FINAL REPORT

PROJECT TITLE:

REGULATION OF NUISANCE BLUE-GREEN ALGAL BLOOMS: RELATIVE IMPORTANCE OF INORGANIC AND ORGANIC NUTRIENT ENRICHMENT

SUBMITTED TO:

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March 1991

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ACKNOWLEDGEMENTS

The research presented in this report would not have been possible without the hard work and input of numerous people who participated in this project over the last 3 years. Seminal discussions and field participation with Dr. Hans Paerl helped formalize many of the objectives and analytical approaches put forth in our original proposal. Mr. Thomas Miller (master's student) and Mr. Lizhu Wang (doctoral candidate) conducted a majority of the field work and laboratory analysis as partial fulfillment for their graduate programs. Both of these individuals contributed significantly to data analysis and presentations in portions of this report. Dr. N. Kangatharalingam, who joined the project in year 2, provided many new insights into relationships between blue-green algal growth and bacterial activity and the regulation of N_2 fixation. Dr. Kangatharalingam also contributed significantly to many of the original contributions presented in the report. Technical support was supplied during various phases of the project by Ms. Lori Dwyer-Hansen, Ms. Patty Denke, Ms. Teresa Galli, Mr. Tom Sharp and Ms. L. Bebout. The assistance of resort and marina managers located on Hebgen Lake enhanced the efficiency of our research efforts.

Finally, I wish to thank all of the members of the Soap and Detergent Association, in particular A. G. Payne and Dr. R. Vashan of the Proctor and Gamble Company, for their financial support and overall interest in our project. As always, Mr. Richard Sedlak played a major role in the success of our project through excellent management and overall flexibility in times of need.

INTRODUCTION

This report presents an extensive documentation of the role of organic and inorganic nutrients on bacterial and phytoplankton (both blue-green algae and other groups) in a naturally eutrophic reservoir (Hegben Lake) in southwestern Montana. Hebgen Lake receives much of its nutrient input from the Yellowstone National Park drainage. The area is rich in phosphorus both as a result of geothermal and natural geologic mineral deposits. Similar mineral deposits (in particular phosphate) occur in much of southwestern Montana and central Idaho. Hence, our results should be a applicable to other aquatic systems in this region which are influenced by naturally high levels of phosphorus.

The overall objectives of our research were:

1. Separate the responses of N_2 fixing blue-green algae from non-N_2 fixing blue-green algae and other algal species to inorganic and organic nutrient enrichment.
2. Examine the effects of various environmental parameters (in particular bacteria and oxygen) on nuisance bloom-forming blue-green algal species.
3. Develop and apply immunological techniques to localize the sites of N_2 fixation in blue-green algae.
4. Determine the effects of nutrient phosphorus, nitrogen and organic carbon enrichment on bacterial growth.
5. Examine interactions between bacteria and blue-green algae in terms of reciprocal growth of both species.
6. Define the role of inorganic nutrient enrichment on toxin production of the blue-green algal species Anabaena flos-aquae.

Results obtained from experiments which addressed these

experiments are presented in this report as a series of manuscripts which we have, or will, submit to publication in international journals. The people responsible for each chapter are listed on the title pages. I have not included the results of our most recent work on toxicity as a separately funded SDA project entitled "Influence of phosphorus and other environmental parameters on toxin production by the blue-green alga Anabaena flos-aquae" is currently underway, the results of which will be detailed in the final report for this project. The manuscripts are grouped into those concerning the algal community (Chapters 1-6), the bacterial community (Chapters 7-10) and those examining interactions between blue-green algae and bacteria (Chapters 11-13). Because each portion of our work is submitted as a manuscript, are differences in style (dictated by the journal we will or have submitted to) and a certain amount of redundancy exists (for example in the References, Study Site and Methods sections). I apologize for this and hope that it does not cause too much of an inconvience.

In addition to this report, I will submit copies of a Master's Thesis and a Doctoral Dissertation to Mr. Sedlak of the SDA upon completion (hopefully within the next year). These documents will contain additional information on results obtained from our study.

Although our results often led to more questions than we could address within the time and funding constraints of this project, we could make some fairly solid conclusions regarding the role of phosphorus, nitrogen and organic carbon inputs on

nuisance blue-green algal blooms as well as on bacterial growth in Hebgen Lake. These conclusions are included in the final Chapter along with statistical models developed with empirical data collected from Hebgen Lake. I recommend that readers not interested in the technical details included within each manuscript proceed to the final chapter for a summary of the major findings and conclusions of this study.

CHAPTER 1

INFLUENCE OF INORGANIC AND ORGANIC NUTRIENT ENRICHMENT ON BLUE-GREEN ALGAL ACTIVITY AND RELATIVE BIOMASS IN A EUTROPHIC SOUTHWEST MONTANA RESERVOIR

INFLUENCE OF INORGANIC AND ORGANIC NUTRIENT ENRICHMENT ON BLUE-GREEN ALGAL ACTIVITY AND RELATIVE BIOMASS IN A EUTROPHIC SOUTHWEST MONTANA RESERVOIR

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ABSTRACT

Nutrient enrichment experiments were conducted seasonally, over 2 ice-free seasons, with natural phytoplankton assemblages in a blue-green algal (cyanobacterial) dominated Southwest Montana reservoir to determine temporal variations in nutrient deficiency. Additional experiments examined the influence of inorganic N and P and organic carbon (mannitol) on the activity of blue-green and non-blue-green components of the community and on relative nitrogen fixing blue-green algal abundance.

The results of our experiments showed that:

1. The whole phytoplankton community (i.e. all size classes) was generally N-deficient.
2. P addition alone stimulated the N_2-fixing blue-green algal dominated community in one experiment only, and in conjunction with N in another.
3. The non-blue-green algal component showed the most consistent stimulations by N, although the nitrogen fixing blue-green algal component was N-stimulated on several occasions.
4. There was a general trend showing that the relative blue-green algal biomass increased with P enrichment and decreased with N enrichment.

Our study provides evidence that P is not the only nutrient that controls productivity of lakes and reservoirs, and that N must also be considered when making water quality decisions even in systems dominated by N_2-fixing blue-green algae.

INTRODUCTION

Phosphorous has traditionally been thought a priori to limit phytoplankton productivity in lakes (Hecky and Kilham 1988; Schindler 1977). This view has been challenged in recent years by studies showing nitrogen deficiency for many freshwater systems (Canfield et al. 1989; Dodds et al. 1989; Elser et al. 1988; Prepas and Trimbee 1988; Priscu and Priscu 1984; Vincent et al. 1984; White et al. 1985). Elser et al.(1990) reviewed phytoplankton nutrient enrichment experiments and found nitrogen to be more important than previously recognized. Competition for nutrients in limited supply plays a significant role in determining phytoplankton community structure (Reynolds 1984). Consequently, an accurate understanding of nutrient deficiencies will provide water quality managers with important information on the development of bloom formation by nuisance algal species.

The ability of scum-forming blue-green algae to outcompete other groups in nitrogen deficient systems, or systems with low nitrogen to phosphorus ratios, allows them to dominate many lakes and reservoirs (McQueen and Lean 1987; Tilman et al. 1986). Systems dominated by these nuisance organisms experience diminished natural resource value with them. Recreational use is hampered by unsightly surface scums and odor reducing the overall aesthetic quality of the water. Fish populations are effected by oxygen depletion following collapse of blue-green algal blooms (Ayles et al. 1976; Barica 1975) and by inefficient transfer of primary production to higher trophic levels (Carpenter et al. 1987; Shapiro 1980). Neuro- and hepato-toxins produced by blue-green algae (Gorham and Carmichael 1988) pose a serious hazard to

ate amount of sample (5-25 ml), depending upon density, and settled for at least 4 h cm^{-1} of water in the chamber. The settled phytoplankton were identified and counted with a calibrated Zeiss inverted microscope (Lund et al. 1957) and measured for biovolume determination. Equations for volumes of geometric shapes that approximated each cell type and appropriate average dimensions for each species were used to determine biovolume, which was converted to biomass under the assumption that the specific gravity of phytoplankton equals that of water.

RESULTS

Initial conditions. Conditions at the inception of each experiment are presented in Table 2. Nitrogen fixing blue-green algae dominated the phytoplankton community at the beginning of all six microcosm experiments, with Anabaena sp. in June 1988, August 1988 and June 1989, and Aphanizomenon sp. in October 1988 and August and October 1989. The diatoms Asterionella sp. and Fragillaria sp. were co-dominant with the blue-green algae in October 1988 and August 1989, respectively. Dissolved inorganic nitrogen (DIN = NH_4^+-N + NO_3^--N) and SRP were highest at the beginning of the August 1988 experiment when the NH_4^+ and NO_3^- additions resulted in an 80% enrichment; PO_4^{-3} addition enriched SRP by 125%. Water for all experiments had low (less than 6 by weight) DIN:SRP ratios; the lowest was 1.1 for June 1989. Although no PP samples were taken for 1988 experiments, data from samples collected within a few days of each experiment show that TN:TP and PN:PP were highest at the start of the June and October 1988 experiments. The highest CHL a concentration encountered was for the October 1988 experiment (144.5 $\mu g\ l^{-1}$), during an Aphani-

zomenon bloom.

June 1988 experiment. Biomass specific rates of photosynthetic carbon fixation (PPR = μg C (μg CHL a h)$^{-1}$) were stimulated to the greatest extent by P enrichment (Fig. 1) which was significantly greater than the unammended control ($p<0.01$). All other treatments were not significantly different from the control ($p>0.05$). The PO_4^{-3} treatment also resulted in significantly greater CHL a values than the control ($p<0.01$) with a maximum 55% increase over control on day 3 (Fig. 2, Table 3). No carbon uptake size fractions were conducted and the NO_3^- microcosm was lost on day 1. Phytoplankton biomass at the end of the experiment was higher than control in the PO_4^{-3} treatment only (Fig. 3). Changes in relative blue-green algal abundance were not apparent.

August 1988 experiment. None of the nutrient amendments stimulated PPR significantly ($p>0.05$) with respect to the control, although all amendments except Mo enhanced PPR on day 1 (Fig. 4). Size-fractionated volume specific photosynthetic carbon uptake rates (e.g. NH_4^+ >20 μm fraction = μg C l^{-1} h^{-1} for phytoplankton in the NH_4^+ microcosm that did not pass through 20 μm mesh) were measured on day 4. The NH_4^+, NO_3^- and NO_3^-+Mo >20 μm and <20 μm fractions were significantly greater than control ($p<0.05$); 1.0 MANN <20 μm and 0.5 MANN >20 μm and <20 μm fractions were significantly less than control (Fig. 5). CHL a increased significantly ($p<0.01$) with NO_3^-+Mo addition (Fig. 6, Table 3). Total phytoplankton biomass increase was greatest with NH_4^+ addition (Fig.7); NO_3^- and NO_3^-+Mo elicited the greatest decrease in relative blue-green algal abundance (Fig. 8).

October 1988 experiment. PPR was stimulated significantly by NO_3^-, NO_3^-+Mo, PO_4^{-3} and 1.0 MANN ($p<0.05$) for the entire experiment; other treatments were not significantly different from the control (Fig. 9). CHL a increased to over 1.6 times the control with NO_3^- addition on day 2 and decreased to control levels thereafter. Only NH_4^+ enrichment resulted in CHL a significantly greater than the control ($p<0.05$) by the end of the experiment (Fig. 10). On day 5, NH_4^+, NO_3^-, Mo and NO_3^-+Mo <20 μm fractions were enhanced significantly ($p<0.05$), and MANN <20 μm fractions were significantly less than control ($p<0.01$). None of the treatments showed an increase in the >20 μm fraction (Fig. 11, Table 3). Phytoplankton biomass increased with NO_3^- and NO_3^-+Mo addition (Fig. 12), and relative blue-green algal abundance was 20% less than control in the NO_3^-+Mo and 0.5 MANN treatments (Fig. 13).

June 1989 experiment. Addition of N (NH_4^+, NO_3^-, NO_3^-+Mo and MANN+NH_4^+) stimulated PPR (Fig. 14) and CHL a (Fig. 15) significantly ($p<0.05$), while PO_4^{-3} and MANN had no significant stimulatory effect. The PPR and CHL a response of NH_4^+ and NH_4^++PO_4^{-3} were similar, as were those for NO_3^- and MANN+NH_4^+. Size fractionations done on day 5 showed the NH_4^+, NO_3^-, NH_4^++PO_4^{-3} and MANN+NH_4^+ <20 μm fractions were significantly ($p<0.05$) greater than control, as were the NH_4^+, NH_4^++PO_4^{-3} and MANN+NH_4^+ >20 μm fractions (Fig. 16, Table 3). N enrichment increased the relative amount of <20 μm fraction uptake and contemporaneously decreased the contribution of the >20 μm fraction (Fig. 17). Phytoplankton biomass was substantially greater than control in all but MANN+NH_4^+, with NH_4^+, NH_4^++PO_4^{-3}, PO_4^{-3} and MANN+PO_4^{-3} all more

than 3 times the control (Fig. 18). The relative abundance of blue-green algae was decreased by N addition (especially NO_3^-) and increased by PO_4^{-3} (Fig. 19).

August 1989 experiment. Stimulation of PPR in the NH_4^++PO_4^{-3} treatment was significant ($P<0.01$), whereas it was not when NH_4^+ or PO_4^{-3} was added alone (Fig. 20). The response of CHL a was similar (Fig.21), with NH_4^++PO_4^{-3} significantly (7 times) greater than the control ($p<0.01$) and NH_4^+ twice the control ($p<0.05$). On day 3, all N addition >20 μm and <20 μm fractions were increased significantly over control ($p<0.01$), but only the >20 μm fraction was significantly ($p<0.01$) stimulated by PO_4^{-3} (Fig. 22, Table 3). The relative contribution of each size fraction was changed by NH_4^++PO_4^{-3} addition that increased carbon uptake in the >20 μm fraction and decreased it in the >20 μm fraction (Fig. 23). Phytoplankton biomass was stimulated by the addition of NH_4^+, NO_3^- and NH_4^++PO_4^{-3} (Fig. 24). PO_4^{-3}, MANN and MANN+PO_4^{-3} increased, while NH_4^+, NO_3^- and NH_4^++PO_4^{-3} decreased the relative abundance of blue-green algae (Fig. 25).

October 1989 experiment. All treatments except NH_4^++PO_4^{-3} and MANN+NH_4^+ (which showed no effect) significantly decreased ($p<0.05$) PPR relative to the control (Fig. 26). Addition of NH_4^+, NO_3^- and NH_4^++PO_4^{-3} resulted in CHL a significantly ($p<0.05$) greater than the control (Fig. 27). N amendments significantly ($p<0.01$) stimulated the <100 μm fraction on day 5, while none of the treatments stimulated the >100 μm fraction (Fig. 28, Table 3). Phytoplankton biomass in the October 1989 microcosm experiment showed the greatest increase with addition of NH_4^++PO_4^{-3}

(Fig. 29). No shifts in relative abundance of blue-green algae were apparent in any treatment.

DISCUSSION

Our microcosm experiments detected phytoplankton nutrient deficiencies for the entire community (i.e. blue-green plus non-blue-green algae) in all six cases. Nitrogen addition most consistently elicited positive responses, stimulating community PPR, CHL *a*, phytoplankton biomass, or all three, in 5 of the 6 experiments. Phosphorus was clearly the deficient nutrient in the June 1988 experiment, when nitrogen showed no effect. Phosphorus addition stimulated phytoplankton PPR in October 1988 but did not result in increased phytoplankton biomass or CHL *a*. PO_4^{-3} addition increased phytoplankton biomass (PO_4^{-3} and MANN+PO_4^{-3} treatments, Fig. 18) without enhancing PPR or CHL *a* in June 1989. Enrichment with both phosphorus and nitrogen was required to stimulate PPR and CHL *a* throughout the August 1989 microcosm experiment. Mannitol treatments, included primarily to examine the effect of dissolved organic carbon enrichment on nitrogenase activity and bacterial activity (Wang et al. 1991; Miller in prep.) resulted in stimulation of PPR in only one case (1.0 MANN, October 1988) without increasing CHL *a* or phytoplankton biomass. Mannitol had several negative effects on carbon uptake (especially the smaller size fraction) that will not be discussed here. The inclusion of Mo treatments in the 1988 experiments was designed to satisfy the requirement of the NO_3^- reductase enzyme for this cofactor (Rueter and Petersen 1987) and to separate responses to Mo from NO_3^- responses. No substantial changes

resulted from Mo enrichment and it was therefore omitted from the 1989 experiments. It is evident that nitrogen and phosphorus can both be of primary importance in limiting phytoplankton production in the Grayling Arm of Hebgen Lake with nitrogen deficiency predominating.

Our nutrient deficiency findings agree with results of a review of experimental enrichments (Elser et al. 1990). They concluded that nitrogen should not be considered a secondary nutrient in freshwaters since the frequency of nitrogen versus phosphorus responses did not differ, and that nitrogen plus phosphorus was often required to elicit substantial phytoplankton growth response. Elser's study supports a growing body of evidence that contradicts the convention that P is the major nutrient controlling primary productivity in freshwater systems that was presented by various researchers (e.g. Hecky and Kilham 1988; Schindler 1975,1977; Smith 1984). It is important for limnologists and lake managers to consider both P and N when studying or managing a system.

The occurrence of simultaneous N and P deficiency, as in the August 1989 experiment, was addressed by Dodds et al.(1989) as attributable to differential N and P deficiencies for distinct members of the phytoplankton community. Differences in requirements and competitive ability of algal species that may explain contemporaneous N and P deficiency have been well documented (Tilman et al. 1986). The lack of stimulation of the <20 μm fraction with concomitant stimulation of the >20 μm fraction following PO_4^{3-} addition, and the requirement for N plus P to

substantially increase community primary productivity in the August 1989 experiment are similar to findings of Dodds et al.(1989). These authors found that the entire Aphanizomenon dominated phytoplankton community in Canyon Ferry Reservoir, Montana, was stimulated by single N or P additions, but when the large blue-green algae were removed, only N stimulated productivity. The view that different fractions of the phytoplankton community can be limited by different nutrients is further supported by the stimulation by N addition of only the <20 μm fraction in October 1988 and <100 fraction in October 1989 (these fractions excluded nitrogen fixing blue-green algae). This, and the concurrent lack of stimulation of the larger size fractions (primarily Aphanizomenon) by N enrichment agrees with the idea that the ability to fix atmospheric nitrogen imparts a competitive advantage to heterocystous blue-green algae in N deficient waters (Schindler 1977; Flett et al. 1980). In our experiments virtually all of the blue-green algae were heterocystous N_2 fixing species. We did however find that both the N_2-fixing blue-green and non-blue-green portions of the community could be N deficient. The shifts in relative carbon uptake towards non-blue-green fractions in the N treatments of June 1989 (Fig. 17) and the N+P treatment in August 1989 (Fig. 23) depict increases in growth of the smaller organisms indicating changes in community structure following changes in nutrient supply.

Substantial shifts in the relative importance of individual phytoplankton groups occurred in 4 out of the 6 experiments after 5 days of nutrient enrichment. Responses included both increases in non-blue-green algal dominance with N enrichment, and in-

creases in blue-green algal dominance with P enrichment. Our nutrient additions caused incipient changes in N:P ratios which have been shown to be important in controlling blue-green algal dominance (Smith 1983; Stockner and Shortreed 1988; Pick and Lean 1987). Observations that N_2 fixing blue-green algae tend to become increasingly dominant as TN:TP drops below about 30 have been taken to indicate that the ability to use atmospheric N_2 explains a large part of blue-green algal dominance. This view was corroborated by our experiments with Grayling Arm water. Sterner (1989) showed experimentally that competition for N was strong in Pleasant Pond, Minnesota, and that addition of N decreased the dominance of blue-green algae, suggesting the important role of N deficiency in promoting blue-green algal abundance. Whole lake manipulations (Barica et al. 1980; Stockner and Shortreed 1988) have been successful in reducing or eliminating blue-green algal blooms by addition of N (increasing N:P). The possibility of obtaining similar results by adding N to the Grayling Arm of Hebgen Lake is suggested by our results. However, our findings also indicate that N enrichment may increase blue-green algal biomass and might intensify blue-green algal blooms there. Furthermore, N-enrichment may stimulate macrophyte growth in the Grayling Arm which can lead to nuisance macrophyte levels while concomitantly stripping N from the water column rendering its effect useless in the control of planktonic blue-green algae.

We have shown that nutrient deficiencies are important in controlling phytoplankton productivity, standing crop and community structure. It is imperative to understand the affects of

changes in nutrient supply to successfully undertake any nutrient removal program to control eutrophication, or any nutrient addition program aimed at increasing lake or reservoir productivity. If a nutrient is in ample supply (deficiency cannot be detected by experimental bioassays) then enrichment with (or removal of) that nutrient may not increase (or decrease) the productivity of the system.

In summary, our results showed nutrient deficiencies in all 6 cases. Nitrogen was found to be limiting more often than phosphorus, but phosphorus was also important in several cases when added alone or in conjunction with nitrogen. The blue-green and non-blue-green components of the community showed different responses to nutrient enrichments. The non-blue-green component was N-deficient more often than the blue-green component whereas the latter was often stimulated by P addition, though the blue-green component was also stimulated by N enrichment in some cases. Organic carbon enrichment did not result in any consistent changes in productivity, biomass or community structure. We found that N addition promoted changes in the phytoplankton community structure in the direction of non-blue-green dominance, and P addition increased blue-green algal dominance.

REFERENCES

Ayles, G. B., J. G. I. Lark, J. Barica, and H. Kling. 1976. Seasonal mortality of rainbow trout (Salmo gardneri) planted in small eutrophic lakes in central Canada. J. Fish. Res. Board Can. 33:647-655.

Barica, J. 1978. Collapses of Aphanizomenon flos-aquae blooms resulting in massive fish kills in eutrophic lakes: effect of weather. Verh. Int. Verein. Limnol. 20:208-213.

Barica, J., H. Kling, and J. Gibson. 1980. Experimental manipulation of algal bloom composition by nitrogen addition. Can. J. Fish. Aquat. Sci. 37:1175-1183.

Canfield, D. E., Jr., E. Philips, and C. M. Duarte. 1989. Factors influencing the abundance of blue-green algae in Florida lakes. Can. J. Fish. Aquat. Sci. 46:1232-1237.

Carpenter, S. R., J. F. Kitchell, J. R. Hodgson, P. A. Cochran, J. J. Elser, M. M. Elser, D. M. Lodge, D. Kretchmer, X. He, and C. N. von Ende. 1987. Regulation of lake primary productivity by food web structure. Ecology 68:1863-1876.

Cooke, G. D. 1986. Lake and reservoir restoration. Ann Arbor Science. Ann Arbor, Michigan.

D'Elia, C. F., P. A. Steudler, and N. Corwin. 1977. Determination of total nitrogen in aqueous samples using persulfate digestion. Limnol. Oceanogr. 22:760-764.

Dodds, W. K., K. R. Johnson, and J. C. Priscu. 1989. Simultaneous nitrogen and phosphorus deficiency in natural phytoplankton assemblages: theory, empirical evidence, and implications for lake management. Lake Reserv. Manag. 5:21-26.

Downes, M. T. 1978. An automated determination of low reactive phosphorus concentrations in natural waters in the presence of arsenic, silicon and mercuric chloride. Water Research 12:743-745.

Elser, J. J., E. R. Marzolf, and C. R. Goldman. 1990. Phosphorus and nitrogen limitation of phytoplankton growth in freshwaters of North America: a review and critique of experimental enrichments. Can. J. Fish. Aquat. Sci. 47:1468-1477.

Elser, J. J., M. M. Elser, N. A. Mackay, and S. R. Carpenter. 1988. Zooplankton-mediated transition between N- and P-limited algal growth. Limnol. Oceanogr. 33:1-14.

Eppley, R. 1978. Nitrate uptake. Pages 401-409 in J. A. Hellebust and J. S. Craigie, eds. Handbook of phycological methods.

Physiological and biochemical methods. Cambridge University Press. Cambridge, England.

Flett, R. J., D. W. Schindler, R. D. Hamilton, and N. E. R. Campbell. 1980. Nitrogen fixation in Canadian Precambrian Shield lakes. Can. J. Fish. Aquat. Sci. 37:494-505.

Gorham, P. R. and W. W. Carmichael. 1988. Hazards of freshwater blue-green algae. Pages 403-420 in C. A. Lembi and J. R. Waaland eds. Algae and human affairs. Cambridge University Press. Cambridge, England.

Hecky, R. E. and P. Kilham. 1988 Nutrient limitation of phytoplankton in freshwater and marine environments: A review of recent evidence on the effects of nutrient enrichment. Limnol. Oceanogr. 33(4):796-822.

Lund, J. W. G., C. Kipling, and E. D. LeCren. 1957. The inverted microscope method of estimating algal numbers and the statistical basis of estimations by counting. Hydrobiologia 11:143-170.

McQueen, D. J., and S. R. Lean. 1987. Influence of water temperature and nitrogen to phosphorus ratios on the dominance of blue-green algae in Lake St. George, Ontario. Can. J. Fish. Aquat. Sci. 44:598-604.

Miller, T. D. (in preparation) Influence of nutrients on blue-green algal productivity, nitrogenase activity and relative biomass in Hebgen Lake, Montana. Master's thesis, Montana State University.

Pick, F. R., and D. R. S. Lean. 1987. The role of macronutrients (C, N, P) in controlling cyanobacterial dominance in temperate lakes. New Z. J. Mar. Freshw. Res. 21:425-434.

Prepas, E. E. and A. M. Trimbee. 1988. Evaluation of indicators of nitrogen limitation in deep prairie lakes with laboratory bioassays and limnocorrals. Hydrobiologia 158:269-276.

Priscu, J. C. and L. R. Priscu. 1984. Inorganic nitrogen uptake in oligotrophic Lake Taupo, New Zealand. Can. J. Fish. Aquat. Sci. 41:1436-1445.

Reynolds, C. S. 1984. The ecology of freshwater phytoplankton. Cambridge University Press. Cambridge, England.

Rueter, J. G., and R. R. Petersen. 1987. Micronutrient effects on cyanobacterial growth and physiology. New Z. J. Mar. Freshw. Res. 21:435-445.

Schindler, D. W. 1977. Evolution of phosphorus limitation in lakes. Science 21:260-262.

Schindler, D.W. 1975. Whole lake eutrophication experiments with phosphorus, nitrogen and carbon. Verh. Int. Verein. Limnol.

19:3221-3231.

Shapiro, J. 1980. The importance of trophic level interactions to the abundance and species composition of algae in lakes. Pages 105-116 in J. Barica and L. R. Mur, eds. Hypertrophic Ecosystems. Den Haag: Junk, the Netherlands.

Smith, S. V. 1984. Phosphorus versus nitrogen limitation in the marine environment. Limnol. Oceanogr. 29:1149-1160.

Smith, V. H. 1983. Low nitrogen to phosphorus ratios favor dominance by blue-green algae in lake phytoplankton. Science 221:669-671.

Solorzano, L. 1969. Determination of ammonia in natural waters by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

Solorzano, L., and J. H. Sharp. 1980. Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol. Oceanogr. 25:754-758.

Sonzogni, W. C., W. M. Repavich, J. H. Standridge, R. E. Wedepohl, and J. G. Vennie. 1988. A note on algal toxins in Wisconsin waters experiencing blue-green algal blooms. Lake Reserv. Manage. 4:281-285.

Stainton, M. P., M. J. Capel, and F. A. J. Armstrong. 1977. The chemical analysis of fresh water. Misc. Special Publ. No. 25. Fisheries and Environment Canada.

Sterner, R. W. 1989. Resource competition during seasonal succession toward dominance by cyanobacteria. Ecology 70:229-245.

Stockner, J. G., and K. S. Shortreed. 1988. Response of Anabaena and Synechococcus to manipulation of nitrogen:phosphorus ratios in a lake fertilization experiment. Limnol. Oceanogr. 33:1348-1361.

Strickland, J. D., and T. R. Parsons. 1972. A practical handbook of seawater analysis. Bull. Fish. Res. Board Can. (2nd ed.)

Tilman, D., R. L. Keisling, R. W. Sterner, S. S. Kilham, and F. A. Johnson. 1986. Green, blue-green, and diatom algae: taxonomic differences in competitive ability for phosphorus, silicon, and nitrogen. Arch. Hydrobiol. 106:473-485.

U.S.G.S. 1984. Water resource data for Montana. Water Data Report MT-84-1.

Uttermohl, H. 1958. Zur Vervollkommnung der quantitativen phytoplankton methodik. Mit. Int. Verein. Limnol. 9:1-38.

Vincent,W.F., W. Wurtsbaugh, C. L. Vincent and P. J. Richerson. 1984. Seasonal dynamics of nutrient limitation in a tropical high altitude lake (Lake Titicaca, Peru-Bolivia): Application of

physiological bioassays. Limnol. Oceanogr. 29:540-542.

Wang, L., T. D. Miller, and J. C. Priscu. 1991. Bacterioplankton nutrient deficiency in a eutrophic lake. (This report).

White, E., K. Law, G. Payne, and S. Pickmere. 1985. Nutrient demand and availability among planktonic communities - an attempt to assess nutrient limitation to plant growth in 12 central volcanic plateau lakes. New Z. J. Mar. Freshw. Res. 19:49-62.

Table 1. Nutrient amendments ($\mu g\ l^{-1}$) to water from Hebgen Lake (Grayling Arm, 0.5 m) for microcosm experiments.

TREATMENT	JUNE 1988	AUGUST 1988	OCTOBER 1988
Control	-- --	-- --	-- --
NH_4^+-N	100.0	100.0	100.0
NO_3^--N	100.0	100.0	100.0
Mo	9.6	9.6	9.6
NO_3^--N + Mo	100.0 9.6	100.0 9.6	100.0 9.6
PO_4^{-3}-P	50.0	50.0	50.0
Mannitol	91.1	91.1	91.1
Mannitol	182.2	182.2	182.2

TREATMENT	JUNE 1989	AUGUST 1989	OCTOBER 1989
Control	-- --	-- --	-- --
NH_4^+-N	140.0	140.0	140.0
NO_3^--N	140.0	140.0	140.0
PO_4^{-3}-P	93.0	93.0	93.0
NH_4^+-N + PO_4^{-3}-P	140.0 93.0	140.0 93.0	140.0 93.0
Mannitol	91.1	91.1	91.1
Mannitol + NH_4^+-N	91.1 140.0	91.1 140.0	91.1 140.0
Mannitol + PO_4^{-3}-P	91.1 93.0	91.1 93.0	91.1 93.0

Table 2. Ambient temperature (°C), CHL *a* ($\mu g\ l^{-1}$) and nutrient concentrations ($\mu g\ l^{-1}$) of water collected from Hebgen Lake (Grayling Arm) for microcosm experiments. Phyto (Ana = *Anabaena*; Aph = *Aphanizomenon*; Ast = *Asterionella*; Frag = *Fragallaria*) refers to the dominant (comprise > 60% of biomass) phytoplankton genus or combination of genera.

	1988			1989		
PARAMETER	22 JUN	21 AUG	23 OCT	20 JUN	08 AUG	19 OCT
°C	16.9	22.0	11.5	15.6	19.2	10.1
CHL *a*	30.3	15.6	144.5	4.5	5.0	48.5
Phyto	Ana	Ana	Aph & Ast	Ana	Aph & Frag	Aph
NH_4^+-N	3.8	61.1	12.5	9.9	4.2	6.0
NO_3^--N	2.6	64.0	5.6	10.2	7.9	94.1
TDN	140.0	570.0	390.0	176.0	146.0	255.0
PN	514.8	299.1	1192.2	137.2	74.2	244.9
TN	654.8	869.1	1582.2	313.0	220.2	499.9
SRP	2.8	39.1	7.7	18.1	5.1	18.1
TDP	10.4	57.1	22.5	28.0	17.8	38.4
PP	-- --	-- --	-- --	16.9	9.9	45.7
TP	-- --	-- --	-- --	44.9	27.7	84.1
DOC	2834.0	7012.0	7749.0	2839.0	6960.0	6552.0
PC	3416.0	2288.4	7751.0	761.0	559.0	1364.0
DIN:SRP	2.3	3.2	2.4	1.1	2.4	5.5
TN:TP	12.9*	9.2*	16.7*	7.0	7.9	5.9
PN:PP	12.5*	8.9*	16.5*	8.1	7.5	5.4
PC:PN	6.6	7.6	6.5	5.5	7.5	5.6
PC:PP	80.3*	47.4*	107.5*	45.0	56.5	29.8

* = sample not from experiment but from same station within 5 days of experiment.

Table 3. Effect of nutrient additions on phytoplankton activity and biomass. Results of one-way ANOVA coupled with least significant difference test. PPR = chlorophyll a specific carbon uptake including all time points. CHL = chlorophyll a concentrations including last two days only. Size fractionated carbon uptake (i.e., >20 = carbon uptake of organisms that did not pass through 20 μm mesh) at one time point. -- = not different from unammended control; +,++ = significantly greater than control at P<0.05, P<0.001; o,oo = significantly less than control at P<0.05, P<0.001, respectively. NA = no data collected.

	JUNE 1988				AUGUST 1988				OCTOBER 1988			
TREATMENT	PPR	CHL	>20	<20	PPR	CHL	>20	<20	PPR	CHL	>20	<20
NH_4^+	--	--	NA	NA	--	--	++	++	--	+	--	++
NO_3^-	LOST				--	--	++	++	+	--	--	++
Mo	--	--	NA	NA	--	--	--	--	--	--	--	+
NO_3^- +Mo	--	--	NA	NA	--	++	+	++	++	--	--	++
PO_4^{-3}	++	++	NA	NA	--	--	--	--	+	--	--	--
0.5 MANN	--	--	NA	NA	--	--	oo	oo	--	--	--	oo
1.0 MANN	--	--	NA	NA	--	--	--	oo	+	--	--	oo

	JUNE 1989				AUGUST 1989				OCTOBER 1989			
TREATMENT	PPR	CHL	>20	<20	PPR	CHL	>20	<20	PPR	CHL	>100	<100
NH_4^+	++	++	++	++	--	+	++	++	o	++	--	++
NO_3^-	--	++	--	++	--	--	++	++	oo	++	--	++
PO_4^{-3}	--	--	--	--	--	--	++	--	oo	--	o	--
$NH_4^+ + PO_4^{-3}$	++	++	++	++	++	++	++	++	--	+	--	++
MANN	--	--	--	--	--	--	--	--	oo	--	--	o
MANN+NH_4^+	+	++	+	+	--	--	++	+	--	--	--	+
MANN+PO_4^{-3}	--	--	--	--	--	--	--	--	oo	--	o	--

FIGURES REFERED TO IN THE TEXT

Note: Error bars are + and - one standard error throughout.

1. Chlorophyll a specific photosynthetic carbon uptake rates of phytoplankton (PPR) in the June 1988 experiment.

2. Chlorophyll a concentrations in the June 1988 experiment.

3. Phytoplankton biomass at the beginning (T=0) and end of the 1988 experiment.

4. PPR in the August 1988 experiment.

5. Size fractionated volume specific carbon uptake in the August 1988 experiment, day 4.

6. Chlorophyll a concentrations in the August 1988 experiment.

7. Phytoplankton biomass at T=0 and end of the August 1988 experiment.

8. Relative abundance of phytoplankton divisions in the August 1988 experiment. (LRGT=unidentified picoplankton <3 μm)

9. PPR in the October 1988 experiment.

10. Chlorophyll a concentrations in the October 1988 experiment.

11. Size fractionated volume specific carbon uptake in the October 1988 experiment, day 5.

12. Phytoplankton biomass at T=0 and termination, October 1988.

13. Relative abundance of phytoplankton divisions, October 1988.

14. PPR in the June 1989 experiment.

15. Chlorophyll a concentrations in the June 1989 experiment.

16. Size fractionated volume specific carbon uptake in the June 1989 experiment, day 5.

17. Percent of total carbon uptake by size fractions in the June 1989 experiment,day 5.

18. Phytoplankton biomass at T=0 and termination, June 1989.

19. Relative abundance of phytoplankton divisions, June 1989.

20. PPR in the August 1989 experiment.

21. Chlorophyll a concentrations in the August 1989 experiment.

22. Size fractionated volume specific carbon uptake in the

Fig. 1

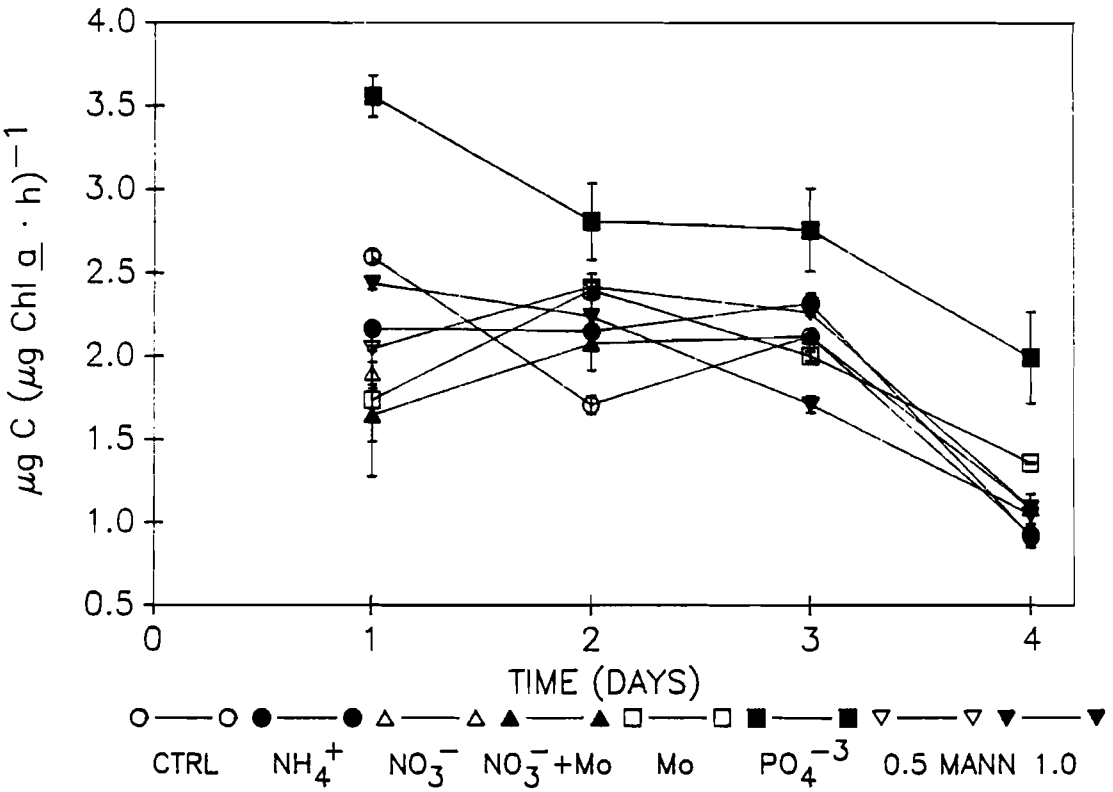

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
μg C (μg Chl a · h)⁻¹
0
1
2
3
4
TIME (DAYS)
CTRL
NH4+
NO3−
NO3−+Mo
Mo
PO4−3
0.5 MANN
1.0

Fig. 2

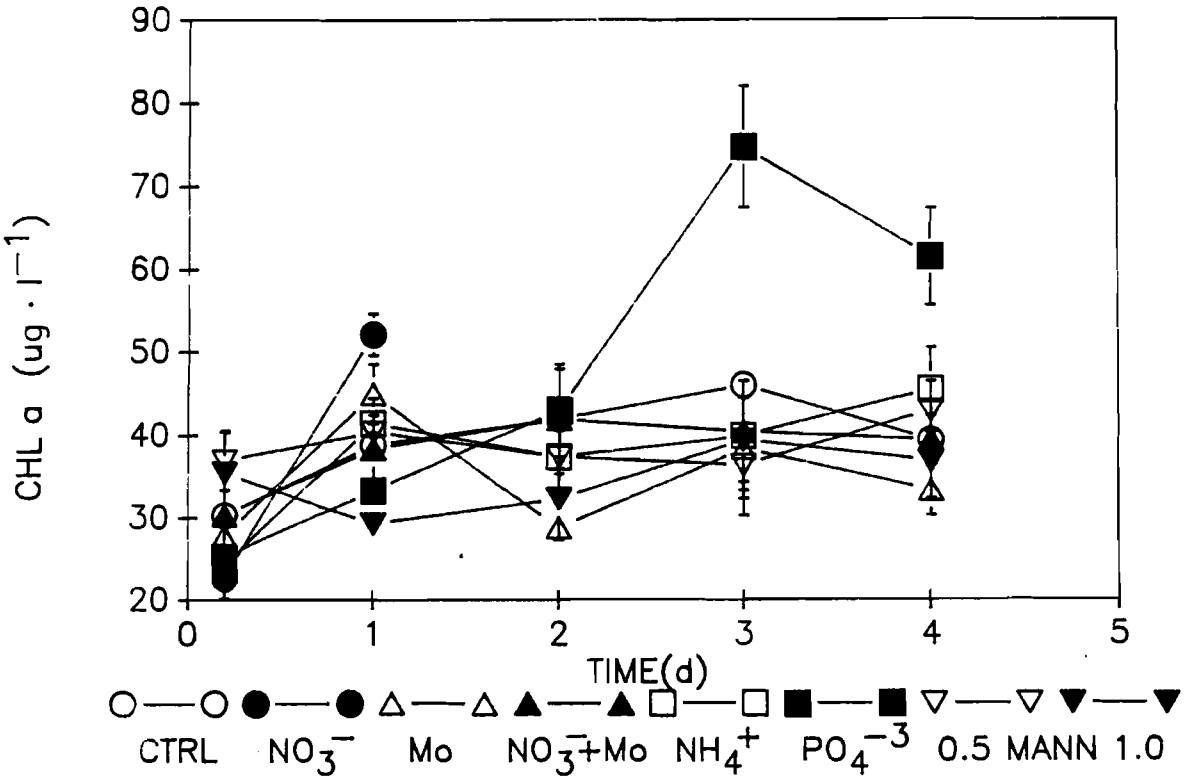
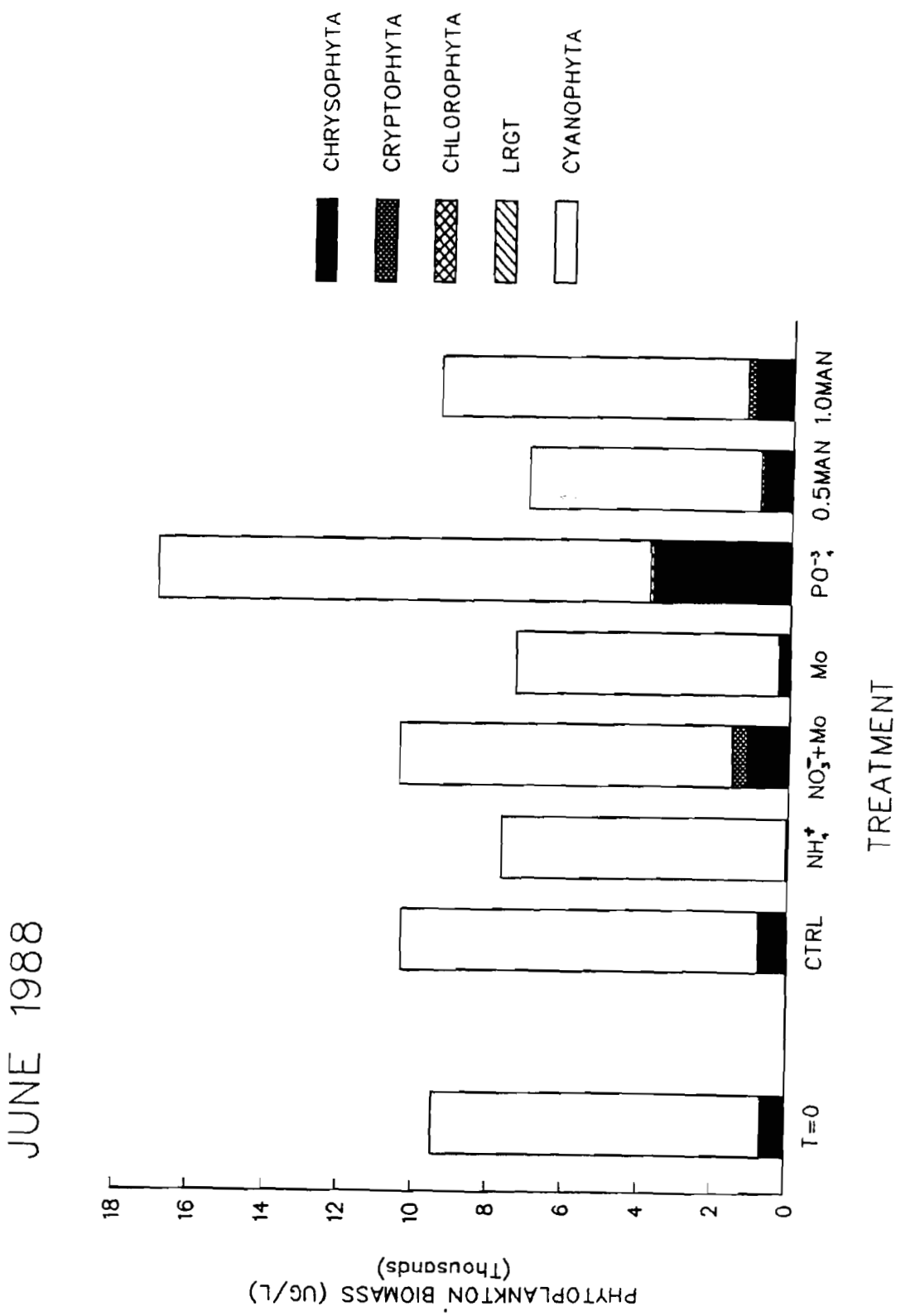

90
80
70
60
50
40
30
20
CHL a (ug · l⁻¹)
0
1
2
3
4
5
TIME(d)
CTRL
NO3−
Mo
NO3−+Mo
NH4+
PO4−3
0.5 MANN
1.0

Fig. 3


JUNE 1988
CHRYSOPHYTA
CRYPTOPHYTA
CHLOROPHYTA
LRGT
CYANOPHYTA
PHYTOPLANKTON BIOMASS (UG/L)
(Thousands)
0
2
4
6
8
10
12
14
16
18
T=0
CTRL
NH4+
NO3-+Mo
Mo
PO-3
0.5MAN
1.0MAN
TREATMENT

0104G

Fig. 4

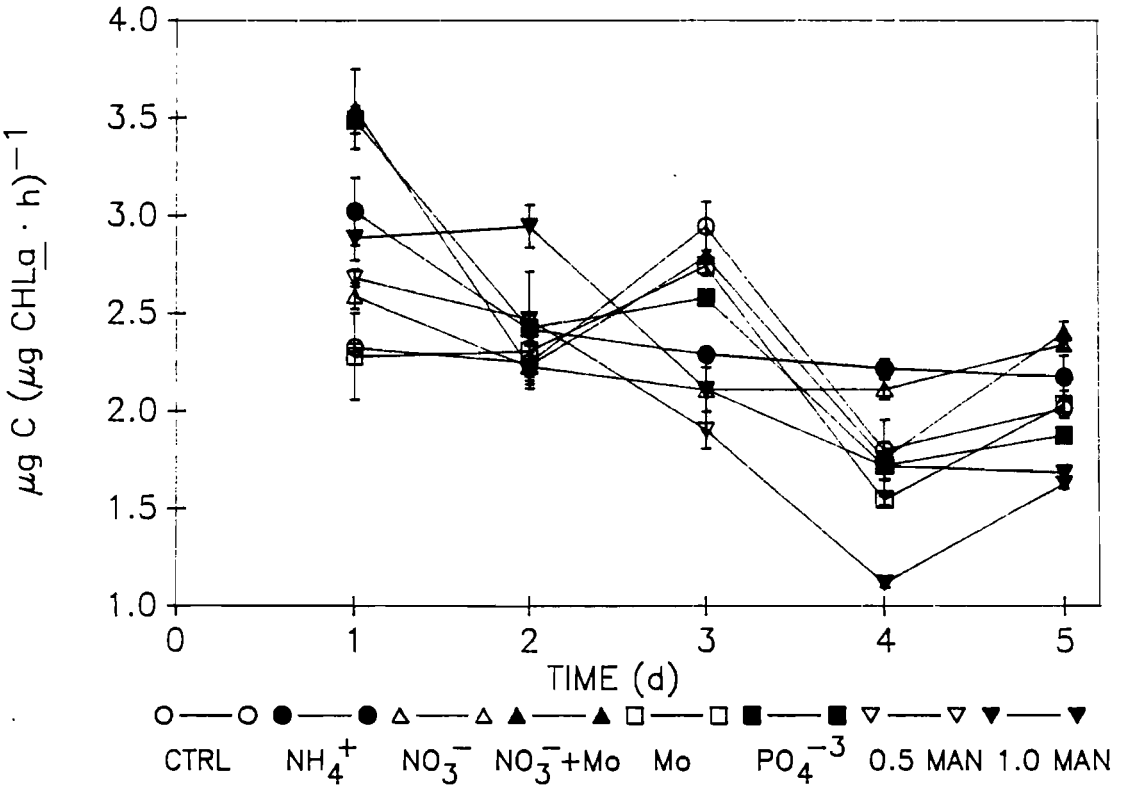

4.0
3.5
3.0
2.5
2.0
1.5
1.0
0
1
2
3
4
5
TIME (d)
μg C (μg CHLa · h)⁻¹
CTRL
NH4+
NO3−
NO3−+Mo
Mo
PO4−3
0.5 MAN
1.0 MAN

Fig. 5

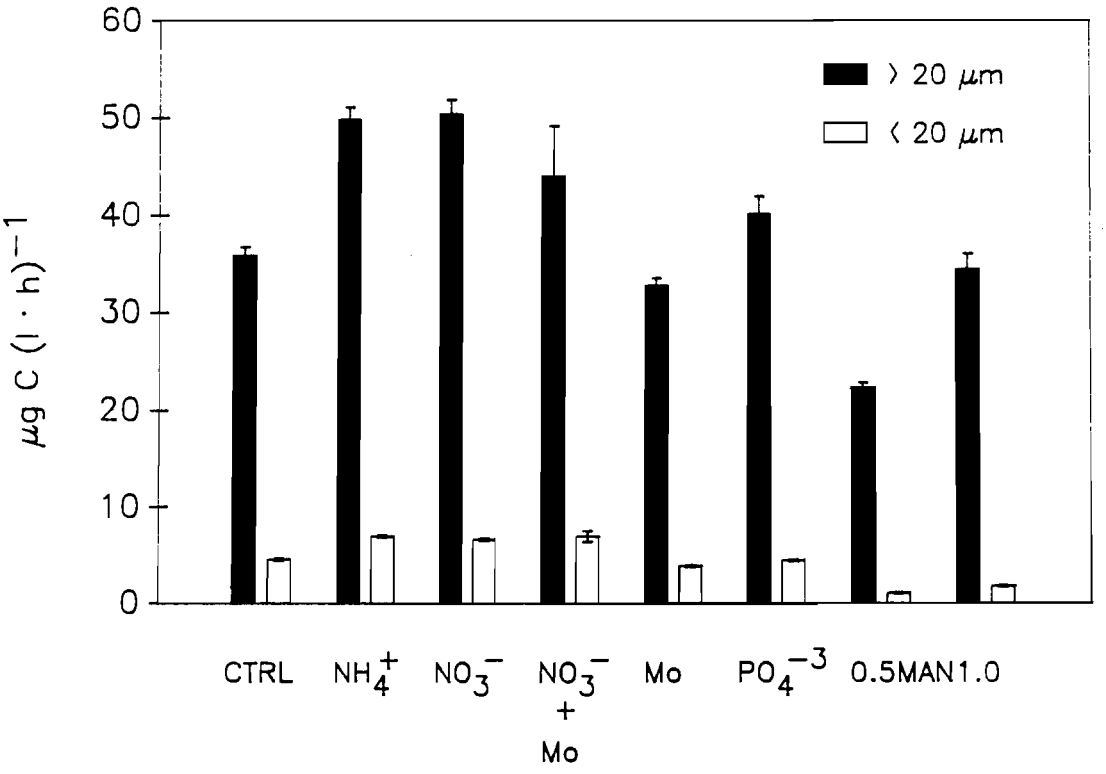
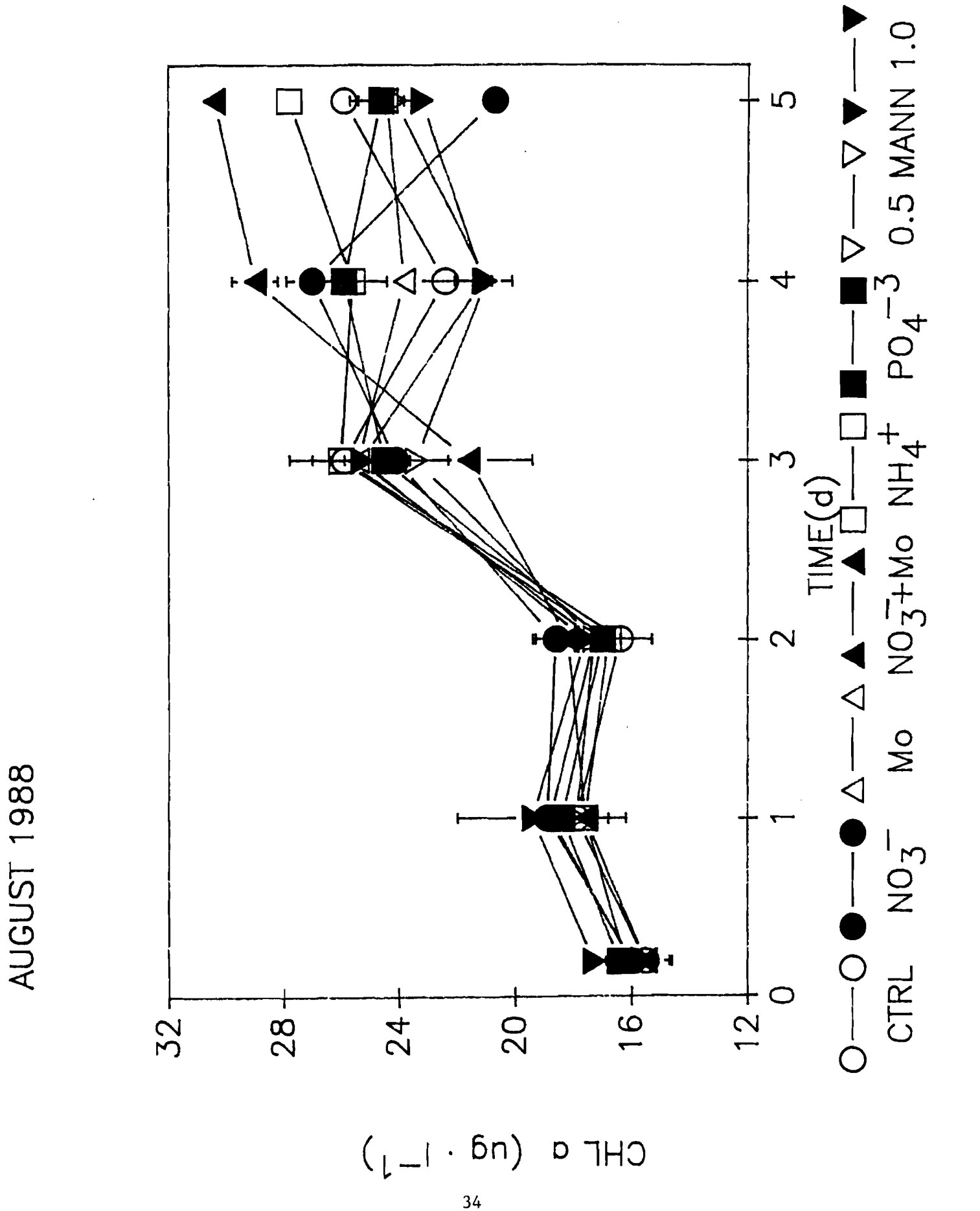
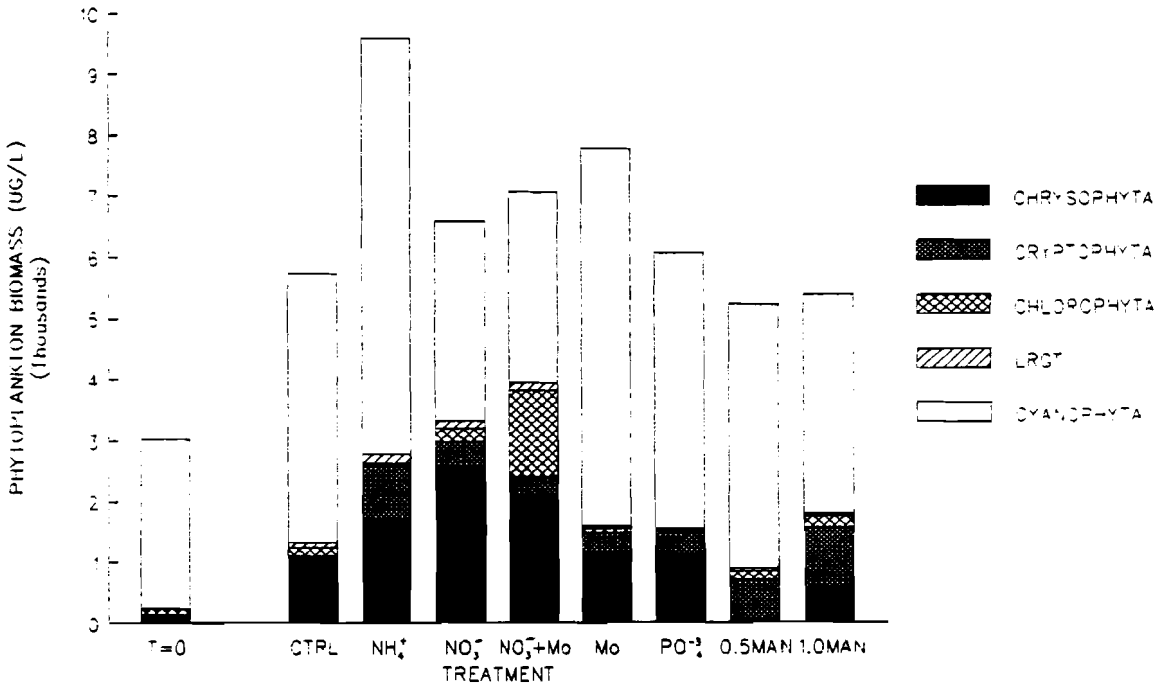

60
50
40
30
20
10
0
μg C (l · h)⁻¹
› 20 μm
‹ 20 μm
CTRL
NH4+
NO3−
NO3− + Mo
Mo
PO4−3
0.5MAN
1.0

Fig. 6


AUGUST 1988
CHL a (ug · l⁻¹)
32
28
24
20
16
12
0
1
2
3
4
5
TIME(d)
CTRL NO3− Mo NO3−+Mo NH4+ PO4−3 0.5 MANN 1.0

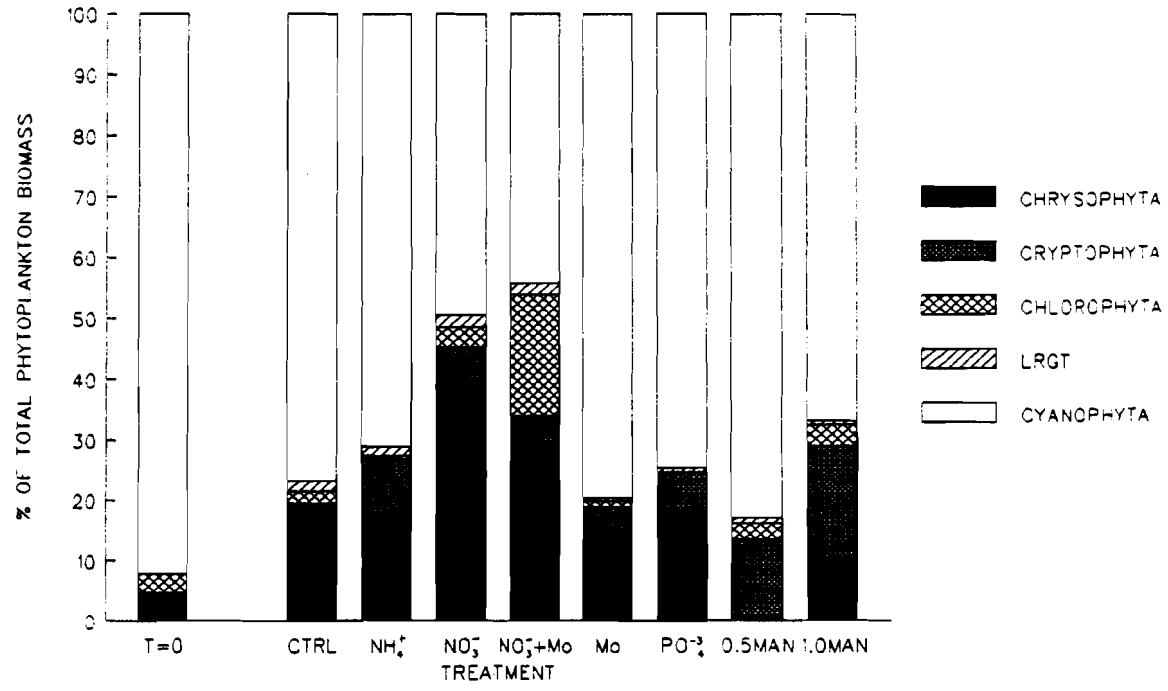
AUGUST 1988

Fig. 7



0107B

Fig. 8



0107C

Fig. 9

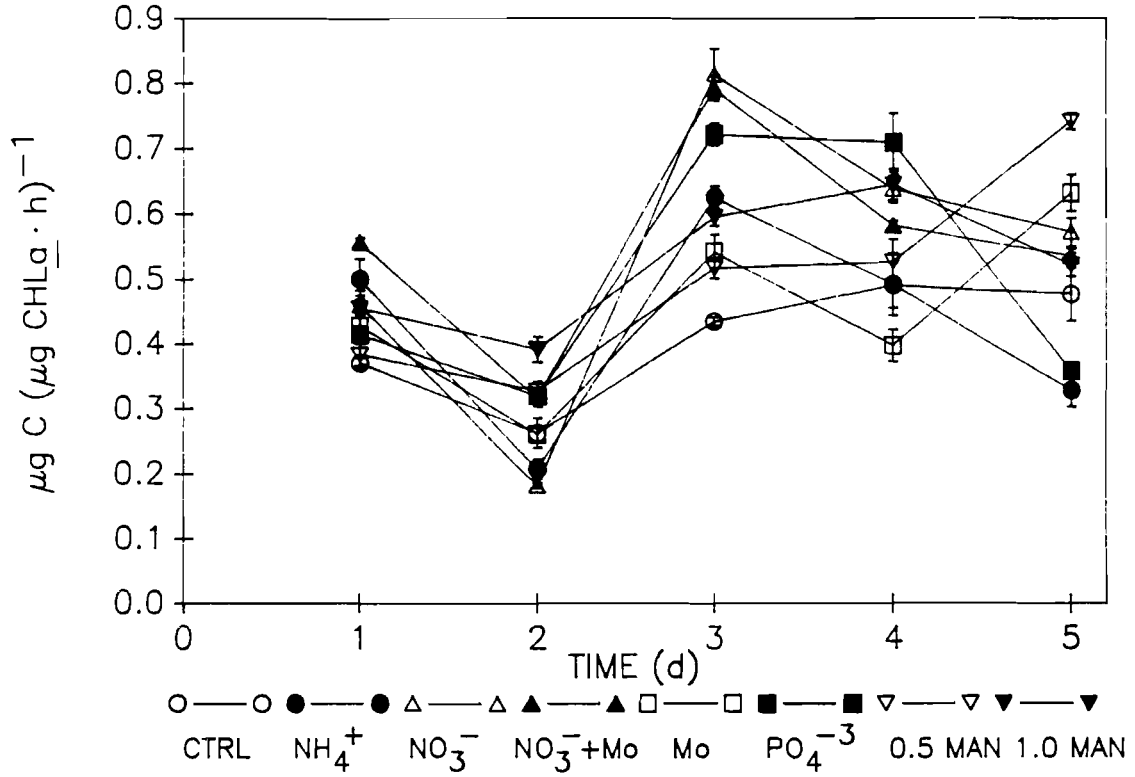

0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
μg C (μg CHLa · h)⁻¹
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
NO3−+Mo
Mo
PO4−3
0.5 MAN
1.0 MAN

Fig. 10

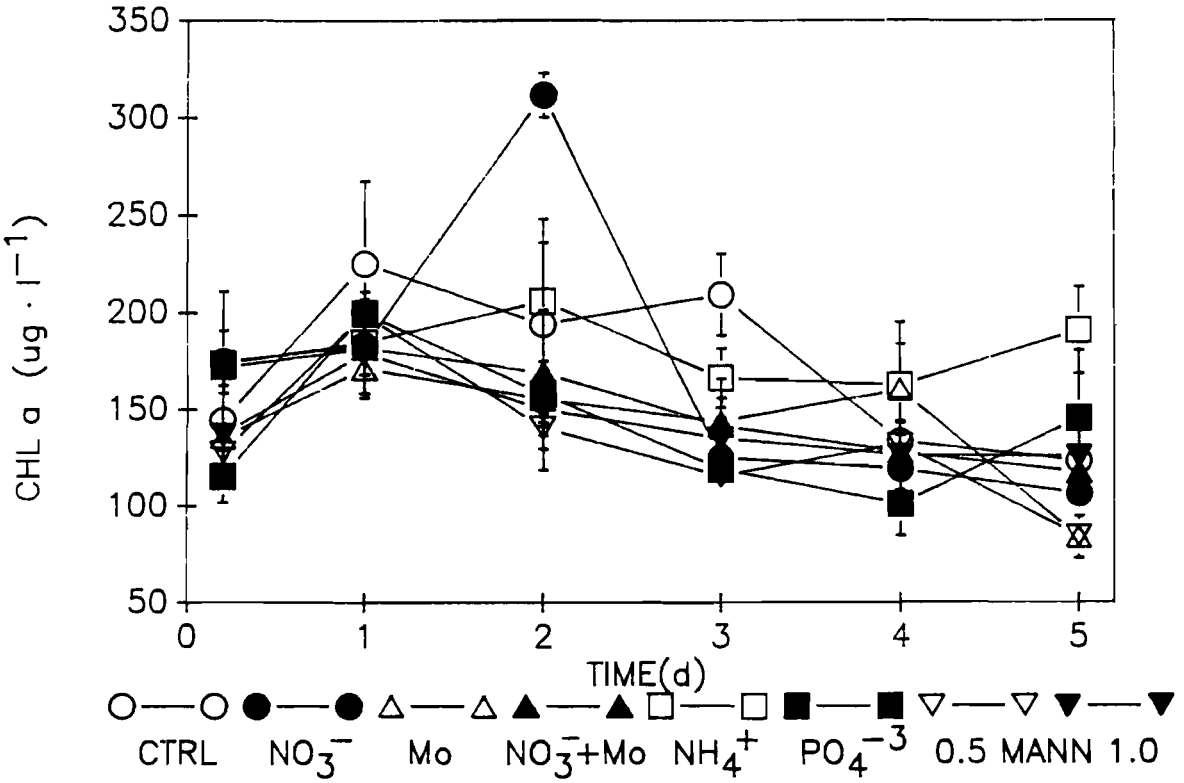
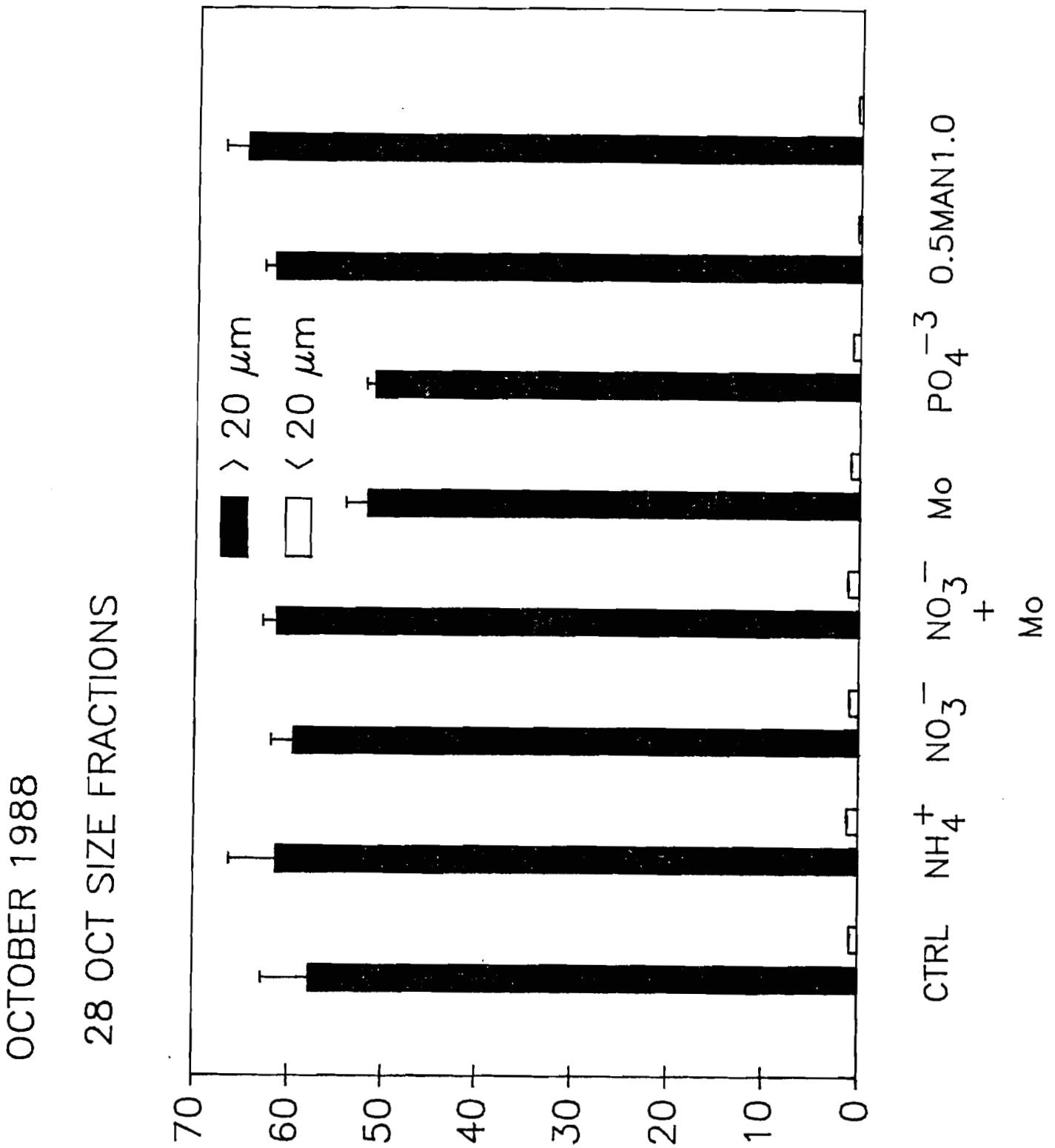
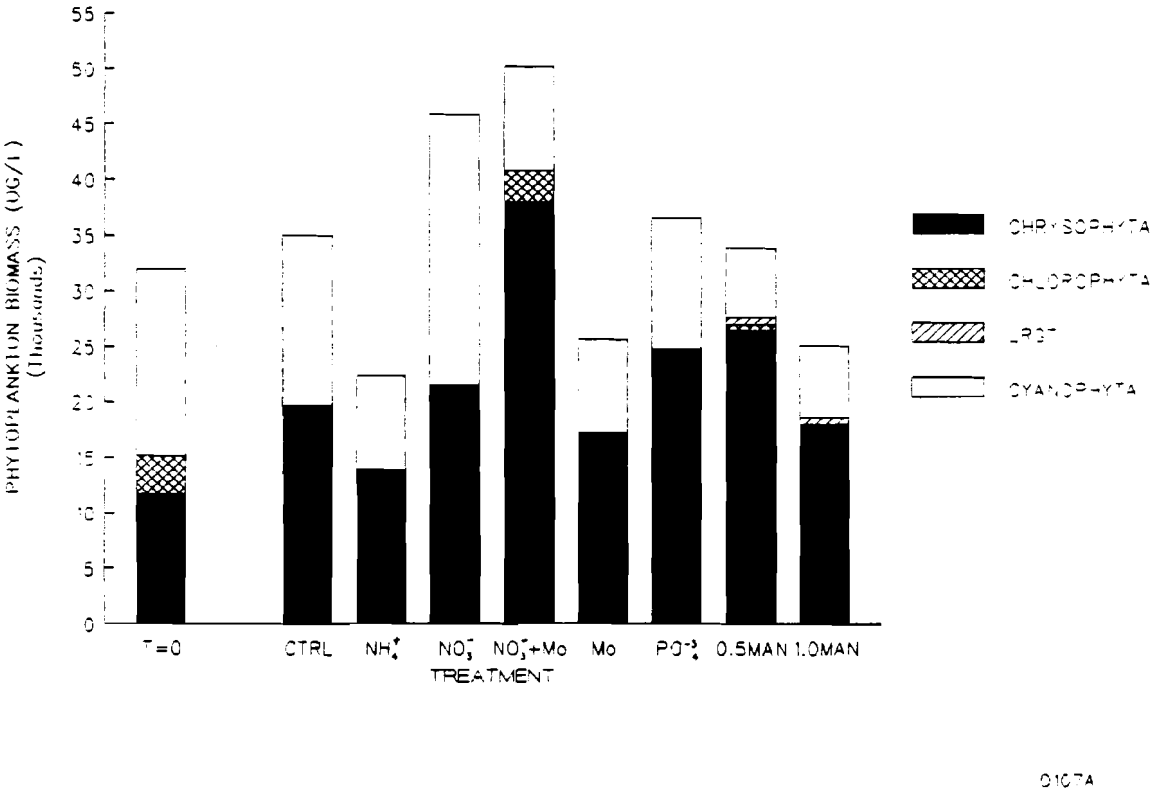

