FIELD AND LABORATORY EVALUATIONS OF BIOASSAYS FOR NITROGEN AND PHOSPHORUS WITH ALGAE AND AQUATIC WEEDS[1]

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ABSTRACT

The rate of absorption of ammonia in the dark and the amount of orthophosphate extracted by boiling water can be used to follow changes in the nutritional status of nitrogen and phosphorus in algae and aquatic weeds with relation to changes in supply of these elements. Measurements of alkaline phosphatase activity carried out in phosphorus-free media can also be used to follow changes in the phosphorus nutrition of aquatic plants; growth under conditions of surplus available phosphorus reduces (by dilution) their alkaline phosphatase activity. Only terminal portions of aquatic weeds should be used for nutritional bioassays because of nutritional differences between young and old portions of the same plant. The importance of testing each species of plants separately is shown by contrasting results obtained with nitrogen-fixing (phosphorus-limited) and nonfixing (nitrogen-limited) blue-green algae from the same environment. These methods provide simple but useful bioassays for studies of eutrophication.

INTRODUCTION

Reversal of the eutrophication of natural waters depends on limiting the available nutrients required for the growth of algae and aquatic weeds. The need for critical analyses for plant-limiting nutrients in aquatic environments has been pointed out (Shapiro and Ribeiro 1965; Gerloff and Krombholz 1966; Oswald and Golueke 1966; Skulberg 1966). Stewart, Fitzgerald, and Burris (1967) suggested that there is a particular need for simple, inexpensive methods for studying nutritional status of algae collected from a given environment.

This report evaluates methods for detecting conditions of surplus or limiting nitrogen (Fitzgerald 1968) and phosphorus (Fitzgerald and Nelson 1966) in algae and aquatic weeds. The test for nitrogen conditions is based on the fact that plants limited by the supply of available nitrogen absorb ammonia-nitrogen (NH_4-N) four to five times more rapidly in the dark than do plants with surplus or adequate nitrogen, and merely requires analyses of NH_4-N before and after incubation. The phosphorus nutrition of these plants can be evaluated either by measuring the amount of orthophosphate (PO_4-P) extracted when they are killed with boiling water or by determining their alkaline phosphatase (alkaline phosphomonoesterase) activity. Algae or aquatic weeds grown with surplus phosphorus will release more than 0.08 mg PO_4-P/100 mg algae after boiling water extraction. Plants that have been limited in growth by the available phosphorus contain little or no extractable PO_4-P and have as much as 25 times the alkaline phosphatase activity of plants grown with surplus phosphate. Alkaline phosphatase activity is determined by incubating the plant material in the presence of a suitable substrate and then measuring the colored end product.

In addition to these methods, a simple, rapid, inexpensive method of measuring *in situ* rates of nitrogen fixation by planktonic and soil blue-green algae has been reported by Stewart et al. (1967).

MATERIALS AND METHODS

Culture media used were modifications of Gorham's medium (Hughes, Gorham, and Zehnder 1958). Details of the culture techniques have been described (Fitzgerald and Nelson 1966; Fitzgerald 1968).

The PO_4-P in algal extracts was analyzed

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by the stannous chloride method and NH_4-N by direct Nesslerization (Am. Public Health Assoc. 1965). All analyses are reported on a dry weight basis. Dry weight of planktonic algae was measured on filtered samples dried in tared vessels. Measurements of the dry weight of plants that could not be sampled by aliquot, such as *Cladophora* or *Myriophyllum*, were made with the actual samples used by filtering and drying them after the PO_4-P extraction or enzymatic tests were completed. Dry weights of samples after extraction with boiling water are not equal to the total dry weight of the original sample but are accurate enough for comparative purposes.

