptake or catabolism of HUFAs, high HUFA levels can be accumulated without a problem. Especially DHA, an essential fatty acid that accumulates in the brain of fish during early development, where it increases neural functions (Bell et al. 1995), is easily incorporated in rotifers, unlike *Artemia*, which catabolise this fatty acid (Dhert et al. 1993; Agh and Sorgeloos 2005). Especially for this last reason, the feeding with DHA-enriched rotifers is often prolonged in flatfish cultures, where the enrichment at an early

stage has been successful in improving pigmentation (Miki et al. 1990; Kanazawa 1993; Reitan et al. 1994).

In contrast to n-3 PUFAs, n-6 PUFAs have been largely neglected in studies on marine fish nutrition. Especially, ARA (20:4n-6), as the preferred substrate for producing eicosanoids (Tocher and Sargent 1987; Sargent et al. 1995), can be blended in an optimal ratio with DHA and EPA and retrieved in approximately the same ratio in rotifers without the same risk of preferential catabolism as in *Artemia* (Estevez et al. 1999). However, the exact balance of DHA, EPA and ARA in the nutrition of larval fish still needs further investigation (Estevez et al. 1999; Sargent et al. 1999). Emulsions with phospholipids have also been used as a more efficient fatty acids source for fish (McEvoy et al. 1995, 1996; Coutteau et al. 1997), but they are immediately broken down in rotifers.

Enrichment with Vitamin C

The water-soluble vitamin C content of rotifers, copepods and cladocerans reflects the dietary ascorbic acid (AA) levels both after culture and during enrichment. Commonly, rotifers, copepods and cladocerans are cultured on, e.g. instant baker's yeast containing low AA levels (150 mg.g^{-1} DW), while the AA content in *Chlorella*-fed rotifers, copepods and cladocerans may vary (from 1000 up to 4000 mg.g^{-1} DW), depending on the quality of the algae. In commercial marine fish and shrimp hatcheries, a wide range of products is used for the culture and subsequent boosting of rotifers, copepods and cladocerans with vitamins. Oil-soluble vitamins or derivatives from water-soluble vitamins [ascorbyl palmitate (AP) for AA] have been formulated in commercial lipid enrichment products. The non-bioactive AP is accumulated by the rotifers together with the oil emulsion and converted to free AA by the enzymes of the rotifers, copepods and cladocerans. Merchie et al. (1997) demonstrated that this process is very effective, since 5% AP (w/w) in the emulsion produced rotifers with an active AA concentration of 1700 mg.g^{-1} DW after 24 h of enrichment, and this high concentration remained in the rotifers, copepods and cladocerans after storage in seawater during the next 24 h. Since the technique has been introduced in Mediterranean bream hatcheries, problems related to stress and operculum deformities have been reduced, which might indicate that vitamin C concentrations in live food may also be critical (Merchie et al. 1997; Agh and Sorgeloos 2005).

Bioencapsulation with Drugs

In addition to the use of enrichment in nutrients, live food-mediated delivery of therapeutic drugs has emerged as a new tool for disease treatment in larval culture. This is normally done through liposomes. The overall nutritional quality of the live food organisms depends on the content and nutritional balance of carbohydrate,

protein and lipids. The content of essential amino acids and protein in live food must meet the requirements for growth and cell maintenance of fish larvae. The enriched live food might be starved in the culture tank before they are consumed by the larvae and, in this period, the protein, lipids and fatty acids decrease. The loss of all these components may be increased with increasing water temperature. The total lipid content loss is of a higher rate than the protein content. The highest loss rate is for DHA and is considerably lower for EPA.

Conclusion

It is concluded that the survivability of marine and freshwater larvae in hatcheries and aquaculture industries depend on the larval nutrition, which, in turn, is due to the quality loss (potency loss of the stock on continuous use, mutation etc.) of live food organisms. Bioencapsulation can be effectively and successfully employed to reduce the larval mortalities in hatcheries.

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Intensive Culture, Biochemical Composition Analysis, and Use of Zooplankton *Tisbe* sp. (Copepoda: Harpacticoida) as an Alternative Live Feed for Shrimp Larviculture

S. Ananth and P. Santhanam

Introduction

Harpacticoid copepods form a vital link in the aquatic food chain. A harpacticoid life begins as fertilized egg and then hatches as a nauplius (6 stages), copepodite (5 stages), and finally becomes an adult. These developmental stages are mainly governed by the environmental parameters. A complete development may take from a week to several months depending upon the species and environmental parameters. The harpacticoid copepod *Tisbe* spp. are able to synthesize essential fatty acids (EFA) like EPA and DHA from short-chain fatty acids, even when fed with EFA-deficient microalgae and yeast (Nanton and Castell 1998). Although copepods constitute 80% of zooplankton in the ocean, only 60 copepods are cultured at laboratory level (Drillet 2010). Owing to the growing importance on copepods as live feed for larviculture, it is important to know the basic knowledge on its biology. Stottrup (2000) suggested that "A basic knowledge of physiological processes and population dynamics of a species is a prerequisite for the development of rearing techniques."

At the same time, mass culture of copepods begins due to the unsatisfactory performance of traditional live feeds *Artemia* and rotifer in larviculture. The key problem in culture of copepods is high yield in the sense of hatchery level on a continuous basis. A number of harpacticoids have been cultured for commercial and laboratory purposes. Kahan (1979) cultured harpacticoids only with vegetables as

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food and achieved a density of 1,69,000 per liter in laboratory condition. Lee and Hu (1981) cultured *Tigriopus japonicus* with production of 204 nauplii per female. Marine fish larvae released from small eggs immediately required live feed because of low vitellin reserve (Drillet 2010). These types of larvae have specific nutrient requirements especially different sizes of food and high amount of EFA like EPA, DHA, etc. which is not met by *Artemia* nauplii and rotifer. Thus, these larvae mainly rely on the live feed. So the scientists, researchers, and entrepreneurs started probing for alternative live feed. Copepods are tiny crustaceans and act as first feed for many larval forms in the wild. Copepods are always better live feed in the sense of nutrition and different sizes than enriched rotifers and *Artemia* spp.

This chapter reveals the survival, nauplii production rate, population density, development time, and generation time of harpacticoid copepod *Tisbe* sp. with reference to different environmental parameters and diets. Based on their optimization, *Tisbe* sp. has been cultured in mass-scale level and analyzed their nutritional profile then the cultured copepod was utilized for white-leg shrimp *L. vannamei* post larvae as a live feed.

Materials and Methods

Culture of Microalgae Refer chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Optimization of Culture Condition for Tisbe *sp.*

Environmental Conditions

To optimize the culture conditions for copepod *Tisbe* sp., a set of experiments were designed, viz., temperature (18 ± 2 °C, 20 ± 2 °C, 23 ± 2 °C, 28 ± 2 °C, 33 ± 2 °C, and 38 ± 2 °C), salinity (18 ± 2‰, 23 ± 2‰, 28 ± 2‰, 33 ± 2‰, and 38 ± 2‰), pH (6.2, 7.2, 8.2, 9.2, and 10.2), diets (*Chlorella marina*, *Dunaliella salina, Isochrysis galbana, Nannochloropsis salina, Tetraselmis suecica*, mixed algae, and yeast), and diet concentrations (15,000 cells/ml, 20,000 cells/ml, 25,000 cells/ml, and 35,000 cells/ml,), and were tested on survival rate (SR), nauplii production rate (NPR), population density (PD), developmental time (DT), and generation time (GT). For all the diet and diet concentration experiments, animals were starved for 36 h before the experiments started.

Survival Rate (SR)

For SR experiment, ten healthy copepods (*Tisbe* sp.) were picked up using fine brush and inoculated in a 100 ml beaker. The beaker was prefilled with sterilized seawater and maintained at different environmental conditions as described above. During experimental period, animals were transferred to a new container for every 2 days. Utmost care has been taken during the transfer of animals. Dissection microscope was used to observe the copepods. Animals with no movement for 30 s were considered dead. The dead animals were removed from the container on a daily basis. The number of live animals were calculated every day, and the experiment was continued for 12 days.

Nauplii Production Rate (NPR)

For NPR experiments, one healthy female with egg sac was inoculated in a Pyrex tube filled with 20 ml of sterilized seawater for each parameter. The tubes were covered with black cloth to avoid illumination and keep algal concentration in a right way. Every 4 h tubes were checked to know the release time of nauplii. After the nauplii released from the egg sac, adult copepods were transferred immediately to avoid predation against nauplii. Nauplii were counted under dissection microscope previously fixed with 5% formalin.

Population Density (PD)

To determine the PD under each parameter, three male and three female copepods were isolated from the stock culture and inoculated in a 250 ml beaker filled with sterilized seawater. After the experimental period (14 days), animals were harvested through a 48 μm mesh and fixed with 5% formalin. Different stages of copepods (nauplii, copepodite, and adult) were counted under the microscope after 14 days of experiment.

Development Time (DT) and Generation Time (GT)

For DT under each parameter, egg-carrying females were inoculated in a 50 ml beaker filled with filtered seawater. Once the nauplii are released from the egg sac, females were transferred to a new beaker and monitored every 30 min under the microscope for metamorphosis of nauplii I (NI) to copepodite I (CI) and copepodite I to copepodite VI (CVI) (adult). For GT, a known number of gravid females were stocked in a 50 ml beaker filled with sterilized seawater maintained with different parameters. The generation time from egg to egg was calculated with reference to the treatment parameters and diet conditions under the microscope.

Mass-Scale Culture and Biochemical Analyses of Live Feeds

Mass Culture of *Tisbe* sp.

Based on the results of optimized parameters in section "Optimization of culture condition for *Tisbe* sp.", the *Tisbe* sp. culture was maintained as batch cultures by maintaining best optimized conditions. Initially stock culture was maintained in a 500 ml glass beaker inoculated with 20–30 adults transferred to 7 l cylindrical plastic containers with appropriate diet and diet concentration. Copepods from these stock cultures were utilized for mass production. For mass culture, approximately 150–200 individuals were stocked in an oval-shaped, flat-bottomed fiberglass tank (0.54 m dia, 0.81 m length) filled with 100 l of filtered seawater, and vigorous aeration was given. Seawater filtered through a membrane filter (1 μm) was used for copepod culture. At the time of rearing period, water quality parameters such as temperature, salinity, pH, and dissolved oxygen were maintained in the ranges of 28 ± 2 °C, 28 ± 2‰, 8.2, and 6.0–7.5 ml/l^{-1}. Copepods were fed with the identified suitable microalgae, i.e., *I. galbana* with a concentration of 25,000 cells/ml once a day. The mass culture experiment was continued over a period of 45 days (when the copepod populations started to decline). The number of nauplii, copepodites, and adults were counted under the microscope. Water quality parameters were monitored on a daily basis. To reduce the risk of contamination, water exchange was done for every 3 days once and the fecal was siphoned out. Copepod populations were calculated using the formula $r = \ln N_t - \ln N_0/t$, where N_0 = initial number of copepods and N_t = total number of copepods after t days (Nandini et al., 2011). The copepods were harvested using a mesh (35 μm) and after being washed with filtered seawater were used for biochemical analysis and fed to the shrimp post larvae. The experimental (copepod) and control feeds (rotifer and *Artemia* nauplii) were given in the concentration of 8–10 ind./ml to shrimp post larvae.

Rotifer Culture Refer chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Artemia **Culture** Refer chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Biochemical Analyses of Live Feed Refer chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production".

Astaxanthin Analysis

The astaxanthin content of the copepod and shrimp larvae were determined by adopting the method of Torrissen and Naevdal (1988). A known amount of sample (0.5 g) was taken and homogenized with a homogenizer and then transferred to

10 ml of pre-weighed glass tubes. First, a 10 ml of dry acetone was added to the samples, which was followed by addition of about 1–1.5 g of anhydrous sodium sulfate. The solutions were centrifuged at 5000 rpm for 5 min and then stored in a refrigerator at 4 °C. After 3 days of incubation in sealed glass tubes, the absorption of the extracts was measured at 476 nm in a UV spectrophotometer (Shimadzu).

Utilization of Tisbe *sp. as Live Feed for Shrimp Larvae*

Larval Rearing of *L. vannamei* Using Different Live Feeds

The larval rearing of *Litopenaeus vannamei* using *Tisbe* sp. was done as discussed in chapter "Assessing the Efficacy of Marine Copepods as an Alternative First Feed for Larval Production of Tiger Shrimp *Penaeus monodon*".

Morphometric Analyses The morphometric analyses of shrimp larvae were done as discussed in chapter "Assessing the Efficacy of Marine Copepods as an Alternative First Feed for Larval Production of Tiger Shrimp *Penaeus monodon*".

Analysis of Nutritional Composition The nutritional parameters such as moisture, protein, lipid, carbohydrate, ash, amino acids, and fatty acids were done according to the standard method explained in chapter "Evaluation of Suitability of Marine Copepods as an Alternative Live Feed in High Health Fish Larval Production". The astaxanthin content was analyzed based on standard procedure presented previously in Astaxanthin Analysis subheading.

Results

Optimization of Tisbe *sp. Culture Condition*

Effects of Temperature, Salinity, pH, Diets, and Diet Concentration on Survival Rate (SR) of Copepod *Tisbe* sp.

Temperature significantly affects the SR of copepod *Tisbe* sp. ($P < 0.05$). The mean SR was >60% in all temperatures tested except for 38 °C. Higher SR was observed in 28 °C (80%) followed by 23 °C (45%). Total mortality was observed earlier in 38 °C by the end of the 9th day (Fig. 1a). Salinity significantly ($P < 0.05$) affects the SR of copepod. More than 50% SR was observed in all salinities tested. The optimum salinity required for the extended survival (80%) was 28‰ salinity. The low survival was recorded in low salinity (18‰) tested (Fig. 1b). The mean SR was above 50% until the 10th day at all pH tested. The SR was high (80%) in the pH 8.2 followed by 7.2 (60%), whereas the pH 10.2 resulted significantly low SR of 25% on the 12th day ($P < 0.05$) (Fig. 1c). The SR of copepod *Tisbe* sp. was significantly

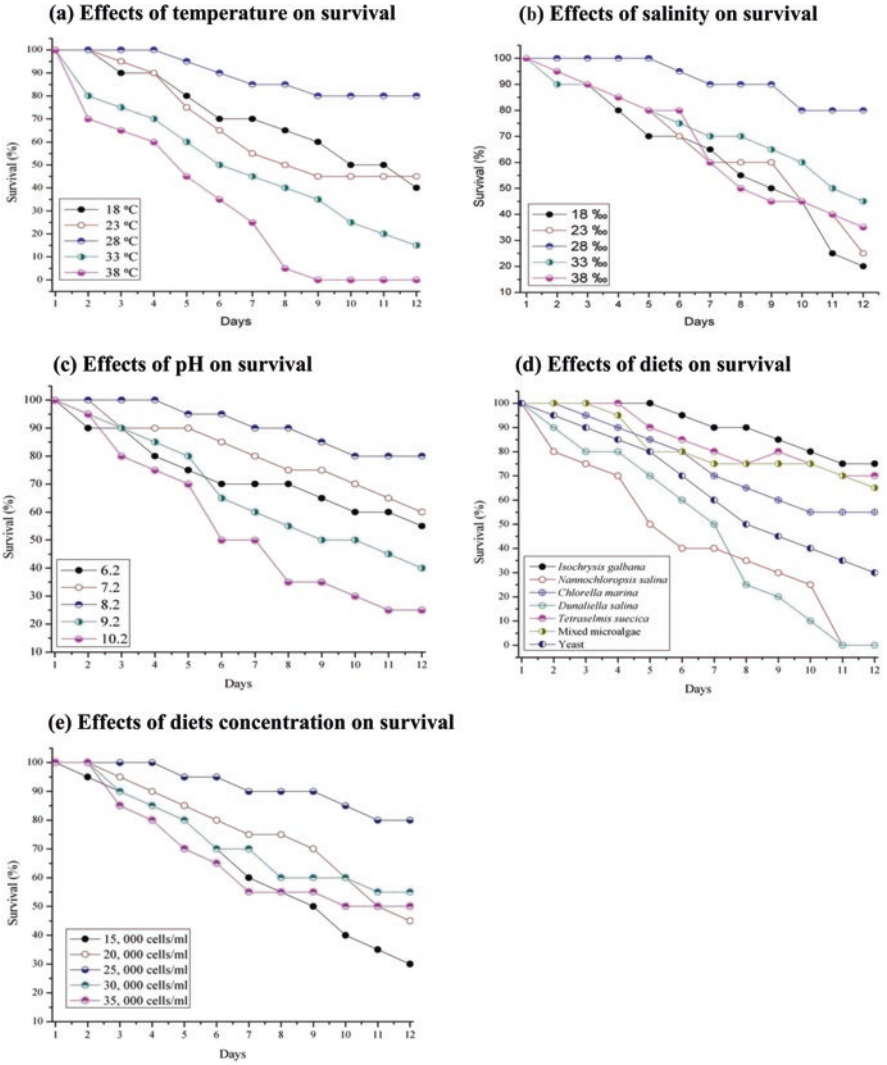

Fig. 1 Survival rate (SR) of copepod *Tisbe* sp. under different parameters: (**a**) temperature, (**b**) salinity, (**c**) pH, (**d**) diet, (**e**) diet concentration

affected by the diets tested ($P < 0.05$). The diet *I. galbana*-fed copepods showed high SR (75%) followed by *T. suecica*-fed copepods (70%). Total mortality was observed in *D. salina*- and *N. salina*-fed copepods on 10th day of the experiment (Fig. 1d). Among the diet concentration tested, the recorded mean SR was 50% up to 9th day of the experiment in all the diet concentrations. The highest SR of 80% was observed in 25,000 cells/ml followed by 30,000 cells/ml (55%). The lowest SR (30%) was recorded in the low diet concentration of 15,000 cells/ml (Fig. 1e).

Effects of Temperature, Salinity, pH, Diets, and Diet Concentration on Nauplii Production Rate (NPR) of Copepod *Tisbe* sp.

The NPR was found to be high in 28 °C with 58 nauplii/female followed by 23 °C with 41.5 nauplii/female, whereas the lowest NPR of 5 nauplii/female was observed in 38 °C. It was about ten times lower than the NPR at 28 °C (Fig. 2a). The highest NPR of 57.5 nauplii/female was found in the salinity of 28‰ followed by 33‰ with 33.5 nauplii/female. The lowest NPR was found in the lowest salinity (18‰) with only 8 nauplii/female (Fig. 2b). Among the pH tested, pH 8.2 resulted the better NPR of 50.5 nauplii/female followed by 7.2 (36.5 nauplii/female), while the poor NPR was found in the pH 10.2 with only 16.5 nauplii/female (Fig. 2c). From the 7 tested diets, microalgae *I. galbana*-fed copepod performed well in NPR with 50 nauplii/female followed by *T. suecica*- and mixed diet-fed copepods with 42.5 and 38.5 nauplii/female respectively. The low NPR was recorded in *N. salina*-fed copepods with 16.5 nauplii/female (Fig. 2d). The maximum NPR (53 nauplii/female) was found in 25,000 cells/ml of microalgae-fed copepod followed by 35,000 cells/ml with 37.5 nauplii/female, while the low NPR was found in the lowest food concentration of 15,000 cells/ml with only 24 nauplii/female (Fig. 2e).

Effects of Temperature, Salinity, pH, Diets, and Diet Concentration on Population Density (PD) of Copepod *Tisbe* sp.

The total PD of *Tisbe* sp. was high at 28 °C, having 211 ind./l comprised of 87 nauplii, 64 copepodites, and 60 adults followed by 23 °C having 175 ind./l. The lowest PD was recorded in highest temperature (38 °C), reaching 53 ind./l which was comparatively much lower than the nauplii obtained at 28 °C (Fig. 3a). At the final day of the experiment (14th day), the total PD of *Tisbe* sp. was maximum at high salinities. At 28‰, 212 ind./l including 91 nauplii, 57 copepodites, and 64 adults were obtained followed by 33‰ with 168 ind./l. The poor PD was recorded at 38‰ and 18‰ attained 75 and 73 ind./l, respectively (Fig. 3b). Among the pH tested, pH 8.2 result the maximum PD of 208 ind./l which includes 83 nauplii, 67 copepodites, and 58 adults. The lowest PD of 46 ind./l was observed in the alkaline pH (10.2), respectively (Fig. 3c). The total PD of *Tisbe* sp. was found highest (218 ind./l) when fed with *I. galbana* with 95 nauplii, 48 copepodites, and 75 adults. A slightly minor variation was observed in the mixed diet-fed copepods (206 ind./l). Copepod population dropped drastically when fed with yeast where the PD was noticed as 75 ind./l (Fig. 3d). The highest PD of 213 ind./l including 93 nauplii, 65 copepodites, and 55 adults were obtained in moderate diet concentration (25,000 cells/ml), while the lowest PD (62 ind./l) including 30 nauplii, 18 copepodites, and 14 adults were observed in the lowest food concentration of 15,000 cells/ml (Fig. 3e).

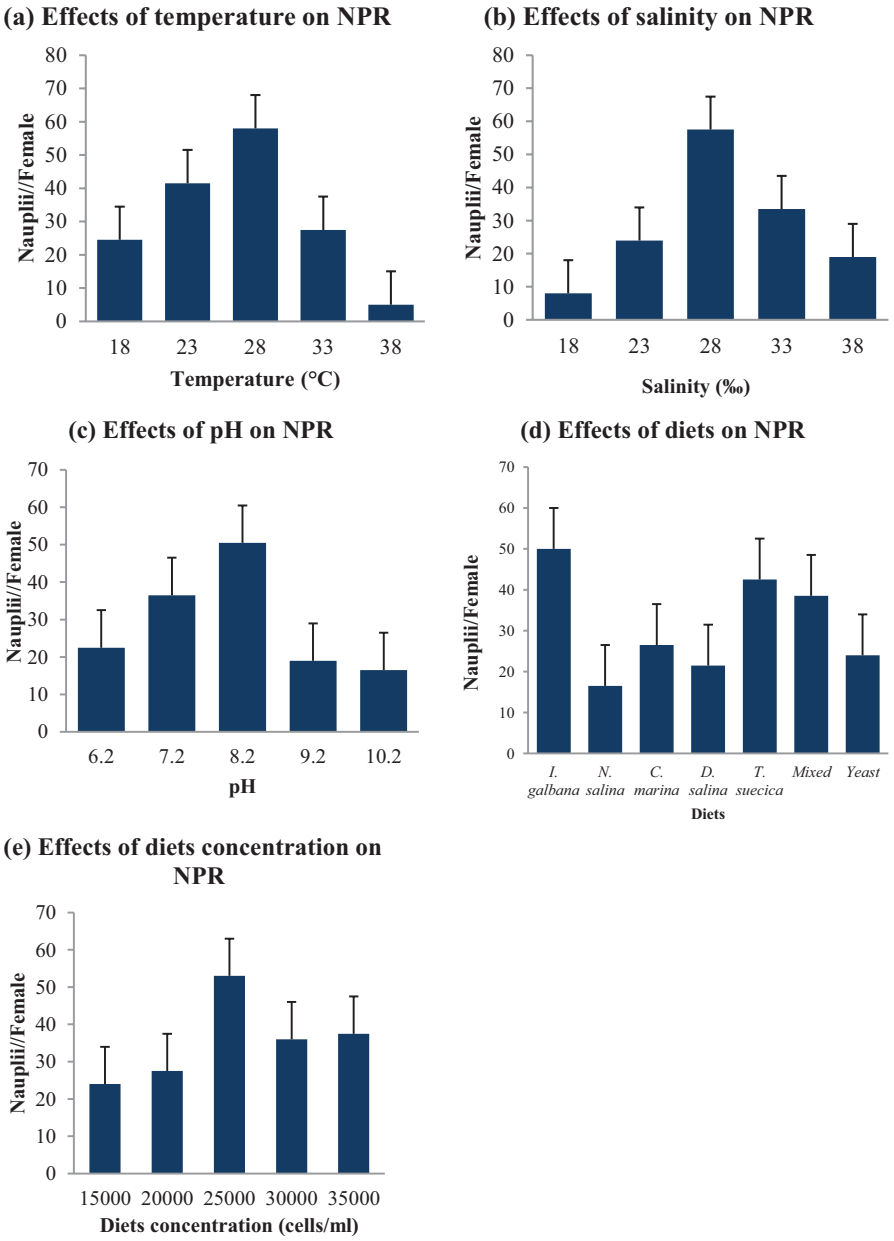

Fig. 2 Nauplii production rate (NPR) of copepod *Tisbe* sp. under different parameters: (**a**) temperature, (**b**) salinity, (**c**) pH, (d) diet, (**e**) diet concentration

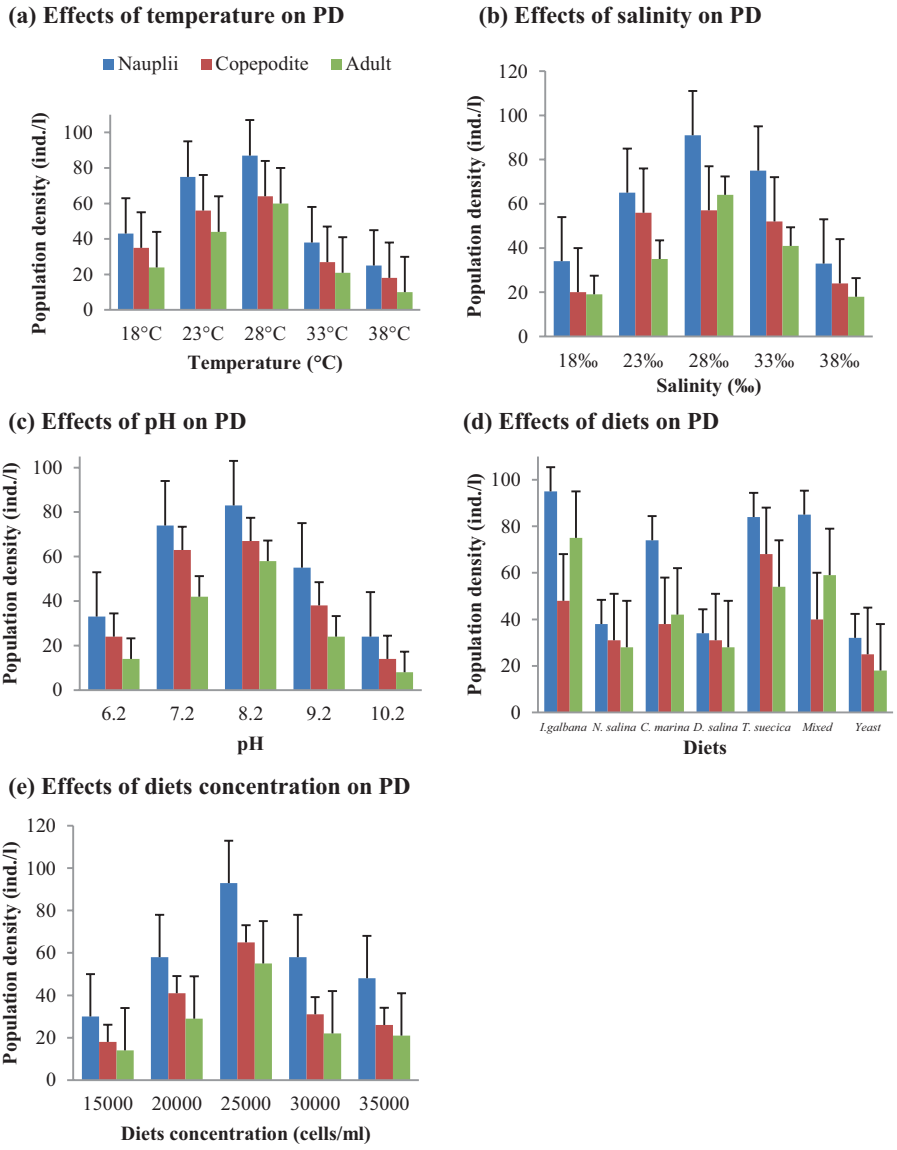

Fig. 3 Population density (PD) of copepod *Tisbe* sp. under different parameters: (**a**) temperature, (**b**) salinity, (**c**) pH, (**d**) diet, (**e**) diet concentration

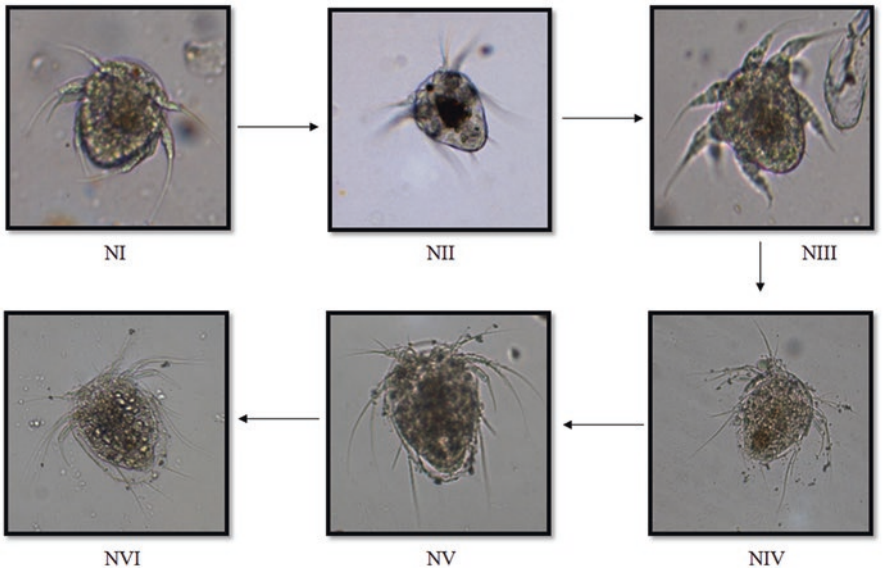

Plate 1 Postembryonic developmental stages of copepod *Tisbe* sp. (nauplii I to nauplii VI)

Effects of Temperature, Salinity, pH, Diets, and Diet Concentration on Development Time (DT) and Generation Time (GT) of Copepod *Tisbe* sp.

The development time of *Tisbe* sp. from nauplius I to copepodite I was shortest at 28 °C which took 4.8 days only, while the longest development time was found at the lowest temperature of 18 °C which took 5.9 days (Plates 1 and 2).