350
300
250
200
150
100
50
CHL a (ug · l⁻¹)
0
1
2
3
4
5
TIME(d)
CTRL
NO3−
Mo
NO3−+Mo
NH4+
PO4−3
0.5 MANN
1.0

Fig. 11


OCTOBER 1988
28 OCT SIZE FRACTIONS
> 20 μm
< 20 μm
μg C (l · h)⁻¹
70
60
50
40
30
20
10
0
CTRL
NH4+
NO3−
NO3− + Mo
Mo
PO4−3
0.5MAN1.0

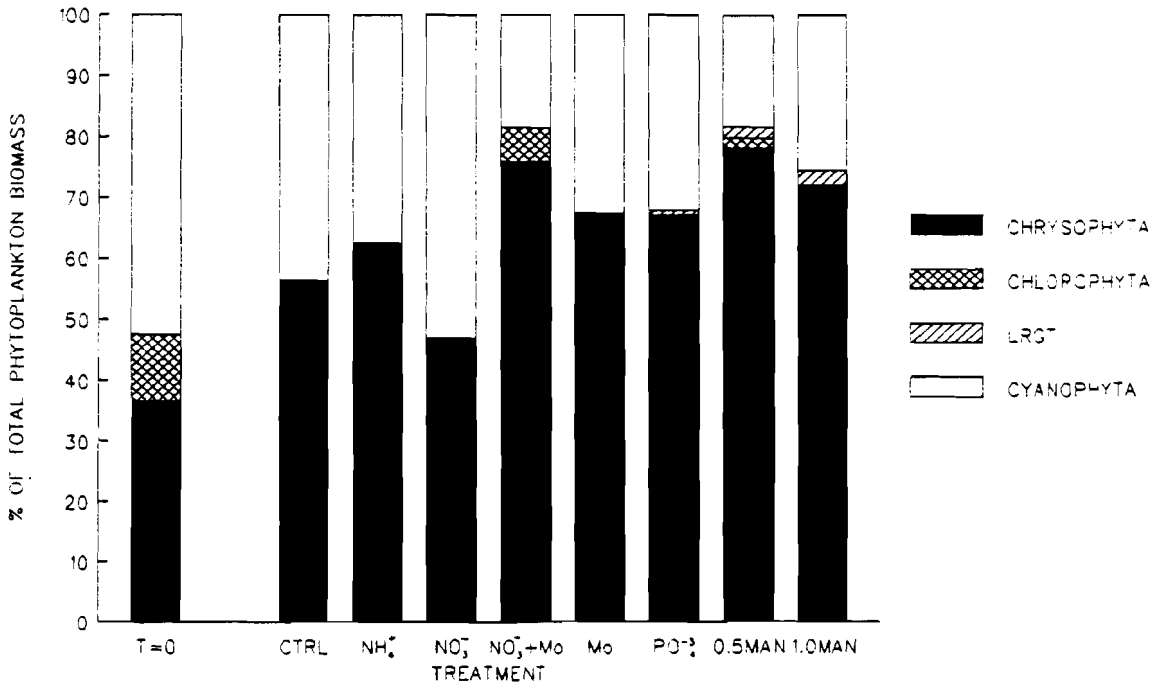
OCTOBER 1988

Fig. 12



0107A

Fig. 13



0104H

Fig. 14

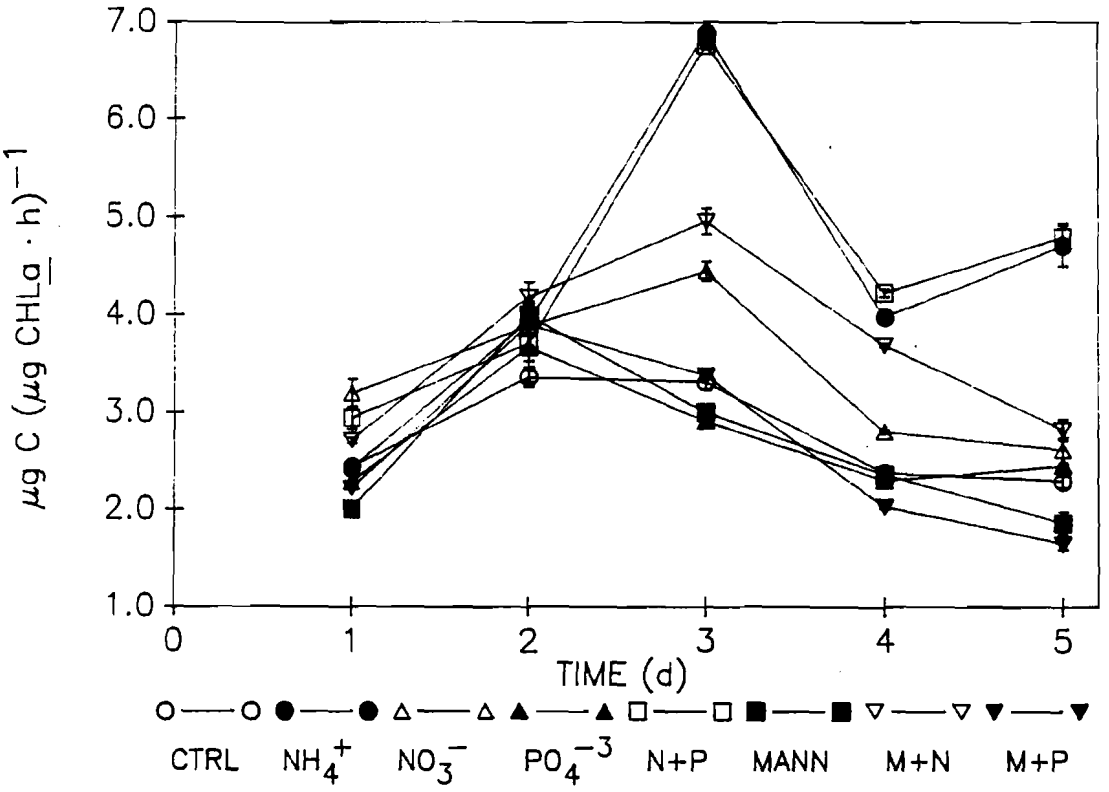

7.0
6.0
5.0
4.0
3.0
2.0
1.0
μg C (μg CHLa · h)⁻¹
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P

Fig. 15

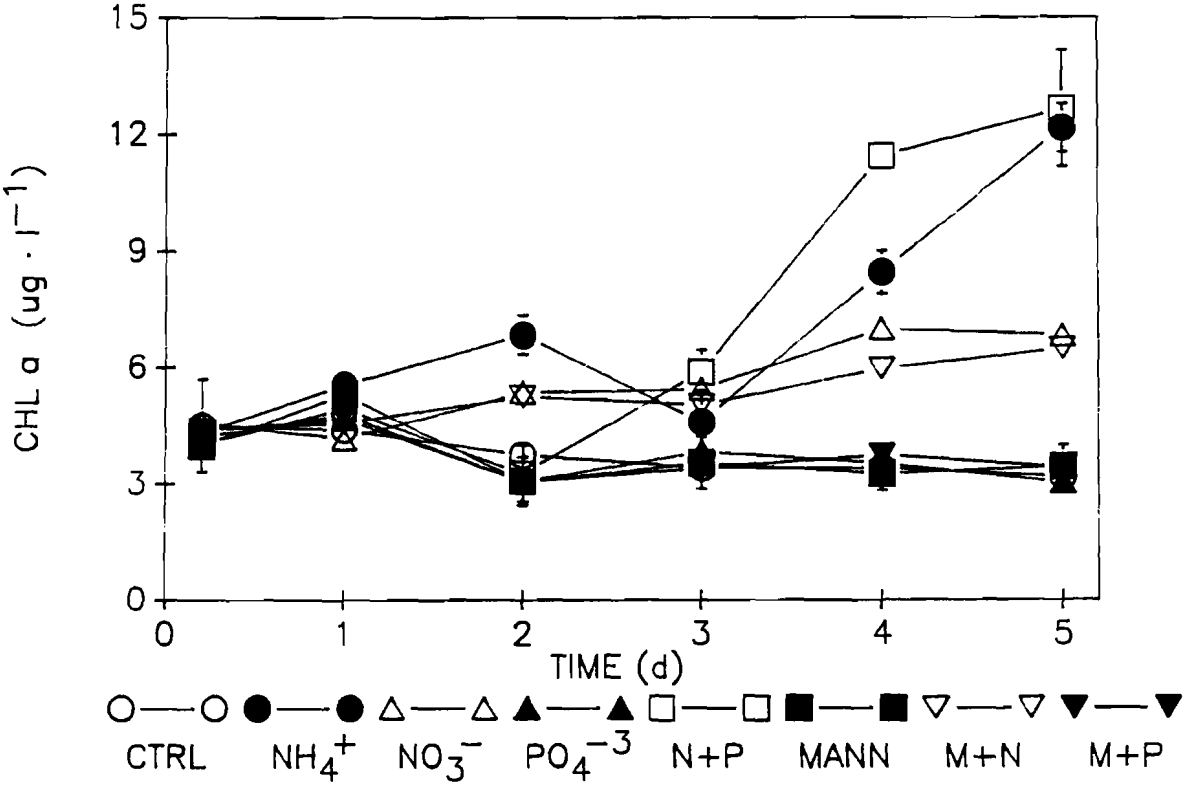

15
12
9
6
3
0
CHL a (ug · l⁻¹)
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P

Fig. 16

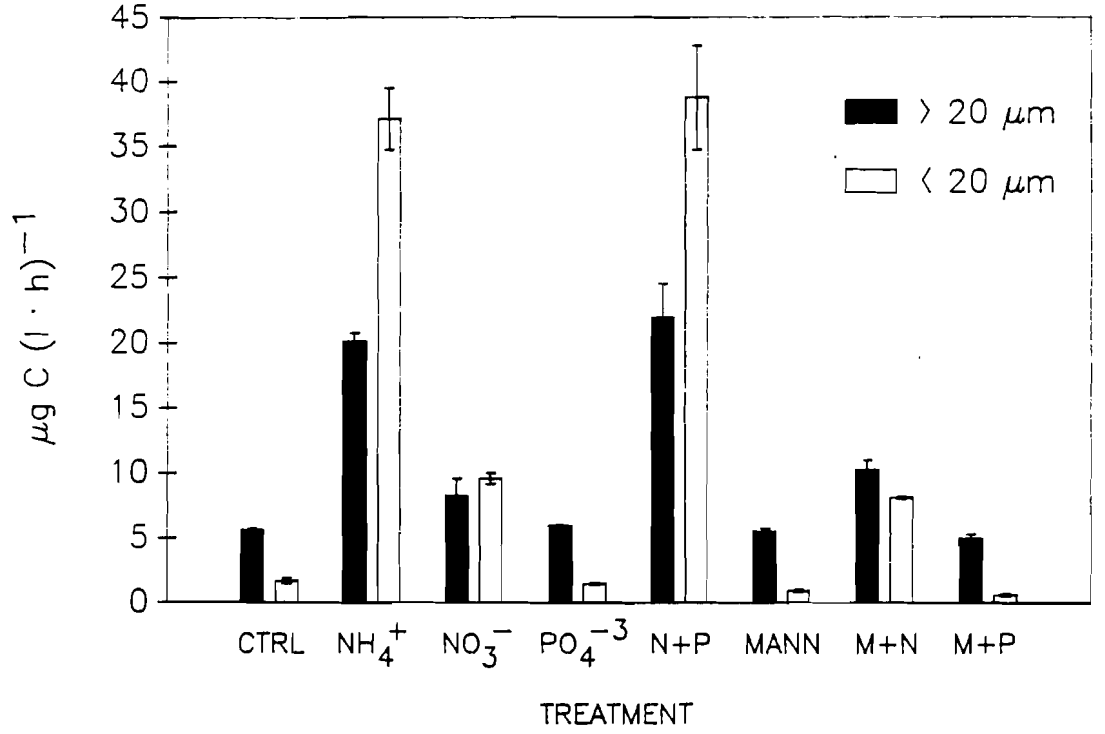
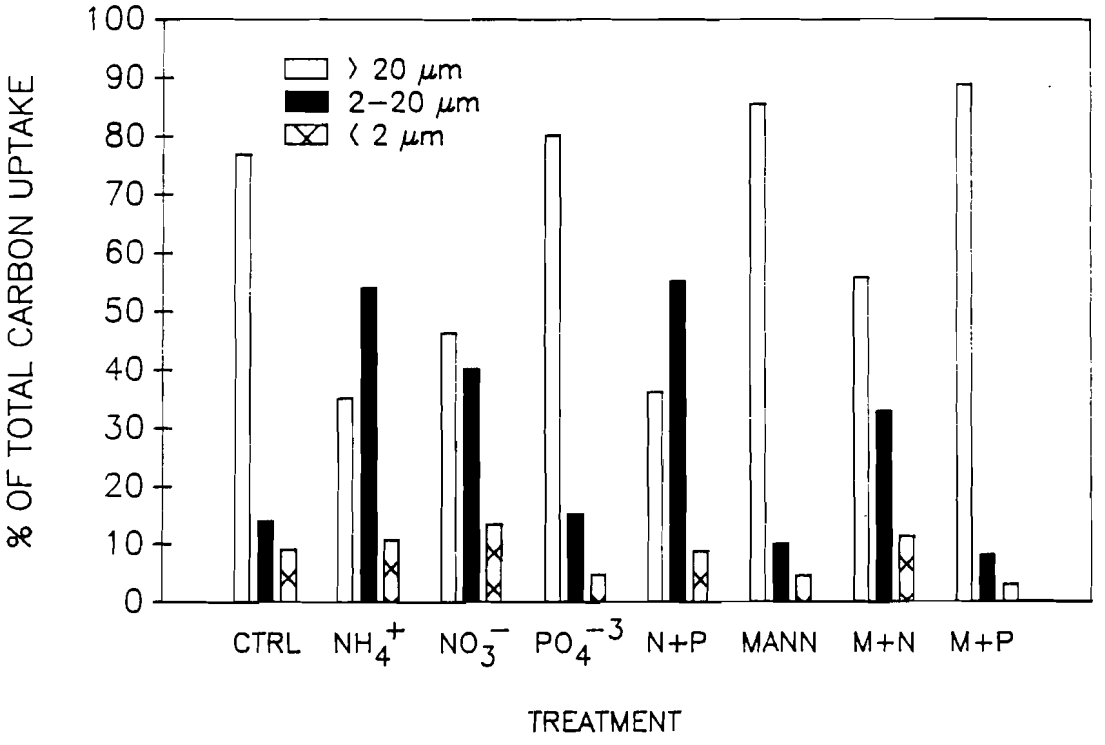
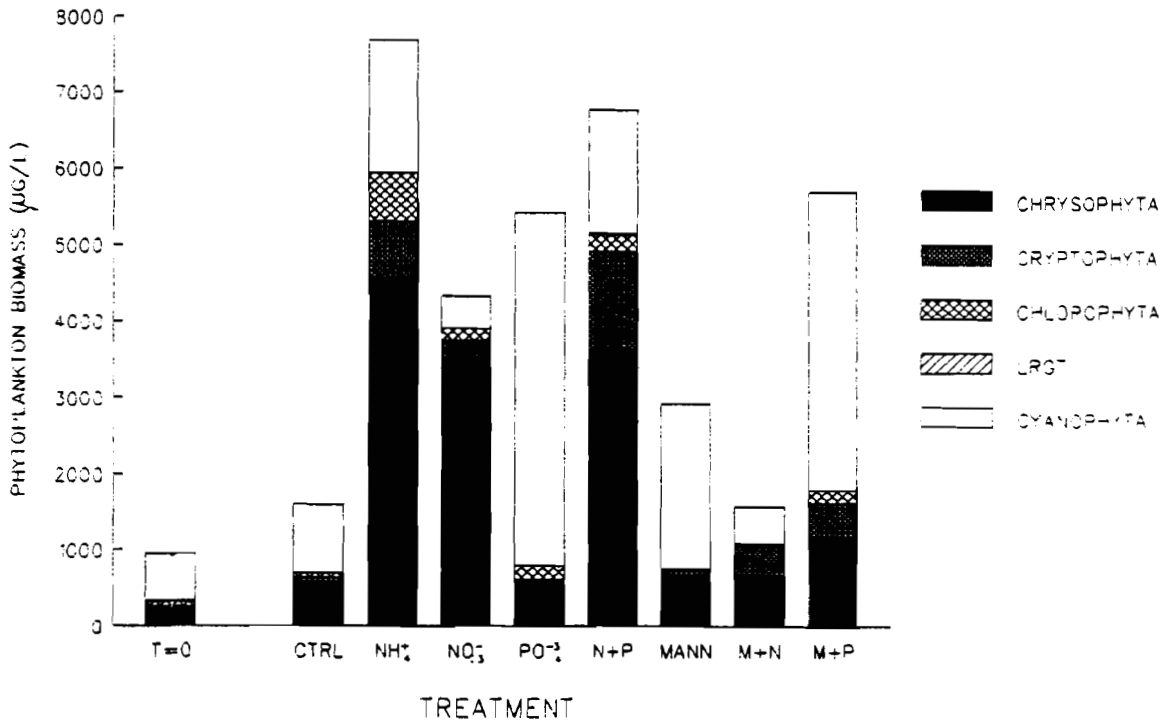

45
40
35
30
25
20
15
10
5
0
μg C (l · h)⁻¹
› 20 μm
‹ 20 μm
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P
TREATMENT

Fig. 17


100
90
80
70
60
50
40
30
20
10
0
% OF TOTAL CARBON UPTAKE
› 20 μm
2–20 μm
‹ 2 μm
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P
TREATMENT

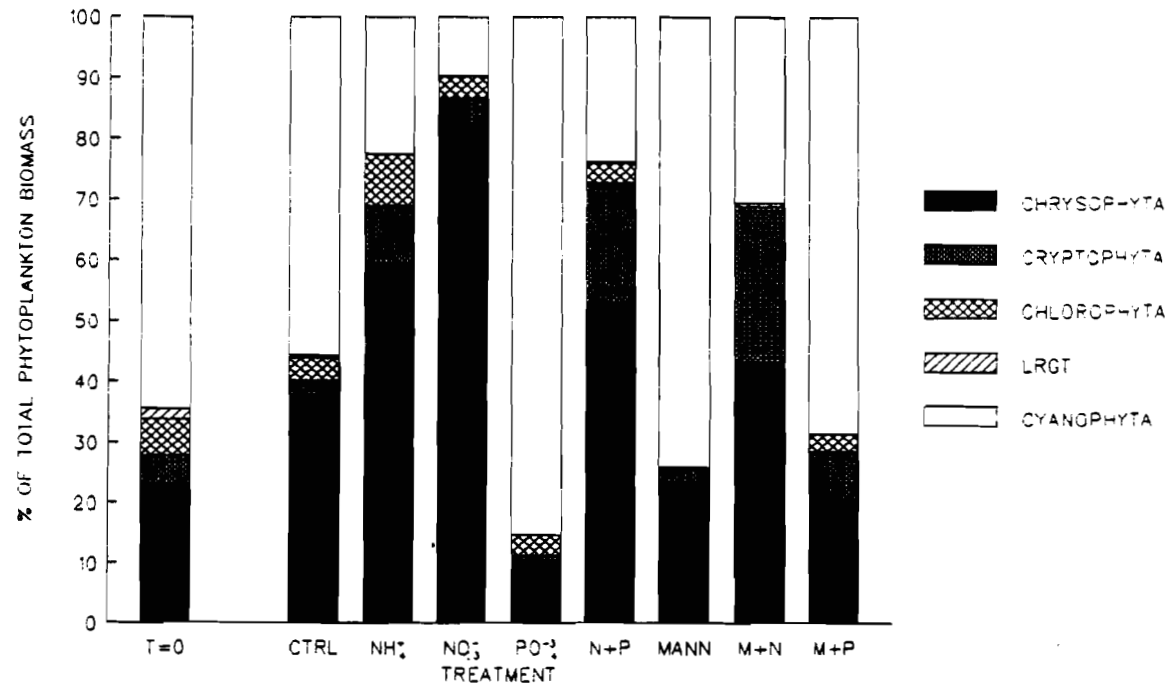
JUNE 1989

Fig. 18



0104D

Fig. 19



0107D

Fig. 20

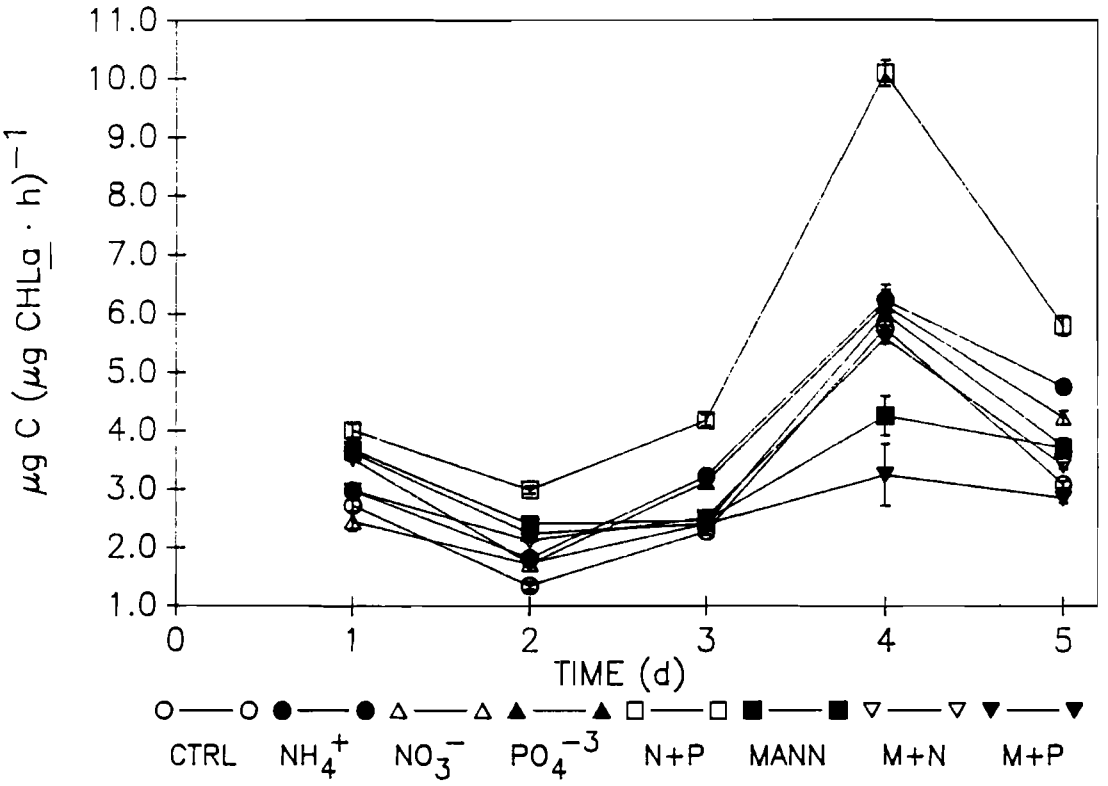

11.0
10.0
9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
μg C (μg CHLa · h)⁻¹
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P

Fig. 21

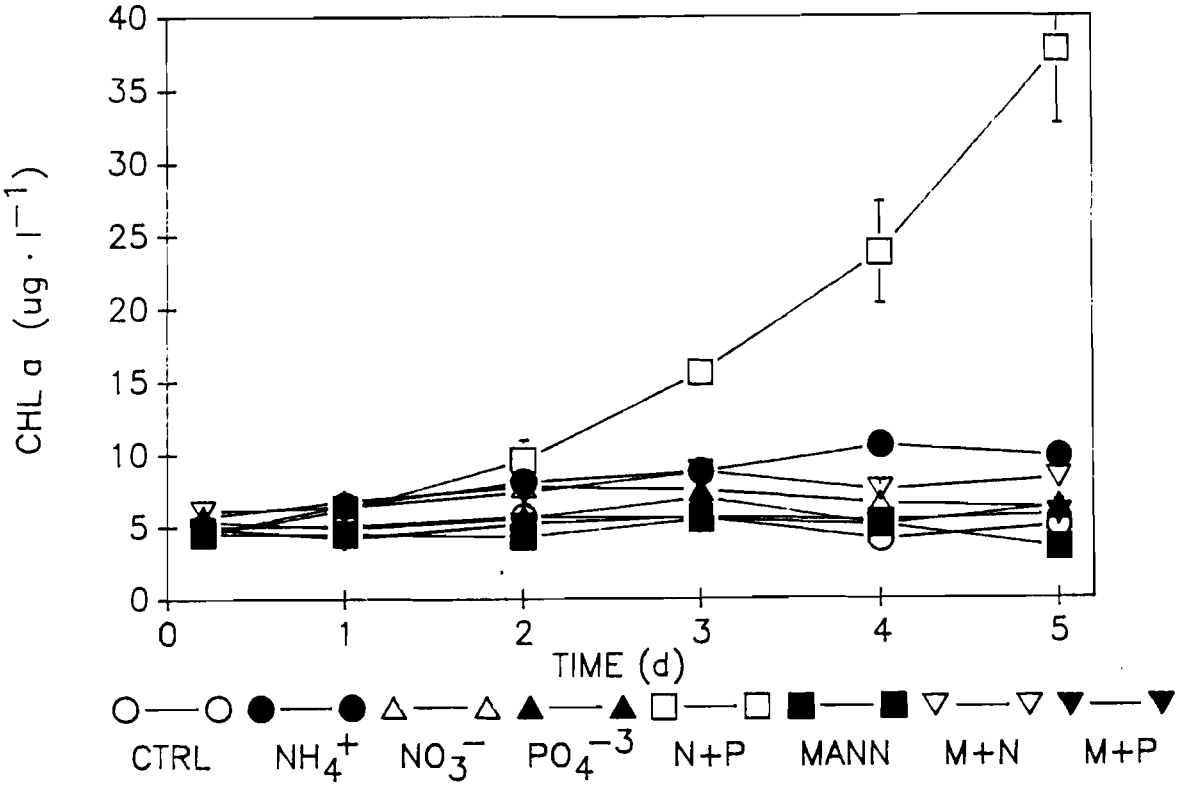

40
35
30
25
20
15
10
5
0
CHL a (ug · l⁻¹)
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P

Fig. 22

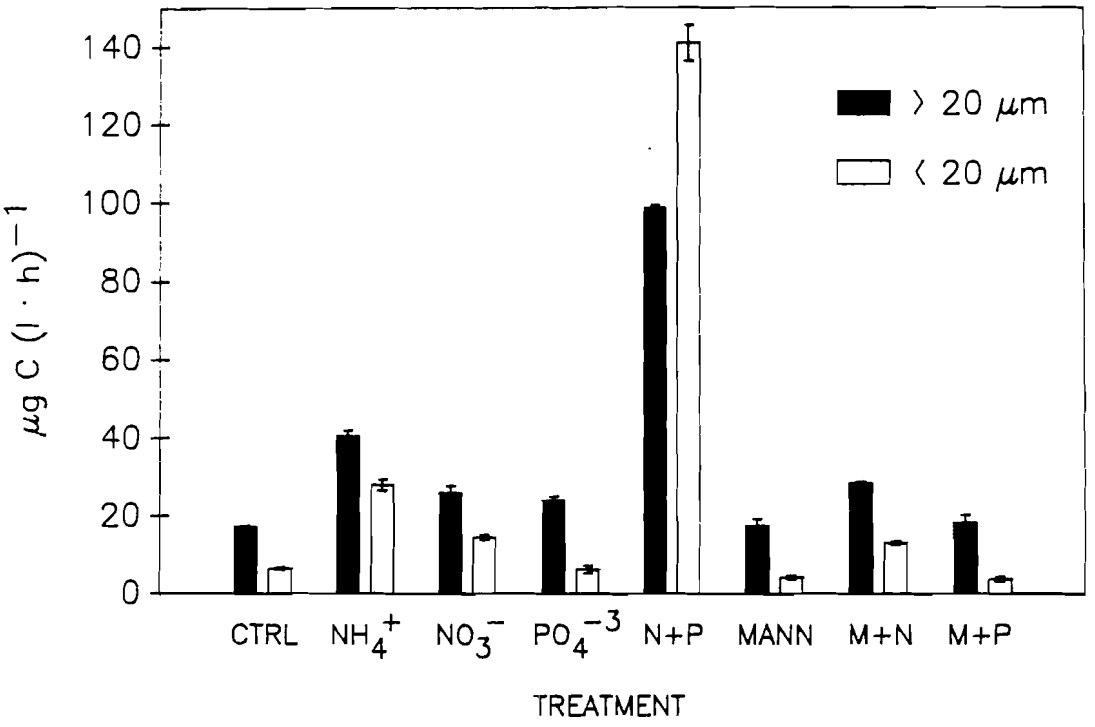
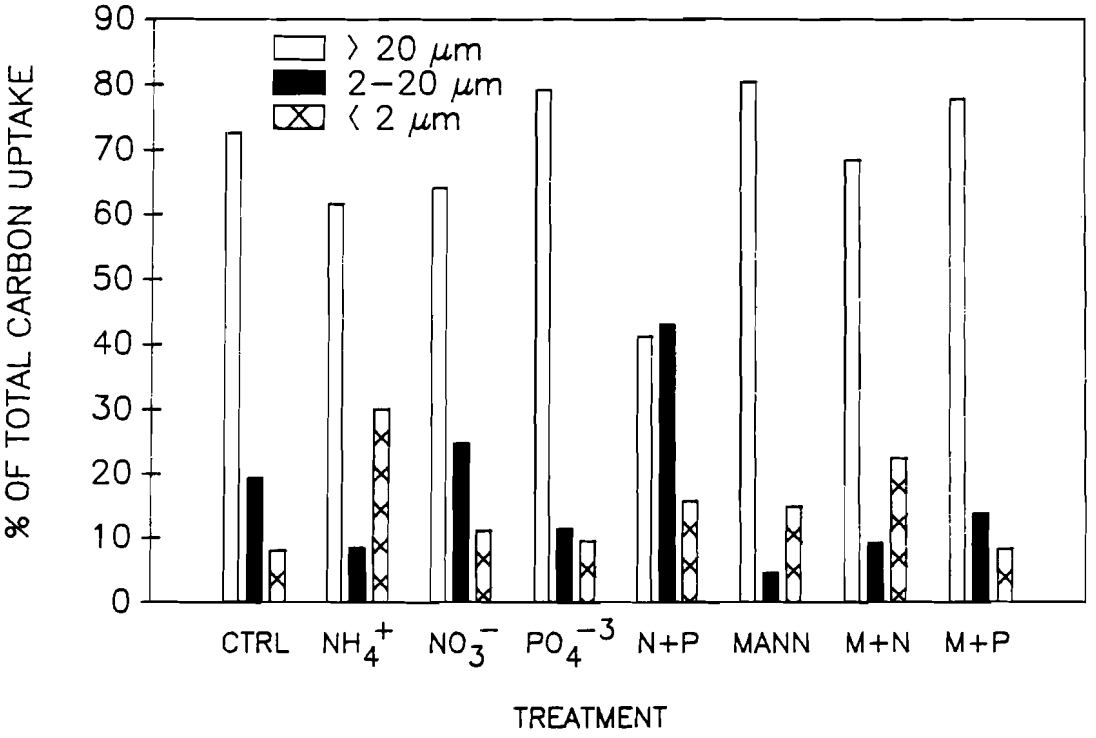
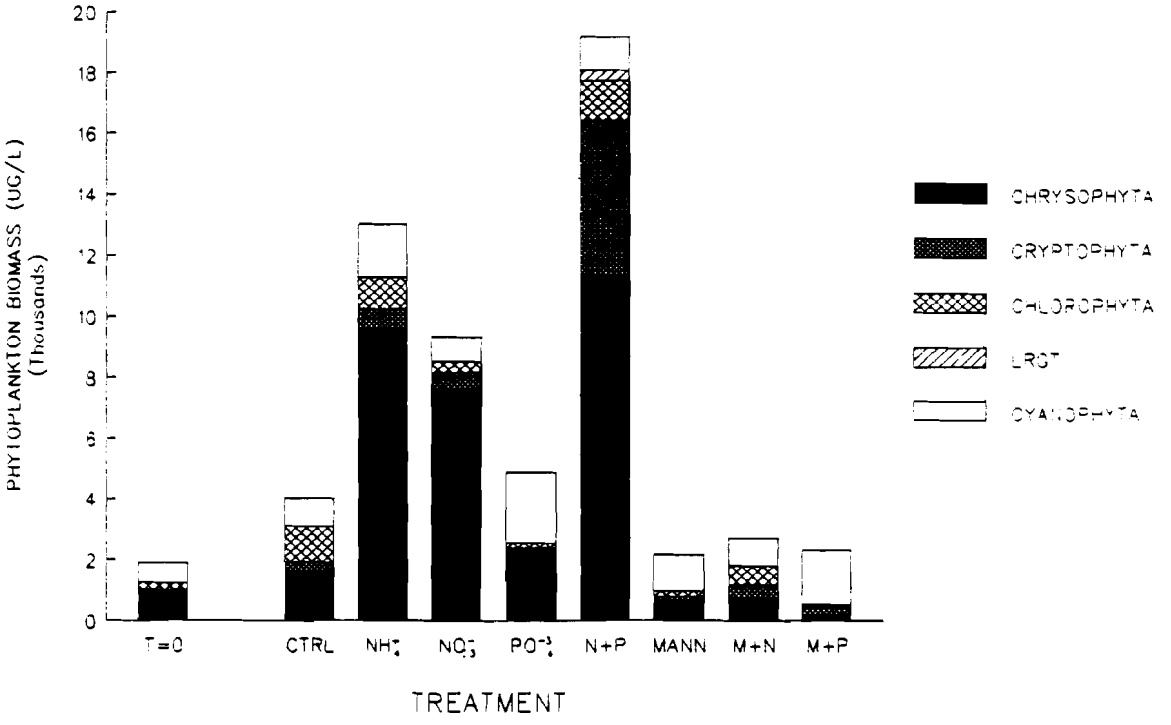

140
120
100
80
60
40
20
0
μg C (l · h)⁻¹
› 20 μm
‹ 20 μm
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P
TREATMENT

Fig. 23


90
80
70
60
50
40
30
20
10
0
% OF TOTAL CARBON UPTAKE
› 20 μm
2–20 μm
‹ 2 μm
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P
TREATMENT

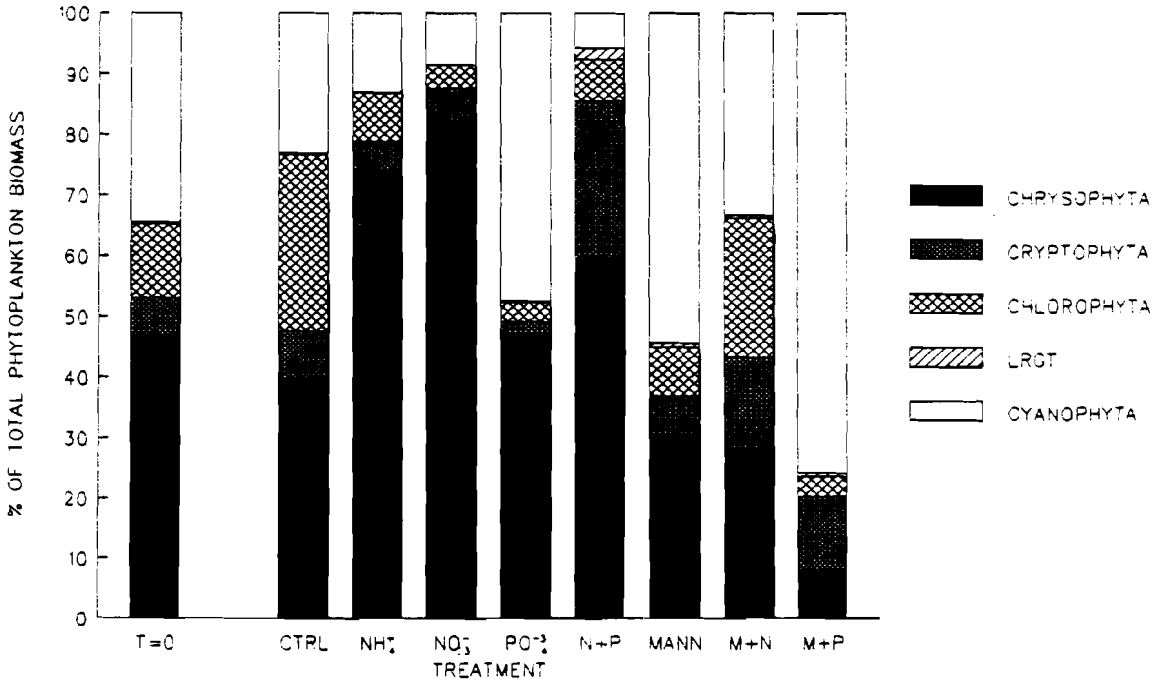
AUGUST 1989

Fig. 24



0104E

Fig. 25



0110C

Fig. 26

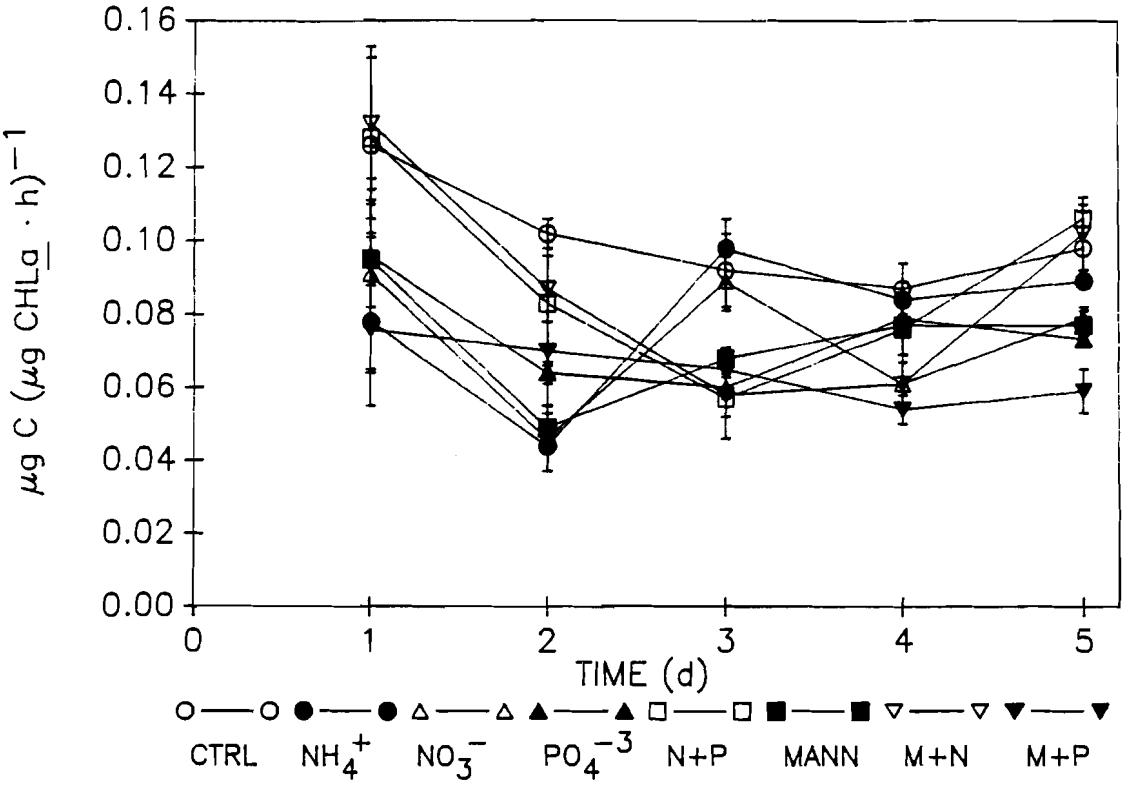
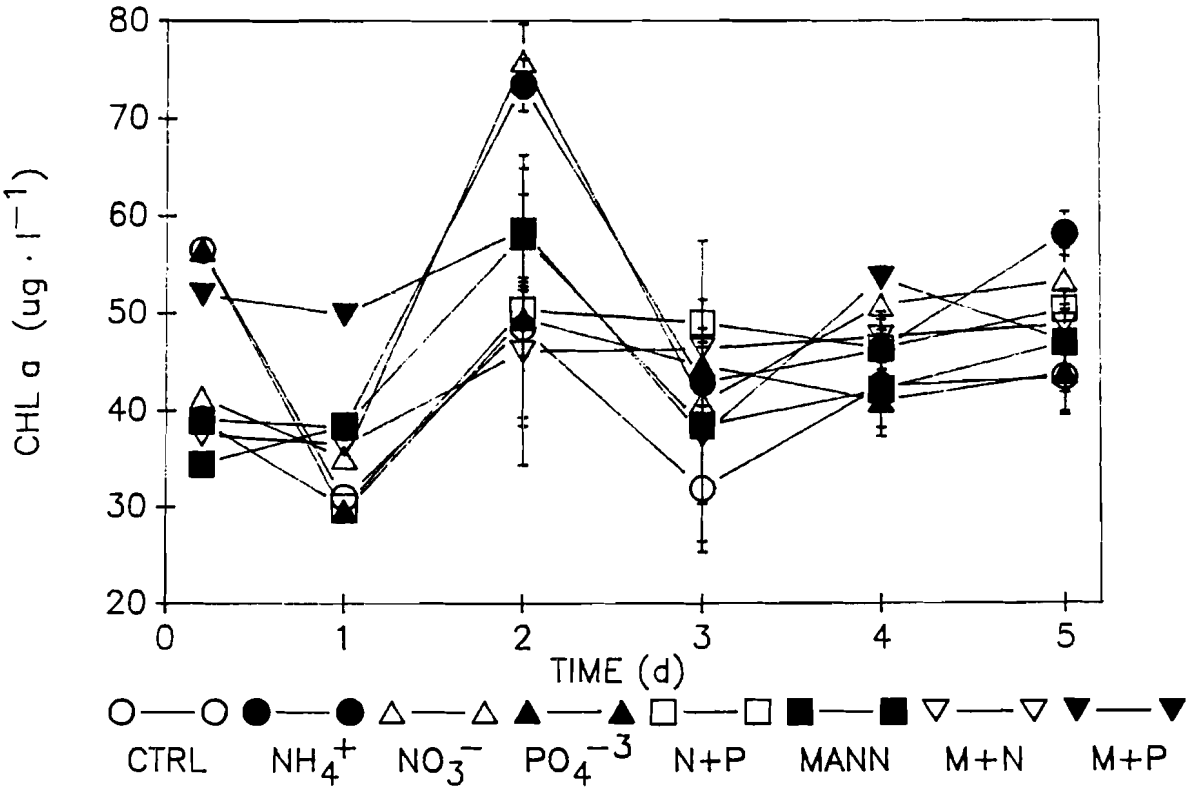

0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
μg C (μg CHLa · h)⁻¹
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
PO4−3
N+P
MANN
M+N
M+P

Fig. 27


80
70
60
50
40
30
20
CHL a (ug · l⁻¹)
0
1
2
3
4
5
TIME (d)
CTRL
NH4+
NO3−
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N+P
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Fig. 28

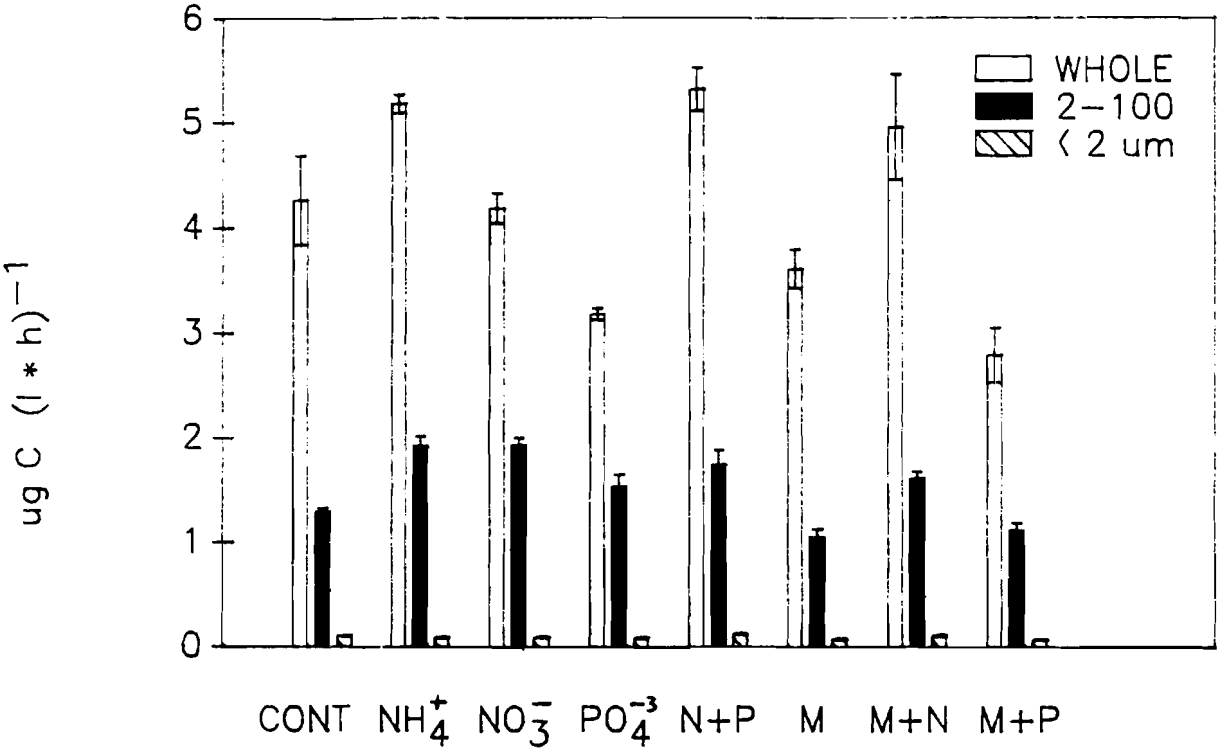
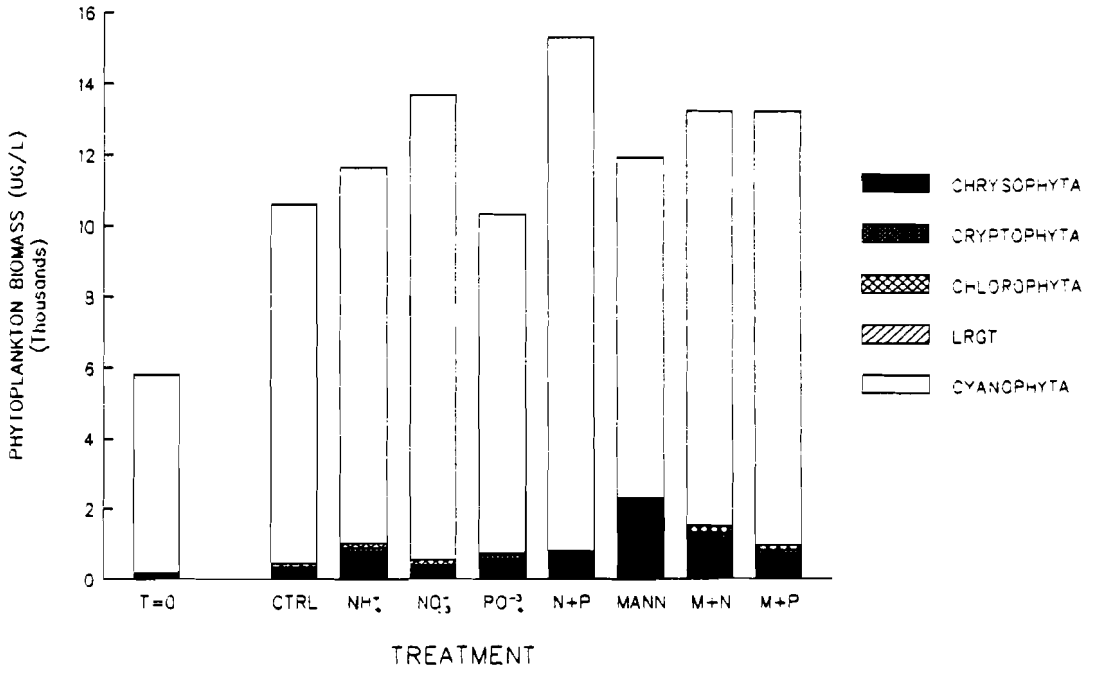


Fig. 29



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CHAPTER 2

IMMUNOCHEMICAL LOCALIZATION OF NITROGENASE IN MARINE *TRICHODESMIUM* AGGREGATES: RELATIONSHIP TO N_2 FIXATION POTENTIAL

(Published: Applied and Environmental Microbiology. 1989. 552:2965-2975)

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Nov. 1989, p. 2965–2975
0099-2240/89/112965-11$02.00/0

Vol. 55, No. 11

Immunochemical Localization of Nitrogenase in Marine *Trichodesmium* Aggregates: Relationship to N_2 Fixation Potential

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Received 22 May 1989/Accepted 16 August 1989

Colonial aggregation among nonheterocystous filaments of the planktonic marine cyanobacterium *Trichodesmium* is known to enhance N_2 fixation, mediated by the O_2-sensitive enzyme complex nitrogenase. Expression of nitrogenase appears linked to the formation of O_2-depleted microzones within aggregated bacterium-associated colonies. While this implies a mechanism by which nonheterocystous N_2 fixation can take place in an oxygenated water column, both the location and regulation of the N_2-fixing apparatus remain unknown. We used an antinitrogenase polyclonal antibody together with postsection immunocolloidal gold staining and transmission electron microscopy to show that (i) virtually all *Trichodesmium* cells within a colony possessed nitrogenase, (ii) nitrogenase showed no clear intracellular localization, and (iii) certain associated bacteria contained nitrogenase. Our findings emphasize the critical role coloniality plays in regulating nitrogenase expression in nature. We interpret the potential for a large share of *Trichodesmium* cells to fix N_2 as an opportunistic response to the dynamic nature of the sea state; during quiescent conditions, aggregation and consequent expression of nitrogenase can proceed rapidly.

The ubiquitous and ecologically significant marine cyanobacterial genus *Oscillatoria* (*Trichodesmium*) supports relatively high rates of light-mediated N_2 fixation in the absence of morphologically differentiated cells termed heterocysts (5, 6, 11). The physiological means by which oxygen-sensitive N_2 fixation occurs contemporaneously with oxygenic photosynthesis in this filamentous genus remains elusive (19, 20). However, field (in situ) and short-term (<1-day) laboratory studies on freshly collected populations have pointed to the importance of a colonial existence, as either fusiform tufts or spherical puffs of aggregated filaments, for N_2 fixation to proceed and be optimized (3, 7, 18). Despite a recent report on the successful isolation and culture of a marine *Oscillatoria* strain which apparently can fix N_2 as single filaments under oxic conditions (16), naturally occurring photosynthetically active populations consistently reveal either a complete lack or severe inhibition of N_2 fixation when aggregates are disrupted (with oxygenic photosynthesis remaining uninhibited) (3, 7, 18). After extensive examinations of natural populations, Carpenter and McCarthy (6) and, later, Carpenter and Price (7) suggested that aggregation of filaments allowed for compartmentalization of O_2-evolving photosynthesis and O_2-inhibited N_2 fixation. Indeed, autoradiographic examinations of photosynthetic $^{14}CO_2$ incorporation reveal this process to be largely confined to terminal regions of filaments (7); hence, internal regions of aggregated filaments could be a likely place for N_2 fixation to occur. Subsequent studies, utilizing the reduction of a low-redox-potential tetrazolium salt, 2,3,5-triphenyl-3-tetrazolium chloride, strongly hinted of highly reduced conditions in internal regions of photosynthetically active aggregates (3, 18). More recently, aggregates have been probed with O_2 microelectrodes, and the results directly demonstrate the existence of O_2-depleted internal microzones within photosynthetically active aggregates (17).

While these studies have implied a mechanism by which N_2 fixation can accompany photosynthesis in aggregates, key questions remain as to the location and regulation of the N_2-fixing apparatus in *Trichodesmium*. Thus far, it has not been conclusively shown that *Trichodesmium* is in fact the site of N_2 fixation as opposed to the diverse array of microflora (both bacteria and microalgae) with which N_2 fixation is commonly found in nature. The question follows that, if N_2 fixation is indeed confined to *Trichodesmium*, what are the intra- and intercellular distributions of the enzyme complex (nitrogenase) responsible for this process? Also, is the distribution of nitrogenase related to its environmental regulation and colonial aggregate formation?

The nitrogenase enzyme complex consists of two distinct polypeptides forming dinitrogenase (Mo-Fe protein) and the dimeric polypeptides of dinitrogenase reductase (Fe protein). To address the above-mentioned questions, polyclonal antibodies against dinitrogenase reductase were used in postsection immuno electron microscopy (IEM) (1) studies to observe intra- and intercellular localization of nitrogenase in *Trichodesmium* aggregates recently obtained from: (i) coastal Atlantic Ocean (Gulf Stream) waters during a late summer 1988 bloom near Beaufort, N.C., and (ii) several open-ocean locations in the western Caribbean Sea.

MATERIALS AND METHODS

Sampling locations and procedures. Samples were collected from a variety of pelagic (offshore) locations in the western Caribbean Sea during a cruise of the R/V *Columbus Iselin* conducted between 4 and 23 November 1988. Samples for immunolocalization of nitrogenase were obtained from a station at 11°20′ N, 82°22′ W during calm sunny conditions. Additional samples were collected in coastal Atlantic Ocean waters 6 km southwest of Beaufort Inlet, N.C., during a late summer bloom in 1988. In both cases, individual *Trichodesmium* aggregates from the same sample as used for immunolocalization studies were examined for N_2-fixing activity

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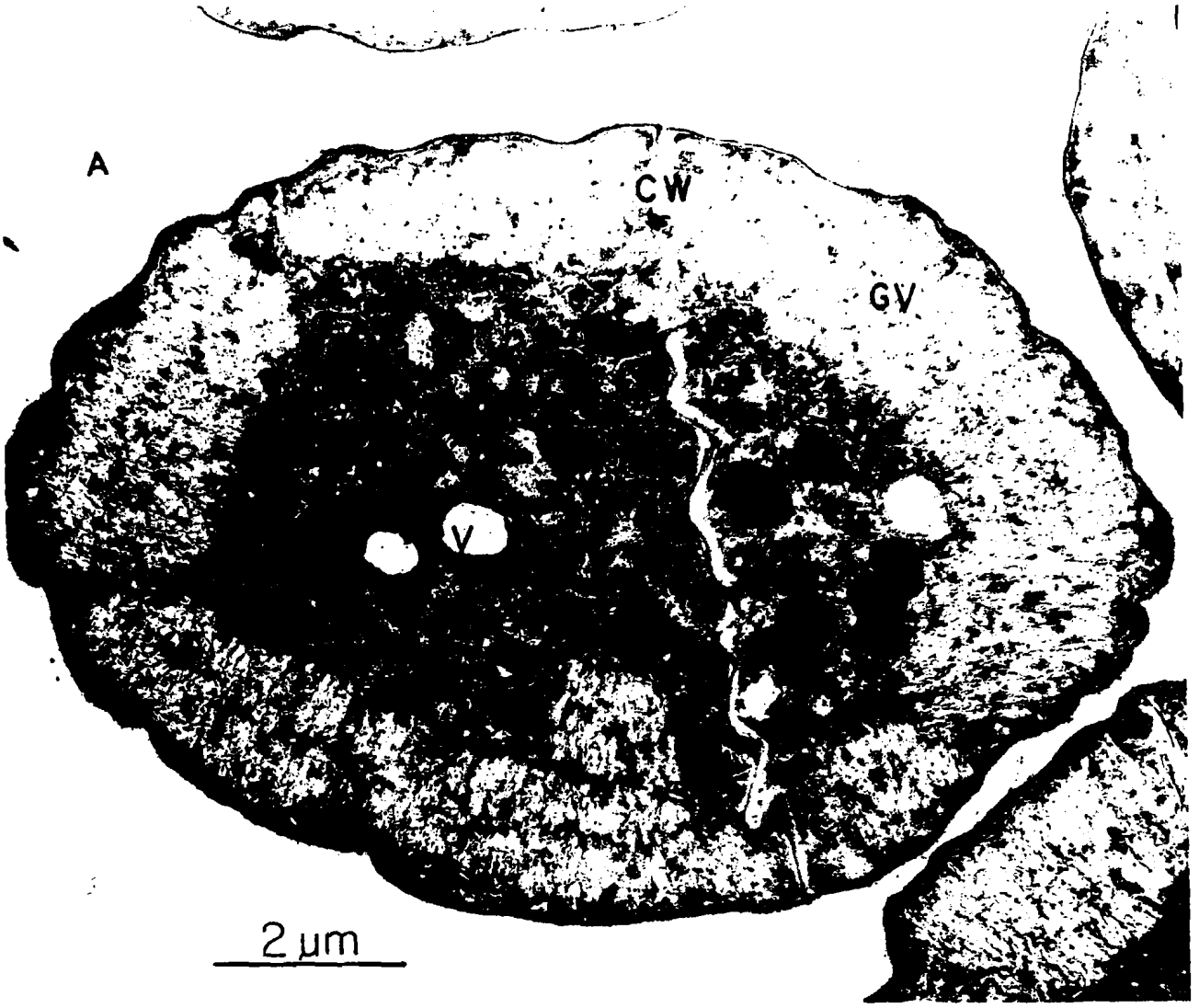

FIG. 1. IEM examinations of CG-labeled *Trichodesmium* spp. filaments. Sample obtained from North Carolina coastal Atlantic waters. (A) Oblique cross-sectional view showing representative morphology of two cells intersected by a cell wall (CW). The nucleoplasmic (N) region, gas vacuoles (GV), other vacuoles (V), and lipid droplets (LD) are indicated. Detailed ultrastructural descriptions are provided by Van Baalen and Brown (21), Gantt et al. (10), and Haxo et al. (12). Electron-dense, spherical CG particles are distributed throughout both nucleoplasmic and gas vacuolate regions, but are difficult to discern at this magnification owing to their small size (10-nm diameter). (B) High-magnification cross-sectional view revealing CG particles (arrows) dispersed throughout nucleoplasmic (N) regions, vacuoles (V), and gas vacuole (GV) regions. Intracellular CG labeling did not appear confined to specific ultrastructural components. However, note that the outer cell wall (OCW) and associated membrane revealed relatively little CG labeling. (C) Longitudinal section. In support of cross-sectional views, the longitudinal section exhibited CG deposition throughout both nucleoplasmic (N) and gas vacuole (GV) regions. In this view, photosynthetic lamellae (P) are evident; generally, little CG was associated with these structures. (D) High magnification longitudinal view of CG deposition (arrows). Note general absence of CG labeling in either the photosynthetic lamellar or outer cell.

by the acetylene reduction assay (5, 17). Relatively high cellular acetylene reduction rates were observed under illuminated conditions in both instances (17; D. G. Capone, unpublished data), indicating the presence of nitrogenase associated with aggregates. Nitrogen-fixing aggregates were gently collected from near the sea surface (0 to 2 m) with a 200-μm mesh plankton net and immediately fixed in 2% (vol/vol) glutaraldehyde for 20 min, washed with phosphate-buffered saline (pH 7.8), and transferred to fresh phosphate-buffered saline for refrigerated (4°C) storage until analysis.

Immunochemical studies. Upon return to the laboratory (within 3 weeks), 5 to 15 aggregated filaments were washed in 0.1 M phosphate-buffered saline at pH 7.2, transferred to BEEM capsule chamber pipettes (8), postfixed for 1 h in aqueous OsO_4, and dehydrated. The dehydrated filaments were then embedded (with care given to orientation of the filaments) and thin sectioned (2). Sections were immunostained with rabbit anti-dinitrogenase reductase polyclonal antibodies, either Rr-2 or universal. Both types of antibodies were kindly provided by P. Ludden, University of Wisconsin, Madison. Rr-2 denotes the anti-rabbit polyclonal anti-

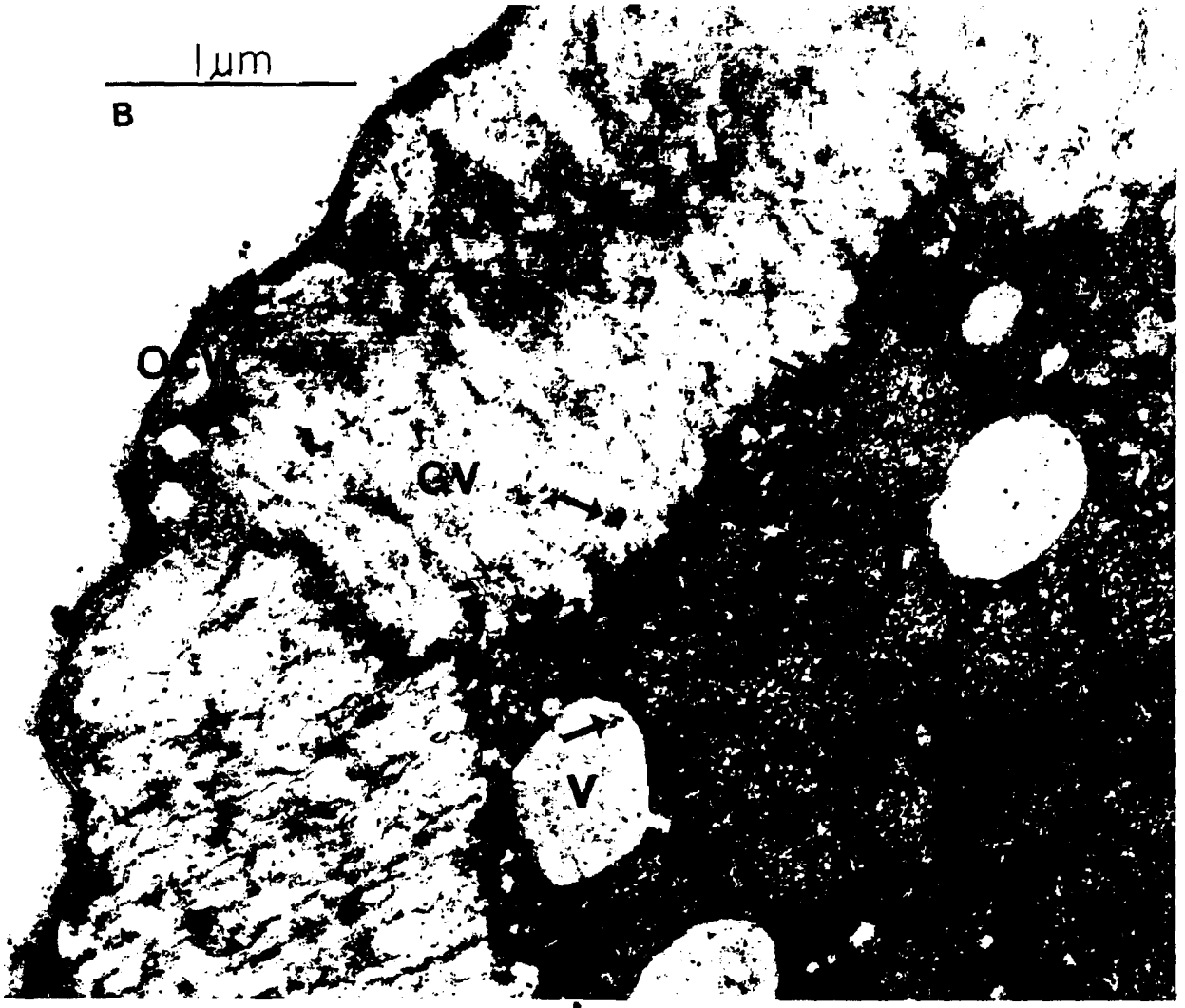

FIG. 1—*Continued*

bodies against dinitrogenase reductase (subunit II or Fe protein) of the nitrogenase complex of *Rhodospirillum rubrum*, a diazotrophic bacterium (14). Universal Fe protein denotes the polyclonal antibodies against the dinitrogenase reductase from a variety of N_2-fixing bacteria, including *R. rubrum*. Previous studies by Ludden et al. (14) critically evaluated the specificity of these polyclonal antibodies toward crude and highly purified Fe protein. Using both Western blotting (immunoblotting) following electrophoretic separation of proteins and Ouchterlony immunodiffusion assays, a highly specific reaction with the Fe protein was observed, with no significant cross-reactions with irrelevant proteins. In addition, these investigators were able to discriminate between active and inactive forms of the Fe protein, based on very small differences in molecular weight, as detected by Western blot assays. We have recently observed similar specificities and small molecular-weight differences in a variety of marine and freshwater N_2-fixing isolates (C. A. Currin, H. W. Paerl, G. Suba, and R. S. Alberte, Limnol. Oceanogr., in press; J. C. Priscu, unpublished data).

Antibodies were applied at dilutions of 1:200 (Rr-2; Caribbean samples), 1:500 (Rr-2; N.C. samples), or 1:50 (universal; Caribbean samples). Antibody dilutions were based on individual antibody titers. Following reaction of the primary antibody with nitrogenase, sections were reacted with 10% (in blocking buffer) goat anti-rabbit gold-conjugated antibody (10-nm particle size; Janssen Life Science Products, Piscataway, N.J.), as described by Brawner and Cutler (2), except that blocking buffer (TTBS) consisted of 7% bovine serum albumin (Sigma Chemical Co., St. Louis, Mo.), 400 mM $(NH_4)_2SO_4$, 1.0 mM $MgCl_2$, 0.1 mM $ZnCl_2$ (J. T. Baker Chemical Co., Phillipsburg, N.J.), and 0.1% Tween 80 (Sigma) in 0.05 M Tris buffer, pH 7.5. A minimum of 50 cells per grid was observed with either a JEOL 100-CX or a Zeiss model EM 10C/CR electron microscope.

Controls to test for nonspecific binding of the goat anti-rabbit gold-conjugated antibody consisted of fetal bovine serum (N.C. samples) and an irrelevant antibody (polyclonal rabbit anti-*Candida albicans* 9938; Caribbean samples) in dilutions matching and replacing that of primary polyclonal serum.

Western blots done by Laemmli's protocol (13) were conducted on lyophilized *Trichodesmium* to determine the

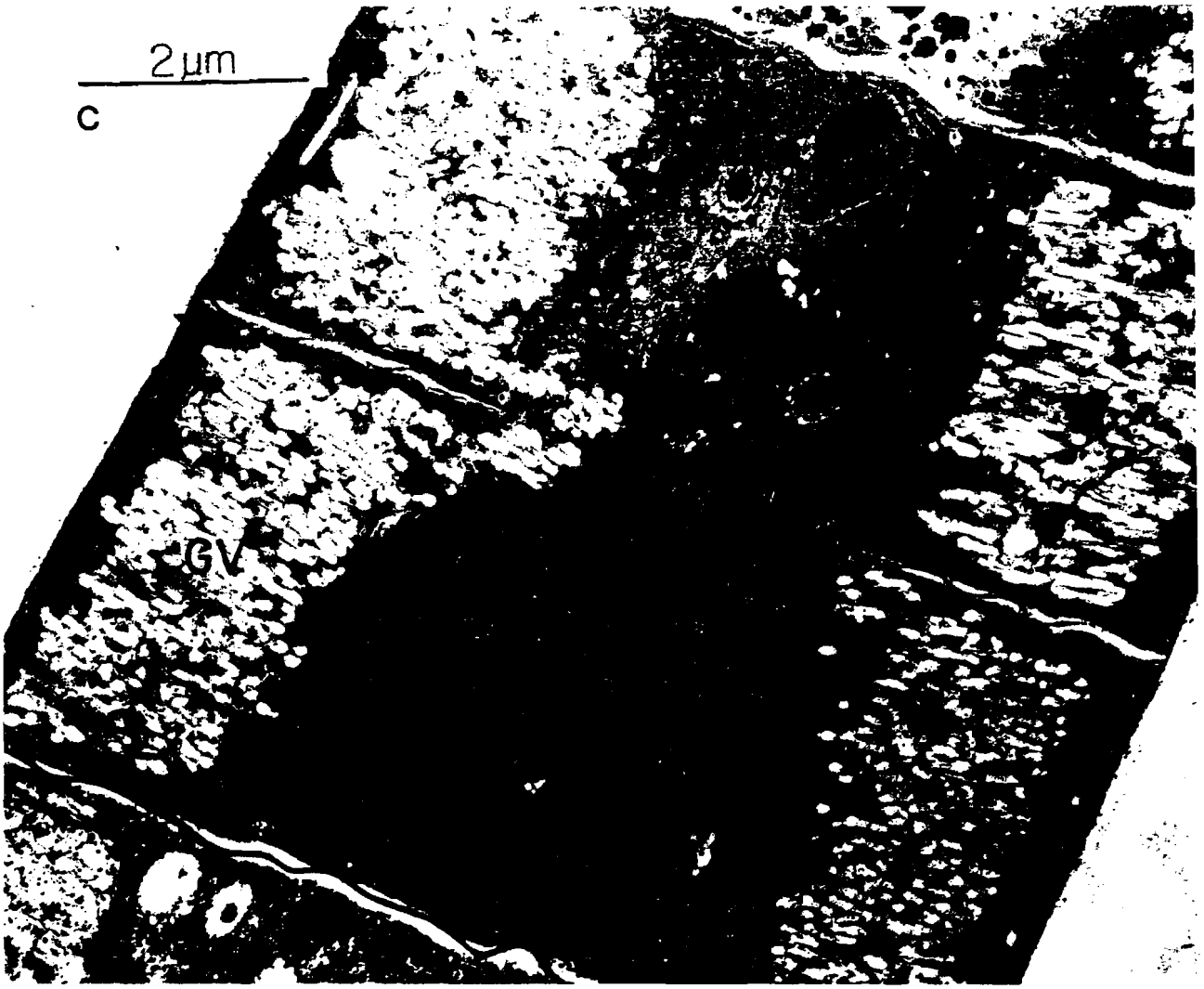

FIG. 1—*Continued*

specific cross-reactivity of the primary antibody. Lyophilized samples were kept at −20°C until use. We suspended 10 to 12 mg of freeze-dried sample in 1.0 ml of Laemmli sample buffer, sonicated twice at low power for 30-s intervals, and heated at 100°C for 5 min. Samples and molecular mass markers (prestained markers, 17 to 130 kilodaltons [kDa]; Bio-Rad Laboratories, Richmond, Calif.) were then loaded onto 7.0-cm 12.5% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gels at 10 μl per well. Gels were electrophoresed for 40 min in a Bio-Rad Mini-PROTEAN II unit supplied with a constant 200 V. Following electrophoresis, gels were equilibrated for 15 min in a methanol transfer buffer (25 mM Tris, 150 mM glycine, 20%, vol/vol, methanol, pH 8.3). Proteins were then electroeluted onto nitrocellulose membranes in a Bio-Rad Mini Trans-Blot transfer cell supplied with a constant 100 V for 1 h. Blocked (1% bovine serum albumin) membranes were then probed for the presence of the nitrogenase enzyme, using either Rr-2 or universal antibodies. Membranes were incubated for 1 h in a 1:10,000 working dilution (in TTBS) of either of these antibodies. Primary antibody was replaced with a conjugated secondary antibody solution (a 1:7,500 dilution of goat anti-rabbit immunoglobulin G conjugated with alkaline phosphatase in TTBS) following several short washes in TTBS, pH 7.5, to remove excess primary probe. Following a 1-h incubation in secondary antibody, membranes were washed in Tris-buffered saline (0.5 M NaCl, 20 nM Tris) at pH 7.5 and placed in an alkaline phosphatase color development system containing Nitro Blue Tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate ptoluidine salt.