The extraction procedure for surplus phosphorus involved placing 10–80 mg of washed [Gorham's minus P(–P) medium] plant material into 40 ml of Gorham's medium (minus P source; *p*H 7), extracting in a boiling water bath for 60 min, centrifuging or otherwise removing plant material, and analyzing the supernatant liquid for orthophosphate. Alternative methods using 5 min direct boiling or autoclaving techniques have not given as complete an extraction as 1 hr in boiling water baths but could be of comparative use. Any extraction method dependent on killing the plant tissue and allowing the PO_4-P to leach to the supernatant liquid would probably be sufficient as long as results could be correlated (Fitzgerald and Faust 1967). The PO_4-P in the extracts was calculated as mg P/100 mg (dry wt) plant material.

Alkaline phosphatase activity was determined by suspending 1–20 mg of washed plant material in 32 ml of Gorham's (minus P) medium, adding 4 ml of buffer solution (1 M Tris, 0.01 M MgCl, adjusted to *p*H 8.5 with acetic acid) and 4 ml of *p*-nitrophenylphosphate solution (30 mg/100 ml), and incubating at 35–37C. The relative activity was measured after 0.25–2 hr by centrifuging 10-ml samples with 10 mg of orthophosphate-P in 0.5 ml (to stop further enzyme activity) and measuring the optical density of the clear supernatant liquid at 395 mμ. An alternative measurement of alkaline phosphatase activity uses commercial enzymatic tablets, one brand of which (Phosphatabs-Alkaline Quantitative, Warner-Chilcott Labs., Morris Plains, N.J.) uses phenolphthalein phosphate and necessary cofactors. Data so obtained are comparable to results with *p*-nitrophenylphosphate as substrate. Activity was recorded as units of enzyme/mg (dry wt) of plant material in 40 ml. One unit of alkaline phosphatase is defined as the amount of enzyme liberating 1 mμmole of nitrophenol/hr under the prescribed conditions.

Comparative rates of NH_4-N absorption in the dark were used to differentiate between plant material from cultures containing surplus nitrogen and those whose growth was limited by the available nitrogen. From 5 to 20 mg (dry wt) of plant material was washed in nitrogen-free medium, placed in 10 to 30 ml of Gorham's medium (minus N), and 0.1 mg NH_4-N was added. After 1-hr incubation at 25 ± 2C in the dark, the NH_4-N content of the supernatant liquid was compared to controls without plant material. Results were calculated as μg N absorbed/(10 mg dry wt × hr). If the plant material settles during incubation, occasional mixing will be required to prevent local depletion of NH_4-N and erroneous rate results. Without such a depletion, the rates of NH_4-N absorption by samples with fourfold differences in weight will be equivalent on a dry weight basis.

RESULTS AND DISCUSSION

To demonstrate how these measurements could be used to follow the transient events of inorganic nutrition in plants, experiments were carried out with cultures of the aquatic weed, *Ceratophyllum* sp.

The data shown in Fig. 1 indicate that the two techniques can be used to closely follow changes in the phosphorus and nitrogen nutrition of *Ceratophyllum*. The amounts of PO_4-P that can be extracted from the plant tissue when it is placed in media with surplus phosphate will, of course, depend upon the relative amounts of tissue and of phosphorus present.

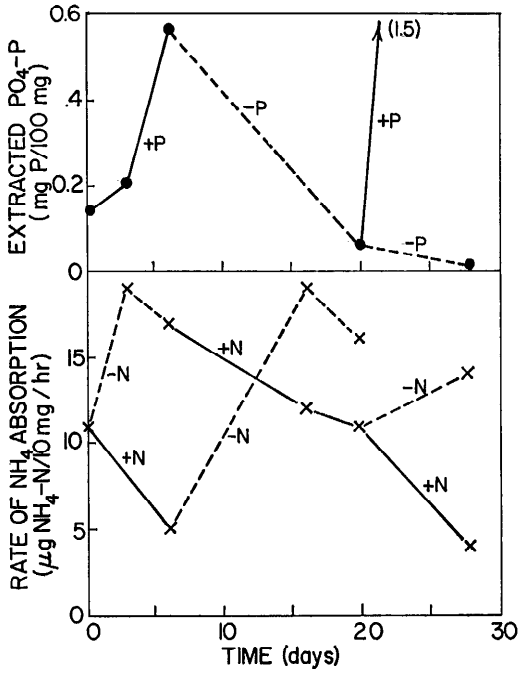

FIG. 1. Extracted PO_4-P and rate of NH_4-N absorption of 5–8-cm tips of *Ceratophyllum* sp. incubated in Gorham's complete medium (solid lines) or media lacking nitrogen or phosphorus (dotted lines). All determinations are averages of triplicate analyses.