The longest development of copepodite I to copepodite VI (adult) took place at 38 °C (7.8 days), and the shortest development was found at 28 °C (7.2 days). The generation time from egg to egg was shortest at 28 °C which took 11.5 days, while the longest GT (15.5 days) was observed at 18 °C (Table 1a). Development time (DT) for nauplius I to copepodite I was observed to be very short at the salinity of 28‰ and 33‰. In both the salinities, DT was only 4.9 days. Under low salinities, DT was lengthened to 6.1 days. Likewise, DT from copepodite I to copepodite VI (adult) was shortest (5.9 days) at 28‰.The fastest GT was achieved in 28‰, which took 11.6 days, while the slowest GT (15.4 days) was found in low salinity of 18‰ (Table 1b). The development of nauplius I to copepodite I was found shortest at pH 8.2 which took 4.4 days, while the longest development time (6.1 days) was procured at alkaline pH 10.2. Likewise, the shortest development (5.7) of copepodite I to copepodite VI (adult) was found at pH 8.2, while the longest DT (7.3 days) was observed at pH 10.2. The GT was shortest at pH 8.2 which took 11.7 days alone, while the longest GT of 15.9 days took place at pH 10.2 (Table 1c). The overall DT and GT were favored by the microalgae *I. galbana*. The shortest development of nauplius I to copepodite I and copepodite I to copepodite VI (adult) and GT

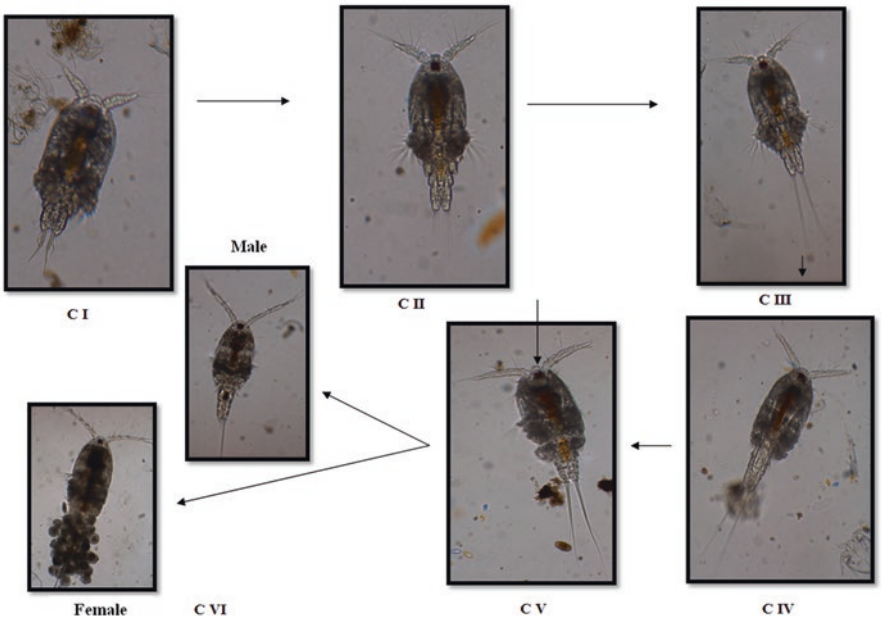

Plate 2 Postembryonic developmental stages of copepod *Tisbe* sp. (copepodite I to copepodite VI)

was observed in the copepod fed with microalgae *I. galbana* which took 4.8, 5.3, and 11.2 days, respectively, whereas the longest development was noticed in *D. salina* as 6.6, 7.8, and 15.8 days, respectively (Table 1d). The shortest development time of 4.7 days was noticed in copepod fed with algal concentration of 25,000 cells/ml, while the longest development was observed in the lowest diet concentration of 15,000 cells/ml which took 5.9 days. The copepodite I to copepodite VI (adult) development was longest at low-diet-concentration (15,000 cells/ml)-fed copepods which took 7.2 days, while the shortest DT was observed at 25,0000 cells/ml diet concentration which took only 5.6 days. The overall GT (11.6 days) was shortest at copepods fed with 25,000 cells/ml, while the longest GT (15.7 days) was noticed at copepod fed with a diet concentration of 15,000 cells/ml (Table 1e).

Mass Culture and Biochemical Profile of Tisbe *sp.*

Indoor Culture and Outdoor Culture

For a period of 15-day culture operation, the indoor culture system produces an average of 18,984 ind./l including 10,952 nauplii, 5158 copepodites, and 2874 adults with an initial inoculum of 20–30 individuals (Fig. 4a). The outdoor culture system of *Tisbe* sp. produced around 1,20,262 ind./l. comprising 55,254 nauplii,

Table 1 Development time (DT) and generation time (GT) of copepod *Tisbe* sp. under different parameters: (a) temperature, (b) salinity, (c) pH, (d) diets, (e) diet concentration

Parameters	DT (NI-CI) (days)	DT (CI-CVI) (adult) (days)	Total DT (days)	GT (days)
(a) Temperature (°C)				
18 ± 2	5.9	7.5	13.4	15.5
23 ± 2	5.1	7.2	12.3	14.1
28 ± 2	4.8	5.6	10.4	11.5
33 ± 2	5.1	7.1	12.2	13.9
38 ± 2	5.2	7.8	13	14.8
(b) Salinity (‰)				
18 ± 2	6.1	7.4	13.5	15.4
23 ± 2	5.4	6.8	12.2	14.3
28 ± 2	4.9	5.9	10.8	11.6
33 ± 2	4.9	6.2	11.1	13.2
38 ± 2	5.3	6.9	12.2	14.3
(c) pH				
6.2	5.8	6.1	11.9	14.3
7.2	4.8	5.9	10.7	12.5
8.2	4.4	5.7	10.1	11.7
9.2	5.9	6.7	12.6	15.1
10.2	6.1	7.3	13.4	15.9
(d) Type of diets				
I. galbana	4.8	5.3	10.1	11.2
N. salina	6.1	7.2	13.3	15.4
C. marina	5.2	6.5	11.7	13.4
D. salina	6.6	7.3	13.9	15.8
T. suecica	5.1	5.7	10.8	11.7
Mixed algae	5.2	5.6	10.8	11.8
Yeast	5.7	7.2	12.9	15.2
(e) Diet concentration (cells/ml)				
15,000	5.9	7.2	13.1	15.7
20,000	5.3	6.1	11.4	12.9
25,000	4.7	5.6	10.3	11.6
30,000	5.1	5.8	10.9	11.8
35,000	5.7	6.5	12.2	14.9

38,387 copepodites, and 26,621 adults per liter with an initial inoculum of 150–200 individuals for a period of 45 days (Fig. 4b).

Biochemical Composition of Live Feeds

The comparative results on biochemical composition of various live feeds were shown in Table 2. The biochemical composition of live feeds showed greater variations. The protein content of copepod *Tisbe* sp. was 62.85%, which was

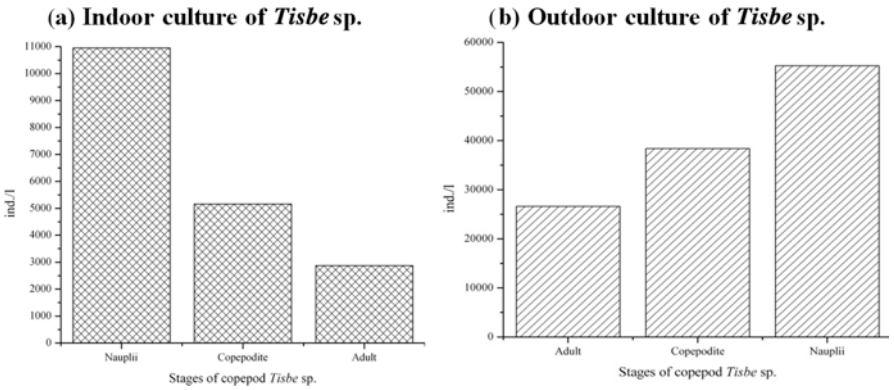

Fig. 4 Copepod *Tisbe* sp. (**a**) Culture density at 15-day culture period. (**b**) Culture density at 45-day culture period

Table 2 Biochemical composition of various live feeds

Live feed	Moisture	Protein	Carbohydrate	Lipid	Ash
Artemia nauplii	85.48 ± 0.264	59.88 ± 0.154	12.33 ± 0.548	13.72 ± 0.120	7.91 ± 0.024
Brachionus plicatilis	87.021 ± 0.402	60.21 ± 0.521	12.08 ± 0.238	10.56 ± 0.251	6.21 ± 0.235
Tisbe sp.	81.24 ± 0.159	62.85 ± 0.214	11.84 ± 0.185	14.97 ± 0.328	6.35 ± 0.261

comparatively higher than *Artemia* nauplii (59.88%) and *B. plicatilis* (60.21%). The carbohydrate value of *Artemia* nauplii (12.33%) showed slightly higher than the rotifer (12.08%) and *Tisbe* sp. (11.84%). The total lipid content of *Tisbe* sp. was 14.97%, whereas the *Artemia* and rotifer showed only 13.72% and 10.56%, respectively.

Amino Acid Composition of Live Feeds

The total amino acid content of different live feeds was given in Table 3. The essential amino acid (EAA) content of copepod *Tisbe* sp. was comparatively higher than the other live feeds tested. The recorded EAA of *B. plicatilis* and *Artemia* nauplii was 28.61% and 25.67%, respectively, whereas the copepod showed 41.73% of essential amino acids. Notably, arginine, histidine, leucine, lysine, and methionine were found to be high in copepod *Tisbe* sp. Phenylalanine was found higher in rotifer *B. plicatilis* (2.8%) and *Artemia* nauplii (3.4%). The NEAA glycine was higher in *B. plicatilis* and *Artemia* nauplii with 4.9% and 3.61%, respectively, whereas the copepod *Tisbe* sp. showed 2.54%.

Table 3 Amino acid content of various live feeds

Amino acids	*B. plicatilis*	*Artemia* nauplii	*Tisbe* sp.
Arginine	1.98	3.1	5.17
Histidine	2.0	2.65	3.98
Isoleucine	2.5	2.1	2.6
Leucine	3.65	4.1	7.1
Lysine	6.12	4.1	6.81
Methionine	2.34	0.72	4.19
Phenylalanine	2.8	3.4	2.01
Threonine	2.1	1.32	2.26
Cystine	–	–	0.71
Valine	5.12	4.18	6.9
Alanine	4.1	4.2	6.7
Aspartic acid	4.98	5.24	6.98
Glutamic acid	6.9	8.2	13.98
Glycine	4.9	3.61	2.54
Serine	4.7	4.5	6.01
Tyrosine	5.1	4.84	7.2
Total NEAA	**30.68**	**27.19**	**46.05**
Total EAA	**28.61**	**25.67**	**41.73**
Total	**59.29**	**56.26**	**85.14**

Fatty Acid Composition of Live Feeds

The fatty acid composition of different live feeds is given in Table 4. The ARA (4.41%) and EPA (3.12%) of *B. plicatilis* were found higher than *Artemia* nauplii and lower than the copepod *Tisbe* sp. (ARA 4.26% and EPA 9.92%). The DHA (5.1%) was found to be dominant in copepod than *B. plicatilis* (0.9%) and *Artemia* nauplii (2.1%). The myristic acid and palmitic acid were found to be dominant in rotifer (3.93 and 22.16%) and *Artemia* nauplii (2.13% and 10.91%) than copepod *Tisbe* sp. (0.51% and 9.56%).

Astaxanthin Content in Live Feeds

The high astaxanthin content was noticed in copepod (27.54 μg/g) compared to *Artemia* (16.95 μg/g) and rotifer (10.06 μg/g). The comparative bar graph of astaxanthin in different live feeds is shown in Fig. 5.

Table 4 Fatty acid content of various live feeds

Fatty acids	*B. plicatilis*	*Artemia* nauplii	*Tisbe* sp.
14:00	3.93	2.13	0.51
14:01	–	0.91	1.02
16:00	22.16	10.91	9.56
16:01	13.12	7.3	5.89
17:00	_	0.85	0.92
18:00	3.91	3.52	9.32
18:01	8.83	16.2	2.4
18:1n-9	_	10.12	0.53
18:2n-6	7.15	1.7	3.85
18:3n-6	1.13	2	2.21
18:3n-3	2.2	18.2	0.98
18:4n-3	1.14	2.48	4.23
20:00	–	0.63	1.64
20:01	4.02	0.82	6.91
20:02	–	1.1	3.71
20:2n-6	0.18	2	2.54
20:4n-6 (ARA)	4.41	1.1	4.26
20:4n-3	1.02	_	0.82
20:5n-3 (EPA)	3.12	2.15	9.92
21:00	–	_	1.13
22:00	0.15	_	0.84
22:01	0.58	_	4.85
22:5n-6	_	_	4.83
22:5n-3	0.91	_	1.62
22:6n-3 (DHA)	0.9	2.1	5.1
24:00	0.26	0.71	3.22
Total	**79.12**	**86.93**	**92.81**

Utilization of Tisbe *sp. as Live Feed for Shrimp Larvae*

Growth and Survival of *L. vannamei* Postlarvae Fed on Different Live Feeds

The copepod *Tisbe* sp.-fed PL showed notable survival of 95%, 87%, and 85% on 7th, 14th, and 21st days, respectively, while PL fed with rotifer *B. plicatilis* had the least survival of 75% on 21st day. The PL fed with *Artemia* nauplii showed 83% survival on 21st day of the experiment. The white-leg shrimp *L. vannamei* postlarvae grew faster when fed with copepod *Tisbe* sp. compared to rotifer and *Artemia* nauplii-fed PL. The mean total length of 2.8 cm with a weight of 20.56 mg on 21st day was obtained in the larvae fed on copepod, while the PL fed on rotifer (2.0 cm and 12.55 mg) and *Artemia* nauplii (2.2 cm and 16.66 mg) result the poor

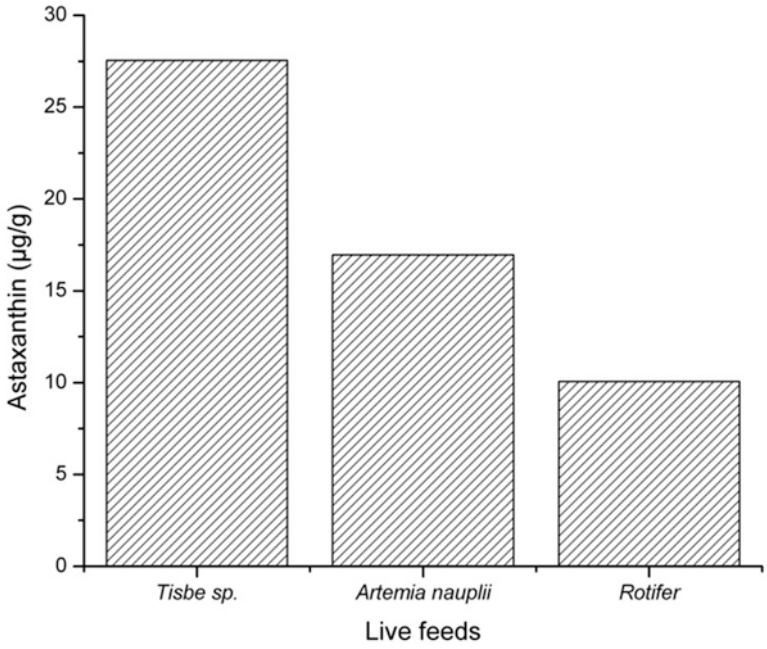

Fig. 5 Astaxanthin content of different live feeds

performance. The detailed result on growth and survival of various feed fed PL is given in Table 5 and comparative growth of shrimp larvae is shown in Plate 3.

Biochemical Composition of *L. vannamei* Postlarvae Fed on Different Live Feeds

The biochemical composition of PL clearly indicated the nutritional performance of copepod. The protein and lipid contents of rotifer and *Artemia* nauplii-fed larvae were comparatively lower than that of copepod-fed larvae. The protein content of *Artemia* nauplii-fed PL (61.78 ± 0.271%) was higher than the rotifer-fed PL (60.84 ± 0.579%) and lower than copepod-fed PL (64.27 ± 0.348%). In the case of carbohydrate, rotifer-fed PL showed high (14.62 ± 0.543%) than *Artemia* nauplii PL (13.75 ± 0.441%) and copepod-fed PL (12.65 ± 0.213%). The high amount of total lipid was recorded in PL fed with copepod (14.10 ± 0.576%), while the low lipid was found in rotifer-fed PL (9.65 ± 0.357%). The detailed results on biochemical composition of PL fed on different live feeds are given in Table 6.

Table 5 Growth and survival of *L. vannamei* PL fed on different live feeds

	Initial day		7th day			14th day			21st day		
Feeding regimes	Length (cm)	Weight (mg)	Length (cm)	Weight (mg)	Survival (%)	Length (cm)	Weight (mg)	Survival (%)	Length (cm)	Weight (mg)	Survival (%)
B. plicatilis-fed PL	0.9	3.66	1.2	5.87	90	1.6	8.08	82	2	12.55	75
Artemia nauplii-fed PL	0.9	3.66	1.2	6.91	95	1.9	12.45	92	2.2	16.66	83
Tisbe sp.-fed PL	0.9	3.66	1.4	6.85	95	2.1	13.18	87	2.8	20.56	85

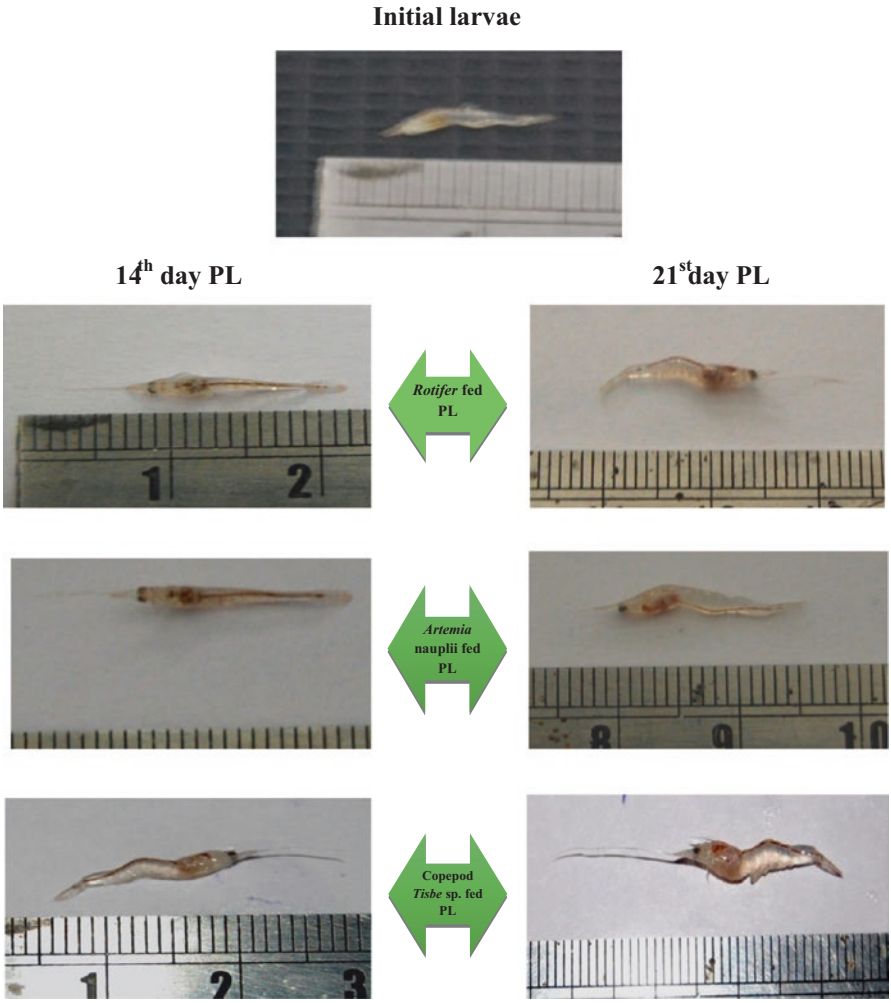

Plate 3 Comparative growth of *L. vannamei* PL fed with different live feeds

Amino Acid Composition of *L. vannamei* PL Fed on Different Live Feeds

The total amino acid content of *L. vannamei* PL fed on different live feeds is given in Table 7. The total amino acid content of copepod-fed PL was comparatively higher than the PL fed with rotifer and *Artemia* nauplii. The recorded EAA of *B. plicatilis*- and *Artemia* nauplii-fed larvae was 27.37% and 32.43%, whereas the copepod-fed larvae showed 32.27% of total amino acids. Notably, leucine, lysine, methionine, alanine, aspartic acid, and glutamic acid were found high in copepod-fed PL. Lysine and alanine were higher in rotifer *B. plicatilis*-fed PL which was lower than the copepod-fed PL.

Table 6 Biochemical composition of shrimp *L. vannamei* PL fed on different live feeds

Feeding regime	Moisture (%)	Protein (%)	Carbohydrate (%)	Lipid (%)	Ash (%)
Initial	79.26 ± 0.24	58.81 ± 0.24	11.61 ± 0.52	9.51 ± 0.66	7.248 ± 0.09
Artemia nauplii-fed PL	80.59 ± 0.68	61.78 ± 0.27	13.75 ± 0.44	10.54 ± 0.24	8.24 ± 0.15
B. plicatilis-fed PL	81.25 ± 0.04	60.84 ± 0.57	14.62 ± 0.54	9.65 ± 0.35	7.54 ± 0.65
Tisbe sp.-fed PL	80.19 ± 0.57	64.27 ± 0.34	12.65 ± 0.21	14.1 ± 0.57	7.543 ± 0.18

Table 7 Amino acid content of *L. vannamei* PL fed on different live feeds

Amino acids	Initial larvae	*Artemia* nauplii-fed larvae	*B. plicatilis*-fed larvae	*Tisbe* sp.-fed larvae
Arginine	3.12	5.92	3.54	3.75
Histidine	2.54	3.01	2.46	2.42
Isoleucine	2.34	1.83	1.96	2.78
Leucine	4.18	4.97	3.98	5.04
Lysine	5.16	5.72	6.81	6.73
Methionine	1.84	2.43	2.13	2.39
Phenylalanine	2.10	2.83	1.02	2.72
Threonine	2.64	2.22	2.74	3.26
Cystine	0.09	0.11	0.10	0.21
Valine	2.69	3.10	2.63	2.97
Alanine	8.98	7.14	9.2	9.18
Aspartic acid	5.65	5.70	5.9	6.44
Glutamic acid	9.69	9.48	10.10	11.33
Glycine	5.13	3.94	1.27	5.25
Serine	2.89	2.74	2.91	3.04
Tyrosine	2.68	2.88	8.48	3.37
Total EAA	**26.7**	**32.43**	**27.37**	**32.27**
Total NEAA	**35.02**	**31.88**	**27.37**	**32.27**
Total AA	**61.72**	**64.31**	**65.23**	**70.88**

Fatty Acid Composition of *L. vannamei* PL Fed on Different Live Feeds

The fatty acid composition of *L. vannamei* PL fed on different live feeds was given in Table 8. The total fatty acid content of copepod-fed PL was comparatively higher than the PL fed with rotifer and *Artemia* nauplii. The DHA (4.91%) of *B. plicatilis*-fed PL was found higher than *Artemia* nauplii-fed PL (1.32%) and lower than the copepod *Tisbe* sp. (DHA 12.41%). The palmitic acid (16:00) was found to be dominant (28.11%) in *Artemia* nauplii-fed PL than *B. plicatilis*-fed PL (19.68%) and copepod-fed PL (11.29%).

Table 8 Fatty acid content of *L. vannamei* PL fed on different live feeds

Fatty acids	Initial larvae	*Artemia* nauplii-fed larvae	*B. plicatilis*-fed larvae	*Tisbe* sp.-fed larvae
12:00	–	0.23	0.10	0.89
14:00	0.12	0.56	0.42	2.88
14:01	9.82	6.82	7.94	8.81
16:00	6.93	28.11	19.68	11.29
16:01	8.95	6.14	6.86	5.13
17:00	10.35	10.73	11.25	6.96
17:01	–	–	–	0.72
18:00	2.89	1.02	6.40	7.13
20:00	–	–	–	0.04
21:00	0.68	1.21	2.62	0.82
22:00	–	–	0.16	0.06
18:01	0.25	2.99	–	6.08
18:1-n 9	4.58	13.11	9.82	12.02
18:3-n 6	0.31	8.83	1.85	1.62
18:2-n 6	6.59	–	6.66	0.21
20:4-n 6 (ARA)	1.38	1.04	2.44	2.24
20:4-n 5	–	0.64	–	1.25
20:5-n 3 (EPA)	3.12	6.06	4.91	12.73
22:6-n 3 (DHA)	1.61	1.32	4.91	12.41
22:01	1.03	–	–	0.66
Total FA	**58.61**	**88.81**	**82.09**	**93.95**

Astaxanthin Content of Shrimp *L. vannamei* PL Fed on Different Live Feeds

The astaxanthin content of *L. vannamei* PL fed on different live feeds is shown in Fig. 6. The pigment astaxanthin was found high in copepod-fed PL (41.6 µg/g) compared to *Artemia* nauplii-fed PL (32.1 µg/g) and rotifer-fed PL (27.4 µg/g).

Discussion

Optimization of Tisbe *sp. Culture Condition*

Copepods that live in neritic and estuarine region have adapted to tolerate a broad range of environmental conditions, particularly harpacticoids. The present results showed that the tested environmental parameters have significant effects on the survival rate (SR), nauplii production rate (NPR), population density (PD),

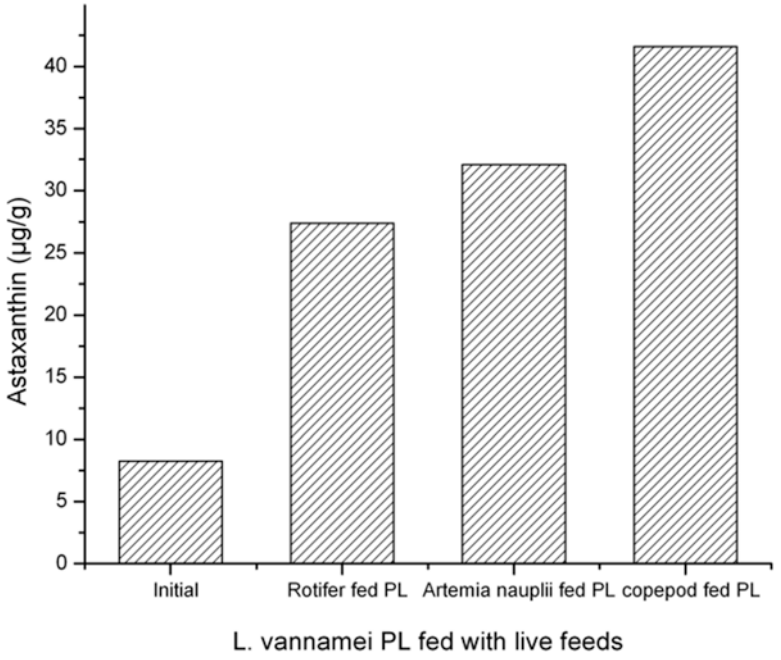

Fig. 6 Astaxanthin content of *L. vannamei* PL fed on different live feeds

development time (DT), and generation time (GT). Previously, some reports stated that temperature is one of the most important factors that affects the production of copepods in the wild (Rhyne et al. 2009). In this study, the optimum temperature for high SR was 28 °C which resulted 80% as supported by Rhyne et al. (2009). Matias-Peralta et al. (2005) also observed high survival in moderate temperatures (30 °C). The present study recorded total mortality at an extreme temperature of 38 °C. In NPR experiment, the copepods exposed to moderate temperature showed more nauplii production rate (58 nauplii/female) in 28 °C, while low NPR was observed in high temperature of 38 °C. This may be because adults are not able to produce nauplii at above optimal temperature due to thermal stress and utilization of energy for survival than reproduction (Anzueto-Sánchez et al. 2014). Miliou and Moraitou-Apostolopoulou (1991) described that the egg sac and offspring were decreased, when the temperature was low in copepod *Tisbe holothuriae.*

The temperature below 28 °C showed extended development time from N1 to adult. The copepod *Tisbe* sp. showed shortest development time and generation time at temperature 28 °C. Matias-Peralta et al. (2005) obtained highest overall reproductive activities and shortest development time at 30–35 °C in *Nitokra affinis*. Williams and Jones (1999) observed that the *T. battagliai* developed rapidly from hatching to the adult stage and have a rapid generation time under optimal temperature. But in temperature of 38 °C, the development time from CI to adult was extended compared to other temperatures tested. Population density (PD) was also

affected by temperature. Population density (PD) of *Tisbe* sp. was elevated at moderate temperature (28 °C) compared to lower temperatures. Milione and Zeng (2008) also observed the better population growth at a temperature of 30 °C and found a decline of PD at 34 °C, while we observed a decline at 32 °C and 36 °C. For *Pseudodiaptomus pelagicus*, the optimal temperature was found as 27.5 °C, and a decline in temperatures above 30 °C was observed due to thermal stress as agreed by Rhyne et al. (2009). Many harpacticoids have showed to tolerate on a wide range of salinities (Matias-Peralta et al. 2005; Chen et al. 2006). However, changes in salinities can alter the nauplii production, development time, and population growth. The present study shows that copepod *Tisbe* sp. can tolerate a wide range of salinities from 18‰ to 38‰. A maximum level of 80% survival in 28‰ of salinity was observed. This result was comparable with the previous study by Matias-Peralta et al. (2005) who reported that survival of copepod *Nitokra affinis* was over 80% under 10 to 35‰ salinities. Finney (1979) found that the *Tisbe* sp. died at high salinity of 48 g L^{-1} and at low salinity of 20 g L^{-1}. Payne and Rippingale (2001) observed that the good survival of *Gladioferens imparipes* was obtained at a range of 18–27 PSU. They also described that the salinity did not affect the nauplii production and remained stable at all salinity level.

The present result on NPR showed better performance of 28‰ and poor NPR on lower salinities as supported earlier by Matias-Peralta et al. (2005). They found that the decrease in reproductive performance in decreased salinities. Chen et al. (2006) found improved egg production at increased salinities. The present results also agreed the same in *Tisbe* sp. Even though *Tisbe* sp. is an estuarine copepod, the PD showed decline trend in low (18‰) and high salinity (38‰). Milione and Zeng (2008) reported that calanoid copepod *Acartia sinjiensis* showed higher population growth at higher salinities of 30 PSU and decreased at lower and too high salinities. Similar trend was also observed in the present study; population density (PD) was found highest at 28‰ with 212 ind./l with initial stocking size of 3 M:3F ratio. The present result on PD clearly indicates that the optimal salinity for high nauplii production was 28‰, but in other salinities, the copepod produces egg sac but did not show high performance in hatching. Chen et al. (2006) also observed that most of the eggs could not hatch at over 30 ppt. Variations in salinity also affect the DT and GT of copepod *Tisbe* sp. The present result showed that the lower salinity has extended DT and GT. The development of NI-CI and CI-CVI was found shortest in optimal salinity of 28‰. This may be due to the estuarine nature of the copepod *Tisbe* sp. Matias-Peralta et al. (2005) also found that minimal GT in salinity of 30 ppt and the maximum was observed at high salinity of 35 ppt. In copepod *Eurytemora affinis*, salinity had no effect on stage NI duration at salinity between 10 and 25 PSU and found that no salinity stress at 15 PSU and NI developed normally to NIII (Devreker et al. 2004).

Copepods can migrate to different depths to encounter various pH adjustments in the wild. Changes in pH may affect the health, survival, and reproduction of marine copepods. It is necessary to understand their biology under captive condition. In the present study, pH significantly affects the SR, NPR, and PD of copepod *Tisbe* sp. The SR was high in the control (8.2) pH than other pH tested. Lewis et al. (2013)

observed the reduction in survival of *O. similis* in high pCO_2 than the control. Kurihara et al. (2004) stated that the survival of *Acartia erythraea* and *Acartia steueri* was not affected by low pH 6.8. Yamada and Ikeda (1999) suggested that the exposure time is an important parameter to check the tolerance. This is true in the present study, because animals exposed to continuous days have effects on the survival. In the present study, alkaline pH caused less SR than the acidic pH. This clearly indicates that an effect of pH is species dependent. The NPR of copepod *Tisbe* sp. was found high in control pH of 8.2. The acidic pH 6.2 has adversely affected the NPR than the alkaline pH 9.2 and 10.2. In the alkaline pH, copepod showed poor performance in reproduction. Weydmann et al. (2012) found that the egg production was unaffected by a pH level of 7.6 and 6.9 in *Calanus glacialis.* They observed a reduction in hatching performance among the eggs that were incubated at the lowest pH level. Zervoudaki et al. (2014) reported that the egg production and hatching success were decreased significantly over the duration of exposure at different pH in *Acartia clausi.* Kurihara and Ishimatsu (2008) reported that egg production and hatching success were unaffected even when adult females were reared for two generations in the CO_2 seawater. The high PD was found in pH 8.2 followed by 7.2 and 9.2. Very low (46 ind./l) PD was observed in alkaline pH 10.2 with 3 M:3F stocking ratio for a period of 14 days. Similar results were also obtained by Venkatesan (2014) in *O. rigida.* He reported that copepods were able to adapt better with acidic pH though copepod exposed to alkaline pH 9.2 yielded poor performance in PD. Dajuan et al. (2011) stated that prolong exposure time have decrease in egg hatching. This will reflect in the population density of the copepod. The DT and GT of *Tisbe* sp. were severely affected by different pH tested. The NI-CI was developed quickly in pH 8.2 (4.4 days), and the DT was extended in the alkaline pH (6.1 days) compared to acidic pH 6.2 (5.8). Pedersen et al. (2013) found that no animals were developed at elevated CO_2 and two animals were arrested as nauplii. Kurihara and Ishimatsu (2008) described that the copepod *Acartia tsuensis* development was not affected in all the CO_2 tested for all the stages.