Bacterial culturing studies. Maruyama et al. (15) reported on N_2-fixing eubacteria associated with *Trichodesmium* aggregates in the Pacific Ocean. We made parallel efforts at isolating and characterizing such eubacteria in this study. Freshly collected (November 1988) Caribbean *Trichodesmium* tuft and puff aggregates were individually picked from water samples, using sterile plastic inoculating loops (10 μl), and given two washes of 0.1-μm filter (Nuclepore Corp., Pleasanton, Calif.)-sterilized seawater. Aggregates were then transferred with loops to sterile culture tubes containing a mannitol (0.8%)–phosphate (100 μM)–Fe-EDTA (30 μM)-amended seawater soft agar. Purified (Difco purified grade; Difco Laboratories, Detroit, Mich.) nitrogen-free agar (5.0 g/liter) was used. This medium was shown previously to

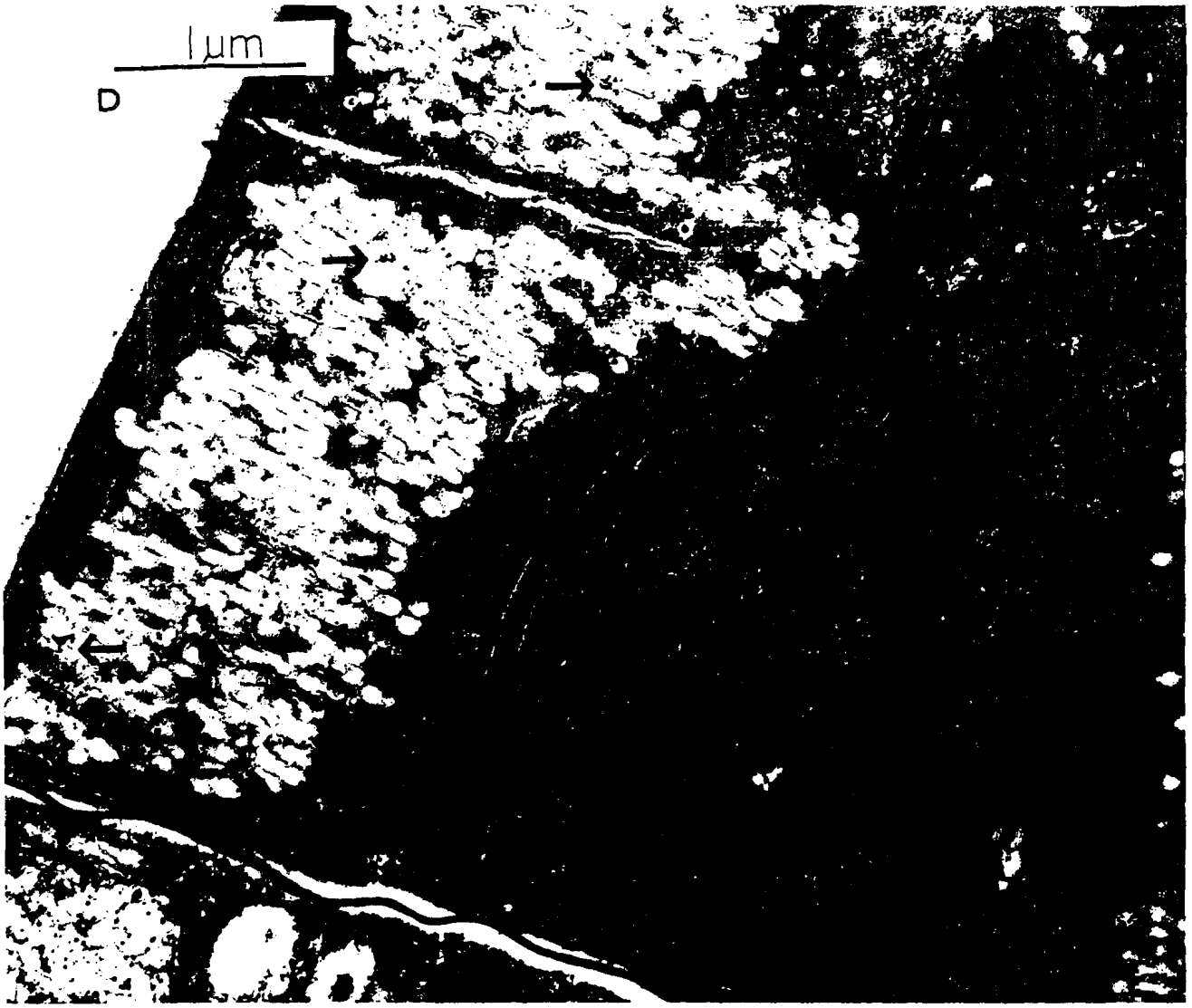

FIG. 1—*Continued*

select for N_2-fixing microheterotrophs in a variety of North Carolina coastal Atlantic and Caribbean habitats (H. W. Paerl, B. M. Bebout, and L. E. Prufert, J. Phycol., in press). Washed *Trichodesmium* aggregates were both placed on the surface and stabbed into the agar. Cultures were incubated for 2 to 7 days in darkness at 25°C.

RESULTS AND DISCUSSION

Immunostained cells viewed by IEM showed deposition of colloidal gold (CG) throughout individual cells collected from both North Carolina coastal Atlantic waters (Fig. 1) and the Caribbean Sea (Fig. 2). High-magnification examinations of samples from both locations revealed no clear intracellular localization of CG deposition. Enumeration of CG deposition in Caribbean samples yielded mean (10 replicate counts) densities of 75 ± 15 gold particles per μm^2 following treatment with primary and secondary antibodies. Examination of over 50 cells in cross section treated in this manner (which encompassed filaments on both the inside and the outside of aggregates) revealed similar total densities of CG in all cells, indicating that nitrogenase was not concentrated or isolated within specific portions of the aggregate. Occasional deposition of CG particles outside the cell wall is presumably an artifact of cell sectioning, rather than nonspecific immunochemical reactions; no evidence exists for nonspecific binding in either the irrelevant antibody or fetal bovine serum controls or in the Western blots (see below).

We conducted additional tests to ensure that CG distribution represented specific binding. Fetal bovine serum and irrelevant antibody controls were consistently negative (less than two CG particles per μm^2 throughout cells), verifying that the secondary antibody was specific for the primary antibody to nitrogenase (Fig. 3). The specificity of the primary antibody is shown in the Western blots (Fig. 4), which indicated a single band occurring at approximately 40 kDa for each sample when calibrated against Bio-Rad prestained markers. We observed prestained markers to migrate further down the gel than comparable molecular-weight

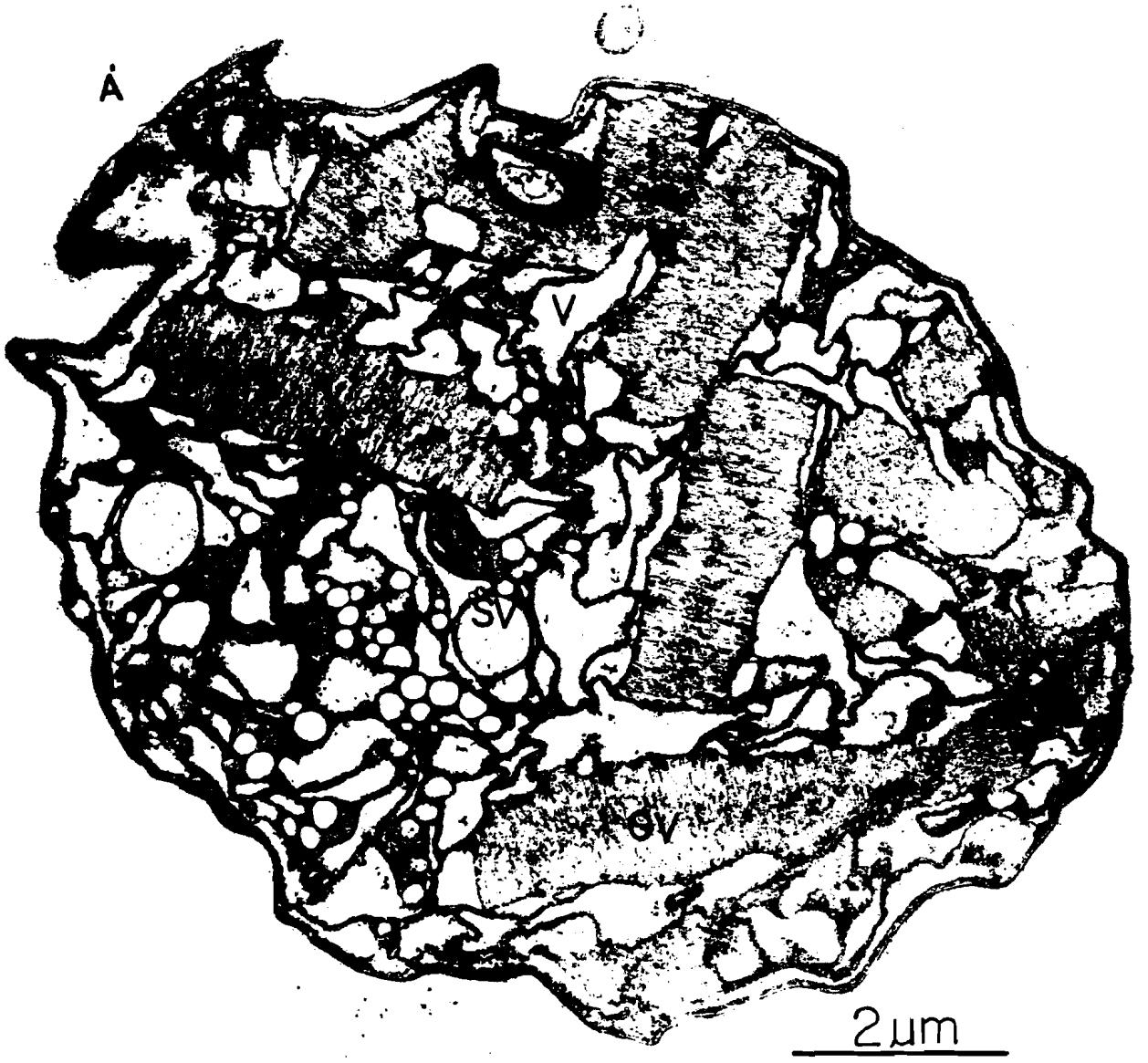

FIG. 2. TEM views of CG-labeled *Trichodesmium* spp. filaments. Filaments were obtained from the Caribbean Sea. (A) Low-magnification cross-sectional view showing CG deposition patterns. Deposition is closely associated with cylindrical, fully inflated gas vacuoles (GV) and spherical vacuoles (SV). We observed additional, although less dense, CG deposition associated with convoluted lamellae (L), while electron-transparent vacuolated (V) regions appeared free of CG particles. (B) High-magnification region of panel A. CG deposition patterns in both gas vacuoles and spherical vacuoles were readily seen at this magnification.

markers stained after electrophoresis. This finding confirms Bio-Rad's precautions (provided with the Mini-PROTEAN II unit) regarding rigorous interpretations of molecular masses solely based on prestained markers. Our cross-calibration of pre- and poststained protein standards reveals a sizable discrepancy (Fig. 4). When calibrated against poststained markers, the Fe protein-specific band appears at approximately 35 kDa. It is known that the dimeric dinitrogenase reductase dissociates in the presence of sodium dodecyl sulfate before electrophoresis, resulting in a monomeric form with a reported molecular mass of ca. 30 to 40 kDa (4, 9). Our results fall within this range.

Additional verifications of antibody specificity have been conducted on Western blots of electrophoresed extracts from other well-documented diazotrophs, including *Azotobacter*, *Klebsiella*, *Vibrio*, and *Anabaena* (Currin et al., in press). When probed with either Rr-2 or universal antinitrogenase antibodies, a single band appeared at approximately 30 to 40 kDa (Currin et al., in press). This band was absent when the same microorganisms were grown on combined nitrogen (nitrogenase activity repressed). In concert, current and previous results consistently showed antibodies to be highly specific for nitrogenase among diverse N_2-fixing genera.

Stab cultures consistently showed the presence of N_2-fixing bacteria, confirmed by positive acetylene reduction assays and serial transfers into N-free mannitol agar tubes which yielded heavy growth. Characteristically, noticeable

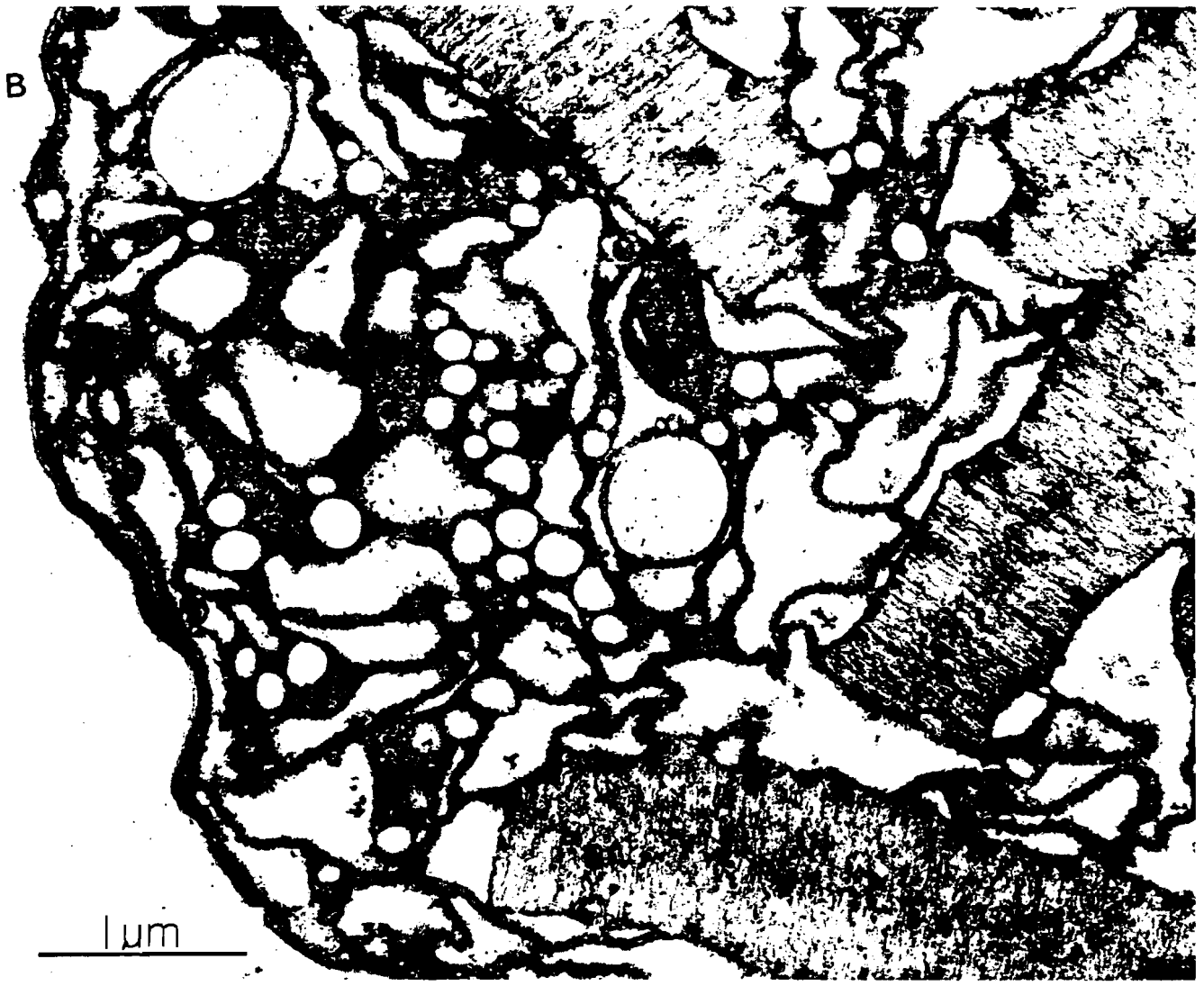

FIG. 2—*Continued*

bacterial growth occurred within 2 to 3 days as white bands or plates situated 0.5 to 1 cm below the agar surface. Oxygen microelectrode profiles revealed that plates represented the oxic-anoxic interface of tubes (Paerl et al., in press). This indicated that N_2-fixing bacterial isolates required microaerophilic or anaerobic conditions or both for expression of nitrogenase.

It was suggested previously that spatial segregation of N_2 fixation and O_2-evolving photosynthesis exists within *Trichodesmium* aggregates (3, 7, 18) and that regions of low oxygen tension within the aggregate may allow N_2 fixation to be localized in cells within the aggregate (i.e., not exposed to the bulk medium) (17). Our present findings lead us to conclude that nitrogenase is present throughout all cells of the aggregate, but that catalytic expression of the nitrogenase is closely regulated by microscale features such as biotically induced low O_2 tension within the aggregate (17).

Several types of eubacteria were associated with aggregates of the Caribbean Sea *Trichodesmium*. Some of these bacteria contained CG, indicating that certain associated bacteria have the potential to fix N_2. By using microaerophilic stab culturing techniques, we were able to isolate N_2-fixing heterotrophic bacteria associated with Caribbean *Trichodesmium* aggregates, confirming the previous observation of Maruyama et al. (15). While we note the presence of such bacteria, they account for <1% of total CG deposition among *Trichodesmium* cells. Furthermore, microaerophilic conditions were apparently required for active N_2 fixation to take place among these bacteria. Such requirements greatly restrict N_2 fixation potentials among bacteria associated with the periphery of *Trichodesmium* aggregates, a region known to be well oxygenated during daytime photosynthetic periods (17). Hence, bacterial contributions to aggregate N_2 fixation are likely to be small. However, these heterotrophic bacteria provide respiratory O_2 consumption which may promote the development and maintenance of low O_2 microzones within aggregates (17). In turn, these microzones facilitate expression of nitrogenase in both

FIG. 3. IEM cross-sectional views of an irrelevant antibody control (conducted on Caribbean *Trichodesmium* spp. filaments) illustrating that the CG-conjugated secondary antibody was specific for the primary antibody to nitrogenase. The use of either fetal bovine serum or anti-*C. albicans* 9938 antiserum in place of the antinitrogenase primary antibodies yielded similar results, namely, no detectable CG labeling of *Trichodesmium* sections. (A) Low-magnification view; (B) high-magnification view.

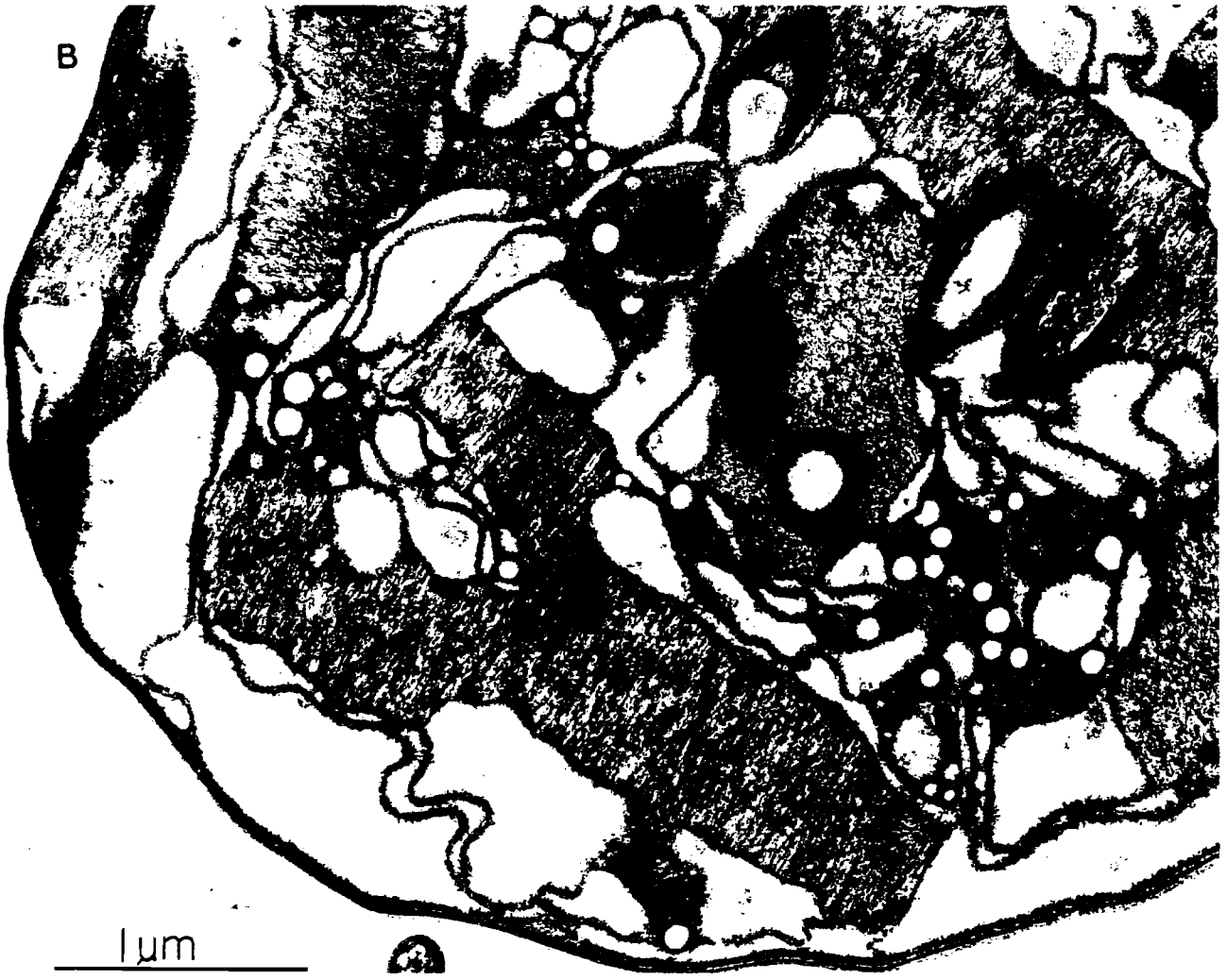

FIG. 3—*Continued*

CHAPTER 3

NITROGENASE ACTIVITY, PHOTOSYNTHESIS, AND THE DEGREE OF HETEROCYST-AGGREGATION IN THE CYANOBACTERIUM *ANABAENA FLOS-AQUAE*

(Submitted: Journal of Phycology)

Nitrogenase Activity, Photosynthesis, and the Degree of Heterocyst-aggregation in the Cyanobacterium *Anabaena flos-aquae*

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Running Title: Heterocyst-aggregation in *Anabaena flos-aquae*.

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1987; Parsons et al. 1987). Many N_2-fixing bacteria and cyanobacteria produce extracellular mucilage which helps protect nitrogenase from O_2 damage by forming a diffusive or solubility barrier to O_2 (Hill 1971; Wilcockson 1977; Bothe 1982). In some aggregate forming non-heterocystous cyanobacteria such as Trichodesmium, nitrogenase is located in most, if not all cells (Paerl et al. 1989b) but the greatest activity is expressed in cells located near the center of the colony where either photosynthetic O_2 production is reduced, its consumption enhanced or both (Carpenter and Price 1976). The net effect is that pO_2 in the center of the colony appears low enough to allow nitrogenase to function; thus spatially separating N_2-fixation from net photosynthetic O_2 evolution (Carpenter and Price 1976; Paerl and Bebout 1988).

Colony or aggregate formation generally promotes growth and survival of cyanobacteria through parallel increases in N_2-fixation rates (Bothe 1982; Paerl and Bebout 1988; Carlton and Paerl 1989). However, an example of increased aggregation that lowered nitrogenase activity (NA) and photosynthesis has also been reported in Nostoc (Dodds 1989a). The precise temporal and spatial relationships among pO_2, N_2-fixation and photosynthesis in cyanobacterial aggregates are probably species specific and regulated by a number of environmental variables including light. Highly specific associations between O_2 consuming bacterial heterotrophs and O_2 evolving cyanobacteria may also be instrumental in the localized enhancement of N_2-fixation in aggregates (Paerl 1982). It has been established that there is a correlation between $^{15}N_2$-fixation and irradiance (Dodds 1989b), and that NA can be inhibited at high irradiances (Lewis and Levine 1984)

which may be related to photosynthesis-induced O_2-supersaturation (Paerl and Kellar 1978; Paerl and Kellar 1979).

Our recent field observations indicate that Anabaena flos-aquae forms cm size flocculent masses during surface blooms in Hebgen Lake, Montana. Further microscopic examination of both field populations and unialgal cultures of this organism showed that flocculation is preceded by heterocyst cohesion. The net effect of this cohesion is an aggregate where the heterocysts are centrally located in a mass of mucilage, leaving the bulk of vegetative cells forming the periphery of the aggregate. This arrangement of biochemically-specialized cells led us to hypothesize that there was spatial separation of oxygenic photosynthesis and O_2-sensitive N_2-fixation within the aggregates. We examined the influence of aggregation on NA and photosynthesis in A. flos-aquae by comparing these activities in aggregated and artificially dispersed samples.

MATERIALS AND METHODS

Organism and culture condition

Unialgal cultures of Anabaena flos-aquae, isolated from eutrophic Hebgen Lake, Montana, were grown in ASM medium (Allen 1968) lacking a fixed nitrogen source (ASM^-). The cultures were maintained at 25 ± 1 ^oC and 100 μmol quanta m^{-2} s^{-1} supplied by "cool white" fluorescent lamps under a 12 h light/dark cycle. Before the experiment, a two week old culture was diluted with ASM^-, and divided into two equal aliquots. One aliquot was gently

RESULTS

Heterocyst aggregation was observed in a two week old A. flos-aquae culture (Fig. 1A); the mucilaginous matrix associated with the heterocysts was revealed by negative staining with India ink (Fig. 1B). Heterocyst cohesion was found in cultures grown in N-free (ASM^-) and N-replete (ASM^+) media (despite a lower heterocyst frequency), indicating that cohesion is found in both nitrogenase suppressed and active conditions (separate experiments showed NA to be suppressed significantly by 5 mM $NaNO_3$, the level of NO_3^- in ASM^+ is 5 mM). Individual heterocyst aggregates induced formation of larger flocculent masses (Fig. 1C). Gentle shaking dispersed the flocculent masses. Our dispersal technique completely separated the trichomes in the aggregates, while not appearing to physically damage the trichomes (Fig. 1D). When aggregates were dispersed and incubated under the culture conditions, they always reaggregated within 2 weeks.

The TTC treatments showed reduced conditions (<0.4 V) in both aggregated and dispersed samples of A. flos-aquae but with more rapid formation of formazan in aggregates (Table 1). Microelectrode measurements made on samples receiving 800 μmol quanta m^{-2} s^{-1} revealed O_2-supersaturation just outside of an aggregate of heterocysts; pO_2 linearly approached saturation about 400 μm away from the aggregate (Fig. 2). No gradients in O_2 concentration existed in the dark (Fig. 2), indicating that respiratory O_2 consumption was not adequate to lower pO_2 below saturation under the conditions of our measurements. Because the O_2 microelectrode could not penetrate the mucilage surrounding the heterocysts,

within heterocyst aggregate pO_2 remains unknown.

Nitrogenase activity of A. flos-aquae at ambient and O_2-supersaturation (30 kPa pO_2) revealed that aggregation increased NA by 55% at ambient pO_2 and 22% at 30 kPa pO_2 relative to dispersed samples (Table 2). Both differences between aggregated and dispersed samples were statistically significant ($P<0.05$). At O_2-supersaturation, heterocyst aggregation provided appreciable increases in nitrogenase activity even though the relative increase was less than half that observed at ambient pO_2.

An aggregated sample of A. flos-aquae exhibited peak NA near 400 μmol quanta m^{-2} s^{-1} and a decline in NA with further increase in irradiance (Fig. 3A, Table 3). In the dispersed samples, maximum NA was about 40% lower, occurred at a lower irradiance (ca. 250 μmol quanta m^{-2} s^{-1}) and showed greater inhibition at high irradiance than that in aggregated samples (Figs. 3A and D). Dark NA was more than two-fold greater in aggregated than dispersed samples (Table 3). Overall, aggregated samples had higher NA at all irradiance levels used in the experiments.

Maximum photosynthetic activities (P_m^B) of both aggregated and dispersed samples of A. flos-aquae were not significantly different ($P>0.05$), indicating minimal physiological damage caused by our dispersal technique (Table 3, Fig. 3E). Bulk phase O_2 concentration was not influenced by irradiance under our experimental conditions (Fig. 3C). Although bulk O_2 concentration was not measured in dispersed samples, we assumed there was no dependence on irradiance during the experiment because the net rate of carbon fixation in dispersed samples was not significantly different from that measured in the aggregated samples. In

tive effect of increased energy supply for NA via photosynthesis, and inhibition of NA by increased O_2 production. This crossover hypothesis can explain why NA was saturated at lower irradiances than photosynthesis in our experiments.

The decrease in the ratio of NA:photosynthesis with increasing irradiance together with rapid light attenuation within the cm size flocculent masses produces a pattern in the ratio of NA:photosynthesis; cells on the outside of the flocculent mass have lower NA:photosynthesis, whereas those deep in the flocculent masses have higher NA:photosynthesis. Irradiance at the surface of Hebgen Lake during summer can be well in excess of 1500 μmole quanta m^{-2} s^{-1} (Priscu, unpublished data). Formation of flocculent masses caused by aggregation can alter the balance of NA and carbon fixation over what it would be if there was no aggregation.

Our results indicate that heterocyst aggregation promotes NA in A. flos-aquae allowing enhanced assimilation of N_2 in N-deficient environments. Other genera such as Aphanizomenon, (Paerl and Carlton, unpublished), Gleotrichia, and Rivularia (Prescott 1982) also exhibit aggregation of heterocysts. It is possible that NA is enhanced by aggregation in these genera as well.

ACKNOWLEDGMENTS

We thank T. Galli for assistance in the laboratory, M. Lizotte, K. Lohman and T. Miller for their critical review of the manuscript. This research was supported by The Soap and Detergent Association.

REFERENCES

Allen, M. M. 1968. Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4:1-4.

American Public Health Association. 1985. Standard methods for the estimation of waste and waste water.

Bergman, B., Lindblad, P. & Rai, A.N. 1986. Nitrogenase in free-living and symbiotic cyanobacteria: immunoelectron microscopic localization. FEMS Microbiology Letters 35:75-8.

Bothe, H. 1982. Nitrogen fixation. In Carr, N.G. & Whitton, B.A. [Eds.] The Biology of Cyanobacteria. Botanical Monographs vol 19. University of California press.

Carlton, R. G., & Paerl, H.W. 1989. Oxygen-induced changes in morphology of aggregates of Aphanizomenon flos-aquae (Cyanophyceae): implications for nitrogen fixation potentials. J. Phycol. 25:326-33.

Carpenter, E. J., & Price, C. C. 1976. Marine Oscillatoria (Trichodesmium): Explanation for aerobic nitrogen-fixation without heterocysts. Science 191:1278-80.

Dodds, W. K. 1989a. Photosynthesis of two morphologies of Nostoc parmelioides (cyanobacteria) as related to current velocities and diffusion patterns. J. Phycol. 25:258-62.

Dodds, W. K. 1989b. Microscale vertical profiles of N_2-fixation, photosynthesis, O_2, chlorophyll a, and light in a cyanobacterial assemblage. Appl. Environ. Microbiol. 55:882-86.

Fay, P., & Cox, R.M. 1967. Oxygen inhibition of nitrogen fixation in cell-free preparations of blue-green algae. Biochim. Biophys. Acta 143:562-69.

Fay, P., & Kulasooriya, S.A. 1972. Tetrazolium reduction and nitrogenase activity in heterocystous blue-green algae. Arch. Mikrobiol. 87:341-52.

Fogg, G. E., Stewart, W.D.P., Fay, P. & Walsby, A.E. 1973. The Blue-green Algae. Academic Press, London. 459 pp.

Hill, S. 1971. Influence of oxygen concentrations on the colony types of Derxia gummosa grown on nitrogen-free media. J. Gen. Microbiol. 67:77-83.

Jassby, A. D., & Platt, T. 1976. Mathematical formulation

of the relationship between photosynthesis and light for phytoplankton. Limnol. Oceanogr. 21:540-47.

Jorgensen, B. B., & Des Marais, D.S. 1986. A simple fiber-optic microprobe for high resolution light measurements: application in marine sediment. Limnol. Oceanogr. 31:1376-83.

Lewis, W. M., & Levine, S.N. 1984. The light response of nitrogen fixation in Lake Valencia, Venezuela. Limnol. Oceanogr. 29:894-900.

Lorenzen, C. J. 1967. Determination of chlorophyll and pheo-pigments: Spectrophotometric equations. Limnol. Oceanogr. 12:343-46.

Meesters, T. M. 1987. Localization of nitrogenase in vesicles of Frankia sp. Cc 1.17 by immunogoldlabelling on ultrathin cryosections. Arch. Microbiol. 146:327-31.

Parsons, R., Silvester, W.B., Harris, S., Gruijters, W.T.M., & Bullivant, S. 1987. Frankia vesicles provide inducible and absolute oxygen protection for nitrogenase. Plant Physiol. 83:728-31.

Paerl, H. W. 1982. Interactions with bacteria. In Carr, N.G. & Whitton, B.A. [Eds.] The Biology of Cyanobacteria. Botanical monographs vol. 19. University of California press.

Paerl, H. W., & Bebout, B.M. 1988. Direct measurement of O_2-depleted microzones in marine Oscillatoria: Relation to N_2 fixation. Science 241:442-45.

Paerl, H. W., Bebout, B.M. & Prufert, L.E. 1989a. Bacterial associations with marine Oscillatoria sp. (Trichodesmium sp.) populations: ecophysiological implications. J. Phycol. 25:773-84.

Paerl, H. W., & Kellar, P.E. 1978. Optimization of N_2 fixation in O_2-rich waters. Proc. 1st Int. Microb. Ecol. Symp. 68-75.

Paerl, H. W., & Kellar, P.E. 1979. Nitrogen-fixing Anabaena: Physiological adaptations instrumental in maintaining surface blooms. Science 204:620-22.

Paerl, H. W., Priscu, J.C. & Brawner, D.L. 1989b. Immunochemical localization of nitrogenase in marine Trichodesmium aggregates: Relationship to N_2 fixation potential. Appl. Environ. Microbiol. 55:2965-75.

Peterson, R. B., & Burris, R.H. 1976. Properties of heterocysts isolated with colloidal silica. Arch. Microbiol. 108:35-40.

Platt, T., Gallegos, C.L. & Harrison, W.G. 1980. Photoinhibition of photosynthesis in natural assemblages of marine phytoplankton. J. Mar. Res. 38:687-701.

Prescott, G. W. 1982. Algae of the Western Great Lakes Area. Otto Koeltz Science Publishers, D 6240 Koenigstein, W. Germany. 977 pp.

Priscu, J. C. 1989. Photon dependence of inorganic nitrogen transport by phytoplankton in perennially ice-covered antarctic lakes. Hydrobiologia 172:173-82.

Revsbech, N. P., & Jorgensen, B.B. 1986. Microelectrodes: their use in microbial ecology. Adv. Microb. Ecol. 9:293-352.

Robinson, J. A. 1985. Determining microbial kinetic parameters using nonlinear regression analysis: advantages and limitations in microbial ecology. Advances in Microbial Ecology 8:61-114.

Robson, R. L., & Postgate, J.R. 1980. Oxygen and hydrogen in biological nitrogen fixation. Ann. Rev. Microbiol. 34:183-207.

Sartory, D. P., & Grobbelaar, J.U. 1984. Extraction of chlorophyll a from fresh water phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-87.

Stewart, W. D. P., Fitzgerald, G.P. & Burris, R.H. 1967. In situ studies on N_2 fixation using the acetylene reduction technique. Proc. US Nat. Acad. Sci. 58:2071-78.

Stewart, W. D. P., Haystead, A. & Pearson, H.W. 1969. Nitrogenase activity in heterocysts of blue-green algae. Nature 224:226-28.

Wetzel, R. G., & Likens, G.E. 1979. Limnological analyses. W. B. Saunders co., Philadelphia. 357 pp.

Wilcockson, J. 1977. The apparent influence of atmospheric oxygen concentration on nitrogenase activity and slime production in Klebsiella pneumoniae grown on a solid medium. J. Gen. Microbiol. 101:311-17.

Wintermans, J. F. G. M., & De Mots, A. 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta 109:448-53.

Table 1. Triphenyl tetrazolium chloride (TTC) localization of reduced zones of O_2 in aggregated and dispersed cultures of A. flos-aquae.

Treatment duration (min)	% Heterocysts containing formazan			
	Aggregated sample		Dispersed sample	
	[a]Ambient pO_2	30kPa pO_2	[a]Ambient pO_2	30kPa pO_2
10	15.4	28.9	0.0	0.0
20	56.1	31.3	0.0	0.0
30	70.8	26.1	8.8	0.0
40	76.9	51.6	66.7	0.0

[a] Ambient pO_2=20 kPa.

Table 2. Nitrogenase activity of the aggregated and dispersed samples of *A. flos-aquae* at ambient pO_2 and O_2-supersaturation.

Treatment	Nitrogenase activity[a]	
	Ambient	30 kPa
Aggregated	2.56±0.36	1.81±0.23
Dispersed	1.65±0.16	1.49±0.31

[a]Expressed in mol C_2H_4 mol Chl *a*$^{-1}$ h^{-1} (±S.E.)

Table 3. Parameters describing the irradiance response of photosynthesis and nitrogenase activity in aggregated and dispersed A. flos-aquae. NF = values that could not be fitted on basis of data. α and β = mol C_2H_4 mol chl a^{-1} h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$ or mol C mmol chl a^{-1} h^{-1} (μmol quanta m^{-2} s^{-1})$^{-1}$. P_s^B, P_m^B, and Dark = mol C_2H_4 mol chl a^{-1} h^{-1} or mol C mmol chl a^{-1} h^{-1}. Standard deviation in parentheses. Parameters are described in Jassby and Platt 1976; and Priscu 1989 (see also Materials and Methods).

Sample	α	P_s^B	β	Dark	P_m^B
Photosynthesis:					
Aggregated	7.71×10^{-4} (1.4×10^{-4})	NF	NF	NF	0.157 (0.018)
Dispersed	5.84×10^{-4} (5.08×10^{-5})	NF	NF	NF	0.142 (0.007)
Nitrogenase:					
Aggregated	0.018 (0.008)	53.7 (5.18)	0.131 (1.32)	1.06 (0.36)	3.65
Dispersed	0.021 (0.005)	6.5 (13.6)	0.018 (0.05)	0.46 (0.11)	2.25

FIG. 1. Aggregated heterocysts under phase contrast (A), bar=14 μm. Heterocyst aggregates negatively stained with India ink (B). Note bright areas of mucilage surrounding heterocyst clumps devoid of stain. Scale bar=15.5 μm. Artificially dispersed A. flos-aquae (C), bar=9.3 μm. A large flocculent mass of A. flos-aquae in unialgal culture (D). Scale bar=1.2 cm.

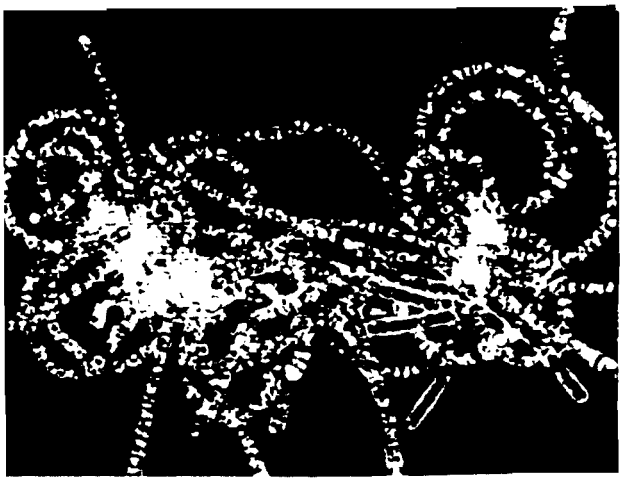
FIG. 2. Profiles of O_2 concentration as a function of distance away from the mucilage surrounding a heterocyst aggregate of A. flos-aquae at 800 umol quanta m^{-2} s^{-1} and in the dark. The plots are each a composite of 3 separate profiles. The error bar=±1 standard deviation.

FIG. 3. Nitrogenase activity, photosynthesis and bulk O_2 concentration versus irradiance in aggregated and dispersed samples of A. flos-aquae. The curves are described with parameters in Table 3. NA versus irradiance in aggregated sample (A), Photosynthesis versus irradiance in aggregated sample (B), Bulk phase O_2 concentration in aggregated sample (C), NA versus irradiance in dispersed sample (D), Photosynthesis versus irradiance in dispersed sample (E). Aggregated and dispersed samples had 0.64 and 0.83 nmol chl a per vial respectively. Note that C-fixation is expressed per mmol chl a, and NA per mol chl a.

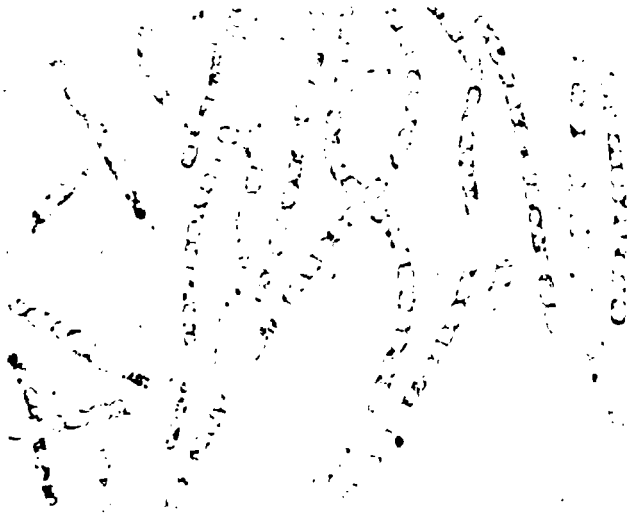
FIG. 4. Molar ratio of ethylene produced to carbon fixed versus irradiance for aggregated samples of A. flos-aquae. The curve was calculated from parameters in Table 3 and represents the ratio of curves in Fig 3A to 3B for aggregated samples. Symbols show actual data for both aggregated and dispersed samples.

Fig 5. Light microelectrode profile from the outside edge of a cm size flocculent mass of A. flos-aquae. Profile represents a composite of 3 separate measurements, error bar=±1 standard deviation.

A



B



C

Fig. 1

D

Fig. 2

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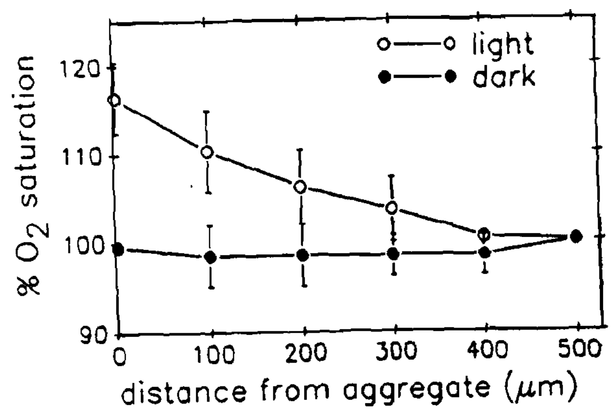


Fig. 3

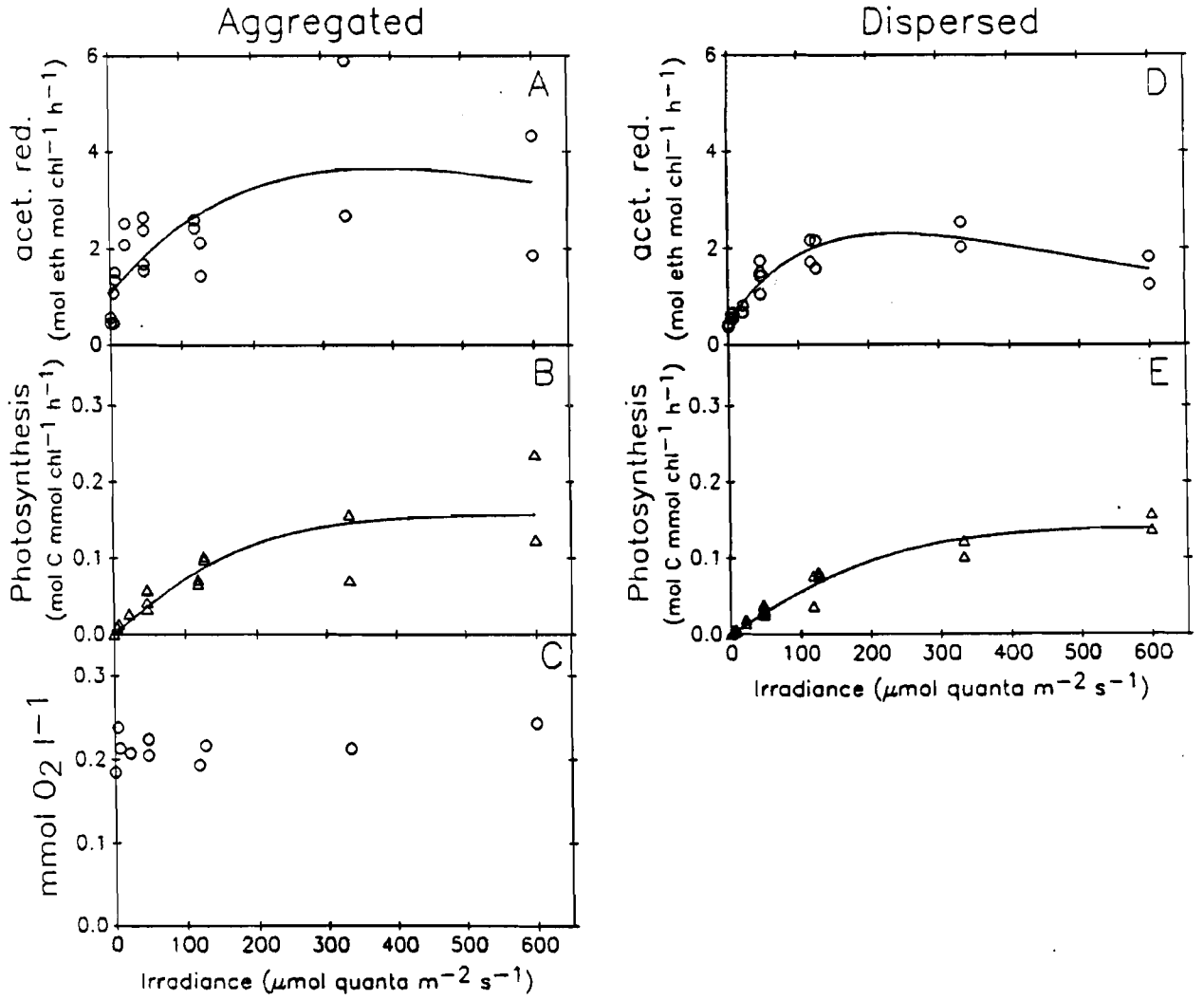

Aggregated
Dispersed
A
B
C
D
E
acet. red.
(mol eth mol chl^{-1} h^{-1})
Photosynthesis
(mol C mmol chl^{-1} h^{-1})
mmol O2 l−1
Irradiance (μmol quanta m^{-2} s^{-1})
0
2
4
6
0.0
0.1
0.2
0.3
100
200
300
400
500
600

Fig. 4

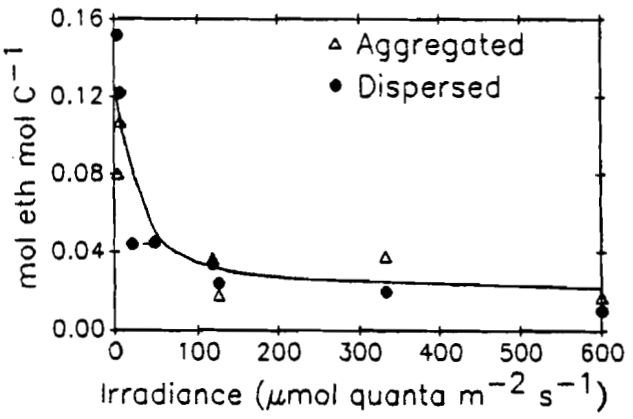
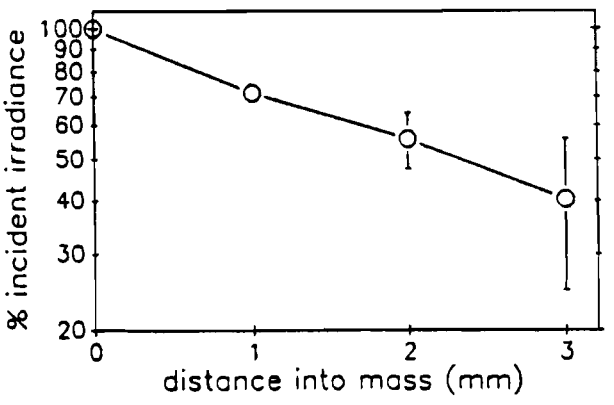

0.16
0.12
0.08
0.04
0.00
mol eth mol C⁻¹
Aggregated
Dispersed
0
100
200
300
400
500
600
Irradiance (μmol quanta m⁻² s⁻¹)

Fig 5


% incident irradiance
100
90
80
70
60
50
40
30
20
0
1
2
3
distance into mass (mm)

CHAPTER 4

HETEROCYST WALL THICKNESS, HETEROCYST FREQUENCY, AND NITROGENASE ACTIVITY IN ANABAENA-FLOS-AQUAE: INFLUENCE OF EXOGENOUS OXYGEN TENSION

(To be submitted to: Canadian Journal of Microbiology)

Heterocyst Wall Thickness, Heterocyst Frequency, and Nitrogenase Activity in *Anabaena flos-aquae*: Influence of Exogenous Oxygen Tension

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Keywords: Cyanobacteria, *Anabaena*, oxygen partial pressure, nitrogen-fixation, heterocyst.

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ABSTRACT

Heterocyst walls of the N_2-fixing cyanobacterium Anabaena flos-aquae were found to thicken as exogenous O_2 partial pressure (pO_2) was increased from 5 to 40 kPa. Such heterocyst wall thickening appears to be an O_2-induced mechanism for providing a greater O_2 diffusion barrier against O_2 inhibition of nitrogenase, the O_2-labile enzyme responsible for N_2-fixation. We observed decreased nitrogenase activity (NA) at pO_2 levels above ambient (20 kPa) indicating that the thicker heterocyst walls were not completely effective as barriers to O_2 diffusion. However, cultures grown at 10 kPa and 40 kPa pO_2, when transferred to ambient pO_2, the culture previously grown at 40 kPa showed higher NA 24 h after transfer compared to those grown at 10 kPa or 20 kPa indicating O_2-protection of nitrogenase by thicker heterocyst walls. In addition to the effect of pO_2 on heterocyst wall thickness, and NA, heterocyst frequency was lowest at 20 kPa O_2 apparently due to faster vegetative cell division. The tradeoff between morphometric and physiological responses to exogenous pO_2 presumably involves a complicated interplay between genetic and physiological flexibility which allows A. flos-aquae to fix atmospheric nitrogen in a variety of environments.

INTRODUCTION

In oxygenic phototrophic cyanobacteria, exogenous oxygen tension is the net product of consumption through respiration and release through oxygenic photosynthesis. The enzyme complex responsible for the conversion of molecular nitrogen to ammonium in diazotrophs, nitrogenase, is readily inactivated by oxygen (7, 22). Various physiological, morphological and ecological strategies exist among different species of diazotrophs to overcome this dilemma (3, 5, 6, 11, 13-19, 22, 25, 28). One of the most important structural features providing protection against oxygen inactivation of nitrogenase in cyanobacteria is the multi-laminated wall of heterocysts (3, 10, 26). However, despite the presence of heterocysts, filamentous cyanobacteria such as Anabaena and Aphanizomenon exhibit some degree of nitrogenase inhibition when exogenous oxygen levels are elevated above ambient. This indicates that the oxygen diffusion barrier afforded by the heterocyst wall is not completely effective against increases in external oxygen tension (12, 16).

Structures analogous to heterocysts, called vesicles in the actinorhizal diazotroph Frankia, are known to have thin walls in cultures grown at low partial pressure of O_2 (pO_2) while having thicker walls at high pO_2 (18; Kangatharalingam and Ensign, unpublished). This phenomenon has not been reported among cyanobacterial heterocysts except for the observation that in Anabaena spp. the heterocyst wall is poorly developed under low pO_2 and anaerobic conditions (9, 21). Among the studies published, only short-term effects of pO_2 or O_2-supersaturation on nitrogenase

activity (NA) in cyanobacteria have been reported (12, 14, 16).

According to Wilcox et al. (30), heterocyst frequency is genetically determined and hence can be changed by mutation. Also, physiological (environmental) factors such as light intensity, combined nitrogen availability and assimilable organic carbon sources are known to affect heterocyst production (8), and hence could affect heterocyst frequency. Heterocyst frequency of A. cylindrica under highly elevated CO_2 levels with no O_2 was shown to be higher than that in air (9). However, we are not aware of any report on the effect of varied pO_2 on heterocyst frequency. Heterocyst frequency is often positively related to corresponding NA (24) presumably because heterocysts are the primary sites of NA (32).

We examined the influence of external pO_2 on heterocyst wall thickness, heterocyst frequency, and NA in A. flos-aquae. This study provides information on the inter-relationships of these factors and the physiological implications as influenced by exogenous pO_2.

MATERIALS AND METHODS

Cultures and general experimental conditions

Anabaena flos-aquae, isolated from Hebgen Lake, Montana, was grown and maintained in batch cultures at 25±1 °C in ASM medium (1) without an inorganic nitrogen source (ASM^-). An irradiance of 200 μmol quanta m^{-2} s^{-1} was supplied by "cool white" fluorescent lamps under a 12 h light/dark cycle.

A total of 9 replicate sealed serum bottles (unit capacity 117.5 ml) per pO_2 treatment, each containing 20 ml ASM^- media and 0.5 ml of 5 day old A. flos-aquae inoculum, were used in this experiment. Temperature and irradiance were the same as for the maintenance of culture. The bottles were incubated with agitation at 60 rpm on a gyrotary shaker. Gas mixtures were replenished every 5 days during the 12 day course of the experiment (see below).

Chlorophyll assay

Chlorophyll a (chl a), used to normalize NA, was determined on samples vacuum filtered onto Whatman GF/C filters. Warm (79 ^oC) 95% ethanol was added to the sample on filter which was then vortexed for 2 min and allowed to cool to room temperature for >12 h (23). Either a spectrophotometer (Varian DMS 80) or fluorometer (Turner fluorometer model 112; Sequoia-Turner Corporation, CA) were employed to quantify the amount of chl a in the supernatant of centrifuged extracts (31). Chl a concentration was calculated using the extinction coefficient reported by Wintermans and De Mots (31); fluorometric determinations were calibrated using pure Anacystis chl a (Sigma Chemical Co., St. Louis, MO) in 95% ethanol.

Gas mixtures

Gas mixtures were made using a two-way gas flowmeter/proportioner (Matheson 7300 series) in which O_2 and N_2 were combined to produce mixtures containing 5, 10, 20, and 40 kPa O_2. The gas mixtures were introduced by gas displacement of the headspace in

the 117.5 ml sealed serum bottles containing 20 ml of culture media. Carbon dioxide was then introduced into the culture bottles using a syringe fitted with a 23 gauge hypodermal needle to a constant level of 0.04 kPa. Filled bottles were then agitated gently for about 1 minute to equilibrate the gas and liquid phases.

Heterocyst wall thickness

On the 12th day of the experiment, a 1 ml sample from each of two replicate serum bottles per pO_2 were removed and viewed under dark field microscopy using a Nikon Labophot photomicroscope. Photomicrographs of the samples on Kodak Panatomic-x film were enlarged to achieve ca. 2000 fold magnification of A. flos-aquae heterocysts. The heterocyst wall thickness in the photomicrographs of samples grown at varied pO_2 was then measured using a calibrated electronic digitizer connected to a microcomputer. Fifteen heterocysts per pO_2 treatment were randomly selected for wall thickness measurements. All measurements were made laterally on each heterocyst, avoiding their polar regions where wall thickness varied considerably.

Nitrogenase Activity

Nitrogenase activity of two replicate samples from each pO_2 was assayed 6 and 12 days after the beginning of the experiment using the acetylene reduction assay (27). Acetylene was added at 10% of the bottle volume and incubations lasted 3 h under the same experimental conditions. Ethylene produced was determined by

flame ionization gas chromatography (Carle AGC series 100) based on ethylene standards. The rate of ethylene production for each sample was normalized to chl a. Nitrogenase activity was also determined at 24 and 48 h for samples grown for 12 days at 10, 20 and 40 kPa pO_2 and transferred to 20 kPa pO_2.

Heterocyst frequency

On the 6th and 12th days of the experiment, heterocyst frequency (number of heterocysts expressed as a percent of total number of heterocysts plus vegetative cells counted) of samples from two replicate incubation bottles per pO_2 was examined microscopically to yield 4 observations per replicate per day. On the 12th day, the replicated pO_2 treatment bottles used for the heterocyst wall thickness study described above were sampled. All cells between 2 or 3 adjacent heterocysts in a trichome were counted. A visible partial septum between vegetative cells was accepted as a complete septum.

Growth rate

Growth rates of A. flos-aquae at various pO_2 were determined by changes in chl a in 2 ml of sample removed just before starting the experiment, on day 6 and day 12 of incubation.

RESULTS

Heterocyst wall thickness of A. flos-aquae following 12 days of incubation at various pO_2 is shown in FIG. 1. Dark field photomicrographs of A. flos-aquae heterocysts in cultures grown

at 10, 20 and 40 kPa pO_2 are shown in FIG 2. The sample grown at 5 kPa is not shown because it was not significantly different from that grown in 10 kPa. The thickest heterocyst walls consistently occurred at 40 kPa pO_2. Heterocyst wall thickness decreased as pO_2 was lowered to 10 kPa. No significant difference in heterocyst wall thickness among pO_2 levels was observed on day 6 of incubation (data not shown). Nitrogenase activity on both 6th and 12th days of incubation was maximal at 20 kPa pO_2 (Fig 3). However, among the cultures grown at 10 kPa, 20 kPa and 40 kPa pO_2, when transferred to 20 kPa pO_2, the culture previously grown at 40 kPa pO_2 showed maximum NA at 24 h after transfer (Table 1).

Heterocyst frequency of A. flos-aquae on the 6th day of incubation was highest at 5 kPa and lowest at 20 kPa pO_2. On the 12th day, 40 kPa pO_2 produced the highest heterocyst frequency though not statistically different from that at 5 kPa; the lowest heterocyst frequency again occurred at 20 kPa pO_2 (Fig 4). The rate of vegetative cell division at 20 kPa pO_2 was significantly higher than at other O_2 levels used. At 5 and 40 kPa pO_2 we found a high proportion of elongated vegetative cells that did not yet show signs of cell division. Such elongated cells were rarely found at 20 kPa (Fig 2, Table 2). In addition, growth of A. flos-aquae determined from changes in chl a showed higher rates at 20 kPa up to 6 days and at 10 kPa O_2 from 6-12 days (Table 3).

DISCUSSION

Heterocyst walls represent a passive O_2 diffusion barrier (3, 10, 12, 26). Our present study indicates that heterocyst wall thickness in the cyanobacterium A. flos-aquae changed in response

to prolonged exposure (12 days) to high exogenous pO_2, an observation similar to that noted in Frankia vesicle wall morphogenesis (18; Kangatharalingam and Ensign, unpublished). Cultures grown at 40 kPa pO_2 with heterocysts having thicker walls, when transferred to 20 kPa pO_2, showed higher NA compared to cultures grown at 10 or 20 kPa pO_2. These results show the inducible nature of heterocyst wall thickness in response to pO_2 suggesting that heterocyst wall thickness is responsive to O_2 concentrations, apparently playing a protective role for nitrogenase, most likely by mediating inward O_2 diffusion. Our observation is in agreement with previous reports of poor development of heterocyst walls in Anabaena species under anaerobic or microaerobic conditions (9, 21). Previous observations of inadequacy of O_2 protection of nitrogenase by heterocysts produced under low external pO_2, and the significant protection afforded by heterocysts induced at high pO_2 (12, 16, 20) also support our results. Producing a thick heterocyst wall would be a metabolically "expensive" process so that organisms presumably only form thick walls when necessary. Our observations of differential heterocyst wall thickening induced by exogenous pO_2 corroborate previous reports and provide additional detail on the regulation of O_2 diffusion into heterocysts.

The fact that optimum NA of A. flos-aquae occurred at 20 kPa under prolonged growth indicates NA is not only regulated by external pO_2 but by other factors as well. Prolonged growth at 40 kPa pO_2 apparently produced physiological stress that would have reduced NA. If optimum NA was entirely due to O_2 protection of

nitrogenase it should have occurred at 5 or 10 kPa pO_2 instead of 20 kPa. Therefore, 20 kPa pO_2 apparently provides a general physiological optima. The observation that the culture transferred to 20 kPa from 40 kPa pO_2 showed higher NA compared to those transferred from 10 and 20 kPa pO_2 implies that thickened heterocyst walls better protect nitrogenase from O_2-inactivation. In addition, this observation also suggests certain degree of recovery from physiological stress at 40 kPa pO_2. It has been shown that short-term anaerobic growth of A. cylindrica with highly elevated CO_2 levels increased NA, followed by a rapid decline in NA (9). In A. cylindrica, azide, an inhibitor of cytochrome activity, strongly inhibited NA under aerobic conditions, suggesting a strong reliance of NA on active respiration (12). In the actinorhizal N_2-fixer, Frankia, respiratory O_2 consumption was higher in N_2-grown cells than in NH_4^+-grown cells (11), increasing with increased rates of NA (Kangatharalingam and Ensign, unpublished). These observations indicate that active ATP production via intermediary metabolism, in addition to that supplied by cyclic photophosphorylation, is required for optimal NA (2, 4, 12). Presumably, the negative effect of energy limitation in A. flos-aquae at low pO_2 (5 and 10 kPa) counters the positive influence of reduced O_2 inhibition of nitrogenase at 5 and 10 kPa pO_2.

High pO_2, apart from its direct inhibitory effect on nitrogenase despite thicker heterocyst walls, could also cause inhibition of inorganic C fixed by photosynthesis through photooxidation and photorespiration which are energy consuming (29). In the presence of reduced photosynthesis, elevated respiration rates,

photorespiration, and consequent limitation of reductant, a corresponding reduction in nitrogenase biosynthesis, and activity of the existing enzyme could occur (12, 29). The observed NA of A. flos-aquae on day 6 and 12 at various pO_2 levels may be the result of any one or a combination of these factors.

Heterocyst frequency in A. flos-aquae was found to be higher at both extremes of pO_2 employed (5 kPa and 40 kPa), compared to 20 kPa, on both days of observation. This resulted from relatively high vegetative cell division at 20 kPa pO_2 (Fig 2). The high proportion of elongated vegetative cells at 5 and 40 kPa pO_2 on both the 6th and 12th days of incubation indicates a delay in cell division supporting our observation of low growth rates (Table 3), and low NA at 5 and 40 kPa pO_2 (Fig 3).

Because heterocyst frequency under N-limitation is directly related to number of heterocysts differentiated and inversely related to the number of vegetative cells present, low rates of vegetative cell division will lead to higher heterocyst frequencies. Therefore, the use of heterocyst frequency as a criterion to predict NA or nitrogen fixation of a cyanobacterium grown under N-limitation at various pO_2 levels may be questionable. Further support for this contention is that all heterocysts in a culture are not equally functional at any point in time. Unless we know the proportion of functional heterocysts in a culture during a particular period, relating heterocyst frequency to NA or nitrogen fixation of the organism becomes inappropriate.

In A. flos-aquae (and perhaps in other heterocystous cyanobacteria) present in N-deficient, O_2-rich environments, it is

important that NA should be optimum for their continued growth, survival and dominance. The biosynthetic processes responsible for altering heterocyst wall thickness appear regulated (either directly or indirectly) by exogenous pO_2. These processes may be of physiological and evolutionary significance when considering long-term increases in oxygenation of the biosphere during the inception and development of oxygenic photosynthesis. The O_2-induced heterocyst wall thickening we have reported is an important mechanism that could contribute to optimum NA of A. flos-aquae under either short or long-term pO_2 changes. Even though pO_2's significantly higher than ambient levels have been observed in the microenvironment of A. flos-aquae during active photosynthesis (Kangatharalingam et al., Submitted), prolonged and continuous oxygenation or microaerobiosis is apparently injurious to A. flos-aquae because the fundamentally important processes of CO_2 and N_2 fixation can be affected. Our results further indicate that A. flos-aquae, appears best adapted to live in an atmospheric pO_2 near 20 kPa despite its ability to produce thicker heterocyst walls at elevated pO_2. The tradeoff between morphogenic and physiological adaptations to varying pO_2 conditions in all likelihood involves a complicated interplay of genetic flexibility, bioenergetics (including the metabolic "costs" of altering and maintaining cell morphology in response to environmental stress) and biochemical limitations (to O_2 stress, N starvation, energy and reductant availability and energy charge=ATP). In essence, these considerations reflect the lengthy evolutionary history of heterocystous cyanobacteria during a 2 billion year transition from anoxic precambrian to contemporary oxic biospheric condi-

tions.

ACKNOWLEDGMENTS

We express our gratitude to T. Galli, and Lizhu Wang for their help in the laboratory, and M. Lizotte for critical review of the manuscript. This research was supported by The Soap and Detergent Association.

LITERATURE CITED

1. Allen, M. M. 1968. Simple conditions for growth of unicellular blue-green algae on plates. J. Phycol. 4: 1-4.

2. Bergersen, F. J., G. L. Turner. 1973. Kinetic studies of nitrogenase from soy-bean root-nodule bacteroids. Biochem. J. 131: 61-75.

3. Bothe, H. 1982. Nitrogen fixation. Chapter 4 in The Biology of Cyanobacteria. Botanical Monographs vol 19. Edited by Carr, N. G. and B. A. Whitton. University of California press.

4. Bottomley, P.J. and W. D. P. Stewart. 1977. ATP and nitrogenase activity in nitrogen-fixing heterocystous blue-green algae. New Phytol. 79: 625-638.

5. Carlton, R. G. and H. W. Paerl. 1989. Oxygen-induced changes in morphology of aggregates of Aphanizomenon flos-aquae (Cyanophyceae): implications for nitrogen fixation potentials. J. Phycol. 25: 326-333.

6. Carpenter, E. J. and C. C. Price. 1976. Marine Oscillatoria (Trichodesmium):Explanation for aerobic nitrogen-fixation without heterocysts. Science 191: 1278-1280.

7. Fay, P. and R. M. Cox. 1967. Oxygen inhibition of nitrogen fixation in cell-free preparations of blue-green algae. Biochim. Biophys. Acta 143: 562-569.

8. Fogg, G. E. 1949. Growth and heterocyst production in Anabaena cylindrica Lemm. II. In relation to carbon and nitrogen metabolism. Ann. Bot. 13: 241-259.

9. Kulasooriya, S. A., N. J. Lang and P. Fay. 1972. The heterocysts of blue-green algae III. Differentiation and nitrogenase activity. Proc. Roy. Soc. London B 181: 199-209.

10. Lang, N. J. and P. Fay. 1971. The heterocysts of blue-green algae. II. Details of ultrastructure. Proc. Roy. Soc. London B 178: 193-203.

11. Murry, M. A., M. S. Fontaine and J. D. Tjepkema. 1984. Oxygen protection of nitrogenase in Frankia sp. HFPArI3. Arch. Microbiol. 139: 162-166.

12. Murry, M. A., A. J. Horne and J. R. Benemann. 1984. Physiological studies of oxygen protection mechanisms in the heterocysts of Anabaena cylindrica. Appl. Environ. Microbiol. 47: 449-454.

13. Paerl, H. W. 1978. Role of heterotrophic bacteria in promot-

ing N_2-fixation by Anabaena in aquatic habitats. Microbial Ecology 4: 215-231.

14. Paerl, H. W. 1978. Light-mediated recovery of N_2-fixation in the blue-green algae Anabaena spp. in O_2 supersaturated waters. Oecologia 32: 135-139.

15. Paerl, H. W. and B. M. Bebout. 1988. Direct measurement of O_2-depleted microzones in marine Oscillatoria: Relation to N_2 fixation. Science 241: 442-445.

16. Paerl, H. W. and P. E. Kellar. 1978. Optimization of N_2 fixation in O_2-rich waters. Proc. 1st Int. Microb. Ecol. Symp. 68-75.

17. Paerl, H. W., and L. E. Prufert. 1987. Oxygen-poor microzones as potential sites of microbial N_2 fixation in nitrogen-depleted aerobic marine waters. Appl. Environ. Microbiol. 53: 1078-1087.

18. Parsons, R., W. B. Silvester, S. Harris, W. T. M. Gruijters and S. Bullivant. 1987. Frankia vesicles provide inducible and absolute oxygen protection for nitrogenase. Plant Physiol. 83: 728-731.

19. Peterson, R. B. and R. H. Burris. 1976. Properties of heterocysts isolated with colloidal silica. Arch. Microbiol. 108: 35-40.

20. Pienkos, P. T., S. Bodmer, and F. R. Tabita. 1983. Oxygen inactivation and recovery of nitrogenase activity in cyanobacteria. J. Bacteriol. 153: 182-190.

21. Rippka, R. and R. Y. Stanier. 1978. The effects of anaerobiosis on nitrogenase synthesis and heterocyst development by Nostocacean cyanobacteria. J. Gen. Microbiol. 105: 83-94.

22. Robson, R. L. and J. R. Postgate. 1980. Oxygen and hydrogen in biological nitrogen fixation. Ann. Rev. Microbiol. 34: 183-207.

23. Sartory, D. P. and J. U. Grobbelaar. 1984. Extraction of chlorophyll a from fresh water phytoplankton for spectrophotometric analysis. Hydrobiologia 114: 177-187.

24. Smith, R. J., S. Hobson and I. R. Ellis. 1987. The effect of abscisic acid on calcium-mediated regulation of heterocyst frequency and nitrogenase activity in Nostoc 6720. New Phytol. 105: 543-549.

25. Stal, L. J. and W. E. Krumbein. 1987. Temporal separation of nitrogen fixation and photosynthesis in the filamentous, non-heterocystous cyanobacterium Oscillatoria sp. Arch. Microbiol. 149: 76-80.

26. Stanier, R. Y. and G. Cohen-Bazire. 1977. Phototrophic proka-

ryotes: The cyanobacteria. Ann. Rev. Microbiol. 31: 225-274.

27. Stewart, W. D. P., G. P. Fitzgerald and R. H. Burris. 1967. In situ studies on N_2 fixation using the acetylene reduction technique. Proc. US Nat. Acad. Sci. 58: 2071-2078.