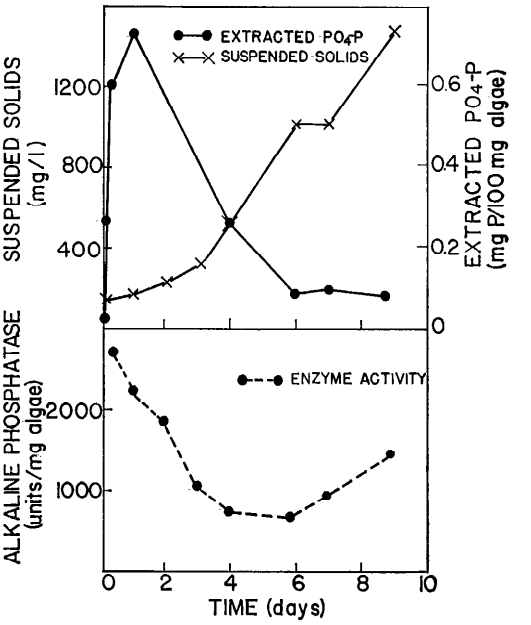

FIG. 2. Relationship between growth, extracted PO_4-P, and alkaline phosphatase activity of phosphorus-limited *Chlorella pyrenoidosa* (Wis. 2005) when placed in a medium containing 7 mg P/liter.

In the experiments shown here relatively small portions (100–300 mg, dry wt) were put in 1,500 ml of Gorham's medium containing 7 mg/liter of phosphorus. In other experiments in which larger portions of plants were used initially, the amounts of PO_4-P that could be extracted after incubation were proportionately less. The tests of the rate of NH_4-N absorption indicate that rates of 15 μg NH_4-N/(10 mg of plant tissue $\times$ hr) or higher represent a condition of nitrogen-limited growth for this plant. With increased culture in the presence of excess nitrogen, NH_4-N absorption rates of less than 5 μg NH_4-N/(10 mg $\times$ hr) can be expected.

The alkaline phosphatase activity of phosphorus-limited algae has been shown to be as much as 25 times that of algae with sufficient phosphorus. To elucidate some of the factors involved when phosphorus-limited algae are placed in medium with adequate or surplus phosphorus, the effects of phosphate added during the alkaline phosphatase analyses and during preliminary incubation were compared.

The green alga, *Chlorella pyrenoidosa* (Wis. 2005) cultured for seven days in Gorham's medium with 0.2 mg P/liter exhibited alkaline phosphatase activity indicative of phosphorus-limited growth conditions. Samples of algae (10 mg dry wt) from this culture were washed into phosphorus-free medium and their alkaline phosphatase activity measured during 1 hr in 0, 5, 10, or 20 mg PO_4-P/liter. In addition, samples of algae were incubated for 1 hr in medium containing 0 and 20 mg PO_4-P/liter and were then washed into phosphorus-free medium for analyses of alkaline phosphatase activity. The results are shown in Table 1. Concentrations of 5 mg PO_4-P/liter or more immediately reduced the activity of the alkaline phosphatase of *Chlorella*. In contrast, the activity of algae incubated in 0 and 20 mg

PO_4-P/liter for 1 hr before enzymatic activity was measured in phosphorus-free medium was 100 and 90%. Thus, the enzymatic activity was not affected by incubating these algae in high phosphate medium for 1 hr.

In another experiment, the effect of added PO_4-P was followed when phosphorus-limited *Chlorella* was added to Gorham's medium containing 7 mg P/liter. Analyses of suspended solids (dry wt), extractable PO_4-P, and alkaline phosphatase activity were carried out over nine days (Fig. 2).