Besides the other water quality parameters, diet and diet concentration also played a significant role in reproductive performance of the copepod. Harpacticoids appear to be ingesting a variety of diets; however, consuming various types of diets has effects in survival and nauplius production (Williams and Jones 1999). In the present study, microalgae *I. galbana*-fed copepods showed better SR and NPR. Williams and Jones (1999) described that the *I. galbana* is suitable sole food for *Tisbe battagliai*, and they observed high survival and better reproductive performance. Low SR was observed in *D. salina*- and *N. salina*-fed copepods; this may be due to the poor nutritional quality of the algae. Guidi (1984) suggested that the survival of *Tisbe cucumariae* was affected by nutritional value of the diet. High survival, growth, and lipid content were significantly higher in *N. spinipes* when fed with diatoms. In the present study, *Tisbe* sp. was able to produce more nauplii when fed with *I. galbana* with a moderate concentration of 25,000 cells/ml. Puello-Cruz et al. (2009) suggested that *I. galbana* is considered as best food for most of the filter feeders mainly copepods, because of high DHA that accelerates the high survival and growth. Payne and Rippinglae (2000) tested *I. galbana* as feed for

G. imparipes and achieved better survival and nauplius production. In too low and too high diet concentrations, SR and NPR decline partially, and this suggested that feeding excessive diet did not bring out the same high NPR response (Ohs et al. 2010). Araújo-Castro and Souza-Santos (2005) reported that the fecundity of *Tisbe biminiensis* was affected by high concentration of diets used for the study. Teixeira et al. (2010) reported that low microalgae densities and low egg production were observed for the three microalgae tested for *Acartia tonsa*. The study on population density is important to achieve the ultimate goal of copepod culture with high density, and also it provides interrelated parameters like nauplii and copepodite development, survival, and egg production (Milione and Zeng 2007). The PD of *Tisbe* sp. was found low when fed with nonalgal diet (yeast) compared to algal diets. This may be due to low nutritional profile of yeast, and it will destroy the water quality. The alga *I. galbana* and *T. suecica* diet with a concentration of 25,000 cells/ml showed better PD for *Tisbe* sp. compared to all other diets and concentrations tested. Milione and Zeng (2007) observed that the *Isochrysis* sp. with a density of 50,000 cells/ml is suitable for *Acartia sinjiensis* population growth. Low concentration of diets leads the animal to search for food and cannibalism.

The present findings showed that the diets significantly affect the PD of *Tisbe* sp. It is confirmed that *I. galbana* is suitable for better population growth (218 ind./l with 3M:3F stocking ratio for a period of 14 days). Copepod *Tisbe* sp. showed that DT and GT were shortest with the diet *I. galbana* and *T. suecica* with a concentration of 25,000 cell/ml as supported by Payne and Rippinglae (2000) for *Gladioferens imparipes*. Low diet concentration resulted in extended DT and GT. High algal concentrations did not elicit fast DT, while the moderate concentration did elicit fast DT and GT. Knuckey et al. (2005) also observed that *I. galbana*-fed *A. sinjiensis* showed better development. The lowest DT was obtained with the diet *Nitzschia closterium* for *T. biminiensis* by Pinto et al. (2001). The GT of *Tisbe* sp. was comparatively shorter (10.8 days) than in *T. biminiensis* (12.2 days). Araújo-Castro and Souza-Santos (2005) found that copepod *T. biminiensis* developed fast when fed on *Navicula* sp. than fed with *Thalassiosira fluviatilis*. Very slow GT was observed in copepod fed on *D. salina*, because of the availability of low essential nutrition especially DHA/EPA (Payne and Rippinglae 2000; Knuckey et al. 2005; Puello-Cruz et al. 2009).

Biochemical Composition of Live Feeds

Harpacticoids are able to culture at high densities than calanoids because of their epibenthic nature (Schipp 2006). The main purpose of the present study is to culture the harpacticoid copepod *Tisbe* sp. with low cost. Over 45 days of operation, our system produced an average of 2672 ind./l/day including nauplii, copepodites, and adults. Our system does not need water exchange throughout the culture period that minimizes the cost, and filtration system is not required. However, detritus were siphoned out every week, and filtered freshwater is added if salinity is increased. At

the initial stage of culture, the systems were unable to achieve high density due to adaptation. Cannibalism was not noticed in the present culture system throughout the study period. The culture system was stocked initially with only adults due to problem in acclimatization of nauplii and copepodite in the new environment. The present results were comparable with Payne and Rippingale (2001), who achieved 878 nauplii/day for calanoid copepod *G. imparipes* for long duration. The present study holds 730 nauplii/day in 15-day culture operation and 1227 nauplii/day for 45-day operation. The harvesting of 28,000 nauplii and copepodites/liter/day was achieved by Ribeiro and Souza-Santos (2011) for *T. biminiensis* over a period of 130 days. Drillet et al. (2014) achieved a high density of *A. tonsa* egg of 11,000–12,000 L^{-1} day^{-1}. Abolghasem et al. (2011) reported a maximum density of 3437 ind./l in *A. clausi* for a period of over 2 weeks in 100 L tanks.

The success of *Tisbe* sp. culture depends on the water quality parameters maintained and diets offered. The biomass increased when the temperature was maintained within the range of thermal tolerance. The biomass of *Tisbe* sp. increased when the temperature was maintained at 28 ° C. Similar results were also reported by Schipp et al. (1999) in calanoid copepod *Acartia* spp. with the temperature range of 28–32 °C and found maximum biomass. Matias-Peralta et al. (2005) stated that overall reproductive performance was good at the temperature ranging from 30 to 35 °C in *N. affinis*. In the present study, the 28‰ salinity was maintained based on the optimization, and high biomass was found. Abolghasem et al. (2011) reported that 35 ppt salinity was good for the biomass achievement in *A. clausi.* Matias-Peralta et al. (2005) reported high offspring was achieved in the salinity range from 30 to 35‰. Raju et al. (2012) yielded high biomass of *O. rigida* in 26–35‰. As *I. galbana* maximizes the growth and production of copepod, *Tisbe* sp. was chosen as diet. Payne and Rippingale (2001) also support the same in *G. imparipes*, and they found that *I. galbana* maximizes the growth and fecundity of the copepod. The mean number of nauplii produced in each brood was higher in copepod *T. biminiensis* fed with *N. closterium* by Pinto et al. (2001). The growth was decreased in the copepod, *Acartia tsuensis* when shortage of food, which was recorded by earlier workers (Deevy 1964; Paffenhofer 1970; Klein Breteler and Gonzalez 1982).

Consideration of biochemical composition in live feeds is essential to understand its suitability to fish larvae. In the present observation, protein and lipid contents of copepod were comparatively higher than that of rotifer and *Artemia* nauplii. The protein was found to be the major biochemical component in the copepod *Tisbe* sp. followed by lipid and carbohydrate. The protein content of rotifer is lower than copepod and higher than *Artemia* nauplii. Higher protein content in copepod *O. rigida* and *A. clausi* was also recorded earlier by Santhanam and Perumal (2012) and Rajkumar and Vasagam (2006). The observed protein variations might be due to the fact that it is utilized as a metabolic substrate (Jeyaraj 2012). The carbohydrate values were slightly higher in *Artemia* nauplii than rotifer and copepod. The recorded carbohydrate values were higher than *A. spinicauda* and *O. similis* reported by Perumal et al. (2009). The lower values of carbohydrate in copepods have been already reported by several researchers (Goswami et al. 1981; Perumal et al. 2009; Santhanam and Perumal 2012; Jeyaraj 2012). This may be due to that animals may

use carbohydrate as glycogen reserve and may utilize it for chitin synthesis in crustaceans (Sumitra Vijayaragavan et al. 1982; Krishnakumari and Goswami 1993). The low carbohydrate content in zooplankton led to contemplations on the other biochemical fractions in their metabolism (Perumal et al. 2009). Lipids are the major constituents of living organic matter, involved in a variety of cellular function including membrane structure (phospholipids and glycolipids) and energy storage (triacylglycerols and wax esters) (Vance and Vance 1985; Wainman and Smith 1997). Lipid content has been found high in copepod *Tisbe* sp. than other live feeds studied. The lipid content of tropical zooplankton when compared to temperate zooplankton is significantly low, and it was proved by the findings of Sreepada et al. (1992). The variations in the lipid content can also be attributed to its storage and utilization during the period of starvation, when it serves as an effective energy reserve (Nageswara Rao and Krupanidhi 2001; Rajkumar and Vasagam 2006; Ananth and Santhanam 2011; Santhanam and Perumal 2012).

Compared to adult stage, fish larvae need protein (amino acids) for very fast growth rates, and this will come from the type of food offered. Hence, the live feed gets more importance in this stage. The amino acid content of copepod *Tisbe* sp. was found higher than *Artemia* nauplii and rotifer. In the present study, a total of 16 amino acids were reported with a dominance of arginine, histidine, leucine, lysine, and methionine in copepod. Rajkumar and Vasagam (2006) also observed 16 amino acids in the cultured copepod, *A. clausi*, with a predominance of lysine, alanine, and glutamic acid. Perumal et al. (2009) recorded 16 and 15 amino acids in wild copepods *A. spinicauda* and *O. similis.* The predominant amino acids were threonine, glutamic acid, alanine, aspartic acid, serine, valine, and methionine. Rønnestad et al. (2003) reviewed that FAA content of *Artemia* depends on species and considerably lower than marine copepods. The amino acid histidine and valine of copepod *Tisbe* sp. was higher than the enriched *Artemia* metanauplii (Santhanm and Perumal, 2001; Aragao et al. 2004). Mitra et al. (2007) found variations in amino acids of zooplankton from pond to pond with copepods as dominant and the amino acids fulfill the requirement of fish larvae. Ronnestad et al. (1998) stated that copepods contain more than twice the amount of FAA per gram wet mass than *Artemia* and mentioned FAA of *Artemia* are strain dependent. From this study, it is clear that the copepod *Tisbe* sp. contains all the amino acids of fish larvae required.

High fatty acid contents of copepod *Tisbe* sp. than rotifer and *Artemia* nauplii suggest the potential of *Tisbe* sp. as live feed for larviculture. In the present study, 18:0 (stearic acid), 20:4 (ARA), 20:5 (EPA), and 22:6 (DHA) were dominant in copepod. Similar results were also experienced by many authors in various copepods (Rajkumar and Vasagam 2006; Drillet et al. 2006; Van der Meeren et al. 2008; Santhanam and Perumal 2012; Nandakumar 2014). DHA content of copepods was several times higher than *B. plicatilis* and *Artemia* nauplii (Rajkumar and Vasagam 2006; Santhanam and Perumal 2012). Stottrup et al. (1999) observed 16:0 as the major peak of saturated FA and18:3 n-3, 20:5 n-3, and 22:6 n-3 as the main unsaturated peaks in *A. tonsa.* Sorensen et al. (2007) described that the mean contents of EPA and DHA made up 12.4–25.7 and 12.5–32.5% of the total percentage of unsaturated fatty acids in *A. tonsa* which was found low in the present study. The recorded

higher EPA and DHA content of the copepod *Tisbe* sp. in the present study may be attributed to the dietary influences of the feed. Influence of the diet on copepod fatty acid composition is already documented by Nanton and Castell (1998). They found that the *Tisbe* sp. contained large amounts of 22:6 n-3 even when the diet has poor essential fatty acids. The requirement of marine fish for long-chain n-3 HUFA has long been recognized as being essential for normal growth and development. The evidence that marine fish are not capable of converting 18:3 n-3 into EPA and DHA and EPA to DHA (Stottrup et al., 1999). The most commonly used live feeds *Artemia* nauplii and rotifer have no or only a limited ability to bioconvert shorter-chain *n*-3 PUFA into the longer-chain EFA (20:5*n*-3) and DHA (22:6*n*-3). However, the copepods, *Tisbe* sp., *A. clausi*, and *O. rigida*, are apparently able to synthesize significant amounts of 20:5*n*-3 and 22:6*n*-3 (Nanton and Castell 1998; Rajkumar and Vasagam 2006; Santhanam and Perumal 2012). The above results were also observed in the present study in *Tisbe* sp. Arachidonic acid, which is essential for marine fish larvae, is also comparatively higher in copepod than rotifer and *Artemia* nauplii (Drillet et al. 2007). The red pigment astaxanthin is one of the strongest antioxidants in nature. In marine pelagic food webs, copepods are the major producers of astaxanthin. The most important function of astaxanthin in copepods is that of an antioxidant for protecting lipids from peroxidation (Van Nieuwerburgh et al. 2005). The copepods can synthesize astaxanthin from the diet, while rotifers cannot synthesize astaxanthin (Dominguez et al. 2005). In the present study, high amount of astaxanthin was noticed in copepod than rotifer and *Artemia* nauplii. This can be attributed to feed supply to the copepod. Dominguez et al. (2005) reported that the amount of astaxanthin accumulated by the rotifers decreased with time and maximal astaxanthin accumulation was observed in short-term (2 h) enrichment experiments. Since astaxanthin cannot be accumulated in their tissues, the rotifers act as carriers of the astaxanthin.

Utilization of Tisbe *sp. as Live Feed for Shrimp Larvae*

Mass-scale seed production of marine fishes is restricted by the production of live feeds available at the time of conversion to exogenous feeding (Cassiano et al. 2012). Successful larval culture of commercially important species is limited by using rotifers and *Artemia* because of their reduced nutritional quality. As alternative, copepods have been considered as a healthy initial feed choice for cultured marine fish larvae (Payne et al. 2001; Olivotto et al. 2006; Cassiano et al. 2011; Santhanam and Perumal 2012). It is well known that copepods are better alternative not only because of their nutritional profile but also for their variety of sizes and movements that improve the feeding activity of fish larvae (Olivotto et al. 2012). Payne et al. (2001) fed a mixture of copepod nauplii and rotifers to dhufish larvae and found increased growth and survival than larvae fed only with rotifers.

Olivotto et al. (2006, 2008, 2010) found that the inclusion of copepods in the feeds was able to improve the survival and growth of ornamental fishes. Cassiano

et al. (2011) reported that feeding copepod nauplii to pompano larvae for as little as the first day of feeding at a daily rate of 2–3 nauplii/ml improved survival and growth. In the present study, the growth and survival of postlarvae of Pacific white shrimp *L. vannamei* fed with rotifer, *Artemia* nauplii, and copepod *Tisbe* sp. were studied. Growth and survival of *L. vannamei* PL were comparatively higher with copepod diet than with rotifers and *Artemia* nauplii. The length and weight of the PL were significantly lower in rotifer diet than *Artemia* nauplii diet. The survival of PL fed with *Artemia* nauplii was similar to copepod-fed ones, but the rotifer-fed PL showed poor survival. The observed difference in survival and growth of PL may attribute to the nutritional quality of the live feeds used. It is well established that copepods have more nutritional value than rotifer and *Artemia* nauplii especially in EPA and DHA (Drillet et al. 2006; Rajkumar and Vasagam 2006; Santhanam and Perumal 2012). The fatty acids of copepods have high content of ARA, EPA, and DHA than other live feeds used. The present results also support the same that the nutritional composition of the live feed could influence the length and weight of the larvae. This is in agreement with the findings of Watanabe et al. (1983), Watanabe and Kiron (1996), Cassiano et al. (2011), Santhanam and Perumal (2012), and Jeyaraj and Santhanam (2011). Payne and Rippingale (2000) have reported the low length and weight in the seahorse *Hippocampus subelongatus* fed with *Artemia* nauplii due to its low content of EPA and DHA. Jeyaraj and Santhanam (2011) stated that the recorded low growth and survival in *Donax faba*-fed *Artemia* nauplii might provide inadequate nutrition for the larvae.

In the present study, the high growth and survival of *L. vannamei* fed with copepods could only reasonably be a reflection of the sufficient nutritional value of copepod *Tisbe* sp. Some authors reported that the percentage of larvae undergoing successful metamorphosis was higher for larvae fed on copepods than larvae fed on enriched *Artemia* sp. (Shields et al. 1999; Evjemo et al. 2003). The high content of EPA and DHA in copepods clearly reflects in the PL growth and survival, while the low content of EPA and DHA in *Artemia* nauplii and rotifers resulted in low nutritional profile and growth of PL. Similar results were also recorded by Evjemo et al. (2003) in halibut larvae, and they also found the live feeds with high content of DHA in the larvae.

Consumption of copepods in the larval stages increased their stress resistance and decreased malpigmentation (Kraul et al.1993; Rajkumar and Vasagam 2006; Santhanam and Perumal 2012). The larvae of mahimahi fed with copepods survived well under the stress conditions. The present study also supports the above facts in *L. vannamei* PL. This may be due to the astaxanthin content of the feed (copepod). Astaxanthin has also demonstrated profound antioxidant properties in fish larvae suppressing lipid peroxidation (Bell et al. 2000; Olivotto et al. 2009). Ananthi et al. (2011) reported that the pigment (astaxanthin) improved in *P. monodon* when fed with marine copepods due to increase in stress resistance of the larvae. Naess and Lie (1998) reared halibut larvae using rotifer and *Artemia* and found abnormal pigmentation. Prey density is also having a major effect on feeding behavior and growth of fish larvae (Kailasam et al. 2002; Olivotto et al. 2006; Rajkumar and Vasagam 2006). Cassiano et al. (2011) reported that the prey density of copepod nauplii was

maintained lower than that of rotifers throughout the experiment, and they concluded that low prey density may have affected feeding performance of early larvae. The growth of larvae fed on copepods was greater than those fed on rotifers. However the prey density was maintained equally for all the preys in the present study.

The amino acid content of the copepod *Tisbe* sp.-fed PL of the *L. vannamei* was comparatively higher than the *B. plicatilis*- and *Artemia* nauplii-fed PL. The high content of histidine, phenylalanine, methionine, and threonine in copepods might be the reason for high growth and survival of *L. vannamei* PL fed with copepods. The essential amino acid content was low in rotifer *B. plicatilis* and *Artemia* nauplii-fed PL than those on copepod-fed PL, and this may be reflected from the live feed consumed by the larvae as agreed by Santhanam et al. (2004) and Ananthi et al. (2011). Safiullah (2001) reported similar result for freshwater prawn, *Macrobrachium rosenbergii*, fed with freshwater copepods *Mesocyclops aspericornis* and *Sinodiaptomus indicus*. The low content of essential amino acids in the rotifers and *Artemia* nauplii might be the reason for the low growth and survival in the PL fed with the same. The present results helped us to understand the value of copepods as alternative live feed for *L. vannamei* postlarvae and showed good results in terms of growth and survival than the traditional live feeds.

Conclusion

The present chapter concluded that the copepod *Tisbe* sp. has been tested for various environmental parameters to optimize the culture conditions. Among the parameters tested, copepod *Tisbe* sp. showed high survival and fecundity at 28 °C temperature, 28‰ salinity, and 8.2 pH, and 25,000 cells/ml concentration of *Isochrysis galbana* were found as a suitable feed and optimum feed concentration to achieve high copepod density, population density, generation time, and developmental time. The biochemical composition of copepod *Tisbe* sp. showed high EAA and PUFA (ARA, EPA, DHA) which are essential for larval nutrition. The recorded EAA of *B. plicatilis* and *Artemia* nauplii was 28.61% and 25.67%, whereas the copepod showed 41.73% of total amino acids. In the present study, copepod *Tisbe* sp.-fed *L. vannamei* showed higher growth and survival than the other live feeds tested. The copepod *Tisbe* sp.-fed PL showed notable survival of 95%, 87%, and 85% on 7th, 14th, and 21st days, respectively. The biochemical composition of PL fed with copepod *Tisbe* sp. showed high amount of EPA, DHA, and astaxanthin content than the rest of the feeds tested presently.

Finally it is revealed that the copepod *Tisbe* sp. is the most suitable candidate species for mass culture as live feed as it attained the high density at small-scale level. Furthermore, it is clear that the rich nutritional profile of *Tisbe* sp. especially PUFA, astaxanthin, and EAA has improved the growth and survival of shrimp *L. vannamei* PL than rotifer (*B. plicatilis)* and brine shrimp (*Artemia* nauplii).

Further it can be cultivated in the pilot-scale level and used as an alternate feed in hatcheries for sustainable production of shrimp seeds.

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A Method of Analysis of Pigments in Copepods

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Introduction

Pigmentation in the animals generally aids in the sexual attraction of partners or camouflage to reduce the risk of predation, but in some planktonic crustaceans, pigments are used as a guard against harmful ultraviolet (UV) radiation (Hansson 2000). Pigments occur almost in all phyla of marine organisms (Goodwin 1976) and are widely present in the zooplankton and micronekton (Cheeseman et al. 1967; Herring 1972). There are so many reports on zooplankton pigmentation, in which most of them focused on crustaceans (Herring 1968; Hairston 1979; Castillo et al. 1982). In marine pelagic food webs, copepods are the major producers of astaxanthin. Firstly, the most important function of astaxanthin in copepods is that it is an antioxidant for protecting lipids from peroxidation. Secondly, pigmentation and thereby photo protection against UV light have been suggested. Thirdly, it could be that astaxanthin esters, since they are lipids, serve as sources of metabolic energy, even if they contribute to only 2% of the total lipid content of a copepod body. In this chapter, some of the common techniques used for the extraction and analysis of pigments in copepods are elaborated.

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Methods

Pigment Extraction from Copepod

Samples of copepods are filtered on 47 mm GF/F filters, and copepods are freeze-dried until only 0.1 ml of water remained in the filter (as determined by weight). Filters should be extracted in 1 ml of 100% HPLC-grade acetone in a glass tissue homogenizer tube, and then after standing at least 0.5 h at −20 °C, samples are grounded for 30–60 s while in an ice bath. Samples should be further extracted for at least 4 h at −20 °C and then centrifuged for 5 min. Subsamples of 50–700 µl from the supernatant should be mixed with Milli-Q H_2O (two parts extract to one part H_2O) immediately before injection.

High-Performance Liquid Chromatography (HPLC) Pigment Analysis

The frozen filters should be cut into small pieces (several mm × 1 cm) and sonicated in centrifuge tubes (on crushed ice), with 2 ml of 100% cold acetone for 2 × 15 s at 50 W using a Labsonic sonicator equipped with a 4 mm diameter probe inserted directly into the solvent. After sonication, samples should be kept for 2 h at 4 °C before centrifuged for 3 min at about 700 g. Then the supernatants should be filtered onto 0.5 µm (Millex SR syringe equipped with 25 mm filter of 0.5 µm porosity), transferred to 1 ml vials, and placed into the autosampler (kept at 4 °C) prior to injection in the HPLC. Reversed-phase HPLC analyses can be conducted based on the ternary gradient method of Wright et al. (1991) as described in Gasparini et al. (2000). Pigments can be detected by absorption at 436 nm and identified based on comparison of their retention time and spectra with standards.

Astaxanthin Analysis in Copepods

The carotenoid pigment astaxanthin in copepods can be analyzed by following the method of Hairston (1976). The pigments in copepods can be extracted from whole animals using approximately 10 mL of 95% ethanol. In each extraction, a minimum of 10–15 adult copepods should be put whole in 95% ethanol and stored in a dark place at room temperature for 24–48 h. Pigmentation intensity (optical density per milligram) can be measured as the optical density of the extract at the wavelength of maximum absorption (474 nm) divided by the total copepod dry mass in milligrams. Absorbance readings should be calibrated to a 95% ethanol blank. Dry mass

can be calculated from the length dry mass relationship according to Dumont et al. (1975). Average visibility deriving from a combination of pigmentation intensity and body size (optical density per animal) can be measured as the optical density of the pigment extract per individual without correcting for mass.

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An Intensive Culture Techniques of Marine Copepod *Oithona rigida* (*Dioithona rigida*) Giesbrecht

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Introduction

Copepods are the main prey for fish and other crustacean larvae in the marine environment compared to other preys (Støttrup 2000; Ostergaard et al. 2005; Sampey et al. 2007). Their dietic value to fish larvae is known to be greater than the rotifer, *Brachionus* spp. and brine shrimp *Artemia* spp., they are the main live prey presently used in aquaculture hatcheries widely (Støttrup 2000; Lee 2003). Using rotifers and *Artemia* during the early fish larval rearing periods of life history not always enhances finest larval growth since these live prey usually have an inadequate fatty acid report and, in some instances, inappropriate size (Kahan et al. 1982; Sargent et al. 1999; Holt 2003; Faulk and Holt 2005). Thus, alternative food sources that do not have these inadequacies and promote larval growth are required. Copepods, copepodites, and naupliar stages are good nominees (Holt 2003), and studies on their mass production have been developed to investigate their efficiency on novel diets in aquaculture (Drillet et al. 2006). The small cyclopoid copepod genus *Oithona* is one of the most prevalent and copious in temperate, tropical, and polar oceans (Gallienne and Robins 2001; Hopcroft et al. 2005; Castellani et al. 2007); *Oithona* sp. can be used as feed transition between Rotifera and *Artemia*, or as a substitution of *Artemia*, recently. The calcium content of *Oithona* sp. is higher than that of *Artemia* (Castellani et al. 2008). The content of eicosapentaenoic acid (EPA)

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and docosahexaenoic acid (DHA)is also higher than that of *Artemia* and Rotifera. The high content of EPA/DHA will be helpful for growth improvement and survival rate and to reduce the occurrence of abnormality on shrimp and fish larvae. *Oithona* sp. contains immunostimulant; attractant and some significant digestive enzyme given the importance of *Oithona* sp. as a substitute for *Artemia* in the aquaculture, and also the sustainable availability of *Oithona* sp., were significant (Diana and Suminto 2015). Furthermore, owing to limitations of mouth gape, newly hatched larvae of some warmwater marine fish species have complexity ingesting Rotifera and *Artemia* nauplii but are able to feed upon copepod nauplii (Støttrup 2003). Optimizing copepod diets to meet their inclination can result in growth, egg production, and successful egg hatching (Milione and Zeng 2008; Rahman and Meyer 2009; Rahman and Verdegem 2010). Based on the commercial availability and production, the aquaculture industry is dominated by rotifers and *Artemia*, even though without enrichment of *Artemia* nauplii and rotifers did not fulfill the HUFA level required by the fish larvae (Raju 2012). The fast growth and higher survival of larvae were noticed when the fish larvae are fed with copepod alone or in combinations with other live feeds (Støttrup 2000, 2003; Payne et al. 2001; Ananthi et al. 2011; Santhanam and Perumal 2012a; Jayaraj 2012; Kathiresan 2013; Nandakumar 2015; Ananth 2015; Dinesh Kumar 2015). For the successful rearing of larvae, nutrition-rich, small-sized feed should be used. Copepods can work on it and considered as a promising live feed for larval stages of shrimp and fish (Santhanam 2002). Temperature, pH, and salinity are the main key factors that ruled the growth and reproductive potential of copepod in aquaculture systems followed by food and food concentration (Sun et al. 2008; Rhyne et al. 2009; Santhanam and Perumal 2012a; Santhanam et al. 2013; Nandakumar et al. 2015, Perumal et al. 2015). Temperature plays a main role throughout the life cycle when the food factor is satisfactory. When pH is low in water, the skeptical damage was found in crustacean (Whiteley 2011), and growth and reproductive success were also affected (Whiteley 2011; Engstrom-Ost et al. 2014). Most of the marine invertebrates including copepods are weak during early developmental and reproductive stages. Previous studies also suggested that the when the pH decreases, egg production, egg-hatching success, and nauplius survival also decreased (Mayor et al. 2007). Only few reports are available on the culture of *Oithona rigida* with reference to environmental condition (Santhanam and Perumal 2012b; Vasudevan et al. 2013). Kahan (1979) optimized the copepod diet with vegetable juice as remedy for algal feed shortages. Though their diet depends on microalgae, we may have reinstated the algal diet with some other edible waste materials (Kahan 1979). The culture materials with various shapes will be given to troubleshoot the various physical barriers such as stable humidity, swimming activity behaviors, etc.; the type of vessels and their shape have been used since the copepod culturing mechanism begins (De Lepiney and Lionelle 1962; Santhanam et al. 2015). Light is also a complex external and ecological factor which includes spectrum of colors, intensity, and periodicity. It is considered to be a critical abiotic factor, influencing biological functions of any

organism. With the above merits and demerits, the present study has been focused on optimization, and culturing *O. rigida* with a series of experiments was conducted to know the effect of temperature, salinity, pH, diets, and diet concentration on the survival, nauplii production rate, population density, development time, generation time, alternate diets, shape of the culture vessels, nature of the culture vessels, different light intensities, and different photo periods which have been analyzed under controlled and sophisticated laboratory condition. The main intention of this study is to develop the intensive culturing technology for copepod *O. rigida* to achieve greater population density of species.

Materials and Methods

Algal Culture

All the microalgae strains used in the present study were developed in Marine Planktonology & Aquaculture Laboratory of Department of Marine Science, Bharathidasan University, Tiruhcirappalli, India. The strain codes of these microalgae were *Chlorella marina* (PSBDU01), *Isochrysis galbana* (PSBDU02), *Dunaliella salina* (PSBDU03), *Tetraselmis suecica* (PSBDU04), and *Nannochloropsis salina* (PSBDU05). These algae were cultured in a temperature-controlled room, using 2 l round bottom conical flasks filled with 0.22 μm filtered seawater (FSW) with a salinity of 30 PSU. Microalgae were fertilized with Conway's medium (Walne 1970) under 14:10 h light-dark cycle with 200 μ mol m^{-2} s^{-1} of light illumination. Algal cultures were supplied to copepods during their exponential growth phases. Yeast was obtained from local market and stirred in water used for experiments.

Copepod Collection, Isolation, and Identification

Copepod samples were collected from Muthupet Lagoon, Southeast coast of India (11° 29′ 23″ N; 79° 46′ 35″ E), using Indian Ocean Expedition (IOE) Standard plankton net (158 μm). After collections the trap was relocated to 5000 mL round bottom plastic container containing 3000 mL (34 PSU) of filtered seawater (1 μM) and provided with aeration. Copepod samples were washed with fresh filtered seawater to avoid contamination of other zooplankton. Copepods with egg sacs were picked up separately and transferred to a new beaker holding 300 mL filtered seawater (1 μM) and provided mixed microalgae as a food which was immediately transferred to laboratory condition, where *O. rigida* was isolated from other zooplankton and gradually scaled up by standard protocols (Davis 1955; Kasturirangan 1963; Santhanam and Perumal 2008; Santhanam et al. 2015).