28. Stewart, W. D. P., A. Haystead and H. W. Pearson. 1969. Nitrogenase activity in heterocysts of blue-green algae. Nature 224: 226-228.

29. Van Liere, L. and A. E. Walsby. 1982. Interactions of cyanobacteria with light. Chapter 2 in The Biology of cyanobacteria. Botanical Monographs vol 19. Edited by Carr, N.G. and B. A. Whitton. University of California press.

30. Wilcox, M., G. J. Mitchison and R. J. Smith. 1975. Mutants of Anabaena cylindrica altered in heterocyst spacing. Arch. Microbiol. 103: 219-223.

31. Wintermans, J. F. G. M. and A. De Mots. 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta 109: 448-453.

32. Wolk, C. P. 1982. Heterocysts. Chapter 14 in The Biology of Cyanobacteria. Botanical Monographs vol 19. Edited by Carr, N. G. and B. A. Whitton. University of California press.

TABLE 1. Nitrogenase activity of *A. flos-aquae* transferred to 20 kPa pO_2 from 10, 20, or 40 kPa pO_2 after 12 days of growth.

Initial Growth Condition (pO_2 in kPa)	NA[a] after transfer	
	24 h	48 h
10	3.20±0.60	2.45±0.45
20	19.40±0.80	20.90±0.30
40	20.90±0.40	22.85±0.75

[a]Mean (±Standard Error; n=2) nitrogenase activity in μmole C_2H_4 h^{-1} mg chl *a* $^{-1}$ at 24 and 48 h after transfer to 20 kPa pO_2.

TABLE 2. Mean[a] (±standard error) vegetative cell length (μm) of *A. flos-aquae* on the 12th day of incubation at various pO_2

	pO_2			
	5 kPa	10 kPa	20 kPa	40 kPa
Length	7.80±0.36	6.25±0.29	5.81±0.23	7.19±0.25

[a] Mean of 20 cells measured at random.

TABLE 3. Growth[a] of A. flos-aquae at various pO_2

	pO_2			
Time interval	5 kPa	10 kPa	20 kPa	40 kPa
0- 6th day	0.004±0.000	0.060±0.000	0.073±0.002	0.023±0.003
6-12th day	0.002±0.001	0.011±0.003	0.008±0.002	0.003±0.002

[a] Change in chl a in $\mu g\ ml^{-1}$ culture day^{-1} ± S.E.

LIST OF ILLUSTRATIONS

Fig. 1

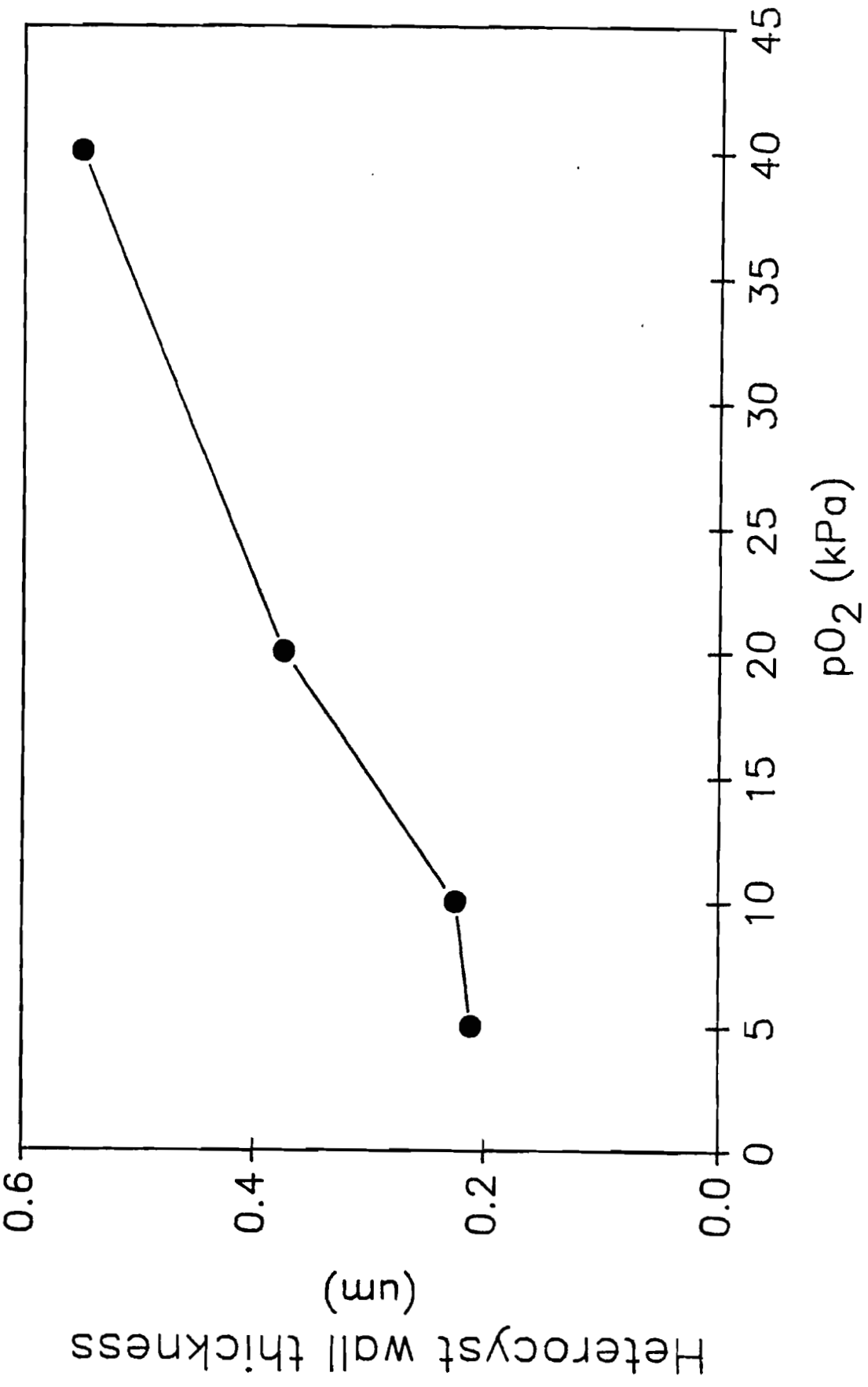
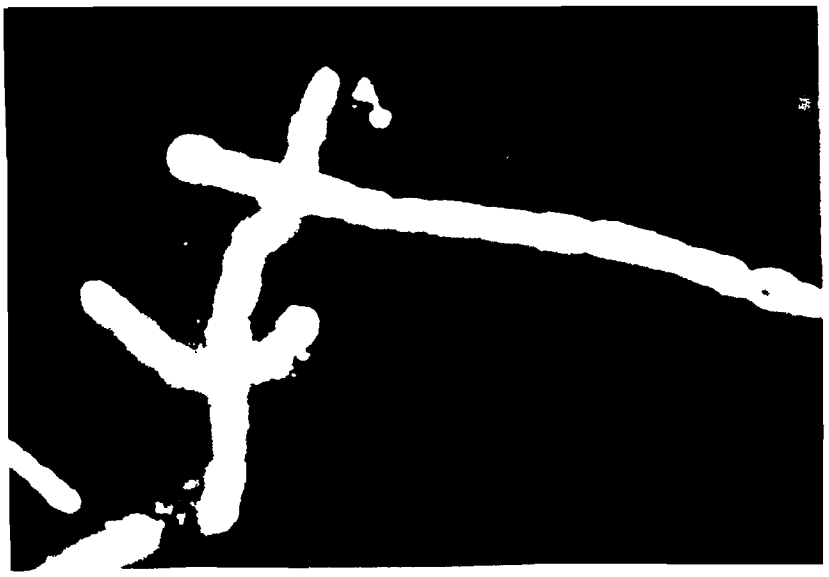

Heterocyst wall thickness
(um)
0.0
0.2
0.4
0.6
0
5
10
15
20
25
30
35
40
45
pO2 (kPa)

Fig. 2

A

B



C

Fig. 3

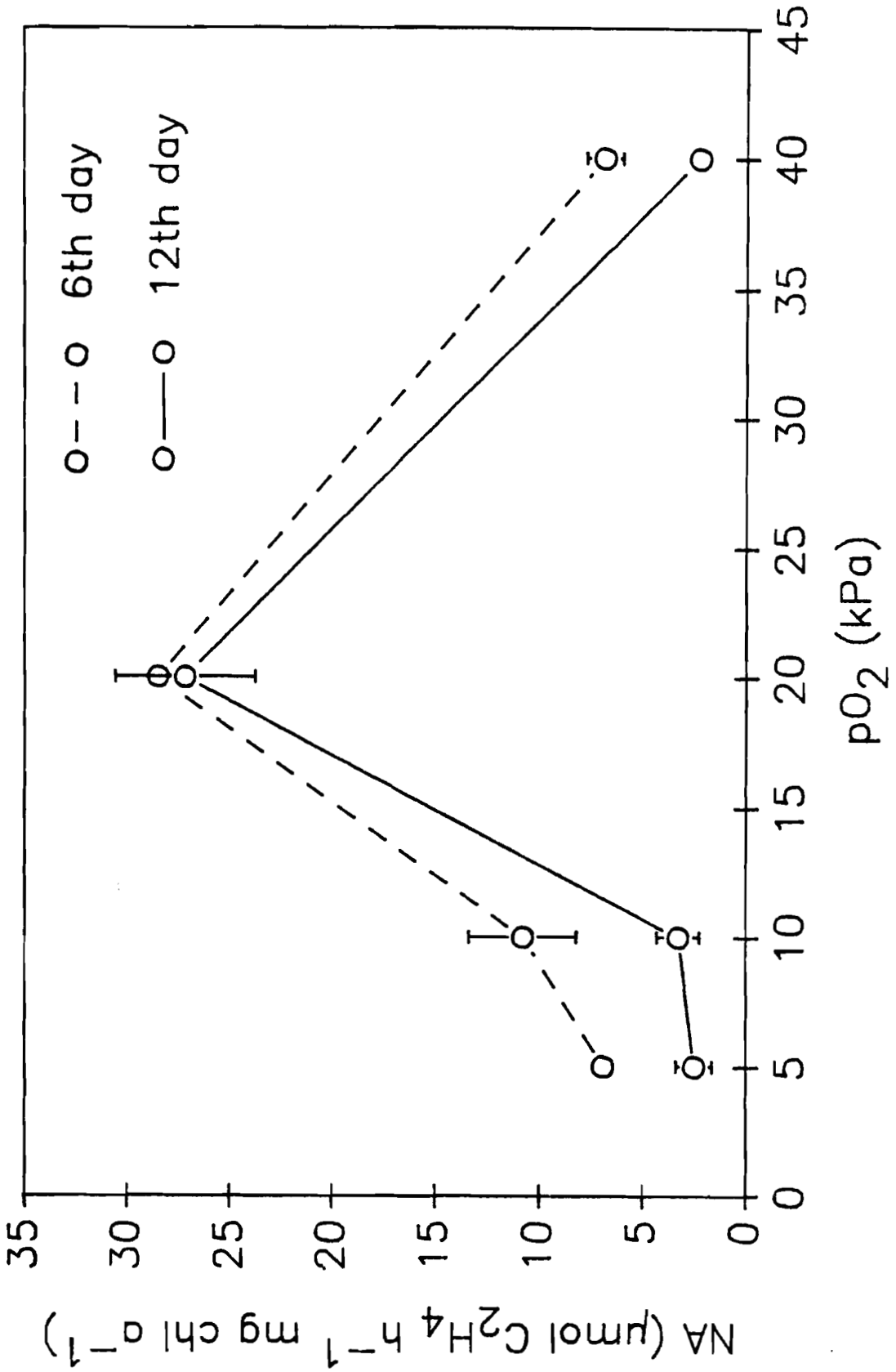
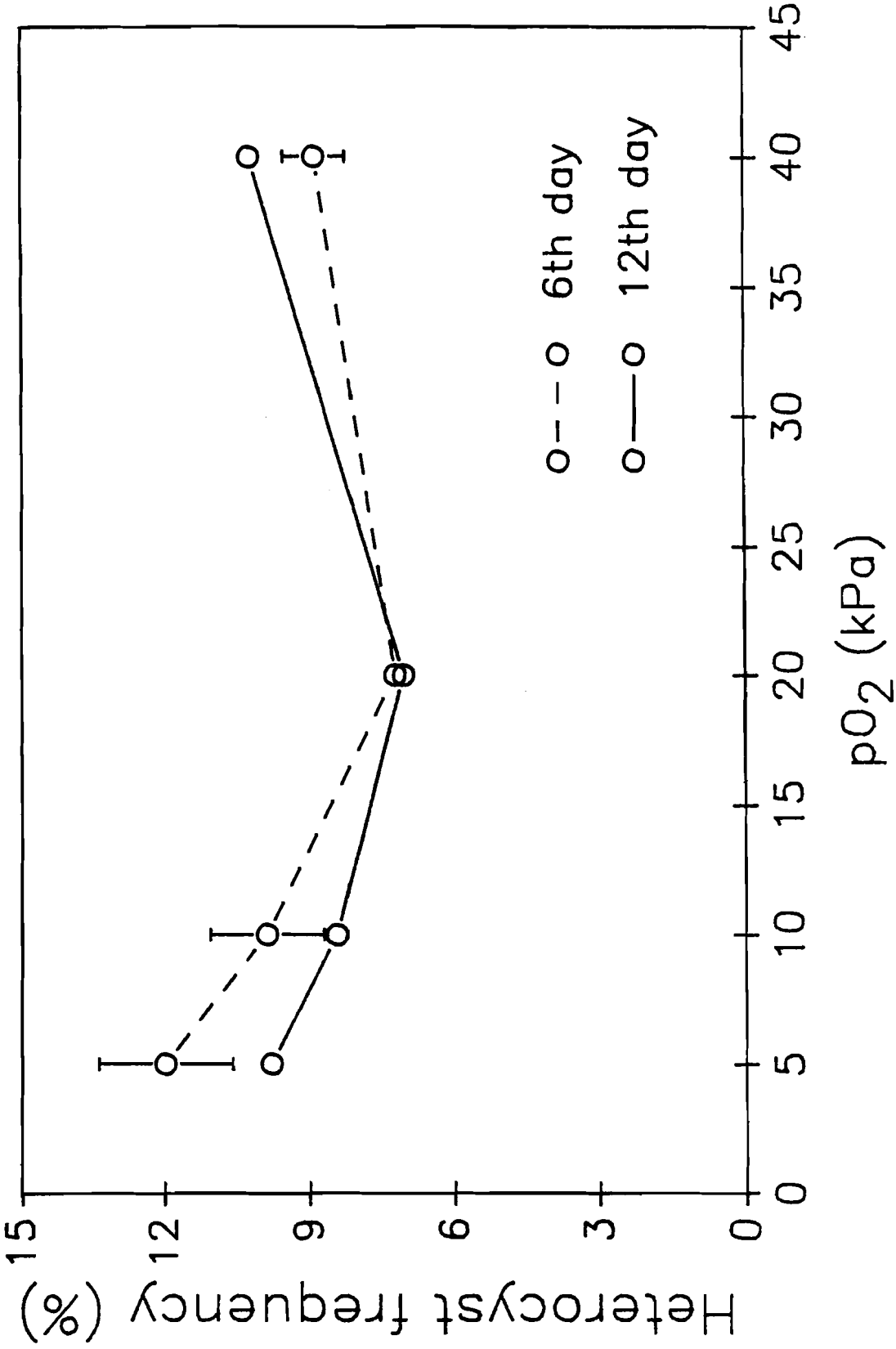

NA (μmol C_2H_4 h^{-1} mg chl a^{-1})
0
5
10
15
20
25
30
35
0
5
10
15
20
25
30
35
40
45
pO_2 (kPa)
o—o 6th day
o—o 12th day

Fig. 4


Heterocyst frequency (%)
0
3
6
9
12
15
0
5
10
15
20
25
30
35
40
45
pO2 (kPa)
6th day
12th day

CHAPTER 5

GLUTAMINE SYNTHETASE ACTIVITY, NITROGENASE ACTIVITY AND HETEROCYSE FREQUENCY IN ANABAENA FLOS-AQUAE (LYNGB.) DE BREB. GROWN UNDER VARIOUS INORGANIC NITROGEN CONCENTRATIONS

(Submitted: Planta)

Glutamine Synthetase Activity, Nitrogenase Activity and Heterocyst Frequency in Anabaena flos-aquae (Lyngb.) De Breb. Grown Under Various Inorganic Nitrogen Concentrations

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Running title: Nitrogen metabolism in Anabaena flos-aquae.

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ABSTRACT

Glutamine synthetase (GS) specific activity [both Mn^{2+} and Mg^{2+} dependent γ-glutamyl transferase (γGT) activities] of the photosynthetic cyanobacterium Anabaena flos-aquae (Lyngb.) De Breb. were compared with nitrogenase activity (NA) and heterocyst frequency following 10 days of growth under various initial concentrations of NH_4Cl and $NaNO_3$. No significant correlations existed between either total GS (γGT with Mn^{2+}) and NA ($r=0.39$; $P>0.05$), or Mg^{2+} dependent GS activity and NA ($r=-0.26$; $P>0.05$). Heterocyst frequency was not significantly correlated with Mn^{2+} dependent GS ($r=0.47$; $P>0.05$) or Mg^{2+} dependent GS activity ($r=-0.32$; $P>0.05$) owing to a strong correlation between heterocyst frequency and NA ($r=0.80$; $P=0.01$). Both NH_4^+ and NO_3^- concentrations decreased during growth indicating that both were used as a N-source by A. flos-aquae. Collectively, our data imply that in A. flos-aquae, GS activity is localized in both heterocysts and vegetative cells, and that NA or nitrogenase biosynthesis is not directly regulated by GS.

Additional key words: Cyanobacteria, glutamine synthetase, heterocyst frequency, nitrogenase activity, nitrogen metabolism.

INTRODUCTION

Among the organisms that have the ability to reduce the dinitrogen molecule to ammonium, the cyanobacteria play a prominent role. The ammonium produced during cyanobacterial N_2-fixation has been shown to be assimilated mainly through the glutamine synthetase (L-glutamate:ammonia ligase [Adenosine 5'-diphosphate forming], EC 6.3.1.2.) and glutamate synthase (L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.7.1.) pathway (Miflin and Lea 1976; Thomas et al. 1975; Wolk et al. 1976; Haystead et al. 1973; Dharmawardene et al. 1973). Glutamine synthetase (GS) activity has been shown to be present in cyanobacteria grown on atmospheric nitrogen and dissolved inorganic nitrogen (i.e. NO_3^- and NH_4^+) (Dharmawardene et al. 1972; 1973; Batt and Brown 1974; Meeks et al. 1977; 1978). Even though GS has been studied intensively in cyanobacteria (Stacey et al. 1977; Rowell et al. 1977; Sampaio et al. 1979; Meeks et al. 1978; Meeks et al. 1977; Lee et al. 1988), little attention has been given to the relationship between GS activity and nitrogenase activity (NA) under different levels of nitrogen fixation. In one of the few published reports, Streicher et al. (1974), working with Klebsiella pneumoniae, showed that GS was positively correlated with NA. We are not aware of any such reports in cyanobacteria. Here we determined GS activity (using the γ-glutamyl transferase assay) present in the cyanobacterium Anabaena flos-aquae grown under various initial extracellular concentrations of inorganic nitrogen. GS activity was compared to NA and heterocyst frequency

(1951) after heat treating the sample at 90 °C for 10 min with an equal volume of 1 N NaOH. Bovine serum albumen served as a standard.

Assay for NH_4^+ and NO_3^-

NH_4^+ and NO_3^- were measured in culture filtrates on day 5 to confirm NH_4^+ and NO_3^- uptake and utilization by A. flos-aquae. Ammonium was determined by the phenol-hypochlorite method (Solorzano 1969) and NO_3^- by the Cd reduction method (Parsons et al. 1984).

RESULTS

Total GS activity and Mg^{2+} dependent GS activity of A. flos-aquae determined on day 10 of growth in media enriched with various levels of NH_4^+ and NO_3^- are presented with NA and heterocyst frequency in Table 1. At 2 mM initial NH_4^+, Mn^{2+} dependent GS declined by 28.6% and NA by 96.7%; at 200 μM NH_4^+ there was 9.5% decline in Mn^{2+} dependent GS, 11.1% decline in Mg^{2+} dependent GS activity, and a 3.0% decline in NA. NA declined by 68.6% with 2 mM NO_3^- but Mn^{2+} dependent GS showed no significant change relative to the control ($P>0.05$). The treatment initially enriched with 200 μM NO_3^- showed 10.6% decline in NA whereas Mn^{2+} dependent GS remained statistically unchanged ($P>0.05$). With both 200 μM and 2 mM NO_3^-, however, there was a 22.2% increase in the Mg^{2+} dependent GS activity. Heterocyst frequency was highest in N_2-grown samples and declined sharply in the presence of 2 mM NH_4^+. Analyses on day 5 showed that up to 70 μmole NH_4^+ or 10 μmole NO_3^- was consumed by A. flos-aquae with an initial inoculum

density of 0.913 μg protein ml^{-1}. Our results showed measureable levels of GS and NA in all treatments on day 10 of incubation when the assays were carried out.

Correlations between Mn^{2+} dependent GS and NA, and Mg^{2+} dependent GS activity and NA were poor ($r=0.39; P>0.05$ and $r=-0.26; P>0.05$, respectively). There was a strong correlation between NA and heterocyst frequency ($r=0.80; P<0.01$); a correspondingly low correlation existed between Mn^{2+} dependent GS activity and heterocyst frequency ($r=0.47; P>0.05$), and between Mg^{2+} dependent GS activity and heterocyst frequency ($r=-0.32; P>0.05$).

DISCUSSION

It is known that A. flos-aquae can utilize NH_4^+ or NO_3^-, and fix molecular N_2 (Turpin et al. 1984; Elder and Parker 1984) a result corroborated by our findings. Work with A. cylindrica has shown that GS activity continues to be present in NH_4^+ grown as well as N_2 grown cells (Dharmawardene et al. 1972; 1973; Batt and Brown 1974; Meeks et al. 1977; 1978) though there were disagreements among the reports as to the relative levels of GS activity when grown on different N sources. We observed a significant decline in NA in A. flos-aquae when grown for 10 days in 2 mM NH_4^+ or NO_3^- (initial concentration) with a simultaneous, significant decline in GS activity in only the 2 mM NH_4^+ treatment. There was no effect on GS activity when shocked with NH_4^+ or NO_3^- for 15 min at a concentration of 5 mM (data not presented). Apparently, the effect of exogenous inorganic nitrogen on GS in A. flos-aquae is not rapid (within the range of concentration used in this study) whereas it has been reported to occur within

7 min in Klebsiella aerogenes (Bender et al. 1977) where concentrations up to 15 mM NH_4^+ were used. Our N concentrations were limited to 2 mM because we observed toxicity in A. flos-aquae (chlorophyll degradation and vegetative cell disintegration within 2 days) beyond 2 mM.

Our NH_4^+ and NO_3^- treatments were used primarily to alter the rates of enzyme activity, allowing us to examine the relationship between NA and GS over a range of activities. Our preliminary observations showed that both Mn^{2+} and Mg^{2+} dependent GS activities were marginal during the early stages of incubation (up to 5 days) in all treatments including N_2, a finding also reported for Anabaena sp. strain CA (Stacey et al. 1977). The assays were conducted on day 10 to ensure detectable levels of GS activity in all treatments.

We estimated heterocyst frequency and correlated it with NA and GS because nitrogenase in heterocystous cyanobacteria is localized in heterocysts (Wolk 1982). Reports on GS localization, though contradictory, have indicated that GS occurs in both heterocysts and vegetative cells in A. cylindrica (Thomas et al. 1977, Bergman et al. 1985). We are unaware of previous reports using the relationship between heterocyst frequency and GS to estimate the localization of GS in a heterocystous cyanobacterium. Our results showing poor correlation between heterocyst frequency and GS activity, and NA and GS activity indicate that GS activity is localized in A. flos-aquae in both heterocysts and vegetative cells.

On day 10, levels of GS remained almost constant among treat-

ments (Table 1) with the exception of the 2 mM NH_4^+ and 2 mM NO_3^- treatments whereas NA varied widely among treatments. However, there was only a poor correlation between GS and NA. This was true for both Mn^{2+} dependent GS and the Mg^{2+} dependent GS activity. These results lead us to contend that GS does not directly regulate NA in A. flos-aquae as reported by Streicher et al. (1974) in K. pneumoniae.

ACKNOWLEDGMENTS

This research was supported by a research grant from The Soap and Detergent Association. We acknowledge the assistance of T. Galli in the laboratory. Reviews by J. Meeks and M. Lizotte improved this manuscript.

Wolk, C.P., Thomas, J., Shaffer, P.W., Austin, S.M., Galonsky, A. (1976) Pathway of nitrogen metabolism after fixation of ^{13}N-labelled nitrogen gas by the cyanobacterium, Anabaena cylindrica. J. Biol. Chem. 251, 5027-5034

Table 1. Mn^{2+} dependent GS activity, Mg^{2+} dependent GS activity, nitrogenase activity and heterocyst frequency of Anabaena flos-aquae on day 10 of growth under various initial inorganic nitrogen concentrations.

Treatment	Mean (±S.E., n=2) Specific activity of Mn^{2+} dependent GS[a].	Mean (±S.E., n=2) Specific activity of Mg^{2+} dependent GS[a].	Mean (±S.E., n=2) Nitrogenase activity[b].	Mean (±S.E., n=5) Heterocyst frequency[c].
N_2-grown	0.42 (±0.02)	0.18 (±0.01)	255.34 (±1.79)	11.00 (±1.01)
100 M NH_4Cl	0.42 (±0.02)	0.18 (±0.00)	261.67 (±2.42)	7.78 (±1.14)
200 M NH_4Cl	0.38 (±0.01)	0.16 (±0.01)	247.82 (±10.16)	8.42 (±0.75)
2 mM NH_4Cl	0.30 (±0.02)	0.18 (±0.01)	8.46 (±1.23)	3.36 (±0.26)
100 M $NaNO_3$	0.40 (±0.03)	0.18 (±0.01)	263.63 (±4.72)	6.86 (±0.81)
200 M $NaNO_3$	0.41 (±0.01)	0.22 (±0.01)	228.18 (±2.59)	6.54 (±0.16)
2 mM $NaNO_3$	0.46 (±0.00)	0.22 (±0.01)	80.28 (±6.76)	5.64 (±0.32)

[a] μmol τ-glutamyl hydroxamate min^{-1} mg^{-1} protein.

[b] nmol C_2H_4 h^{-1} mg^{-1} protein.

[c] Percent of total number of cells.

CHAPTER 6

GLUTAMINE SYNTHETASE ACTIVITY IN THE CYANOBACTERIUM *ANABAENA FLOS-AQUAE* (LYNGB.) DE BREB.: EVIDENCE FOR REGULATION OF FEEDBACK INHIBITION AND ENERGY CHARGE

(To be submitted to: Archives of Microbiology)

Glutamine Synthetase Activity in the Cyanobacterium Anabaena flos-aquae (Lyngb.) De Breb.: Evidence for Regulation by Feedback Inhibition and Energy Charge

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Additional Key words: Cyanobacterium, N_2-metabolism, glutamine synthetase, γ-glutamyl transferase assay

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ABSTRACT

Mn^{2+}, and Mg^{2+} dependent glutamine synthetase (GS) activities of cell-free extracts of the cyanobacterium Anabaena flos-aquae (Lyngb.) De Breb., were estimated by the γ-glutamyl transferase (γGT) assay with either Mn^{2+} or Mg^{2+}. N_2-grown cells failed to show GS inhibition when pulsed with NH_4^+ whereas growth for 10 days in medium containing an initial concentration of 2 mM NH_4^+ significantly inhibited GS activity; nitrate failed to inhibit GS activity under similar conditions. Comparison of Mn^{2+} dependent GS activity assayed between pH 6.8 and 8.3 showed little indication of adenylylation of GS in N_2-grown, and NH_4-pulsed cells of A. flos-aquae even though an "isoactivity point" existed at pH 7.4. When the extract was incubated for 1 h with 5 mM L-Methionine-DL-sulfoximine a GS inhibitor, GS was significantly inhibited. Snake venum phosphodiesterase (0.02% w/v final concentration) which transforms adenylylated GS into non-adenylylated GS showed no significant effect on Mn^{2+} dependent GS activity of A. flos-aquae, further indicating that regulation of GS by adenylylation/deadenylylation was insignificant. The addition of 1 mM L-alanine, glycine and L-serine to the reaction mixture inhibited Mn^{2+} dependent GS activity by 47%, 16% and 13%, respectively, relative to unammended controls. ATP inhibited Mn^{2+} dependent GS activity by 42% whereas only 3.5% inhibition occurred with AMP. In concert, the results of these experiments imply that GS is regulated primarily by feedback inhibition and energy charge in A. flos-aquae.

INTRODUCTION

Glutamine synthetase [GS; EC 6.3.1.2.; L-glutamate:ammonia ligase (adenosine 5'-diphosphate (ADP)) forming] is an important nitrogen assimilating enzyme found in both prokaryotes and eukaryotes (Tyler 78). In N_2-fixing organisms, GS is of particular importance owing to its role in converting NH_4^+ produced through N_2-fixation into glutamine which plays a vital role in the synthesis of nucleotides, other amino acids and eventually proteins (Johansson et al. 83; Dharmawardene et al. 73; Stewart et al. 75; Meeks et al. 77). The GS/glutamate synthase [L-glutamine:2-oxoglutarate aminotransferase, EC 1.4.7.1.] pathway has been shown to be the primary route of NH_4^+ assimilation in cyanobacteria (Brown et al. 74; Wolk et al. 76; Miflin and Lea 76).

Several mechanisms of regulation of GS exist (Shapiro and Stadtman 70; Tyler 78). However, little work has been done to determine the specific regulatory mechanisms of GS in cyanobacteria, particularly Anabaena flos-aquae, an important planktonic N_2-fixing and toxin producing cyanobacterium (e.g. McMaster et al. 80). Even though adenylylation has been shown to be an important GS regulatory mechanism in N_2-fixing bacteria (Bender et al. 77; Foor et al. 75; Kingdon et al. 67; Adler et al. 75), adenylylation has not been shown equivocally to regulate cyanobacterial GS. We present the results of an investigation of the GS regulatory mechanisms existing in A. flos-aquae emphasizing the role of feedback inhibition and energy charge.

MATERIALS AND METHODS

Organism and Growth Conditions

Anabaena flos-aquae (isolated from Hebgen Lake, Montana) was maintained in N_2-fixing batch cultures in ASM medium (Allen 68) lacking combined nitrogen (ASM^-) at 25°C under a photosynthetic photon flux density of 200 μmole photons m^{-2} s^{-1} supplied by "cool white" fluorescent lamps (14:10 h light/dark cycle). Ten day old cultures grown in ASM^- or ASM^- amended with selected levels of NH_4^+ or NO_3^- were used for GS assays.

Glutamine Synthetase Assay

Before the assay, the cells were treated for 10 min and washed twice under gentle vacuum on a 8 μm membrane filter with a solution containing equal volumes of 0.5 M immidazole-HCl (pH 7.4) and cetylammonium bromide (CTAB; 0.5 mg ml^{-1}). Cells were then suspended (2 replicates) in the same solution at 4°C and extracted by vortexing with acid-washed glass beads (<150 μm mesh; 0.5 g ml^{-1}). The extracts were centrifuged at 4°C to remove the cell debris before GS determination. Glutamine synthetase was assayed on cell-free extracts using the γ-glutamyl transferase (γGT) assay described by Bender et al. (Bender et al. 77) with either 0.27 mM Mn^{2+} (transfer assay for total GS) or with 60 mM Mg^{2+} (for Mg^{2+} dependent GS activity) added to the reaction mixture (Stadtman et al. 70; Bender et al. 77). The reaction mixture was incubated for 30 min at 37°C. After the reaction was terminated the mixture was centrifuged to remove any precipitate

before absorbance (540 nm) was measured.

Because the Mn^{2+} dependent GS activity increases with increasing adenylylation of GS within a range of pH (Bender et al. 77) the assay was first carried out at various pH levels between 6.8 and 8.3 to determine the isoactivity point, the pH at which γGT activity is the same independent of the adenylylation state of GS. The γGT reaction is a non-physiological expression of GS activity and is routinely used to estimate GS activity. In this reaction, glutamine and hydroxylamine in the presence of ADP and arsenate produce γ-glutamyl hydroxamate (γGH) which is then quantified by absorbance measurements at 540 nm relative to γGH standards. The specific activity of GS is defined as μmole γGH produced min^{-1} mg^{-1} protein.

To examine mechanisms regulating GS activity, the γGT assay was also conducted on cell-free extracts pre-incubated with L-methionine-DL-sulfoximine (MSX; 5 mM final concentration for 1 h), snake venum phosphodiesterase (SVPD; final concentration of 0.02 % w/v in immidazole-HCl buffer for 1 h) and with either L-alanine, glycine, L-serine, AMP, or ATP (1 mM final concentration for 30 min) before adding glutamine to start the reaction. The forward reaction assay (reverse γGT assay; Bender et al. 77) was used as a measure of biosynthetic GS activity.

Protein Assay

Total protein in all samples was determined as described by Lowry et al. after heat treating samples at 90°C for 10 min with an equal volume of 1 N NaOH (Lowry et al. 51). Bovine serum albumen was used as standard.

(Meeks 79), limitations in the nitrate reduction process in A. flos-aquae, or small amounts of NH_4^+ produced through NO_3^--reduction is presumably assimilated rapidly and therefore unavailable to affect the biosynthesis of GS (Stacey et al. 77).

MSX, which inhibits NH_4^+ assimilation into glutamine (Singh et al. 83; Kleiner and Castorph 82;), inhibited Mn^{2+} dependent GS activity by 47.5% compared to an unamended control without MSX. Despite previous reports of total inhibition of GS by 1 mM MSX in culture in 1 h (Turpin et al. 84), our results indicate that GS in A. flos-aquae extracts is not completely inhibited by MSX within 1 h incubation. The previous reports of total inhibition of GS in culture (Turpin et al. 84), may not be a direct effect of MSX on GS but perhaps includes GS biosynthesis in vivo, or there was a ATP deficiency in our reaction mixture which prevented total effect of MSX.

SVPD pre-incubation which transforms adenylylated GS into non-adenylylated GS (Bender et al. 77; Rowell et al. 77; Kingdon et al. 67), showed no significant difference in the GS activity compared to controls without SVPD. These results further suggest lack of adenylylation/de-adenylylation of GS in A. flos-aquae. Even though our results are in agreement with previous reports on cyanobacterial GS (Stacey et al. 77; Stacey et al. 79; McMaster et al. 80), the reason for Mg^{2+} inhibition of γGT assay in the absence of adenylylation/de-adenylylation as found in this study is not clear. Similar observations were reported previously (McMaster et al. 80). However, Mg^{2+} inhibition of the γGT assay may be due to the fact that biosynthetic GS activity in γGT assay

has cation specificity for Mg^{2+} whereas the γGT assay for total GS activity has specificity for Mn^{2+} as reported for Anabaena spp. (Stacey et al. 79; Sawhney and Nicholas 78). Because we used CTAB, a known adenylyl transferase inhibitor (Mura and Stadtman 81) before and during our harvesting and extraction processes, any possibility of transformation in the original state of adenylylation/non-adenylylation of the enzyme during the harvesting and extraction processes is unlikely (Bender et al. 77). Our data imply that adenylylation/de-adenylylation of GS in A. flos-aquae is not an important regulatory mechanism of GS activity though further work needs to be done to determine whether adenylylation is completely absent or present in marginally detectable levels in this cyanobacterium.

Synthesis of glutamine via GS forms the initial step in the biosynthesis of several different compounds such as amino acids (e.g. L-alanine, glycine, L-serine), nucleotides (e.g. AMP, GMP, CTP), complex polysaccharides, p-aminobenzoate, and carbamyl-phosphate (Johansson et al. 83; Shapiro and Stadtman 70). Amino acids such as L-alanine, glycine, L-serine, and AMP therefore, represent potential feedback inhibitors of GS (Stacey et al. 79; Rowell et al. 77; McMaster et al. 80). Our results indicate greatest inhibition of GS by L-alanine, followed by glycine and L-serine. This inhibition shows GS regulation via feedback inhibition by amino acids in A. flos-aquae. Even though ATP significantly inhibited Mn^{2+} dependent GS activity, there was insignificant inhibition by AMP which supports previous findings (McMaster et al. 80). This lack of AMP inhibition must be due to the direct involvement of AMP in the energy charge of the enzyme activity

(Weissman 76). Of the two nucleotides tested, ATP, with the higher number of phosphate groups, produced a more pronounced inhibition of GS, implying that the enzyme is regulated by energy charge of the cell (Stacey et al. 79; Weissman 76; McMaster et al. 80). A further indication of the involvement of energy charge in GS regulation comes from the forward reaction assay for GS we conducted (Bender et al. 77) using ATP as the reagent to start the reaction. The forward reaction assay gave very poor results in our studies.

Our results show that feedback inhibition by amino acids and ATP, and energy charge are important regulators of GS in the cyanobacterium A. flos-aquae. Even though there is indication of GS inhibition by high initial levels of NH_4^+ in prolonged cultures, evidence for direct NH_4^+ induced repression of GS was not found. Furthermore, the lack of evidence for adenylylation regulation of GS activity in A. flos-aquae raises the following questions. Firstly, is adenylylation totally absent or occurs in very limited extent at all in this cyanobacterium. Secondly, the enzyme phosphodiesterase that deadenylylates adenylylated GS (Kingdon et al. 67; Stacey et al. 79) whether present or absent, and if present, at what level and under what conditions. The cyanobacterium A. flos-aquae being a gram negative organism, the presence, and the possible expression of the genome for the adenylylation system of GS at least under certain specified conditions may not be ruled out unless shown otherwise.

ACKNOWLEDGMENTS

We express our gratitude to T. Galli for assistance in the laboratory, J. Meeks and M. Lizotte for reviewing the manuscript. This work was supported by The Soap and Detergent Association.

LITERATURE CITED

Alder SP, Purich D, Stadtman ER (1975) Cascade control of Escherichia coli glutamine synthetase. J Biol Chem 250: 6264-6272

Allen MM (1968) Simple conditions for growth of unicellular blue-green algae on plates. J Phycol 4: 1-4

Bender RA, Janssen KA, Resnick AD, Blumenberg M, Foor F, Magasanik B (1977) Biochemical parameters of glutamine synthetase from Klebsiella aerogenes. J Bacteriol 129: 1001-1009

Brown CM, MacDonald-Brown DS, Meers JL (1974) Physiological aspects of microbial inorganic nitrogen metabolism. Advances in Microbial Physiology 11: 1-52

Deuel T, Ginsburg A, Yeh J, Shelton E, Stadtman ER (1970) Bacillus subtilis glutamine synthetase. J Biol Chem 245: 5195-5205

Dharmawardene MWN, Haystead A, Stewart WDP (1973) Glutamine synthetase of the nitrogen-fixing alga Anabaena cylindrica. Arch Mikrobiol 90: 281-295

Foor F, Janssen K, Magasanik B (1975) Regulation of the synthesis of glutamine synthetase by adenylylated glutamine synthetase. Proc Natl Acad Sci USA 72: 4844-4848

Hubbard JS, Stadtman ER (1967) Regulation of glutamine synthetase VI Interactions of inhibitors for Bacillus licheniformis, glutamine synthetase. J Bacteriol 94: 1016-1024

Janssen KA, Magasanik B (1976) Glutamine synthetase of Klebsiella aerogenes: genetic and physiological characterization of mutants in the adenylylation system. J Bacteriol 129: 993-1000

Johansson BC, Nordlund S, Baltscheffsky H (1983) Nitrogen fixation and ammonia assimilation. Chapter 7 In: Studies in microbiology vol 4. The phototrophic bacteria: Anaerobic life in the light. Ormerod JG (Ed). University of California press, LA. 246 pp

Kingdon HS, Shapiro BM, Stadtman ER (1967) Regulation of glutamine synthetase VIII ATP:glutamine synthetase adenylyltransferase, an enzyme that catalizes alterations in the regulatory properties of glutamine synthetase. Proc Natl Acad Sci USA 58: 1703-1710

Kleiner D, Castorph H (1982) Inhibition of ammonium (methylammonium) transport in Klebsiella pneumoniae by glutamine and glutamine analogues. FEBS Lett 146: 201-203

Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with Folin phenol reagent. J Biol Chem 193: 262-275

McMaster BJ, Danton MS, Storch TA, Dunham VL (1980) Regulation of glutamine synthetase in the blue-green alga Anabaena flos-aquae. Biochem Biophys Res Commun 96: 975-983

Meeks JC (1979) NO_3-assimilation by two Anabaena species. In: Nichols JM (Ed) Abstracts of the third international symposium on photosynthetic prokaryotes, Oxford. p D-15. Univ. Liverpool, England

Meeks JC, Wolk CP, Thomas J, Lockau W, Shaffer PW, Austin SM, Chien WS, Galonsky A (1977) The pathways of assimilation of $^{13}NH_4$ by the cyanobacterium, Anabaena cylindrica. J Biol Chem 252: 7894-7900

Miflin BJ, Lea PJ (1976) The pathway of nitrogen assimilation in plants. Phytochem 15: 873-885

Mura U, Stadtman ER (1981) Glutamine synthetase adenylylation in permeabilized cells of Escherichia coli. J Biol Chem 256: 13014-13021

Prival MJ, Brenchley JE, Magasanik B (1973) Glutamine synthetase and the regulation of histidine formation in Klebsiella aerogenes. J Biol Chem 248: 4334-4344

Rowell P, Enticott S, Stewart WDP (1977) Glutamine synthetase and nitrogenase activity in the blue-green alga Anabaena cylindrica. New Phytol 79: 41-54

Sawhney SK, Nicholas DJD (1978) Some properties of glutamine synthetase from Anabaena cylindrica. Planta 139: 289-299

Schutt H, Holzer H (1972) Biological inactivation of glutamine synthetase in Escherichia coli. Eur J Biochem 26: 68-72

Shapiro BM, Stadtman ER (1970) Glutamine synthetase (Escherichia coli). Methods in Enzymology 17A: 910-922

Singh HN, Rai UN, Rau VV, Bagchi SN (1983) Evidence for ammonia as an inhibitor of heterocyst and nitrogenase formation in the cyanobacterium Anabaena cycadea. Biochem Biophys Res Commun 111: 180-187

Stacey G, Tabita FR, Van Baalen C (1977) Nitrogen and ammonia assimilation in the cyanobacteria: Purification of glutamine synthetase from Anabaena sp. strain CA. J Bacteriol 132: 596-603

Stacey G, Van Baalan C, Tabita FR (1979) Nitrogen and ammonia assimilation in the cyanobacteria: Regulation of glutamine synthetase. Arch Biochem Biophys 194: 457-467

Stadtman ER, Ginsburg A, Ciardi JE, Yeh J, Hennig SB, Shapiro BM (1970) Multiple molecular forms of glutamine synthetase produced by enzyme catalyzed adenylylation and deadenylylation reactions. Adv Enzyme Regul 8: 99-118

Stewart WDP, Haystead A, Dharmawardene MWN (1975) Nitrogen assimilation and metabolism in blue-green algae. Stewart WDP (Ed) In: Nitrogen fixation in free living microorganisms. pp 129-158. Cambridge Univ press, UK

Turpin DH, Edie SA, Canvin DT (1984) In vivo nitrogenase regulation by ammonium and methylamine and the effect of MSX on ammonium transport in Anabaena flos-aquae. Plant Physiol 74: 701-704

Tyler B (1978) Regulation of the assimilation of nitrogen compounds. Ann Rev Biochem 47: 1127-1162

Weissman GS (1976) Glutamine synthetase regulation by energy charge in sunflower roots. Plant Physiol 57: 339-343

Wolk CP, Thomas J, Shaffer PW, Austin SM, Galonsky A (1976) Pathway of nitrogen metabolism after fixation of ^{13}N-labelled nitrogen gas by the cyanobacterium, Anabaena cylindrica. J Biol Chem 251: 5027-5034.pa

Table 1. Gamma-GT activity of the variously treated 10 day old *Anabaena flos-aquae* with Mn^{2+}, Mg^{2+}, Mn^{2+}+MSX and Mn^{2+}+SVPD.

Treatment	Specific Activity of GS[a]			
	$+Mn^{2+}$	$+Mg^{2+}$	$+Mn^{2+}$+MSX	$+Mn^{2+}$+SVPD
N_2	0.411±0.014	0.178±0.003	0.216±0.004	0.393±0.004
NH_4^+-Shocked	0.419±0.009	0.193±0.002	0.226±0.002	0.403±0.002
100 μM NH_4^+	0.415±0.021	0.181±0.003	0.177±0.012	0.492±0.002
200 μM NH_4^+	0.377±0.008	0.157±0.011	0.161±0.021	0.431±0.002
2 mM NH_4^+	0.298±0.023	0.174±0.002	0.166±0.001	0.309±0.007
100 μM NO_3^-	0.391±0.025	0.178±0.004	0.179±0.003	0.397±0.006
200 μM NO_3^-	0.402±0.004	0.212±0.006	0.203±0.001	0.397±0.005
2 mM NO_3^-	0.457±0.000	0.219±0.012	0.188±0.006	0.469±0.008

[a] μmole γ-glutamyl hydroxamate min^{-1} mg^{-1} protein ±S.E.(n=2).

Table 2. Mn^{2+} dependent GS activity of 10 day old N_2-grown Anabaena flos-aquae treated with various amino acids and nucleotides.

Treatment	Specific Activity of GS[a]	% Inhibition[b]
Control	0.433±0.002	
L-Alanine	0.230±0.002	46.9
Glycine	0.362±0.001	16.4
L-Serine	0.377±0.002	12.9
AMP	0.418±0.001	3.5
ATP	0.250±0.001	42.3

[a] μmole glutamyl hydroxamate min^{-1} mg^{-1} protein ±S.E.(n=2)

[b] Percent inhibition of the specific activity of Mn^{2+} dependent GS compared to control.

LIST OF ILLUSTRATIONS

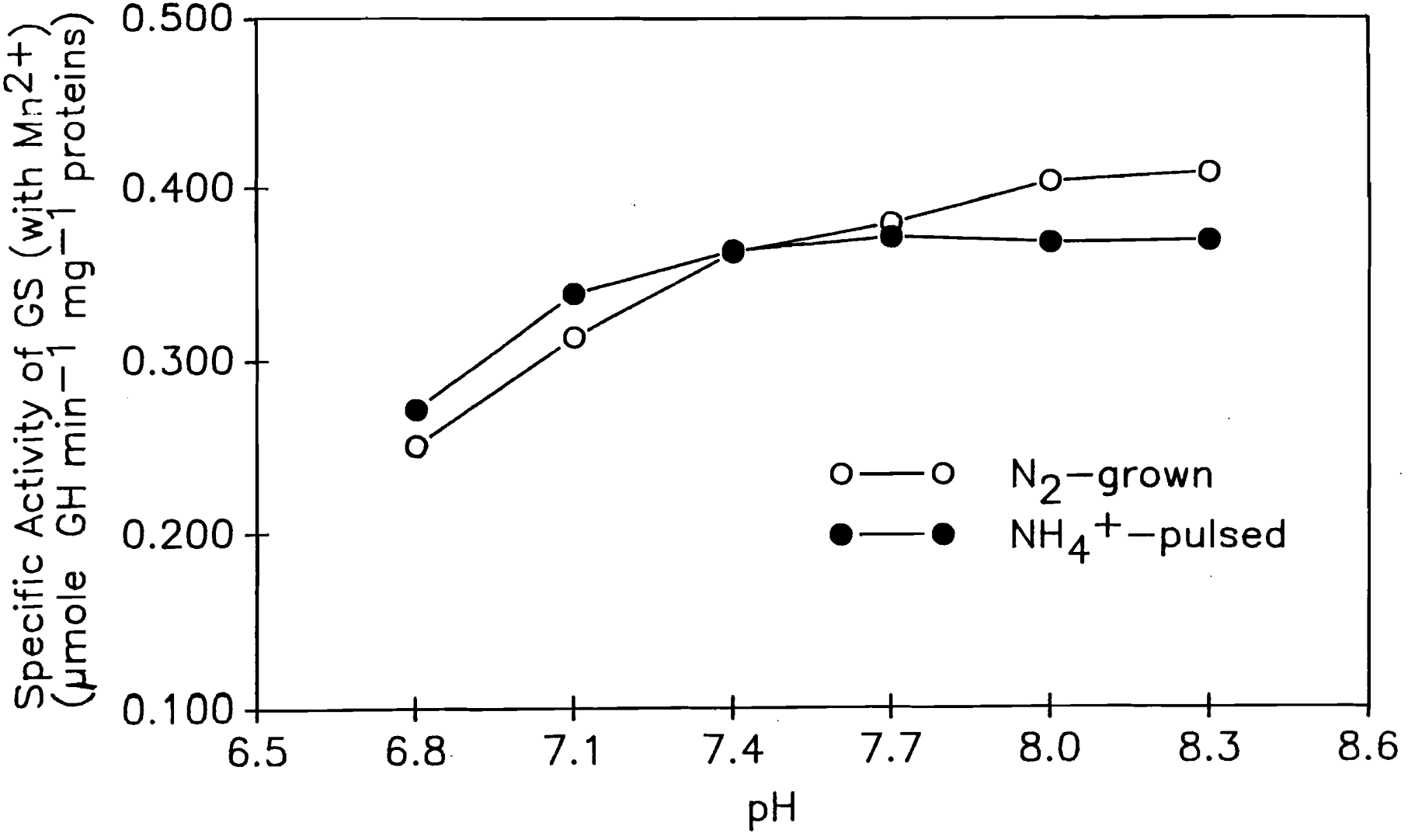
Specific Activity of GS (with Mn^{2+})
(μmole GH min^{-1} mg^{-1} proteins)
0.500
0.400
0.300
0.200
0.100
6.5
6.8
7.1
7.4
7.7
8.0
8.3
8.6
pH
N_2–grown
NH_4^+–pulsed

Fig. 1

CHAPTER 7

BACTERIOPLANKTON NUTRIENT DEFICIENCY IN A EUTROPHIC LAKE

(Part of a Ph.D. dissertation: Lizhu Wang)

BACTERIOPLANKTON NUTRIENT DEFICIENCY IN A EUTROPHIC LAKE

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Abstract

The responses of bacterioplankton and phytoplankton were measured in 20-l microcosms enriched with NH_4^+, PO_4^{-3}, and mannitol (singly and in various combinations) and incubated *in situ* for 5 days during June, August, and October in a eutrophic lake. Bacterial cell number and activity (^{3}H-thymidine uptake) increased significantly ($p<0.05$) with addition of PO_4^{-3} in June, PO_4^{-3}, NH_4^+, and mannitol in August, and mannitol in October during 1988. Bacterial cell number and activity increased significantly ($p<0.05$) with all nutrient additions during 1989 except PO_4^{-3} alone, which showed no stimulation in June. During 1988 $^{14}CO_2$ uptake (phytoplankton photosynthesis) and chlorophyll *a* increased significantly ($p<0.05$) with addition of PO_4^{3-} in June; $^{14}CO_2$ uptake increased significantly ($p<0.05$) with addition of NH_4^+ in August and October. The addition of NH_4^+ in June, and NH_4^+ and PO_4^{-3} in August significantly ($p<0.05$) increased $^{14}CO_2$ uptake and chlorophyll *a* during 1989.

Introduction

Heterotrophic bacteria are largely responsible for the degradation of organic matter and regeneration of minerals in aquatic ecosystems (Marvalin et al. 1989; Robarts and Wicks 1990). It has been suggested that bacterial production is controlled by, or is directly related to, the supply of decomposable organic matter (Cole et al. 1988). Correlations between mean bacterioplankton production and chlorophyll a concentration or photosynthesis (Fuhrman and Azam 1980; Bjornsen et al. 1989; Marvalin et al. 1989; Roberts and Wicks 1990) suggest that phytoplankton products are the dominant source of bacterial substrate. However, other studies have shown that bacteria have a substantially higher specific phosphorus requirement than do phytoplankton, and act as primary consumers of inorganic phosphorus (Vadstein et al. 1988). It has also been found that bacterioplankton can effectively compete with phytoplankton for inorganic nitrogen and phosphorus, not only in oligotrophic water but also in eutrophic water (Priscu and Downes 1985; Vadstein and Jensen 1988; Vadstein and Olsen 1989). Those findings indicate that inorganic nutrients may be as important as organic nutrients in regulating bacterioplankton growth in aquatic systems.

The purpose of this study was to examine experimentally the response of bacterioplankton to the supply of dissolved organic carbon (DOC), inorganic nitrogen and inorganic phosphorus in microcosms in a eutrophic lake. The following specific questions were addressed: Can inorganic nutrient enrichment stimulate bacterioplankton activity? And if so, is inorganic nutrient

stimulation of bacterial activity direct or indirect (resulting from stimulation of phytoplankton)?

Materials and Methods

Study site. Experiments were conducted in the Grayling Arm of Hebgen Lake, a eutrophic reservoir located on the upper Madison River, Montana. The Grayling Arm is one of the three bays of Hebgen Lake with its own water inlet and a narrow connection with the main lake. The Grayling Arm has an area of 8 km^2 and a maximum depth of 6 m at the time of our experiments. Phytoplankton in this portion of the lake are dominated by N_2 fixing cyanobacteria over most of the ice-free season.

Experimental procedures. Bioassay experiments were conducted in microcosms during June, August, and October of 1988 and 1989, each extending up to 5 days. The experimental microcosms were 20-L polyethylene collapsible carboys (cubitainers) attached to floats anchored at a 0.5 m in a location near the deepest part in the Grayling Arm. Water for October 1988 and 1989 experiments was incubated in a laboratory incubator using the same kind of cubitainer as in the lake at lake temperature when the water was collected. A photon flux density about 150 $\mu E\ m^{-2}\ s^{-1}$ was provided by cool white fluorescent lamps on a 12:12 h light:dark cycle. Water for our experiments was collected from 0.5 m depth, filtered with 280 μm pore size Nitex mesh to remove large grazers (we did not filter water for experiments of October 1988 and August and October 1989 because of the presence of large cyanobacteria filaments and aggregates), and directly added to the microcosms. Three similar nutrient treatments were used in all

1988 experiments; 6 similar treatments were employed in all 1989 experiments (Table 1). Unamended controls were included with each experiment. Nutrients were added on day 1 only during the 1988 experiments, and were added every sampling interval during 1989 experiments with the exception of mannitol, which was pulsed on day 1 and day 3 only. Water chemistry, phytoplankton $^{14}CO_2$ uptake, chlorophyll a, bacterioplankton thymidine uptake, and bacterioplankton number were determined on each day of the experiment. Bottles (3 light and 1 dark) for $^{14}CO_2$ uptake were incubated in the lake alongside the cubitainer; the thymidine uptake bioassay was incubated in the dark at the same temperature as the cubitainer.

Nutrient analysis. NO_3^- was determined by Cd reduction method (Eppley 1978), NH_4^+ by the phenol hypochlorite method (Solorzano 1969), and soluble reactive phosphorus (SRP) by the molybdate method modified for AsO_4^- interference (Downes 1978), and total dissolved phosphorus (TDP) using the acid hydrolysis procedure (Solorzano and Sharp 1980) followed by orthophosphate determination (Stainton et al. 1977). Dissolved organic phosphorus (DOP) was computed from the difference between TDP and SRP. Total dissolved nitrogen (TDN) was measured by persulfate digestion (D'Elia et al. 1977) followed by determination of nitrate by Cd reduction. Dissolved organic nitrogen (DON) was computed by subtracting the sum of NO_3^- and NH_4^+ from TDN. All of the above samples were prefiltered with Whatman GF/C filters and frozen before analysis.

Particulate matter determination. Chlorophyll a was determined by fluorometry on 90% acetone extracts. Fluorescence was

measured with a Turner model 112 fluorometer on pre- and post-acidified samples and compared to a chlorophyll a standard (Sigma) treated in the same manner (Strickland and Parsons 1972). Particulate organic carbon (POC) and particulate organic nitrogen (PON) were measured with a Carlo-Erba model 1106 elemental analyzer calibrated with standard amounts of acetanilide. Particulate organic phosphorus (PP) was determined with the acid hydrolysis procedure of Solorzano and Sharp (1980) followed by orthophosphate measurement of the digest (Stainton et al. 1977). Dissolved organic carbon (DOC) was analyzed with a Dorhmann Carbon Analyzer. Total organic carbon (TOC) is the sum of DOC and POC; total nitrogen (TN) is the sum of TDN and PON, and total phosphorus (TP) is the sum of SRP and PP.

Isotope experiments. Phytoplankton $^{14}CO_2$ uptake was determined by adding ^{14}C-$NaHCO_3$ stock (ICN Radiochemical Inc.) to lake water to a final ^{14}C activity of about 0.05 μCi ml^{-1}. The samples were incubated in situ for about 4 h near midday. Photosynthesis was terminated by filtration of the entire sample through Whatman GF/C filters. Eight ml of Scintiverse E (Fisher) was added to the filter in a 20-ml scintillation vial and counted with a Beckman LS-100C scintillation spectrometer. Efficiency was computed by the external standard channels ratio. Bacterioplankton thymidine uptake was determined by adding methyl-^3H thymidine stock (ICN Radiochemical Inc.) to 10 ml lake water in a 20 ml glass scintillation vial yielding a final concentration of 10 nM thymidine. The ^3H-thymidine stock (in 70% ethanol) was evaporated to dryness and rehydrated with deionized water before use to eliminate

products of self radiolysis and to remove ethanol. The inoculated sample was incubated under lake temperature in the dark for 20 to 30 minutes. Activity was terminated by adding 10 ml of 10% ice-cold trichloroacetic acid (TCA) to each vial. The sample was then extracted over night at about 4 ^{o}C followed by filtration onto 0.2 μm polycarbonate filters which were rinsed 5 times with 2 ml each of ice-cold 5% TCA (Fuhrman and Azam 1980). Bacterial cell numbers were determined on samples filtered onto 0.2 μm polycarbonate filters with epifluorescence microscopy using the acridine orange direct count technique (Hobbie et al. 1977).

Results

Initial conditions of the experiments.

Initial conditions for all 1988 and 1989 experiments are presented in Table 2. Dominant phytoplankton, in percent of total biovolume at the time of our 1988 nutrient enrichment experiments, were Anabaena spiroides (73%) and A. flos-aquae (17%) in June, A. circinalis (92%) in August, and Asterionella sp. (35%) and Aphanizomenon sp. (52%) in October. The dominant phytoplankton during the 1989 experiments were A. circinalis (64%) in June, Fragilaria sp. (47%) and Aphanizomenon sp. (34%) in August, and Aphanizomenon sp. (97%) in October 1989 experiments. The maximum chlorophyll a concentrations occurred in October of both 1988 and 1989. The concentrations of NH_4^+ and SRP in June 1988 and August 1989 were about 1.5 to 16 times lower than the other experiments. The ratios of TN:TP were 2 to 6 fold higher in June and October 1988 than the other experiments. The highest TOC:TN was found in August 1989 and TOC:TP in June 1988 experiments.

Nutrient Response of Bacterioplankton and Phytoplankton.

June 1988 Experiment. PO_4^{-3} addition significantly ($p<0.01$) increased both bacterioplankton and phytoplankton production. The bacterial thymidine uptake maximum was 1.64 times and phytoplankton $^{14}CO_2$ uptake maximum was 1.21 times higher than those of the control. The bacterial thymidine uptake maximum occurred 1 day before that of phytoplankton $^{14}CO_2$ uptake. NH_4^+ and mannitol additions significantly ($p<0.01$, and $p<0.05$, respectively) reduced bacterioplankton thymidine uptake, and did not show a significant effect on phytoplankton $^{14}CO_2$ uptake (Figure 1, Table 3). PO_4^{-3} addition significantly increased bacterioplankton cell number ($p<0.01$) and chlorophyll a concentration ($p<0.01$); other treatments induced no significant effects on bacterial cell number compared with the control (Figure 1, Table 3).

August 1988 Experiment. Mannitol and PO_4^{-3} additions significantly ($p<0.05$) increased bacterioplankton thymidine uptake. NH_4^+ addition had no significant effect on bacterioplankton thymidine uptake whereas it significantly ($p<0.01$) increased $^{14}CO_2$ uptake by phytoplankton. Mannitol addition significantly ($p<0.01$) decreased $^{14}CO_2$ uptake of phytoplankton. PO_4^{-3} had no significant effect on $^{14}CO_2$ uptake (Figure 2, Table 3). None of the nutrient treatments significantly affected chlorophyll a concentration. NH_4^+ and PO_4^{-3} additions significantly ($p<0.01$) increased bacterial cell number (Figure 2, Table 3).

October 1988 Experiment. Mannitol addition significantly increased both thymidine uptake and bacterial cell number ($p<0.01$). The PO_4^{-3} addition significantly decreased thymidine

uptake and showed no significant effect on bacterial cell number. NH_4^+ addition did not affect either bacterial thymidine uptake or bacterial cell number (Figure 3, Table 3). $^{14}CO_2$ uptake increased significantly ($p<0.01$) with NH_4^+ addition, but decreased significantly ($p<0.01$) with mannitol addition. PO_4^{-3} had no effect on $^{14}CO_2$ uptake. Both mannitol and PO_4^{-3} additions had negative effects on chlorophyll a concentration while NH_4^+ showed no significant effect (Figure 3, Table 3).

June 1989 Experiment. All treatments, except PO_4^{-3}, significantly increased thymidine uptake and bacterial cell numbers ($p<0.01$). Maximum thymidine uptake occurred on days 3 and 4 in lake water enriched with mannitol plus NH_4^+ with levels exceeding the control by more than 3 fold (Figure 4, Table 3). All treatments, except mannitol, PO_4^{-3}, and mannitol plus PO_4^{-3}, significantly ($p<0.01$) increased both $^{14}CO_2$ uptake and chlorophyll a concentration (Figure 4, Table 3).

August 1989 Experiment. All nutrient additions significantly increased thymidine uptake ($p<0.01$) and bacterial cell number ($p<0.01$). All nutrient additions except mannitol and mannitol plus PO_4^{-3} significantly ($P<0.01$) increased $^{14}CO_2$ uptake and chlorophyll a concentration (Figure 5, Table 3). Bacterioplankton and phytoplankton were enhanced most in the treatment containing PO_4^{-3} plus NH_4^+.

October 1989 Experiment. All nutrient additions significantly ($p<0.01$) increased thymidine uptake and bacterial cell numbers except PO_4^{-3} plus NH_4^+, which had no effect on bacterial cell numbers (Figure 6, Table 3). $^{14}CO_2$ uptake was negatively influenced by PO_4^{-3} ($p<0.01$), mannitol ($p<0.05$), and mannitol plus

PO_4^{-3} ($p<0.05$) whereas NH_4^+, PO_4^{-3} plus NH_4^+, and NH_4^+ plus mannitol additions had no significant effect. None of the nutrient additions significantly affect chlorophyll a concentration except NH_4^+ which showed a significant positive effect on chlorophyll a (Figure 6, Table 3).

Relationships between bacterioplankton and phytoplankton.

Correlations grouped by treatment and experiment showed that in 13 of the 132 cases the bacterioplankton parameters (thymidine uptake, cell number) were significantly ($p<0.05$) correlated with the phytoplankton parameters ($^{14}CO_2$ uptake, chlorophyll a) (Table 4). Among those, 2 of the 33 cases (22 positive, 11 negative) of bacterial thymidine uptake and 2 of the 33 cases (19 positive, 14 negative) of bacterial cell number showed significant positive correlation with $^{14}CO_2$ uptake ($p<0.05$). Five (2 positive, 3 negative) of the 33 cases (14 positive, 19 negative) of bacterial thymidine uptake and 3 (1 positive, 2 negative) of the 33 cases (16 positive, 17 negative) of bacterial cell number were significantly ($p<0.05$) correlated with chlorophyll a (Table 4).

Discussion

Inorganic phosphorus and nitrogen enrichments increased bacterial activity and number in our microcosm experiments confirming that inorganic nutrient enrichment can stimulate bacterial activity. However, the limiting nutrient changed substantially among the experiments. Bacterioplankton were stimulated by organic carbon, inorganic phosphorus, and inorganic nitrogen in 3 of the 6 experiments. Inorganic phosphorus alone increased bacterio-

plankton in 1 of the 6 experiments; so did organic carbon. Changes in the nutrient limiting bacterioplankton activity appears to be related to the initial nutrient condition of the experiments. It has been reported that bacterial growth was phosphorus limited when TOC:TP was between 8.3 and 58.8 in a chemostat study (Vadstein and Olsen 1989). During our experiments, all initial TOC:TP ratios exceeded those reported by Vadstein and Olsen (1989) except that in August 1988 which was within the range. Therefore, it is not surprising that 10 of our 12 PO_4^{-3} additions showed significant stimulation of bacterioplankton growth. NH_4^+ and amino acids are generally considered to be the primary nitrogen sources for bacterioplankton in natural waters. When ambient concentration of free amino acids are low, bacteria may be forced to utilize NH_4^+ as a nitrogen source (Wheeler and Kirchman 1986). Chemostat studies have also shown that NH_4^+ and NO_2^- additions stimulated heterotrophic bacterial activity (Horrigan et al. 1988). The reported bacterial C:N content of 2.3-8.3 (Linley and Newell 1984, cited in Vadstein and Olsen 1989) is lower than the initial ambient TOC:TN level in all our experiments implying that nitrogen is in short supply for bacterioplankton compared with organic carbon. High TOC:TN in Hebgen Lake may help to explain why 10 of 12 NH_4^+ additions significantly stimulated bacterioplankton activity.

Our results indicate that inorganic nutrient can stimulate bacterioplankton activity through direct utilization. Bacterioplankton thymidine uptake was out of phase (1 day before) with phytoplankton $^{14}CO_2$ uptake and chlorophyll *a* concentration following PO_4^{-3} addition in June 1988. That 95% (125 out of 132) of

the correlations between bacterioplankton and phytoplankton variables were insignificant or negative supports this conclusion. Our results corroborate previous reports that bacterioplankton growth can be limited by inorganic nitrogen in natural waters (Wheeler and Kirchman 1986; Horrigan et al. 1988; Vadstein et al. 1988; Vadstein and Olsen 1989).

Although inorganic nutrient enrichments directly stimulated bacterioplankton activity in certain cases, indirect stimulation via phytoplankton products also appears to exist. That 5% (7 out of 132) of the correlations between bacterioplankton and phytoplankton variables were significantly positive supports this contention. It has been found that bacterioplankton growth and phytoplankton production are well correlated in both coastal waters (Fuhrman et al. 1980) and fresh waters (Chrzanowski and Hubbard 1988; Marvalin et al. 1989; Robarts and Wicks 1990). Our result that the number of positive and negative correlation coefficients were essentially equal (65 vs. 67) indicates that indirect support of bacterial growth by phytoplankton products is important at only certain times; direct uptake of inorganic nutrient presumably is responsible for the lack of a consistent trend.

That bacterioplankton have been shown to compete with phytoplankton for inorganic phosphorus in cultures (Rhee 1972; Brown et al. 1981) and inorganic phosphorus and nitrogen in natural waters (Vadstein and Jensen 1988; Vadstein and Olsen 1989; Parker et al. 1975; Wheeler and Kirchman 1986; Fuhrman et al. 1988) may explain why 6 of the 132 correlations between bacterial and

phytoplankton variables were significantly negative in Hebgen lake. Conversely, the finding that bacterioplankton production correlated well with phytoplankton production (Fuhrman at al. 1980; Robarts and Wichs 1990) can explain 7 of the 132 correlations between bacterial and phytoplankton variables in Hebgen Lake that were significantly positive. Previously published information (discussed above), in concert with our results, indicate that bacterioplankton compete with or benefit from phytoplankton depending upon the environmental conditions, particularly TOC:TP, TOC:TN, and TN:TP ratios and the physiological state of the phytoplankton.

In summary, organic carbon, inorganic nitrogen and inorganic phosphorus enrichments significantly increased bacterioplankton thymidine uptake and cell number in a eutrophic lake. The deficient nutrient varied over each season and between seasons. During June 1988, the nutrient which stimulated the greatest bacterioplankton activity was PO_4^{-3}. DOC was most stimulating in August and October 1988. During June 1989, DOC and NH_4^+ showed the greatest stimulation whereas in August and October 1989 PO_4^{-3}, NH_4^+, and DOC enrichments all stimulated bacterioplankton thymidine uptake and cell numbers. Inorganic nutrient enrichments appeared to stimulate bacterioplankton activity both directly, and indirectly via phytoplankton products.

References

Bjornsen, P. K., B. Riemann, J. Pock-Steen, T. G. Nielsen, and S. J. Horsted. 1989. Regulation of bacterioplankton production and cell volume in a eutrophic estuary. Appl. Environ. Microbiol. 55:1512-1518.

Brown, E. J., D. K. Button, and D. S. Lang. 1981. Competition between heterotrophic and autotrophic microplankton for dissolved nutrients. Microb. Ecol. 7:199-206.

Chrzanowski, T. H., and J. G. Hubbard. 1989. Bacterial utilization of alga extracellular products in a southwestern reservoir. Hydrobiologia 179:61-71.

Cole, J. J., S. Findlay, and M. L. Pace. 1988. Bacterial produc tion in fresh and saltwater ecosystems: across-system over view. Mar. Ecol. Progr. Ser. 43:1-10.

D'Elia, C. F., P. A. Steudler, and N. Corwin. 1977. Determination of total nitrogen in aqueous samples using persulfate digestion. Limnol. Oceanogr. 22:760-764.

Downes, M. T.. 1978. An automated determination of low reactive phosphorus concentrations in natural waters in the presence of arsenic, silicon acid and mercuric chloride. Water Research 12:743-745.

Eppley, R. 1978. Nitrate uptake. In J. A. Hellebust and J. S. Craigie, (eds.). Handbook of Phyological Methods. Physiological and Biological methods. pp 401-409. Cambridge University Press. Cambridge, England.

Fuhrman, J. A., and F. Azam. 1980. Bcaterioplankton secondary production estimates for coastal water of British Columbia, Antarctic, and California. Appl. Environ. Microbiol. 39: 1085-1095.

Fuhrman, J. A., and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. 66:109-120.

Fuhrman, J. A., J. W. Ammerman, and F. Azam. 1980. Bacterioplankton in the coastal eutrophic zone: distribution, activity and possible relationships with phytoplankton. Mar. Biol. 60: 201-207.

Fuhrman, J. A., S. G. Horrigan, and D. G. Capone. 1988. Use of ^{13}N as tracer for bacterial and algal uptake of ammonium for seawater. Mar. Ecol. Prog. Ser. 45:271-278.

Hobbie, J. E., J. Daley, S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Horrigan, S. G., A. Hagstrom, I. Koike, and F. Azam. 1988. Inorganic nitrogen utilization by assemblages of marine bacteria in seawater culture. Mar. Ecol. Prog. Ser. 50: 147-150.

Linley, E., and R. C. Newell. 1984. Estimates of bacterial growth yields based on plant detritus. Bull. Mar. Sci. 35:409-429.

Lorenzen, C. J. 1967. Determination of chlorophyll and phaeopigments: spectrophometric equations. Limnol. Oceanogr. 12:343-351.

Marvalin, O., L. Aleya, and H. J. Hartman. 1989. Coupling of the seasonal patterns of bacterioplankton and phytoplankton in an eutrophic lake. Can. J. Microbiol. 35:706-712.

Parker, R. R., J. Sibert, and T. J. brown. 1975. Inhibition of primary productivity through heterotrophic competition for nitrate in a stratified estuary. J. Fish. Res. Board Can. 32:409-425.

Priscu, J. C., and M. T. Downes. 1985. Relationship among nitrogen uptake, ammonium oxidation and nitrous oxide concentration in the coastal waters of western Cook Strait. New Zealand. Estuarine, Coastal and Shelf Science 20:529-542.

Rhee, G.. 1972. Competition between an alga and aquatic bacterium for phosphate. Limnol. Oceanogr. 17:505-514.