Within 2 hr, the extractable PO_4-P had changed from 0.015 to 0.26% P and in 5 hr to 0.60% P, with no decrease in alkaline phosphatase activity. At 24 hr, the dry weight of the culture had increased from 110 to 145 mg/liter, the extractable PO_4-P was 0.72% P, and there had been a slight decrease in enzyme activity. With increasing growth, the extractable PO_4-P gradually decreased and the enzyme activity also decreased. After six days, phosphorus appeared to become limiting: Extractable PO_4-P had decreased to 0.08% P and on the seventh and ninth days the enzyme activity again increased.

These data indicate that the alkaline phosphatase activity of algae does not stop or decrease immediately when they are placed in medium containing adequate phosphorus. The activity of the enzyme must be reduced by growth (or dilution) under conditions of surplus substrate, as in the case of the nitrogenase system of nitrogen-fixing blue-green algae (Stewart, Fitzgerald, and Burris, unpublished). Thus, aquatic plants that have been recently exposed to a relatively high concentration of available phosphorus could absorb enough to indicate surplus phosphorus conditions by their total phosphorus content (Gerloff and Krombholz 1966) or extracted PO_4-P, but still have high alkaline phosphatase activity. This difference could possibly be used to advantage in ecological studies of the effects of high dosage of nutrients or accidental spills in river systems since the relative rate of growth of plants would be a function of the time required for their enzymatic activity to decrease to its lowest level. The effects of different dosages of nutrients, conditions during treatment, or conditions during the subsequent growth period could be evaluated readily by a simple enzymatic assay.

TABLE 1. *The effect of PO_4-P on measurements of alkaline phosphatase activity**

PO_4-P treatment (mg/liter)	Relative phosphatase activity (%)	
	Analysis during PO_4-P treatment	Analysis after PO_4-P treatment
0	100	100
5	35	—
10	10	—
20	0	90

* *Chlorella pyrenoidosa* (Wis. 2005), 10 mg algae/40 ml.

The relative changes in total nitrogen content and the rate of NH_4-N absorption when algae are transferred from relatively high to limiting nitrogen conditions or vice versa have been reported (Fitzgerald 1968). *Chlorella pyrenoidosa* from nitrogen-limited cultures contained only 2.6% N and had an NH_4-N absorption rate of approximately 40 μg N/(10 mg $\times$ hr), whereas *Chlorella* from Gorham's complete medium had a total nitrogen content of 7.3% and an NH_4-N absorption rate of 14 μg N/(10 mg $\times$ hr). With increasing time of incubation in complete medium of nitrogen-limited *Chlorella*, the total nitrogen content increased in 22 hr from 2.6 to 6.9% N and the rate of NH_4-N absorption decreased from 40 to 6 μg N/(10 mg $\times$ hr). *Chlorella* from complete medium when placed in nitrogen-free medium exhibited a gradual increase in the NH_4-N absorption rate from 14 to 45 μg N/(10 mg $\times$ hr) after 22 hr. However, *Chlorella* from the complete medium was not able to grow enough in the nitrogen-free medium within 22 hr to bring about a significant decrease in the percentage of total nitrogen. Therefore, changes in the nitrogen nutrition of algae can be related to changes in the rate of absorption of NH_4-N in the dark before the alga grows enough to change its chemical composition. Changes in the rate of NH_4-N absorption are dependent on the nitrogen

nutrition of the algae at the time of sampling, whereas the total nitrogen content is dependent on further growth in the case of algae under suddenly limited nitrogen conditions.