Experiment Design

Before the experiments, healthy copepods were starved overnight. A known amount of unicellular algal cells were dissolved in wide-mouth beaker consisting of 100 mL of filtered seawater (1 μM). Three replicates were performed for each set of treatments. In each beaker the following numbers of copepod were inoculated for each experiment (nauplii production rate, 1 female with egg sac; survival, 10; population density, 3 males and 3 females; development and generation time, 3 females). There were six treatment levels for water temperature (16, 20, 24, 28, 32, and 36 °C), seven salinities (10, 18, 22, 26, 30, 35, and 40 PSU), five pH (5.2, 6.2, 7.2, 8.2, and 9.2), five diet varieties (*Chlorella marina*, *Isochrysis galbana*, *Dunaliella salina*, *Tetraselmis suecica*, *Nannochloropsis salina*), and six diet concentrations (15,000, 25,000, 35,000, 50,000, 75,000, 100,000 cells/mL) that were conducted. All experiments were conducted in 31 wide-mouth Borosil glass bowls (Model-IH22MB05913). The following environmental settings were used during each experiment except when the variable served as an independent variable (see above): 28 °C as water temperature, 30 PSU as salinity, and 8.5 as pH, mixed feed as a diet, and 45,000 cells/mL as a diet concentration.

The experiments are also consequences of various edible waste materials as alternate diets on *O. rigida*, impact of different culture vessel materials to culture *O. rigida*, different shapes of the culture vessels, and different light intensities and photo periods. For the alternate diet experiment, four different dietary organic wastes such as, cow dung, rice bran, shrimp shell waste, and vegetable juice, have been chosen, and their extract was used to feed the *O. rigida*. Ten adult species were taken from the stock culture and transferred to the new 100 mL beaker filled with filtered seawater 32 ± 1‰ of salinity; each beaker was fed by its respective extract for 18 days; then the copepodite, nauplii, and adults were calculated during which the experiment water was ultimately changed every 2 days; and the dead animals and detritus have been removed simultaneously.

The population density was determined under a stereomicroscope using Sedgewick Rafter Counting Cell which was following the standard procedure and recorded from the beginning of the experiment. For the study of different culture vessels or containers, five different container materials such as plastic, glass, fiber, polythene bags, and clay pot, which are filled with 100 mL of filtered seawater (32 ± 1‰); all the containers have been stocked with ten healthy adults copepods, taken from the stock culture which are fed with *I. galbana* daily in the concentration of 30,000 cells/mL for 18 days. Furthermore, different shapes of the culture vessels and the same numbers of individuals were taken, and physicochemical parameters were maintained as previously used for other experiments. Then the shapes were chosen as rectangular, circular, conical, and oval, and the experiments were conducted according to the standard method (Syuhei and Takashi 1994; Rajthilak et al. 2014).

The experiments of light intensity and photo period were conducted with four different light intensities, viz., 1500, 3000, 4500, and 6000 lx, and five photoperiod series of 8L:16D; 10L:14D; 12L:12D; 14L:10D; and 16L:8D were established. Production of nauplii, copepodite, and adult was monitored. All the study has been carried over 18 days to observe the growth of the desired species. Moreover, in experiments the animals were fed with *I. galbana* daily in the concentration of approximately 30,000 cells/mL. All the experiments were carried as triplicate and the results were statistically analyzed.

Evolutions

For survival (SR), the replicate was covered with a 100 mL beaker which contained 60 mL of filtered seawater (1 μM). A total of ten nauplii were stocked into each beaker. Water exchange has been made every day and daily mortality was recorded carefully. For nauplii production rate (NPR), one healthy female was inoculated in 50 mL beaker consisting of 30 mL of filtered seawater (1 μM). To avoid photosynthesis of algal feed over light illumination, the tubes were covered with thick block cloth. Every 4-h interval, the beakers were checked for nauplii production, if produced adult copepod was separated from nauplii for avoiding predation. After fixing with 5% formalin, nauplii were counted under a dissection microscope. For population density (PD), three females with egg sacs and male copepods were inoculated in 250 mL of round bottom beaker with 200 mL of filtered seawater (1 μM). Copepod was filtered by using 48 μM mesh and fixed with 5% formalin at the end of the experiment. Filtered copepods were counted stage-wise, viz., nauplii, copepodite, and adult. For development time (DT), female copepods with eggs were inoculated to 50 mL of round bottom beaker with 30 mL of filtered seawater (1 μM). After nauplii production, the female copepod was transferred into a new beaker immediately, and the changes were observed every 30 min to know the metamorphosis of nauplii I (N1) to copepodite I (C1) and copepodite I (C1) to copepodite VI (C VI). For generation time (GT), a known number of female copepods were inoculated into 50 mL of round bottom beaker with 30 mL of filtered seawater (1 μM). The time was counted from the stage of egg to egg. The experiment lasted for 15 days.

Statistical Analyses

An analysis of variance (ANOVA) was used to determine if there were statistically significant differences between treatments for survival. The means were separated by the Tukey's procedure of SPSS 17.0.

Result and Discussion

Effects of pH

The effects of pH on the survival, nauplii production rate, population density, post embryonic development and generation time of the *Oithona rigida* in 15 days are summarized in Table 1 and Fig. 1a–c. High survival rates (>65%) ($p < 0.001$) were found in pH 7.2 and 8.2 at the end of the experiment (15th day), whereas low survival (20%) was found in pH 9.2 ($p < 0.05$). The other pH had resulted above 50% up to the eighth day of the experiment (Fig. 1a). Among the various pH tested, the better nauplii production rate of 56 nauplii/female and 47 nauplii/female was obtained in pH 8.2 and 7.2, respectively (Fig. 1b). The poor NPR (15 nauplii/female) was found in pH 5.2. The maximum population density (176 ind./l) was recorded in pH 8.2 consisting of 73 nauplii, 56 copepodites, and 47 adults, whereas the minimum population density (66 ind./l) was recorded in pH 9.2 with 29 nauplii, 22 copepodites, and 15 adults (Fig. 1c). pH 8.2 resulted in shortest duration (3.9 days) for developing the stage of nauplius I to copepodite I, whereas pH 9.2 derived the longest duration (5.1 days) of development. Besides, the precise development (7.2 days) of copepodite I to copepodite VI (adult) was found at pH 8.2, while protracted development time (8.2 days) was observed at pH 5.2. pH 8.2 took the short duration (11.6 days) for generation, and pH 5.2 took the long duration (14.2 days) (Table 1). The copepod species *O. rigida* has been proposed in aquaculture as a live feeds (Raju 2012; Kaviyarasan 2012; Santhanam and Perumal 2012a; Kathiresan 2013; Dinesh Kumar 2015). Therefore, it is important to find better conditions to rear this copepod with reduced efforts in terms of costs and time. Due to adjustment of pH, copepod can migrate in the ocean (Engstrom-Ost et al. 2014). The effect of pH on the metabolism of marine copepods is more limited in our facts (Marshall et al. 1935; Li and Gao 2012) especially *O. rigida*. Zervoudaki et al. (2013) report that the pH can affect negatively the egg production and hatching success of marine copepod. In this stage, understanding their biology under laboratory condition with reference to pH is very important. This study might be treated as a first report of the pH combined with temperature, salinity, and diets on growth and reproductive parameters of *O. rigida*. The present study revealed that the survival, nauplii production rate, population density, and development and generation time of *O. rigida* under different pH can be affected significantly. Higher survival was recorded in pH 8.2 compared to other pH tested (5.2, 6.2, 7.2, and 9.2). Yamada and Ikeda (1999), Kurihara et al. (2004), Lewis et al. (2013), and Ananth (2015) had suggested that the intermediate pH did not affect the survival of copepod if the culture duration is short. From the results of the present study, least survival was found in higher and lower pH (9.2 and 5.2). The same time above results revealed that the threats of pH on copepod survival varied in different species. Likewise survival and nauplii production rate of *O. rigida* were low in higher and lower pH compared to pH of 8.2. Egg production of *Calanus glacialis* is not affected in the pH levels of 6.9 and 7.6 (Weydmann et al. 2012). They advised that when the copepods were incubated in low pH, egg-hatching performance was reduced. Under various pH, pH 8.2 had

Table 1 Effects of pH on development and generation time of *Oithona rigida*

	pH				
Duration (days)	5.2	6.2	7.2	8.2	9.2
Development time (N1–C1)	4.9	4.2	4	3.9	5.1
Development time (C1–C6) (adult)	8.2	7.8	7.4	7.2	8.1
Total development time	13.1	12	11.4	11.1	13.2
Generation time	14.2	13.1	12	11.6	14.1

Note: *N1–C1* nauplius 1 to copepodite 1, *C1–C6* copepodite 1 to copepodite 6 (adult)

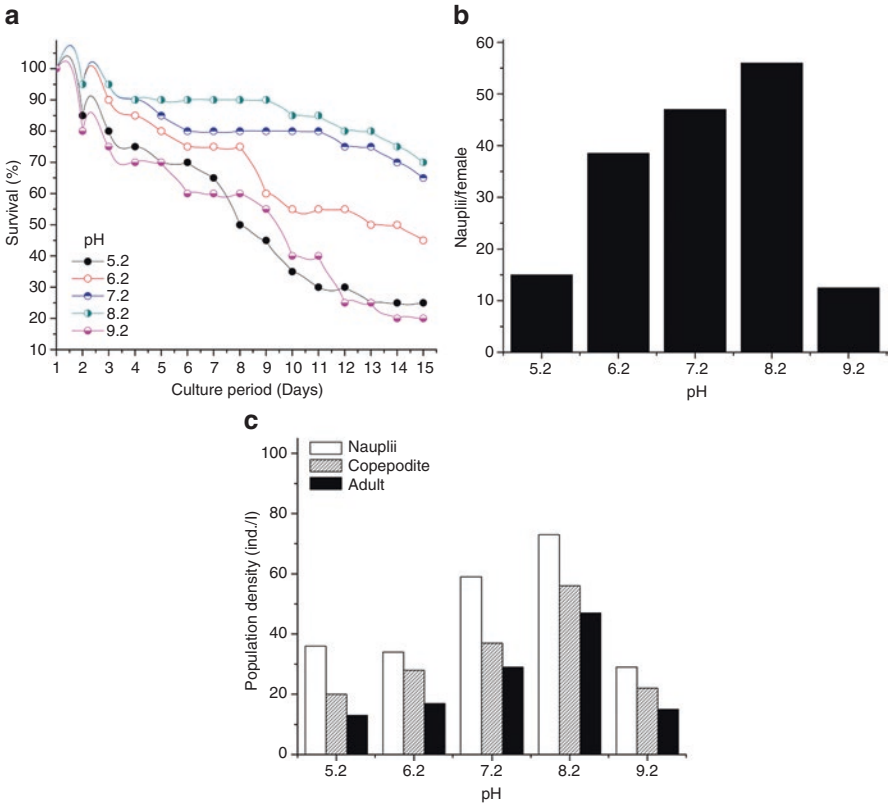

Fig. 1 Effects of pH on the survival, nauplii production rate, and population density of *Oithona rigida*

resulted in maximum population density followed by 7.2 and 6.2. Among the five pH, the lowest population density was observed in the highest pH (9.2). Generally copepods can adopt and survive in acidic pH compared to alkaline pH (Vengatesan 2014), and at the same time, long duration of culture will affect the egg-hatching and population density. Based on our results, pH can affect the development and generation time of copepod severely. The stages of N1–C1 and C1–C6 were

developed in short duration (3.9 and 7.2 days) at pH 8.2, while pH 5.2 and 9.2 resulted in long duration (totally 13.1 and 13.2 days). Previous researchers (Kurihara and Ishimatsu 2008; Pedersen et al. 2013) suggested that the high pH can arrest the copepod as nauplii and didn't allow to develop further.

Effects of Salinity

Figure 2a–c shows survival, nauplii production rate, population density, development and generation time with reference to different salinities. Among the seven salinities (10, 18, 22, 26, 30, 35, and 40 psu) tested, higher survival rate (75%) ($p < 0.001$) was found in 26 PSU followed by 30 PSU (50%) ($p < 0.01$) at the end of the experiment. The low survival (10%) ($p < 0.05$) was recorded in higher salinity (40 PSU) (Fig. 2a). The surpassing nauplii production rate (55.5 nauplii/female) was recorded in 26 PSU of salinity, and inferior nauplii production rate (20.5 nauplii/female) was recorded in higher salinity (40 PSU) (Fig. 2b). At the end of the experiment (15th day), the total population density (197 ind./l) was high in 26 PSU of salinity consisting of 83 nauplii, 66 copepodites, and 48 adults. The second best population density (180 ind./l) was found in 30 PSU of salinity. The lower population density (91 ind/l.) was found in higher salinity (40 PSU) (consisting of 52 nauplii, 23 copepodites, and 16 adults) (Fig. 2c). Development time for nauplius I to copepodite I was observed very short at the salinity of 26 PSU, and the development time was only 3.8 days. Under higher salinities (40 PSU), the development time was lengthened to 4.9 days. Likewise, the development time from copepodite I to copepodite VI (adult) was shortest (7 days) at 26 PSU. The quick generation time was achieved in 26 PSU, which took 11.9 days, while the clumsy GT (14.8 days) was found in higher salinity of 40 PSU (Table 2). In copepod research, the relation between salinity and their feeding behavior is very rare. This may be due to salinity in the marine environment which is relatively stable, and copepods living in the estuarine area have adapted to the seasonal change in salinity. If salinity increases due to agricultural and domestic runoff to the coastal area, marine copepods are capable of regulating their osmotic balance and internal ionic concentration. Few studies have been indicating that the salinity could influence the survival and reproduction of copepods. Likely, when salinity decreased from 30 ppt to 10 ppt, *A. tonsa* spawning rate will be decreased (Ambler 1986). Liu and Chen (1995) stated that the 7–28 ppt of salinity is best for devolving *Apocyclops royi* culture. In the present study, higher survival was found in 26 PSU of salinity, and lower survival was found in higher salinity (40 PSU) as well as least salinity (10 PSU) too. Higher survival of copepod in different salinities is depending on their osmotic acclimatization (Roddie et al. 1984). Santhanam and Perumal (2012a, b) have stated that the optimal salinity for culturing *Oithona rigida* is 28–34 PSU. According to the interpretation of findings, the results stated that the best (75%) and second best (50%) survivals were found at 26 and 30 psu of salinity. Maximum nauplii production was attained at 26 PSU of salinity, and minimum production was found to be 40 PSU of salinity.

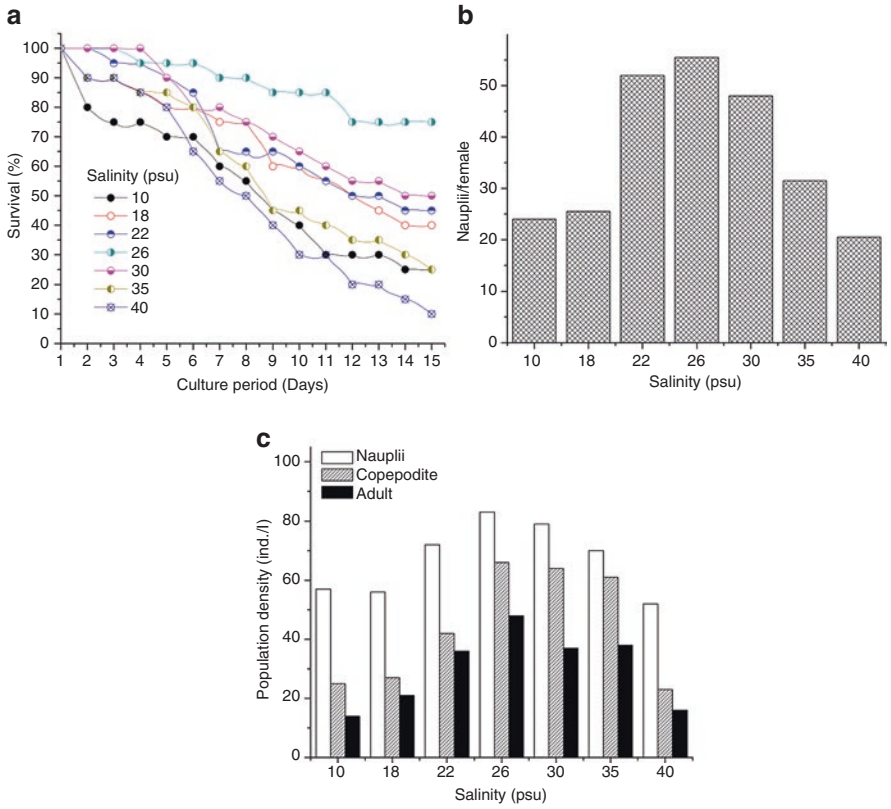

Fig. 2 Effects of salinity on the survival, nauplii production rate and population density of *Oithona rigida*

Table 2 Effect of salinity on development and generation time of *Oithona rigida*

	Salinity (psu)						
Duration (days)	10	18	22	26	30	35	40
Development time (N1–C1)	4.6	4.5	4.2	3.8	4.4	4.8	4.9
Development time (C1–C6) (adult)	8.2	8	7.8	7	7.9	8.4	8.8
Total development time	12.8	12.5	12	10.8	12.3	13.2	13.7
Generation time	13.4	13.2	13	11.9	14.3	14.6	14.8

Note: *N1–C1* nauplius 1 to copepodite 1, *C1–C6* copepodite 1 to copepodite 6 (adult)

The second lowest nauplii production was found at 10 PSU of salinity, these trends were observed in earlier works (Matias-Peralta et al. 2005), and they stated that the salinity reduction could decrease their reproductive performance. If the salinity level is increased, an egg production will also be increased but it should not be exceeded than the permisible limit (Chen et al. 2006). The current study revealed

that after optimum salinity level (26 PSU), nauplii production rate will be decreased. Population density under different salinities revealed that the decreasing trend was observed in too low (10 PSU) and high (40 PSU) salinities. In current observation the highest population density (197 ind./l) was found in 26 psu of salinity. Higher population growth was observed in higher salinity in *Acartia sinjiensis* culture, and their population was decreased when the salinity level was increasing and decreasing (Milione and Zeng 2008), and the same trend was observed in the present study. Salinity levels of 22–30 PSU were found to provide the best condition for the development and generation of *Oithona rigida* from N1 to C1 and from C1 to C6. Under these salinities, copepods were developed in the stage of N1–C1 and C1–C6 shortly (3.8 and 7.0 days) under 26 PSU, and this may be due to the coastal environmental conditions of the *Oithona* sp. The minimum duration of copepod generation was found at 26 PSU of salinity, and the same trend was observed by Devreker et al. (2004). They stated that salinity between 10 and 25 PSU didn't make any stress in *Eurytemora affinis* generation.

Effects of Temperature

The percentage of survival and mean nauplii production rate, population density, and development and generation time as a function of temperature are shown in Fig. 3a–c and Table 3. Temperature significantly affects the survival of the *Oithona rigida* ($p < 0.001$). The maximum (75%) survival was found recorded in 28 °C at the end of the experiment, and the second best (45%) was found in 24 °C, while the minimum survival (30%) was found in 16 °C. Total mortality was observed earlier in 36 °C by the end of 9th day of the experiment (Fig. 3a). The 28 °C resulted in higher nauplii production rate (58.5 nauplii/female) compared to other temperatures tested (16, 20, 24, 32, and 36 °C), while the lower (12 nauplii/female) nauplii production rate was found in higher temperature (36 °C) (Fig. 3b). The overall population density of *O. rigida* was low at the highest temperature (36 °C), reaching 64 ind./l which was comparatively much lower than the nauplii obtained at 28 °C, while high PD of *O. rigida* at 28 °C, having 201 ind./l, comprises of 86 nauplii, 67 copepodites, and 47 adults followed by 24 °C having 138 ind./l (Fig. 3c). From nauplius I to copepodite I was shortest at 28 °C which took 3.9 days only for *O. rigida*, while the longest development time was found at the highest temperature of 36 °C which took 5.1 days. The shortest time for developing stage of copepodite I to copepodite VI (adult) was found at 28 °C (7.1 days), and the longest development was found at 38 °C (7.8 days). The generation time from egg to egg was shortest at 28 °C which took 12.1 days, while the longest GT (13.6 days) was observed at 16 °C (Table 3). Temperature is often the most important environmental factor affecting the productivity of copepods in natural systems (Rhyne et al. 2009; Ananth 2015). From the study of growth and reproductive parameters, the *O. rigida* was examined under various temperatures (16, 20, 24, 28, 32, and 36 °C). Survival, population density, and nauplii production rate were highest at 28 °C, and the lowest was found in 36 °C. The present study found that the optimum temperature (28 °C)

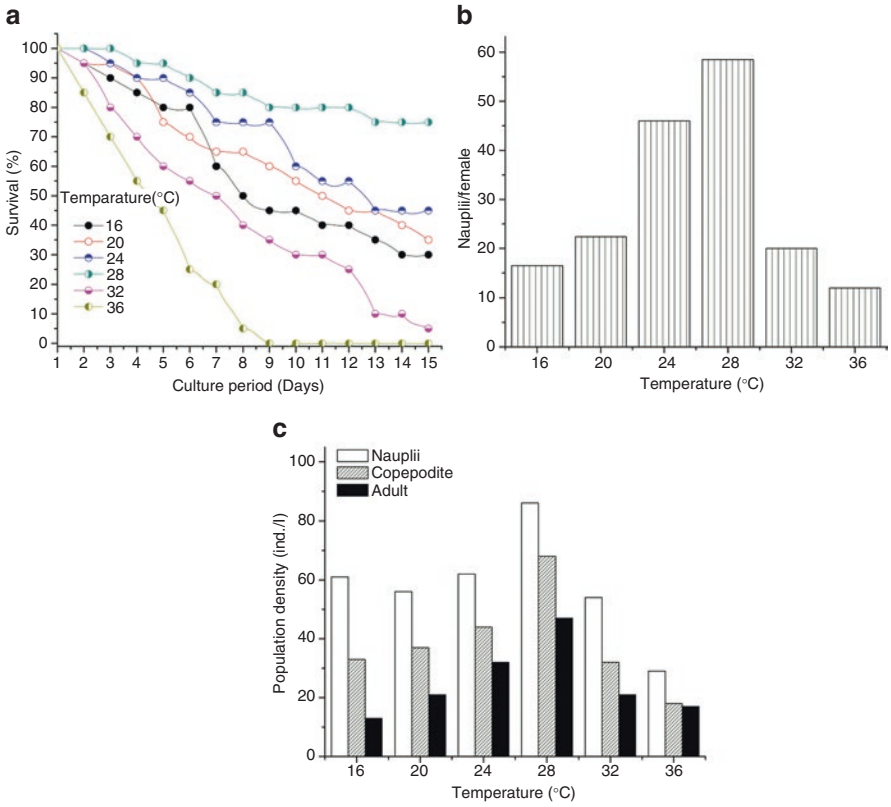

Fig. 3 Effects of temperature on the survival, nauplii production rate, and population density of *Oithona rigida*

Table 3 Effect of temperature on development and generation time of *Oithona rigida*

	Temperature (°C)					
Duration (days)	16	20	24	28	32	36
Development time (N1–C1)	4.8	4.4	4.2	3.9	4.3	5.1
Development time (C1–C6) (adult)	8.1	8	7.8	7.1	7.7	8.4
Total development time	12.9	12.4	12	11	12	13.5
Generation time	13.6	13.2	12.8	12.1	13.2	14.6

Note: *N1–C1* nauplius 1 to copepodite 1, *C1–C6* copepodite 1 to copepodite 6 (adult)

for higher survival (<75%) and results were supported by early works (Gonzalez and Bradley 1994; Amarasinghe et al. 1997; Carlotti et al. 1997; Rhyne et al. 2009). The results of the present study show clearly that the mortality of adult *Oithona rigida* is increased at elevated temperatures. Moore et al. (1996) also suggested that copepods and cladocerans have better survival in low and median temperatures compared to higher temperatures. Based on their findings, the present study also showed that the total mortality (100%) was found at higher temperature (36 °C)

from the 9th day onward. While temperature increased from favorable or optimal, the energy was used to extend their living purpose, so automatically nauplii production rate will be decreased (Williams and Jones 1999; Santhanam and Perumal 2012a; Anzueto-Sánchez et al. 2014; Ananth 2015). At the same time, low temperature also affected the nauplii production rate with decreasing activity of egg sac and offspring (Miliou and Moraitou-Apostolopoulou 1991). Temperature variation affected the population density of *O. rigida* significantly and the higher population density were observed in 28 °C and lower was 36 °C second lower was found in 16 °C. Based on the results obatined from the effects of temperature the higher and lower temperatures affected the population density severely while the modrate was motivated. We found that population density increased when temperature increased from 24 °C to 28 °C and decreased more often than that. The report of Milione and Zeng (2008) also supported our findings; they found that the temperature below 30 °C resulted in high density, while temperature increase more than 30 °C resulted in low density. 27.5 °C is the optimal temperature for culturing *Pseudodiaptomus pelagicus*, and density was decreased if temperature was increased beyond that (Rhyne et al. 2009). Development and generation time were short in 28 °C temperature while long in 36 °C temperature. An Increased temperature have also been affected the duration of development period and generation of *O. rigida*. When the temperature was increased to 34°C from the optimal temperature (28 °C) it will elangate the duration of development time from 11 days to 13.5 days and generation time from 12.1 days to 14.6 days. These findings are consistent with early works on *B. hamata* which showed that clutch size decreased with increased temperature (Jamieson 1988; Jamieson and Burns 1988). Williams and Jones (1999) suggested that under optimal temperature, egg hatching will go through rapidly without barrier compared to higher and lower temperatures.

Effects of Diets

The percentage of survival and mean value of nauplii production rate, population density, development time, and generation time for *Oithona rigida* copepods fed with seven diets are reported in Fig. 4a–c and Table 4. Each diet induced variables on each experiment during the 15-day culture period with lowest (20%) ($p < 0.05$) survival rate found in yeast given as a diet, highest survival (80%) ($p < 0.0001$) found in *Isochrysis galbana*, and second best (70%) ($p < 0.001$) found in *Tetraselmis suecica* at the end of the experiment (Fig. 4a). With a diet of *I. galbana*, *O. rigida* produced highest (60 nauplii/female) nauplius followed by mixed diet (58.5 nauplii/female). Yeast resulted in lowest (12 nauplii/female) nauplii production rate at the end of the experiment (15 days) (Fig. 4b). The total population density of *Oithona rigida* was found highest (204 ind./l) when fed with *Isochrysis galbana* with 84 nauplii, 52 copepodites, and 65 adults. A slightly minor variation was observed in the mixed-diet-fed copepods (203 ind./l). *O. rigida* population was discarded exceedingly when they are fed with yeast where the population density was noticed as 74 ind./l (Fig. 4c). *I. galbana* is a favorable source of food for development and

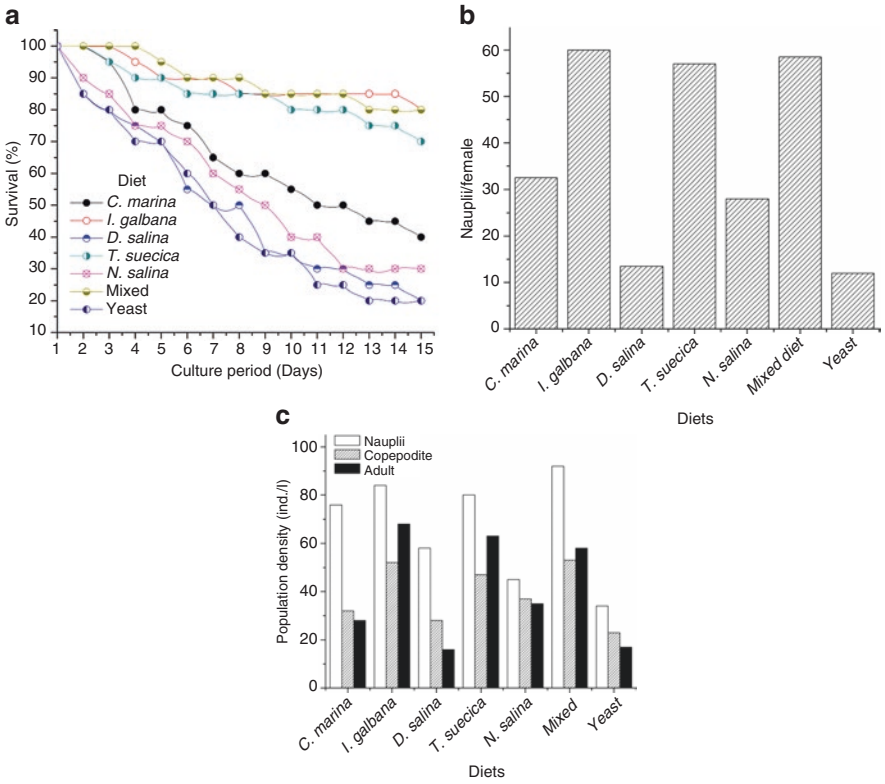

Fig. 4 Effects of diet on the survival, nauplii production rate, and population density of *Oithona rigida*

Table 4 Effect of diet on development and generation time of *Oithona rigida*

	Diets						
Parameters	*C. marina*	*I. galbana*	*D. salina*	*T. suecica*	*N. salina*	Mixed diet	Yeast
Development time (N1–C1)	4.2	3.8	4.9	4.1	4.6	4	5.2
Development time (C1–C6) (adult)	7.8	7.1	8.4	7.2	8	7.1	8.3
Total development time	12	10.9	13.3	11.3	12.6	11.1	13.5
Generation time	13.2	11.9	14.6	12.1	13.8	11.9	14.8

Note: *N1–C1* nauplius 1 to copepodite 1, *C1–C6* copepodite 1 to copepodite 6 (adult)

generation of *O. rigida*; quickest development time from nauplius I to copepodite I and from copepodite I to copepodite VI (adult) and generation time were observed in the copepod fed with microalgae *I. galbana* which took 3.1, 7.1, and 11.9 days, respectively, whereas the longest development was noticed in *D. salina* as 4.9, 8.4, and 14.6 days, respectively (Table 4). Copepods have an effect on survival and

nauplii production rate when they consume different diets (Williams and Jones 1999). The total concert of a diet depends on its digestibility, composition, and available energy content. Survival, nauplii production rate, population density, and development and generation time of *Oithona rigida* resulted in maximum when they are fed with *Isochrysis galbana*. We confirmed *Isochrysis galbana* as an excellent diet for *Oithona rigida*, which is in agreement with results, and they contained the highest proportion of DHA and had a high DHA and EPA ratio, as was recorded previously by researchers (Pillsbury 1985; Dunstan et al. 1993). In survival, maximum was observed in *Isochrysis galbana* and minimum was found in *Dunaliella salina* and yeast. The poor performance of the diets is depending on their cell wall (Dhont and Lavens 1996; Payne and Rippingale 2000) or by its small size and low weight and energy content. If size is small, nutrient content will be less only, and their nutritional value could affect the copepod survival. Watanabe et al. (1983) and Brown et al. (1997) suggested that the *C. muelleri* and *I. galbana* are best food for filter feeders, because they consist higher EFA for promoting survival and growth of copepod. Higher nauplii production was observed using *I. galbana* as diet and second best in mixed diet. Arnott et al. (1986) and Payne and Rippingale (2000) found that *Isochrysis* sp. is the best diet for copepod nauplii production among the diets tested, because, compared to other diets, *Isochrysis* sp. sustained viable egg production through multiple generations, and also they suggest that the *Isochrysis* consisted essential micronutrients (HUFA and DHA) not present in *Dunaliella* and other algal diets. The successful copepod culture is explaining higher population density with nauplii, copepod development, survival, and egg production. The population density of *O. rigida* was high in mixed diet (203 ind./l) followed by *Isochrysis galbana* (202 ind/l), and least density was found in yeast (74 ind./l). The reason may be due to water quality of cultured tank which destroyed the yeast (Ananth 2015). *Oithona rigida* showed that the shortest development and generation time were found when they are fed with *Isochrysis galbana* (DT, 10.9; GT, 11.9 days) as a diet followed by mixed diet (DT, 11.1; GT, 11.9 days) compared to others as supported by Knuckey et al. (2005).