Robarts, R. D., and R. J. Wicks. 1990. Heterotrophic bacterial production and its dependence on autotrophic production in a hypertrophic African reservoir. Can. J. Fish. Aquat. Sci. 47:1027-1037.

Solorzano, L.. 1969. Determination of ammonia in natural water by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

Solorzano, L., and J. H. Sharp. 1980. Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol.Oceanogr. 25:754-758.

Stainton, M. P., M. J. Capel, and F. A. J. Armstrong. 1977. The chemical analysis of fresh water. Misc. Special Publ. No. 25. Fisheries and Environment Canada.

Strickland, J. D., and T. R. Parsons. 1972. A practical handbook of seawater analysis. Bull. Fish. Res. Board Can. (2nd ed.).

Vadstein, O., and A. Jensen. 1988. Growth and phosphorus status of limnetic phytoplankton and bacteria. Limnol. Oceanogr. 33:489-503.

Vadstein. O., and Y. Olsen. 1989. Chemical composition and phosphate uptake kinetics of limnetic bacterial communities cultured in chemostats under phosphorus limitation. Limnol. Oceanogr. 34:939-946.

Wheeler, P. A.. 1985. Nutrients. P.53-66, In: M. M. Littler and D. S. Littler (eds.), Handbook of phycological methods; Ecological field methods: Macroalgae. Cambridge.

Wheeler, P. A., and D. L.Kirchman. 1986. Utilization on inorganic and organic nitrogen by bacteria in marine systems. Limnol. Oceanogr. 31:998-1009.

Table 1. Nutrient additions ($\mu g\ L^{-1}$ for NH_4^+ and PO_4^{-3}, $mg\ L^{-1}$ for mannitol) for microcosm experiments in the Grayling Arm of Hebgen Lake. NA = not incorporated into the experiment, see Table 2 for ambient concentrations before each addition.

	1988			1989		
Treatment	Jun 22	Aug 21	Oct 23	June 20	Aug 8	Oct 19
Control	0	0	0	0	0	0
NH_4^+-N	100	100	100	140	140	140
PO_4^{-3}-P	50	50	50	93	93	93
NH_4^+-N &	NA	NA	NA	140	140	140
PO_4^{-3}-P	NA	NA	NA	93	93	93
Mannitol	91	91	91	91	91	91
NH_4^+-N &	NA	NA	NA	140	140	140
mannitol	NA	NA	NA	91	91	91
PO_4^{-3}-P &	NA	NA	NA	93	93	93
mannitol	NA	NA	NA	91	91	91

Table 2. Initial temperature (^{o}C), chlorophyll *a* (Chl *a*) (g L^{-1}) and nutrient levels (g L^{-1}) for the microcosm experiments in the Grayling Arm of Hebgen Lake. Symbols are defined in "Material and Methods", Phytoplankton refers to the dominant genus representing more than 60% of the biovolume (or the sum of two dominant genera more than 60%). Ana = *Anabaena*, Aph = *Aphanizomenon*.

	1988			1989		
Nutrient	Jun 22	Aug 21	Oct 23	Jun 20	Aug 8	Oct 19
Temperature	17	22	12	16	19	10
Chl. *a*	30.3	15.6	144.5	4.5	5.0	48.5
Phyto-plankton	Ana.	Ana.	Asteri-onella Aph.	Ana.	Fragi-laria Aph.	Aph.
NH_4^+-N	3.8	61.1	12.5	9.9	4.2	6.0
NO_3^--N	2.6	64.0	5.6	10.2	7.9	94.1
TDN	140.0	570.0	390.0	176.0	146.0	255.0
DON	133.7	444.9	371.9	155.9	133.9	155.0
PON	514.8	299.1	1192.2	137.2	74.2	244.9
SRP	2.8	39.1	7.7	18.1	5.1	18.1
TDP	10.4	57.1	22.5	28.0	17.8	38.4
DOP	8.2	18.0	14.8	9.9	12.7	20.2
PP	14.7[a]	166.8[a]	72.1[a]	16.9	9.9	45.7
DOC	2834	7012	7749	2839	6960	6552
POC	3416	2288	7751	761	559	1364
TN:TP	26	4	17	9	8	6
TOC:TN	10	11	10	9	34	16
TOC:TP	249	42	164	80	271	94

a. Data were from surface of lake water within 10 days before the experiment.

Table 3. Effect of nutrients on bacterioplankton and phytoplankton in the microcosm experiments conducted in Grayling Arm of Hebgen Lake. Significance refers to comparison with amended controls by Multi-factor Analysis of Variance. NS=not significant at $p>0.05$, **=significant at $p\leq0.05$, ***=significant at $p\leq0.01$, +=significant increase, -=significant decrease, NA= treatment not included in experiment. Thym=bacterioplankton thymidine uptake; Cell=bacterial cell number; PPR = phytoplankton ^{14}C uptake; Chl=chlorophyll *a*.

	1988												1989											
	June				August				October				June				August				October			
Treatment	Thym	Cell	PPR	Chl	Thym	Cell	PPR	Chl	Thym	Cell	PPR	Chl	Thym	Cell	PPR	Chl	Thym	Cell	PPR	Chl	Thym	Cell	PPR	Chl
NH_4^+	-***	NS	NS	NS	NS	+***	+***	NS	NS	NS	+***	NS	+***	+***	+***	+***	+***	+***	+***	+***	+***	NS	NS	+***
PO_4^{-3}	+***	+***	+***	+***	+***	+***	NS	NS	-***	NS	NS	-**	NS	NS	NS	NS	+***	+***	+***	NS	+***	NS	-***	NS
NH_4^+ & PO_4^{-3}	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+***	+***	+***	+***	+***	+***	+***	+***	+***	+***	NS	NS
Mannitol	-**	NS	NS	NS	+***	NS	-***	NS	+***	+***	-***	-***	+***	+***	NS	NS	+***	+***	NS	NS	+***	+***	-**	NS
NH_4^+ & mannitol	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+***	+***	+***	+***	+***	+***	+***	+***	+***	+***	NS	NS
PO_4^{-3} & mannitol	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	+***	+***	NS	NS	+***	+***	NS	NS	+***	+***	-**	NS

Table 4. Correlation coefficients (r) between bacterioplankton variables (thymidine uptake, bacterial cell number), and phytoplankton variables ($^{14}CO_2$ uptake, chlorophyll *a*) from the microcosm experiments in the Grayling Arm of Hebgen Lake.

		1988						1989					
		June		August		October		June		August		October	
Treatment	Bacterioplankton	PPR	CHL	PPR	CHL	PPR	CHL	PPR	CHL	PPR	CHL	PPR	CHL
Control	Thym	0.93*	0.25	0.11	0.67	-0.47	-0.93*	-0.22	0.46	0.05	-0.20	0.54	0.10
	Cell	0.99*	0.41	-0.44	-0.23	-0.16	-0.89*	0.37	0.59	-0.43	0.28	-0.26	-0.26
NH_4^+	Thym	0.87	-0.72	0.52	0.83	-0.32	-0.21	0.88*	0.99*	-0.51	-0.82	-0.11	-0.22
	Cell	0.64	-0.40	0.42	0.22	0.21	0.49	0.50	0.60	-0.73	-0.94*	0.59	-0.25
PO_4^{-3}	Thym	0.11	-0.27	-0.40	0.55	-0.26	-0.64	0.58	0.51	-0.10	-0.61	0.67	-0.02
	Cell	0.08	0.17	-0.21	-0.43	0.52	-0.65	-0.47	-0.73	-0.67	-0.39	0.89*	0.47
NH_4^+ & PO_4^{-3}	Thym	NA	NA	NA	NA	NA	NA	0.21	0.31	0.80	0.44	0.32	0.68
	Cell	NA	NA	NA	NA	NA	NA	0.12	0.04	-0.40	-0.72	-0.38	0.61
Mannitol	Thym	-0.92*	-0.98*	-0.07	0.90*	0.22	-0.61	0.01	-0.64	0.08	-0.29	-0.16	-0.08
	Cell	0.51	-0.49	0.02	0.89*	0.29	-0.72	-0.35	0.18	-0.35	0.17	-0.34	0.01
NH_4^+ & mannitol	Thym	NA	NA	NA	NA	NA	NA	0.85	0.19	0.24	-0.89*	-0.33	0.75
	Cell	NA	NA	NA	NA	NA	NA	0.18	-0.17	-0.59	-0.71	-0.55	0.86
PO_4^{-3} & mannitol	Thym	NA	NA	NA	NA	NA	NA	-0.85	-0.39	-0.27	-0.67	-0.70	-0.31
	Cell	NA	NA	NA	NA	NA	NA	-0.62	0.28	-0.63	-0.84	-0.67	-0.21

PPR=phytoplankton ^{14}C uptake; CHL=chlorophyll *a*; Thym=bacterioplankton thymidine uptake; Cell=bacterial cell number; NA=treatment not included in experiment; *=significant correlation at $p \leq 0.05$.

Figure Captions

Figure 1. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of June 1988. ○ control, ● NH_4^+, △ PO_4^{-3}, ▲ mannitol enrichments. Point is the mean of 3 observations ± 1 SE.

Figure 2. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of August 1988. The symbols are the same as in Fig. 1.

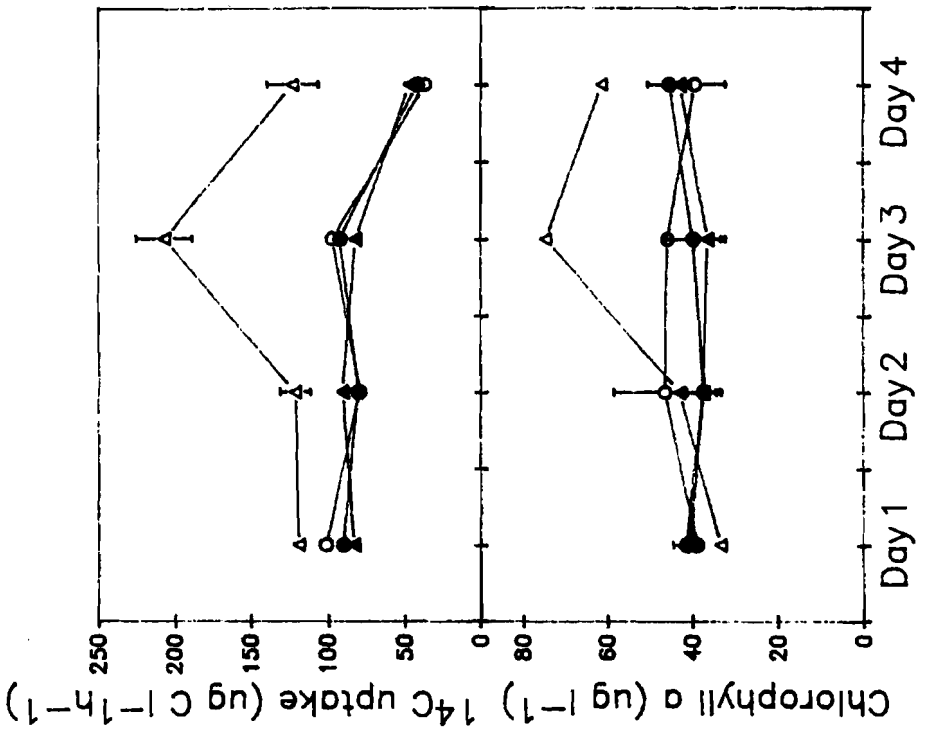
Figure 3. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of October 1988. The symbols are the same as in Fig. 1.

Figure 4. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of June 1989. ○ control, ● NH_4^+, △ PO_4^{-3}, ▲ NH_4^+ plus PO_4^{-3}, □ mannitol, mannitol plus NH_4^+, ■ and mannitol plus PO_4^{-3}. Point is the mean of 3 observations ± 1 SE.

Figure 5. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of August 1989. The symbols are the same as in Fig. 4.

Figure 6. Bacterioplankton thymidine uptake, bacterial cell number, phytoplankton ^{14}C uptake, and chlorophyll *a* concentration in the experiment of October 1989. Symbols are the same as in Fig. 4.

Fig. 1


Chlorophyll a (ug l−1)
14C uptake (ug C l−1h−1)
Day 1
Day 2
Day 3
Day 4

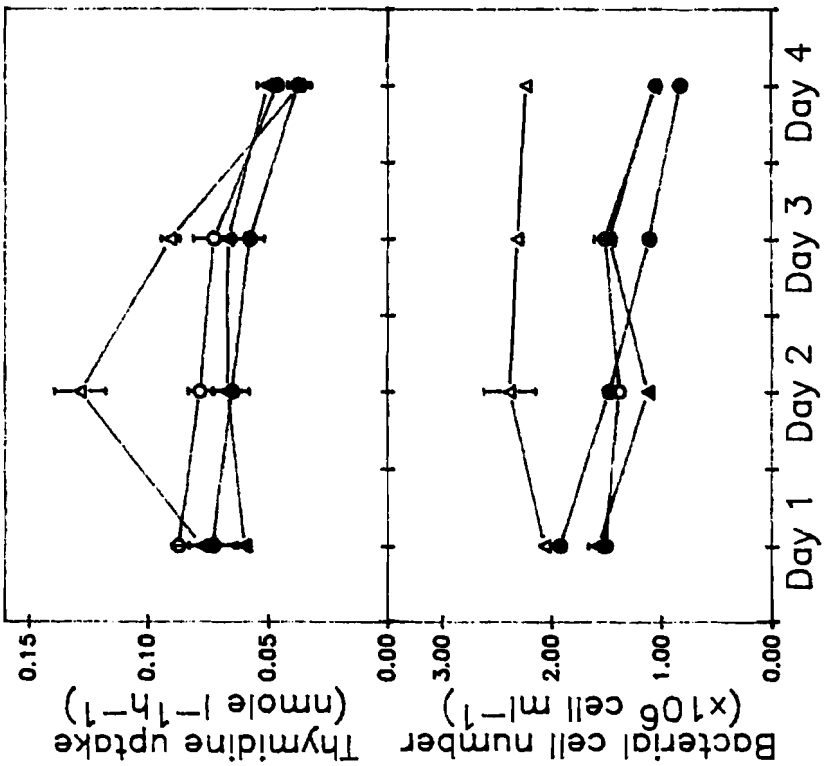
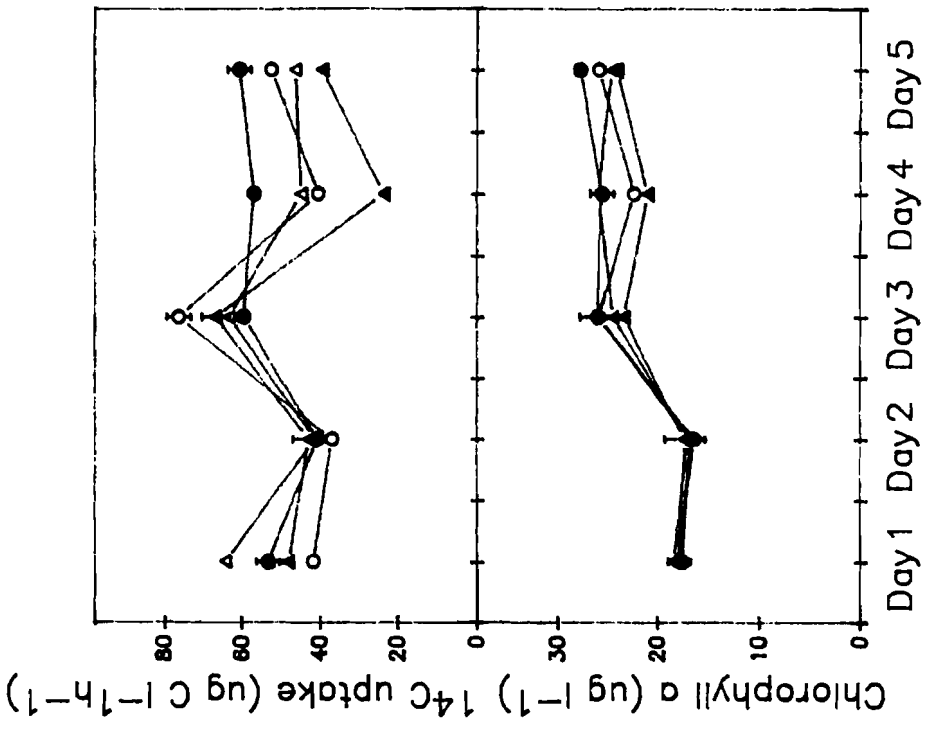

Bacterial cell number (x10⁶ cell ml−1)
Thymidine uptake (nmole l−1h−1)
Day 1
Day 2
Day 3
Day 4

Fig. 2


Chlorophyll a (ug l−1)
14C uptake (ug C l−1h−1)
Day 1
Day 2
Day 3
Day 4
Day 5

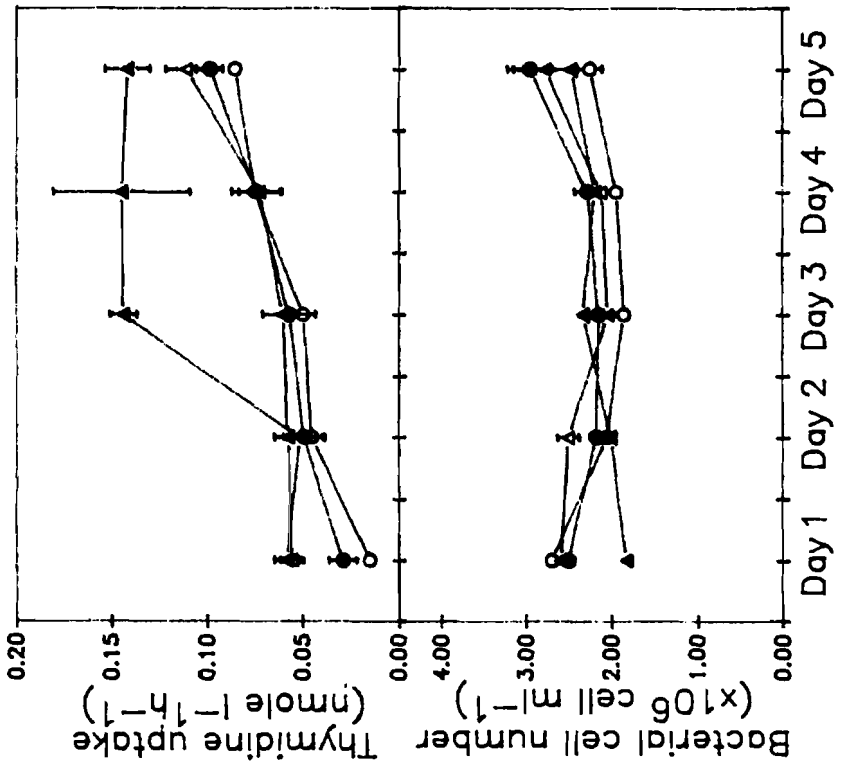

Bacterial cell number (x10⁶ cell ml−1)
Thymidine uptake (nmole l−1h−1)
Day 1
Day 2
Day 3
Day 4
Day 5

Fig. 3

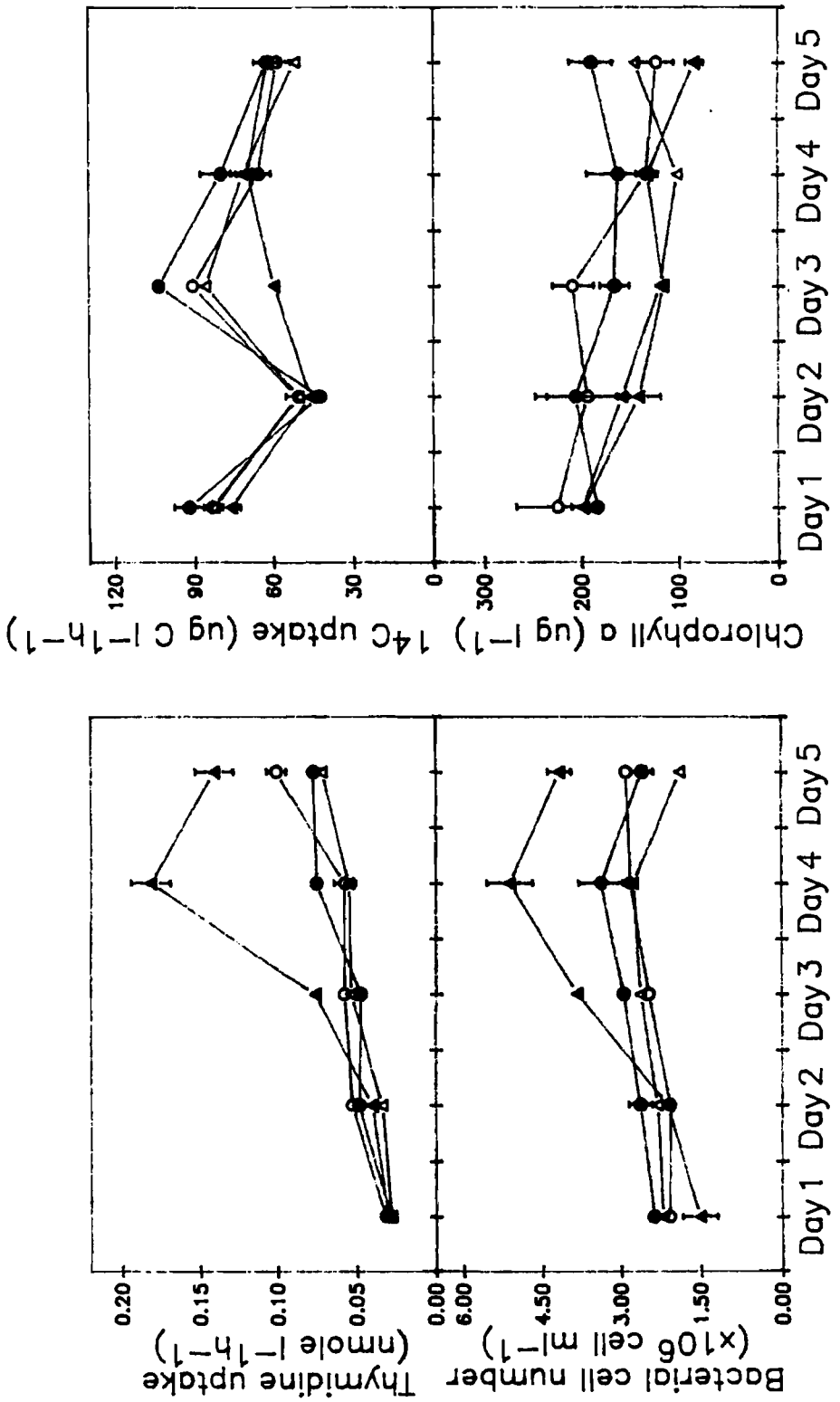
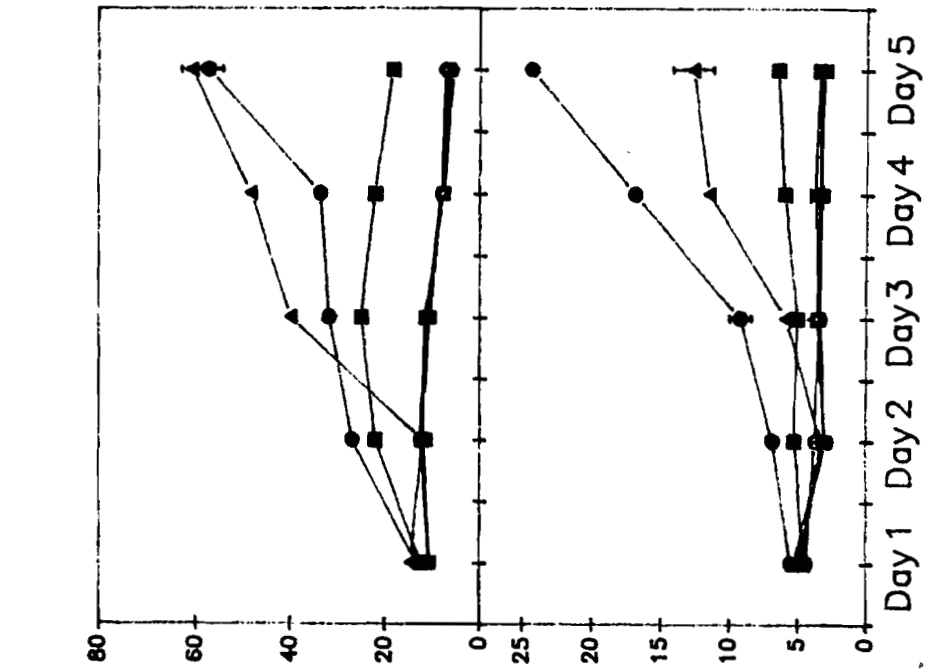

Bacterial cell number (x10⁶ cell ml⁻¹)
Thymidine uptake (nmole l⁻¹h⁻¹)
Chlorophyll a (ug l⁻¹)
¹⁴C uptake (ug C l⁻¹h⁻¹)
Day 1
Day 2
Day 3
Day 4
Day 5

Fig. 4


Chlorophyll a (ug l−1)
14C uptake (ug C l−1h−1)
Day 1
Day 2
Day 3
Day 4
Day 5

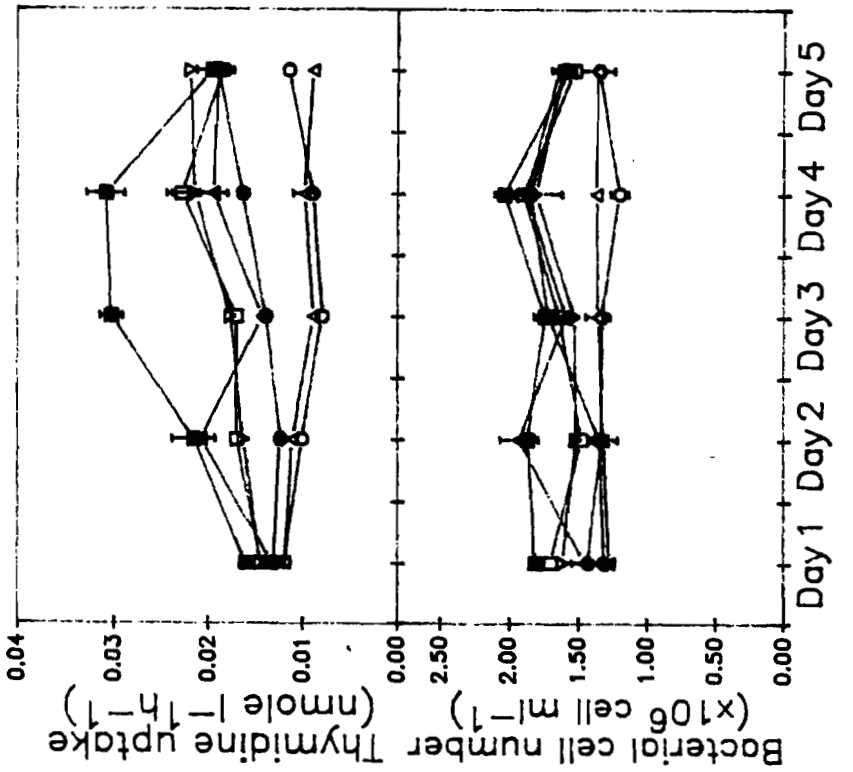

Bacterial cell number (x10⁶ cell ml−1)
Thymidine uptake (nmole l−1h−1)
Day 1
Day 2
Day 3
Day 4
Day 5

Fig. 5

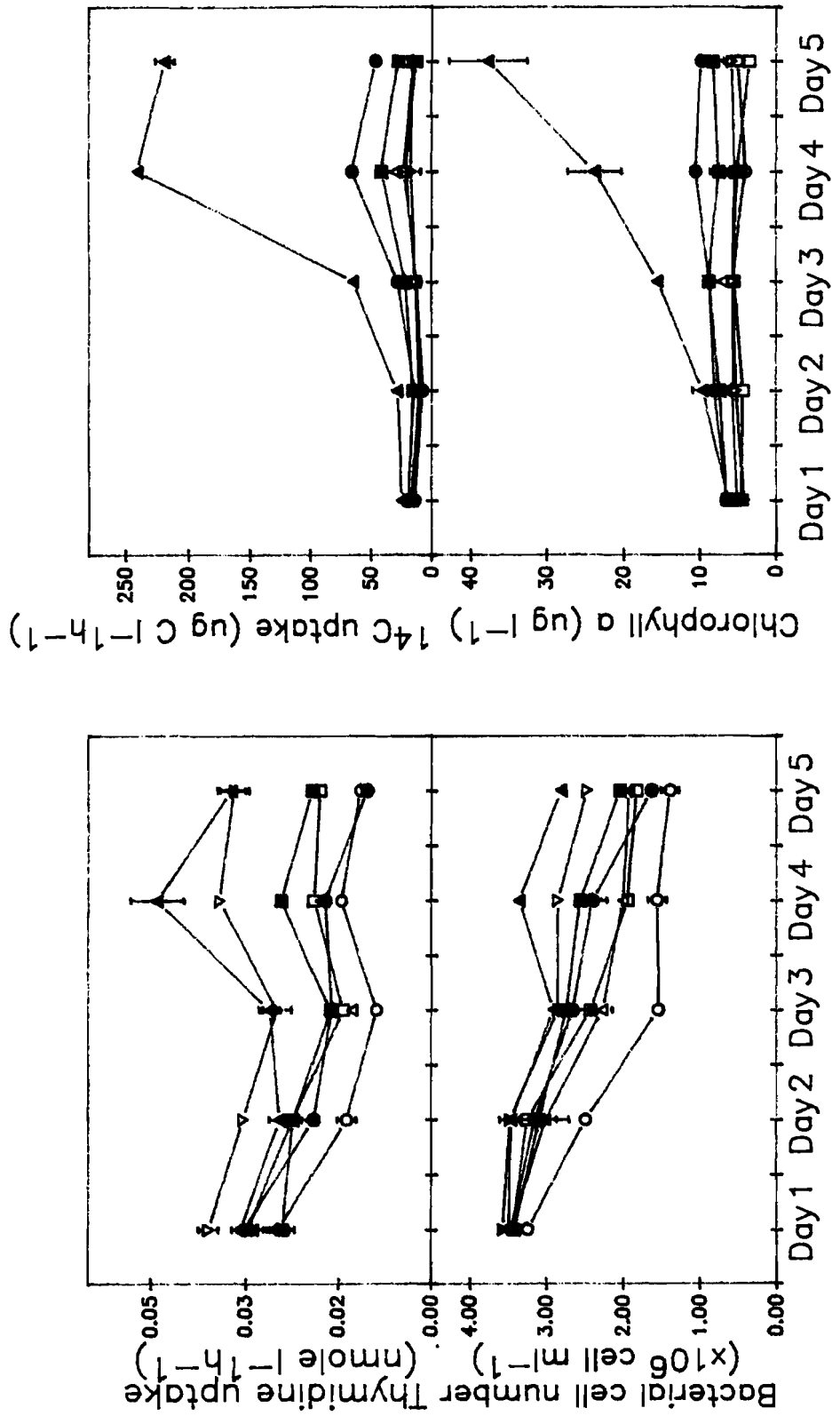
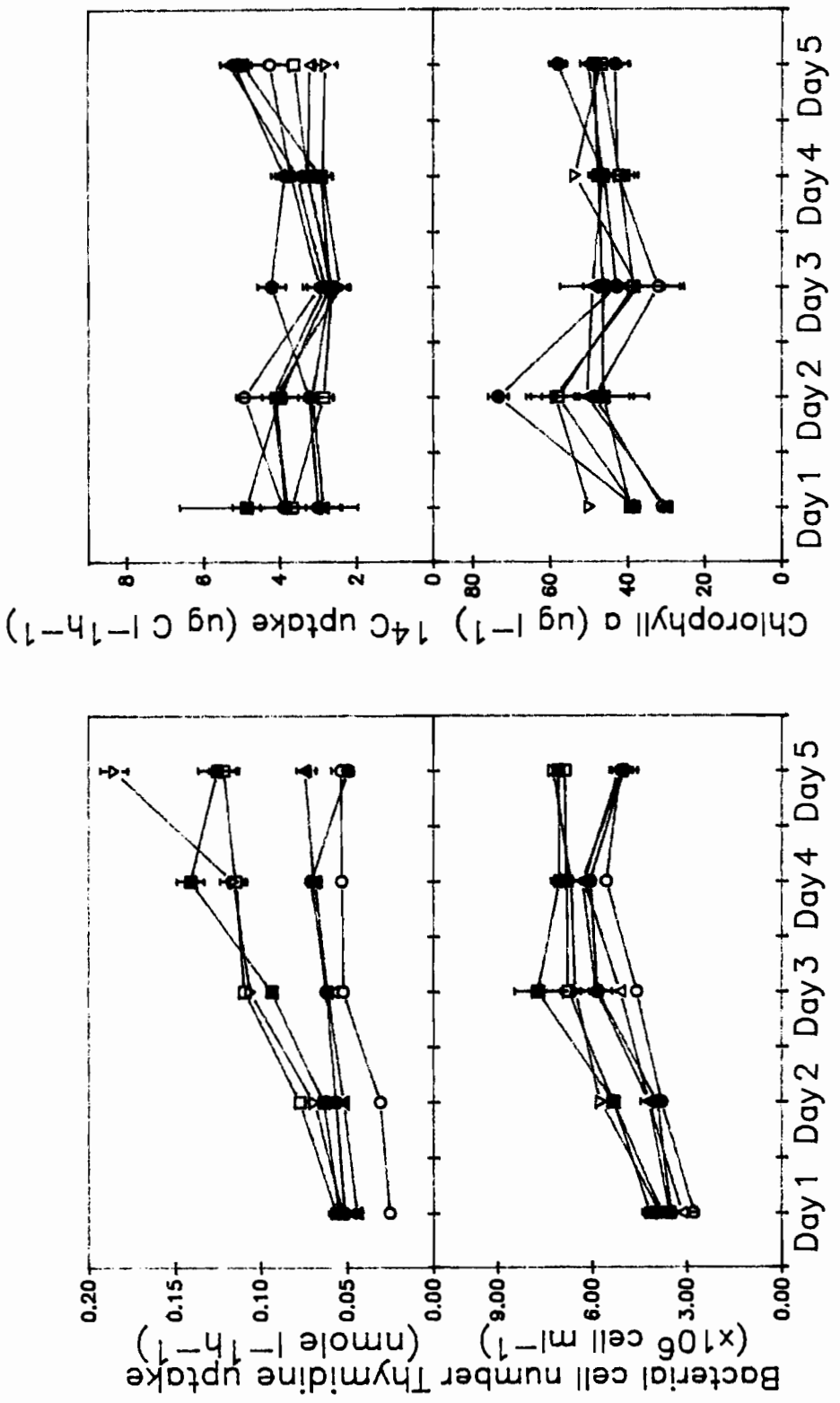

Chlorophyll a (ug l−1)
14C uptake (ug C l−1h−1)
Day 1
Day 2
Day 3
Day 4
Day 5
Bacterial cell number (x10⁶ cell ml−1)
Thymidine uptake (nmole l−1h−1)

Fig. 6


Bacterial cell number (x10⁶ cell ml⁻¹)
Thymidine uptake (nmole l⁻¹h⁻¹)
Chlorophyll a (ug l⁻¹)
¹⁴C uptake (ug C l⁻¹h⁻¹)
Day 1
Day 2
Day 3
Day 4
Day 5

CHAPTER 8

RESPONSE OF BACTERIOPLANKTON PRODUCTION TO INORGANIC NITROGEN AND PHOSPHORUS IN THE PRESENCE AND ABSENCE OF PHYTOPLANKTON

(Part of a Ph.D. dissertation: Lizhu Wang)

RESPONSE OF BACTERIOPLANKTON PRODUCTION TO INORGANIC NITROGEN AND PHOSPHORUS IN THE PRESENCE AND ABSENCE OF PHYTOPLANKTON

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Abstract

Laboratory experiments were conducted to test the effects of nutrient enrichment on bacterioplankton growth in the presence and absence of phytoplankton photosynthesis. In the first series of experiments, bacterioplankton growth, measured as ^{3}H-thymidine uptake and change in cell number, was greater in unfiltered lake water than in water from which phytoplankton had been removed, regardless of the nutrient enrichments (+140 μg NH_4^+-N l^{-1}, +93 μg PO_4^{-3}-P l^{-1}, +140 μg NH_4^+-N l^{-1} plus 93 μg PO_4^{-3}-P l^{-1}, +91 mg mannitol l^{-1}). Bacterioplankton growth in the mannitol enrichment was greater than in other enrichments in both filtered and unfiltered lake water. In the second series of experiments, bacterioplankton growth in unfiltered lake water was greater than in 2 treatments designed to inhibit photosynthetic activity (+DCMU and dark), regardless of nutrient enrichment (+140 μg NH_4^+-N l^{-1}, +93 μg PO_4^{-3}-P l^{-1}, +140 μg NH_4^+-N l^{-1} plus 93 μg PO_4^{-3}-P l^{-1}, +90 mg glucose l^{-1}). Bacterioplankton response to nutrient addition was greatest in the NH_4^++PO_4^{-3} enrichment under all 3 conditions (lake water, lake water amended with DCMU, and lake water incubated in the dark). These experiments indicate that bacterioplankton growth is enhanced in the presence of phytoplankton photosynthesis.

Introduction

Bacterioplankton have long been assumed to be responsible for nutrient recycling in both freshwater and marine ecosystems (Wetzel 1982). Several recent studies have suggested that bacterioplankton might also be significant consumers of inorganic nutrients and may compete with phytoplankton for certain elements. Direct uptake of inorganic nutrients by bacterioplankton has been reported in chemostat experiments (Horrigan et al. 1988; Vadstein and Olsen 1989) and in nutrient tracer studies (Pearl and Lean 1976; Wheeler and Kirchman 1986; Fuhrman et al. 1988). Field studies have also indicated that bacterioplankton may be limited by inorganic nutrients and may compete directly with phytoplankton for their uptake (Parker et al. 1975; Vadstein and Jensen 1988; Wehr 1989).

Our study was conducted to determine whether nutrient stimulation of bacterioplankton growth is affected by the presence of phytoplankton. Two types of experiments were designed to test this hypothesis. In the first, bacterioplankton response to nutrient enrichment was compared in unfiltered lake water and in lake water from which phytoplankton were removed. In the second, bacterioplankton growth in treatments where photosynthesis was inhibited was compared with growth in lake water with a photosynthetically viable phytoplankton assemblage. All treatments were subjected to both organic or inorganic enrichment.

Material and Methods

Experiments were conducted with water collected from Hebgen Lake, a 50 km^2 eutrophic reservoir located on the upper Madison River 33 km west of Yellowstone National Park. At the time of our experiments, 22 September 1989 (experiment 1) and 19 June 1990 (experiment 2), this lake had chlorophyll *a* concentration of 1.1 and 181.3 $\mu g\ l^{-1}$; soluble reactive phosphorus (SRP) and NH_4^+-N of 8 and 10, and 9 and 11 $\mu g\ l^{-1}$; and dissolved organic carbon (DOC) of 75.4 and 66.6 mg l^{-1}, respectively. In both experiments, 900 ml lake water was placed in autoclaved 1-l flasks on a G10 Gyrotory Shaker set at 60 rpm. A photon flux density of 120 $\mu E\ m^{-2}\ s^{-1}$ was provided by 40 W cool white fluorescent lamps. Water temperature was maintained at approximately 25 ^oC.

Experiment 1.

Two treatments, one with and one without phytoplankton, were used in experiment 1. Water was filtered through 1.0 μm membrane filters, then examined under an epifluorescence microscope to verify the absence of algae in phytoplankton-free treatments. In treatments with phytoplankton, water was filtered through 280 μm size Nitex mesh to remove larger grazers. In both treatments (with and without phytoplankton), 5 initial nutrient levels were established: control (ambient levels), +140 $\mu g\ NH_4^+$-N l^{-1}, +93 $\mu g\ PO_4^{-3}$-P l^{-1}, +140 $\mu g\ NH_4^+$-N plus 93 $\mu g\ l^{-1}\ PO_4^{-3}$-P l^{-1}, and 91 mg mannitol l^{-1}.

Concentrations of NH_4^+, SRP, DOC, and chlorophyll *a* were measured in each flask at the start of the incubation, on day 1 and at the end of the experiment on day 5. NH_4^+ was determined by the phenol hypochlorite method (Solorzano 1969), and SRP by the

molybdate method (Downes 1978). DOC was analyzed using a Dorhmann Carbon Analyzer. Chlorophyll a was measured by initial extraction in 95% ethanol heated to boiling (79 ^{o}C) and extracted overnight at 4 ^{o}C (Sartory and Grobbelaar 1984). Fluorescence of the extract was measured with a Turner model 112 fluorometer (Lorenzen 1965) and compared to a chlorophyll a standard curve made using pure Anacystis chlorophyll a (Sigma Chemical Co.).

Bacterial ^{3}H-thymidine uptake and cell number were measured 4 h after starting the incubation, and on day 1, 3, and 5. Bacterial thymidine uptake was determined by adding high activity (55 Ci mmole^{-1}) methyl-^{3}H thymidine (ICN Radiochemical INC.) to 10 ml water samples (final concentration 10 nM) in 20 ml glass scintillation vials. ^{3}H-thymidine was evaporated to dryness and rehydrated with deionized water before use to eliminate products of self radiolysis and to remove ethanol. The inoculated sample was incubated at 25 ^{o}C in the dark for 30 minutes. Activity was terminated by adding 10 ml ice-cold 10% trichloroacetic acid (TCA) to each vial. Following overnight extraction at 4 ^{o}C, samples were filtered onto 0.2 μm membrane filters (Poretics Corporation). After rinsing 5 times (2 ml each rinse) with ice-cold 5% TCA, the filter was transferred to a 20 ml polyethylene scintillation vial with 7.0 ml Cytoscint scintillation cocktail (ICN Radiochemical, Irvine, CA). Radioactivity in each sample was determined by standard scintillation spectrometry using a Beckman LS-100C liquid scintillation counter. Counting efficiency was determined by the external standard ratio method using ^{3}H-toluene as reference and acetone as the quenching agent. Samples for

bacterial counts were fixed with formaldehyde (3% final concentration) and stored at 4 ^{o}C. Bacterial cell counts were made using the acridine orange direct count technique (Hobbie et al. 1977) with a Nikon epifluorescence microscope.

Experiment 2.

Three treatments (lake water, lake water + photosynthetic inhibitor, and lake water in the dark) were used in the second experiment. All water was first filtered through 280 μm size Nitex mesh to remove large zooplankton. Photosynthetic inhibitor, 3-(3,4-Dichlorophyenyl) 1, 1-Dimethyl urea (DCMU), was added to a final concentration of $5x10^{-6}$ M in the DCMU-amended treatment. Flasks in the darkened treatment were covered with 2 layers of aluminum foil. All 3 treatments were subjected to 5 nutrient levels: control (ambient levels), +140 μg NH_4^+-N l^{-1}, +93 μg PO_4^{-3}-P l^{-1}, +140 μg NH_4^+-N plus 93 μg PO_4^{-3}-P l^{-1}, and +90 mg glucose l^{-1}. DOC and chlorophyll a concentrations were measured 4 h after the start of incubation, and on day 1, 3, and 5; SRP and NH_4^+ were analyzed before incubation and on day 1 and 5; bacterial thymidine uptake and cell number were measured daily over the entire 5 days of the experiment. Analytical methods were the same as described for Experiment 1.

Results

Experiment 1

At the time of our experiment, the dominant phytoplankton species in the unfiltered treatment were the Cyanobacterium Anabaena spiroides (69%) and the Chrysophyta Ochromonas sp. (13%) in biovolume. The chlorophyll a concentration in the lake was about 3.5 μg l^{-1}.

Bacterial thymidine uptake and cell number in all nutrient enrichments with phytoplankton present, except mannitol, were significantly higher ($p<0.05$) than in the same enrichment with phytoplankton absent (Fig. 1). Average thymidine uptake and bacterial cell number were 1.8 and 1.2 times higher in the treatment with phytoplankton than in the treatment without phytoplankton.

All nutrient enrichments, except PO_4^{-3}, significantly ($p<0.01$) increased bacterial thymidine uptake and bacterial cell numbers in both treatments with and without phytoplankton compared to the control (Fig. 1). The most significant increase in bacterial thymidine uptake in both treatments occurred in the mannitol enrichments.

Concentration of DOC on day 5 was higher in the treatment with phytoplankton than in the treatment without phytoplankton when individual nutrient enrichments were compared (Table 1). Concentration of NH_4^+ on day 5 in the treatment with phytoplankton in control, NH_4^+, PO_4^{-3}, and NH_4^+ plus PO_4^{-3} enrichment was 5.8, 2.4, 4.3, and 8.5 times lower than those in the treatment without phytoplankton when the same nutrient enrichments were compared (Table 1). Concentration of SRP on day 5 in treatments with phytoplankton was about one half of that in the treatments without phytoplankton when the same nutrient enrichments were compared (Table 1). The highest chlorophyll *a* increase in the treatment with phytoplankton occurred in the PO_4^{-3} plus NH_4^+ enrichment, followed by the NH_4^+ and mannitol enrichments. Chlorophyll *a* in the treatment without phytoplankton was always

undetectable (Table 1).

Experiment 2.

The dominant phytoplankton in the lake at the time of our experiment were the Schroederia sp. (48%) and Closterium sp. in biovolume. Chlorophyll a concentration was 178 $\mu g\ l^{-1}$.

Bacterial thymidine uptake and bacterial cell numbers of PO_4^{-3} and PO_4^{-3} plus NH_4^+ enrichments were significantly higher ($p<0.05$) in the treatment with active photosynthesis than in treatments where photosynthesis was inhibited (i.e. DCMU, dark). There were no statistical difference between treatments with and without inhibition of photosynthesis on bacterial thymidine uptake and bacterial cell number in controls, NH_4^+, and glucose enrichments (Figs. 2, 3, 4). Bacterial thymidine uptake and bacterial cell numbers showed no significant difference between treatments amended with DCMU and incubated in the dark in any the nutrient enrichments except that the bacterial cell number of the NH_4^+ enrichment was lower ($p<0.05$) in the treatment amended with DCMU than that incubated in the dark (Fig. 3, 4).

The PO_4^{-3} and PO_4^{-3} plus NH_4^+ enrichments significantly ($p<0.01$) increased bacterial thymidine uptake and bacterial cell number compared with controls in all 3 experimental treatments. The NH_4^+ enrichment significantly increased bacterial thymidine uptake in the treatment incubated in the dark, and bacterial thymidine uptake and bacterial cell number in the treatment amended with DCMU, relative to the control ($p<0.01$). The NH_4^+ enrichment in the unamended lake water treatment had no significant ($p>0.1$) effect on bacterial thymidine uptake and cell number compared with the control. Bacterial thymidine uptake and bacte-

rial cell number in the glucose enrichments were not significantly ($p>0.1$) different than the controls in all 3 experimental treatments (Fig. 2, 3, 4). The most significant increase in bacterial thymidine uptake and bacterial cell number occurred in the PO_4^{-3} plus NH_4^+ enrichment in all 3 experimental treatments (i.e. photosynthetic regimes) ($p<0.01$).

Chlorophyll *a* in NH_4^+ and NH_4^+ plus PO_4^{-3} enrichments increased more than 3-fold from day 0 to day 5 (Fig. 5a). In the other nutrient enrichments, chlorophyll *a* initially increased about 2-fold, but did not increase after day 3. Chlorophyll *a* in treatments where photosynthesis was inhibited changed relatively little ($p>0.1$) (Fig. 5b,c). DOC in PO_4^{-3} and PO_4^{-3} plus NH_4^+ enrichments was lowest in all 3 experimental treatments except the PO_4^{-3} enrichment in the photosynthetically active treatment (Fig. 6). Concentrations of NH_4^+ were lower (5 to 16 times) in the PO_4^{-3} plus NH_4^+ enrichment than in NH_4^+ enrichment alone under all photosynthetic regimes although the same amount of NH_4^+ was added (Table 2). SRP on day 5 was lowest in NH_4^+ enrichments with photosynthetic inhibition compared with the other nutrient enrichments (Table 2).

Discussion

Our results indicate that bacterioplankton growth is enhanced in the presence of photosynthetically active phytoplankton even under apparent non-DOC limited conditions. In a study of algal-bacterial biofilms, Murray et al. (1986) found that bacterial thymidine uptake was 4 to 16 fold greater in the presence of

phytoplankton than in their absence. When bacteria were incubated with algae in an arctic river, Peterson et al. (1985) found that the bacterial activity incubated in light was significantly higher than in dark. These findings are consistent with our results implying a syntrophic relationship between these organisms.

Our study demonstrates that bacterioplankton activity and cell number in Hebgen Lake can be stimulated directly by inorganic N and P addition without a substantial effect of phytoplankton. Approaches used in our study were to eliminate phytoplankton by filtration and to inhibit photosynthesis with DCMU and dark incubation. The undetectable chlorophyll _a_ in the first approach and the statistically unchanged chlorophyll _a_ concentration in the second approach indicate that such methods are fairly successful in minimizing phytoplankton effects. When phytoplankton are excluded, the limiting nutrients for bacterial growth were DOC and NH_4^+ in our first experiment and NH_4^+ plus PO_4^{-3} in our second experiment. The difference between our two experiments may be due to background levels of total N and P, and organic carbon. Such difference may also be caused by different species of bacteria and phytoplankton between the 2 experiments. Recent findings that bacterioplankton are net consumers of inorganic N and P in both marine and freshwater (Wheeler and Kirchman 1986; Horrigan et al. 1988; Fuhrman et al. 1988; Vadstein and Olsen 1989) corroborate our results. Such findings indicate that bacterioplankton may respond to inorganic N and P as well as DOC in aquatic ecosystems.

There are two common ideas concerning the relationship among nutrients, bacterioplankton and phytoplankton. The first assumes that phytoplankton are P or N limited, while bacterioplankton are organic C limited. Therefore P or N determines phytoplankton abundance, which in turn can determine bacterioplankton abundance via extracellular release of DOC. The second idea assumes that both bacterioplankton and phytoplankton are P or N limited, and organic C limitation on bacterial growth is switched on and off depending upon the abundance of phytoplankton, which results a bacterioplankton-phytoplankton competition or dependency relationship. The latter idea, proposed by Currie (1990), postulates that inorganic P or N can directly influence both algal and bacterial abundance, with a concomitant direct interaction between algae and bacteria.

It has been found that bacteria are often more efficient competitors for inorganic nutrients than algae (Parker et al. 1975; Parson et al. 1981; Currie and Kalff 1984; Wehr 1989). Bacteria cannot outcompete phytoplankton completely, however, because this would remove their C source (Rhee 1972). In our study, bacterioplankton growth was limited by both organic C and inorganic N (experiment 1), implying that bacterial growth was dependent on phytoplankton products to provide organic C. The N stimulation apparently resulted indirectly from an increase in photosynthetic activity. This contention is consistent with our results that inorganic N and P were lower and organic C was higher in the treatment with phytoplankton than that without phytoplankton, when the same nutrient enrichments were compared. In our second experiment, bacterioplankton and phytoplankton

growth were most consistently limited by inorganic P and secondly by N. This result would indicate a potential for competition between bacteria and phytoplankton for inorganic N and P.

In summary, our experiments showed:

(1) Bacterioplankton grew better when phytoplankton were presence.

(2) limiting nutrients for bacterioplankton growth were DOC and inorganic N in our first experiment and were inorganic P and N in our second experiment.

(3) Inorganic N and P (singly or in combination) increased bacterial activity and abundance directly without involving phytoplankton.

Together, these results indicate that bacterial activity was stimulated directly by the addition of in organic N and P and indirectly from products associated with phytoplankton.

References

Bratbak, G., and T. F. Thingstad. 1985. Phytoplankton-bacteria interactions: an apparent paradox? Analysis of a model system with both competition and commensalism. Mar. Ecol. Prog. Ser. 25:23-30

Currie, D. J. 1990. Large-scale variability and interactions among phytoplankton, bacterioplankton, and phosphorus. Limnol. Oceanogr. 35:1437-1455.

Currie, D. J., and J. Kalff. 1984. A comparison of the abilities of freshwater algae and bacteria to acquire and retain phosphorus. Limnol. Oceanogr. 29:298-310

Downes, M. T.. 1978. An automated determination of low reactive phosphorus concentrations in natural waters in the presence of arsenic, silicon acid,and mercuric chloride. Water research 12:743-745

Fuhrman, J. A., S. G. Horrigan, and D. G. Capone. 1988. Use of ^{13}N as tracer for bacterial and algal uptake of ammonium for seawater. Mar. Ecol. Prog. Ser. 45:271-278

Hobbie, J. E., J. Daley, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228

Horrigan, S. G., A. Hagstrom, I. Koike, and F. Azam. 1988. Inorganic nitrogen utilization by assemblages of marine bacteria in seawater culture. Mar. Ecol. Prog. Ser. 50: 147-150

Laws, E. A., W. G. Harrison, and G. R. DiTullio. 1985. A comparison of nitrogen assimilation rates based on ^{15}N uptake and autotrophic protein synthesis. Deep Sea Res. 32:85-95

Myklested, S., O. Holm-Hansen, K. M. Varum, and B. E. Volcani. 1989. Rate of release of extracellular amino acids abd carbohydrates from the marine diatom Chaetoceros affinis. J. Plankton Res. 11:763-773.

Parker, R. R., J. Sibert, and T. J. Brown. 1975. Inhibition of primary productivity through heterotrophic competition for nitrate in a stratified estuary. J. Fish. Res. Board Can. 32:409-425

Parsons, T. R., L. J. Albright, F. Whitney, C. S. Wong, and P. J. William. 1981. The effect of glucose on the productivity of seawater: An experimental approach using controlled aquatic ecosystems. Mar. Environ. Res. 4:229-242

Pearl, H. W., and D. R. S. Lean. 1976. Visual observations of

phosphorus movement between algae, bacteria, and abiotic particles in lake water. J. Fish. Board Can. 33:2805-2813

Peterson, B. J., J. E. Hobbie, A. E. Hershey, M. A. Lock, T. E. Ford, R. M. Ventullo, and G. S. Volk. 1985. Transformation of a tundra river from heterotrophy to autotrophy by the addition of phosphorus. Science 229:1383-1386.

Rhee, G. 1972. Competition between an alga and aquatic bacterium for phosphate. Limnol. Oceanogr. 17:505-514.

Sartory, D. P., and J. U. Grobbelaar. 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-187

Solorzano, L.. 1969. Determination of ammonia in natural water by the phenol hypochloride method. Limno. Oceanogr. 14: 799-801

Vadstein, O., and A. Jensen. 1988. Growth and phosphorus status of limnetic phytoplankton and bacteria. Limno. Oceanogr. 33:489-503

Vadstein, O., and Y. Olsen. 1989. Chemical composition and phosphate uptake kinetics of limnetic bacterial communities cultured in chemostats under phosphorus limitation. Limno. Oceanogr. 34:939-946

Wehr, J. D.. 1989. Experimental tests of nutrient limitation in freshwater picoplankton. Appl. Environ. microbiol. 55: 1605-1611

Wetzel, R. G. 1982. Limnology. Saunders Publishing, Philadelphia.

Wheeler, P. A., and D. L. Kirchman. 1986. Utilization on inorganic and organic nitrogen by bacteria in marine systems. Limno. Oceanogr. 31:998-1009

Table 1. Chlorophyll a, inorganic, and organic nutrient concentrations in experiment 1.

Nutrient additions	DOC (mg l^{-1})		NH_4^+-N ($\mu g\ l^{-1}$)		SRP ($\mu g\ l^{-1}$)		Chlorophyll a ($\mu g\ l^{-1}$)	
	Day 1	Day 5	Day1	Day 5	Day 1	Day 5	Day 1	Day 5
Unfiltered-water								
Control	62.5	13.1	7	6	7	7	3.5	4.1
NH_4^+	97.1	12.5	83	95	7	7	3.7	6.5
PO_4^{-3}	71.5	215.7	9	7	10	14	3.5	5.7
PO_4^{-3} & NH_4^+	83.4	138.8	74	26	10	10	3.8	9.6
Mannitol	203.0	500.0	6	7	6	2	3.7	7.0
Filtered-water								
Control	132.6	12.5	14	36	13	15	0.0	0.0
NH_4^+	118.6	10.2	119	227	13	14	0.0	0.0
PO_4^{-3}	132.4	8.3	11	30	18	23	0.0	0.0
PO_4^{-3} & NH_4^+	67.7	5.6	98	220	17	23	0.0	0.0
Mannitol	221.9	389.2	12	4	12	3	0.0	0.0

Table 2. The concentrations of NH_4^+ and SRP in the experiment 2.

Nutrient addition	NH_4^+-N (ug l^{-1})						SRP (ug l^{-1})					
	Day 1			Day 5			Day 1			Day 5		
	Algal-set[a]	DCMU-set[b]	Dark-set[c]	Algal-set	DCMU-set	Dark-set	Algal-set	DCMU-set	Dark-set	Algal-set	DCMU-set	Dark-set
Control	2	2	4	2	3	3	7	6	7	9	8	8
NH_4^+	169	160	210	129	157	174	7	7	7	8	7	7
PO_4^{-3}	5	4	6	4	4	6	7	7	7	10	11	9
PO_4^{-3} & NH_4^+	12	22	41	8	8	11	7	7	7	13	11	9
Glucose	2	1	1	2	3	3	7	6	7	8	8	8

a. Unamended lake water treatment
b. Treatment amended with DCMU
c. Treatment incubated in dark

Figure Captions

Figure 1. Bacterial thymidine uptake rate and bacterial cell number in experiment 1. A. Unfiltered lake water treatment. B. Filtered lake water treatment. ○ control, ● NH_4^+, △ PO_4^{3-}, ▲ NH_4^+ plus PO_4^{3-}, □ mannitol. Error bars are ± 1SE (n=3) and are shown only if larger than the symbol.

Figure 2. Bacterial thymidine uptake rate and bacterial cell number of the unamended lake water treatment in experiment 2. ○ control, ● NH_4^+, △ PO_4^{3-}, ▲ NH_4^+ plus PO_4^{3-}, □ glucose. Error bars are ± 1SE (n=3) and are shown only if larger than the symbol.

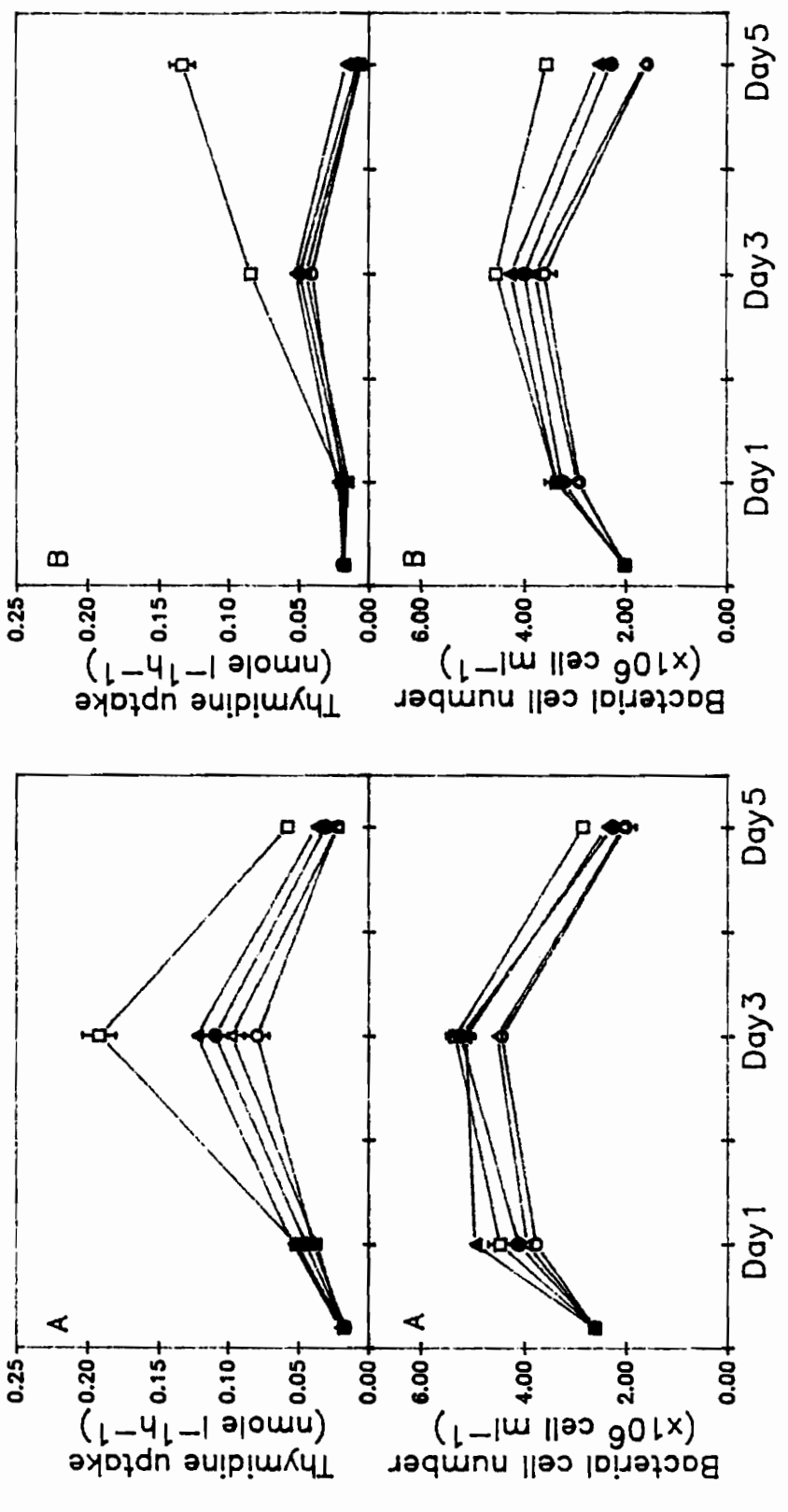
Figure 3. Bacterial thymidine uptake rate and bacterial cell number of the DCMU treatment in experiment 2. The error bars and symbols are the same as in Fig. 2.

Figure 4. Bacterial thymidine uptake rate and bacterial cell number of the dark treatment in experiment 2. The error bars and symbols are the same as in Fig. 2.

Figure 5. The concentration of chlorophyll a of (A) lake water treatment, (B) lake water amended with DCMU, and (C) lake water incubated in dark in experiment 2. The error bars and symbols are the same as in Fig. 2.

Figure 6. The concentration of dissolved organic carbon of (A) lake water treatment, (B) lake water amended with DCMU, and (C) lake water incubated in dark in experiment 2. Error bars and symbols are the same as in Fig. 2.

Fig. 1


A
B
Thymidine uptake
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0.20
0.15
0.10
0.05
0.00
Bacterial cell number
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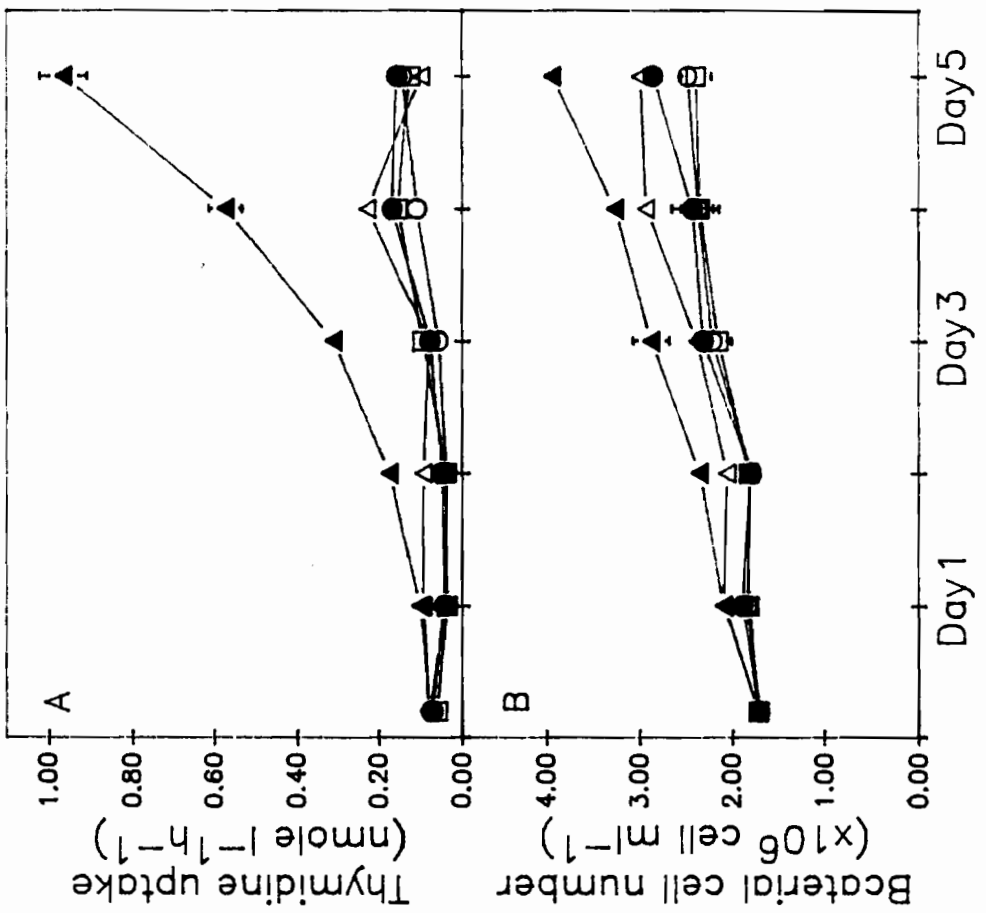
4.00
2.00
0.00
Day1
Day3
Day5

Fig. 2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B
Thymidine uptake
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Bacterial cell number
(x10⁶ cell ml⁻¹)
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0.20
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0.80
1.00
1.00
2.00
3.00
4.00
Day1
Day3
Day5

Fig. 3

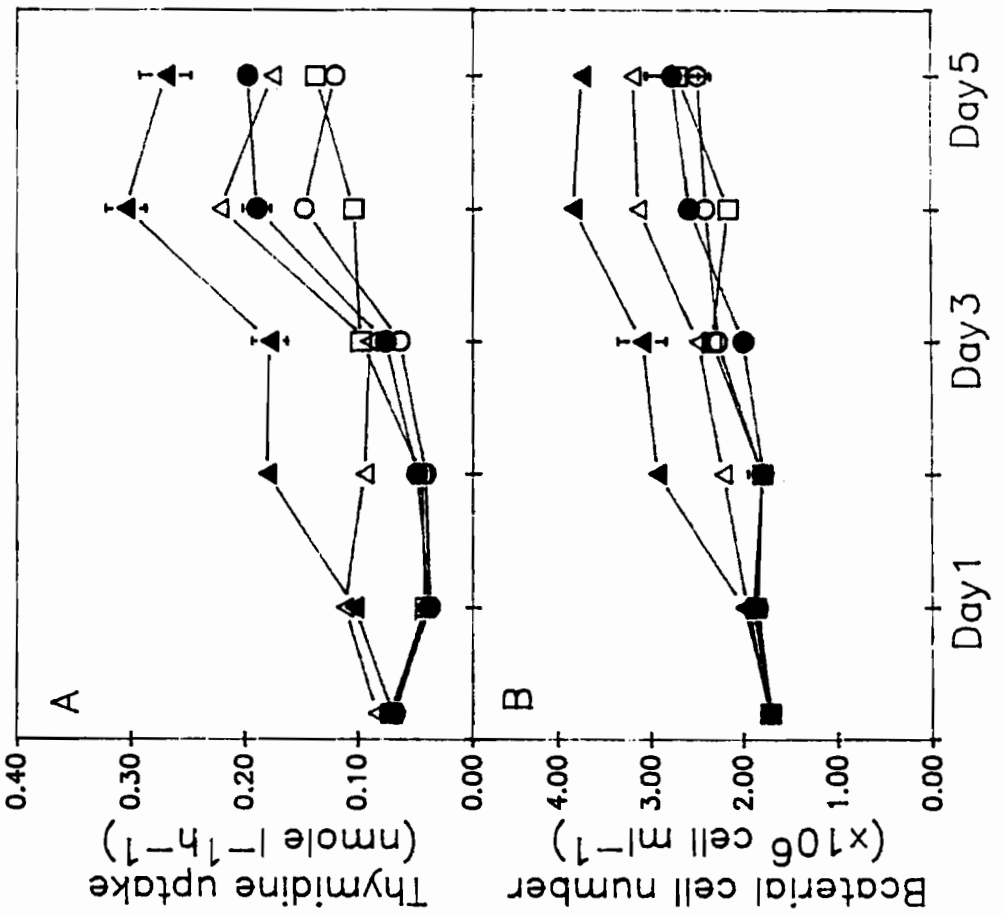
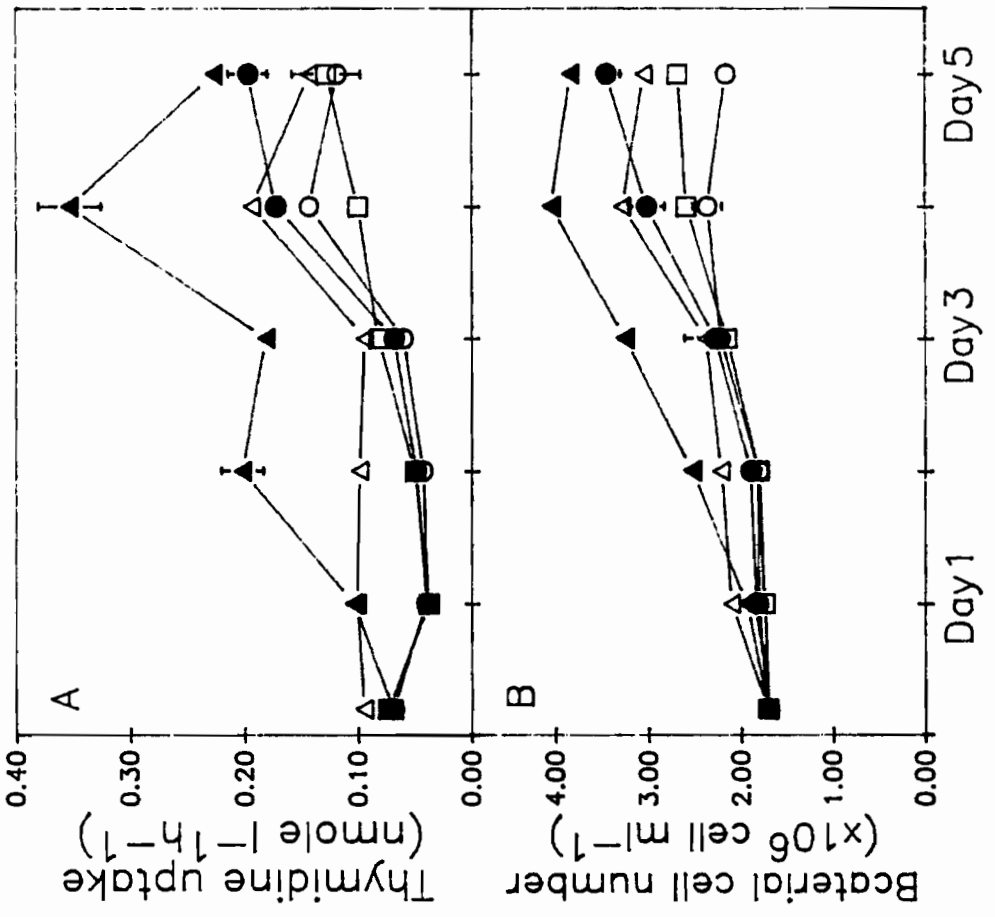

A
B
Thymidine uptake
(nmole l⁻¹h⁻¹)
Bacterial cell number
(x10⁶ cell ml⁻¹)
0.40
0.30
0.20
0.10
0.00
4.00
3.00
2.00
1.00
0.00
Day1
Day3
Day5

Fig. 4


A
B
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0.00
4.00
3.00
2.00
1.00
0.00
Thymidine uptake
(nmole l⁻¹h⁻¹)
Bacterial cell number
(x10⁶ cell ml⁻¹)
Day1
Day3
Day5

CHAPTER 9

EPILIMNETIC BACTERIOPLANKTON PRODUCTION AND ITS RELATION WITH BIOTIC AND ABIOTIC FACTORS IN A EUTROPHIC RESERVOIR

(Part of a Ph.D. dissertation: Lizhu Wang)

EPILIMNETIC BACTERIOPLANKTON PRODUCTION AND ITS RELATION WITH BIOTIC AND ABIOTIC FACTORS IN A EUTROPHIC RESERVOIR

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Abstract

Bacterioplankton numbers and their incorporation of ^{3}H-thymidine into DNA were measured during 2 consecutive ice-free seasons in a eutrophic reservoir. Bacterioplankton number ranged between 0.62 and 2.9 x 10^{6} cell ml^{-1} (average = 1.68 x 10^{6}) while their production varied between 0.09 and 0.82 μg C L^{-1} h^{-1} (average = 0.28). Regression analysis on data from the entire study period showed that the bacterioplankton variables (production, activity/cell, biomass, cell numbers) were most strongly correlated to water temperature ($p<0.01$), chlorophyll *a* ($p<0.05$), and PON ($P<0.05$). Bacterioplankton biomass and cell numbers were also strongly correlated phosphorus variables (TDP, DOP, PP) ($p<0.01$). Factors regulating bacterioplankton activity and biomass were water temperature in spring, TP and TN in summer, and TN and TOC in the fall. River water had higher proportions of TP and TN than TOC, compared with epilimnetic lake water. Bacterioplankton activity and cell numbers were stimulated when lake water was enriched with river water. Our study revealed a close coupling between bacterioplankton production and autotrophic production and temperature. However, low bacterioplankton compared with phytoplankton production (epilimnetic bacterioplankton production averaged 2% of daily phytoplankton production), together with the substantial TOC:TN:TP difference between river water and lake water, and the stimulation of bacterioplankton activity by river water enrichment, suggest that the supply of TP and TN from river water plays an important role in regulating bacterioplankton growth.