The portion of a plant used for nutritional analyses can be important. When the rate of NH_4-N absorption of samples of terminal portions of *Cladophora* filaments from Lake Mendota was compared to that of more basal sections no difference could be detected. However, when analyses of leaves from within 20 cm of the tips of sprigs of *Myriophyllum* sp. were compared with those of older leaves from more basal positions, there were obvious differences. The rate of NH_4-N absorption by young leaves (14–15 μg N/10 mg per hr) indicated they were probably nitrogen deficient, whereas the amount of PO_4-P extracted from them showed surplus phosphorus (0.19–0.20 mg PO_4-P/100 mg). Older leaves appeared to have more nitrogen (8 μg NH_4-N absorbed/10 mg per hr) but they were deficient in phosphorus (0.05 to 0.07 mg PO_4-P extracted/100 mg). Thus, analyses made on whole plants or even on all the plant above the mudline can give confusing results because one portion may have surplus phosphorus while another is limited in phosphorus but has relatively high levels of alkaline phosphatase activity. One must be aware of the basic patterns of nutrient mobilization in a particular species or for a particular nutrient when dealing with complex situations. Only terminal portions of plants should be used for comparative analyses until more information on nutrient mobility is available.

The nutrients in rainfall have been reviewed (Putnam and Olson 1960; Mackenthun and Ingram 1967; Fruh 1967). Studies of the rate of NH_4-N absorption in the dark by algae revealed that the nitrogen in rainfall had dramatic effects on the green algae, *Spirogyra* sp. and *Cladophora* sp. Samples of *Spirogyra* obtained on 7 June 1967 from the surface of a small gravel-lined pond near Madison (Salmo Pond) were a pale yellowish-green, compared to dark green samples of *Spirogyra* obtained from a nearby eutrophic creek (Black Earth Creek). The rates of NH_4-N absorption of the samples were 16 and 8 μg N/(10 mg $\times$ hr), respectively. After a rainfall (1 cm) the night of 7 June, samples of *Spirogyra* collected from the pond on 8 June were bright green and the rate had fallen to 8 μg N/(10 mg $\times$ hr). Samples of *Spirogyra* from Black Earth Creek obtained after the rain were not affected by it—their absorption rate was unchanged. Similarly, 11 samples of *Cladophora* from the rocks along the shoreline of Lake Mendota collected and tested during a dry period, 1 to 6 June 1967, averaged 13 μg N/(10 mg $\times$ hr). Between 7 and 10 June, the area received over 5 cm of rain. Eight samples of *Cladophora* collected on 10 and 11 June had an average rate of 5 μg N/(10 mg $\times$ hr). In the later part of July, after a week or more without rain, 11 samples of *Cladophora* from Lake Mendota averaged 25 μg N/(10 mg $\times$ hr). Two days later, after 1.65 cm of rain, the average rate of nine samples was 9 μg N/(10 mg $\times$ hr).

These data indicate that rainfall influences the nitrogen nutrition of algae in natural environments. Whether the effect is due to nutrients in the rain itself or to nutrients washed into the lakes or ponds from the shore by the rain is not known.

The immediate local environment has an effect on the nitrogen nutrition of algae. Six samples of the green alga, *Hydrodictyon* sp., collected from the surface of a small pond near University Bay, Lake Mendota, on 11 and 12 July 1967, had an average NH_4-N absorption rate of 44 μg N/(10 mg $\times$ hr), whereas six samples of the same alga collected from a small shaded outlet stream, which also received underground discharges from a nearby irrigation project, absorbed little or no NH_4-N; the *Hydrodictyon* from the pond surface was nitrogen-limited, whereas the *Hydrodictyon* from the outlet stream had sufficient or surplus nitrogen available. Samples of *Hydrodictyon* collected on 14 July from the surface of the pond had small

TABLE 2. *Comparisons of the nitrogen and phosphorus nutritional status of algae from mixed blooms in Lake Monona*