Effects of Diet Concentration

Figure 5a–c and Table 5 show survival, nauplii production rate, population density, and development and generation time of *Oithona rigida* under different diet concentrations. The recorded mean survival rate was 40% up to the 7th day of the experiment in all the diet concentrations. The maximum survival (75%) ($p < 0.001$) was observed in 50,000 cells/mL followed by 75,000 cells/mL (70%) ($p < 0.001$) at the end of the experiment (15th day), while the lowest survival (10%) ($p > 0.05$) was recorded in the low diet concentration of 15,000 cells/mL (Fig. 5a). The highest nauplii production rate (57.5 nauplii/female) was found in 50,000 cells/mL of microalgae-fed *O. rigida* followed by 75,000 cells/mL with 47.5 nauplii/female,

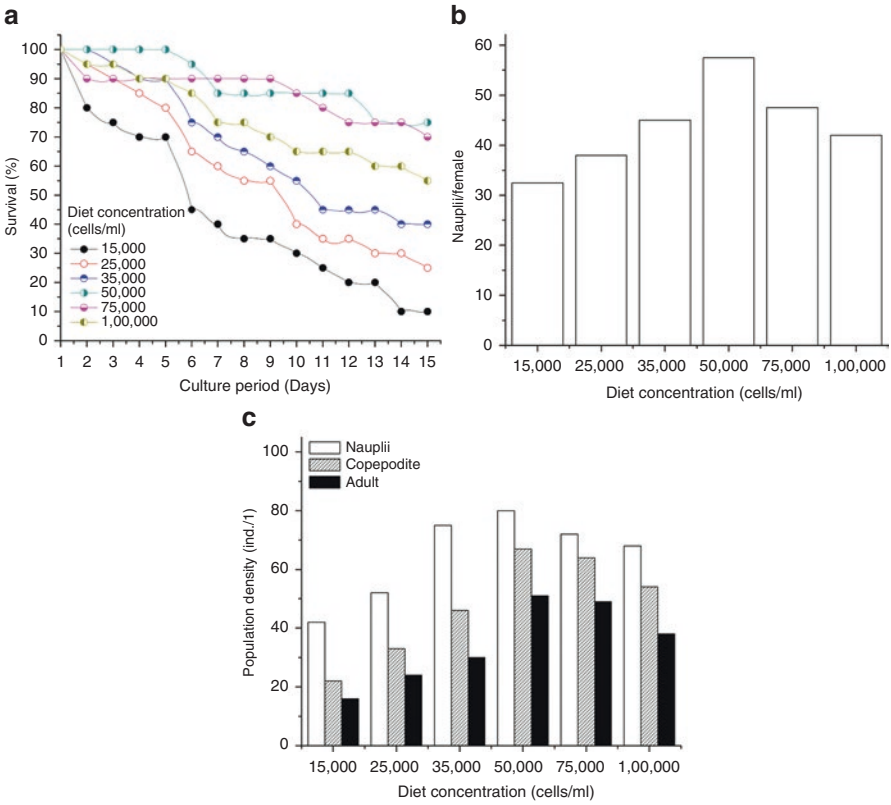

Fig. 5 Effects of diet concentration on the survival, nauplii production rate, and population density of *Oithona rigida*

Table 5 Effect of diets concentration on development and generation time of *Oithona rigida*

	Diet concentration (cells/ml)					
Duration (days)	15,000	25,000	35,000	50,000	75,000	100,000
Development time (N1–C1)	5.1	4.6	4.2	3.9	4.1	4.4
Development time (C1–C6) (adult)	8.2	8	8	7.1	7.3	7.6
Total development time	13.3	12.6	12.2	11	11.4	12
Generation time	14.6	13.4	13.1	11.8	12.6	12.8

Note: *N1–C1* nauplius 1 to copepodite 1, *C1–C6* copepodite 1 to copepodite 6 (adult)

while the low nauplii production rate was found in the lowest food concentration of 15,000 cells/mL with only 32.5 nauplii/female (Fig. 5b). Higher population density (198 ind./l) was found in 50,000 cells/mL of diet concentration including 80 nauplii, 67 copepodites, and 51 adults, while the lowest population density (80 ind./l) was found in 15,000 cells/mL of diet concentration (Fig. 5c). Under different diet

concentrations, the quickest development time (3.9 days) for nauplii I to copepodite I was found in 50,000 cells/mL, and the second best (4.1 days) was found in 75,000 cells/mL. For copepodite I to copepodite VI (adult), the shortest time duration of development (7.1 days) was recorded in 50,000 cells/mL, and the longest time duration (8.2 days) was recorded in 15,000 cells/mL of diet concentration. The overall generation time (11.8 days) was shortest at copepods fed with 50,000 cells/mL, while the longest generation time (14.6 days) was noticed at copepod fed with a diet concentration of 15,000 cells/mL (Table 5). Diet concentration is probably the key factor regulating the productivity of copepod culture (Rhyne et al. 2009; Sun et al. 2008). Still, copepod size and species affected significantly their population growth. In the present study, higher survival (75%) was found in 50,000 cells/mL followed by 75,000 cells/mL (70%) and lower (10%) survival in 15,000 cells/mL. It might be due to the lack of nutrition and energy from the diets (Santhanam and Perumal, 2012a,; Ananth 2015). Nauplii production rate of *O. rigida* was high in 50,000 cells/mL and low in 10,000 cells/mL. Survival and nauplii production rate decreased partially when diet concentration is too low and high. Ohs et al. (2010) suggest that compared to moderate concentration, others didn't cause any positive variance on survival and nauplii production rate of copepod. The highest population density (198 ind./l) was observed in 50,000 cells/mL as agreed by Milione and Zeng (2007). They found that while culturing, *Acartia sinjiensis* optimum diet concentration is 50,000cells/mL. Meanwhile, compared to higher diet concentration (75,000 and 1,00,000 cells/mL), low diet concentration (35,000, 25,000, and 15,000 cells/mL) has resulted in lower population density. This might be due to the lack of food for copepod and cannibalism activities of copepod (Milione and Zeng 2007). Development and generation time were short (11.0 and 11.8 days) in moderate concentration (50,000 cells/mL) and long (13.3 and 14.6 days) in low concentration (15,000 cells/mL). From the results we understood that the low diet concentration has extended the development and generation time. At the same time, higher diet concentrations didn't bring out quick development and generation time, and the moderate concentration diet can induce development and generation time quickly as supported by Ananth (2015).

In the observation after 18 days of culture under different dietary organic waste extracts, the average final mean of copepod population productions is illustrated in Fig. 6a. The distribution of various life stages (nauplii, copepodites, and adults) of *O. rigida* within the populations cultured at various waste extracts as diets was shown in Fig. 6b and Table 6 when compared to all other waste extracts; rice bran has reached the highest mean population density on various life stages, the number of individuals per 100 mL; and 69.00 ± 3.65, 48.33 ± 2.51, and 30.66 ± 4.93 were recorded in respect to nauplii, copepodites, and adults. The results revealed that the different diets showed a significant difference in the total population density of *O. rigida* ($p < 0.001$). Through the various edible waste extracts (Fig. 6b) tested, the final number of *O. rigida* population attained peak at rice bran diet (148 ± 9.10 ind./100 mL), and the lowest population density of 80.66 ± 7.09 ind./100 mL was noticed in cow dung diet, which is dissimilar to

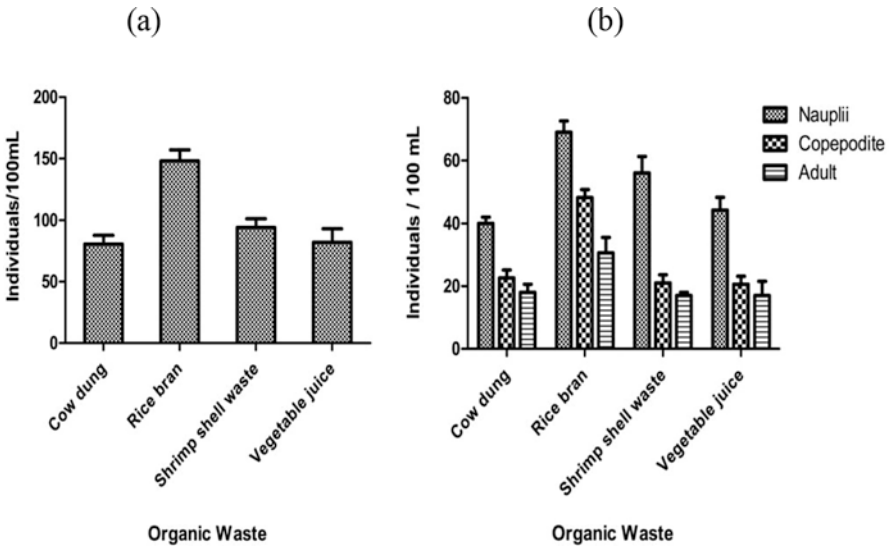

Fig. 6 (**a**) Total mean population production of *O. rigida* fed with various dietary organic wastes cultured for 18 days. (**b**) Mean population of three life stages (nauplii, copepodites, and adults) of *O. rigida* fed with different dietary organic wastes

Table 6 Effects of dietary organic waste extract diets on the mean population density (ind/100 mL) of adults, copepodites, and nauplii of *O. rigida* based on one-way ANOVA results

	Stages		
Diets	Nauplii	Copepodite	Adult
Cow dung	***	***	ns
Rice bran	**	***	**
Shrimp shell waste	***	***	ns
Vegetable juice	***	***	ns
Treatment mean ± SD			
Cow dung	40. 00 ± 2.00	22.68 ± 2.51	18. 00 ± 2.64
Rice bran	69.00 ± 3.65	48.33 ± 2.51	30.66 ± 4.93
Shrimp shell waste	56.00 ± 5.29	21.00 ± 2.64	17.00 ± 1.00
Vegetable juice	44.33 ± 4.04	20.66 ± 2.51	17.0 ± 4.58

Values were expressed as mean ± SD of three replicates in each group. ANOVA was followed by Tukey test; *, **, ***, and ns indicate $p < 0.05$, $p < 0.005$, $p < 0.0001$, and not significantly different, respectively

Rajthilak et al.'s (2014) report for *Nitokra affinis*; even though the highest population density was observed in microalgal diet as recorded by Pinto et al. (2001) and Rajthilak et al. (2014), Rajkumar and Rahman (2016) also concluded that the waste extract has to be used as alternative feed when the microalgal feeds are out of stock. For the experiment of culture vessels to rectify the physical barriers and to encounter the culture vessel shortages with readily availing materials such as plastic, glass,

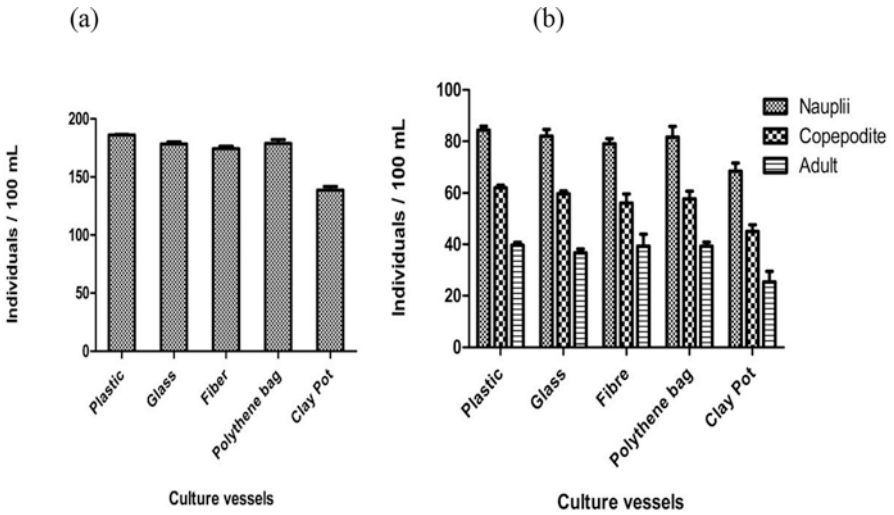

Fig. 7 (**a**) Total mean population of *O. rigida* cultured for 18 days, using different culture vessels. (**b**) Mean population of three life stages (nauplii, copepodites, and adults) of *O. rigida* cultured at culture vessels

Table 7 Effects of culture vessel types on the mean population density (ind/100 mL) of adults, copepodites, and nauplii of *O. rigida* based on one-way ANOVA results

	Stages		
Culture vessels	Nauplii	Copepodite	Adult
Plastic	***	***	***
Glass	***	***	***
Fiber	***	***	**
Polythene	***	***	***
Pot	***	***	***
Treatment mean ± SD			
Plastic	84.34 ± 1.52	62.0 ± 1.0	39.67 ± 1.15
Glass	82.00 ± 2.64	59.67 ± 1.15	36.67 ± 1.52
Fiber	79.00 ± 2.00	56.0 ± 3.61	39.34 ± 4.62
Polythene	81.67 ± 4.16	57.67 ± 3.06	39.34 ± 1.52
Pot	68.34 ± 3.21	45.00 ± 2.64	25.34 ± 4.16

Values were expressed as mean ± SD of three replicates in each group. ANOVA was followed by Tukey test; *, **, ***, and ns indicate $p < 0.05$, $p < 0.005$, $p < 0.0001$, and not significantly different, respectively

fiber, polythene bags, and clay pot with those materials, the total mean of the copepod population production was depicted in Fig. 7a. The distribution of various life stages (nauplii, copepodites, and adults) of *O. rigida* within the populations cultured at different culture vessels was showed in Fig. 7b and Table 7; there was no significant difference between them while analyzed. Although the highest popula-

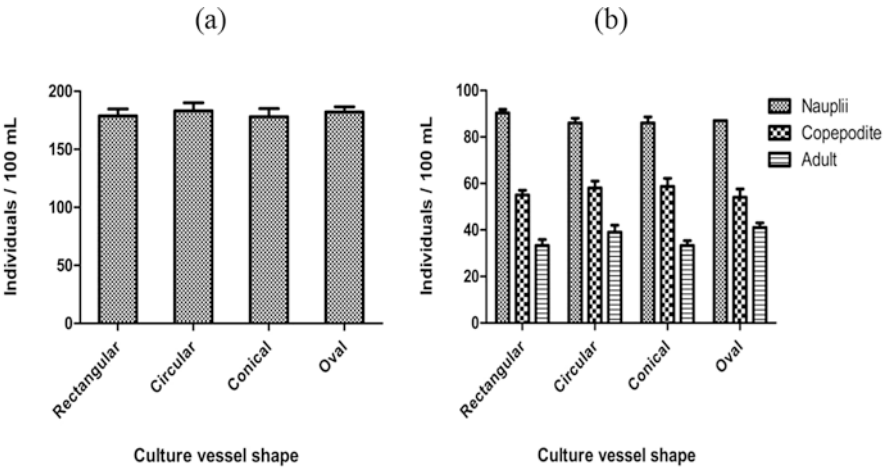

Fig. 8 (**a**) Total mean population of *O. rigida* reared at different shaped vessels. (**b**) Mean population of three life stages (nauplii, copepodites, and adults) of *O. rigida* reared at different shaped vessels

tion was observed in plastic containers among other materials, 84.34 ± 1.52 nauplii, 62.0 ± 1.00 copepodite, and 39.67 ± 1.15 adults were recorded for plastic vessels per 100 mL of seawater. Ribeiro and Souza-Santos (2011) also found that the plastic is a good material for copepod production in a mass culture level. From the statistical analysis, the greater significances were noted for all the vessels ($p < 0.0001$). Furthermore the total mean of the population density also obtained peak in the plastic vessel experiment which was noted as 186.00 ± 1.00 ind./100 mL, whereas the lowest density was observed in the culture vessel of pot 138.66 ± 5.510 ind./100 mL. The quality of culture vessels may have graded as plastic > polythene bags > glass > fiber > clay pot with suggestion to the present study carried. In different shapes of the culture vessel experiments, we could not take the best accurate shape because the acquired result of all the shapes we used may have shared the even density of population; in fact the highest (183.00 ± 7.00 ind./100 mL) total population density was obtained in circular shape of the vessels (Fig. 8a), because most probably the swimming behavior will be in circular form in all the aquatic environment perhaps. But when looked onto the distribution of different life stages of targeted species that varies from one another to the shapes, the rectangular vessel has produced 90.33 ± 1.52 ind./100 mL of nauplii, and the conical vessel has produced 58.66 ± 3.51 ind./100 mL of copepodite (Fig. 8b and Table 8). De Lepiney and Lionelle (1962) also have good agreement with rearing copepods in conical flask under laboratory condition, and oval-shaped vessel produced 41.00 ± 2.00 ind./100 mL of adults in maximum level during the experiment. It was statistically highly significant ($p < 0.0001$) while analyzing the experiment. Analysis of population density is probably more relevant to the ultimate goal of improving productivity of copepod culture for hatcheries because it provides

Table 8 Effects of different vessel shapes on the mean population density (ind/100 mL) of adults, copepodites and nauplii of *O. rigida* based on one-way ANOVA results

	Stages		
Shapes	Nauplii	Copepodite	Adult
Rectangular	***	***	***
Circular	**	***	***
Conical	***	***	***
Oval	***	***	**
Treatment mean ± SD			
Rectangular	90.33 ± 1.52	55.00 ± 2.00	33.33 ± 2.51
Circular	86.00 ± 2.00	58.00 ± 3.00	39.00 ± 3.00
Conical	86.00 ± 2.64	58.66 ± 3.51	33.33 ± 2.08
Oval	87.00 ± 0.004	54.00 ± 3.60	41.00 ± 2.00

Values were expressed as mean ± SD of three replicates in each group. ANOVA was followed by Tukey test; *, **, ***, and ns indicate $p < 0.05$, $p < 0.005$, $p < 0.0001$, and not significantly different, respectively

a summary of the dietary effects on a range of interrelated parameters, including egg production, egg-hatching rates, and nauplii and copepodite development time and survival. Although photoperiod is a major environmental condition that can be easily controlled with less costs in aquaculture hatcheries (Chinnery and Williams 2003), the effects of photoperiod on copepod productivity have not been well examined (Peck and Holste 2006). Photoperiod is considered as an important cue for seasonality in nature (Hairston and Kearns 1995). Changes in relative length of diurnal light and dark period are an essential component of the seasonal calendar by which many aquatic organisms seem to regulate their reproductive activities. The present experiment was conducted to analyze the effect of light intensity and photoperiod on production of marine copepod. The 1500 lux was encountered as an optimum intensity of copepod population growth which is illustrated in Fig. 9a. (185 ± 3.15 ind./100 mL) comprising of 88 ± 2.00 nauplii, 53 ± 2.00 copepodite, and 44 ± 3.51 adults, whereas low in 6000 lx which resulted in 57 ± 2.88 ind./100 mL comprising of 33 ± 1.53 nauplii, 17 ± 2.00 copepodite, and 7 ± 2.00 adults (Fig. 9b and Table 9). From the statistical analysis, no relative significances were observed for light intensity ($p > 0.05$). Low population density in copepods might be due to the low light adaptation level, which in turn causes low grazing index (Stearns 1986).The results illustrate that, the light intensity showed descending population density. Figure 10a shows the photoperiod of 10L:14D showed highest total mean of population density. (190 ± 2.68 ind./100 mL) comprising of 91.33 ± 1.52 nauplii, 64.00 ± 1.52 copepodite, and 34.66 ± 4.16 adults (Fig. 10b and Table 10). As stated by Miliou (1992), constant light generally inhibits growth, maturation, and reproduction of aquatic invertebrates. This could be the reason for low density of copepods in 16L:8D and 14L:10D. In addition changes in illumination are also found to affect the endocrine activity. Omori and Ikeda (1984) showed that endocrine activities affect reproduction behavior, egg production, molting, and death in copepods.

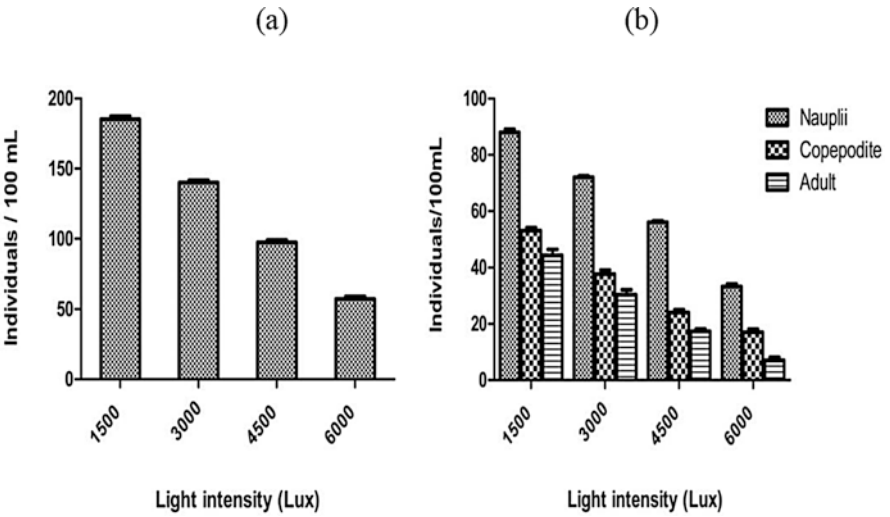

Fig. 9 (**a**) Total mean population of *O. rigida* reared at different light intensities. (**b**) Mean population of three life stages (nauplii, copepodites, and adults) of *O. rigida* reared at different light intensities

Table 9 Effects of light intensity on the mean population density (ind/100 mL) of adults, copepodites, and nauplii of *O. rigida* based on one-way ANOVA results

	Stages		
Light intensity (lux)	Nauplii	Copepodite	Adult
1500	***	***	*
3000	***	***	*
4500	***	***	**
6000	***	***	***
Treatment mean ± SD			
1500	88.00 ± 2.00	53.00 ± 2.00	44.33 ± 3.51
3000	72.00 ± 1.00	37.67 ± 2.51	30.33 ± 3.05
4500	56.000 ± 1.00	24.00 ± 1.73	17.33 ± 1.52
6000	33.33 ± 1.53	17.00 ± 2.00	7.0 ± 2.00

Values were expressed as mean ± SD of three replicates in each group. ANOVA was followed by Tukey test; *, **, ***, and ns indicate $p < 0.05$, $p < 0.005$, $p < 0.0001$, and not significantly different, respectively

And the photoperiod shows high significances ($p < 0.0001$). The results of a number of previous studies suggest that light regime can markedly influence diet rates of egg production in *Acartia tonsa* (Stearns et al. 1989; Cervetto et al. 1993; Peck and Holste 2006).

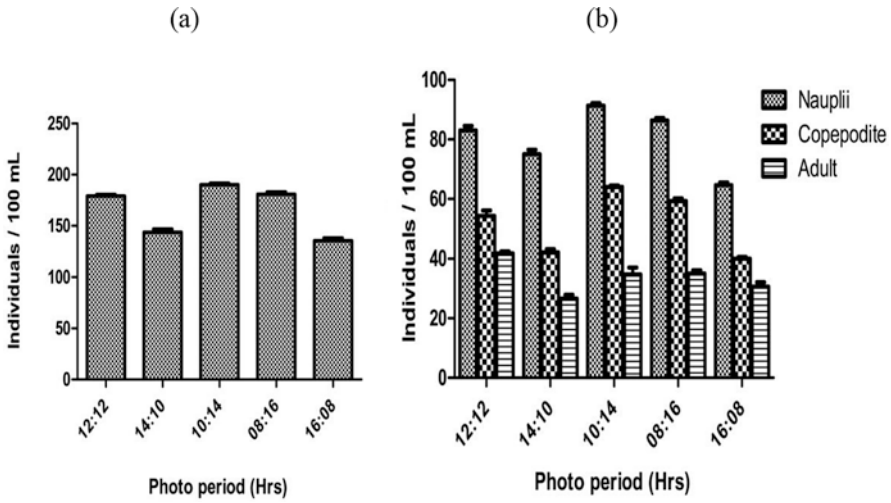

Fig. 10 (**a**) Total mean population of *O. rigida* reared at different photo periods. (**b**) Mean population of three life stages (nauplii, copepodites, and adults) of O. rigida reared at different photo periods

Table 10 Effects of different photo periods on the mean population density (ind/100 mL) of adults, copepodites and nauplii of *O. rigida* based on one-way ANOVA results

	Stages		
Photo period(Hrs)	Nauplii	Copepodite	Adult
12:12	***	***	*
14:10	***	***	*
10:14	***	***	**
08:16	***	***	***
16:08	***	***	**
Treatment mean ± SD			
12:12	83.00 ± 2.64	54.33 ± 3.05	41.66 ± 3.51
14:10	75.00 ± 2.64	42.00 ± 2.00	26.67 ± 2.08
10:14	91.33 ± 1.52	64.00 ± 1.52	34.66 ± 4.16
08:16	86.33 ± 1.53	59.33 ± 2.00	35.00 ± 2.00
16:08	64.66 ± 1.53	40.00 ± 1.00	30.67 ± 2.51

Values were expressed as mean ± SD of three replicates in each group. ANOVA was followed by Tukey test; *, **, ***, and ns indicate $p < 0.05$, $p < 0.005$, $p < 0.0001$, and not significantly different, respectively

Conclusion

The main aim of this present study was to find out the optimum conditions for maximum production of cyclopoid copepod *Oithona rigida*. The pH, salinity, temperature, diets, and diet concentration significantly affect the growth and productivity of

copepod. The optimum pH, salinity, temperature, diets, and diet concentration for successful culture of *Oithona rigida* are 8.2, 26 PSU, 28 °C, *Isochrysis galbana*, and 50,000 cells/mL, respectively. All the information accumulated from this study is imperative and precious for the mass production. *Oithona rigida* has good potential as live food source for commercially important species of tropical fish and crustaceans. Different diets of waste extracts were significantly different on *O. rigida* growth performance. Among them, rice bran waste extract gave the best growth performance on *O. rigida*, when compared to other waste extracts used for copepod feeding in this study; the highest mean population was also documented for rice bran extract for three life stages of copepods; hence, we could recommend the rice bran waste to cultivate *O. rigida* when there is lesser availability of microalgal feed. In various culture vessels tested, greater significances were observed for all the materials used in this experiment, but plastic containers containing samples were showing maximum growth performance compared with others obtained. In addition plastic containers containing copepods show good mean population of three various life stages of copepod than the other four materials used in this experiment. The study revealed that the plastic containers which are the suitable material to grow such organism were evidently proven. From the experiment of different shaped vessels, the fact revealed that the shapes should not be a much barrier for the culture of desired species, which can be adapted to culture condition, the four shapes we used for this experiment. However, the circular-shaped vessels might be the most appropriate to achieve maximum population yield as evidenced in the present experiment. The light intensity and photoperiod both equally and significantly influence the population growth of *O. rigida*. This study has great potential interest for copepod culture because they provide indication on the best illumination and photoperiod conditions for optimizing copepods in order to increase the production for mariculture activities and needs.

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A Microcosm Study on the Impact of Acidification on Feeding, Survival, Nauplii Production Rate, Post-embryonic Development and Nutritional Composition of Marine Copepod

T. Jayalakshmi and P. Santhanam

Introduction

Ocean acidification is the ongoing decrease in the pH of the oceans, caused by their uptake of anthropogenic carbon dioxide from the atmosphere. Increased carbon dioxide (CO_2) from the burning of fossil fuels and other human activities continues to affect our atmosphere, resulting in global warming and climate change. Less well known is that this carbon dioxide is altering the chemistry of the surface oceans and causing them to become more acidic. From scientists and marine resource managers to policy- and decision-makers, there is a growing concern that the process called ocean acidification could have significant consequences on marine organisms which may alter species composition, disrupt marine food webs and ecosystems and potentially damage fishing, tourism and other human activities connected to the seas.

CO_2 entering the ocean alters seawater carbonate equilibrium, decreasing pH and shifting dissolved inorganic carbon away from carbonate (CO_3^{2-}) towards carbonic acid (H_2CO_3), bicarbonate (HCO_3^-) and H^+. Since the beginning of the Industrial Revolution, the pH of surface ocean waters has fallen by 0.1 pH units. Since the pH scale, like the Richter scale, is logarithmic, this change represents approximately a 30% increase in acidity. Future predictions indicate that the oceans will continue to absorb carbon dioxide and become even more acidic. Estimates of future carbon dioxide levels, based on business-as-usual emission scenarios, indicate that by the

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end of this century, the surface waters of the ocean could be nearly 150% more acidic, resulting in a pH that the oceans haven't experienced for more than 20 million years.

The Carbon Cycle

In order to understand ocean acidification and its possible impacts, one needs to understand the behaviour of carbon in nature. Carbon, as other elements, is circulating in different chemical forms and between different parts of the Earth system (Fig. 1) (atmosphere, biosphere and the oceans). These fluxes of carbon in inorganic (e.g. CO_2) and organic forms (sugar and more complex carbohydrates in the biosphere) constitute the carbon cycle. In a very short time span, human activities use an old reservoir of carbon (fossil fuels) which took millions of years to accumulate, thus creating a new and massive flux of CO_2 into the atmosphere. The oceans can mitigate this additional carbon dioxide flux and thus help moderate global warming, but this is not without consequences. The world's oceans play a fundamental role in the exchange of CO_2 with the atmosphere and constitute an important sink for atmospheric CO_2. Once dissolved in seawater, carbon dioxide is subject to two possible fates. It can either be used by photosynthesis or other physiological processes or remain free in its different dissolved forms in the water and later leads to ocean acidification.

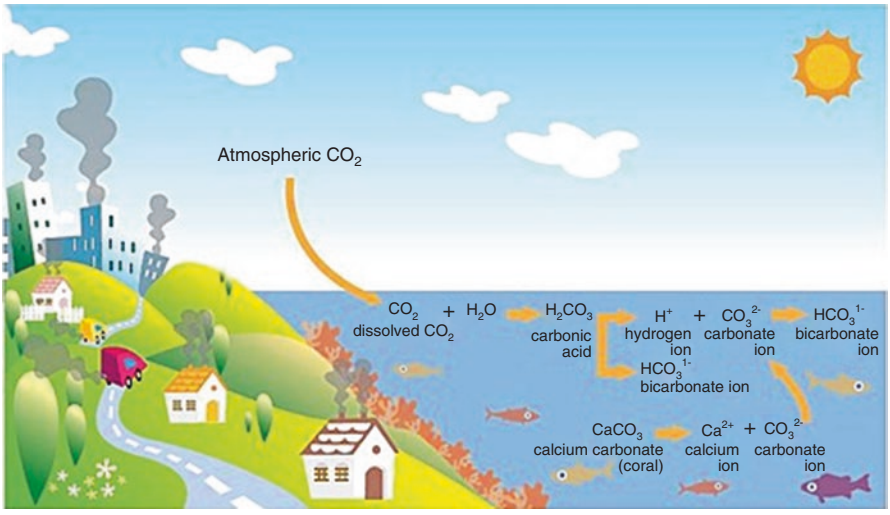

Fig. 1 The simplified chemistry of ocean acidification (Figure reprinted from the National Academy of Sciences, Engineering, and Medicine, courtesy of the National Academies Press, Washington, D.C. (National Resource Council, 2010). https://www.nap.edu/catalog/12904/ocean-acidification-a-national-strategy-to-meet-thechallenges-of). Arrows indicating the precipitation and dissolution of calcium carbonate from bedrock to the ocean are added by A. Bailey

The Chemical Process of Ocean Acidification

There is a constant exchange between the upper layers of the oceans and the atmosphere. Nature strives towards equilibrium and thus for the ocean and the atmosphere to contain equal concentrations of CO_2. Carbon dioxide in the atmosphere therefore dissolves in the surface waters of the oceans in order to establish a concentration inequilibrium with that of the atmosphere. As CO_2 dissolves in the ocean, it generates dramatic changes in seawater chemistry. CO_2 reacts with water molecules (H_2O) and forms the weak acid H_2CO_3 (carbonic acid). Most of this acid dissociates into hydrogen ions (H^+) and bicarbonate ions (HCO_3^-). The increase in H^+ ions reduces pH (measure of acidity) and the oceans acidify, that is, they become more acidic or rather less alkaline since although the ocean is acidifying, its pH is still greater than 7 (that of water with a neutral pH).