The significance of the bacterial microheterotrophic community, both as a potential food resource for larger consumer organisms and in the regeneration of nutrients necessary to support primary production, has attracted considerable attention in recent years. A number of studies have shown a close coupling between heterotrophic bacterial growth or biomass and phytoplankton primary production or chlorophyll a (Fuhrman et al. 1980; Chrzanowski and Hubbard 1989; Marvalin et al. 1989). Other studies have attempted to quantify the transport of organic carbon from phytoplankton to bacterioplankton (e.g.. Cole et al. 1982; Jensen 1983; Jensen and Sondergaard 1985; Feuillade et al. 1988; Chrzanowski and Hubbard 1989; Sundh 1989; Tranvik 1989). It has been suggested that bacterial production is controlled by, or is directly related to, the supply of decomposable matter (Cole et al. 1988), which has been confirmed by the study of a hypertrophic lake (Robarts and Wicks 1989). In studies emphasizing factors controlling bacterial production, Marvalin et al. (1989) found that epilimnetic phytoplankton productivity and temperature played major roles in controlling bacterial development. Bjornsen et al. (1989), studying a eutrophic estuary, found that phytoplankton was the dominate source of bacterial substrate. Gebre-Mariam and Taylor (1989) also found that no significant positive correlation existed between bacterial variables and inorganic phosphorus, nitrogen, and total dissolved solids. However, Vadstein et al. (1988) showed that bacteria have a substantially higher phosphorus requirement than do phytoplankton and act as

net consumers of inorganic phosphorus. Bacterial abundance and production have also been shown to be correlated with particulate organic carbon and nitrogen in the Chesapeake Bay (Jonas and Tuttle 1990). Other studies have shown that bacteria are net consumers of inorganic nitrogen and phosphorus, and they may compete for such nutrients with phytoplankton (Rhee 1972; Parker et al. 1975; Wheeler and Kirchmen 1986; Horrigan et al. 1988). In general, these studies have revealed that bacterioplankton production may be limited by phosphorus and nitrogen in addition to organic carbon.

The objectives of our study were to determine the major factors controlling bacterioplankton production in a eutrophic system, with an emphasis on the effects of nutrient supply.

Materials and Methods

Study site

Data were collected at 3 permanently buoyed stations (Fig. 1) in Hebgen Lake, located on the upper Madison River at latitude $44^{o}51'55'$ and longitude $111^{o}20'05'$ (Martin 1967). The maximum depth was 6.5 m for station I, 20 m for station II, and 10 m for station III during the study period. The upper 5 m depths were chosen to represent the epilimnion depth for the stations. Lake water samples were collected biweekly at 0, 3, and 5 m for bacterial variables, and at 0, 1, 3, and 5 m for other variables during the 1988 ice-free season at station I, II, and III. Lake water samples were collected every 2 to 3 weeks at 0, 1, 3, and 5 m for all variables during the 1989 ice-free season at station I, and on 8 June, 19 July, and 8 October 1989 at station II. River

inflow samples were collected at the time of lake sampling during 1988, and on 21 May, 26 July, 13 August, 20 September, and 8 October during 1989. The sampling sites are shown in Fig. 1.

Field procedures

Samples were collected between 1100 and 1200 h using a 4-l Van-Dorn water sampler. The $^{14}CO_2$ uptake bioassay was incubated at the location of water collecting for 4-6 h. The ^{3}H-thymidine uptake bioassay was incubated in a dark box at the temperature of collection for 20-30 min. Samples for bacterial abundance were fixed with particle-free formaldehyde (3% final concentration) immediately after collection. All samples were transported on ice to the laboratory for final analysis.

Laboratory procedures

Dissolved nutrients. Nitrate was determined by cadmium reduction (Eppley 1985), ammonium by the phenol hypochlorite method (Solorzano 1969), and soluble reactive phosphorus (SRP) by the molybdate method (Downes 1978). Total dissolved phosphorus (TDP) was measured using the acid hydrolysis procedure of Solorzano and Sharp (1980) followed by orthophosphate determination (Stainton et al. 1977); dissolved organic phosphorus (DOP) was computed from the difference between TDP and SRP. Total dissolved nitrogen (TDN) was measured by persulfate digestion (D'Elia et al. 1977) followed by determination of nitrate by Cd reduction. Dissolved organic nitrogen (DON) was computed by subtracting the sum of nitrate and ammonium from TDN. All of the above samples were prefiltered through Whatman GF/C filters and frozen before analyzing.

Particulate matter. Chlorophyll a was determined by fluorome-

try on 90% acetone extracts (Strickland and Parsons 1972). Fluorescence was measured with a Turner model 112 fluorometer on pre- and post-acidified samples and compared to a chlorophyll a standard (Sigma) treated in the same manner. Particulate organic carbon (POC) and particulate organic nitrogen (PON) were measured with a Carlo-Erba model 1106 elemental analyzer calibrated with standard amounts of acetanilide. Particulate organic phosphorus (PP) was determined with the acid hydrolysis procedure of Solorazano and Sharp (1980) followed by orthophosphate measurement of the digestion (Stainton et al. 1977). Dissolved organic carbon (DOC) was analyzed using Dorhmann Carbon Analyzer. Total nitrogen (TN) was the sum of TDN and PON; total phosphorus (TP) was the sum of TDP and PP; and total organic carbon (TOC) was the sum of DOC and POC.

Radioisotope bioassay. Phytoplankton $^{14}CO_2$ uptake was measured by adding ^{14}C-$NaHCO_3$ stock (ICN Radiochemical Inc.) to lake water to a final ^{14}C activity of about 0.05 μCi ml^{-1}. The photosynthetic reaction was terminated by filtration of the entire sample through Whatman GF/C filters. Seven ml of CytoScint scintillation cocktail (ICN Radiochemical, Irvine, CA) was added to the filter in a 20-ml scintillation vial and counted with a Beckman LS-100C scintillation spectrometer. Efficiency was computed by the external standard channels ratio.

Bacterial ^{3}H-thymidine uptake was determined by adding high activity (55 Ci $mmole^{-1}$) methyl-^{3}H-thymidine (in 70% ethanol) (ICN Radiochemical INC.) to a 10 ml water sample (final concentration 10 nM) in a 20-ml glass scintillation vial. The thymidine

was evaporated to dryness and rehydrated with deionized water before use to eliminate products of self radiolysis and to remove ethanol. Thymidine uptake was terminated by adding 10 ml ice-cold 10% (w/v) trichloroacetic acid (TCA) to each vial followed by extraction overnight at 4 ^{o}C. The extracted samples were then filtered onto 0.2 μm membrane filters (Poretics Co.). After rinsing the filter 5 times (2 ml each rinse) with 5% ice-cold TCA, the filter was transferred to a 20 ml polyethylene scintillation vial with 7.0 ml CytoScint scintillation cocktail (ICN Radiochemical, Irvine, CA). Radioactivity in each sample was determined by standard scintillation spectrometery using a Beckman LS-100C liquid scintillation counter. The counting efficiency was determined by the external standard ratio method using ^{3}H-toluene as a reference and acetone as the quench agent.

Bacterial conversions. The portion of ^{3}H-thymidine incorporated into DNA was determined by the method of Servais et al. (1987). A conversion factor from mole ^{3}H-thymidine incorporated into DNA to number of cells produced was $1x10^{18}$ (Moriarty 1988). Bacterial cell counts were made using the acridine orange direct count technique (Hobbie et al. 1977) with a Nikon labophot epifluorescence microscope. Bacterial size was estimated with a calibrated ocular micrometer. The corresponding biomass and production were calculated, in terms of carbon, using the coefficient of 560 fg C μm^{-3} (Bratbak 1985).

Nutrient and river water enrichment experiments.

Three laboratory experiments were conducted during May (experiment 1), July (experiment 2), and October (experiment 3) 1989. Surface water enriched with ammonium, phosphorus, mannitol,

and river water (singly and in various combinations, see Table 1) were incubated at the temperature of collection (Table 1) in a laboratory incubator in 1-l polyethylene bottles. A photon flux density of 150 $\mu E\ m^{-2}s^{-1}$ was provided by cool-white fluorescent lamps with 12 h light/dark cycle. The incubation period for May was 5 days and for June and October was 4 days. River water used to enrich the lake water was a mixture from sampling sites 1 and 2 (1:1) for experiment 1 and 2, and was a mixture from sampling sites 1, 2, and 3 (1:1:1) for experiment 3 (see Fig. 1 for sampling sites). The mixed river water was filtered through 0.45 μm filter to remove particles before mixing with lake water collected from surface of station I. Bacterial ^3H-thymidine uptake and cell number were measured before starting the incubation for all 3 experiments, on day 1, 3, and 5 for experiment 1, and on day 2 and 4 for experiment 2 and 3 using the methods described above.

Results

Bacterioplankton production, biomass, abundance, and doubling time

Integrated epilimnion bacterioplankton production varied throughout the sampling seasons. The minimum production (<0.1 $\mu g\ C\ l^{-1}\ h^{-1}$) was found in both early spring and late fall; the maximum (>0.8 $\mu g\ C\ l^{-1}\ h^{-1}$) was found in late July and early August for all the 3 sampling stations in both 1988 and 1989 (Fig. 2). The minimum bacterioplankton biomass (<20 $\mu g\ C\ l^{-1}$) occurred in the spring (May and June) and the maximum (>50 $\mu g\ C\ l^{-1}$) in mid-summer (early August). Biomass was about 40 $\mu g\ C\ l^{-1}$ during fall 1988 and 1989 in all 3 stations (Fig. 2). Bacterio-

plankton abundance had the same trends as biomass (Fig. 3) owing to the presence of similar sized bacterial cell over the season. Bacterioplankton doubling time was highest during late spring and fall, and lowest during early spring and summer of both 1988 and 1989 at all 3 stations (Figure 3).

Phytoplankton production, chlorophyll a, and organic and inorganic nutrients

The depth integrated mean epilimnetic phytoplankton production was 12.4 μg C l^{-1} h^{-1} (range 2.5-36.6) at station 1, 8.1 μg C l^{-1} h^{-1} (range 1.8-13.6) at station 2, and 8.9 μg C l^{-1} h^{-1} (range 3.9-13.1) at station 3 over the sampling period. The integrated annual mean epilimnion chlorophyll a concentration was 9.8 μg l^{-1} (range 1.5-21.1) at station 1, 5.84 μg l^{-1} (range 1.2-12.0) at station 2, and 5.8 μg l^{-1} (range 0.7-27.2) at station 3 during the sampling season. The seasonal patterns of phytoplankton production and chlorophyll a are shown in Fig. 4.

Seasonal trends of inorganic nitrogen and phosphorus, organic carbon, and organic nitrogen and phosphorus are shown in Figs. 5, 6, and 7. Phosphorus parameters (SRP, DOP, TDP, PP) had the similar seasonal patterns for the 3 stations during 1988 (Fig. 5). The concentration of SRP was less than 10 μg l^{-1}, DOP and PP were less than 30 μg l^{-1} during most of the study period (Fig. 5). Nitrogen parameters (NH_4^+, NO_3^-, TDN, PON, DON) varied substantially throughout the season; their concentrations were higher at station 1 during most of the study period (Figs. 6, 7). Maximum DOC concentration occurred during late summer and fall and did not have the same seasonal pattern as POC (Fig. 7). Water

temperature showed the same seasonal pattern in all the 3 stations in both 1988 and 1989 (Fig. 7).

Correlations among bacterioplankton variables, phytoplankton variables, and other biotic and abiotic nutrients.

Bacterioplankton production and specific activity (thymidine uptake per unit bacterial cell number) were most strongly correlated to water temperature ($r>0.5$, $p<0.001$) followed by PON ($r>0.26$, $p<0.05$) and chlorophyll a ($r>0.23$, $p<0.05$). Bacterioplankton production and specific activity were inversely correlated with TOC:TN and TOC:TP ratios ($p<0.001$) followed by DOC and SRP ($p<0.05$) (Table 2). Bacterioplankton biomass and abundance were most strongly correlated with chlorophyll a ($r>0.39$, $p<0.001$) followed by water temperature ($r>0.36$, $p<0.01$). No significant negative correlations were found between bacterioplankton biomass or abundance and other variables (Table 2).

PP, PON, POC, TN, TP, and TC were strongly correlated ($p<0.01$) to chlorophyll a concentration and phytoplankton biovolume. The ratio of TN:TP was moderately correlated with chlorophyll a and phytoplankton biovolume ($p<0.05$) while water temperature, inorganic nitrogen and inorganic phosphorus showed no significant correlation with chlorophyll a or phytoplankton biovolume (Table 2). Phytoplankton production was significantly ($p<0.01$) correlated with water temperature, POC, and PON. Chlorophyll a concentration, phytoplankton production and biovolume were all correlated strongly ($p<0.01$) (Table 2).

Effects of stratification on epilimnion bacterioplankton growth and abundance

Integrated average epilimnetic bacterioplankton production

and biomass during the summer (July-August) were significantly higher ($p<0.05$) at station I than at station II. Bacterioplankton doubling time was significantly shorter ($p<0.01$) at station I than station II (Fig. 8). Phytoplankton variables (chlorophyll, biovolume, PPR) were significantly higher ($p<0.05$) at station I than at station II (Table 3). All inorganic nitrogen, inorganic phosphorus, organic nitrogen, organic phosphorus and organic carbon were consistently higher at station I than at station II except ammonium which showed little difference between stations (Table 3). The ratios of TOC:TP and TOC:TN were higher at station II than at station I (Table 3).

Seasonal variation of factors regulating bacterioplankton activity and abundance

Correlation among bacterial variables, phytoplankton variables, nutrient variables and temperature. During spring (May-June), bacterioplankton production, biomass, and numerical abundance was significantly ($p<0.05$) correlated with water temperature. All other measured variables showed no significant ($p>0.05$) correlation with bacterial variables (Table 4). During summer (July-August), bacterial variables were most strongly correlated to phosphorus variables. Bacterial variables were significantly negatively correlated to the TN:TP ratio whereas carbon variables showed no significant correlation with bacterial variables (Table 4). During autumn (September-November), bacterial production and specific activity were strongly correlated with nitrogen variables ($r>0.41$, $p<0.05$) and temperature ($r>0.43$, $p<0.01$), and bacterial biomass and abundance were strongly correlated with

certain nitrogen variables (PON, TN) ($r>0.32$, $p<0.05$) and phytoplankton biovolume and chlorophyll a ($r>0.39$, $p<0.05$). Phosphorus variables showed no significant correlation ($p>0.05$) with bacterial variables (Table 4).

Nutrient and river water enrichment experiment. During the May experiment, river water and combinations of DOC, NH_4^+, and PO_4^{-3} enrichments increased both bacterial thymidine uptake and cell numbers (Fig. 9a). All nutrient (singly and in combinations) and riverwater enrichments stimulated bacterial thymidine uptake and cell numbers during the July experiment (Fig. 9b). PO_4^{-3} enrichments did not stimulate bacterial thymidine uptake or cell number during the October experiment (Fig. 9c).

Nutrient differences between river inflow and lake water.

The annual mean concentration of phosphorus in lake water was essentially the same as river water. However, the concentrations of nitrogen and organic carbon in lake water were consistently higher than the river water. The ratio of TOC:TN:TP (by weight) was 147.5:8.0:1 in lake water and 80.6:3.7:1 in river water (Table 5).

DISCUSSION

Bacterioplankton variables

The mean epilimnetic bacterial production (0.28 μg C l^{-1} h^{-1}, range 0.09-0.82) of Hebgen Lake is low compared with that for the freshwater and saltwater systems (average 1.1, range 0.02-6.38 μg l^{-1} h^{-1}) summarized by Cole et al. (1988). The average epilimnion bacterial biomass and abundance (41.49 μg C l^{-1}; 1.86×10^9 cell l^{-1}) are also low compared with other eutrophic lake data of 80-

130 μg C l^{-1} (Riemann et al. 1982) and 3.9-31 X 10^9 cell l^{-1} (Gebre-Mariam and Taylor 1989; Marvalin et al. 1989; Robarts and Wicks 1990). Bacterioplankton production as a percent of phytoplankton production in Hebgen lake (2%) is much lower than reported mean value (20%) from a range of aquatic systems (Cole et al. 1988) although it is within the range reported by Robarts and Wicks (1990) of 2% for Hartbeesport Dam, hypertrophic reservoir Africa.

Relation between bacterioplankton and phytoplankton

The strong correlation ($r>0.23$, $p<0.05$) between bacterial variables and chlorophyll a over the entire study period implies a close coupling between bacterioplankton and phytoplankton biomass in the epilimnion of Hebgen Lake. The lack of a significant correlation between bacterial variables and phytoplankton production may be due to the variable effect of underwater irradiation because the regression was done on discrete samples down the water column, which does not take light attenuation into effect. However, the non-significant correlation between bacterial and all phytoplankton variables during spring and summer indicates that epilimnetic bacterial production is affected by phytoplankton during only part of the ice-free season. Positive correlations between bacterial and phytoplankton variables have been reported for a number of other marine and freshwater environments (Fuhrman et al. 1980; Chrzanowski and Hubbard 1989; Marvalin et al. 1989; Robarts and Wicks 1990). Lack of correlation between bacterial and phytoplankton variables were also found in some marine environments in which water temperature was

strongly correlated with bacterial variables (Coffin and Sharp 1987; Joint and Pomoroy 1987). Our findings support these latter result in that bacterial variables were significantly ($p<0.05$) correlated with temperature but not phytoplankton variables during the spring.

Factors regulating bacterioplankton.

Entire study period. That bacterial biomass and abundance were highly correlated with organic phosphorus and particulate organic nitrogen and carbon (Table 2) infers that these are the main substrates regulating bacterioplankton in Hebgen Lake. The strong correlation between these organic nutrients and phytoplankton variables (Table 2) further implies that phytoplankton are an important source of such nutrients. The negative correlations of bacterial production and specific activity with SRP and DOC indicate that high bacterial activity can effectively reduce these nutrients. Therefore, high concentrations of essential nutrients do not necessarily indicate high bacterial production. The correlations between bacterial variables and TOC, TN, and TP suggest that they are important bacterial substrates. When these 3 nutrients are compared, TN and TP appear to be the most deficient forms. This is supported by the strong negative correlations between bacterial production or specific activity and ratios of TOC:TN and TOC:TP.

Seasonal difference. Our correlations infer that bacterioplankton in Hebgen Lake are most closely associated with water temperature during spring, with phosphorus followed by nitrogen during summer, and nitrogen followed by organic carbon during fall. This conclusion is supported by our laboratory experiments,

which showed essentially the same results (Fig. 9). Seasonal variation in factors controlling bacterial production and abundance has also been reported for other aquatic environments (Jonas and Tuttle 1990).

Influence of nutrient input from river on lake bacterioplankton and nutrient composition

External nutrient input from river inflow is an important bacterial nutrient source in Hebgen Lake. This conclusion is supported by all of our river water bioassays which showed increased bacterial thymidine uptake and bacterial abundance with the addition of river water (Fig. 9).

The lower ratio of TOC:TN:TP in river water relative to lake water (Table 5) indicates that lake water has relatively less organic carbon. It has been reported that bacteria are phosphorus limited when the C:P ratio is 8.3-58.8 by weight (Vadstein and Olsen 1989). Reported bacterial C:N and N:P weight ratios are 2.3-8.3 and 4.7-8.7 (Linley and Newell 1984 cited in Vadstein and Olsen 1989; Vadstein and Olsen 1989). Hebgen Lake water had lower proportions of nitrogen and phosphorus compared with organic carbon (TOC:TN=19:1; TOC:TP=148.1) than the reported ratios. However, the ratio of TN:TP (8:1) of the lake water is in the range of the reported values. Difference in these ratios can be used to explain why river water enrichments to Hebgen Lake water stimulated bacterial activity and abundance in our laboratory experiments.

Conclusions

1. Epilimnetic bacterioplankton production and abundance in Hebgen Lake were low relative to the degree of autotrophic production when compared with other aquatic environments.

2. Factors most highly correlated with bacterial variables were temperature, chlorophyll, and TON.

3. Potential substrates regulating epilimnetic bacterial production were (in the order of importance) TP, TN, and TOC during 1988 and 1989.

4. Factors regulating bacterial production and abundance varied seasonally. The regulating factors were water temperature in spring, TP and TN in summer, and TN and TOC in the fall.

5. River water had a higher proportion of phosphorus and nitrogen than organic carbon compared to epilimnetic lake water, and proved to be an important bacterial nutrient source for bacterial production in the latter.

References

Bjornsen, P. K., B. Riemann, J. Pock-Steen, T. G. Nielsen, and S. J. Horsted. 198 . regulation of bacterioplankton production and cell volume in a eutrophic estuary. Appl. Environ. Microbiol. 55:1512-1518.

Bratak, G. 1985. Bacterial biovolume and biomass estimations. Appl. Environ. Microbiol. 49:1488-1493.

Chrzanowski, T. J., and Hubbard. 1989. Bacterial utilization of algal extracellular productions in a southwestern reservoir. Hydrobiologia 179:61-71.

Cole, J. J., G. E. Likens, and D. L. Strayer. 1982. Photosynthetically produced dissolved organic carbon: An important carbon source for planktonic bacteria. Limnol. Oceanogr. 27:1080-1090.

Cole, J. J., S. Findlay, and M. L. Pace. 1988. Bacterial production in fresh and saltwater ecosystems: a cross-system overview. Mar. Ecol. Prog. Ser. 43:1-10.

D'Elia, C. F., P. A. Steudler, and N. Corwin. 1977. Determination of total nitrogen in aqueous samples using persulfate digestion. Limnol. Oceanogr. 22:760-764.

Downes, M. T. 1987. An automated determination of low reactive phosphorus concentrations in natural waters in the presence of arsenic, silicon acid and mercuric chloride. Water Research 12:743-745.

Epply. R. 1978. Nitrate uptake. In J. A. Hellebust and J. S. Graigie (eds.). Handbook of Phyological Method, Physiological and Biological Methods, pp 401-409. Cambridge university Press. Cambridge, England.

Feuillade, M., Ph. Dufour, and J. Feuillade. 1988. Organic carbon release by phytoplankton and bacterial reassimilation. Schweiz. Z. Hydrol. 50:115-135.

Fuhrman, J. A., J. W. Ammerman, and F. Azam. 1980. Bacterioplankton in the coastal euphotic zone: distribution, activity and possible relationship with phytoplankton. Mar. Biol. 60:201-207.

Gebre-Mariam, Z, and W. D. Taylor. 1989. Seasonality and spatial variation in abundance, biomass and activity of heterotrophic bacterioplankton in relation to some biotic and abiotic variables in an Ethiopian rift-valley lake (Awassa). Freshwater Biology 22:355-368.

Hobbie, J. E., J. Daley, s. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Horrigan, S. G., A. Hagstrom, I. Koike, and F. Azam. 1988.

Inorganic nitrogen utilization by assemblages of marine bacteria in seawater culture. Mar. Ecol. Prog. Ser. 50:147-150.

Jesen, L. M. 1983. Phytoplankton release of extracellular organic carbon, molecular weight composition, and bacterial assimilation. Mar. Ecol. Prog. Ser. 11:39-48.

Jesen, L. M., and M. Sondergaard. 1985. Comparison of two methods to measure algal release of dissolved organic carbon and the subsequently uptake by bacteria. J. Plankton Res. 7:41-56.

Jonas, R. B., and J. H. Tuttle. 1990. Bacterioplankton and organic carbon dynamics in the lower mesohalin Chesapeake Bay. Appl. Environ. Microbiol. 56:747-757.

Linley, E., and R. C. Newell. 1984. Estimates of bacterial growth yields based on plant detritus. Bull. mar. Sci. 35:409-425.

Martin, D. B. 1967. Limnology studies on Hebgen Lake, Montana. MS. thesis. Montana State University, Bozeman, Montana.

Marvalin, O., L. Aleya, and H. J. Hartmann. 1989. Coupling of the seasonal patterns of bacterioplankton and phytoplankton in a eutrophic lake. Can. J. Microbiol. 35:706-712.

Parker, R. R., J. Sibert, and T. J. Brown. 1975. Inhibition of primary productivity through heterotrophic competition for nitrate in a stratified estuary. J. Fish. Res. Board. Can. 32:72-77.

Riemann, B., M. Sondergard, H. m. Schierup, S. Bosselmann, G. Christensen, J. Hansen, and B. Neilsen. a982. Carbon metabolism during a spring diatom bloom in the eutrophic Lake Mosso. Int. Rev. Gegamten Hydrobiol. 67:145-185.

Rhee, G-Yull. 1972. Competition between an alga and an aquatic bacterium for phosphate. Limnol. Oceanogr. 17:505-514.

Robarts, R. D., and R. J. Wicks. 1990. Heterotrophic bacterial production and its dependence on autotrophic production in a hypertrophic African reservoir. Can. J. Fish. Aquatic. Sci. 47:1027-1037.

Servais, P., J. Martinez, G. Billen, and J. Vives-Rego. 1987. Determining [^{3}H]Thymidine incorporation into bacterioplankton DNA: improvement of the method by DNase treatment. Appl. Environ. Microbiol. 53:1977-1979.

Solorzano, L. 1969. Determination of ammonia in natural water by the phenolhypochlorite method. Limnol. Oceanogr. 14:799-801.

Solorzano, L., and J. H. Sharp. 1980. Determination of total dissolved phosphorus and particulate phosphorus in natural waters. Limnol. Oceanogr. 25:754-758.

Stainton, M. P., M. J. Capel, and F. A. J. Armstrong. 1977. The chemical analysis of freshwater. Misc. Special Publ. No. 25. Fisheries and Environment Canada.

Strickland, J. D., and T R. Parsons. 1972. A practical handbook of seawater analysis. Bull. Fish. Res. Board. Can. (2nd ed.).

Sundh, I. 1989. Characterization of phytoplankton extracellular products and their subsequent uptake by heterotrophic organisms in a mesotrophic forest lake. J. Plankton Res. 11:463-486.

Tranvik, L. J. 1989. bacterioplankton growth, grazing mortality and quantitative relationship to primary production in a humic and a clearwater lake. J. Plankton Res. 11:985-1000.

Vadstein, O., A. Jensen, Y. Olsen, and H. Reinertsen. 1988. Growth and phosphorus status of limnetic phytoplankton and bacteria. Limnol. Oceanogr. 33:489-503.

Vadstein, O., and Olsen. 1989. chemical composition and phosphate uptake kinetics of limnetic bacterial communities cultured in chemostats under phosphorus limitation. Limnol. Oceanogr. 34:939-946.

Wheeler, P. a., and D. L. Kirchman. 1986. Utilization of inorganic and organic nitrogen by bacteria in marine systems. Limnol. Oceanogr. 31:998-1009.

Table 1. Nutrient and river water enrichments in the laboratory experiments. Control presents ambient levels, NH_4^+ and PO_4^{-3} are in the units of μg N or P l^{-1}, mannitol in mg l^{-1}, and river in % of lake water. NA=treatment not applicable.

Treatment	May	July	October
Temp (^oC)	14	18	15
Control	0	0	0
NH_4^+-N	NA	NA	200
PO_4^{-3}-P	NA	NA	50
NH_4^+-N &	NA	NA	200
PO_4^{-3}-P	NA	NA	50
Mannitol	91	91	91
River water	50	50	50
NH_4^+-N &	100	100	NA
mannitol	91	91	NA
PO_4^{-3}-P &	50	50	NA
mannitol	91	91	NA
NH_4^+-N &	100	100	NA
PO_4^{-3}-P &	50	50	NA
mannitol	91	91	NA

Table 2. Linear correlations (r) of bacterioplankton and phytoplankton variables against inorganic and organic nutrients in Hebgen Lake during 1988 and 1989 (n=86). See text for variable description. NS not significant at $p>0.05$; r values are listed only when $p<0.05$. All data were transformed (ln) before analysis.

	Bacterial production (BP)	Bacterial activity/cell (BAPC)	Bacterial biomass (BB)	Bacterial abundance (BA)	Chlorophyll (CHL)	Phytoplankton biovolume (PHYB)	Phytoplankton production (PPR)
Temp	0.60	0.52	0.40	0.36	NS	NS	0.35
CHL	0.28	0.23	0.41	0.39	1.00	0.62	0.41
PHYB	NS	NS	NS	NS	0.62	1.00	0.36
PPR	NS	NS	NS	NS	0.41	0.36	1.00
SRP	-0.25	-0.35	NS	NS	NS	NS	NS
TDP	NS	NS	0.28	0.28	0.83	0.45	0.26
DOP	NS	NS	0.32	0.29	0.30	NS	NS
PP	0.28	NS	0.29	0.28	0.83	0.45	0.26
NH_4^+-N	NS	NS	NS	NS	NS	NS	NS
NO_3^--N	NS	NS	0.26	NS	NS	NS	NS
TDN	NS	NS	NS	NS	0.41	0.24	NS
DON	NS	NS	NS	NS	NS	NS	NS
PON	0.33	0.26	0.30	0.24	0.83	0.56	0.31
DOC	-0.28	-0.43	NS	NS	0.29	NS	NS
POC	0.28	NS	0.30	0.29	0.89	0.61	0.42
TN	0.30	NS	0.30	0.28	0.79	0.51	NS
TP	0.28	NS	0.29	0.29	0.83	0.45	0.26
TOC	NS	NS	0.26	0.29	0.72	0.41	NS
TN:TP	NS	NS	NS	NS	0.25	0.40	NS
TOC:TN	-0.44	-0.54	NS	NS	NS	NS	NS
TOC:TP	-0.40	-0.47	NS	NS	NS	NS	NS

Table 3. Comparison of mean (± 1SE) summer season epilimnion phytoplankton variables and nutrient concentrations at station I and II in Hebgen Lake during 1988 and 1989. Symbols are as in Table 2. Errors on TP, TN, and TOC were obtained by propagating errors associated with each form.

	Station I	Station II
Temp (^{o}C)	18.1±0.5	19.2±0.3
CHL (g l^{-1})	10.8±1.8	7.5±1.2
PHYB (mg l^{-1})	6.4±1.2	1.8±0.8
PPR (g C l^{-1} h^{-1})	19.5±2.8	11.3±0.8
SRP (g l^{-1})	6.3±1.5	2.7±0.4
TDP (g l^{-1})	38.9±9.2	23.9±4.8
PP (g l^{-1})	20.6±2.3	8.3±0.8
NH_4^+-N (g l^{-1})	9.2±2.0	11.0±5.1
NO_3^--N (g l^{-1})	28.7±11.0	3.6±1.2
TDN (g l^{-1})	250 ±33	147 ±19
PON (g l^{-1})	291 ±66	128 ±15
DOC (g l^{-1})	4470 ±750	2890 ±660
POC (g l^{-1})	1733 ±363	1136 ±130
TP (g l^{-1})	59 ±9.5	32 ±4.9
TN (g l^{-1})	541 ±73.8	274 ±24.2
TOC (g l^{-1})	6203 ±833	4026 ±673
TOC:TN:TP (weight)	103:9:1	126:9:1

Table 4. Linear correlations of bacterial variables against various biotic and abiotic variables for different seasons in Hebgen Lake. Symbols are as in Table 2. NS not significant at $p>0.05$; r values are listed only when they are significant at $p<0.05$. All data were transformed (ln) before analysis.

	May-Jun (N=32)				Jul-Aug (N=34)				Sep-Oct (N=38)			
	BP	BAPC	BB	BA	BP	BAPC	BB	BA	BP	BAPC	BB	BA
Temp	0.48	NS	0.35	0.38	NS	NS	NS	NS	0.43	0.40	NS	NS
CHL	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.47	0.43
PHYB	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.45	0.39
PPR	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
SRP	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS
TDP	NS	NS	NS	NS	0.38	NS	0.44	0.44	NS	NS	NS	NS
DOP	NS	NS	NS	NS	0.39	NS	0.45	0.47	NS	NS	NS	NS
PP	NS	NS	NS	NS	0.34	0.38	NS	NS	NS	NS	NS	NS
NH_4^+-N	NS	NS	NS	NS	0.34	0.42	NS	NS	NS	NS	-0.36	NS
NO_3^--N	NS	NS	NS	NS	NS	NS	0.39	NS	0.41	0.42	NS	NS
DON	NS	NS	NS	NS	NS	NS	NS	NS	0.54	0.51	NS	NS
TDN	NS	NS	NS	NS	NS	0.38	NS	NS	0.53	0.57	NS	NS
PON	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.45	0.34
DOC	NS	NS	NS	NS	NS	NS	NS	NS	-0.49	-0.53	NS	NS
POC	NS	NS	NS	NS	NS	NS	NS	NS	NS	NS	0.43	0.40
TP	NS	NS	NS	NS	0.41	NS	NS	NS	NS	NS	NS	NS
TN	NS	NS	NS	NS	NS	NS	-0.40	-0.35	0.51	0.41	0.41	0.32
TOC	NS	NS	NS	NS	NS	NS	NS	NS	-0.43	-0.51	NS	NS
TN:TP	NS	NS	NS	NS	-0.32	NS	-0.34	-0.34	0.48	0.43	NS	NS
TOC:TN	NS	NS	NS	NS	NS	NS	NS	NS	-0.66	-0.68	NS	NS
TOC:TP	NS	NS	NS	NS	-0.30	NS	NS	NS	-0.56	-0.60	NS	NS

Table 5. The mean (± 1SE) nutrient concentrations ($\mu g\ l^{-1}$) of the epilimnetic water column and river inflows in Hebgen Lake during 1988 and 1989. Symbols are the same as in Table 2. Errors on TP, TN, and TOC were obtained by propagating errors associated with each form.

	River inflow	Epilimnetic lake water
SRP	10.8±1.9	9.4±2.7
TDP	23.9±2.9	29.0±4.6
PP	15.1±4.3	15.9±3.0
NH_4^+-N	9.6±3.1	11.6±4.0
NO_3^--N	14.2±3.7	27.2±10.3
TDN	109.0±11.0	200.9±19.9
PON	35.9±6.5	157.4±38.1
DOC	2515 ±475	5490 ±364
POC	626 ±79	1140 ±165
TP	39.0±5.2	44.9±5.5
TN	144.4±12.8	358.3±43.0
TOC	3141 ±482	630 ±399
TOC:TN:TP (weight)	80.6:3.7:1	147.5:8.0:1

Figure Captions

Figure 1. Map of Hebgen Lake showing the 3 permanent lake sampling stations (I, II, III) and the 4 river water sampling sites (1, 2, 3, 4). Map is modified from (Martin 1967).

Figure 2. Hebgen Lake seasonal distribution of epilimnetic bacterioplankton production and biomass during 1988 and 1989. ○ Station I 1988, △ station I 1989, ▽ station II 1988, and □ station III 1988.

Figure 3. Hebgen Lake seasonal distribution of epilimnetic bacterioplankton cell number and doubling time during 1988 and 1989. Symbols are the same as in Fig. 1.

Figure 4. Hebgen Lake seasonal distribution of epilimnetic phytoplankton production and chlorophyll a concentration during 1988 and 1989. Symbols are the same as in Fig. 1.

Figure 5. Hebgen Lake seasonal distribution of epilimnetic phosphorus variables (SRP, DOP, TDP, PP) during 1988 and 1989. ● Station I 1988, ○ station I 1989, ▲ station II 1988, △ station III 1988.

Figure 6. Hebgen Lake seasonal distribution of epilimnetic nitrogen variables (NH_4^+, NO_3^-, TDN, PON) during 1988 and 1989. Symbols are the same as in Fig. 5.

Figure 7. Hebgen Lake seasonal distribution of epilimnetic DOC, POC, DON, and temperature during 1988 and 1989. Symbols are the same as in Fig. 5.

Figure 8. Mean summer (Jul-Aug) epilimnetic bacterioplankton production, doubling time, biomass, and cell number at stations I and II of Hebgen Lake during 1988 and 1989. Open

boxes are station I, and crossed boxes are station II

Figure 9. Bacterioplankton ^{3}H-thymidine uptake and cell number in nutrient enriched lake water. In Fig. 9a and b, ○ Control, ● mannitol, △ mannitol+NH_4^+, ▲ mannitol+PO_4^{-3}, □ mannitol+NH_4^++PO_4^{-3}, ■ 50% river water. In Fig. 9c, ○ Control, ● mannitol, △ NH_4^+, ▲ PO_4^{-3}, □ NH_4^++PO_4^{-3}, ■ 50% river water.

Fig. 1

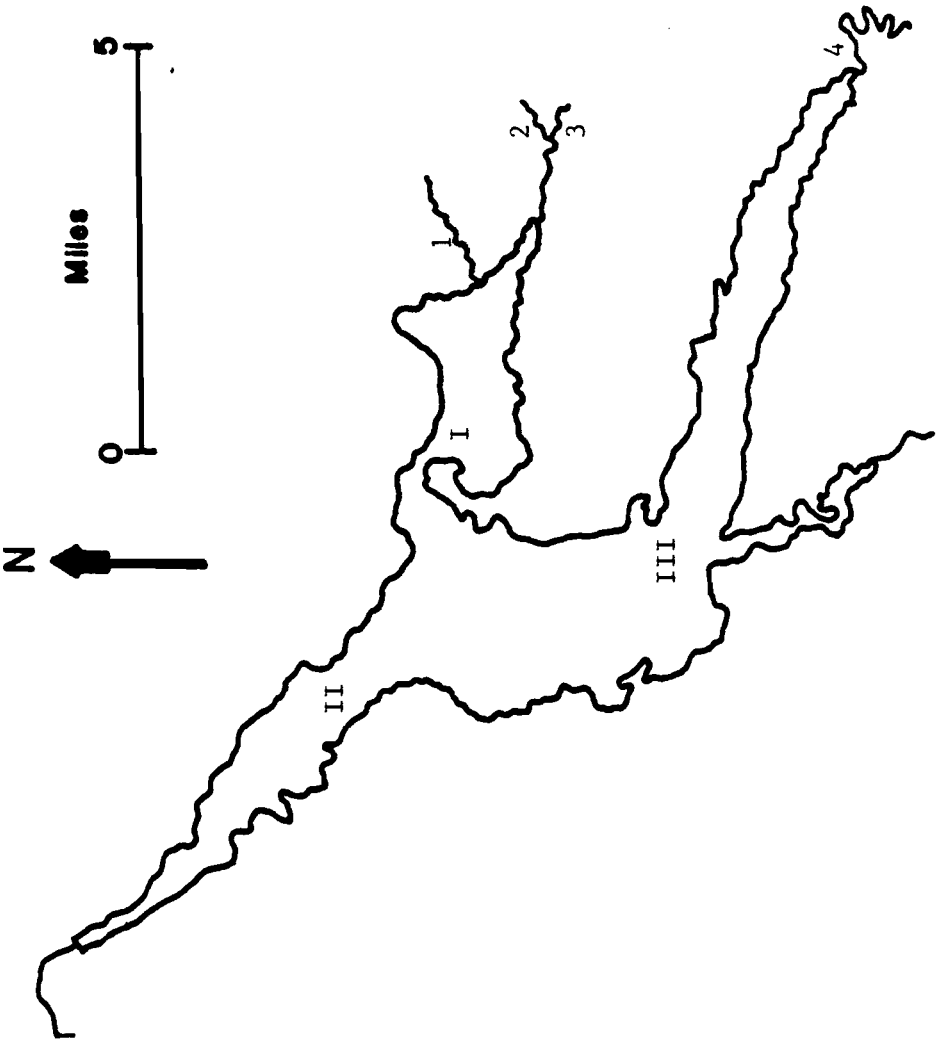


Fig. 2

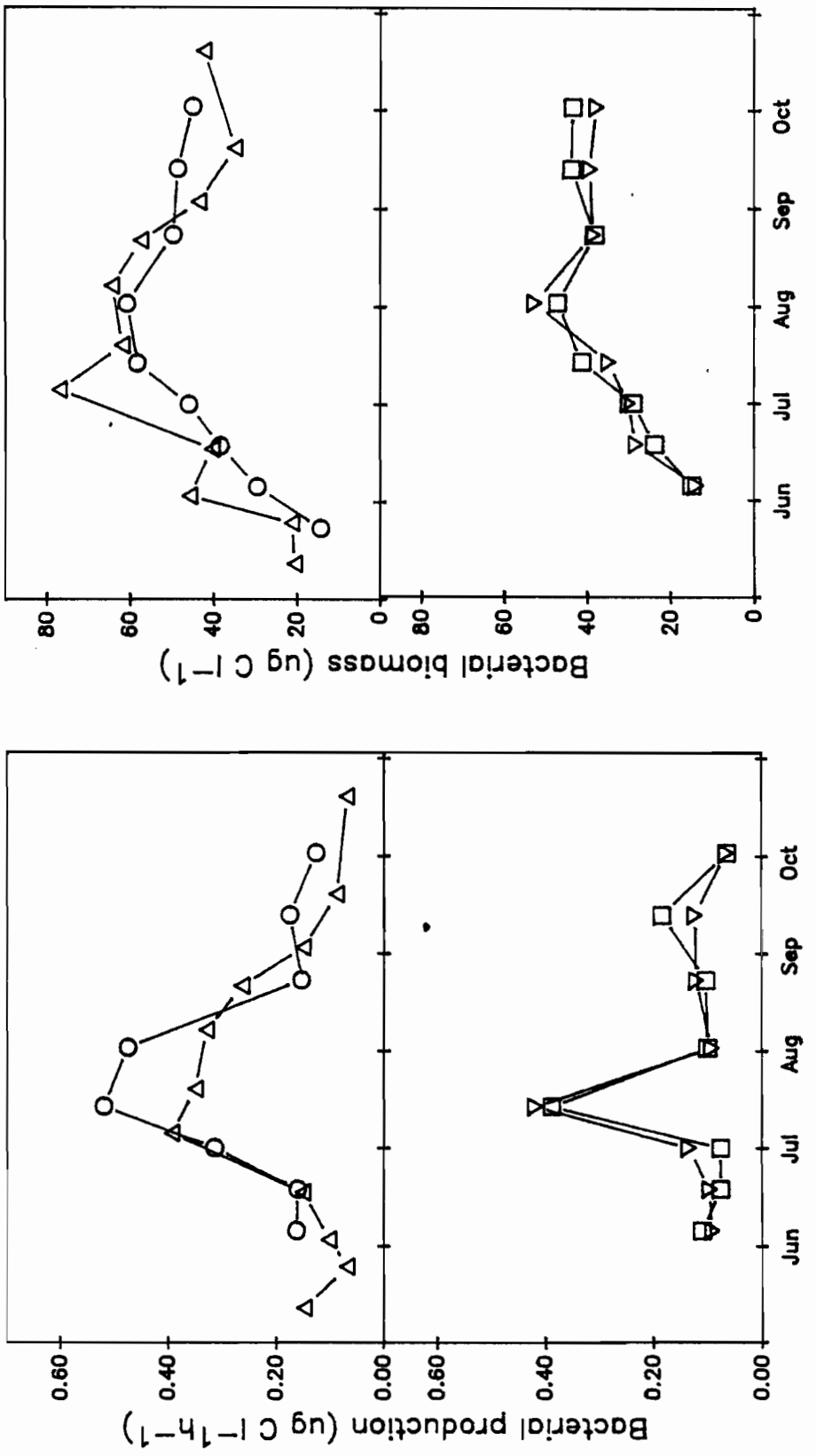

Bacterial biomass (ug C l⁻¹)
Bacterial production (ug C l⁻¹h⁻¹)
0
20
40
60
80
0.00
0.20
0.40
0.60
Jun
Jul
Aug
Sep
Oct

Fig. 3

Bacterial cell number (x10⁶ ml⁻¹)
0
2
3
Jun
Jul
Aug
Sep
Oct
Bacterial doubling time (h)
0
200
400

Fig. 4

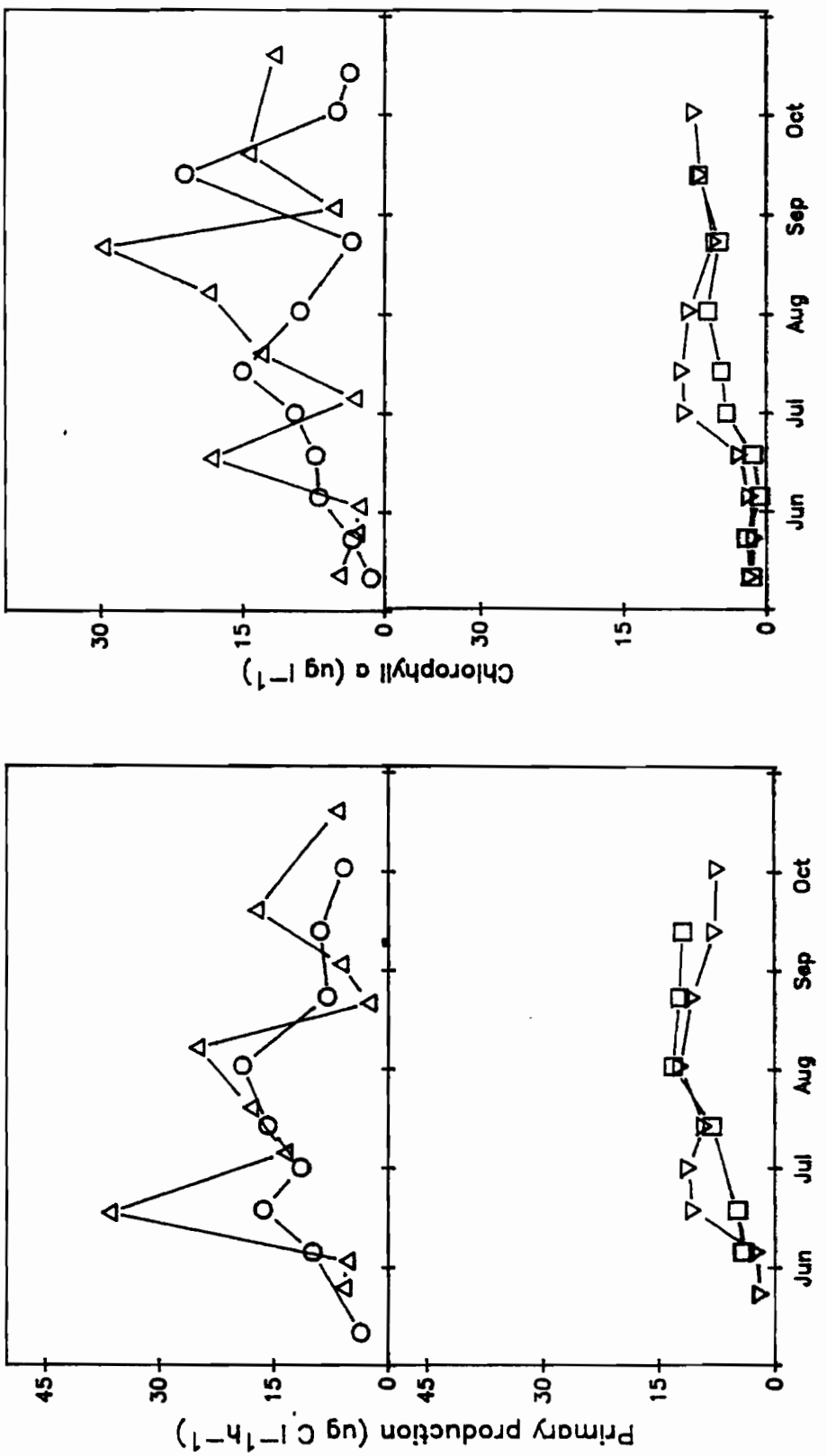

Chlorophyll a (ug l⁻¹)
Primary production (ug C l⁻¹h⁻¹)
0
15
30
45
Jun
Jul
Aug
Sep
Oct

Fig. 5

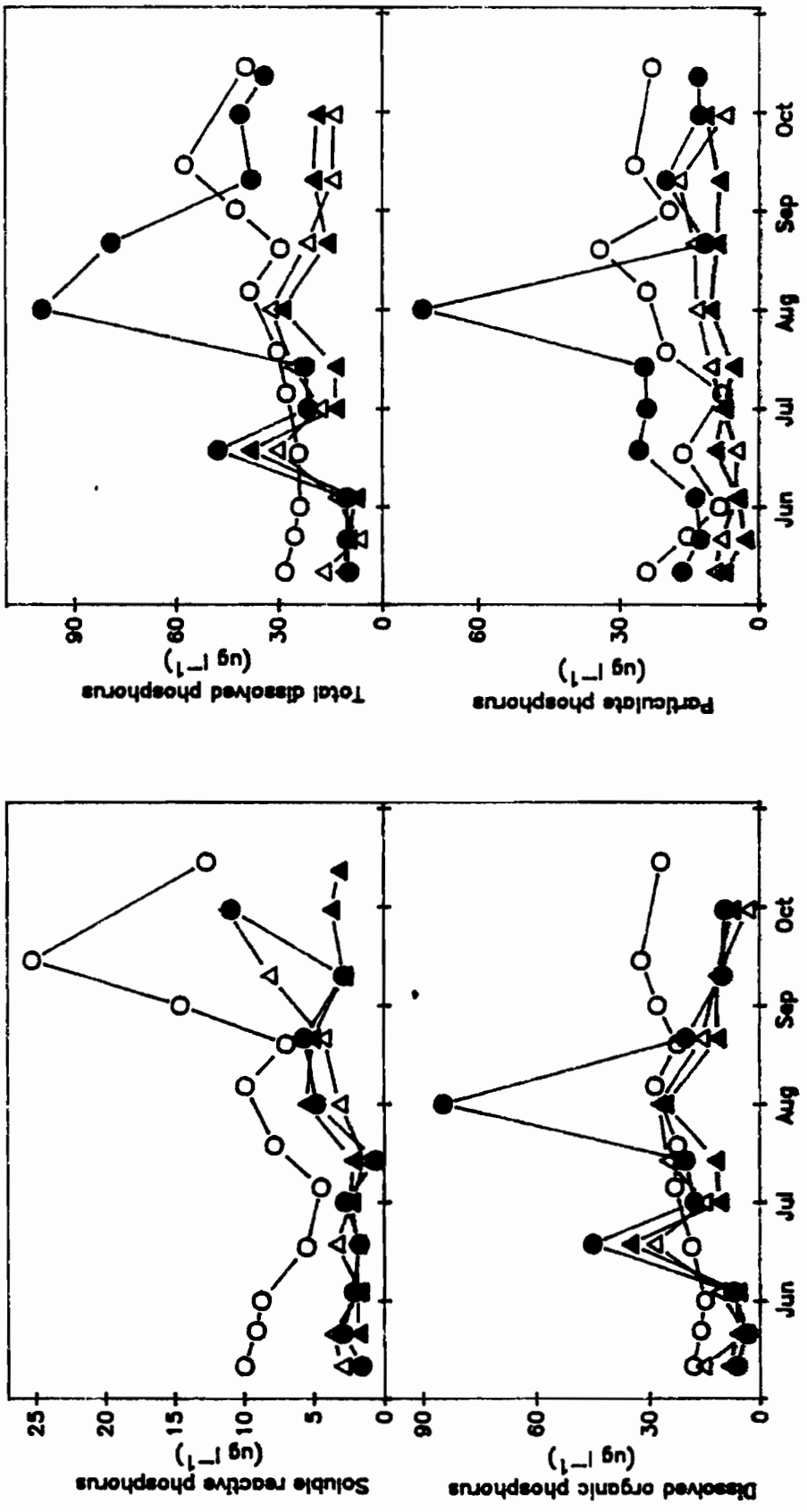

Particulate phosphorus
(ug l⁻¹)
Total dissolved phosphorus
(ug l⁻¹)
Dissolved organic phosphorus
(ug l⁻¹)
Soluble reactive phosphorus
(ug l⁻¹)
0
30
60
90
0
5
10
15
20
25
Jun
Jul
Aug
Sep
Oct

Fig. 5

Fig. 6

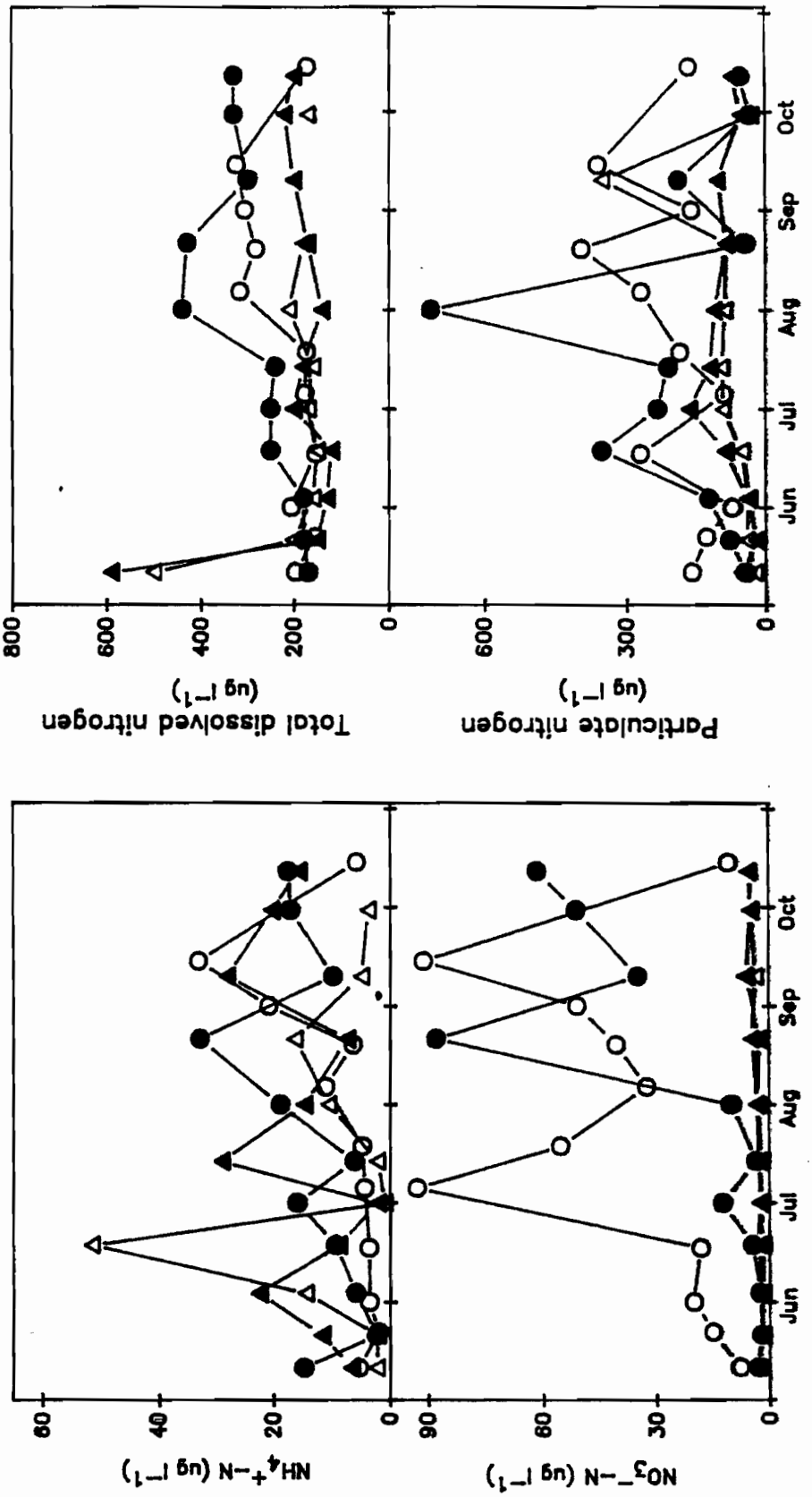

Particulate nitrogen (ug l⁻¹)
Total dissolved nitrogen (ug l⁻¹)
NO3⁻–N (ug l⁻¹)
NH4⁺–N (ug l⁻¹)
Jun
Jul
Aug
Sep
Oct

Fig. 7

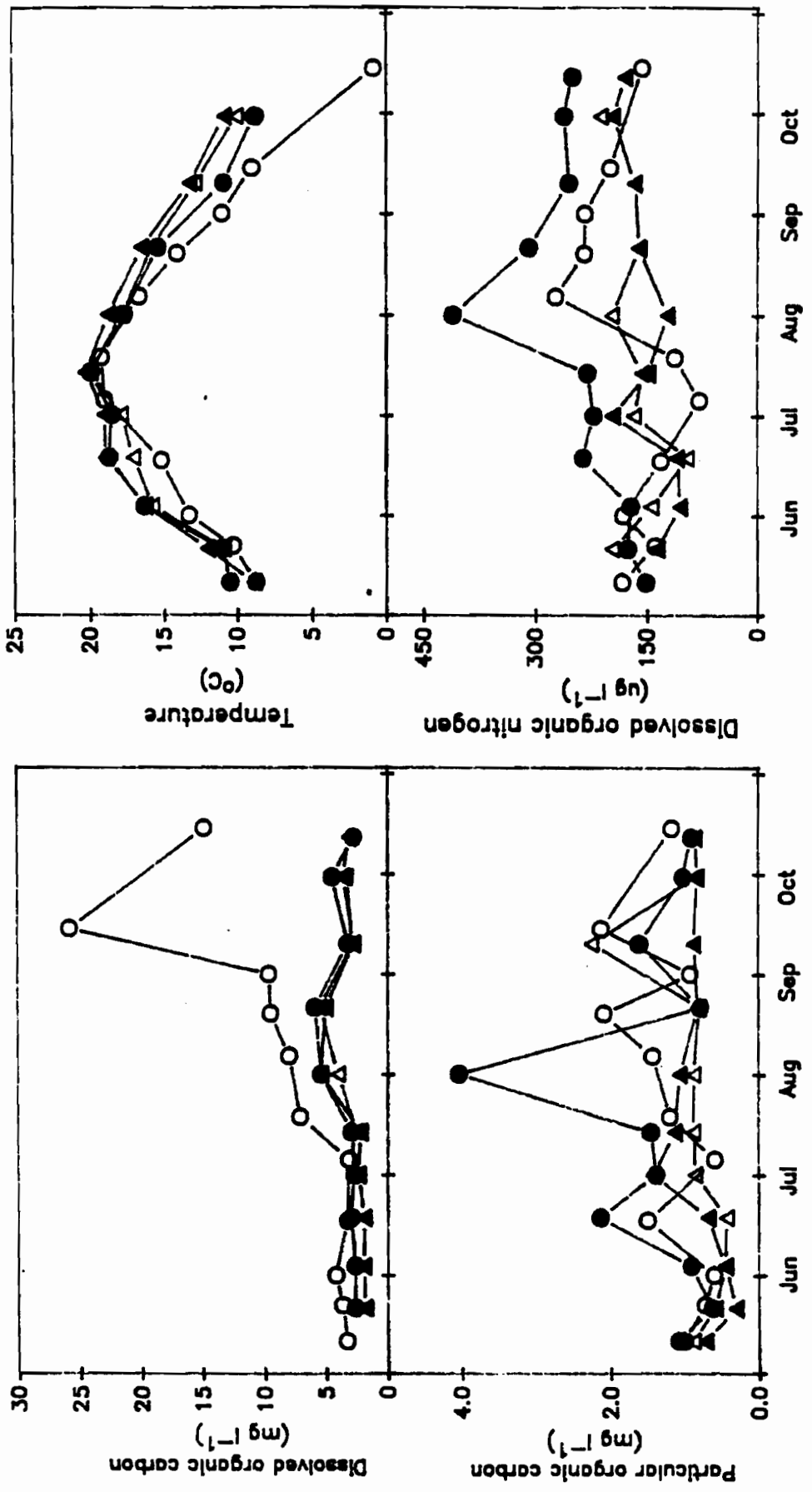
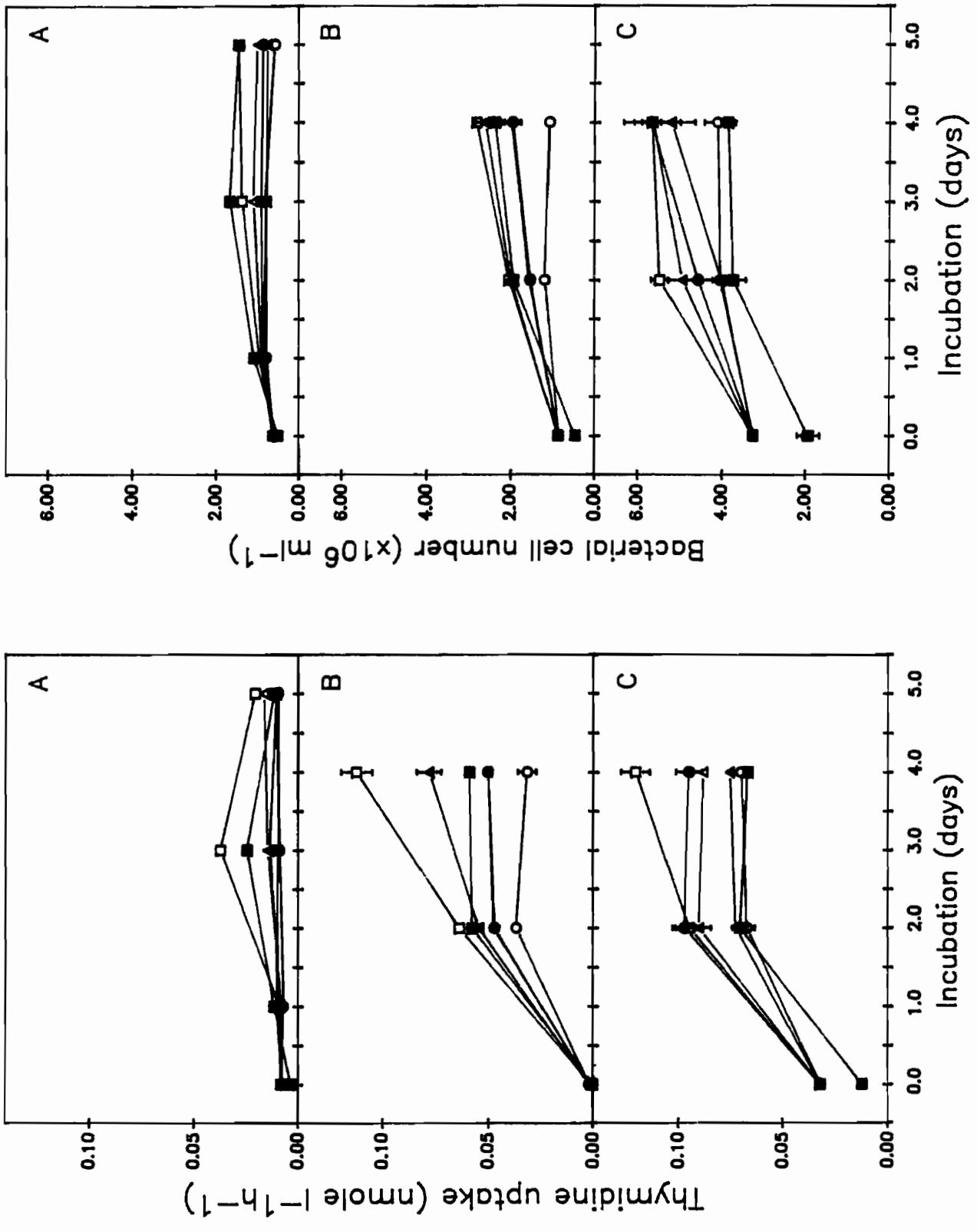

Particular organic carbon
(mg l⁻¹)
Dissolved organic carbon
(mg l⁻¹)
Dissolved organic nitrogen
(ug l⁻¹)
Temperature
(°C)
Jun
Jul
Aug
Sep
Oct

Fig. 8

Bacterial doubling time
(h)
0
100
200
300
400
Bacterial production
(ug C l−1h−1)
0.00
0.30
0.60
0.90
Bacterial cell number
(x10⁶ml−1)
0.0
1.0
2.0
3.0
Bacterial biomass
(ug C l−1)
0
20
40
80

Fig. 9


Thymidine uptake (nmole l⁻¹h⁻¹)
Bacterial cell number (x10⁶ ml⁻¹)
Incubation (days)
A
B
C
0.00
0.05
0.10
2.00
4.00
6.00
0.0
1.0
2.0
3.0
4.0
5.0

CHAPTER 10

AN IN SITU TECHNIQUE TO MEASURE BACTERIAL CHEMOTAXIS IN NATURAL AQUATIC ENVIRONMENTS

(Published: Microbial Ecology. 1990. 20: 3-10)

Microb Ecol (1990) 20:3–10

MICROBIAL ECOLOGY

An in situ Technique to Measure Bacterial Chemotaxis in Natural Aquatic Environments

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Received: April 24, 1990; Revised: June 29, 1990

Abstract. A simple and reliable technique to study bacterial chemotaxis in natural aquatic environments is reported. This technique uses the test chemicals in known volumes of semi-solid agar media placed in double layered, highly porous, polyester tubes. Following in situ incubation, bacteria attracted by the test chemicals are enumerated with fluorescence microscopy following acridine orange staining. Studies in an eutrophic reservoir showed that significant numbers of bacteria were attracted to D-glucose and glycine; no significant effects were observed with L-serine, sodium succinate, or sodium chloride.

Introduction

Bacterial chemotaxis, the movement of bacteria in response to chemicals, was discovered in the eighteenth century [11]. Since then, there have been numerous reports on this subject [1–4, 8, 9, 12]. Bacterial chemotactic behavior can play an important role in bacterial survival and microbial community structure [3]; the various assay methods and techniques reported thus far to study bacterial chemotaxis reflect its importance [1, 8, 10, 11]. Unfortunately, all of these studies were conducted in vitro in prepared liquid media and may not reflect actual responses in nature. Except for the unpublished work of Walsh and Mitchell [as reviewed by Chet and Mitchell (3)] in flowing streams, we are not aware of any reports of in situ assays for bacterial chemotaxis in natural aquatic environments. The paucity of published in situ experimental data is presumably due to lack of available assay techniques that are reliable and effective in natural environments. We present a simple and reasonably easy technique to study bacterial chemotaxis in situ in lentic environments. The technique utilizes direct counting of bacterial cells using fluorescence microscopy of acridine orange-stained cells [5] in semi-solid agar amended with various test chemicals.

Materials and Methods

Laboratory Experiments

Two laboratory experiments were set up to verify the efficacy of our technique. The first examined the extent of the chemical gradient produced outside the assay tubes through diffusion. The second

experiment determined whether 3 hour lake incubation of the assay tubes with the various chemical treatments at 12°C (experimental conditions of the lake experiment) could result in an increase in bacterial numbers through simple multiplication.

The chemicals used in the lake experiment (details presented below) were made up to the same concentration (3 mM) in filtered lake water (0.2 μm polycarbonate membrane, Poretics Corporation). Two types of bacteria most frequently isolated from Hebgen Lake water, *Aeromonas hydrophila* and an unidentified motile bacillus, were grown for 2 days in 0.05% peptone broth in filtered lake water and mixed in equal proportion. A 100 μl aliquot (total bacterial count = 1.7×10^5) of the mixed species bacterial suspension was introduced into each of two replicate conical flasks (125 ml) containing 50 ml of medium at 12°C. The inoculated flasks were then incubated for 3 hours at 12°C. Aliquots (100 μl) of the bacterial suspension were introduced into two separate flasks, each containing 50 ml filtered lake water, and immediately treated with formaldehyde (final concentration, 1.2% w/v) to determine the initial bacterial counts. After 3 hours incubation, the samples were treated with the same level of formaldehyde as above. The bacterial numbers in the various treatments were then enumerated using the acridine orange technique described above. The final bacterial counts were compared with the initial counts using analysis of variance.

To verify the extent of the chemical gradient around the assay tubes, semi-solid agar media (described below in the lake experiment) were prepared with 5 mM sodium chloride or 0.05% w/v phenolphthalein. Assay tubes were then prepared with these agar media. The tube containing sodium chloride-agar was immersed vertically in a beaker containing deionized water at 12°C, and the tube with phenolphthalein-agar was immersed in a beaker containing 0.03 N sodium hydroxide. Samples of water (1–1.5 ml) within one cm of the surface of the sodium chloride-agar tube were carefully drawn using a micropipette every 10 min and tested with a few drops of dilute silver nitrate (2% w/v). The tube containing phenolphthalein was observed for color changes in the bulk sodium hydroxide medium.

Lake Experiment

Six aliquots of semi-solid agar were prepared by autoclaving 0.75 g purified agar (Difco) in 100 ml filtered lake water (0.2 μm polycarbonate membrane, Poretics Corporation, Livermore, CA). When the agar cooled to ca 50°C, five of the aliquots were enriched with either glycine, D-glucose, L-serine, sodium chloride, or sodium succinate (Sigma Chemical Co., St. Louis, MO) to a final concentration of 3.0 mM. The sixth, unamended agar aliquot served as a control. All aliquots were cooled to 4°C for ca 1 hour with periodic brisk hand-agitation so that the media remained a viscous semi-solid. The lake water was obtained from Hebgen Lake, Montana, a highly productive reservoir on the upper Madison River [see (7) for details of the physical, chemical, and biological properties]. All experiments were conducted in this lake during October 1989 on calm days.

Assay tubes were made with double layers of 100% polyester interfacing material (Pellon-Sew in white interfacing 998) sewn together to form a uniformly cylindrical tube with a diameter of 1.1 cm (Fig. 1). They were cut to 11.0–11.5 cm lengths and one end was tied closed with polyester cord. The tubes were then autoclaved for 10 min at 1.1 kg cm^{-2} pressure.

The assay tubes were kept on ice and the agar media, previously cooled to 4°C, was cooled further on ice for at least 1 hour before starting the experiment. Just before starting the experiment, the assay tubes were filled separately with 10.0 ml of the various agar media using individual sterile, wide-mouthed pipettes. The open ends of the tubes were then tied closed using polyester twine and a lead weight of about 10 g was hung at least 20 cm away from the lower end of the tubes (Fig. 2). The assay tubes were then suspended randomly about 20 cm apart from a horizontal beam floating on the surface of water so that the tubes were immersed vertically in water at a depth of 50 cm below the surface. Three such units (replicates) were deployed in a close proximity (randomized complete block design) in the littoral zone of the lake. After a 3 hour incubation, the assay tubes were removed from the lake and individually immersed in 10.0 ml of 1.2% formaldehyde solution in separate sterile polyethylene bags (Nasco-Whirlpak) and sealed.

Fig. 1. Photomicrograph of the polyester material used to make the chemotaxis assay tubes (Bar = 20 μm).