Samples	Rate of NH_4-N absorbed (μg N/10 mg)	PO_4-P extracted (mg PO_4-P/100 mg)	Alkaline phosphatase activity: *p*-Nitrophenyl phosphate test (units/mg)	Alkaline phosphatase activity: Phosphatabs test (units/mg)
1967 *Microcystis* sp.	20–23	0.42	—	—
Aphanizomenon sp.	6 or less	0.10	—	—
1966 *Microcystis* sp.	—	0.22	2,200	0.4
Anabaena sp.	—	0.06	18,000	20.0

green spots among the network of *Hydrodictyon* cells. These green spots were a species of *Nostoc*, found to be capable of active nitrogen fixation. The NH_4-N absorption rate of the *Hydrodictyon* after it was cleaned of *Nostoc* decreased from 44 to 16 μg N/(10 mg $\times$ hr). This decrease in NH_4-N absorption rate was presumably due to the effect of combined nitrogen that the *Hydrodictyon* obtained from the nitrogen-fixing *Nostoc* in its immediate environment; Stewart (1967) and Jones and Stewart (1968) showed that *Nostoc* and *Calothrix* supplied combined nitrogen to higher plants, nonnitrogen-fixing algae, fungi, and bacteria, which either grew in close association with or grew through layers of surface soil rich in the nitrogen-fixing algae.

The influence of nitrogen-fixing algae on the nitrogen nutrition of the phytoplankton and aquatic weeds has been the subject of much conjecture. To determine if nitrogen-fixing, bloom-forming blue-green algae would contribute available nitrogen to other bloom-forming algae in mixed blooms, samples of each were analyzed (Table 2). On two separate occasions during 1967 Lake Monona contained mixed blooms of *Microcystis* sp. and *Aphanizomenon* sp. from which clumps of either alga could be separated. Measurements of the rate of NH_4-N absorption indicated that the *Aphanizomenon* (a nitrogen-fixing alga) was not nitrogen-limited and that the *Microcystis* was. The amounts of PO_4-P extracted by boiling water indicated that the *Aphanizomenon* was probably phosphorus-limited, whereas the *Microcystis* contained surplus phosphorus. Analyses carried out in 1966 on mixed blooms of *Microcystis* sp. and *Anabaena* sp. indicated that the *Microcystis* had surplus available phosphorus, whereas the *Anabaena* was phosphorus-limited. Thus, it can be assumed that the *Aphanizomenon* and *Anabaena* had sufficient or surplus nitrogen unavailable to the *Microcystis*, and that the *Microcystis* had surplus phosphorus unavailable to the *Aphanizomenon* and *Anabaena*. Further, the sources of nitrogen and phosphorus for these algae must have been other than the immediate environment of the blooms, since the *Microcystis* was nitrogen-depleted but still had surplus phosphorus and the *Aphanizomenon* and *Anabaena*, although able to fix nitrogen, were phosphorus-depleted. If both species had absorbed surplus nutrients from bottom deposits and then grew in the photic zone until starved by lack of some nutrient, such a situation could result. We do not yet know how algal nutrients in the surface waters change as blooms of algae develop and decline, or whether algae such as *Microcystis* can absorb available phosphorus compounds at the expense of nitrogen-fixing algae such as *Aphanizomenon* or *Anabaena*.

SUMMARY

These studies demonstrate the usefulness of simple bioassay procedures for the nitrogen and phosphorus nutrition of algae and aquatic weeds. The procedures are supplemented by the procedure for determining the rate of nitrogen-fixation by blue-green algae reported by Stewart et al. (1967).

The characteristics of each type of analy-

sis make it possible to obtain valuable ecological information by comparing their results; recent phosphate fertilization of an environment can be assumed from data showing high extractable PO_4-P and high alkaline phosphatase activity. Similarly, high total nitrogen analyses in combination with high NH_4-N absorption rates indicate that the plants have recently been exposed to comparatively less available nitrogen than previously.

The effect of the local environment or of recent changes in that environment must be evaluated, since certain factors (such as recent rains or unusual circulation patterns in lakes) cause changes in the distribution of algae that must be taken into account when interpreting the results of nutritional tests.

It is of utmost importance to record the portion of the plant or the species of alga analyzed. Mixed algal samples are of no more value than total plant analyses since different results can be obtained from different species or parts of a plant. The data indicate that nonnitrogen-fixing algae can be limited by available nitrogen in the same environment in which nitrogen-fixing algae are limited by available phosphorus. Thus, the various types of plankton or aquatic weeds from each local environment must be considered individually and no total environmental predictions made unless all situations have been evaluated.

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