Reason for OA

The consequences of man's use of fossil fuels (coal, oil and natural gas) in terms of global warming have not escaped any ones attention. Ocean acidification is another, and much less known, result of the approximately 79 million tons of carbon dioxide (CO_2) released into the atmosphere every day, not only as a result of fossil fuel burning but also of deforestation, vehicle emissions and production of cement (IPCC 2007). Since the beginning of the Industrial Revolution, about one third of the CO_2 released in the atmosphere by anthropogenic (human-caused) activities has been absorbed by the world's oceans, which play a key role in moderating climate change (Sabine et al. 2004). Without this capacity of the oceans, the CO_2 content in the atmosphere would have been much higher and global warming and its consequences more dramatic.

Marine Copepods

The word copepod originates from the Greek words kope ("oar") and podos ("foot"), referring to how their antennae and legs move like oars as the copepod is swimming. Copepods are small crustacean found in almost all aquatic environments. They are most abundant in the marine habitat, but many species also occupy the freshwater or estuarine environment as well. The orders of copepods that are most interesting for aquaculture are the Calanoida, Harpacticoida and Cyclopoida. The Harpacticoida make up more than 50% of the total copepod species. They are primarily benthic, free-living organisms (Støttrup 2003; Conceiçao et al. 2010).

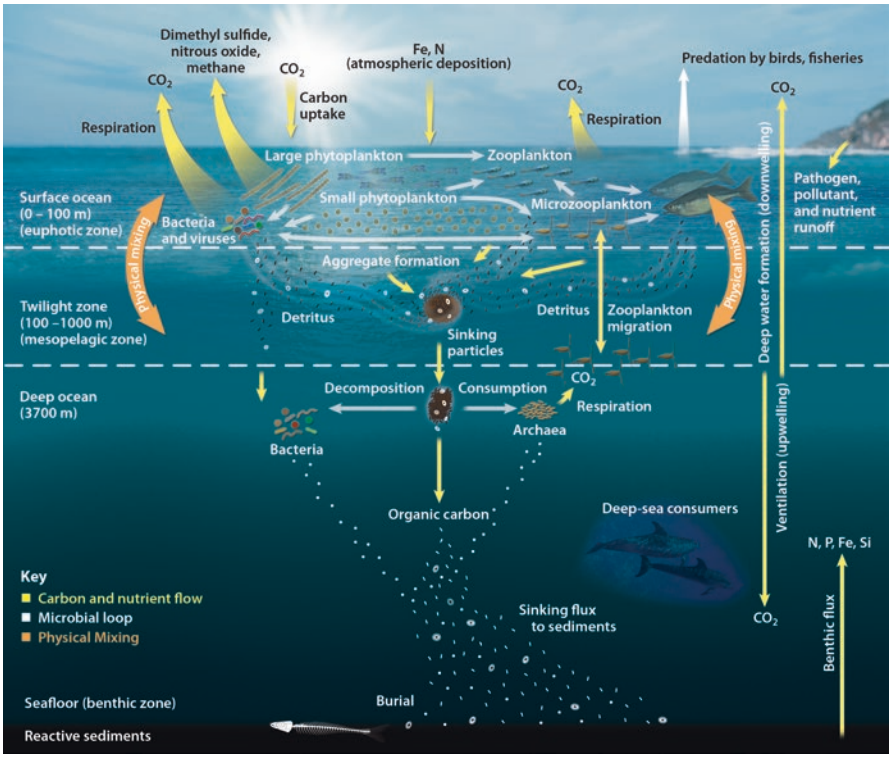

Fig. 2 US DOE 2008. Carbon Cycling and Biosequestration: Report from the March 2008 Workshop, DOE/SC-108, U.S. Department of Energy Office of Science. (p. 81) (website)

The copepods are the dominant forms of the marine plankton and constitute the secondary producers in the marine environments and a fundamental step in the trophodynamics of the oceans. They are found in abundance and constitute an important source of protein in the oceans. Their faecal pellets also accumulate onto the ocean floor and greatly accelerate the flow of nutrients and minerals from the ocean surface to the ocean floor. Copepods represent an important food source for a number of fish species. Most copepod species live in the benthic environment (Fig. 2).

Importance of Copepods

Copepods serve as the link between phytoplankton and larval fish. They have the right size range and biochemical profile, and they trigger the appropriate hunting behaviour in fish larvae due to their zigzag swimming patterns which the larvae find attractive. It represents an important alternative food to present classical live feed organisms in marine fish hatcheries. Their use is known to improve survival, growth and development of fish larvae. One of the most important mechanisms by which carbon is transported from the euphotic layer to the sea floor is the vertical flux of

zooplankton faecal pellets and large organic aggregates, commonly referred to as marine snow. Like phytoplankton, zooplankton is significantly sensitive to changes in their environment. A change in zooplankton concentrations can indicate a variation in the environment. Zooplankton has developed special adaptations to avoid their main predator, the fish. To avoid being eaten by fish, zooplankton has developed transparent bodies. According to marine biological organisms, zooplankton species have evolved to occupy specific marine habitats. Certain species are particularly adapted to external features, including temperature, light, salinity, turbulence and others. These characteristics can sometimes help scientists differentiate between masses of water.

Marine copepods (Crustacea) are an important part of this meiobenthos throughout the world and are known to feed on a wide variety of food sources (Hicks and Coull 1983). Marine copepods can be held and reproduced in captivity at high densities, and there are a number of published protocols for routine laboratory-scale culture. Harpacticoids are meroplanktonic with free-swimming early stages and benthic later stages. Despite their benthic mode of life as adults, there is some experimental evidence that these can be a suitable live feed for marine fish larvae and that their use leads to the same types of improvements in larval performance as seen with calanoid copepods. Although harpacticoids are rarely the dominant taxon in marine sediments, they are known to be the primary food source for bottom or phytal feeding juvenile and small fish. As harpacticoids are important grazers on primary production, they represent an important link between microalgal primary production and higher trophic levels. Harpacticoids play an important trophic role in coral sands because of their numerical dominance, capacity to recycle nitrogen and high bacterial ingestion rates (Gray 1985; Moriarty et al. 1985).

Effects of OA on Copepods

Carman et al. (2004) found that the abundances of several micro-metazoan taxa (including benthic copepods) were not reduced at or near (2 m from) the source of CO_2, compared to a distant (40 m) site, after 29 days. Thistle et al. (2005) noted that, in the same experiment, sediment pH was reduced and that significantly more dead copepods were collected near the CO_2 source. As elevated pH can also affect inorganic nutrient dynamics, which are potentially toxic for copepod cultures (Buttino 1994; Jepsen et al. 2015), addressing pH tolerance is of vital importance for the aquaculture industry.

The responses of copepods may yield insight into CO_2 effects because they more adequately represent a broad range of marine animals. Copepods are typically sensitive of chemical contamination (Peterson et al. 1996). They have different physiologies regarding oxygen tolerance (Wetzel et al. 2001), and exposure potentials of copepods have a relatively impermeable chitinous exoskeleton. Thus, to ultimately draw conclusions on ocean acidification effects on marine copepods, it is essential to characterize the pH level and its natural variability in their habitat.

In the past years, numerous national and international research initiatives have been launched to investigate the effects of ocean acidification. Up to now, results suggest that the process most sensitive to low pH and decreased carbonate availability is calcification (Dupont et al. 2010; Hendriks et al. 2010). Furthermore, various studies focused on eukaryotic phytoplankton, some suggesting that increased carbon availability due to ocean acidification will have a fertilizer effect on primary production and concomitant effects on marine harpacticoid copepods.

Many scientific studies shown that a wide range of marine organisms are sensitive to pH changes of such magnitude, affecting their feeding, survival, fecundity and nutritional profiling in a negative way as shown in Fig. 3. Studies on impact of acidification are mainly focused on macro-organisms. However, effects of acidification on key marine plankton which are major players of entire coastal and ocean bioresources are not well represented due to lack of reliable information.

Ocean acidification changes seawater chemistry in ways that are mostly well understood. What is much less known is the impact that those chemical changes will have on marine organisms and ecosystems, particularly copepod that plays a crucial role in marine food chain and food web, with potential use as live feeds in fish production. Due to anthropogenic influences, the oceans are undergoing rapid changes, with possibly severe consequences for marine life. Ocean acidification is a relatively new field of research, with the overwhelming majority of studies carried out over the last decade. While the topic is attracting increasing attention among policy makers, international leaders and the media, there is still much to be understood about the fundamental biogeochemical, physiological and ecological processes, interactions with other stressors (notably temperature change) and the consequences of ocean acidification for society. By an ecosystem-scale microcosm study, during which ecological and physiological responses of the copepods were monitored, this study aims to provide a comprehensive overview on the sensitivity of an important meiobenthic copepod to ocean acidification.

Ocean Acidification and Zooplankton

Ocean acidification (OA) is considered among the most important environmental changes associated with the human-induced rise in atmospheric CO_2 concentration (IPCC 2013). Predictions of long-term change in pCO_2 and ocean mean surface pH have decreased from 8.13 to the present day 8.05, and consequences for marine life have driven intense research activity into the effects of these drivers on marine organisms (Caldeira and Wickett 2003; Fabry et al. 2008; Dupont and Portner 2013). Marine organisms function in close interplay with the surrounding water. They are adapted to handle challenges visited upon them by predictable changes in water chemistry, and any environmental change is followed by physiological regulation of intracellular chemistry (Thor and Dupont 2015). While OA is known to have various effects on processes such as calcification (Comeau et al. 2015; Gao et al. 1993; Ries et al. 2009), other physiological processes in zooplankton such as respiration (Li and Gao 2012; Thomsen and Melzner 2010), pH regulation (Pörtner

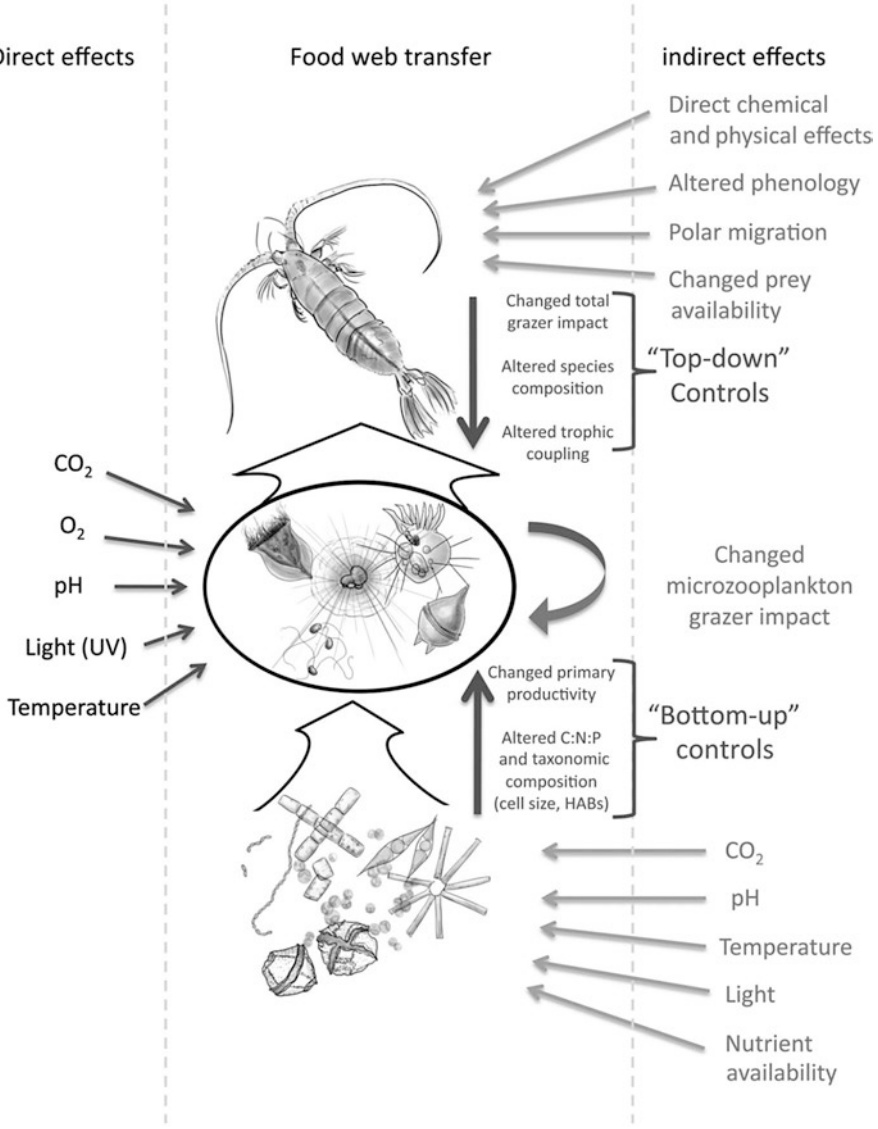

Fig. 3 Effects of ocean acidification on plankton (Source: https://www.csmonitor.com/Science/Science-Notebook/2015/0722/Plankton-threatened-by-ocean-acidification-Why-that-matters)

et al. 2010), feeding (Dupont and Thorndyke 2008; Li and Gao 2012), egg production and nauplius development (Kurihara et al. 2004a, b) were well documented in temperate countries.

Zooplankton is the major link between pelagic primary producers and fish (Mauchline 1998). Therefore, when predicting effects of ocean acidification on pelagic ecosystems, it is crucial to address zooplankton responses. Copepods are

one of the most important components of zooplankton and play major roles in the structure and functioning of marine food webs, which have generally been found resilient to OA levels projected about a century ahead, so that they appear as potential "winners" under the near-future CO_2 emission scenarios. Copepods have a mainly chitinous exoskeleton, so they are not as vulnerable to calcium carbonate under saturation as other calcifying organisms, such as pteropods (Comeau et al. 2010). Even under severe acidification, relevant for upward "leakage" of deep-ocean CO_2 sequestration, impacts on the survival of adult copepods have rarely been demonstrated and seem to be mainly species-specific (Watanabe et al. 2006; Zhang et al. 2011; Cripps et al. 2014). Other studies show that the ability of crustaceans to adjust acid-base imbalances during high CO_2 exposure could be metabolically expensive and affect growth and survival (reviewed by Whiteley 2011; Fitzer et al. 2012).

Generally, marine organisms exposed to changes in environmental conditions will be vulnerable for stress that could lead to changes in reproduction, growth and development (Hendriks et al. 2010). Moreover, harpacticoids are more sensitive to pollutants, which make them good indicators of pollution (Coull and Chandler 1992; McLachlan and Brown 2006). Several studies (Shiryama and Thorton 2005; Byrne 2011) have revealed adverse effects on marine life due to elevated CO_2 at levels that are within projections for the near future (Caldeira and Wickett 2003, 2005). Hence the present study is made to explore the metabolic approach to understand the direct impacts of OA on marine copepod. In this study I assessed the impacts of short-term exposure to elevated pH levels on several vital rates (survival, feeding, nauplii production, development and population density) of marine harpacticoid copepod *Parastenhelia* sp., under an OA scenario relevant for the end of this century.

Copepods are the most abundant zooplankton in the oceans. They have a high reproductive potential, short generation time and high population growth, are flexible in diet and tolerate a wide range of environmental factors such as temperature and salinity (Sun and Fleeger 1995). Due to its wide range of sizes, high levels of protein, digestive enzymes and a rich source of fatty acids compared to commercial and conventional larval feeds (*Artemia* and rotifers), they are preferentially used by the aquaculture industries as an initial feed for the early larval stages of fishes (Barroso et al. 2015). There are reports that indicate the availability of enzyme in harpacticoid copepods which enable the organisms to convert any type of their organic food into lipids stored in their body (Nanton and Castell 1998; Drillet et al. 2011; Camus and Zeng 2012). Many studies have reported that fish larvae fed with early stages of copepods attain improved survival, better growth and pigmentation (Støttrup and Norsker 1997; Copeman et al., 2002; Cutts 2003; Meeren et al. 2008; Vizcaíno-Ochoa et al. 2010; Ajiboye et al. 2011; Santhanam and Perumal 2013). This is generally due the availability of high level of essential fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) in copepods as key nutrients that influence food quality and also enhance somatic growth and reproductive rates of larval fish and invertebrates (Tocher et al. 2010). Polyunsaturated fatty acids (PUFA) are essential metabolites for copepods. Certain PUFA have specific roles in central processes of copepod reproduction including egg production (20:5 EPA), egg hatching (22:6 DHA) and development

(18:3 and 18:5) (Jónasdóttir et al. 2009). In recent years the need of alternative marine lipid sources for use in fish feed in the aspiring aquaculture industry has been prominent (FAO 2012). This has led to an interest to use the n-3 highly unsaturated fatty acids contained in the lipid storage in copepod (Hanssen 2014).

Ocean acidification, caused by the increased uptake of anthropogenic CO_2, can cause a reduction in pH and alteration in the seawater chemistry. In addition, high CO_2 levels interact with other climate change-related factors that may have negative effects on marine organisms (Kroeker et al. 2013; Talmage and Gobler 2012; Almén et al. 2016). Therefore, when predicting effects of climate change on pelagic ecosystems, it is crucial to address copepod responses. It can induce oxidative stress in marine organisms mainly female copepod antioxidant capacity decreased (Tomanek et al. 2011; Kaniewska et al. 2012; Vehmaa et al. 2012). They can also affect the biochemical and elemental composition of organisms (Sato et al. 2003; Torstensson et al. 2013; Bermudez et al. 2016) and consequently the nutritional value for higher trophic levels that depend upon them as a source of essential biomolecules (Rossoll et al. 2012).

Lipids, carbohydrates and proteins have crucial structural and physiological roles in all living organisms (Mayor et al. 2015). The biochemical composition of individual copepod species is influenced by environmental variables such as feed availability, temperature, seasons and CO_2 concentration (Meeren et al. 2008; Bundy et al. 2009). In addition, fish larvae use proteins, carbohydrates and lipids for energy, larval development and survival. The lowered pH may disturb the acid-base balance, thereby altering turnover of lipids, carbohydrates and proteins to be elevated in our ocean acidification treatments owing to the additional metabolic demands (Rossoll et al. 2012).

Most studies have investigated the isolated effects of temperature increase (Richardson 2008) or ocean acidification (Fabry et al. 2008), whereas some have considered interactions between a plethora of biotic and abiotic climate-related variables (Byrne et al. 2009; Gao and Zheng 2010; Vehmaa et al. 2012; Kroeker et al. 2013; Reymond et al. 2013). Several studies stated that ocean acidification tends to reduce the fatty acid composition of copepods (Leu et al. 2013; Zaleha et al. 2014). Effects of acidification on lightly calcified species, such as planktonic crustaceans, are less known (Vehmaa et al. 2013). Hence the present experiment was conducted to discover the changes in biochemical composition of copepod *Parastenhelia* sp., under different pH levels in vitro condition.

Sample Collection and Acclimatization

Copepod samples were collected from the Point Calimere coastal waters (Lat. 10°15.21′ N; Long. 78°48.50′ E), Southeast coast of India, during early in the morning by using plankton net with 158 μm mesh by horizontal towing. The collected samples were immediately transported to the laboratory provided with sufficient aeration by using battery aerators, and the samples were thoroughly rinsed to reduce the contamination from other zooplankters. At the laboratory, the copepod was

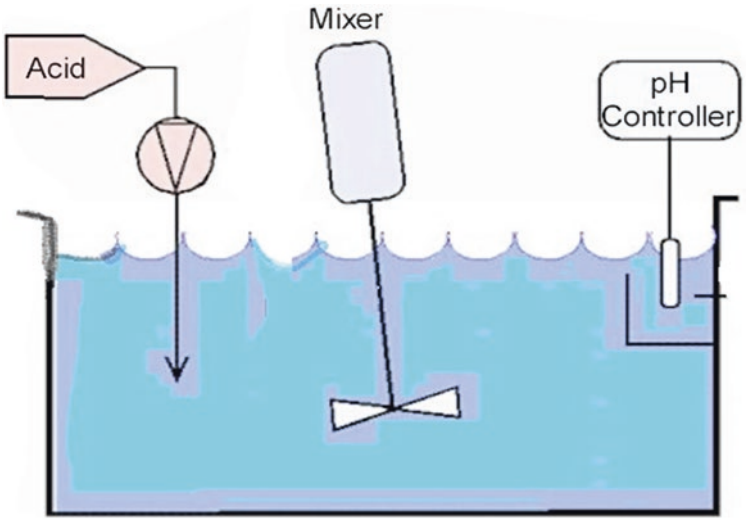

Fig. 4 Experimental setup

isolated using Stempel pipette and identified using the morphological characters by referring to the standard book and manual (Davis 1955). The stock culture of *Parastenhelia* sp. was maintained using the method of Santhanam et al. (2015) and acclimatized in indoor culture condition at 28 °C temperature and 32‰ salinity, fed with *Isochrysis galbana*.

Preparation of Acidified Seawater

Seawater pH was adjusted by adding calculated amounts of HCl (0.1 N), $NaHCO_3$ (0.1 N) and/or Na_2CO_3 (0.1 N) solutions (Lavigne and Gattuso 2011) successively. The changes in salinity due to the addition of the acid and salts were negligible. Salinity was checked with a portable refractometer (accuracy ±0.2). The combined addition of acid and bicarbonate and/or carbonate that was used to manipulate the carbonate chemistry closely mimics the ongoing and future changes in seawater carbonate chemistry due to the invasion of anthropogenic CO_2 into the surface ocean (Gattuso and Lavigne 2009; Schulz et al. 2009) (Fig. 4).

Experimental Setup

For studying the effect of pH on copepods, the preferred methodology usually includes (Kurihara et al. 2004a, b; Kurihara and Ishimatsu 2008; Mayor et al. 2007; Pedersen et al. 2013, 2014). In brief, for survival experiment ten adults of

Parastenhelia sp. were placed into new 50 ml plastic container covered using black chart filled with different pH (4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3) and control pH (8 ± 0.3) in triplicates corresponding water salinity at 32 ppt. The water in the plastic container was changed every 2 days. During the experiment, algal concentration was maintained at 0.18 µg/l and at a constant temperature of 28 °C. The dead organisms and faecal pellets were removed with a pipette. Survival of the animals was determined under a stereomicroscope and recorded from the start of the experiment.

For fecundity experiment, one egg carrying female *Parastenhelia* sp. was picked with pipette under the stereomicroscope and inoculated in 50 ml plastic containers filled with filtered seawater. The number of nauplii produced was calculated. Water quality parameters were maintained at constant temperature of 28 °C and salinity of 32‰ and exposed to different pH (4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3) and control pH (8 ± 0.3) in triplicates. Salinity was measured using hand refractometer (Atago, Japan) and pH using Elico pH meter (model LC-120). All the experimental containers were covered with black chart to avoid light penetration, and the container was mixed three times a day, and their place on the shelf was changed randomly. Every day the copepods were checked to ascertain the time of their nauplii release, and the culture media were refreshed every alternate days. Once the copepod released its nauplii, it was transferred to a new 50 ml plastic containers filled with a fresh culture medium. The copepod nauplii released from the eggs were counted under a stereomicroscope and their viability checked before preserving them adding a drop of 5% formalin.

For determining the population density at different pH (4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3) and control pH (8 ± 0.3), an initial of 15 adults of *Parastenhelia* sp. were stocked into plastic container (1 L) covered with black colour chart. The estimation of population density of copepod was made according to standard procedure as explained in Chap. III.

For assessing the post-embryonic development, an ovigerous female of *Parastenhelia* sp. carrying first egg sacs was placed in 20 ml vial filled with filtered seawater at different pH (4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3) and control pH (8 ± 0.3). After hatching and release of nauplii from ovisac, the female and nauplii were separated, and the development of nauplii was followed by fixing them in 5% formalin at regular intervals of 6–12 h and copepodid development at intervals of 12–24 h. The experiments were continued until the copepodid attained adult stage. The different stages were found using a cavity slide and a high-powered microscope (Optika). All work was done in dark room.

Feeding experiment was done according to Santhanam and Perumal (2012). In brief, for estimation of effect of pH on feeding rate, different pH, viz. 4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3, and control pH (8 ± 0.3) were maintained; the *Isochrysis galbana* were given in the concentrations of 0.18 µg/l to feed the copepod control (algal suspension without copepod). One healthy adult copepod was inoculated into each experimental flask. The feeding rate of copepod was measured by subtracting the final Chl "a" concentration from the initial value (Strickland and Parsons 1972). The hourly difference in algal population was estimated by removing 10 ml of cell suspension at an hourly interval for 7 h.

Experimental Setup

A total of 200 healthy adult individuals of *Parastenhelia* sp. were transferred from stock culture to serially arranged glass bowls (250 ml). Water quality parameters were maintained at constant temperature of 28 °C and salinity of 32‰, and copepods were exposed to different pH (4 ± 0.3, 5 ± 0.3, 6 ± 0.3 ± 0.3, 7 ± 0.3) and control (8 ± 0.3) in triplicates. Salinity was measured using hand refractometer (Atago, Japan) and pH using Elico pH meter (model LC-120). The percentage mortality of experimental copepods was recorded every 3 days interval (mortality ranged between 10% and 60%). After 30 days of experimental period, the copepods were harvested and oven-dried for biochemical analysis.

Moisture Estimation

One gram of copepod was taken and the excess moisture was removed using a filter paper (Rajendran 1973). Then the sample was dried in a hot air oven at a constant temperature of 60 °C until the wet sample was dried completely. The moisture content was estimated by subtracting the dry weight of the sample from the wet weight of the sample. The percentage of moisture content was calculated as follows:

$$\text{Moisture}\% = \frac{\text{Wet weight of sample} - \text{Dry weight of sample}}{\text{Wet weight of the sample}} \times 100$$

Protein Estimation

Protein was estimated according to the Lowry method described by Lowry et al. (1951). One gram of copepod sample was homogenized with double-distilled water, and the extract was centrifuged at 4000 rpm for 10 min. To 1 ml of the supernatant, 4.5 ml of alkaline copper sulphate reagent was added and incubated for 10 min. Then 0.5 ml of reagent Folin-Ciocalteu solution was added and incubated for 20 min. The optical density (OD) of the colour developed was read at 640 nm using spectrophotometer, and the protein was calculated by referring to the standard graph of bovine serum albumin. The result was expressed in the dry weight. The percentage of protein content was calculated as follows:

$$\text{Protein}(\%) = \frac{\text{OD of sample} \times \text{standard value} \times \text{total volume}}{\text{Weight of sample} \times \text{Volume of extract}} \times 100$$

Lipid Estimation

For the estimation of lipid, chloroform-methanol method was followed (Folch et al. 1956). The copepod (400 mg) was homogenized with 5 ml of chloroform-methanol mixture and filtered by a fat filtering unit. The filtered solution was poured into a previously weighed 10 ml beaker and kept in an oven dried at 70 °C. The difference in weight between the empty beaker and the beaker containing fat was expressed as the amount of fat in the sample analysed. The percentage of lipid content was calculated as follows:

$$\text{Lipid}(\%) = \frac{\text{Amount of lipid in the sample}}{\text{Weight of the sample taken}} \times 100$$

Carbohydrate Estimation

Carbohydrate was estimated according to the procedure of Dubois et al. (1956).The copepod (25 mg) was homogenized with double-distilled water and centrifuged. 1 ml of 5% phenol solution and 5 ml of concentrated sulphuric acid were added and it was allowed to react for 30 min, and then the OD value was measured at 490 nm in spectrophotometer. The standard value was obtained by using glucose and the carbohydrate percentage was then calculated. The percentage of carbohydrate content was calculated as follows:

$$\text{Carbohydrate}(\%) = \frac{\text{Standard value} \times \text{OD of the sample}}{\text{Weight of the sample taken}} \times 100$$

Fatty Acid Estimation

The copepod samples were homogenized with chloroform-methanol (2:1 v/v) mixture, and they were extracted using the method of Bligh and Dyer (1959). After the fat extraction, they were esterified with 1% H_2SO_4, and fatty acid methyl esters were prepared by following the procedure of AOAC (1995). Identification and quantification of fatty acids were done by using a gas chromatography (Hewlett Packard 5890 model).

Statistical Analysis

ANOVA was used to determine if there were statistically significant differences between treatments for survival, nauplius production rate, population density, development time and feeding rate. The means were separated by the Tukey's procedure. Statistical significance occurred in all analyses when the calculated *p*-value was <0.05. All mean values are reported as mean ±S. All statistical analyses were performed using Graph Pad Prism version 6.

Results

Effects of Acidification on Parastenhelia *sp.*

Survival

The mean survival was found above 50% at all tested pH (Fig. 5a), although survival rate was found maximum at control pH 8 ± 0.3 (80%) followed by neutral pH 7 ± 0.3 with final survival of 60%. Though pH had considerable effect on survival, maximum effects were observed at acidified pH. Lowest survival of 5% was observed for copepod maintained at acidified pH of 4 ± 0.3 followed by pH 5 ± 0.3 with survival of 10% (Plate 1).

Nauplius Production Rate

pH significantly ($P < 0.05$) affects the nauplius production rate of *Parastenhelia* sp. (Fig. 5b). The results showed that *Parastenhelia* sp. was adaptive under varying pH conditions, although the nauplius production decreased beyond the tolerance range. NPR was found to be highest (67 ± 25.16) when the pH was maintained at 8 ± 0.3 followed by 7 ± 0.3 pH with an NPR of 63 ± 27.4, respectively. The lowest nauplius production (17.33 ± 4.72) was recorded at 4 ± 0.3 pH. The acidified pH appeared to have adverse effect on nauplius production of *Parastenhelia* sp. as evidenced by the present results (Plate 2).

Population Density

The pH also had significant impact on population growth of copepods ($p < 0.05$) (Fig. 6a). Mean population density of *Parastenhelia* sp. was found to be highest at 8 ± 0.3 pH, reaching 780 ± 13.31 ind L^{-1} from an initial population of 15 adults.