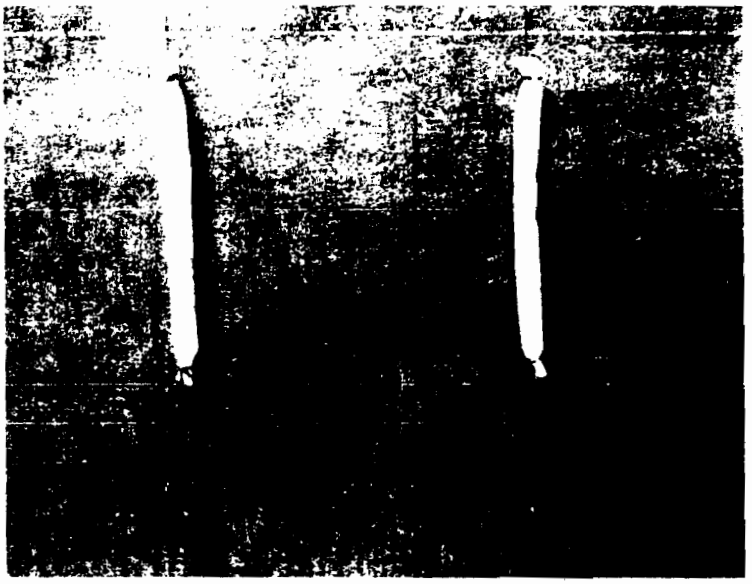

Fig. 2. Chemotaxis assay tubes ready to deploy with their contents of test chemicals incorporated in semi-solid agar (Bar = 2.6 cm).

Bacterial Quantification

Within 48 hours after the samples were fixed in formaldehyde, the assay tubes were cut logitudinally (after cutting the ends) and placed in 125 ml flasks maintained at 60°C in a water bath to soften the medium. Each tube was rinsed (with brisk agitation) with five 10 ml aliquots of distilled water (at 60°C); the rinse was added to the contents of the flask bringing the final volume to 70 ml (10 ml agar, 10 ml 1.2% formaldehyde solution and 50 ml distilled water). All of the material in the flask was then passed serially through 20 and 23 gauge hypodermic needles attached to a 20 ml syringe to make a thin uniform suspension. Aqueous acridine orange solution [1.25 ml of 0.1% (w/v); J. T. Baker Chemical Co., New Jersey] was added to the 70 ml suspension and incubated at 60°C for 30 min. Filtered (0.2 μm) distilled water (70 ml at 60°C) was then mixed with the suspension to make a total volume of 140 ml and incubation at 60°C was continued for an additional hour. Three 500 μl subsamples from each flask were then filtered at 12.0 cm Hg vacuum through 0.2 μm polycarbonate filters (25 mm diameter, Poretics Corporation, Livermore, CA) prestained with Irgalan black. This experimental design yielded 3 subsamples to be counted from each of 3 replicates per treatment. The filters were air dried for about 10 min and placed on microscopic

slides. A drop of immersion oil was added to the filter before adding a cover slip. A counting grid mounted on the eye piece of an epifluorescence microscope (Nikon Labophot, fitted with a Nikon DM 510 B-3A filter cube) was used to make ten separate bacterial cell counts per subsample (under oil immersion at 1250×) following the procedure of Hobbie et al. [5]. Ten counts per subsample (30 counts per replicate) were then analyzed with a three way analysis of variance to determine statistical differences among treatments. This design yielded a pooled total of 90 bacterial counts per treatment.

The temperature of the lake water at the experimental locations was measured within 1.5 hours after starting the lake incubation and samples of lake water were fixed with formaldehyde (1.2% final concentration) for quantification of ambient bacterial numbers. Bacterial counts in the lake water samples collected from the experimental sites were made following the technique described by Hobbie et al. [5].

Results

Laboratory experiments showed no significant difference ($P = 0.46$) between the bacterial counts after 3 hours incubation at 12°C in the various chemical treatments compared to the initial counts. After 20 min, the aliquots collected within one cm from the surface of the sodium chloride-agar tube gave a visually detectable cloudy white precipitate of silver chloride when treated with silver nitrate solution, indicating diffusion of sodium chloride from the agar medium. Samples collected >2 cm from the surface of the tube within about 30 min of the experiment did not produce the characteristic precipitation. The phenolphthalein-agar tube showed red on the surface within 5 min, but after about 15 min a diffusion zone of red extended about one cm from the tube surface, indicating diffusion of phenolphthalein from the agar media and the formation of a gradient. Hence, our technique should provide reliable results when applied to lake water.

The D-glucose and glycine treatments showed 2.6 and 1.8 times higher bacterial numbers, respectively, than the control (Table 1). Attraction to the amino acid L-serine, sodium chloride, and sodium succinate was not significantly greater than that to unamended lake water.

A three way analysis of variance showed that bacterial cell counts among subsamples had greater variation than that among replicates (Table 2). In fact, the source of highest variation (with the exception of among treatment differences) was within subsamples (i.e., associated with bacterial enumeration); the next highest variance was among subsamples (MS = 5.9×10^{13}) and then replicates (MS = 4.07×10^{13}) (Table 2). Despite this error distribution, the method as outlined was still sensitive enough to detect differences among treatments.

The mean temperature (±SE) of the experimental site was 12.0(±0.0)°C and the mean total bacterial number (±SE) at the same sites was 0.90×10^6 ($\pm 0.04 \times 10^6$) cells ml^{-1}.

Discussion

Our observation that D-glucose and glycine showed a highly significant ($P < 0.001$) chemotactic effect despite the high variation observed within and among

Table 1. Number of bacterial cells (mean ± SE) attracted to various chemicals during 3 hours *in situ* incubation in Hebgen Lake, Montana

Treatments	Mean[a] (±SE) counts ($\times 10^6$ cells ml^{-1})
Control	3.285 ± 0.356
Glycine	5.997 ± 0.507[b]
D-glucose	8.530 ± 0.578[b]
L-serine	3.701 ± 0.425[c]
Sodium chloride	2.692 ± 0.231[c]
Sodium succinate	3.206 ± 0.197[c]

[a] Mean of three replicates with each replicate comprised of 3 subsamples with 10 counts each.
[b] Significant from control; $P < 0.001$
[c] Not significant from control; $P > 0.05$

Table 2. Results from a three way analysis of variance of data from chemotaxis experiment in Hebgen Lake, Montana

Sources of variation	DF	MS	*P*
Replicate	2	4.07×10^{13}	0.0219
Treatment	5	4.59×10^{14}	0.0001
Subsample	2	5.90×10^{13}	0.0040
Treatment × replicate	10	1.67×10^{13}	0.1079
Replicate × subsample	4	4.27×10^{12}	0.8056
Treatment × subsample	10	9.09×10^{12}	0.5705
Treatment × replicate × subsample	20	1.19×10^{13}	0.3161
Residual	486	1.06×10^{13}	

Df = degree of freedom; MS = mean square; *P* = probability level; the residual MS corresponds to the errors associated with bacterial counts within subsamples

subsamples and replicates (Table 2) demonstrated that the technique was effective under the conditions in which it was used. That there was no significant ($P > 0.05$) treatment-replicate or treatment-replicate-subsample interaction (Table 2) further verifies the efficacy of the technique with respect to detecting differences among treatments. Even though L-serine did not produce a statistically significant chemotactic effect ($P > 0.05$), it attracted, on average, 13% more bacterial cells than the control. Sodium chloride and sodium succinate treatments apparently repulsed 18% and 2% bacteria, respectively, compared to the control; these decreases were statistically not significant ($P > 0.05$). The nearly 3.5-fold higher total counts of bacterial cells found in control tubes compared to the surrounding water may be due to chance entanglement on the viscous surface of the tubes, to an unknown attraction to the surface of the tubes, or to possible attraction to low molecular weight contaminants present in the purified agar.

This study, aimed at developing a technique to assay bacterial chemotaxis in a natural aquatic environment, did not examine the various factors known

to affect chemotaxis, such as temperature, concentration of test chemicals, bacterial speciation, the chemical nature of the surrounding medium, bacterial counts in the surrounding medium, and percent motile cells [1–3]. The concentration of 3.0 mM was selected on the basis of previous laboratory studies which reported optimal chemotaxis towards organic compounds near this concentration [1, 4]. However, by manipulating test chemical concentration, time of incubation, and other selected variables, our technique could be used to examine mechanisms responsible for the observed tactic responses. Laboratory verification experiments using sodium chloride and the pH indicator chemical phenolphthalein showed that these chemicals formed a concentration gradient in the bulk medium further indicating that chemotaxis is the mechanism being measured in this technique.

Even though the bacterial species found in the various treatments were not characterized, we noted that there was more than one morphological type that showed positive chemotaxis. The involvement of more than one bacterial species, the use of a fixed concentration of 3.0 mM for all test chemicals, and a set incubation time of 3 hours may be partially responsible for the apparently insignificant chemotactic response shown towards L-serine, which has been shown by others to be a positively chemotactic substance for bacteria [2–4, 9]. More detailed experiments are required in Hebgen Lake to verify the apparent lack of chemotaxis, particularly to L-serine and perhaps to sodium succinate.

The material of the assay tubes used in this technique was durable, very porous (Fig. 1), and easy to manipulate while still retaining fully the semi-solid agar under the test conditions when two layers were used. The semi-solid agar or the agar-filled assay tubes were maintained at <5°C before starting the experiment to prevent excessive softening of the medium.

Even though a duration of 3 hours was used for the experiment, reduced time periods may be equally effective [1, 12]. Adler in his *in vitro* assays [1] used a constant incubation period of 1 hour, whereas, Wellman and Paerl [12] found a steady increase in numbers of attracted bacteria with times at 15, 30, and 60 min. These results are also dependent on other factors such as bacterial density, temperature, and ambient chemical composition. Time-course experiments could be conducted with our method to provide information on optimum incubation time and to examine potential mechanisms involved with the tactic responses observed. Our laboratory observation that there was no significant increase of bacterial numbers during the 3 hour incubation at 12°C further verified that chemotaxis was responsible for the differential accumulation of bacteria in the chemotaxis assay tubes. Even though we used only two species of motile bacteria, they were the most frequently isolated types from Hebgen Lake water at the time of our lake experiment, and therefore, should be good representatives for this laboratory verification.

Five rinses of the evacuated assay tubes, each in 10.0 ml portions of distilled water at 60°C, was adequate to remove all the agar medium and bacteria. The efficacy of rinsing was demonstrated by trial washings, followed by microscopic examination. Warming the semi-solid agar medium to 60°C and passing it through 20 and 23 gauge needles and eventually bringing it to 14-fold dilution proved effective in dispersing the agar, thus making a fairly uniform bacterial

suspension. However, from the relatively high variation observed within subsamples, the technique could be improved by increasing the number of counts per subsample, or further dispersing the cells by increased dilution or by more than one serial passage through the needles. It should be noted that our error distribution is specific for the Hebgen Lake experiment; systems with different bacterial abundances, both ambient and attracted to the test chemicals, may show a different distribution of errors.

The modified fluorescence microscopic technique for bacterial enumeration utilized in this study proved effective under the protocols used. Most of the bacterial cells appeared as distinct green or greenish-orange particles thus making it easy to differentiate bacteria from other nonbacterial particles. Acridine orange staining is particularly applicable to our technique in that it does not require accurate pH controls, as opposed to the use of other fluorochromes such as DAPI and bisbenzimide (Hoechst dye 33258) which require accurate pH control for optimal results [6].

Although chemotactic assays have been conducted primarily in nonflowing liquid media under laboratory conditions, the unpublished work of Walsh and Mitchell revealed nonrandom linear movement of bacteria towards attractants injected into chemotactic assay chambers in flowing streams [3]. Their observations have been the only available information we are aware of which indicates that bacterial chemotaxis indeed occurs in natural environments. Our study, conducted in a lake, corroborates the findings of Walsh and Mitchell and provides a simple and tractable technique to study bacterial chemotaxis in lentic environments *in situ*.

Acknowledgments. We thank T. Tonkovich for her assistance in the laboratory, W. Dodds, M. Lizotte, and K. Lohman for their critical review of this manuscript. This study was conducted under a grant from The Soap and Detergent Association to JCP.

References

1. Adler J (1973) A method for measuring chemotaxis and use of the method to determine optimum conditions for chemotaxis by *Escherichia coli*. J Gen Microbiol 74:77–91
2. Adler J (1975) Chemotaxis in bacteria. Ann Rev Biochem 44:341–356
3. Chet I, Mitchell R (1976) Ecological aspects of microbial chemotactic behavior. Ann Rev Microbiol 30:221–239
4. Gallucci KK, Paerl HW (1983) *Pseudomonas aeruginosa* chemotaxis associated with blooms of N2-fixing blue-green algae (cyanobacteria). Appl Environ Microbiol 45:557–562
5. Hobbie JE, Daley RJ, Jasper S (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33:1225–1228
6. Kangatharalingam N, Ferguson MW (1984) A simple and rapid technique for fluorescence staining of fungal nuclei. Current Microbiology 10:99–104
7. Martin DB, Arenson RD (1978) Comparative limnology of a deep-discharge reservoir and a surface-discharge lake on the Madison River, Montana. Freshwater Biol 8:33–42
8. Migas J, Anderson KL, Cruden DL, Markovetz AJ (1989) Chemotaxis in *Methanospirillum hungatei*. Appl Environ Microbiol 55:264–265
9. Paerl HW, Gallucci KK (1985) Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association. Science 227:647–649

10. Palleroni NJ (1976) Chamber for bacterial chemotaxis experiments. Appl Environ Microbiol 32:729–730
11. Pfeffer W (1884) Locomotorische Richtungsbewegungen durch chemische Reize. Untersuchungen aus dem Botanischen Institute in Tubingen 1:363–482
12. Wellman AM, Paerl HW (1981) Rapid chemotaxis assay using radioactively labelled bacterial cells. Appl Environ Microbiol 42:216–221

CHAPTER 11

EVIDENCE FOR BACTERIAL CHEMOTAXIS TO CYANOBACTERIA FROM A RADIOASSAY TECHNIQUE

(Submitted: Applied and Environmental Microbiology)

Assay Organisms and Media

The motile bacterium Aeromonas hydrophila (identity confirmed by Microbial ID, Inc., Newark, DE) was used as the test bacterium. This species was the numerically dominant type (based on gram stain, cell and colony morphology during dilution plating) among those isolated from eutrophic Hebgen Lake, Montana. The non-N_2-fixing cyanobacterium Lyngbya birgei and N_2-fixing cyanobacterium Aphanizomenon flos-aquae isolated from the same lake were used in this study. These cyanobacteria dominated the water column of Hebgen Lake during September and October 1989 when our study was conducted (Kangatharalingam and Priscu, unpublished data).

Aeromonas hydrophila was grown in peptone agar (0.5% bacto-peptone with 1.5% or 2.0% Difco bacto-agar), or lake water (pH 7.9-8.0) filtered through a polycarbonate membrane filter (0.4 μm; Poretics Corporation, Livermore, CA.) amended with either 0.5% or 0.25% bacto-peptone (Difco). When grown in liquid media, 50 μM EDTA (J.T. Baker Chemical Co., Phillipsburg, NJ) and 3 mM methionine (Calbiochem, LA, CA) were added to ensure motility of the bacteria (1, 7).

The cyanobacteria were used for experiments within two days of collection from the upper 20 cm of Hebgen Lake. Upon collection, the cyanobacteria were transferred to filtered (0.2 μm) lake water and stored for up to 2 days at 12 ^{o}C and a photosynthetic photon flux density of 200 μmol quanta m^{-2} s^{-1} supplied by "cool white" fluorescent tubes. Bacteria were isolated from lake water samples and cyanobacterial washings (cyanobacteria were expressed 3 times through 20 and 22 gauge needles) by plating on nutrient agar (Difco) and amended lake-water agar contain-

ing 0.1% bacto-yeast extract, 0.25% bacto-dextrose, 0.2% bacto-peptone, and 2.0% bacto-agar in lake water filtered through a 0.4 μm filter. Both media were autoclaved at 1.1 kg cm^{-2} pressure for 15 min.

Radiolabeling Bacteria

Aeromonas hydrophila was grown in 0.5% bacto-peptone in filtered (0.4 μm) and autoclaved lake water in a 125 ml Erlenmeyer flask at 23$\pm$1 ^{o}C on a G10 Gyrotory shaker (New Brunswick Scientific Co. Inc., NJ) at 60 rpm to a bacterial density of approximately 1 X 10^{10} cells ml^{-1}. One ml of this culture was transferred to 10 ml of 0.25% bacto-peptone in lake water with 3 mM methionine and 50 μM EDTA, and grown with shaking as described above to obtain a cell count of approximately 1 X 10^{10} ml^{-1}. The bacterial culture was again transferred (1 ml per 10 ml medium) into filter-sterilized (0.2 μm) lake water (lacking bacto-peptone) with 3 mM methionine, 50 μM EDTA and 20 μCi methyl-^{3}H thymidine (specific activity 80.7 Ci $mmol^{-1}$; Dupont, Wilmington, DE) and grown as described above for 4 h. This dilution series gradually lowered the concentration of bacto-peptone which was necessary for efficient ^{3}H labeling of the cells. The labeled bacterial suspension was then filtered onto a sterile 0.2 μm membrane filter under gentle vacuum, washed three times with filter-sterilized lake water (0.2 μm) and sufficient cells were resuspended in filter-sterilized lake water with 50 μM EDTA and 0.005% (w/v) Tween 80 (Sigma Chemical Co., St. Louis, MO) to bring the bacterial counts to 8.0 X 10^{8} cells ml^{-1}. The specific

activity of a 0.5 ml aliquot of bacterial suspension was determined by standard liquid scintillation spectrometry following the addition of 7 ml scintillation cocktail (Cytoscint, ICN Radiochemicals) using a Beckman LS-100C liquid scintillation counter. Because certain heavy metals are known to affect bacterial motility, the chelating agent EDTA was added; Tween 80 was used to prevent bacterial attachment to surfaces (7). Methionine was added to the bacterial suspension because it has been shown that chemotaxis of at least some strains or mutants of bacteria are stimulated in the presence of methionine (1).

Preparation of Cyanobacterial Samples for Chemotaxis Assay

Samples from essentially uni-algal blooms of L. birgei, and A. flos-aquae (confirmed by microscope observation) from Hebgen Lake, were gently filtered onto polycarbonate membrane filters (pore size 8 μm), rinsed from the filter with filter-sterilized (0.2 μm) lake water and gently passed through 18 and 20 gauge sterile hypodermic needles fitted on a 20 ml syringe. The expressed sample was further washed two times on an 8 μm membrane filter with filter-sterilized (0.2 μm) lake water. This procedure removed most of the bacteria and debris associated with the cyanobacteria while dispersing the aggregates without causing visible damage to the filaments. The dispersed cyanobacteria were suspended in filter-sterilized lake water and pre-incubated at 25 ^{o}C at 200 μmol quanta m^{-2} s^{-1} supplied by "cool white" fluorescent lamps for 2-3 h before the chemotaxis assay.

Chlorophyll Assay

Assay for chlorophyll a (chl a) was by initial extraction in 95% ethanol at 79 ^{o}C for ca. 30 min; extraction continued overnight at 4 ^{o}C (15). Either a spectrophotometer (Varian DMS 80) or fluorometer (Turner fluorometer model 112; Sequoia-Turner Corporation, CA) was used to quantify the concentration of chl a in the extract (10, 17). Spectrophotometric chl a concentration was determined using the extinction coefficient given by Wintermans and De Mots (17); fluorometrically measured concentration was computed from comparison with pure chl a from Anacystis (Sigma Chemical Co., St. Louis, MO) in 95% ethanol.

Chemotaxis Assay

Tygon tubing (formulation R-3603, VWR Scientific) having an inner diameter of 0.8 cm, wall thickness of 0.16 cm was used as the reservoir for labeled bacterial cells. Holes of approximately 3.5 mm diameter were made along the length of the tube using heated steel wire. The distance between adjacent holes was 5 cm, leaving a length of 3 cm on one end and 5 cm on the other (Fig 1). The tube was then autoclaved at 1.1 kg cm^{-2} pressure for 10 min and positioned on a rigid styrofoam sheet using adhesive tape over layers of absorbing paper so that the holes were parallel to the plane of the sheet. The end of the tube with the 3 cm extension from the last hole was closed with a metal clamp. The other end was raised slightly using a piece of the rigid styrofoam.

After 2-3 hours of pre-incubation, the cyanobacterial suspensions were degassed using a vacuum of 12 cm of Hg for about 5 min to remove existing bubbles and prevent their formation in the

media. After gentle mixing to ensure homogeneous suspensions, 0.5 ml each of the L. birgei and A. flos-aquae samples were drawn into sterile, disposable glass syringes (Glaspak B-D, Becton Dickinson, Rutherford, NJ) of 2.5 ml capacity without gas bubble formation which otherwise caused erratic results. The tips of two replicate syringes per species were then randomly inserted into holes on the tube along with two control syringes that contained 0.5 ml each of degassed filter-sterilized (0.2 μm) lake water. The six syringes were mounted horizontally on the styrofoam sheet following adjustment to ensure that the syringe contents completely filled the tips of the syringes. The freshly prepared radiolabeled bacterial cell suspension was then slowly introduced into the tube through the open end using a sterile pipette without introducing air bubbles so that it filled all but the last 1 cm of the tube. Care was taken to ensure that no air bubbles were introduced into the tube which could trap bacteria. The open end of the tube was sealed using a metal clamp. The whole apparatus (Fig. 1) was incubated horizontally at 23$\pm$1 ^{o}C for 3 h in the dark.

Two 0.5 ml portions of the initial radiolabeled bacterial sample were immediately fixed using 0.05 ml formalin to determine the initial radioactivity. Bacterial cell counts of the same initial sample were also conducted using a hemocytometer to determine the initial specific activity of the bacterial suspension on a per cell basis.

Before initiating the actual experiments, the equipment was tested for over 4 h with neutral red solution in the syringes and water in the Tygon tube, and vice versa, to verify that there was

no significant bulk transport of the liquid from the syringes into the tube or from the tube into the syringes. We also verified that more than 95% of the bacterial cells in the suspension were motile when examined under the microscope at the beginning of the experiment.

After 3 h of incubation, the syringes were removed, the tips carefully rinsed with distilled water and the contents transferred to separate scintillation vials. Formalin (3% final concentration) was added to each vial to kill cells immediately before the addition of 7.0 ml Cytoscint scintillation cocktail (ICN Radiochemicals, Irvine, CA). The radioactivity in each sample was determined by standard scintillation spectrometry using a Beckman LS-100C liquid scintillation counter; the counting efficiency was determined by the external standard ratio method using ^{3}H-toluene as a reference. The data from 2 observations/ treatment for 3 experiments (yielding a total of 6 observations per treatment) were combined in an analysis of variance to determine statistical differences among treatments.

Two additional controls were examined in a separate series of experiments to determine whether the chemical substances exuded from the cyanobacteria were present in the suspending media after pre-incubation. These additional controls consisted of suspending medium exposed to L. birgei (lake water which was filter sterilized (0.2 μm) following 24 h incubation at 12 ^{o}C and 300 μmole photon m^{-2} s^{-1} with L. birgei and used in place of the lakewater control described previously), and suspending medium exposed to A. flos-aquae under similar conditions. To further verify that

bacterial motility led to the accumulation of labeled bacteria in the syringes, the chemotaxis assay was repeated using radiolabeled bacteria immobilized with $HgCl_2$ (final concentration 0.05% w/v). Microscopic observation confirmed that this concentration of $HgCl_2$ eliminated motility.

The chemotaxis assay was also carried out following the above procedure to determine the effect of cyanobacterial biomass on bacterial chemotaxis. After pre-incubating the cyanobacteria, four different concentrations (i.e. chl a levels) of cyanobacteria (2 replicates at each concentration), together with a lake water control free of cyanobacteria, were used to determine the differential chemotactic response of A. hydrophila to cyanobacterial biomass.

RESULTS AND DISCUSSION

The chemotactic response of radiolabeled A. hydrophila, towards L. birgei and A. flos-aquae showed significant differences ($P=0.025$) from lake water controls containing no cyanobacteria (Table 1). Differences between cyanobacterial species were compared after normalization to chl a. No statistically significant difference between L. birgei and A. flos-aquae occurred in the chemotactic attraction of A. hydrophila when the observations from the three experiments were pooled (Table 2). Within each individual experiment, bacterial attraction to A. flos-aquae was always greater than to L. birgei. The lack of significant differences between the cyanobacterial species, despite almost two-fold greater attraction to A. flos-aquae, results from relatively high variances associated with the L. birgei treatment. Greater at-

traction was expected to A. flos-aquae because Hebgen Lake has been shown to be strongly N-deficient (J. C. Priscu, unpublished data); extracellular N-products resulting from N_2-fixation by this organism may result in greater attraction of microorganisms incapable of N_2-fixation.

Microscopic observation at the end of the experiment showed that active motility of the bacterial cells was maintained in the suspension for at least 3 h. Degassing the cyanobacterial suspensions just before starting the experiment, and avoiding gas bubbles in the syringe contents were essential because surface tension and physical blockage were shown to impede bacterial movement. This experiment was conducted in the dark because preliminary experiments showed that gas bubbles were produced by cyanobacterial samples in the syringes when incubated under light (presumably through photosynthesis). The formulation of Tygon tube used in this study was tested for apparent toxic effects on the bacteria by growing the bacteria in cultures mixed with pieces of the tube material for 3 days. We found no significant difference in growth rates or motility of the bacteria in the treatment compared to control.

The suspending medium from either species showed no significant ($P>0.05$) bacterial chemotactic attraction compared to the lake water control. The lake water control, therefore, appears to be an appropriate control for this assay. This observation implies that cyanobacterial products, presumably amino acids and carbohydrates as reported previously (8, 13), eliciting chemotaxis may be localized in the mucilaginous outer wall layers of the

ACKNOWLEDGEMENTS

We thank T. Galli for assistance in the laboratory, T. Miller for his useful discussions during our experiments, and R. Sedlak, M. Lizotte and K. Lohman for their critical review of the manuscript. This research was supported by a grant from The Soap and Detergent Association.

LITERATURE CITED

1. Adler, J. 1973. A method for measuring chemotaxis and the use of the method to determine optimum conditions for chemotaxis by Escherichia coli. J. Gen. Microbiol. 74:77-91.

2. Adler, J. 1975. Chemotaxis in bacteria. Ann. Rev. Biochem. 44:341-356.

3. Arora, D.K., A.B. Filonow, and J.L. Lockwood, 1983. Bacterial chemotaxis to fungal propagules in vitro and in soil. Can. J. Microbiol. 29:1104-1109.

4. Bunt, J.S. 1961. Isolation of bacteria-free cultures from hormogone-producing blue-green algae. Nature 192:1275-1276.

5. Chet, I., and R. Mitchell. 1976. Ecological aspects of microbial chemotactic behavior. Ann. Rev. Microbiol. 30:221-239.
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6. Delucca, R., and M. D. McCracken. 1977. Observations between naturally collected bacteria and several species of algae. Hydrobiologia 55: 71-75.

7. Gallucci, K.K., and H.W. Paerl. 1983. Pseudomonas aeruginosa chemotaxis associated with blooms of N_2-fixing blue-green algae (cyanobacteria). Appl. Environ. Microbiol. 45:557-562.

8. Herbst, V., and J. Overbeck. 1978. Metabolic coupling between the alga Oscillatoria redekei and accompanying bacteria. Naturwissenschaften 65:598-599.

9. Kangatharalingam, N., L. Wang and J.C. Priscu. 1990. An in situ technique to measure bacterial chemotaxis in natural aquatic environments. Microb. Ecol. 20:3-10.

10. Lorenzen, C.J. 1967. Determination of chlorophyll and pheopigments: Spectrophotometric equations. Limnol.Oceanog. 12:343-346.

11. Murray, R.E., K.E. Cooksey, and J.C. Priscu. 1986. Stimulation of bacterial DNA synthesis by algal exudates in attached algal-bacterial consortia. Appl. Environ. Microbiol. 52: 1177-1182.

12. Paerl, H.W. 1982. Interactions with bacteria: In The Biology of Cyanobacteria - Botanical Monographs Vol. 19. Edited by Carr, N.G. and B.A. Whitton. University of California press, Los Angeles. 688 pp.

13. Paerl, H.W., and K.K. Gallucci. 1985. Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association. Science 227:647-649.

14. Pfeffer, W. 1884. Locomotorische Richtungsbewegungen durch

chemische Reize. Untersuchungen aus dem Botanischen Institute in Tubingen 1:363-482.

15. Sartory, D.P., and J.U. Grobbelaar. 1984. Extraction of chlorophyll a from fresh water phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-187.

16. Wellman, A.M., and H. W. Paerl. 1981. Rapid chemotaxis assay using radioactively labeled bacterial cells. Appl. Environ. Microbiol. 42:216-221.

17. Wintermans, J.F.G.M., and A. De Mots. 1965. Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim. Biophys. Acta 109:448-453.

Table 1. Chemotactic attraction of *Aeromonas hydrophila* to *Lyngbya birgei* and *Aphanizomenon flos-aquae*, based on pooled data from three experiments. Significance was determined using a one-way analysis of variance coupled with a least significant difference comparison.

Treatment	[a]N	[b]DPM (±S.E.)
Control	6	74.04±7.45
L. birgei	6	*224.60±58.76
A. flos-aquae	6	*295.90±77.51

[a] Number of observations.

[b] Mean disintegrations min^{-1} ±Standard Error.

* Significant from control at P=0.025.

Table 2. Comparison of chemotactic attraction of *Aeromonas hydrophila* to *Lyngbya birgei* and *Aphanizomenon flos-aquae* based on pooled data from three experiments. Significance was determined using a one-way analysis of variance coupled with a least significant difference comparison.

Treatment	[a]N	[b]Net DPM (μg chl *a*)$^{-1}$ ($\pm$S.E.)
L. birgei	6	8.54$\pm$3.22
A. flos-aquae	6	16.46$\pm$3.70

[a] Number of observations.

[b] Mean disintegrations min^{-1} (minus that of control) normalized to chlorophyll *a* $\pm$Standard Error.

LIST OF ILLUSTRATION

Fig. 1

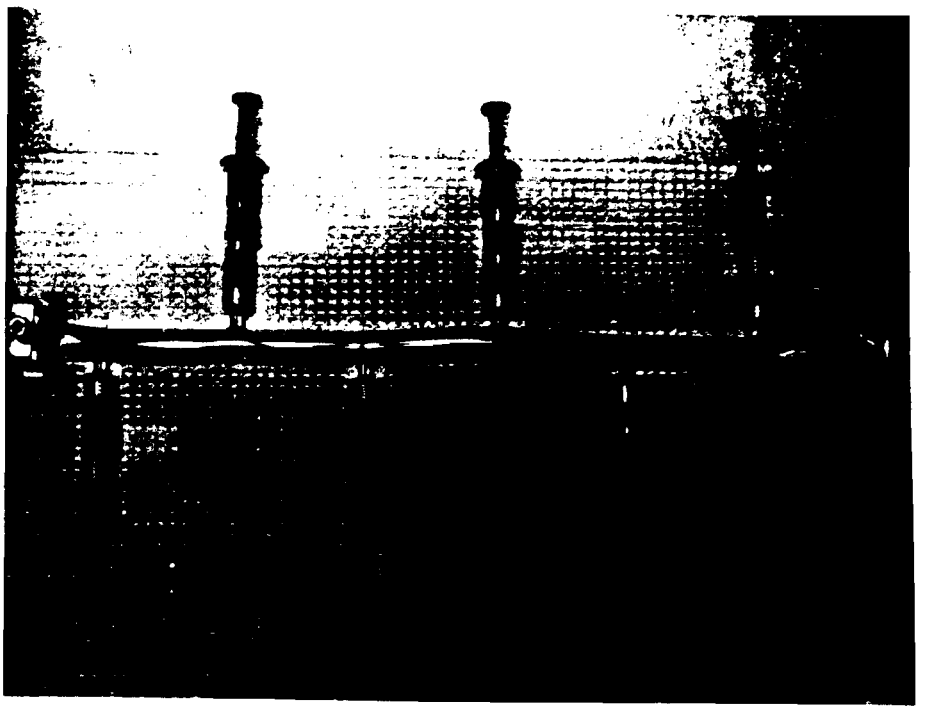
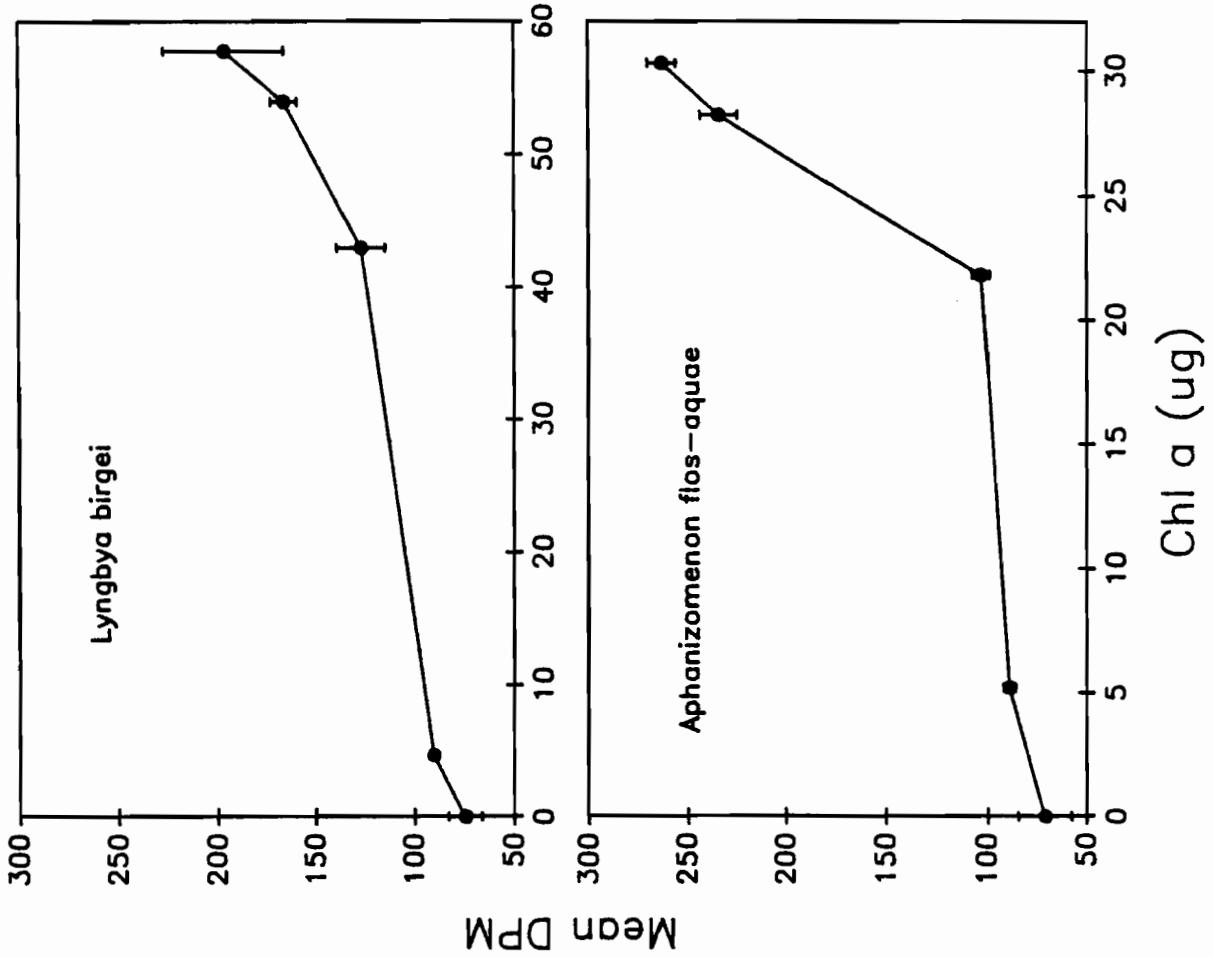


Fig. 2


Lyngbya birgei
Aphanizomenon flos-aquae
Mean DPM
Chl a (ug)
300
250
200
150
100
50
0
10
20
30
40
50
60
5
15
25

CHAPTER 12

STIMULATION OF BACTERIOPLANKTON ACTIVITY BY CYANOBACTERIA IN A LABORATORY EXPERIMENT

(Part of a Ph.D. dissertation: Lizhu Wang)

STIMULATION OF BACTERIOPLANKTON ACTIVITY BY CYANOBACTERIA IN A LABORATORY EXPERIMENT

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ABSTRACT

The response of a natural bacterioplankton population to various densities of the filamentous cyanobacteria Aphanizomenon flos-aquae and Lyngbya birgei collected from a eutrophic lake is reported. The cyanobacteria were separated from bacterioplankton by dialysis membranes which allowed cyanobacterial products to pass. ^{3}H-thymidine uptake and cell number of bacteria isolated from lake water and cyanobacterial phycosphere, and resuspended in lake water, significantly ($p<0.05$) correlated with cyanobacterial density. Bacterial cell numbers on outer surface of the tubular membrane containing the cyanobacteria (on the side exposed to the test bacteria) also significantly ($p<0.01$) correlated with densities of cyanobacteria. Our results indicate that cyanobacterial products including exudates and other forms of releases have a potentially important role in regulating bacterioplankton productivity in aquatic systems.

Introduction

Phytoplankton cells can release a substantial portion of their photosynthate to their environment under certain condition (Larsson and Hagstrom 1979; Feuillade et al. 1988; Sundh 1989). Such release can result from actively growing and senescing phytoplankton and may arise from mechanical breakage by grazers (Nalewajko 1977; Lampert 1978). Operational distinctions between production by healthy cells and other modes of organic carbon loss by phytoplankton are controversial (Cole et al. 1982). The term "photosynthetically produced dissolved organic carbon (PDOC)" has been used by Wiebe and Smith (1977), Cole et al. (1982), and Sundh (1989) to include releases from phytoplankton. We use the term "cyanobacterial products" to include all soluble products released by the intact and senescing cells and perhaps products of decay of cyanobacteria.

Studies have shown that phytoplankton products can be utilized by bacteria (Larsson and Hagstrom 1979; Bell and Sakshaug 1980; Cole et al. 1982; Feuillade et al. 1988; Chrzanowski and Hubbard 1989; Sundh 1989) and can account for 30 to 90% of bacterial carbon uptake (Coveny 1982; Larsson and Hagstrom 1982). Therefore, phytoplankton products are potentially important substrates and energy sources for bacterioplankton growth.

Methods of investigating the pathway from phytoplankton products to bacteria include correlations between phytoplankton and bacterial activity (Larsson and Hagstrom 1982; Marvalin et al. 1989; Robarts and Wicks 1990), size fractionation and radio isotope labeling of bacteria and phytoplankton (Derenbach and Williams 1974; Berman and Gerber 1980; Larsson and Hagstrom 1982;

Cole et al. 1982; Wolter 1982; Feuillade et al. 1988; Chrzanowski and Hubbard 1989; Sundh 1989) and antibiotic inhibition of bacterial activity (Chrost 1978; Jensen 1983; Jensen and Sondergaard 1985). However, there are many problems associated with these methods (Jensen and Sondergaard 1985; Sundh 1989). Problems associated with size fractionation techniques include size overlap of bacterioplankton and phytoplankton, difficulties in separating attached bacteria from phytoplankton, and difficulties in estimating bacterial respiration (Jensen and Sondergaard 1985). The use of antibiotics is often complicated by secondary effects on phytoplankton (Derenbach and Williams 1974; Berman 1975; Chrost 1978), which makes calculations of gross release and uptake difficult (Jensen 1983; Jensen and Sondergaard 1985). Factors influencing direct activity or biomass correlation between phytoplankton and bacterioplankton include losses owing to grazing on bacterioplankton and phytoplankton which can lead to misleading relationships.

A substantial amount of work has been done on the release of phytoplankton products and its subsequent assimilation by bacteria (e.g. Mague et al. 1980; Cole et al. 1982; Chros and Faust 1983; Jensen 1983; Jensen and Sondergaard 1985; Feuillade et al. 1988; Sundh 1989). However, most of the studies have been qualitative in their approach or have compared the heterotrophic utilization of phytoplankton products with primary production, and very few have treated the quantitative role of phytoplankton products in relation to bacterial growth (Riemann and Sondergaard 1984). The purpose of our study was to examine the stimulation of

bacterioplankton activity and growth by different densities of cyanobacterial aggregates using dialysis membrane to physically separate the bacteria from cyanobacteria.

Materials and Methods

Field collection

The cyanobacteria, Lyngbya birgei and Aphanizomenon flos-aquae were collected from the surface water of Hebgen Lake, Montana, a 50 km^2 eutrophic reservoir located on the upper Madison River. The cyanobacteria were transported under darkness to the laboratory and maintained in filter-sterilized (0.2 μm) lake water for 3 to 5 days for them to get acclimatized to the experimental conditions. Surface lake water containing natural bacterioplankton populations was collected from the same lake and transported on ice to the laboratory. Samples were collected during September 1989 and again in August 1990.

Experimental conditions.

All incubations were at room temperature (25 $\pm$ 1 ^{o}C) on a G10 Gyrotory Shaker (New Brunswich Scientific Co.) set at 60 rpm. Light was provided at a 12 h light/dark cycle by four 40-w cool white fluorescent tubes with a photosynthetic photon flux density of 120 $\mu E\ m^{-2}\ s^{-1}$. Incubations were conducted in 1000 ml (experiments 1 and 2) and 250 ml (experiment 3) flasks for 5 days. Duplicate flasks were used for each treatment.

Experimental design

Experiment 1 September 1989. Lyngbya. birgei and A. flos-aquae were filtered (30 mm Hg pressure) onto 8.0 μm polycarbonate

membrane filters (Poretics Co.) and resuspended in filter-sterilized (0.2 μm) lake water to obtain chlorophyll a concentrations of 368 and 507 μg l^{-1}, respectively. Spectra/por molecular-porous membrane tube (2.5 cm diameter; MW cut off of 12,000-14,000) was used to partition the test bacteria from cyanobacteria. Control tubes were filled with lake water filtered through 1.0 μm membrane filters to eliminate grazers and phytoplankton. Tubes were filled separately with concentrated L. birgei and A. flos-aquae using only one density per species in initial tubes. Five 1 l conical flasks for each species were filled with 900 ml lake water filtered through 1.0 μm filters.

Cyanobacterial density for different treatments were adjusted by varying the ratio of the number of tubes containing cyanobacteria, to control tubes. By using this method, we achieved 4 cyanobacterial densities (low, mid-low, mid-high, and high).

The final concentration of chlorophyll a at each cyanobacterial density was calculated based on the total amount of chlorophyll a in the tubes and the mean water volume in each of the flasks during the incubation. Such calculations were based on number of tubes with cyanobacteria in each flask to get an average cyanobacterial density per flask.

Experiment 2 September 1989. Lyngbya birgei and A. flos-aquae were concentrated to 147 and 206 μg l^{-1} chlorophyll a respectively as described in experiment 1. The cyanobacterial tubes were prepared and the final chlorophyll a concentrations were determined as in experiment 1 except that the "high" cyanobacterial density was omitted. The bacteria employed in experiment 2 were

those associated with the cyanobacteria.

To isolate the bacteria from cyanobacteria, L. birgei and A. flos-aquae samples were rinsed 3 times with filtered lake water (0.2 μm) on 8.0 filters and resuspended in filtered lake water. The cyanobacterial suspensions were then expressed 3 times through 22, 23, and 26 gauge needles to remove as much associated bacteria as possible. Suspensions (0.5 ml) were plated on amended lake water agar (ALWA) containing 0.1% bacto-yeast extract, 0.25% bacto-dextrose, 0.2% bacto-peptone, and 0.2% bacto-agar in lake water filtered through 0.2 μm filter. The agar medium was autoclaved at 1.1 kg cm^{-2} pressure for 15 min. Agar plates were inoculated with the bacterial suspensions using spread plate technique and incubated at room temperature (25 ± 1 ^{o}C) for 48 h. Two morphologically distinct bacterial colony types, which accounted for >95% total colony forms, were isolated from each of L. birgei and A. flos-aquae cultures. The 2 selected types of colonies were transferred separately into amended lake water broths (ALWA without bacto-agar). The broth cultures were incubated at room temperature for 48 h. Lake water filtered through 1.0 μm membrane filters and autoclaved under 1.1 kg cm^{-2} pressure for 40 min were prepared in two 10 l containers. Equal amounts of both broth cultures isolated from the same cyanobacteria were pipetted into the autoclaved lake water to obtain a bacterial density of $1.6x10^{6}$ cells ml^{-1} determined microscopically. Sufficient lake water with inoculated bacteria was transferred to the experimental flasks.

Experiment 3 August 1990. Lyngbya birgei and A. flos-aquae were concentrated into 4 different densities (Table 3) as de-

scribed in experiments 1 and 2. Thirty ml per tube of each cyanobacterial density was added to each of 4 spectra/por molecular-porous membrane tubes with the same MW cutoff as in experiment 1 and 2 but 2.86 cm in diameter. Lake water (200 ml) prepared as in experiment 1 was added to each of five 250-ml flasks. The 4 tubes containing different concentrations of cyanobacteria were then suspended in each of the 4 flasks. One additional tube filled with 1.0 μm filtered lake water was suspended in a fifth flask as a control.

Routine procedures.

In experiment 1 and 2, bacterial cell numbers and bacterial activities were determined at 6 h, 1 day, 3 day, and 5 day from the beginning. The bacterial cell numbers in the surrounding water medium and the outer surface of the tubes in experiment 3 were enumerated on day 5. Samples for bacteria attached to the surface of the tubes were prepared by gently taking out the tube from experimental flask. Places 1 cm from each end of the tube were tied using fine string. The extra parts of the 2 ends of the tube were cut off. The shorted tube was then placed into autoclaved 40 ml 0.2% sodium dodecyl sulfate (SDS) in 3% aqueous formaldehyde followed by mixing on a G10 Gyrotory Shaker at 175 rpm for 5 min. Microscopic examination showed that this procedure removed virtually all the bacteria from the surface of the tubes.

NH_4^+ and dissolved organic carbon (DOC) were sampled on day 1 and day 5. To avoid significant reduction of the water volume in the flasks, aliquots of sample from replicate flasks were mixed

for chemical analysis.

Determination of nutrients, chlorophyll a, and bacteria.

Concentration of NH_4^+ was determined by the phenol- hypochlorite method (Solorzano 1969). DOC was analyzed with a Dorhmann Carbon Analyzer. All of the above samples were prefiltered through Whatman GF/C filters before analysis. Chlorophyll a was measured by 95% ethanol extraction method (Sartory and Grobbelar 1984).

Bacterial ^{3}H-thymidine uptake was determined by adding high activity (55 Ci m mole^{-1}) methyl-^{3}H thymidine (ICN Radiochemical INC.) to 10 ml samples (final concentration of 10 nM) in 20 ml glass scintillation vials. The ^{3}H-thymidine stock (in 70% ethanol) was evaporated to dryness and rehydrated with deionized water before use to eliminate products of self radiolysis and to remove ethanol. The inoculated sample was incubated at room temperature (25 $\pm$ 1 ^{o}C) in the dark for 30 min. Bacterial activity was terminated by adding 10 ml of ice-cold 10% (W/V) trichloroacetic acid (TCA) to each vial. Following overnight extraction at 4 ^{o}C, the samples were filtered onto 0.2 μm polycarbonate filters (Poretics Corporation). After rinsing 5 times with 2 ml each of ice-cold 5% TCA, the filter with labeled bacteria was transferred to a 20 ml polyethylene scintillation vial with 7.0 ml Cytoscint scintillation cocktail (INC Radiochemical, Irvine, CA.). Radioactivity in each sample was determined by standard scintillation spectrometry using a Beckman LS-100C liquid scintillation counter. The counting efficiency was determined by the external standard ratio method using ^{3}H-toluene as reference and

acetone as the quenching agent. Bacterial cell samples were fixed with formaldehyde (3% final concentration) and stored at 4 ^{o}C until analysis. Bacterial cell numbers were determined on samples filtered onto 0.2 μm polycarbonate filters counted with a Nikon Labophot epifluorescence microscope using the acridine orange direct count technique (Hobbie et al. 1977).

Results

Influence of cyanobacterial density on bacteria.

Time course bacterial responses to different cyanobacterial biomass (chlorophyll a concentration) was measured by bacterial thymidine uptake, and cell number. Bacterial thymidine uptake and cell numbers over the course of the experiment significantly ($p<0.05$) correlated with chlorophyll a concentrations of both L. birgei and A. flos-aquae for all the 3 experiments (Figs. 1-6, Table 1). The only exception was that bacterial cell number did not significantly correlate with L. birgei chlorophyll a at 6 h after incubation in experiment 1 (Fig. 2, Table 1). The degree of correlation increased with time of incubation for A. flos-aquae treatment (Fig. 1; Table 1) and was highest on day 1 for L. birgei (Fig. 2; Table 1). The thymidine uptake and cell numbers of the 2 unidentified attached bacteria isolated from A. flos-aquae in experiment 2 highly correlated ($p<0.01$) with chlorophyll a of A. flos-aquae at all measured incubation periods (Fig. 3; Table 1). Those isolated from L. birgei significantly correlated ($p<0.05$) with chlorophyll a of L. birgei. The highest correlation between the bacteria isolated from cyanobacteria and cyanobacte-

ria were found on day 1 and 5 for A. flos-aquae and during the first 24 h for L. birgei (Fig. 4; Table 1). The bacterial numbers in water outside and attached to the cyanobacterial tubes significantly ($p<0.01$) correlated with chlorophyll a of both A. flos-aquae and L.birgei (Figs. 5, 6; Table 1). The slope of the regression between chlorophyll a and attached bacterial numbers were 2.8 times greater for A. flos-aquae and 2.5 times greater for L. birgei, respectively than for bacteria in the bulk water phase outside the cyanobacterial tubes (Table 1).

Average (mean of measurements at 6 h, day 1, day 3, and day 5 for experiment 1 and 2, and day 5 for experiment 3) bacterial thymidine uptake and cell numbers at different densities of cyanobacteria are listed in Table 2. Bacterial thymidine uptake and cell numbers were significantly higher at all densities of A. flos-aquae and L. birgei than those in controls, except thymidine uptake at the lowest density of L. birgei in both experiment 1 and 2 (Table 2). In experiment 3, bacterial cell numbers in the water outside the cyanobacterial tubes and on the outside surfaces of the tubes at different densities of A. flos-aquae and L. birgei were significantly higher than those in controls; free-living cells at the low and mid-low densities and attached cell in the low density of L. birgei were exceptions (Table 2). The responses of bacteria to cyanobacteria were weaker in the lowest density than in the highest density of both species of cyanobacteria in all the 3 experiments (Table 2).

The slopes of regressions between bacterial growth variables and cyanobacterial chlorophyll a over the incubation period are shown in Table 4. The response of bacteria to A. flos-aquae and

L. birgei were tested by comparing slopes of these regressions. Twelve out of 18 regressions between chlorophyll a of L. birgei and bacterial growth variables were greater than those regressed between A. flos-aquae and bacteria; 8 of the 12 were significantly ($p<0.05$) higher (Table 4).

Dynamics of chlorophyll a, DOC, and NH_4^+ during the experiments.

Chlorophyll a concentrations of A. flos-aquae and L. birgei remained essentially unchanged during the 5 day incubation in both experiment 1 and 2 (Table 3). In experiment 3, chlorophyll a concentration on day 5 was unmeasurable after the SDS wash due to apparent chlorophyll degradation by SDS. Concentration of DOC consistently decreased from day 1 to day 5 in all tested cyanobacterial densities in both experiment 1 and 2 (Table 3). The concentrations of NH_4^+ increased in A. flos-aquae and decreased in L. birgei treatments from day 1 to 5 at all tested cyanobacterial densities in both experiments 1 and 2 (Table 3).

The concentration of DOC on day 5 decreased at higher densities of both A. flos-aquae and L. birgei in experiment 1 and A. flos-aquae in experiment 2 (Table 3); DOC increased in the L. birgei treatment. The concentration of NH_4^+ on day 5 showed an overall increase with density of A. flos-aquae in both experiments 1 and 2 (Table 3). The concentration of NH_4^+ on day 5 did not show any systematic change with the density change of L. birgei in experiment 1 and 2 (Table 3).

Discussion

Our experiments demonstrated that a significant positive correlation exits between cyanobacterial density and bacterioplankton thymidine uptake and cell numbers. Similar findings have also been reported from natural populations (Furhman et al. 1880; Larsson and Hagstrom 1982; Murry et al. 1986; Chrzanowski and Hubbard 1989). Net bacterial increase attributed to cyanobacterial products in our experiments can be obtained by subtracting bacterial growth variables in treatments from the control. Such calculations can reveal the role of cyanobacterial products in relation to bacterial growth. This approach has the following advantages as compared with the methods used in the previous studies: (i) artifacts of size fractionation are minimized, (ii) radio labeling of phytoplankton products is not required, and the problems associated with $^{14}CO_2$ labeling can be avoided, and (iii) the secondary effects of heterotrophic inhibitors on phytoplankton is eliminated.

The 1.0 μm filters used to isolate the bacterial inoculum in the experiments eliminated phytoplankton and bacterial grazers, but may also exclude some bacteria larger than 1.0 μm. The MW cut off of the tubing allowed compounds smaller than 12,000-14,000 molecular weight to pass through the membrane barrier. Although it has been reported that 95% of the dissolved organic carbon compounds released by marine phytoplankton had MW smaller than 3,500 (Wiebe and Smith 1977) and the utilization of high MW compounds was slower than lower MW compounds (Saunders 1972), the utilization of larger molecules with 10,000 to 100,000 and larger than 100,000 MW size were also observed (Ogura 1975). However, our selected MW cut off range would allow most of the simple

water-soluble compounds expected be released by the cyanobacteria while effectively providing a barrier to the direct interplay between the two groups of test organisms. The potential bacterial growth inside the tubes may contribute to the consumption of part of the products from the cyanobacteria, thereby causing certain degree of under-estimation of effect of cyanobacterial products on bacteria out side of the tubes.

During the 5 day incubations, chlorophyll a concentration showed no significant changes in all 3 experiments. This indicates that the cyanobacteria did not grow or that their growth was balanced by death. Despite the physiological state of the cyanobacteria, higher densities of cyanobacteria did produce increasingly higher bacterial growth and activity.

The consistent decrease in DOC concentration from day 1 to day 5 in experiments 1 and 2 for all the different densities of cyanobacterial treatments indicates that no net photosynthetically produced DOC accumulation occurred during the experiments. The decreases of DOC concentrations with the increase of cyanobacterial densities on day 5 for both A. flos-aquae and L. birgei in experiment 1 and for A. flos-aquae in the experiment 2 indicate that higher bacterial activity can control DOC to a low level even with a high DOC supply rate under our experimental conditions. Several other studies have indicated that bacterial utilization of phytoplankton products is rapid. Using axenic algal and 2 different bacteria cultures to test release and utilization rates of phytoplankton products, Herbst and Overbeck (1978) found that little such products is present in natural water in their

released form, because the bacterial utilization is immediate. Bell and Sakshaug (1980) also reported that a 4-fold increase in bacterial activity was enough to prevent a large accumulation of dissolved extracellular products in an aquatic system.

The significantly higher bacterial activity stimulated by L. birgei than by A. flos-aquae on day 1 and day 3 in experiment 1, and from day 1 to day 5 in experiment 2 indicates that higher levels of cyanobacterial products were released from L. birgei relative to A. flos-aquae during our experiments. Such difference may also be due to toxins or inhibitory products released from A. flos-aquae. Mechanisms for greater stimulation of bacterial activity by L. birgei with respect to A. flos-aquae cannot be explained by our data. Further work may be needed on the cyanobacterial species difference for the quality and quantity of phytoplankton products released.

In summary, stimulation of bacterioplankton activity and cell numbers by increasing densities of cyanobacteria was documented. Details are presented below.

1. Thymidine uptake and cell number of bacterioplankton from a eutrophic lake were increased by increasing densities of A. flos-aquae and L. birgei.

2. Thymidine uptake and cell number of bacteria, isolated from A. flos-aquae and L. birgei and resuspended in lake water, were elevated by increasing densities of A. flos-aquae and L. birgei.

3. Bacterial cell number (both free-living and attached on the outer surface of dialysis tubes) increased at elevated densities of A. flos-aquae and L. birgei. Slopes of regressions

between the cell number of bacteria attached on the outer surface of the dialysis tubes and cyanobacterial chlorophyll a was greater than that between free-living bacterial cell number and cyanobacterial chlorophyll a for both species of cyanobacteria tested.

4. L. birgei stimulated bacterial activity to a greater extent than by A. flos-aquae.

5. A 7-fold increase of bacterial thymidine uptake and 2-fold increase of bacterial cell number were apparently enough to prevent accumulation of DOC under our experimental condition.

References

Azam, F., T. Fenchel. J. G. Field, J. S. Gray, L. A. Meyer-Reil, and F. Thingstad. 1983. The ecological role of water-column microbes in the sea. Mar. Ecol. Prog. Ser. 10:257-263.

Bell, W. H. and E. Sakshaug. 1980. Bacterial utilization of algal extracellular products. 2. A kinetic study of natural populations. Limnol. Oceanogr. 25:1021-1033.

Berman, T. 1975. Size fractionation of natural aquatic populations associated with autotrophic and heterotrophic carbon uptake. Mar. Biol. 33:215-220.

Berman, T. and C. Gerber. 1980. Differential filtration studies of carbon flux from living algae to microheterotrophs, microplankton, size distribution in the lake Kinneret. Microb. Ecol. 6:189-198.

Chrost, R. J. 1978. The estimation of extracellular release by phytoplankton and heterotrophic activity of aquatic bacteria. Acta. Microbiol. Pol. 27:139-146.

Chrost, B. and M. Sondergaard. 1983. Organic carbon release by phytoplankton: its composition and utilization by bacterioplankton. J. Plankton Res. 5:477-493.

Chrzanowski, T. H. and J. G. Hubbard. 1989. Bacterial utilization of algal extracellular products in a southwestern reservoir. Hydrobiologia 179:61-71.

Cole, J. J., G. E. Likens, and D. L. Strayer. 1982. Photosynthetically produced dissolved organic carbon: An important carbon source for planktonic bacteria. Limnol. Oceanogr. 27:1080-1090.

Coveney, M. F. 1982. Bacterial uptake of photosynthetic carbon from freshwater phytoplankton. Oikos 38:8-20.

Derenbach, J. B. and P. J. Leb. Williams. 1974. Autotrophic and bacterial production: fractionation of plankton populations by different filtration of samples from the English Channel. Mar. Biol. 25:263-269.

Downes, M. T. 1978. An automated determination of low reactive phosphorus concentration in natural waters in the presence of arsenic, silicon acid, and mercuric chloride. Water Research 12:743-745.

Feuillade, M., Ph. Dufour, and Feuillade. 1988. Organic carbon release by phytoplankton and bacterial reassimilation. Schweiz. Z. Hydrol. 50:115-135.

Furhman, J. A. and F. Azam. 1980. Bacterioplankton secondary

production estimates for coastal water of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. 39:1085-1095.

Fuhrman, J. A. and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. 66:109-120.

Fuhrman, J. A., J. W. Ammerman, and F. Azam. 1980. Bacterioplankton in the coastal eutrophic zone: distribution, activity and possible relationship with phytoplankton. Mar. Biol. 60:201-207.

Herbst, V. and Y. Overbeck. 1978. Metabolic coupling between alga Oscillatoria redekei and accompanying bacteria. Naturwissenschaften 65:598-608.

Hobbie, J. E., J. Dalay, and S. Jasper. 1977. Use of nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Jensen, L. M. 1983. Phytoplankton release of extracellular organic carbon, molecular weight composition, and bacterial assimilation. Mar. Ecol. Prog. Ser. 11:39-48.

Jensen, L. M. and M. Sondergaard. 1985. Comparison of two methods to measure algal release of dissolved organic carbon and the subsequent uptake by bacteria. J. Plankton Res. 7:41-56.

Lampert, W. 1978. Release of dissolved organic carbon by grazing zooplankton. Limnol. Oceanogr. 23:831-834.

Larsson, U. and A. Hagstrom. 1979. Phytoplankton exudate release as an energy source for the growth of pelagic-bacteria. Mar. Biol. 52:199-296

Larsson, U. and A. Hagstrom. 1982. Fractionated phytoplankton primary production, exudate release, and bacterial production in a Baltic eutrophication gradient. Mar. Biol. 67:57-70.

Mague, T. H., E. Friberg, D. J. Hughes, and I. Morris. 1980. Extracellular release of carbon by marine phytoplankton: A physiological approach. Limnol. Oceanogr. 25:262-279.

Marvalin, O., L. Aleya, and H. J. Hartman. 1989. Coupling of the seasonal pattern of bacterioplankton and phytoplankton in a eutrophic lake. Can. J. Microbiol. 35:706-712.

Murry, R. E., K Cooksey, and J. C. Priscu. 1986. Stimulation of bacterial DNA synthesis by algal exudates in attached algal-bacterial consortia. Appl. Enverc. Microbiol. 52:1177-1182.

Nalewajko, C. 1977. Extracellular release in freshwater algae and bacteria: extracellular products of algae as a source of

carbon for heterotrophs, pp 589-624. In J. Cairns (ed.), Aquatic Mocrobial Communities. Garland Publ., New York.

Nalewajko, C., T. G. Dunstal, and H. Sheer. 1976. Kinetics of extracellular release in axenic algae and in mixed algal-bacterial cultures: significance in estimation of total (gross) phytoplankton excretions rates. J. Phycol. 12:1-5.

Ogura, N. 1975. Further studies on decomposition of dissolved organic matter in coastal sea water. Mar. Biol. 31:101-111.

Riemann, B. and M. Sondergaard. 1984. Bacterial growth in rela tion to phytoplankton primary production and extracellular release of organic carbon, pp 233-248. In J. E. Hobbie and P. B. Williams (eds.). Heterotrophic Activity in the Sea. NATO Publ. New York and London.

Roberts. R. D. and R. J. Wicks. 1990. Heterotrophic bacterial production and its dependence on autotrophic production in a hypertrophic African reservoir. Can. J. Fish. Aquat. Sci. 47: 1027-1037.

Sartory, D. P. and J. U. Grobbelaar. 1984. Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114:177-187.

Saunders, G. W. 1972. The kinetics of extracellular release of soluble organic matter by plankton. Verh. Int. Verein. Limnol. 18:140-146.

Solorzano, L. 1969. Determination of ammonia in natural water by the phenol hypochloride method. Limnol. Oceanogr. 14:799-801.

Sundha, I. 1989. Characterization of phytoplankton extracellular products (PDOC) and their subsequent uptake by heterotrophic organisms in a mesotrophic forest lake. J. Plankton Res. 11: 463-486.

Wiebe, W. J. and D. F. Smith. 1977. Direct measurement of dissolved organic carbon released by phytoplankton and incorporation by microheterotrophs. Mar. Biol. 42:213-223.

Wolter, K. 1982. Bacterial incorporation of organic substances released by natural phytoplankton productions. Mar. Ecol. Prog. Ser. 7:287-295.

Table 1. Correlation coefficient (r) between cyanobacterial chlorophyll a and bacterial thymidine uptake, and cell number in the 3 experiments.

	Experiment 1				Experiment 2				Experiment 3	
Treatment	6 h	day 1	day 3	day 5	6 h	day 1	day 3	day 5	water	surface
Thymidine uptake										
A. flos-aquae	0.553**	0.817***	0.923***	0.944***	0.932***	0.936***	0.897***	0.986***	NA	NA
L. birgei	0.534**	0.960***	0.837***	0.857***	0.840***	0.862***	0.791**	0.785**	NA	NA
Cell number										
A. flos-aquae	0.712**	0.922***	0.930***	0.941***	0.840***	0.962***	0.841***	0.923***	0.904***	0.960***
L. birgei	0.480	0.957***	0.745**	0.822***	0.867***	0.924***	0.808**	0.813**	0.852***	0.907***

** Significant at $p<0.05$
*** Significant at $p<0.01$
NA Treatment not included in the experiment

Table 2. Bacterial thymidine uptake (nmole l^{-1} h^{-1}) and cell number ($x10^6$ ml^{-1} or $x10^6$ cm^{-2}) and their statistical significance compared with controls in the 3 experiments[a]. Values represent the time course means from 2 replicate flasks.

Treatment	Chlorophyll a gradient				
	control	low	mid-low	mid-high	high
Experiment 1					
A. flos-aquae					
Thymidine uptake	0.008	0.010**	0.011***	0.014***	0.017***
Cell number	2.937	3.305**	3.807***	4.180***	4.753***
L. birgei					
Thymidine uptake	0.008	0.088	0.013***	0.016***	0.018***
Cell number	2.937	3.340**	3.713***	3.931***	4.204***
Experiment 2					
A. flos-aquae					
Thymidine uptake	0.038	0.066***	0.083***	0.095***	NA
Cell number	4.627	5.728***	6.431***	7.121***	NA
L. birgei					
Thymidine uptake	0.030	0.044	0.079**	0.225***	NA
Cell number	4.859	6.228***	6.904***	7.880***	NA
Experiment 3					
A. flos-aquae					
Free-living cell	1.151	1.594**	1.875***	2.222***	NA
Attached cell	0.798	1.672***	2.451***	3.598***	NA
L. birgei					
Free-living cell	1.269	1.582	1.585	1.891**	2.212***
Attached cell	1.106	1.815	2.903***	3.613***	4.561***

a All the significant levels were tested by multi-factor variance analysis.
** Significant compared with control at $p<0.05$
*** Significant compared with control at $p<0.01$
NA Treatment not included in the experiment

Table 3. The concentrations of chlorophyll *a*, DOC, and NH_4^+ at different densities of *A. flos-aquae* and *L. birgei* in the 3 experiments. NA=treatment not included in the experiment.

Treatment	Chlorophyll *a* ($\mu g\ l^{-1}$)		DOC ($mg\ l^{-1}$)		NH_4^+-N ($\mu g\ l^{-1}$)	
	day 1	day 5	day 1	day 5	day 1	day 5
Experiment 1						
A. flos-aquae						
Control	0.00	0.00	71.1	76.2	25.0	23.9
Low	19.92	20.60	76.4	75.0	16.9	40.3
Mid-low	39.84	41.20	78.2	71.4	8.8	22.3
Mid-high	59.76	61.80	77.9	61.8	12.4	53.8
High	79.68	82.40	60.5	43.9	11.5	66.4
L. birgei						
Control	0.00	0.00	77.1	76.2	25.0	23.9
Low	14.82	14.72	71.7	66.5	23.2	11.5
Mid-low	29.62	29.44	65.1	57.6	25.0	16.9
Mid-high	44.46	44.16	65.2	55.4	22.3	10.6
High	59.28	58.88	63.5	48.8	18.9	13.3
Experiment 2						
A. flos-aquae						
Control	0.00	0.00	171.4	154.3	259.7	16.0
Low	13.07	14.27	137.4	128.9	244.4	305.6
Mid-low	26.13	28.53	112.5	97.2	205.8	615.9
Mid-high	41.12	42.81	90.0	78.4	186.0	739.9
L. birgei						
Control	0.00	0.00	107.8	94.0	121.2	13.3
Low	9.00	10.57	105.3	91.3	127.5	13.3
Mid-low	18.00	21.14	144.5	118.0	118.5	15.1
Mid-high	27.00	31.71	140.0	103.6	76.3	7.9
Experiment 3						
L. birgei						
Control	0.00	NA	NA	5.8	NA	91.7
Low	0.99	NA	NA	26.3	NA	20.0
Mid-low	2.68	NA	NA	27.6	NA	23.2
Mid-high	7.20	NA	NA	26.1	NA	142.7
High	NA	NA	NA	NA	NA	NA
A. flos-aquae						
Control	0.00	NA	NA	4.9	NA	50.2
Low	1.24	NA	NA	16.1	NA	5.2
Mid-low	3.88	NA	NA	11.8	NA	7.7
Mid-high	6.62	NA	NA	13.0	NA	2.8
High	16.51	NA	NA	14.9	NA	4.4

Table 4. Regression slopes of bacterial thymidine uptake ($x10^{-4}$), bacterial cell number ($x10^{-2}$) versus cyanobacterial chlorophyll *a* for each time the experiments were sampled.

	Experiment 1				Experiment 2				Experiment 3	
Treatment	6 h	day 1	day 3	day 5	6 h	day 1	day 3	day 5	Free-living cell	Attached cell
Thymidine uptake										
A. flos-aquae	0.07	0.50	0.93	2.75	3.22	0.47	11.78	36.62	NA	NA
L. birgei	0.10	2.07***	1.57***	3.68	2.01	6.52**	36.54***	207.7***	NA	NA
Cell number										
A. flos-aquae	0.36	2.31	2.72	3.50	1.31	5.95	8.16	8.55	13.16	36.51
L. birgei	0.31	3.56***	1.61	3.01	2.18	11.21***	12.14**	14.29	7.94	19.70

a A T-test was used to compare slopes between cyanobacterial species.
** Significant at $p<0.05$.
*** Significant at $p<0.01$.
NA Treatment not included in our experiment.

Figure Captions

Figure 1. Bacterial thymidine uptake and cell number in different densities of A. flos-aquae at different length of incubation in experiment 1. ○ 6 h, ● day 1, △ day 3, ▲ day 5 beginning incubation.

Figure 2. Bacterial thymidine uptake and cell number in different densities of L. birgei at different length of incubation in experiment 1. Symbols are the same as Fig. 1.

Figure 3. Bacterial thymidine uptake and cell number in different densities of A. flos-aquae at different length of incubation in experiment 2. Symbols are the same as Fig. 1.

Figure 4. Bacterial thymidine uptake and cell number in different densities of L. birgei at different length of incubation in experiment 2. Symbols are the same as Fig. 1.

Figure 5. Bacterial cell number (free-living in the water outside of the tubing and attached on the outside surface of the tube) in different densities of A. flos-aquae on day 5 after incubation.

Figure 6. Bacterial cell number (free-living in the water outside of the tubing and attached on the outside surface of the tubing) in different densities of L. birgei on day 5 after incubation.