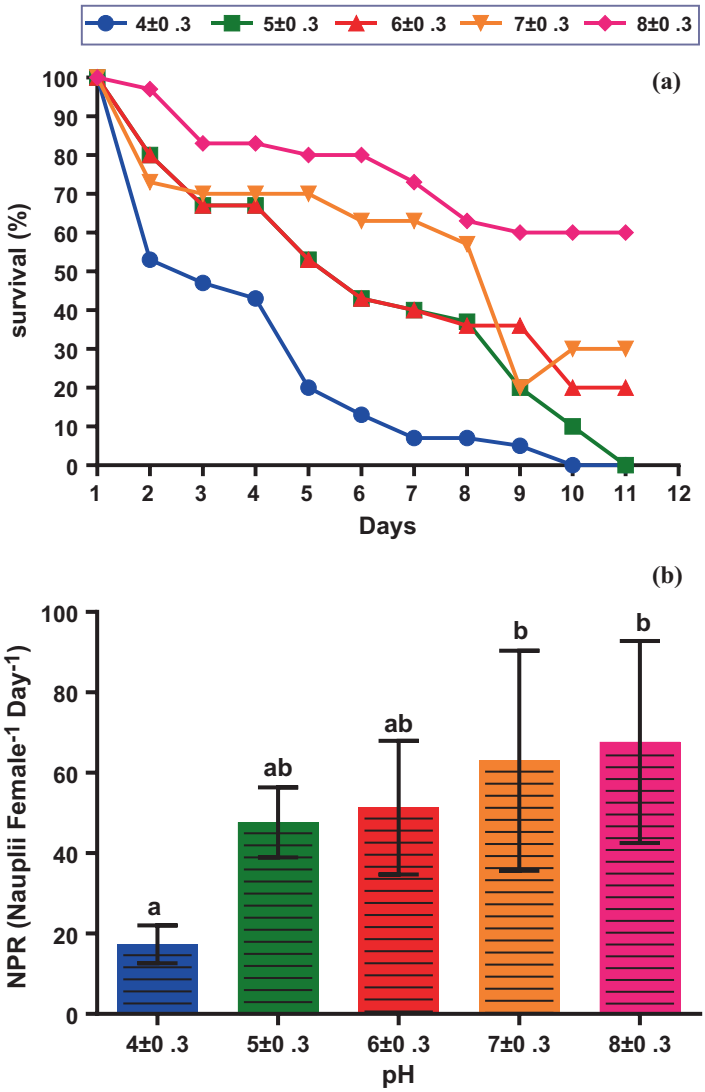

Fig. 5 (**a**) Survival; (**b**) Nauplius production rate of copepods exposed to different pH. The values expressed as mean ± S.E

Lowest population mean (145 ± 12.70 ind L^{-1}) was obtained when copepods were maintained at acidified pH 4 ± 0.3. Copepods survived for a longer period at pH between 8 ± 0.3 and 7 ± 0.3 compared to other levels. pH had significant effect on nauplius production. An average nauplius production of 17 nauplii $female^{-1}$ day^{-1} was recorded for all the pH tested presently (Plate 3).

Plate 1 Survival of *Parastenhelia* sp. exposed to different pH

Plate 2 Nauplius production rate of *Parastenhelia* sp. exposed to different pH

Development Time

Effects of pH on post-embryonic development of *Parastenhelia* sp. are shown in Table 1. Development time and generation time were significantly lengthened ($p < 0.05$), and the longevity of female was significantly shortened ($p < 0.05$) compared to higher pH. The embryonic developmental time was shortest at normal seawater pH 8 ± 0.3, which hatched within 18.33 ± 0.29 h, followed by neutral pH 7 ± 0.3 with a EDT of 20 ± 0.57 h. The embryonic developmental time was longest in acidified pH 4 ± 0.3, which was around 25 ± 0.58 h. The nauplius to

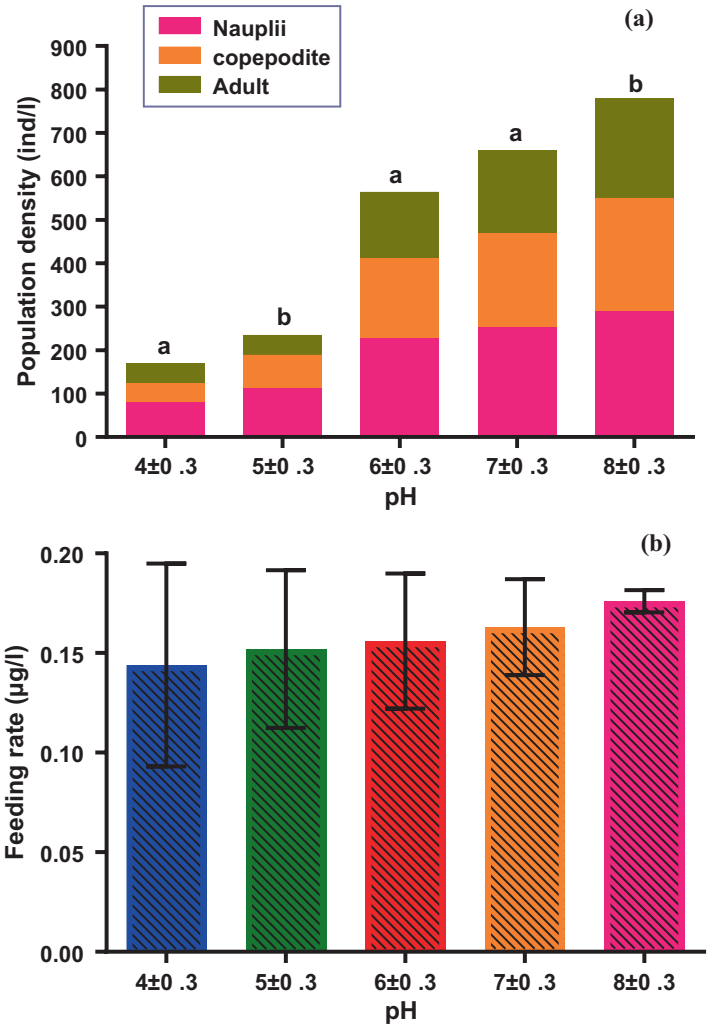

Fig. 6 (**a**) Population density; (**b**) Feeding rate of copepods exposed to different pH. The values expressed as mean ± S.E

copepodite transformation was shortest at pH 8 ± 0.3 which took only 2.67 ± 0.33 days, while the longest period was 6.82 ± 0.21 days occurred at acidified pH 4 ± 0.3. The copepodite to adult transformation was longest in acidified pH 4 ± 0.3 which took place at around 7.67 ± 0.33, while it was shortest (2.67 ± 0.33) in normal pH 8 ± 0.3. The shortest generation time of 8.0 ± 0.15 days was observed at 8 ± 0.3 pH, while it was longer (13.33 ± 0.88) at acidified pH 4 ± 0.3. The female longevity was longer for about 29 ± 0.58 in 8 ± 0.3 pH while lower for about 6.33 ± 0.33 at acidified pH 4 ± 0.3.

Plate 3 Population density of *Parastenhelia* sp. exposed to different pH

Table 1 The mean ± SE embryonic development time (EDT), development time from nauplii I to copepodid I (N1–C1) and from copepodid I to adult (C1-A), generation time (GT) and female longevity (FL) of *Parastenhelia* sp. with reference to different pH

pH					
Parameters	4 ± 0.3	5 ± 0.3	6 ± 0.3	7 ± 0.3	8 ± 0.3
EDT (hrs)	25.0 ± 0.58^{c}	24.33 ± 0.33^{c}	21.33 ± 0.28^{bc}	20.0 ± 0.57^{ab}	18.33 ± 0.29^{a}
N1-C1 (days)	6.82 ± 0.21^{b}	6.33 ± 0.33^{b}	4.21 ± 0.58^{ab}	3.62 ± 0.38^{ab}	2.67 ± 0.33^{a}
C1-A (days)	7.67 ± 0.33^{c}	7.21 ± 0.58^{bc}	4.27 ± 1.00^{ab}	4.11 ± 0.15^{ab}	2.67 ± 0.33^{a}
GT (days)	13.33 ± 0.88^{b}	12.67 ± 0.67^{b}	9.67 ± 0.21^{a}	8.67 ± 0.33^{a}	8.0 ± 0.15^{a}
FL (days)	6.33 ± 0.33^{a}	11.0 ± 0.58^{b}	21.67 ± 0.89^{c}	25.0 ± 1.15^{c}	29.0 ± 0.58^{d}

Note: Different small letters in superscript within a row represent a significant difference among groups

Feeding Rate

The pH significantly ($P < 0.05$) affects the feeding rate of *Parastenhelia* sp. (Fig. 6b). Feeding rate was maximum (0.168 µg/l) in normal pH 8 ± 0.3, while acidified pH 4 ± 0.3 did not elicit a good feeding response as evidenced by lower feeding by copepods (0.138 µg/l). Better feeding rates were also observed in 7 ± 0.3 and 6 ± 0.3 diets with values of around 0.166 and 0.144 µg/l. On the whole a gradual decrease in feeding rate along with the pH was observed (Table 2 and Plate 4).

Table 2 Feeding rate of *Parastenhelia* sp. at different pH

Parameters (pH)	4 ± 0.3	5 ± 0.3	6 ± 0.3	7 ± 0.3	8 ± 0.3
Initial con. (hrs) (µg/l)					
Hourly feeding rate	0.180	0.180	0.180	0.180	0.180
1	0.160	0.142	0.136	0.130	0.097
2	0.118	0.074	0.151	0.064	0.091
3	0.097	0.069	0.138	0.052	0.048
4	0.083	0.052	0.093	0.042	0.041
5	0.066	0.047	0.082	0.038	0.029
6	0.051	0.044	0.062	0.024	0.021
7	0.042	0.036	0.028	0.014	0.012
Total feeding rate (µg/l)	0.138	0.144	0.152	0.166	0.168

Plate 4 Feeding rate of *Parastenhelia* sp. exposed to different pH

Moisture

The moisture content was changed significantly in respect to pH (Fig. 7a). The average moisture percentage at different pH treatments were 4 ± 0.32 (71.42 ± 0.95), 5 ± 0.32 (78.57 ± 0.82), 6 ± 0.32 (82.05 ± 0.79), 7 ± 0.32 (86.91 ± 0.52) and 8 ± 0.32 (89.76 ± 0.57). One-way ANOVA runs $p < 0.001$ (n = 3).

Protein

The amount of protein in different pH treatments stayed unsteady over the course of storage (ANOVA $p < 0.001$) with an average percentage of 71.42 ± 0.95(4 ± 0.32), 78.57 ± 0.82 (5 ± 0.32), 82.05 ± 0.79 (6 ± 0.32) and 86.91 ± 0.52 (7 ± 0.32) and

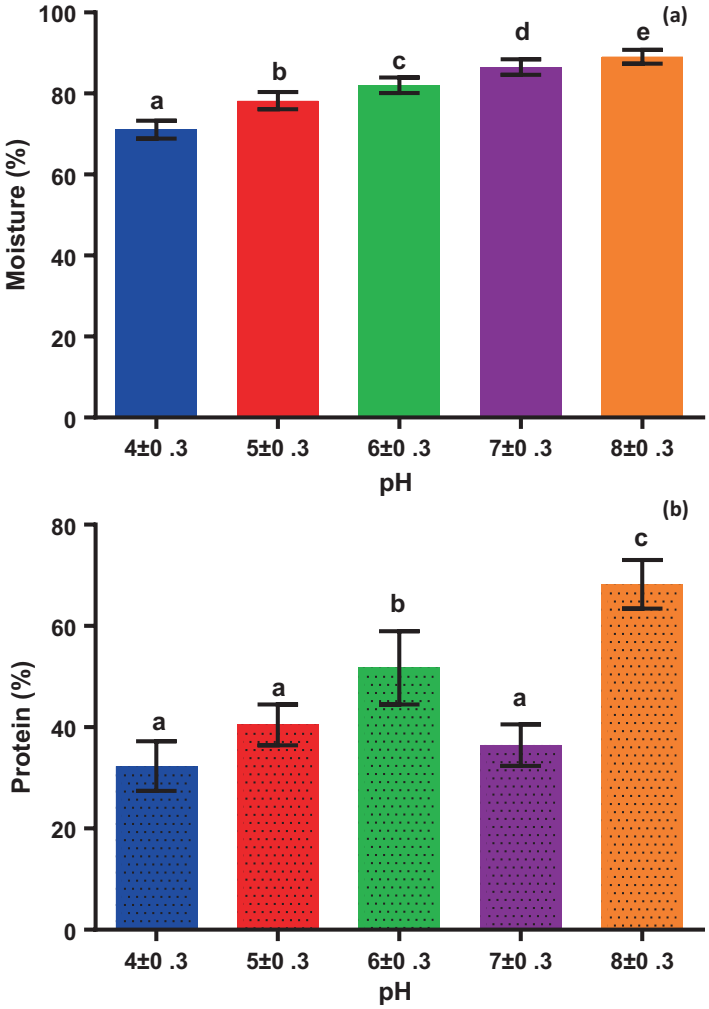

Fig. 7 Effect of different pH on moisture (**a**) and protein (**b**) contents of copepod *Parastenhelia* sp. The values expressed as mean ± SE

89.76 ± 0.57 (8 ± 0.32), (Fig. 7b). The amount of protein in the different pH experiments was significantly varied between experiments indicating that acidification affects the protein content of copepod *Parastenhelia* sp.

Lipid

Analyses of lipid in different pH treatments showed significant changes (Fig. 8a). The different pH treatment copepods indicate an increase in lipid content at control pH 8 ± 0.32 (68.23 ± 4.79), but there was a corresponding decrease in acidic and

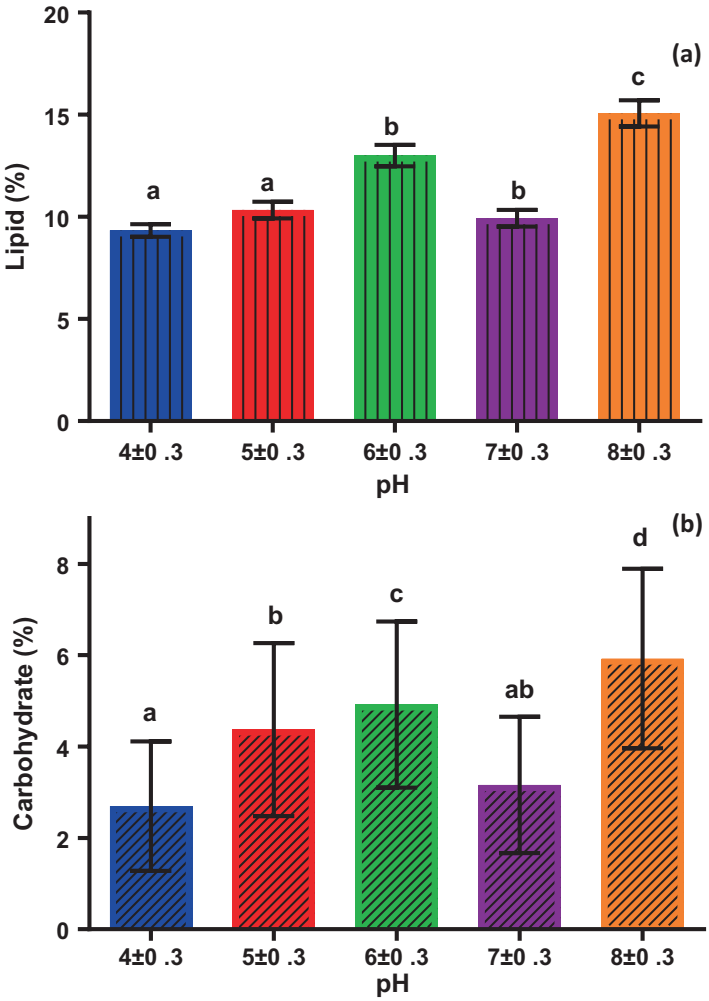

Fig. 8 Effect of different pH on lipid (**a**) and carbohydrate (**b**) contents of copepod *Parastenhelia* sp. The values expressed as mean ± SE

neutral pH 4 ± 0.32 (32.32 ± 4.89), 5 ± 0.32 (40.49 ± 4.04), 6 ± 0.32 (51.74 ± 7.23) and 7 ± 0.32 (36.48 ± 4.10) (one-way ANOVA $p < 0.001$ (n = 3)).

Carbohydrate

Carbohydrate concentration remained unsteady over the experiment period (Fig. 8b). The carbohydrate content per pH treatment remained essentially different over the course of the experiments (one-way ANOVA $p = 0.412$ (n = 3)) with an average percentage of 2.03 ± 1.69 (4 ± 0.32), 3.04 ± 3.15 (5 ± 0.32), 3.59 ± 3.15

(6 ± 0.32), 3.16 ± 1.49 (7 ± 0.32) and 5.93 ± 1.97 (8 ± 0.32). Since the carbohydrate was significantly different across experiments, it appears that pH affect the amount of carbohydrate content in the resultant *Parastenhelia* sp.

Fatty Acids

Wide ranges of essential fatty acids (EFAs) and non-essential fatty acids (nEFAs) were detected in different pH treatments with the carbon numbers ranging from C_4 to C_{24} with a significant difference at $p < 0.05$. Present results showed that the quantitative and qualitative expressions of fatty acids increased in pH 8 ± 0.3 (alkaline) followed by 7 ± 0.3, 6 ± 0.3, 5 ± 0.3 and 4 ± 0.3 (acidic) treatments. EFAs such as *cis*-11,14,17-eicosatrienoic acid and *cis*-8,11,14-eicosatrienoic acid contributed in lower percentage (0.40–1.25%), while *cis*-11,14-eicosadienoic acid (9.10–17.30%) followed by linolenic acid (4.24–16.26%) was comparatively in higher levels in different pH levels. Generally highly unsaturated fatty acids such as EPA, DHA and arachidonic acid were higher in control pH (8 ± 0.3) while lower in acidic pH (4 ± 0.3) resulted in 0.04–13.71%. The erucic acid was found in ranged from 2.94% to 9.40%, and *cis*-11-eicosenoic acid was found only in pH 6 ± 0.3, 7 ± 0.3 and 8 ± 0.3 (0.46–5.51%) (Table 3).

Table 3 Mean percentage composition of essential fatty acids detected in benthic harpacticoid copepod *Parastenhelia* sp. exposed to different pH treatments (data represented in mean ± SD)

		Samples				
Group	Common Names	pH: 4 ± 0.3	pH: 5 ± 0.3	pH: 6 ± 0.3	pH: 7 ± 0.3	pH: 8 ± 0.3
EFA	Linolenic acid (C18)	$4.24 \pm .69^{a}$	$10.73 \pm .08^{b}$	$13.53 \pm .79^{c}$	$14.41 \pm .60^{c}$	16.26 ± 1.23^{d}
	Cis-11-eicosenoic acid (C20)	–	–	0.46 ± 0.20^{a}	0.53 ± 0.13^{a}	5.51 ± 0.71^{b}
	Cis-11,14-eicosadienoic acid (C20)	9.10 ± 0.50^{a}	14.69 ± 0.81^{b}	15.39 ± 0.92^{bc}	16.64 ± 1.12^{cd}	$17.30 \pm .47^{d}$
	Cis-8,11,14-eicosatrienoic acid (C20)	0.40 ± 0.10^{ab}	0.36 ± 0.08^{a}	0.63 ± 0.05^{abc}	0.65 ± 0.09^{bc}	0.80 ± 0.28^{c}
	Erucic acid (C22)	2.94 ± 0.67^{a}	8.34 ± 1.13^{b}	8.62 ± 0.65^{b}	9.10 ± 0.44^{b}	$9.40 \pm .61^{b}$
	Cis-11,14,17-eicosatrienoic acid (C20)	0.98 ± 0.02^{a}	1.10 ± 0.17^{a}	1.19 ± 0.13^{a}	1.20 ± 0.22^{a}	$1.25 \pm .18^{a}$
	Arachidonic acid (C20)	2.29 ± 0.59^{a}	3.91 ± 0.22^{b}	4.55 ± 0.59^{bc}	4.93 ± 0.61^{c}	$5.15 \pm .53^{c}$
	Eicosapentaenoic acid (EPA)	0.04 ± 0.02^{a}	0.14 ± 0.05^{a}	1.38 ± 0.15^{b}	10.97 ± 0.13^{c}	$11.98 \pm .82^{d}$
	Docosahexaenoic acid (DHA)	0.88 ± 0.60^{a}	5.56 ± 0.30^{b}	9.21 ± 0.18^{c}	11.09 ± 0.03^{d}	$13.71 \pm .63^{e}$

Note: Different letters within a row represent a significant difference among groups

Table 4 Mean percentage composition of non-essential fatty acids detected in benthic harpacticoid copepod *Parastenhelia* sp. exposed to different pH treatments (data represented in mean ± SD)

Group	Common names	Samples				
		pH: 4 ± 0.3	pH: 5 ± 0.3	pH: 6 ± 0.3	pH: 7 ± 0.3	pH: 8 ± 0.3
nEFA	Palmitic acid (C16)	12.70 ± 0.5^{c}	0.92 ± 0.06^{b}	–	–	–
	Palmitoleic acid (C16)	37.32 ± 0.21^{e}	17.28 ± 0.98^{d}	9.06 ± 1.36^{c}	5.45 ± 1.38^{b}	0.85 ± 0.42^{a}
	Cis-10-heptadecenoic acid (C17)	0.36 ± 0.11^{b}	0.55 ± 0.52^{ab}	0.73 ± 0.45^{b}	–	–
	Elaidic acid (C18)	0.73 ± 0.54^{a}	1.32 ± 1.01^{a}	1.45 ± 0.45^{a}	1.23 ± 0.34^{a}	1.08 ± 0.23^{a}
	Linolelaidic acid (C18)	2.10 ± 1.80^{a}	2.74 ± 1.49^{a}	3.63 ± 1.01^{a}	3.39 ± 1.10^{a}	3.39 ± 0.63^{a}
	Arachidic acid (C20)	2.00 ± 0.50^{a}	6.67 ± 1.31^{b}	8.02 ± 0.98^{bc}	8.40 ± 1.24^{bc}	9.68 ± 0.63^{c}
	Heneicosanoic acid (C21)	15.51 ± 1.14^{a}	16.74 ± 0.94^{ab}	17.74 ± 0.84^{b}	18.23 ± 1.16^{b}	18.39 ± 0.24^{b}
	Behenic acid (C22)	1.75 ± 0.64^{b}	1.52 ± 0.86^{ab}	1.48 ± 0.24^{ab}	0.77 ± 0.33^{ab}	0.58 ± 0.14^{a}
	Tricosanoic acid (C230)	3.55 ± 0.65^{a}	3.22 ± 0.87^{b}	2.72 ± 1.06^{bc}	1.42 ± 1.47^{ab}	0.46 ± 0.21^{a}
	Lignoceric acid (C24)	1.34 ± 0.06^{a}	1.40 ± 0.56^{a}	1.11 ± 0.01^{a}	0.58 ± 0.77^{a}	0.83 ± 0.32^{a}
	Nervonic acid (C24)	0.67 ± 0.14^{a}	0.67 ± 0.25^{a}	0.59 ± 0.28^{a}	0.42 ± 0.01^{a}	0.41 ± 0.10^{a}

Note: Different letters within a row represent a significant difference among groups

In case of nEFAs, palmitoleic acid was contributing higher percentage in acidic pH 4 ± 0.3 (37.32%) and lower percentage in alkaline pH 8 ± 0.3 (0.85%). Palmitic acid was observed only at acidic pH such as 4 ± 0.3 and 5 ± 0.3. Arachidic acid was quantified in all the pH tested with highly significant level of concentration ($p < 0.001$). Heneicosanoic acid and tricosanoic acid were quantitated in all the treatments with significantly varying concentration of 3.55–18.39% at $P < 0.05$. Low percentage composition of elaidic acid and nervonic acids was noticed in pH 8 ± 0.3, 7 ± 0.3, 6 ± 0.3, 5 ± 0.3 and 4 ± 0.3, and cis-10-heptadecenoic acid was completely absent in pH 7 ± 0.3 and pH 8 ± 0.3. Elaidic acid, linolelaidic acid and lignoceric acid were quantified in all pH levels tested with no significance (Table 4). However, the method adopted in this research shows quality peaks in chromatograms at the same retention time for each fatty acid detected. Thus, the method used in this study is highly reproducible.

The FA composition of *Parastenhelia* sp. was calculated from Tables 3 and 4. In different pH levels, the expression of C_{20} was higher (>30%) followed by C_{21} (>18%). The higher level of C16 was noticed in acidic pH 4 ± 0.3 (>50%) followed by 5 ± 0.3, 6 ± 0.3, 7 ± 0.3 and 8 ± 0.3 (<1%). However, C_{17} was not detected in pH 7 ± 0.3 and pH 8 ± 0.3 levels (Fig. 9a). C_{23} and C_{24} were recorded in lower

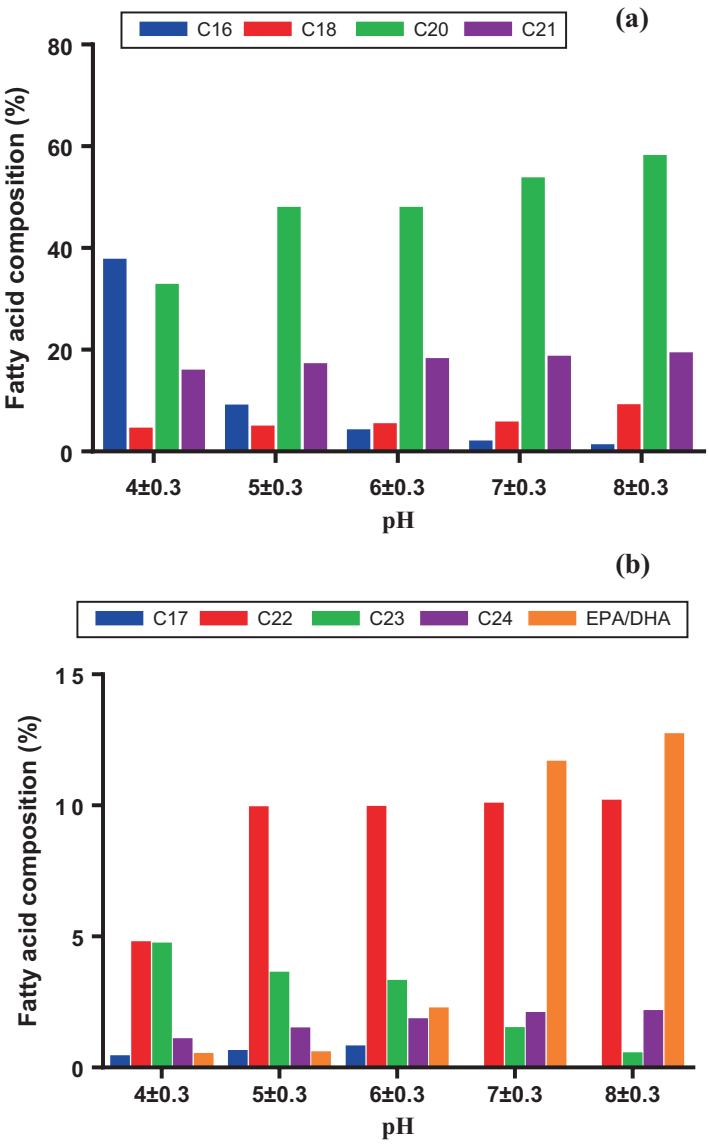

Fig. 9 (**a**, **b**) Comparison of percentage fatty acid composition in benthic harpacticoid copepod (*Parastenhelia* sp.) exposed to different pH levels

percentage (<4%).The higher expression level of C_{22} was reported in pH 4 ± 0.3, 5 ± 0.3, 6 ± 0.3, 7 ± 0.3 and 8 ± 0.3 levels (Fig. 9b).

Overall, the observation showed that the expression of fatty acids was higher at the normal seawater pH condition (pH 8 ± 0.3) compared to acidic pH treatments. This might be probably due to the minimal stress undergone by the cultured copepods at these conditions that eventually reflected in rich FA profile.

Discussion

The results of present study found a significant acidification-dependent effect in *Parastenhelia* sp. which increases risk of survival in copepods (and possibly other organisms). Point Calimere region is shallow (maximum depth, 18 m) and always dominated by high levels of suspended sediments (resulting from bottom resuspension) and effluents caused by tides, alongshore currents and winds; because of this action, the pH of the water sometime reduced up to 3.9 as reported earlier in Cochin waters (Robin et al. 2012). Hence in the present experiment, the diapason for experiment was set up to pH 4 ± 0.3. The nauplius production rate was severely reduced by acidified seawater. From the viewpoint of seawater chemistry, reduction in pH affects the carbonate system, the chemical form of metals and the characteristics of substances dissolved in it. Thus, drop in pH can reduce or increase the toxicity and availability for uptake of many substances, in particular weak acids and bases. The results on survival observed in the present study reflect the sensitivity of the most sensitive developmental stage(s). And this may be due to the copepod exoskeleton contains little $CaCO_3$, so no direct impairment by shell dissolution or decreased rates of calcification is expected. However, variations in other physiological processes in affected organisms, calcifiers and non-calcifiers alike may instigate diminished performance. CO_2 can permeate the cell membrane more easily, and once it diffuses intracellularly, it reacts with water to form carbonic acid, which immediately dissociates into H^+ and HCO^{-3} ions and so induces hypercapnia. The organisms suffering from acidified seawater could transport the extra hydrogen irons and CO_2 through passive buffering of intra- and extracellular fluids, transporting and exchanging of relevant ions, transporting of CO_2 in the blood in those species that have respiratory pigments and depressing the metabolism. Once the compensation of pH failed, mortality of organisms will be observed and increases with the level of pCO_2 (Fabry et al. 2008). Otherwise a species can't tolerate the wide changes in water chemistry. But some studies showed positive results that changes to metabolic processes in response to acidification are generally followed by an increase in energy acquisition or metabolic cost (Thor and Oliva 2015; Thomsen and Melzner 2010), a mechanism that has been shown to increase survival in several taxa (Burnell et al. 2013; Tunnicliffe et al. 2009), and the response that is tested could be mediated by food concentration in the same species within different populations (Thor and Oliva 2015). We might suppose that the impacts of acidified seawater on copepod survival would be related to body size, and so for the bigger copepods, which have a relatively smaller surface area, the quantity of CO_2 permeated across the membrane might be less; thus, the impacts would be slight. However, this is just a hypothesis and needs further investigation.

The present experiment shows that the nauplii production of copepod *Parastenhelia* sp. seems to be affected by gradually decreasing pH in an otherwise optimal environment. During the periods of adverse environmental conditions, however, low fitness of female copepods and their offspring may further impaired by additional OA effects. Previous studies investigating the direct effect of ocean

acidification on the reproduction of different copepod species revealed that egg production and hatching are significantly inhibited by elevated pCO_2 levels greatly exceeding what are projected for the coming decades (Kurihara et al. 2004a, b; Zervoudaki et al. 2016). Our results also are in accordance with that direct effect of acidification on copepod reproduction. An explanation for this is that acidified pH could reduce the development rate, because the acidic pH reduce the intracellular pH of the copepod egg. Also, the shift in energy source from endogenous yolk to exogenous food represents a critical phase that may explain much of the high mortality rate observed among the early life stages and may be attributed to the suppression in metabolic activity through decreased protein synthesis consequently decreasing the reproductive output, which could explain the decline in female carbon production and also to increase the oxidative stress from the maternal parent in copepods, which can subsequently be passed down to the offspring. The decrease in body carbon resulted due to higher energetic costs under OA for the acid-base regulation. The stoichiometry (C:P) of food resources is important for copepod developmental rates, and less balanced nutrient ratios can lead to slower development and growth or increased respiration rates. The decrease of nauplii production under acidic pH, especially when coupled to a decline in the fitness of these nauplii, could significantly alter population dynamics of copepods in the future, with potential impacts for both higher and lower trophic level interactions. But some studies showed that no significant reduction in hatching was observed in the acidified treatment. It is possible that the embryo inside the eggs might be less vulnerable to high pCO_2 exposure, at least compared to the first nauplii stages (Pedersen et al. 2014). In low pH, organisms were more vulnerable due to lack of food, and it affects the physiological rates at the individual level which reduces the ability of the species to allocate resources for coping with multiple stressors (Kletou and Hall-Spencer 2012).

Exposure of adults to acidic pH prior to mating has influence the outcome of the future progeny in copepods. In adult harpacticoid copepods, mortality rates are significantly higher when the seawater carbonate chemistry is manipulated through HCl addition. The direct increase in nauplii mortality, coupled with the declines in nauplii recruitment upon parental exposure to different pH levels, indicates that these early ontogenetic stages may act as a bottleneck for copepod populations in the near future. These early developmental nauplii (NII–NIII) undergo critical physiological changes, switching energy sources from the endogenous yolk to exogenous food available. It may be the additive energetic demand required to maintain metabolic homeostasis under acidic pH for copepods.

In the present experiment, the development time of *Parastenhelia* sp., shows deleterious effects by acidification. It might be acidic pH probably affecting the copepod development by altering the enzyme activity, or inhibiting protein synthesis might be due to an inability of the copepods to adapt to different pH levels. The fact that only a moderate effect of acidic pH on development rate was observed in the present study may be due to the use of ad libitum feeding conditions. This may potentially have reduced any negative effects related to a reduction of the energy budgets of the animals. These stresses may also affect the physiology and lead to

sublethal effects such as retarded growth and reduced metabolic activity. The implication is that future generations will experience dampened reactions of development time also to natural short term (<1 generation) variations in pH. This is mirroring the result of Kelly et al. (2012) in *Tigriopus californicus* and *Scottolana canadensis*.

The population density would have been mostly attributable to direct impacts of greater acidity. Low population density may be due to unsuitable pH conditions encountered by copepod adults of *Parastenhelia* sp. It's moved abruptly from control pH condition to acidified pH conditions. It is likely that this sudden change constitutes much greater physiological stress than the gradual change, thus supporting the view that the population density of copepods is rigid to acidified pH. But under extended or repeated heating events, increased individual survival would come at the expense of population growth through reduced reproductive output (Hofmann and Todgham 2010). It appears may be that under OA the interplay between pH and phytoplankton growth, with knock-on implications for biochemical stoichiometry and subsequent prey quality, will collectively generate the potential for significant changes in the multi-stressor environment for copepod populations.

In the present study acidified conditions may potentially moderate the negative effects on feeding. An explanation for this may be that acidified pH could reduce the feeding rate and thereby give the animals additional time to grow and lipid accumulation, a function central for the development in *Parastenhelia* sp. This may be due to the changed pH gradient of *Parastenhelia* sp. between intracellular and extracellular compartments. A possible explanation could be an effect of acidification on a physiological process such as enzymatic activity. The pH may affect the oxidation of amino acids to ammonia; two types of enzymes play a role: transaminase and glutamate dehydrogenase (GDH) in the copepods.

In the open sea, copepods are the major portion of zooplankton biomass and natural food resource for juvenile fishes (Lahnsteiner et al. 2009). Many attempts were made to determine the protein, carbohydrate, lipid classes (LCs) and fatty acid (FA) composition of wild and hatchery reared copepods (Nageswara Rao and Krupanidhi 2001; Evjemo et al. 2003; Drillet et al. 2006; Rajkumar et al. 2008; Perumal et al. 2008; Parrish et al. 2012; Santhanam and Perumal 2013; Mayor et al. 2015). However, those attempts failed to address the effect of different pH on the expression level of protein, lipid, carbohydrate, LCs (lipid classes) and FAs (fatty acids). A recent review on the reproductive biology of hatchery reared copepod has clearly proven that the lower pH could be the limiting factor for the biochemical composition of copepod (Hanssen 2014). The present study provides a direct negative effect of ocean acidification on biochemical composition of copepod *Parastenhelia* sp.

The protein content of copepod showed greater variations. The protein formed the major fraction compared with lipid and carbohydrate, indicating the usefulness as energy reserve (Conover and Corner 1968). In the present result, the control pH showed higher percentage of protein compared to acidic and neutral pH. The

observed marked variations in the protein content might be due to imbalance pH that it is utilized as a metabolic substrate.

In the present experiment the carbohydrate content of the copepod decreased towards the acidified pH. It may be because of glycogen content reduced influence of pH in the copepods body. In general low carbohydrate content in zooplankton led to contemplations on the functional role of other biochemical fractions in their metabolism. In the present study acidification also influence the carbohydrate content of the copepods. Carbohydrate from the food might be oxidized directly by copepod and that fats might be oxidized on need or stored as principal reserve food, but in the study the carbohydrate was used for tolerating the acidification.

In the present experiment, the alkaline pH 8 ± 0.3 which showed higher biochemical profile compared to acidified pH tested might be due to the minimal stress undergone by the cultured copepods at these optimum conditions that eventually reflected in rich FA profile as reported earlier (Zaleha and Farahiyah 2010; Zaleha and Bursa 2012; Zaleha et al. 2014) where the copepod had undergone higher pH during the culture period and produced good survival and egg-hatching success. In this study, ARA (~5.15%) was found lower compared to EPA (~11.98%) and DHA (~13.71%) in normal pH 8 ± 0.3. This suggests that ARA is mainly used in harpacticoid metabolism (Lima et al. 2013).

In the present observation, C_{21}–C_{24} was not detected in pH 5 and pH 7 treatments that could be ascribed to the lower physiological and biochemical response by the cultured copepod under high-stress conditions (Zaleha and Farahiyah 2010; Zaleha et al. 2014). These findings agree with the present result that cis-11-eicosenoic acid was not noticed at pH 4 ± 0.3 and 5 ± 0.3 but found to be higher at pH levels such as 6 ± 0.3, 7 ± 0.3 and 8 ± 0.3. It shows that the *Parastenhelia* sp. was unable to produce these fatty acids at higher acidific levels.

In the present study, palmitic acid was produced at lower pH levels of 4 ± 0.3 and 5 ± 0.3 but not at the higher pH levels of 6 ± 0.3, 7 ± 0.3 and 8 ± 0.3. In the present study, cis-10-heptadecenoic acid was noticed at lower pH levels of 4 ± 0.3, 5 ± 0.3 and 6 ± 0.3, while it was absent at pH 7 ± 0.3 and 8 ± 0.3, respectively. The copepod *Parastenhelia* sp. is stressed at higher pH levels to produce these two fatty acids.

Conclusion

Among the different pH tested, the copepod's survival, fecundity, nauplius production rate, population density, post-embryonic development and feeding rate were higher at control pH (pH 8 ± 0.3) whereas lower in acidic pH. The nutritional profile analyses on different pH treatments clearly indicate that the acidification can adversely affect the moisture, protein, carbohydrate, lipid and fatty acid profiling of marine harpacticoid copepod *Parastenhelia* sp. that can be considered as one of the candidate live feed for fish and shrimp larval production in wild and aquaculture industry.

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The Impact of Microplastics on Marine Copepods

P. Raju, S. Gunabal, and P. Santhanam

Introduction

Marine atmospheres are exposed to a widespread of anthropogenic pollutants, including radionuclides, nanoparticles, sewage, endocrine disruptors, hydrophobic contaminants and plastic debris. Plastic debris is a wide range contaminant of both freshwater and marine ecosystems, where it can accumulate over time and pose a risk to the health of aquatic organisms (Barnes et al. 2009; Derraik 2002). In the last 60 years, there has been a rapid growing in plastic manufacture, and in 2012 over 288 million tonnes of plastic was produced globally (Plastics Europe 2013) which demonstrates 2.8% development upon the previous year (Plastics Europe 2013). It is held on that 10% of plastics mass produced are likely to end up in the marine environment (Thompson 2006).

Microplastics are small plastic fragments, varying in shape and size, less than 5 mm in diameter (Arthur et al. 2009), which enter the marine environment in one of two ways. Microplastics that are manufactured to be of microscopic size, such as those used in air blasting or "microbeads" or "micro-exfoliates" in cosmetic products (Fendall and Sewell 2009), are referred to as primary microplastics (Cole et al. 2011). Secondary microplastics refer to microplastics that are made by the degradation and breakdown of larger plastic debris (Cole et al. 2011) by photodegradation, oxidation and chemical abrasion (Andrady 2003; Browne et al. 2007). The microplastics can enter the surroundings directly via run-off or indirectly as a result of activities including fishing and shipping (Andrady 2011).

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The Impacts of Microplastic on Marine Copepod

Microplastics have become widespread and ubiquitous information on the biological impact of this pollutant on organisms in the marine environment is only just emerging (Barnes et al. 2009; Gregory 1996; Ryan et al. 2009). The possibility that microplastics pose a threat to biota, as their small size makes them available to a wide range of marine organisms, is of increasing scientific concern (Barnes et al. 2009; Derraik 2002; Fendall and Sewell 2009; Lozano and Mouat 2009; Thompson et al. 2004). In addition to potential adverse effects from ingesting the microplastics themselves, toxic responses could also result from (a) inherent contaminants leaching from the microplastics and (b) extraneous pollutants, adhered to the microplastics, disassociating.

Microplastic is the most prevalent type of marine debris found in our ocean. Microplastic debris can come in different shapes, sizes, and compositions. As an emerging field of study, nothing is known about microplastics distribution and their impacts on keystone secondary producers, i.e copepods as they are base of marine food web. Microplastics have direct negative impacts on organisms in marine systems at both the physical and molecular levels. Recent research suggests that plastic particles can interfere with copepods and consequently affect the feeding, reproduction, survival and biochemical composition of copepods. Microplastic consumed by copepods can move through the food web and end up in species that are important to economy and ecology of marine system. Since microplastics occupy the same size range as many planktonic organisms, they can easily be mistaken for food and thus may affect a wide range of copepods and concomitantly affects commercial fisheries. The few studies carried out to date in developed countries have shown that microplastics are ingested by copepods and that ingestion of microplastics may result in reduced feeding, breeding, energetic deficiencies, injury, or death. This paper explains the methods of culture optimizations and assessing the impact of ingestion of microplastic on feeding, function, fecundity and body composition of marine copepod.

Materials and Methods

Collection, Isolation and Morphological Identification of Marine Copepods

The zooplankton samples are collected from the coastal waters by using 158 μm mesh. The collected samples are immediately transferred into the sterile container by providing with vigorous aeration using battery aerators, and the samples are transported to the laboratory. In the laboratory, the mixed zooplankton samples are screened through a set of superimposed sieves with varying mesh sizes with decreasing mesh size from upstream to downstream. Zooplankton samples are screened

coarsely through a 500 μm mesh to remove fish and prawn larvae. Then the samples are screened through 190 μm mesh to remove rotifers and nauplii of copepods. Finally samples containing predominantly adult, late-stage copepodids and egg-bearing female copepods are collected using fine brush, needles and Stempel pipette under the microscope adopting the standard method of Santhanam et al. (2015). The isolated copepod species are identified morphologically using the standard keys (Davis 1955; Kasturirangan 1963; Perumal et al. 2008) and adopting standard molecular technique (Bucklin 2000).

Culture of Marine Copepods

The copepod culture is done according to Santhanam et al. (2015). In brief, a known number of copepods including male and female or gravid females are isolated using fine brush, needles and Stempel pipette. Isolated copepods are stocked initially in 250 ml glass beakers and conical flasks provided with mono-microalgae diets or mixed microalgae diets without aeration. Latter copepods are subcultured into 5 or 7 L of plastic or carboys containers filled with filtered seawater, and vigorous aeration is given. Then copepods are transferred to oval-shaped, flat-bottomed fibreglass tank filled with 100 L of filtered seawater with vigorous aeration for mass culture. The water quality parameters such as temperature, salinity, pH and dissolved oxygen are maintained in the range of 26–30° C, 28–32‰, 7.5–8.5 and 5.0–7.5 mL/L, respectively. Copepods are fed with a daily ration of microalgae, viz. *C.marina*, *D.salina*, *I.galbana* and *Nannochloropsis* sp., or mixed microalgae in the concentration of 25,000 cells/ml.

Optimization of Environmental Conditions

To optimize the culture conditions for copepod, a set of experiments, viz. temperature (18 ± 2 °C, 20 ± 2 °C, 23 ± 2 °C, 28 ± 2 °C, 33 ± 2 °C and 38 ± 2 °C), salinity (18 ± 2‰, 23 ± 2‰, 28 ± 2‰, 33 ± 2‰ and 38 ± 2‰), pH (6.2, 7.2, 8.2, 9.2 and 10.2), diets (*Chlorella marina*, *Dunaliella salina*, *Isochrysis galbana*, *Nannochloropsis salina*, *Tetraselmis suecica*, mixed algae and yeast) and diet concentrations (15,000 cells/ml, 20,000 cells/ml, 25, 00 cells/ml and 35, 000 cells/ml), are designed.

Growth Rate

For growth experiment, ten individuals of copepods are maintained separately in each bowl for each algal concentration. They were transferred daily to a new bowl with freshly filtered seawater and feed. The daily mortality is recorded carefully. The

experimental sets are maintained at 28 ± 2 °C till the death of all animals. For the experiment of food concentration effects, the concentration of unicellular algae quantified by cell counts using a Sedgwick counting chamber under compound microscope, the different cell concentrations, viz. 10,000 and 20,000 cells/ml, are used for the present experiment. For the growth estimation, the nauplii, copepodite and adult copepods fed with different type of algae are collected from the respective tanks. The total length of the different stages of copepods are measured under a microscope at a magnification of 4 × 10 by using ocular and stage micrometre, from the tip of the prosome to the end of the caudal rami, excluding the caudal setae.

Survival Rate

For survival experiment, ten healthy copepods are picked up using fine brush and inoculated in a 100 mL beaker. The beaker is prefilled with sterilized seawater and maintained at differential environmental conditions as described above. During experimental period, animals are transferred to a new container for every 2 days. Utmost care is taken during the transfer of animals. Dissection microscope is used to observe the copepods. Animals with no movement for 30 s are considered dead. The dead animals are removed from the container daily basis. The numbers of live animals are calculated for every day, and the experiment should be continued for 12 days.

Fecundity

Egg production rate is measured in 24 h incubations following the procedure described in Bellantoni and Peterson (1987) on 12 of the 14 cruises. Females with clearly developed ovaries are chosen for the experiments. However, one male copepod is placed in each bottle to ensure fertilization of the females. The bottle is held in a temperature-controlled environmental chamber with a 12 L: 12D light cycle for 24 h. The temperature of the chamber is set to correspond to that of the water from where the animals were collected (range of temperature = 12–21 QC). At the end of the incubations, all eggs, hatched nauplii and females from each bottle are counted and the daily fecundity expressed as eggs female^{-1} day^{-1} (a hatched nauplii is counted as an egg).

Nauplii Production Rate

For NPR experiments, one healthy female with egg sac is inoculated in apex tubes filled with 20 mL of sterilized seawater for each parameter. The tubes are covered with black cloth to avoid illumination and keep algal concentration in right way.

Every 4 h tubes are checked to know the release time of nauplii. After the nauplii are released from the egg sac, adult copepod is transferred immediately to avoid predation against nauplii. Nauplii are counted under dissection microscope previously fixed with 5% formalin.

Population Density

To determine the population density under each parameter, three male and three female copepods are isolated from the stock culture and inoculated in a 250 mL beaker filled with sterilized seawater. After the experimental period (14 days), animals are harvested through 48 μm mesh and fixed with 5% formalin. Different stages of copepods (nauplii, copepodite and adult) are counted under the microscope after 14 days of experiment.

Development Time (DT) and Generation Time (GT)

For development time under each parameter, egg-carrying females are inoculated in a 50 mL beaker filled with filtered seawater. Once the nauplii are released from the egg sac, females are transferred to new beaker and monitored every 30 min under the microscope form metamorphosis of nauplii I (NI) to copepodite I (CI) and copepodite I to copepodite VI (CVI) (adult). For Generation time, a known number of gravid females are stocked in a 50 mL beaker filled with sterilized seawater maintained with different parameters. The generation time from egg to egg is calculated with reference to the treatment parameters and diet conditions under the microscope.

Determination of the Effect of Microplastics on Copepods

Ingestion of Microplastics into Marine Copepods

To determine the impact of microplastic on copepod, the selected marine copepod species is ingested with microplastics using fluorescent polystyrene beads and used microscopy to assess uptake. Microplastic suspensions is made up by pipetting 20 μl of different sized (e.g. 7.3, 20.6 or 30.6 μm diameter) fluorescently labelled polystyrene spheres into glass vials containing 20 mL of filtered seawater, and then mixed through repeated inversion. Then directly inoculate the individual copepod to the vial containing microplastic and fit to a rotating plankton wheel (<5 RPM) for 24 h. The copepods are exposed to microplastic suspensions in Petri dishes at ambient sea temperature for 1 h. Post-exposure, copepods are washed with filtered

seawater and transferred to Eppendorf tubes containing 1 mL of 4% formalin. Ingestion can be ascertained by viewing specimens at ×40–100 magnification with an inverted light microscope with fluorescence to determine the presence of polystyrene beads (fluorescing yellow-green) within the alimentary canal or body cavity of the copepod. To better understand the interactions between copepod and microplastics, both live and preserved copepods can be viewed under the microscope for varying lengths of time to observe the feeding process, ingestion, gut passage and egestion of polystyrene beads.

Interactions Between Microplastics and Copepods

The interactions between microplastics and copepods are studied by adopting the method of Cole et al. (2015). In brief, to explore the internal distribution and external adherence of microplastics in copepod, copepods should be exposed to polystyrene beads and then employed microscopy observation to visualize their uptake. Microplastic suspensions are formulated by adding 12 µl of 0.4, 1.7 or 3.8 µm diameter non-labelled polystyrene spheres to 24 mL of filtered seawater (0.05% v/v, 1×106 beads ml^{-1} (0.4 µm), 380×103 beads ml^{-1} (1.7 µm) and 40×103 beads ml^{-1} (3.8 µm)), 44 of which are mixed through inversion and sonication. Individual copepod is added to each vial, rotated at <5 RPM at ambient sea temperature for 24 h. Post-exposure, specimens are poured onto a 200 µm mesh suspended in filtered seawater (to prevent damage to the copepods), washed gently, preserved in 4% formalin and then transferred to the bioimaging using microscope.

Impact of Microplastics on Copepod Feeding

The impact of microplastics on copepod feeding is examined by following the method of Cole et al. (2015). In brief, the copepod is exposed to feeding of algae with and without microplastics and compared algal ingestion rates between treatments. First experiment is designed to identify the size of microplastic that would have the greatest impact on copepod feeding, the individual copepod specimens are exposed to a known volume of natural seawater containing a known volume of different sizes (µm) fluorescent polystyrene beads, rotated at <5 RPM for 24 h. To quantify algal concentrations within the natural seawater pre- and post-exposure, the exposure media is vacuum-filtered through a glass fibre filter, and then transferred the filter to 7 mL of acetone, held at 4 °C in the dark for 24 h. The chlorophyll levels within the acetone solution are measured using a UV-spectrophotometer of fluorometer. Based on the result obtained at first experiment on feeding, the ideal-sized microplastics beads are used to conduct a further experiment to establish a dose-response relationship between microplastic concentration and food uptake. Microplastic suspensions consisted of different volume (µl) additions of suitable

size (μm) fluorescent polystyrene beads in a known volume (mL) of natural seawater are prepared. A 1.8 mL aliquot of natural seawater is taken from all vials at T0 and fixed with 40 μl of 50% glutaraldehyde (4% final concentration), inverted for 2 min, refrigerated at 4 °C for 30 min and subsequently snap-frozen in liquid nitrogen and stored in a −80 °C freezer prior to analysis using analytical flow cytometry. Individual copepod (n = ≥6 per exposure) is added to experimental vials, while controls (with no copepod) are set up to determine natural growth or decline of algae over the exposure period. The vials are incubated on a rotating plankton wheel (5 RPM) for 24 h in the dark. Post-exposure (T24), a further 1.8 mL aliquot is fixed (as with T0). Flow cytometric analysis is carried out on thawed natural seawater samples using a flow cytometer according to Tarran et al. (2006). Particle abundance data is subsequently used to calculate the ingestion rates of algae by copepod (Frost 1972).

Impact of Microplastics on Feeding, Function and Fecundity in the Copepod

Copepod Sampling

Copepods are collected from coastal or oceanic waters using 158 μm plankton nets. Samples are transported within insulated boxes, containing 2 L of natural seawater, to laboratory within 3 h of sampling. Adult female copepod is identified under a dissecting microscope and transferred to experimental chambers using stork-billed forceps. Experiments are conducted in controlled temperature laboratories matched to the ambient sea surface temperature (SST) from where the animal is collected.

Microalgal Prey

Marine microalgae will be collected from coastal areas using 48 μm plankton mesh made up of bolting silk cloth. Collected microalgae will be transported to the laboratory. In the laboratory, different species of microalgae will be isolated by adopting different techniques, viz. agar plating and serial dilution under sterile conditions using laminar air flow chamber. The stock cultures for different marine microalgae species are maintained separately in 250 mL, 500 mL, 1L and 2L conical flasks, 5L plastic jars and 19L transparent carboys cans containing filtered seawater at optimum temperature, salinity and light intensity using automated probes and fertilized with different culture mediums. The algal stock culture is maintained in air conditioned room. The seawater will be filtered using filter bag (1 micron), the filtered seawater is sterilized by using automatic autoclave, and after cooling water will be transferred to the culture flasks. Culture flasks are plugged with cotton or covered

by aluminum foil. All vessels used for algal culture are sterilized properly and dried in an oven before use. Mild continuous aeration is provided throughout the culture period. When the algae reach the exponential phase, it is harvested to feed copepods. Cultures of microalgae are maintained on F/2 media with silica, at 20–25 °C under a 12:12 light-dark regimen. Media is refreshed weekly to allow for optimal growth conditions. Algal size, cell density and biovolume are quantified daily using a coulter counter. Carbon biomass of algal prey is estimated using a literature-derived conversion factor of 5 nL biovolume ≈1 μg C (Jones et al. 2002).

Treatments

For all exposures, selected copepod is maintained in 0.2 μm filtered seawater containing only microalgae (250 μg C L^{-1}) for controls or microalgae (250 μg C L^{-1}) and microplastics (75 beads mL^{-1}) for microplastic treatments. A 20.0 μm unlabelled polystyrene (PS) bead is used for the experiment since polystyrene is ubiquitous within sea surface samples collected from across the globe (Hidalgo-Ruz et al. 2012), and this size of microplastics has previously been shown to be readily ingested by the copepods (Cole et al. 2013). Stock solutions (10 L) are prepared daily, and algal cell density, algal biovolume and the microplastic concentration of each stock solution are verified using a multisizer counter prior to experimentation.

Ingestion Rate

The experiment on copepod ingestion rate with reference to microplastics is done according to Cole et al. (2015). A 24-h feeding study is conducted to measure the impact of microplastics on copepod ingestion rates. A 500 ml glass bottles are filled to the brim with either control or microplastic-enriched stock solution (n = 5 per treatment). Five adult copepods are added to each bottle. Additional bottles (n = 3) containing no copepods or microplastics are set up to measure algal growth without predation. All bottles are secured to a rotating plankton wheel (<5 rpm) and left for 24 h in the dark at ambient temperature. Post-exposure, 20 mL subsamples are taken from each bottle and immediately analysed using coulter counter to quantify final algal density, algal biovolume and microplastic concentration. The equation of Frost (1972) is applied to calculate copepod ingestion rates of both algae (cells copepod^{-1} day^{-1} and μg C copepod^{-1} day^{-1}) and microplastics (beads copepod^{-1} day^{-1}). To reveal size selectivity, ingestion rates are calculated for five 1.1 μm size intervals, encompassing the size range of algal cells.

Impact of Microplastics on Copepod Fecundity

The impact of microplastics on copepod fecundity is studied according to Cole et al. (2015). A 9-day exposure is employed to measure the impacts of microplastics on copepod egg production rates, egg size, hatching success, respiration rates and survival. To ensure only healthy, fertile copepods are used, a known number of copepods are individually placed in 25 mL beakers containing filtered seawater fed with microalgae and left overnight; only copepods that survived and produced eggs are selected for subsequent exposures. On initial setup, 1.8 L of control stock solution is poured into 2 L beakers. Egg production chambers (plexiglass cylinders with a 200 μm mesh base) are inserted into each beaker; these chambers allow eggs and faecal pellets to sink to the bottom of the beaker but preclude adult copepods, thereby minimizing egg cannibalism. Groups of four healthy, egg-producing copepods (approximately prosome length, 2.24 ± 0.1 (control); 2.27 ± 0.1 mm (microplastic treatment)) are transferred to each beaker (n = 10) and chambers covered with loosely fitting lids to prevent airborne contamination. Exposures are conducted under a 16:8 light-dark regimen at ambient temperature. Every 24 h egg production chambers (containing the copepods) are tapped to displace eggs and then transferred to beakers containing fresh media. On days 1–3, all copepods are maintained on microalgae (without microplastics) to acclimate copepods to experimental conditions and ascertain baseline egg production, hatching success and egg size. From day 4, treatments diverged, with half the copepod groups maintained on only microalgae as controls (n = 5) and the other half exposed to microalgae and microplastics (n = 5). Microplastic uptake is verified by visually checking the faecal pellets egested by the copepods.

Egg Production Rate, Egg Size and Hatching Success

The average egg production rate (eggs copepod^{-1} day^{-1}) of the copepods is assessed daily. Eggs and nauplii are collected by pouring the contents of each beaker (after removal of copepods) through a 50 μm mesh. Retained material is carefully washed into gridded Petri dishes, and then eggs and nauplii are systematically quantified under a dissection microscope. Mean egg size (μm) is determined on days 3, 5, 7 and 9. Eggs are visualized under high magnification microscope and ten healthy eggs (i.e. circular with no obvious signs of deformation) per replicate selected for assessment. Egg diameter is measured across two planes, using software or micrometres. Average hatching success (%) is assessed using eggs collected on days 2, 4, 6 and 8. Following egg counts, Petri dishes are loosely covered to avoid evaporative loss and then stored at ambient temperature under a 16:8 light-dark regimen. After 48 h a dissection microscope is used to visualize and quantify any unhatched eggs present and values compared with initial egg and nauplii numbers.

Respiration Rate

The oxygen consumption rate (μL O_2 copepod^{-1} day^{-1}) of copepods is assessed on day 10 as a proxy for standard metabolic rate. Small glass vials (volume: 2.14 mL) fitted with oxygen-sensitive optical sensor patches are filled with well-aerated control or microplastic-enriched stock solution and individual copepods introduced (n = 10 per treatment). Additional vials are set up without copepods (i.e. blanks) to establish the oxygen consumption rates of the algae present within the filtered seawater (n = 5). Bungs are fitted carefully, ensuring air bubbles are excluded, and vials transferred to a water bath maintained at optimum temperature. The internal oxygen concentration (μmol O_2 L^{-1}) of each vial is noninvasively measured by scanning the optical sensor patches with an optrode. Measurements are taken every 30 min until oxygen saturation is <70%. The vials in which copepods had died are excluded from further analysis. The oxygen consumption rate of each copepod is calculated for the time range (≥60 min) in which oxygen depletion is most consistent (i.e. $R^2 \geq 0.99$), taking into account comparative mean oxygen decline measured in blanks.

Survival Rate

The number of live copepod specimens remaining in each chamber is recorded daily. Dead copepods are removed from treatments. The survival rate of copepod in respect to experimental and control treatments are measured by subtracting the final density of copepods from initial density of copepods.

Carbon Budget

Biomass and energetic transfer can be estimated using carbon according to Mauchline (1998). Values for ingested carbon biomass are calculated as previously described (*algal prey*). Further, applied literature-derived conversion factors to copepod experimental data to estimate the energetic costs (μg C copepod^{-1} day^{-1}) of (A) reproduction, (B) metabolism and (C) egestion. The average carbon biomass of the eggs is estimated using their mean equivalent spherical volume (day 7) and a literature-derived conversion factor of 0.14 pg C μm^{-3} (Kiørboe and Sabatini 1995). The reproductive costs are calculated by multiplying egg carbon biomass with average egg production rate (day 7). Metabolic carbon consumption is calculated using average respiration rates (day 9) and established conversion metrics (Harris et al. 2000). Elemental (CHN) analysis of collected faecal pellets is confounded by the presence of the PS microplastics. Therefore estimated losses through egestion as 40% of ingested carbon biomass, based upon a food absorption factor of 0.60 estimated for the copepod fed upon microalgae at concentrations of 250 μg C L^{-1} (Thor and Wendt 2010).

Impact of Microplastics on Copepods Faecal Pellet

Experimental Setup

Copepods are incubated in 2 L glass beakers filled with 1750 mL of filtered seawater fed with cultured algal prey (~10,000 cells mL^{-1}) for experiments, with microplastics added for the plastic treatments. An egg production chamber, designed to limit egg cannibalism and coprophagy by separating adult copepods from their eggs and faecal pellets, and an air stone are added to each beaker.

Faecal Pellet Analysis

Five adult copepods are introduced to each beaker (n = 5 beakers per treatment). Exposures to microplastics are conducted in the dark at ambient temperature for 18.5 h. Post-exposure, the contents of each beaker are carefully poured through a 20 μm mesh (suspended in filtered seawater) to retain faecal pellets. Faecal pellets are examined under a dissecting microscope and the number of whole and fragmented pellets recorded. The length and diameter of a subsample of intact faecal pellets (n > 10 per replicate) are measured using an ocular micrometre in conjunction with an inverted light microscope. Measurements are used to calculate the equivalent cylindrical volume of the selected faecal pellets. Following volumetric measurement, the sinking rates (m day^{-1}) of the subsampled faecal pellets are assessed using standard methods (Fowler and Small 1972; Smayda 1969) which pellets are individually transferred via micropipette to a 1 L glass measuring cylinder, filled with filtered seawater and maintained at 15 °C within a controlled temperature laboratory. Low-energy lights and coloured backing sheets are arranged to aid visualization of the faecal pellets. Pellets are allowed to sink for 100 mm to achieve a constant velocity, and then their descent is timed over a 33 mm distance (i.e. between horizontal graticules on the measuring cylinder). The density of each faecal pellet is calculated using Stoke's law, as modified for use with cylindrical shapes (i.e. faecal pellets) with low Reynolds numbers (Komar et al. 1981).

Coprophagy

Ten adult copepods are added to 1 L exposure vessels (n = 8 per treatment). Microplastic exposures are conducted in the dark at ambient temperature for 24 h. Post-exposure, the contents of each vessel are carefully poured through a 40 μm mesh to collect faecal pellets and rinsed with filtered seawater to remove the polystyrene beads. Faecal pellets are visualized under a fluorescent microscope to confirm microplastic incorporation and to ascertain that no waterborne polystyrene

beads remained. Each set of faecal pellets is subsequently transferred to a 23 mL glass bottle (n = 8 bottles per treatment), filled to the brim with filtered seawater. A single animal (a copepod which can display coprophagy) is added to each bottle, and the vessels then gently rotated on a plankton wheel (<5 rpm) at ambient temperature for 2 h. Post-exposure, the contents of each bottle are fixed (4% formalin) and subsequently viewed under an inverted light microscope with fluorescence microscope to identify whether copepod had ingested the microplastic-laden faecal pellets.

Effect of Microplastics on Nutritional Profile of Copepods

The separate experiment is done to know the effects of microplastics on dietary nutritional profile of marine copepods. For this, a known numbers of copepods are exposed to microplastics with one control (without microplastic) as explained previously *(faecal pellet experiment)*. After the stipulated experimental period, the copepods are harvested by draining the content through 100 μm mesh. Then copepod samples are washed with double-distilled water and subjected to the analyses of nutritional compositions by analysing the parameters, viz. moisture, protein, carbohydrate, lipid, ash, amino acids, fatty acids, pigments, vitamins, enzymes and minerals by adopting standard methods such as AOAC (1995); Raymont et al. (1964); Dubois et al. (1956); Folch et al. (1957); AOAC (1995); Yamamoto et al. (1994); AOAC (1995); Pan and Chien (2003); Ueberschär (1988); and AOAC (1995).

Statistical Analysis

Data is presented as mean ± standard error. Student's t tests are used to compare ingestion rates, reproductive outputs and respiration rates between treatments and dates, with significant difference attributed where $P \leq 0.05$. Regression analysis is used to analyse oxygen consumption rates.

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