Fig. 1

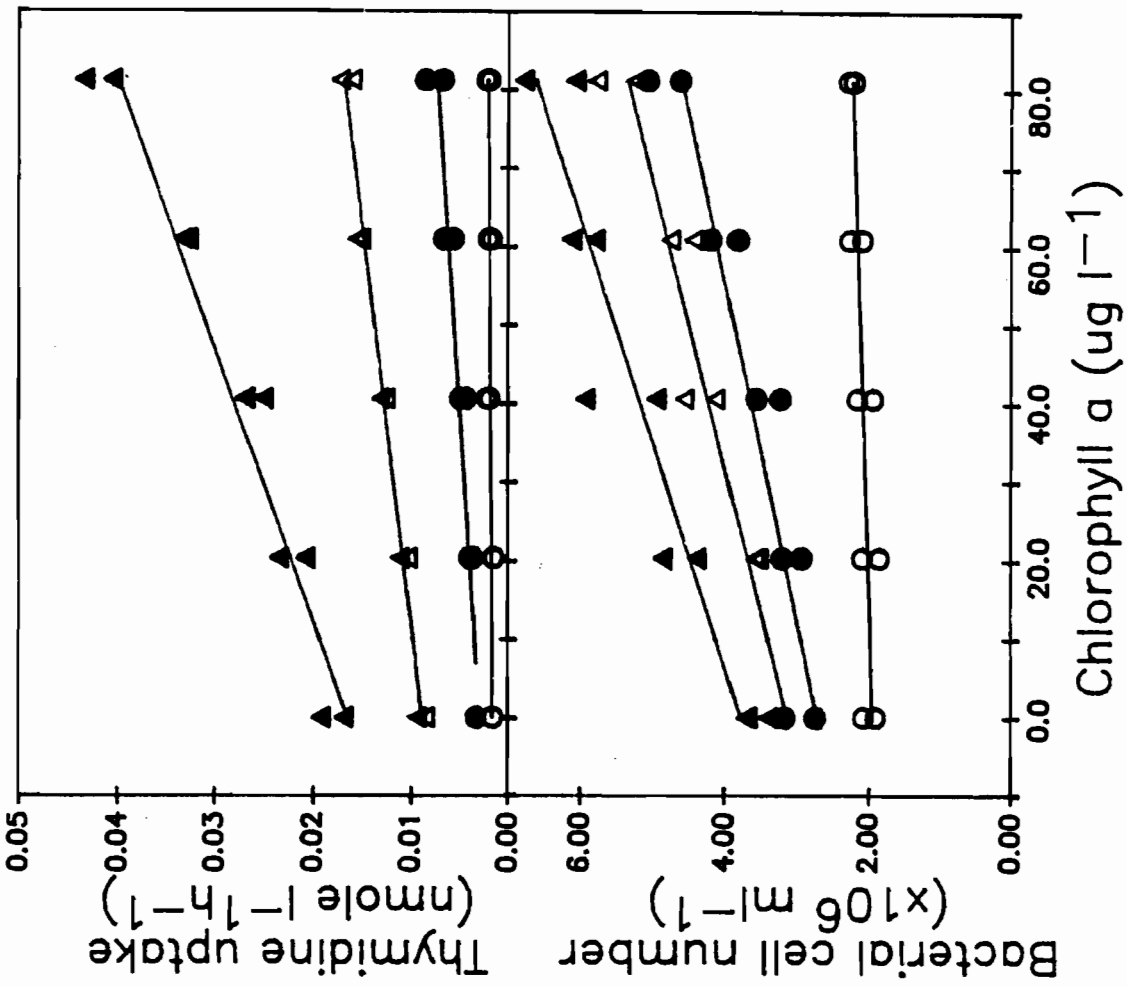

Thymidine uptake
(nmole l⁻¹h⁻¹)
0.05
0.04
0.03
0.02
0.01
0.00
Bacterial cell number
(x10⁶ ml⁻¹)
6.00
4.00
2.00
0.00
0.0
20.0
40.0
60.0
80.0
Chlorophyll a (ug l⁻¹)

Fig. 2

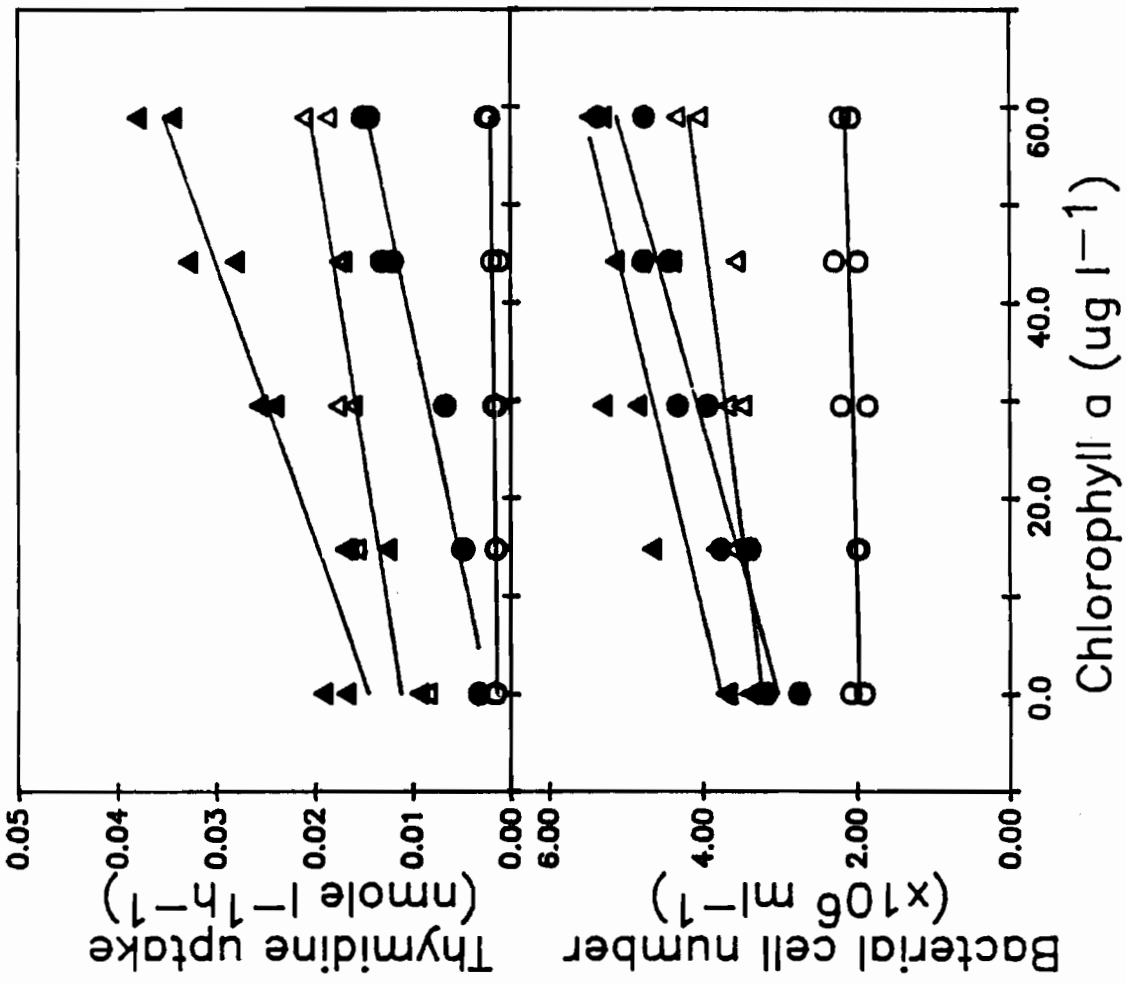

Thymidine uptake
(nmole l−1h−1)
0.05
0.04
0.03
0.02
0.01
0.00
Bacterial cell number
(x10⁶ ml−1)
6.00
4.00
2.00
0.00
0.0
20.0
40.0
60.0
Chlorophyll a (ug l−1)

Fig. 3

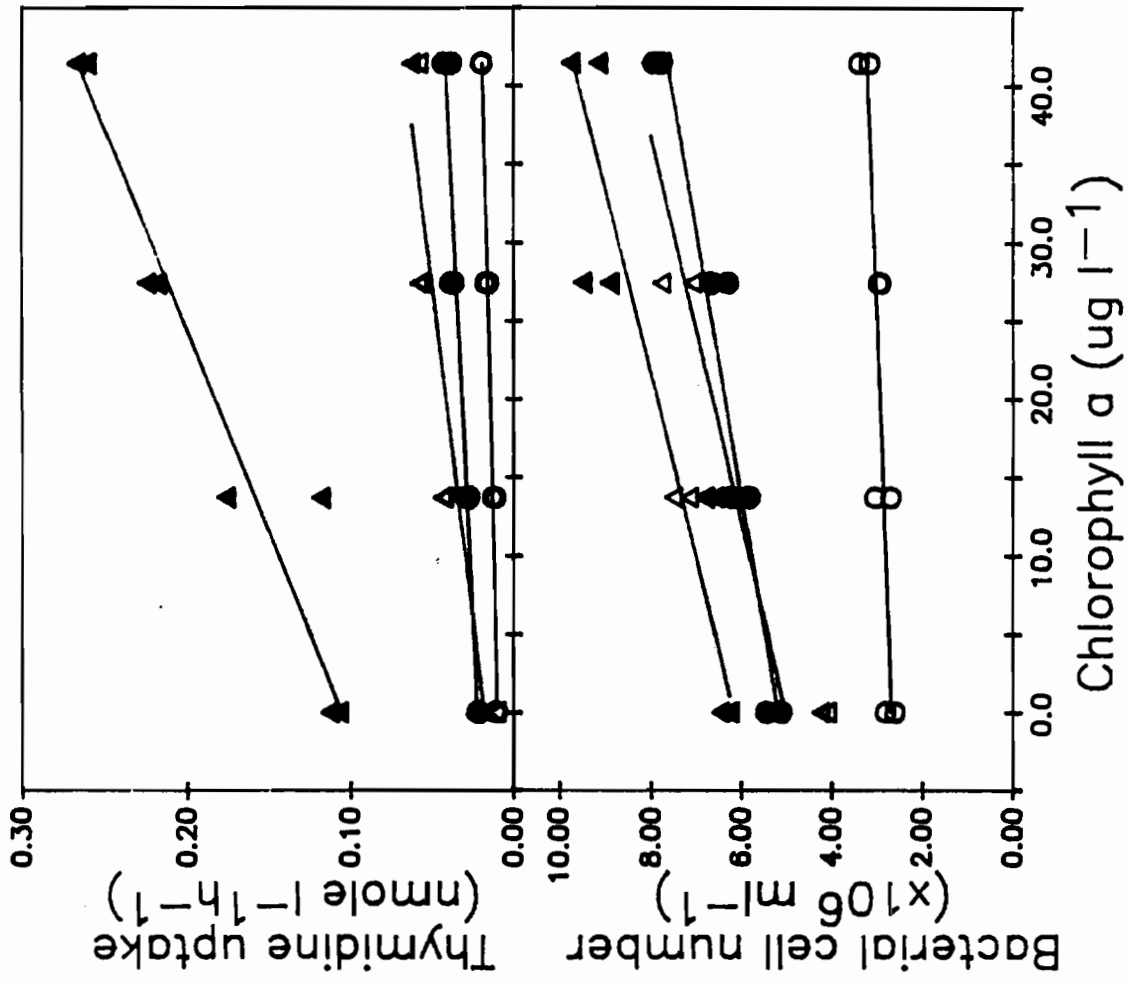

Thymidine uptake
(nmole l−1h−1)
0.30
0.20
0.10
0.00
Bacterial cell number
(x106 ml−1)
10.00
8.00
6.00
4.00
2.00
0.00
0.0
10.0
20.0
30.0
40.0
Chlorophyll a (ug l−1)

Fig.4

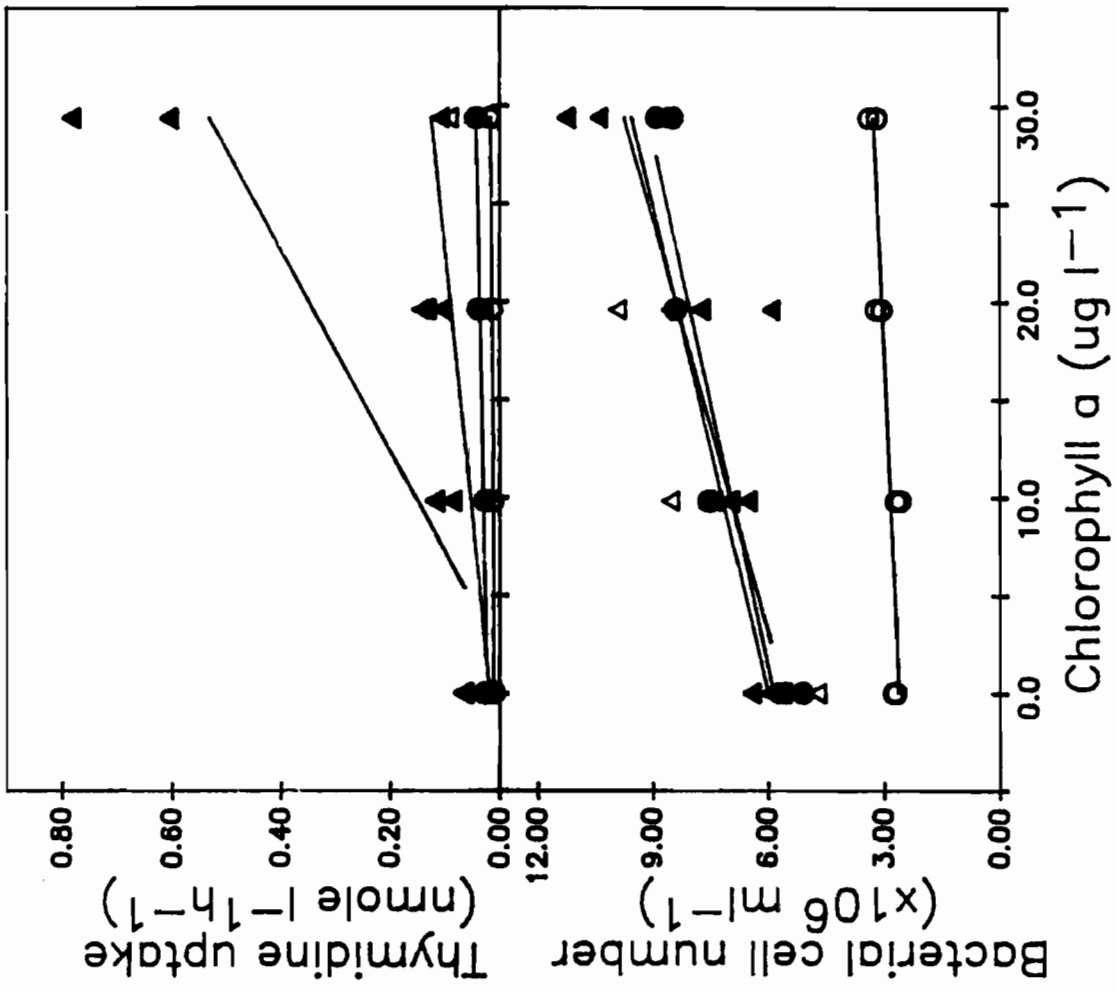

Thymidine uptake
(nmole l−1h−1)
0.80
0.60
0.40
0.20
0.00
Bacterial cell number
(x10⁶ ml−1)
12.00
9.00
6.00
3.00
0.00
0.0
10.0
20.0
30.0
Chlorophyll a (ug l−1)

Fig. 5

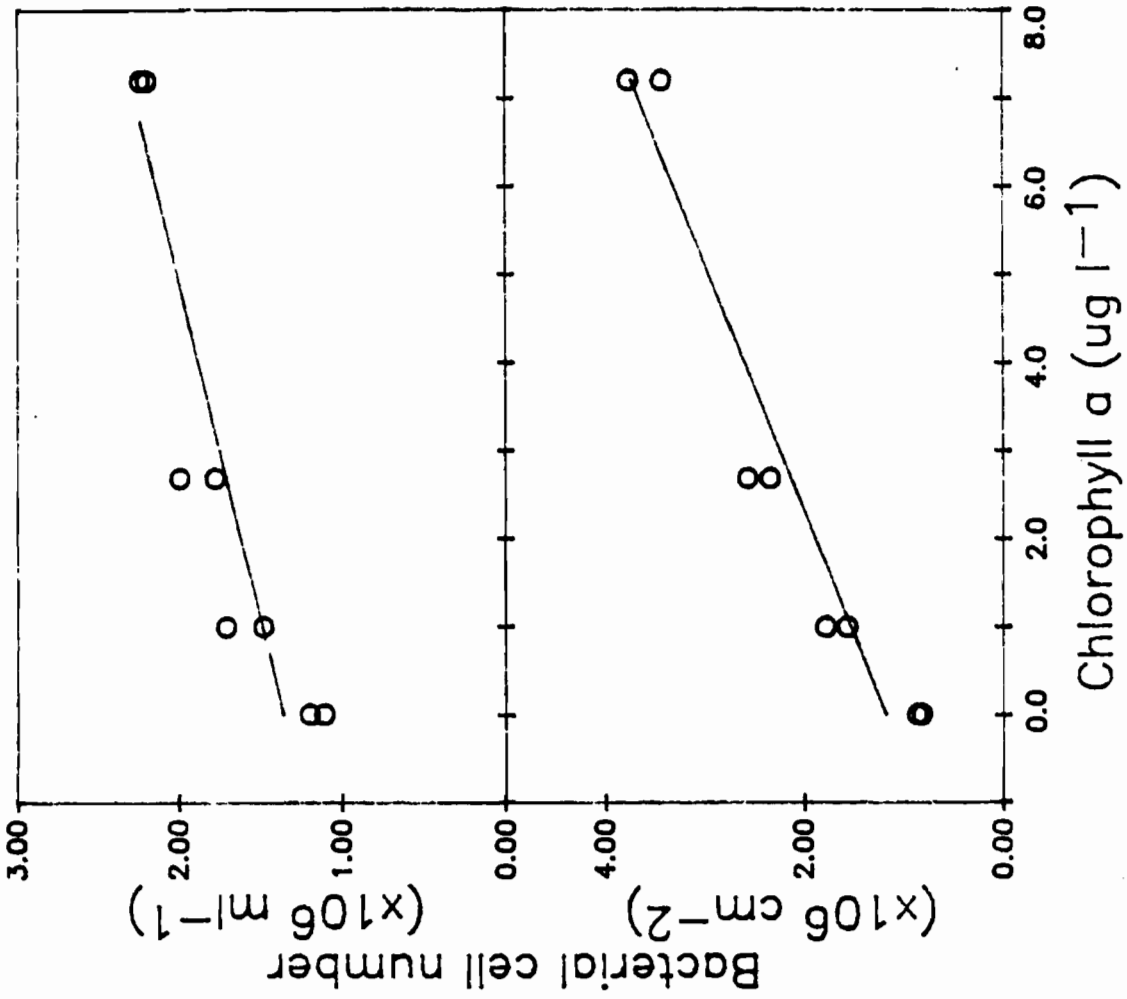
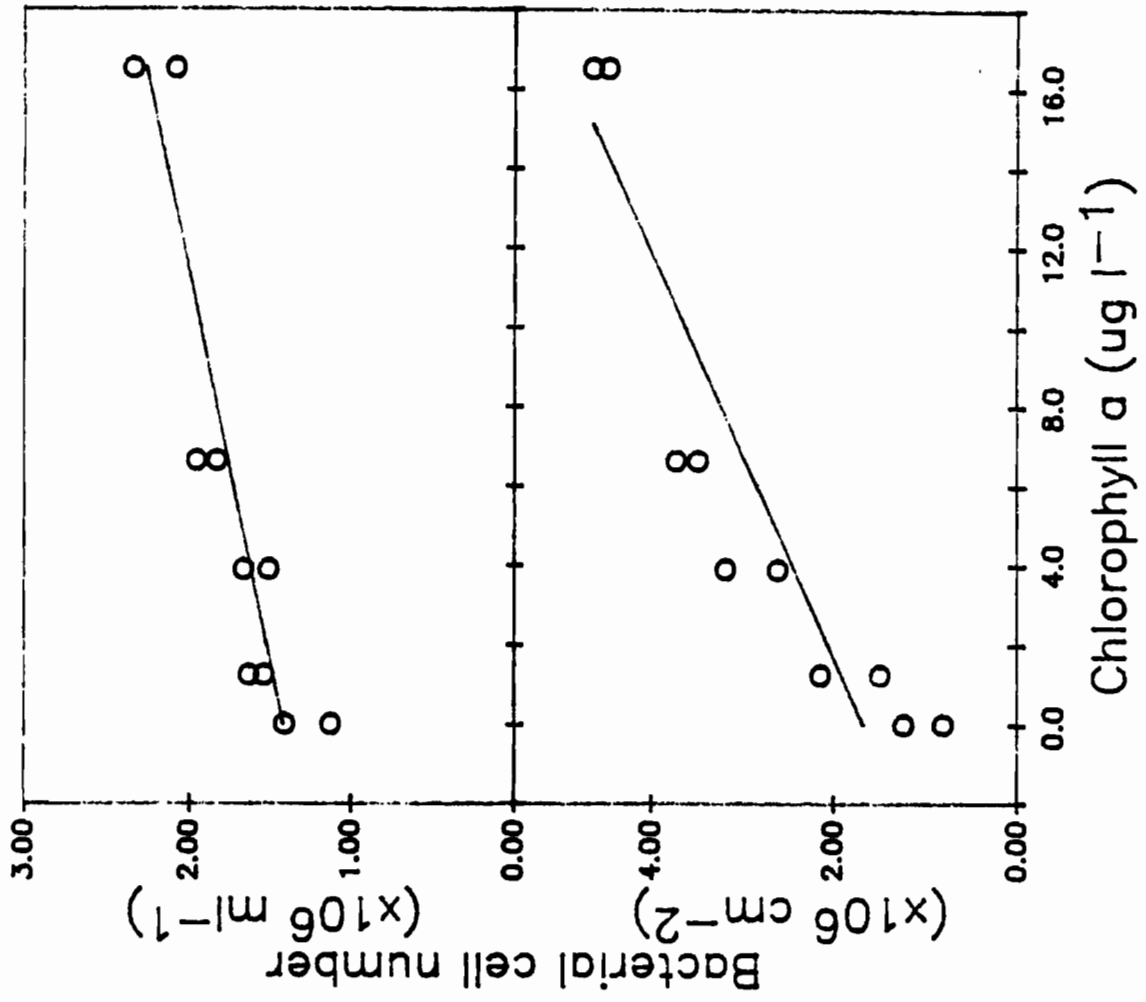

Bacterial cell number
(x10⁶ ml⁻¹)
(x10⁶ cm⁻²)
Chlorophyll a (ug l⁻¹)
3.00
2.00
1.00
0.00
4.00
2.00
0.00
0.0
2.0
4.0
6.0
8.0

Fig. 6


Bacterial cell number
(x10⁶ cm⁻²)
(x10⁶ ml⁻¹)
Chlorophyll a (ug l⁻¹)
0.00
2.00
4.00
0.00
1.00
2.00
3.00
0.0
4.0
8.0
12.0
16.0

CHAPTER 13

MUCILAGINOUS INVESTMENTS OF CYANOBACTERIA AS A FACTOR INFLUENCING CYANOBACTERIAL-BACTERIAL ASSOCIATIONS

(To be submitted to: Microbial Ecology)

Mucilaginous Investments of Cyanobacteria as a Factor Influencing Cyanobacterial-Bacterial Association

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Additional Key Words: Cyanobacteria, Outer wall layers, Mucilaginous layers, Cyanobacterial-bacterial association.

Running Head: Role of cyanobacterial mucilaginous investments in bacterial association.

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ABSTRACT

Numerical bacterial association with species of cyanobacteria, Anabaena flos-aquae, Aphanizomenon flos-aquae and Lyngbya birgei from the same location in Hebgen Lake, Montana with an ambient bacterial count of 2.55×10^{6} cells ml^{-1} was investigated in relation to cyanobacterial mucilaginous investments (visible outer layers). Bacterial numbers were normalized to cyanobacterial surface area, and chlorophyll a (chl a) respectively. Thickness of cyanobacterial mucilaginous investments was measured using a micrometer eye piece after Indian ink treatment of filaments. Among the species of cyanobacteria studied, there was a highly significant (P=0.0001) difference in the bacterial numbers per surface area, and a strong correlation was found (r=0.99) between bacterial numbers per cyanobacterial surface area and thickness of mucilaginous investments. Bacterial numbers associated with Anabaena flos-aquae and L. birgei based on chl a showed significant difference (P=0.05), but Aphanizomenon flos-aquae did not show significant difference between either one of the other two species. However, there was a strong correlation (r=0.70) between the bacterial numbers normalized to chl a and the thickness of mucilaginous investments among the cyanobacterial species studied. Direct microscopic observations further support our numerical data. Mucilaginous investments of cyanobacteria therefore, are an important factor that influences cyanobacterial-bacterial association in aquatic environments.

INTRODUCTION

The cell wall of cyanobacteria in most species is surrounded by one or more external layers (Drews and Weckesser 83; Lang 68). Among cyanobacteria, there is a great variety of form and structure of these external layers (Fritsch 45) which appears to be mainly due to their morphology, physical and chemical characteristics, and ultra structure. Due to difficulties in terminology of these external layers in the past, they have been named cell wall layers, sheaths, capsules, cell envelope layers, slime layers, mucilaginous layers, and mucilaginous investments (Drews and Weckesser 83; Drews 73; Stanier and Cohen-Bazire 77). Many filamentous cyanobacteria possess tubular, fibrous material of mostly uniform thickness called sheath immediately surrounding the cell wall (Lamont 69; Tuffery 69). Outer to the sheath, filamentous cyanobacteria often produce slime layers which can be visualized by Indian ink treatment (Martin and Wyatt 74). The amorphous, probably low viscous slime layers just outer to the sheath do not always reflect the shape of the cell or filament in contrast to the sheath (Drews and Weckesser 83). In this report we follow Martin and Wyatt (74) in calling sheath and visible slime layers collectively as mucilaginous investments.

Environmental chemical gradients, and bacterial chemotaxis are known to play a role in cyanobacterial-bacterial associations (Paerl 83; Wellman and Paerl 81). Extracellular mucilaginous investments of cyanobacteria though observed by many workers to contain diverse bacteria (Paerl 83; Kessel and Eloff 75; Caldwell and Caldwell 78), no known effort has ever been made to determine whether the mucilaginous investments of cyanobacteria is a factor

that influences cyanobacterial-bacterial association. Bacterial chemotaxis in response to metabolites exuded from cyanobacteria whereby motile bacterial cells would move into the microenvironment of cyanobacteria have been reported (Paerl and Gallucci 85; Gallucci and Paerl 83). Bacteria (both motile and nonmotile) in aquatic environments can also reach the microenvironment of planktonic cyanobacteria by passive movements and accidental landing particularly due to mass flow, water currents and turbulence in the environment. No matter how the bacteria reached the microenvironment of cyanobacteria, unless they either get entangled, anchored or attached to some substrate, a major proportion of them would soon leave that environment actively or passively so that there would be little stagnation of bacteria in that microenvironment. If however, they remain in that microenvironment, they would then multiply increasing the bacterial population associated with the cyanobacteria.

After preliminary studies of several species of planktonic cyanobacteria, we hypothesized that mucilaginous investments of cyanobacteria is a factor that influences cyanobacterial-bacterial association. We tested this hypothesis by microscopic examinations of a) bacterial numbers per unit cyanobacterial surface area, b) bacterial numbers normalized to cyanobacterial chlorophyll a, and these two enumerations respectively were related to thickness of the mucilaginous investments of the particular species. Filamentous cyanobacteria, Anabaena flos-aquae, Aphanizomenon flos-aquae and Lyngbya birgei belonging to two different orders of Cyanophyta, Nostocales and Oscillatoriales collected from the same location in Hebgen Lake, Montana

were used in this study. The results presented in this report indicates the significance of cyanobacterial mucilaginous investments in forming cyanobacterial-bacterial association.

MATERIALS AND METHODS

Cyanobacterial samples

Samples of Anabaena flos-aquae, Aphanizomenon flos-aquae, and Lyngbya birgei were collected during cyanobacterial blooms from the same location in Hebgen Lake, Montana in August 1990. Lake water was collected from the sampling site to determine the ambient bacterial numbers.

Microscopy and bacterial enumeration

Within 24 h after sample collection, untreated filaments of cyanobacteria were viewed under phase contrast microscopy using a Nikon Labophot photomicroscope. Photographs of the above three species of cyanobacteria that were predominant in our samples, and Anabaena spiroides and Gomphosphaeria sp. that occurred sporadically were made to show their bacterial association in situ. Kodak Technical Pan film was used for photography. Thickness of the cyanobacterial mucilaginous investments were measured using a micrometer eye piece after Indian ink treatment of the samples. Ten measurements per species were made at random and analyzed using analysis of variance.

Bacterial enumeration on the cyanobacterial surface was carried out under epifluorescence microscopy after acridine orange staining (0.01% w/v final concentration) to make the

bacterial cells more clear and conspicuous. Number of bacterial cells in a constant surface area of the cyanobacteria (known length and breadth) was counted on 3 random samples (replicates) to make 10 separate counts per sample thus making a total of 30 counts per species. Analysis of variance and correlation analysis were used to analyze the data.

Bacterial enumeration was also done on the unialgal samples of Anabaena flos-aquae, Aphanizomenon flos-aquae and Lyngbya birgei (confirmed by microscopic examination) suspended in filter sterilized lake water after 3 passages serially through each of 18, 22, and 23 gauge hypodermic needles fitted on a 20 ml syringe to remove the bacteria from cyanobacterial surface. Two 1 ml portions of the samples were used for chlorophyll a (chl a) assay. Three 0.5 ml aliquots (replicates; 0.5 ml diluted in 1 ml filter sterilized distilled water to disperse the sample uniformly on the filter membrane) per species of the samples after acridine orange treatment were then used to count the bacterial numbers under epifluorescence microscopy following the procedure of Hobbie et al. (77). Ten random bacterial counts per replicate were made, making a total of 30 bacterial counts per species. The bacterial counts were normalized to cyanobacterial chl a. The data was analyzed using analysis of variance and correlation analysis.

Chlorophyll a determination

Cyanobacterial chl a was determined on samples vacuum filtered onto Whatman GF/C filters. The samples on filter were extracted with warm 95% ethanol (70^{o}C) by vortexing for few min

and allowing to stand at room temperature for over 5 h (Sartory and Grobbelaar 84). Either a spectrophotometer (Varian DMS 80) or fluorometer (Turner fluorometer model 112) was employed to quantify chl *a* in the supernatant of centrifuged extracts (Wintermans and De Mots 65). Chl *a* concentration was calculated using the extinction coefficient of chl *a* (Wintermans and De Mots 65). Fluorometric determinations were calibrated using pure *Anacystis* chl *a* (Sigma Chemical Co., St. Louis, MO) in 95% ethanol.

RESULTS

A microscopic view of the *in situ* bacterial association with *Anabaena flos-aquae*, *Aphanizomenon flos-aquae*, *Lyngbya birgei*, *Anabaena spiroides*, and *Gomphosphaeria* sp. are presented in Fig. 1. Even though *A. spiroides* and *Gomphosphaeria* sp. were not studied in detail, because they showed thick mucilaginous investments, remarkable bacterial association, and found in the same location where the other species of cyanobacteria were collected, they were included for further visual comparison. Photomicrographs of cyanobacteria treated with Indian ink to show the thickness of mucilaginous investments are presented in Fig. 2. The mean ambient bacterial count at the location where the samples were collected was 2.55×10^6 cells ml^{-1}.

Among the cyanobacteria studied, there was a highly significant ($P=0.0001$) difference in the bacterial numbers per surface area. Correlation between the bacterial numbers per surface area and thickness of mucilaginous investments was also very high ($r=0.99$). When the bacterial numbers were normalized to cyanobacterial chl *a*, there was significant ($P=0.05$) difference between

Anabaena flos-aquae and L. birgei. Aphanizomenon flos-aquae did not show significant difference between either one of the other two species. However, there was a strong correlation (r=0.70) between the bacterial numbers normalized to chl a and the thickness of mucilaginous investments among the cyanobacterial species (Table 1).

DISCUSSION

Our results have clearly indicated that there is a strong correlation between the thickness of cyanobacterial mucilaginous investments and the number of bacterial cells associated with them. There was a comparatively weaker indication of this fact among cyanobacteria by the bacterial counts normalized to cyanobacterial chl a. Because the wavelength at which chl a has peak absorption depends on the amount and composition of other pigments and chemicals associated with it. The position of peak absorption would therefore be different with different species (Lehninger 71), and this could cause considerable inaccuracy in the inter-species comparisons of bacterial counts based on chl a. In this study, bacterial numbers per surface area would be a more reliable data compared to that normalized to chl a. This data is further supported by the direct visual observations of bacterial association with cyanobacterial filaments as revealed by the photomicrographs in Fig. 1.

Even though thickness of the mucilaginous investments is found to be important, its physico-chemical properties too may be contributing to bacterial association. The extent of the fibril-

lar nature of the mucilaginous investments would be an important characteristic in the anchoring of bacterial cells on cyanobacterial surface layers (Paerl 83). The viscosity of the mucilaginous investments with its chemical constituents may be another factor that contributes to the binding of bacterial cells to the mucilaginous substrate. The watery matrices in some species of cyanobacteria that usually disperse in the surrounding media and become invisible, probably having poor viscosity, less fibrillar, and amorphous (Drews and Weckesser 83; Martin and Wyatt 72) would be an insignificant contributing factor in cyanobacterial-bacterial association and therefore, was not considered in this study. The chemical composition of the mucilaginous investments particularly the polysaccharide constituents (Lang 68) would be important for survival and multiplication of the bacteria that reached and stagnate in the substrate. Motile bacterial cells on reaching the surface layers of cyanobacteria are known to impart rapid and violent rotational movements (Paerl 83; Adler 76) before complete cessation of motility, and onset of cell division (Paerl 83). This may be due to their entanglement or anchoring (Paerl 83) in the viscous and fibrillar mucilaginous investments that mechanically resist their dislodging. Bacteria that reach the cyanobacterial surface either through chemotaxis, passive movements or accidental landing due to mass flow of the aquatic system, would find themselves in a microenvironment that affords certain degree of physical constraint to their escape or dislodging. Chemotaxis, even though known to play an important role in cyanobacterial-bacterial associations (Paerl and Kellar 78; Paerl 78), would bring only the motile bacteria to the microenvironment of cyano-

LITERATURE CITED

1. Adler J (1976) The sensing of chemicals by bacteria. Scient Am 234: 40-51

2. Caldwell DE, Caldwell SJ (1978) A Zoogloea sp. associated with blooms of Anabaena flos-aquae. Can J Microbiol 24: 922-931

3. Drews G (1973) Fine structure and chemical composition of the cell envelopes. In: Carr NG, Whitton BA (Eds.) The biology of blue-green algae. 676 pp. Blackwell, Oxford

4. Drews G, Weckesser J (1983) Function, structure and composition of cell walls and external layers. In: Carr NG, Whitton BA (Eds.) The biology of cyanobacteria. 688 pp. University of California press, USA

5. Fritsch FE (1945) Myxophyceae (Cyanophyceae). The structure and reproduction of the algae, vol II, 939 pp. Cambridge University press, UK

6. Gallucci KK, Paerl HW (1983) Pseudomonas aeruginosa chemotaxis associated with blooms of N_2-fixing blue-green algae (cyanobacteria). Appl Environ Microbiol 45: 557-562

7. Hobbie JE, Daley RJ, Jasper S (1977) Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl Environ Microbiol 33: 1225-1228

8. Kangatharalingam N, Wang L, Priscu JC (1990) An in situ technique to measure bacterial chemotaxis in natural aquatic environments. Microb Ecol 20: 3-10

9. Kessel M, Eloff JN (1975) The ultrastructure and development of the colonial sheath of Microcystis marginata. Arch Microbiol 106: 209-214

10. Lamont HC (1969) Shear-oriented microfibrils in the mucilaginous investments of two motile oscillatoriacean blue-green algae. J Bacteriol 97: 350-361

11. Lang NJ (1968) The fine structure of blue-green algae. Ann Rev Microbiol 22: 15-46

12. Lehninger AL (1971) Biochemistry. The molecular basis of cell structure and function. 833 pp. Worth publishers, NY, USA

13. Martin TC, Wyatt JT (1974) Extracellular investments in blue-green algae with particular emphasis on the genus Nostoc. J Phycol 10: 204-210

14. Paerl HW (1978) Role of heterotrophic bacteria in promoting N_2-fixation by Anabaena in aquatic habitats. Microb Ecol 4: 215-231

15. Paerl HW (1983) Interactions with bacteria. In: Carr NG, Whitton BA (Eds.) The biology of cyanobacteria. 688 pp. University of California press, USA

16. Paerl HW, Gallucci KK (1985) Role of chemotaxis in establishing a specific nitrogen-fixing cyanobacterial-bacterial association. Science 227: 647-649

17. Paerl HW, Kellar PE (1978) Significance of bacterial-Anabaena (cyanophyceae) associations with respect to N_2-fixation in freshwater. J Phycol 14: 254-260

18. Sartory DP, Grobbelaar JU (1984) Extraction of chlorophyll a from freshwater phytoplankton for spectrophotometric analysis. Hydrobiologia 114: 177-187

19. Stanier RY, Cohen-Bazire G (1977) Phototrophic prokaryotes: the cyanobacteria. Ann Rev Microbiol 31: 225-274

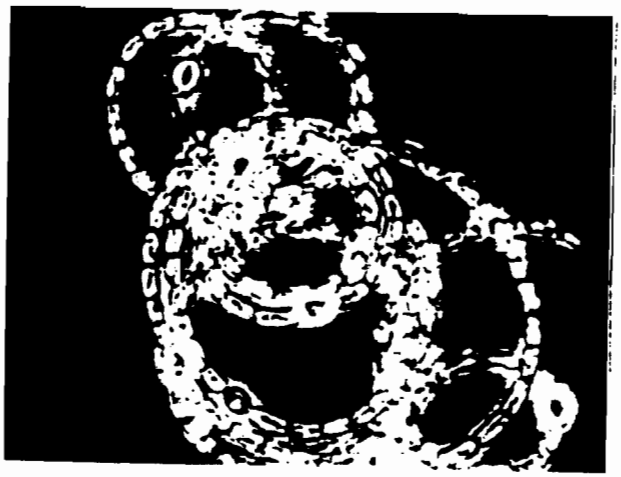
20. Tuffery AA (1969) Light and electron microscopy of the sheath of a blue-green alga. J Gen Microbiol 57: 41-50

21. Wellman AM, Paerl HW (1981) Rapid chemotaxis assay using radioactively labeled bacterial cells. Appl Environ Microbiol 42: 216-221

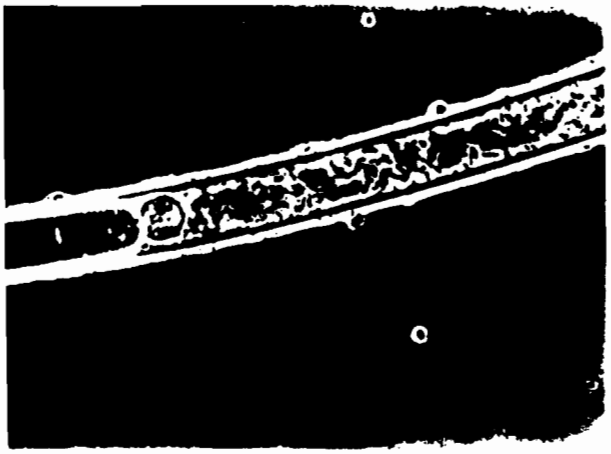
22. Wintermans JFGM, De Mots A (1965) Spectrophotometric characteristics of chlorophylls a and b and their pheophytins in ethanol. Biochim Biophys Acta 109: 448-453

Fig. 1

A



B



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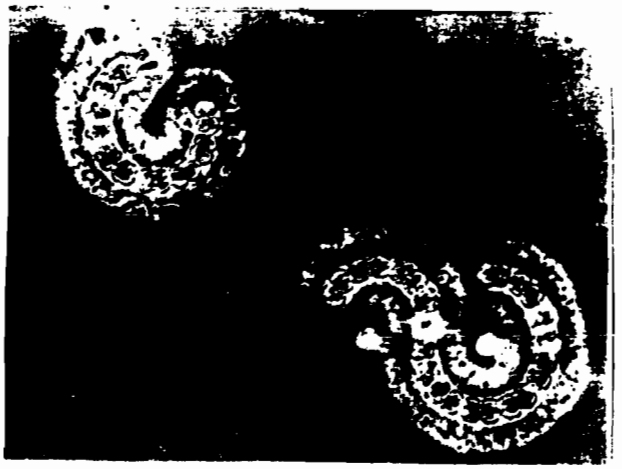

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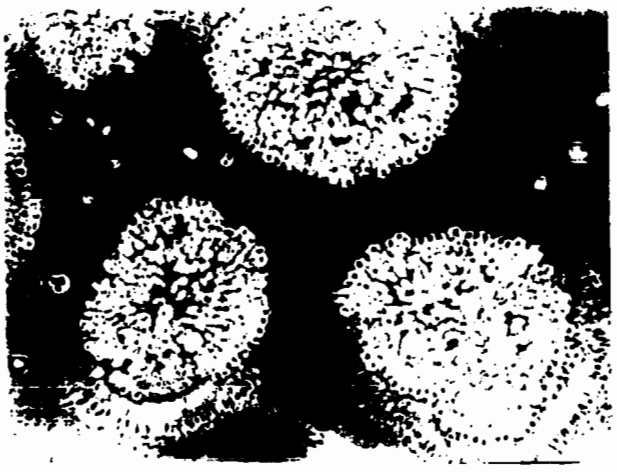
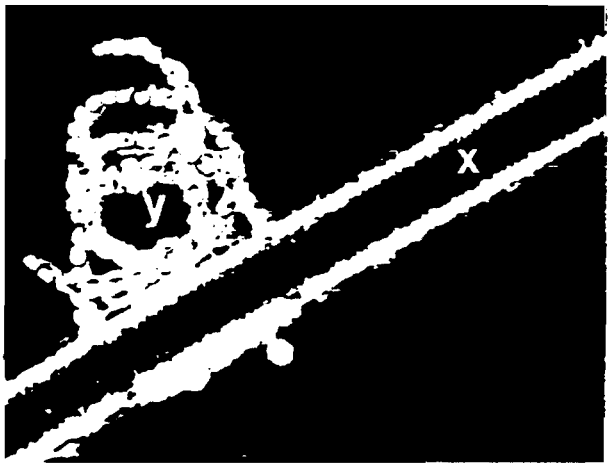


E

Fig. 2



A

B

CHAPTER 14

STATISTICAL MODELS

where cattle and dog deaths attributable to algae have been reported. Interestingly, temperature was not selected as a major factor controlling blue-green dominance. This may be related to the fact that the Grayling Arm very rarely thermally stratifies, although experimental verification would be required to verify this claim.

Models for Bacterial Biomass

Transformations of the data used in these models are the same as those described above for blue-green algae, except that the ARCSIN-square root transformation was not employed because no proportional variables were included. The dependent variable in all models was bacterial biovolume (BB = $x10^6$ μm^3 l^{-1}) which is directly related to bacterial biomass.

Whole Lake

FINAL MODEL:

BB = 0.092 (ln nitrate) + 0.859 (ln TEMP) + 0.121 (ln TOC) - 6.125

R^2 = 0.50; n = 104; $p < 0.01$

Standardized partial regression coefficients:

nitrate-N = 0.391
temperature = 0.709
total-C = 0.312

The R^2 for this model indicates that nitrate, temperature (TEMP) and total organic carbon (TOC = dissolved organic-C + particulate organic-C) accounts for 50 % of the variation observed in bacterial biomass (BB). All of these variables were positively related to the bacterial biomass present in the lake. The standardized partial regression coefficients show that tem-

perature had the greatest effect on bacterial biomass. From a management standpoint, one would have to remove nitrate or total organic carbon (presumably by reducing the dissolved organic carbon component) from the system. As for the blue-green algal models, the model for the entire lake may not be the best one for managing bacterial biomass, owing to the unique characteristics of the Grayling Arm. The models presented below for the lake excluding Grayling Arm and the Grayling arm alone are the most accurate in terms of variables relating to bacterial biomass.

Non-Grayling Arm Portion of Hebgen Lake

FINAL MODEL:
BB = 0.115 (ln particulate-P) + 0.107 (ln PPR) + 0.102 (ln nitrate-N) - 3.235

$R^2 = 0.41$; $n = 44$; $p < 0.01$

Standardized partial regression coefficients:

particulate-P = 0.256
PPR = 0.344
nitrate-N = 0.324

The R^2 for this model indicates that particulate-P (PP), primary productivity (PPR) and nitrate-N accounts for 41 % of the variation observed in bacterial biomass (BB). All of these variables were positively related to bacterial biomass present in the lake. The model infers that both phosphorus and nitrate regulate bacterial biomass in this portion of the lake. That the standardized regression coefficient for nitrate was about 30 % greater than for PP further infers that nitrate was most closely coupled to bacterial growth. PPR presumably contributes to bacterial growth and biomass via excretion products which contain both nitrogen and phosphorus (see also Chapters 8, 11 and 12).

Grayling Arm of Hebgen Lake

FINAL MODEL:

$BB = 0.185\ (\ln TEMP) + 0.529\ (\ln CHL) + 0.682\ (\ln POC) + 0.688$

$R^2 = 0.26;\quad n = 60;\quad p < 0.01$

Standardized partial regression coefficients:

temperature = 0.156
CHL = 0.207
POC = 7.236×10^{-3}

The R^2 for this model indicates that temperature, algal biomass (CHL = chlorophyll a) and particulate organic carbon (POC) account for 26 % of the variation observed in bacterial biomass (BB). This model is the only one where Ridge Trace analysis showed significant colinearity among the independent variables, specifically between CHL and POC, both of which are indicators of algal biomass. The standardized regression coefficients were adjusted to compensate for this colinearity and should provide a fairly accurate assessment of the contribution of the variables toward explaining variations in bacterial biomass. The magnitude of the adjusted standardized partials shows that chlorophyll and temperature alone can explain virtually all of the variation observed in bacterial biomass. The relationship with chlorophyll, as stated previously, presumably arises from extracellular release of organic carbon, and organic and inorganic forms of N and P. Consequently, regulation of bacterial biomass in the Grayling Arm of Hebgen Lake is closely related to regulation of blue-green algae biomass (the dominant algal group present). Experimental evidence for the close coupling between bacteria and blue-green algae is presented throughout this report

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(see Chapters 8, 9, 10, 11 and 12). Therefore, nutrient management of algal biomass should be an effective method of regulating bacterial biomass in systems similar to Hebgen Lake.

REFERENCES

Hoerl, A.E. and R.W. Kennard. 1970. Ridge regression: biased estimation for non-orthogonal problems. Technometrics. 12:55-67.

Priscu, J.C. 1987. Environmental factors regulating the dynamics of blue-green algal blooms in Canyon Ferry Reservoir, Montana. Report No. 159. Montana water Resources Center.

Steele, R.G.D. and J.H. Torrie. 1960. Principles and procedures of statistics. McGraw-Hill Co., New York.

CHAPTER 15

SUMMARY AND CONCLUSIONS

This section presents a short, concise summary along with the general conclusions reached during this study. Evidence supporting these conclusions can be gleaned from data presented in Chapters 1 through 14; references to specific chapters will be given when possible.

SUMMARY.

We have completed a 3 year study aimed at understanding the role of nutrients (specifically phosphorus, inorganic nitrogen and organic carbon) on nuisance blue-green algae growth. The first year of the study was spent examining blue-green algal responses to nutrient enrichment in a variety of southwestern Montana lakes and reservoirs. Based on results from the first year, we selected Hebgen Lake as our primary study site in which to conduct detailed experiments over the ensuing 2 years. The preliminary results from year 1 also pointed out that bacterial activity may have a role in mediating blue-green algal blooms. Hence, a bacterial component was added to our studies on Hebgen Lake. This lake receives minimal impact from human activities (most of the drainage lies within Yellowstone National Park), is located in a geological area rich in natural phosphate and is dominated by several species of blue-green algae, many of which are toxic.

We approached our study of Hebgen Lake from both a monitoring and an experimental perspective. The monitoring data were used to determine ambient conditions at the time of our experiments and to develop statistical models to corroborate and expand upon our experimental results. Our field experiments consisted

of nutrient enrichment bioassays where organismal responses (i.e. bacteria, blue-green algae, non-blue-green algae) were monitored over time. Routine experiments were conducted in 20 liter microcosms anchored in the lake; several experiments were also conducted in mesocosms (>2,000 liters) to reduce potential "container effects".

In addition to field experiments, we conducted a number of laboratory experiments on various aspects of bacterial nutrition, blue-green algal physiology and morphological adaptations, and interactions between bacteria and blue-green algae. These laboratory experiments were all conducted on organisms isolated from Hebgen Lake except those employing immunochemical techniques, developed in our laboratory under the auspices of SDA, which used natural populations of blue-green algae collected from the Atlantic Ocean (see Chapter 2).

Our study represents one of the more intense examinations of nutrient-blue-green algae interactions we are aware of. Data ~~from~~ produced during the last 2 years of our study are included in 13 manuscripts (see Chapters 1 through 13) which are being refined for publication in international journals (or have already been submitted or published). In addition, a Ph.D. dissertation and a Master's thesis based on results from this project are in their final stages of completion. We expect that several more manuscripts will materialize as the data are analyzed further.

CONCLUSIONS

1. Overall, the phytoplankton community (i.e. all size classes) of Hebgen Lake was N-deficient (indicated by both experiments and statistical models). Much of the experimental response to nitrogen was from the smaller, non-blue-green algal component of the community; the blue-green algal component of the community could be stimulated by the addition of P alone at certain times of the year. Organic carbon addition had little effect on algal growth (Chapter 1).

2. In situ experiments revealed a general trend in which relative blue-green algal biomass increased with P enrichment and decreased with N enrichment (Chapter 1).

3. The ability of blue-green algae to fix N_2 apparently allows them to outcompete non-N_2 fixers in the overall N-deficient environment in Hebgen Lake. Consequently, P addition alone can stimulate their growth when all other factors were favorable (we observed a clear P stimulation of N_2 fixing blue-green algae only once during our study, whereas P addition never stimulated the growth of non-N_2 fixing blue-green algae). See Chapter 1 for details.

4. Even though P addition has the potential to enhance the growth of N_2 fixing blue-green algal species, a host of other factors must exist for them to express their nitrogenase activity, in particular oxygen (nitrogenase, the enzyme responsible for N_2 fixation is highly oxygen sensitive). We showed that morphological adaptations by blue-green algae (e.g. micro- and macros-

cale aggregation) and association with oxygen consuming (through respiration) bacteria can allow (and may be necessary for) blue-green algae to express their N_2 fixing potential (Chapters 2, 3, 4, 8, 9, 11, 12 and 13).

5. Bacterial activity and biomass was stimulated by both organic-C, inorganic-N and inorganic-P additions. This stimulation showed no clear seasonal trend but appeared to be related to the type and condition of the phytoplankton present (Chapters 7, 8, 9, and 13).

6. Stimulation of bacterial growth by inorganic nutrient addition was both direct and indirect. Indirect stimulation resulted from enhanced blue-green algal activity upon nutrient addition and subsequent extracellular nutrient leakage. Apparent bacterial enhancement with P addition may result from products of N, obtained from N_2 fixation, which are excreted by the blue-green algae, i.e. the bacteria may in reality be responding indirectly to N enrichment from blue-green algal products, rather than to P addition (Chapters 7, 8, 11, 12).

7. Bacteria isolated from Hebgen Lake were shown to have positive chemotaxis to various species of blue-green algal species isolated from the same lake (Chapter 11), presumably in response to nutrients released from the blue-green algae (Chapter 10).

8. Management of aquatic systems dominated by N_2 blue-green algae, is complex. In general, the blue-green algae are present because the system is N-deficient, a problem they can overcome

with their ability to acquire N from the atmosphere. Hence, these systems tend to harbor simultaneously phytoplankton populations that are N-deficient (non-N_2 fixers, including blue-greens and non-blue-greens) and P-deficient (N_2 fixing blue-green algae). Despite this apparently clear distinction, the addition of P did not always stimulate N_2 fixing blue-green algae (at times N addition alone stimulated these species). This lack of P stimulation apparently resulted from other environmental factors which regulate the ability of N_2 fixing blue-green algae to fix atmospheric N_2. Our results indicate that the most probable causes for this lack of expression are (i) the presence of high levels of inorganic-N which suppresses N_2 fixation (Chapters 5 and 6), (ii) lack of appropriate morphological configuration required to protect the oxygen sensitive enzyme, nitrogenase, which mediated N_2 fixation) (Chapters 2, 3, 4 and 13), (iii) lack of minor nutrients such as molybdenum and iron which are required for nitrogenase to function (see Phase I Progress Report) and (iv) lack of sufficient bacterial activity to maintain a low oxygen environment in the region where nitrogenase exists (Chapter 2).

Based on the evidence we present in this study, one cannot <u>a priori</u> assume that an aquatic system will become dominated by blue-green algae (N_2 fixing or non-fixing) if it is enriched with P or if N is removed. Evidence for this is present in the statistical models presented in Chapter 14 which infer that nitrogen and temperature also play a significant role in regulating blue-green algal biomass. Lake managers must consider the effects of environmental factors other than P when planing strategies to

reduce nuisance blue-green algal dominance (and perhaps other algal groups as well). This is particularly apparent in regions where phosphate is present in naturally high levels due to local mineral deposits such as those present in southwestern Montana and much of Idaho.

APPENDIX

DATA COLLECTED DURING OUR MONITORING PROGRAM

KEY TO SYMBOLS USED TO PRESENT RESULTS

SYMBOL	DEFINITION
SRP	SOLUBLE REACTIVE PHOSPHORUS
TDP	TOTAL DISSOLVED PHOSPHORUS
DOP	DISSOLVED ORGANIC PHOSPHORUS (DOP = TDP - SRP)
PART-P	PARTICULATE PHOSPHORUS
TOT-P	TOTAL PHOSPHORUS (TOT-P = TDP + PART-P)
NH4-N	AMMONIUM NITROGEN
NO3-N	NITRATE NITROGEN
DIN	DISSOLVED INORGANIC NITROGEN (DIN = NH4-N + NO3-N)
TDN	TOTAL DISSOLVED NITROGEN
DON	DISSOLVED ORGANIC NITROGEN (DON = TDN - DIN)
PART-N	PARTICULATE NITROGEN
TOT-N	TOTAL NITROGEN (TOT-N = TDN + PART-N)
DOC	DISSOLVED ORGANIC CARBON
PART-C	PARTICULATE CARBON
PPR	PRIMARY PRODUCTIVITY (PHOTOSYNTHETIC CARBON UPTAKE)
CHL a	CHLOROPHYLL a
PHYTBIO	PHYTOPLANKTON BIOMASS
B.G.BIO	BLUE-GREEN ALGAL BIOMASS
FIXING B.G.BIO	ATMOSPHERIC NITROGEN FIXING BLUE-GREEN ALGAL BIOMASS
nmol C2H4/l*h	NITROGENASE ACTIVITY (ACETYLENE REDUCTION)
% B.G.	% OF TOTAL PHYTOPLANKTON BIOMASS COMPRISED OF BLUE-GREEN ALGAE
FIXING %B.G.	% OF TOTAL PHYTOPLANKTON BIOMASS COMPRISED OF ATMOSPHERIC NITROGEN FIXING BLUE-GREEN ALGAE
BPR	BACTERIAL THYMIDINE UPTAKE
BAPC	CELL-SPECIFIC BACTERIAL THYMIDINE UPTAKE
BV	BACTERIAL BIOVOLUME
BN	BACTERIAL CELL NUMBERS

Grayling Arm 1988

1988 DATA

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
13MAY-0m	11.3	1.01	5.22	4.21	15.33	20.55	1.13	2.50	3.63	0.17	166.37
-1m	11.1	1.01	5.22	4.21	15.95	21.17	1.88	2.30	4.18	0.17	165.82
-3m	11	1.01	5.22	4.21	21.14	26.36	1.13	2.40	3.53	0.16	156.47
-5m	8.5	.	.	.	.	.	.	.	.	.	.
20MAY-0m	12.2	3.62	10.38	6.76	14.29	24.67	3.38	3.60	6.98	0.13	123.02
-1m	10.5	2.75	10.38	7.63	16.36	26.74	3.38	2.63	6.01	0.15	144.00
-3m	10	3.62	10.38	6.76	17.54	27.92	4.87	2.98	7.85	0.2	192.15
-5m	9.5	3.62	6.94	3.32	17.61	24.55	57.19	3.50	60.69	0.14	79.31
02JUN-0m	11	6.11	11.75	5.64	12.41	24.16	11.29	1.80	13.09	0.25	236.91
-1m	11.1	7.88	10.02	2.14	12.66	22.68	1.13	2.16	3.29	0.15	146.71
-3m	10.9	6.11	10.02	3.91	13.23	23.25	1.91	2.18	4.09	0.22	215.91
-5m	10.9	6.11	10.02	3.91	12.47	22.49	0.35	2.16	2.51	0.13	127.49
16JUN-0m	17	0.47	11.75	11.28	14.66	26.41	5.56	1.76	7.32	0.16	152.68
-1m	16.9	1.4	10.02	8.62	16.19	26.21	4.74	2.47	7.21	0.19	182.79
-3m	16.6	3.25	11.75	8.50	14.87	26.62	6.39	3.26	9.65	0.21	200.36
-5m	14.2	6.03	6.55	0.52	8.21	14.76	6.39	2.11	8.50	0.12	111.50
30JUN-0m	18.9	2.32	30.27	27.95	23.01	53.28	16.31	3.17	19.48	0.23	210.52
-1m	18.8	2.32	47.73	45.41	23.18	70.91	7.22	3.17	10.39	0.33	319.61
-3m	18.6	3.25	49.48	46.23	25.81	75.29	6.39	3.62	10.01	0.2	189.99
-5m	18.2	2.32	52.97	50.65	32.07	85.04	14.65	9.15	23.80	0.23	206.20
14JUL-0m	18.5	5.48	41.09	35.61	26.5	67.59	15.14	16.90	32.04	0.27	237.96
-1m	18.6	3.65	23.84	20.19	26.5	50.34	15.99	10.71	26.70	0.25	223.30
-3m	18.5	3.65	18.67	15.02	24.03	42.70	17.68	12.44	30.12	0.26	229.88
-5m	18.5	1.83	13.49	11.66	20.57	34.06	12.6	12.50	25.10	0.21	184.91
28JUL-0m	20	1.83	22.12	20.29	24.05	46.17	16.84	3.29	20.13	0.2	179.87
-1m	20	0.91	18.67	17.76	24.91	43.58	4.14	3.21	7.35	0.25	242.65
-3m	19.9	1.83	23.84	22.01	26.28	50.12	4.98	3.64	8.62	0.23	221.38
-5m	19.5	2.74	23.84	21.10	22.16	46.00	5.83	4.03	9.86	0.24	230.14
17AUG-0m	17.5	9.91	32.6	22.69	166.78	199.38	10.07	2.99	13.06	0.53	516.94
-1m	17.8	9.03	129.79	120.76	68.44	198.23	5.01	3.00	8.01	0.38	371.99
-3m	17.8	10.8	96.82	86.02	74.11	170.93	16.82	2.99	19.81	0.43	410.19
-5m	17.5	32.92	91.61	58.69	24.28	115.89	48.02	38.15	86.17	0.47	383.83
09SEP-0m	15.2	55.04	73.72	18.68	12.51	86.23	34.53	85.29	119.82	0.4	280.18
-1m	15.5	60.35	73.72	13.37	11.8	85.52	30.31	80.76	111.07	0.42	308.93
-3m	15.5	58.58	79	20.42	11.35	90.35	34.53	91.96	126.49	0.41	283.51
-5m	15.5	58.58	89.58	31.00	12.33	101.91	32.84	92.31	125.15	0.51	384.85
01OCT-0m	11.5	16.66	26.04	9.38	72.09	98.13	8.01	14.81	22.82	0.26	237.18
-1m	11.5	17.53	26.04	8.51	14.38	40.42	7.17	15.14	22.31	0.28	257.69
-3m	10.6	33.18	48.94	15.76	14.2	63.14	8.85	39.89	48.74	0.31	261.26
-5m	10.2	38.4	40.13	1.73	15.42	55.55	16.4	65.49	81.89	0.35	268.11
22OCT-0m	8.8	30.4	36.61	6.21	12.56	49.17	18.49	52.41	70.90	.	.
-1m	8.8	31.3	36.61	5.31	13.94	50.55	17.63	51.86	69.49	0.41	340.51
-3m	8.8	30.4	40.13	9.73	12.9	53.03	16.77	51.55	68.32	0.31	241.68
-5m	8.8	35.84	52.46	16.62	13.08	65.54	15.9	49.77	65.67	0.27	204.33
04NOV-0m	5	27.39	31.41	4.02	8.44	39.85	16.18	67.78	83.96	0.31	226.04
-1m	5	25.52	33.18	7.66	8.78	41.96	21.79	64.57	86.36	0.35	263.64
-3m	5	25.52	33.18	7.66	17.87	51.05	15.25	58.46	73.71	0.3	226.29
-5m	5	28.32	38.46	10.14	14.27	52.73	16.18	61.16	77.34	0.33	252.66

GRAYLING ARM 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
13MAY-0m	64.7	234.7	.	1251.2	4.10	2.57	.	.	.	0
-1m	36.1	206.1	.	799.6	4.00	2.51	.	.	.	0
-3m	74.2	234.2	.	1120.5	1.60	2.99	.	.	.	0
-5m	.	.	.	.	.	.	.	.	.	.
20MAY-0m	27.3	157.3	.	737.8	.	1.14	557.95	0	0	0
-1m	42.4	192.4	.	1095	.	1.51	.	.	.	0
-3m	48.6	248.6	.	1110.1	.	1.75	1654.98	0	0	0
-5m	40.5	180.5	.	1262.3	.	0.9	1976.38	30.69	30.69	0
02JUN-0m	74.6	324.6	2.776	584.7	6.33	6.28	2305.09	0	0	23.76
-1m	79.5	229.5	.	607.1	6.76	2.85	.	.	.	14.74
-3m	77	297	2.599	573.9	2.13	2.97	.	.	.	16.56
-5m	71.9	201.9	2.668	820.7	0.28	3.98	15969.35	360.58	360.58	12.81
16JUN-0m	183.9	343.9	2.786	1180.1	19.05	4.74	2579.85	0	0	540.59
-1m	141.7	331.7	.	1026	24.53	10.08	.	.	.	496.12
-3m	140.6	350.6	2.691	1017.1	1.61	7.22	3595.65	1357.72	1357.72	209.79
-5m	33.4	153.4	2.398	450	0.18	2.84	2702.13	1558.41	1558.41	679.02
30JUN-0m	405.6	635.6	2.835	2569.7	15.30	9.55	6632.59	6348.98	6348.98	257.49
-1m	373.6	703.6	.	2241.9	39.40	6.05	.	.	.	508.04
-3m	359.3	559.3	3.164	2114.3	7.50	7.8	6663.36	5424.52	5424.52	240.25
-5m	281.5	511.5	2.69	1821.1	0.20	7.22	3892.13	3300.31	3300.31	150.89
14JUL-0m	200.1	470.1	2.864	1309	12.00	5.88	9085.95	7142	4894	50.21
-1m	290	540	.	1565.5	23.10	11.17	.	.	.	54.12
-3m	215.7	475.7	2.683	1367.6	7.80	9.78	3511.47	1916.02	1594.82	56.4
-5m	202.7	412.7	2.727	1265	0.90	8.1	3353.067	2111.09	2111.09	12.26
28JUL-0m	214.9	414.9	3.053	1471.8	32.60	11.13	2448.4	797.87	797.87	225.24
-1m	219.1	469.1	.	1519	31.90	16.38	.	.	.	243.88
-3m	203.9	433.9	2.905	1462.5	6.70	14.83	2846.83	1311.84	1270.11	104.99
-5m	214.3	454.3	2.985	1429.6	1.30	15.45	4782.34	2392.5	2392.5	41.22
17AUG-0m	1484.1	2014.1	4.632	7902.4	101.20	116.75	3290.36	3275.89	3275.89	1159.94
-1m	809.4	1189.4	.	4373.4	28.20	86.72	.	.	.	632.22
-3m	751.5	1181.5	5.772	4241.8	1.40	85.14	3475.36	3382.68	3382.68	184.79
-5m	105.5	575.5	5.994	1234.7	0.00	8.81	514.48	451.11	451.11	0.16
09SEP-0m	70.9	470.9	6.411	921.2	12.60	3.67	1031.92	471.1	471.1	6.49
-1m	47.8	467.8	.	841.4	14.60	4.53	.	.	.	5.49
-3m	41.9	451.9	5.497	771.6	5.80	3.01	1154.1	223.97	223.97	3.32
-5m	32.6	542.6	6.487	728.5	0.40	2.73	319.71	84.95	84.95	0.18
010CT-0m	1534.9	1794.9	2.276	9038.3	22.10	151.51	35492.11	33595.12	33595.12	560.82
-1m	39.4	319.4	.	962.8	16.50	13.91	.	.	.	35.83
-3m	22.4	332.4	2.517	680	4.40	3.91	1543.27	0	0	8.26
-5m	86.7	436.7	6.639	799.5	0.10	1.22	410.96	0	0	0.19
220CT-0m	23.9	.	5.11	886.1	7.70	5.76	2108.33	0	0	42.37
-1m	35.1	445.1	.	1128.5	13.80	5.39	.	.	.	4.73
-3m	41.6	351.6	4.433	1078.3	2.10	5.64	3248.99	177.63	177.63	5.44
-5m	23.6	293.6	4.035	905.3	0.40	2.85	1210.16	0	0	9.47
04NOV-0m	54.5	364.5	2.576	811	.	3.91	902.62	278.03	278.03	.
-1m	40.5	390.5	.	699.3	.	3.26	.	.	.	.
-3m	75.7	375.7	2.871	1176.6	.	4.28	1035.85	0	0	.
-5m	32.3	362.3	3.123	820.8	.	3.22	.	.	.	.

GRAYLING ARM 1988 INTEGRATED 0-5 METERS

DATE	TEMP AVG	SRP mg/m^2	TDP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
13MAY88	11.11	3.03	15.66	12.63	52.73	68.39	4.52	7.10	11.62	0.50	488.39
20MAY88	10.37	16.80	48.46	31.67	84.38	132.84	73.69	15.19	88.88	0.83	741.12
02JUN88	10.97	33.21	50.96	17.76	64.13	115.09	11.51	10.66	22.17	0.92	897.83
16JUN88	16.14	14.87	50.96	36.09	69.57	120.52	29.06	13.20	42.26	0.91	862.74
30JUN88	18.60	13.46	238.66	225.20	129.97	368.63	46.42	22.72	69.14	1.24	1170.86
14JUL88	18.53	17.35	107.14	89.79	121.63	228.77	79.52	61.90	141.41	1.24	1098.59
28JUL88	19.84	8.68	110.59	101.91	124.11	234.70	30.42	17.77	48.19	1.18	1126.81
17AUG88	17.68	73.02	496.23	423.22	358.55	854.79	94.21	50.13	144.34	2.17	2020.66
09SEP88	.	293.79	395.02	101.24	58.99	454.01	164.63	440.02	604.65	2.16	1555.35
01OCT88	10.88	139.39	190.09	50.71	101.44	291.53	48.86	175.39	224.25	1.52	1295.75
22OCT88	8.80	158.79	205.94	47.15	66.07	272.01	85.13	256.86	341.99	1.71	1368.46
04NOV88	5.00	131.34	170.30	38.96	67.40	237.70	87.46	308.83	396.28	1.61	1213.72

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C g/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	FIXING % B.
13MAY88	160.70	660.70	.	2.95	9.65	8.04	.	.	.	0.00	.	
20MAY88	214.95	1044.95	.	5.49	.	7.24	6.95	0.03	0.03	0.00	0.44	0.4
02JUN88	382.45	1302.45	13.33	3.17	17.85	17.34	45.69	0.90	0.90	79.92	1.97	1.9
16JUN88	619.10	1524.10	13.30	4.61	49.72	34.77	15.56	4.95	4.95	2113.08	31.83	31.8
30JUN88	1763.30	3003.30	14.85	10.70	81.95	36.67	30.50	26.39	26.39	1522.20	86.51	86.5
14JUL88	1169.15	2409.15	13.73	7.00	57.15	47.36	25.76	17.61	13.44	231.35	68.38	52.1
28JUL88	1058.20	2233.20	14.83	7.37	78.85	75.25	15.57	6.87	6.76	729.64	44.11	43.4
17AUG88	3564.65	5729.65	27.37	20.23	95.70	367.55	14.14	13.82	13.82	1898.04	97.76	97.7
09SEP88	223.55	2383.55	29.85	3.99	40.20	17.38	4.75	1.35	1.35	18.30	28.44	28.4
01OCT88	958.05	2478.05	16.35	8.12	44.70	105.66	23.56	16.80	16.80	350.87	71.30	71.3
22OCT88	171.40	1886.95	22.78	5.20	29.15	25.10	12.50	0.44	0.44	48.63	3.55	3.5
04NOV88	271.70	1881.70	14.16	4.63	.	18.63	4.98	1.45	1.45	.	29.17	29.1

DATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
13MAY88	3.83	9.66	3.05	2.34
20MAY88	5.29	7.87	2.55	0.90
02JUN88	0.67	11.32	5.96	0.32
16JUN88	2.84	12.65	8.90	0.89
30JUN88	5.14	8.15	13.57	1.69
14JUL88	8.15	10.53	9.61	3.57
28JUL88	5.55	9.52	8.53	2.05
17AUG88	1.98	6.70	9.94	0.69
09SEP88	2.06	5.25	3.79	1.50
01OCT88	1.61	8.50	9.44	1.26
22OCT88	2.15	6.94	2.59	1.62
04NOV88	3.02	7.92	4.03	2.35

MADISON ARM 1988

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (µg/l)	DON (µg/l)
20MAY -0	12	1.01	8.66	7.65	10.76	19.42	1.88	3.684	5.564	130.0	124.44
-1	12	1.88	8.66	6.78	11.73	20.39	2.63	2.938	5.568	360.0	354.43
-3	9.3	1.88	29.32	27.44	7.99	37.31	1.88	3	4.88	690.0	685.12
-5	9	1.01	8.66	7.65	8.54	17.2	2.63	3.15	5.78	510.0	504.22
-10	7.8	.	.	.	.	.	.	.	.	.	.
02JUN -0	11.1	7.88	13.49	5.61	7.11	20.6	0.35	1.802	2.152	190.0	187.85
-1	11	0.81	4.82	4.01	7.75	12.57	0.35	1.815	2.165	150.0	147.84
-3	11	2.58	6.55	3.97	9.98	16.53	1.91	2	3.91	250.0	246.09
-5	10.4	4.35	6.55	2.2	5.45	12	5.82	2.162	7.982	150.0	142.02
-10	9	.	.	.	.	.	.	.	.	.	.
16JUN -0	16.9	1.4	18.68	17.28	4.56	23.24	4.74	1.762	6.502	160.0	153.50
-1	16	1.4	15.22	13.82	4.94	20.16	5.56	1.809	7.369	130.0	122.63
-3	15.5	2.32	11.75	9.43	4.69	16.44	8.04	2.111	10.151	190.0	179.85
-5	14.6	3.25	10.02	6.77	5.9	15.92	45.23	2.114	47.344	130.0	82.66
-10	12	5.11	8.29	3.18	3.67	11.96	9.7	2.894	12.594	110.0	97.41
30JUN -0	18	1.4	42.49	41.09	5.49	47.98	.	2.114	.	190.0	.
-1	18	1.4	44.24	42.84	4.75	48.99	6.39	2.17	8.56	190.0	181.44
-3	17.6	1.4	26.78	25.38	4.67	31.45	10.41	2.463	12.873	130.0	117.13
-5	17.5	3.25	11.06	7.81	5.25	16.31	.	4.581	.	130.0	.
-10	15.6	7.89	11.06	3.17	5.82	16.88	.	5.787	.	120.0	.
14JUL -0	18	2.74	13.49	10.75	8.21	21.7	0	2.548	2.548	120.0	117.45
-1	18	0.91	15.22	14.31	8.13	23.35	1.6	2.562	4.162	180.0	175.84
-3	17.9	4.57	25.57	21	7.72	33.29	1.6	2.464	4.064	180.0	175.94
-5	17.8	1.83	8.32	6.49	7.96	16.28	2.44	2.499	4.939	150.0	145.06
-10	16	10.05	15.22	5.17	6.73	21.95	18.53	5.856	24.386	160.0	135.61
28JUL -0	20	1.83	22.12	20.29	7.75	29.87	2.44	2.112	4.552	150.0	145.45
-1	20	0.91	13.49	12.58	11.35	24.84	3.29	2.142	5.432	190.0	184.57
-3	20	0	39.37	39.37	10.67	50.04	1.6	2.196	3.796	150.0	146.20
-5	19.8	0.91	18.67	17.76	9.98	28.65	1.6	1.785	3.385	140.0	136.62
-10	18	10.05	20.39	10.34	11.87	32.26	28.69	5.824	34.514	130.0	95.49
17AUG -0	17.8	4.6	39.54	34.94	12.77	52.31	5.86	2.98	8.84	190.0	181.16
-1	17.8	6.37	43.02	36.65	12.43	55.45	8.39	3.429	11.819	210.0	198.18
-3	17.8	3.72	25.66	21.94	15.18	40.84	15.13	2.991	18.121	220.0	201.88
-5	17.8	4.6	25.66	21.06	12.6	38.26	6.7	3.002	9.702	230.0	220.30
-10	17.1	8.14	23.29	15.15	10.02	33.31	20.19	5.987	26.177	90.0	63.82
09SEP -0	15.8	5.49	19.07	13.58	15.55	34.62	15.98	4.116	20.096	190.0	169.90
-1	15.8	5.49	20.84	15.35	14.66	35.5	16.82	3.333	20.153	.	.
-3	15.8	5.49	20.84	15.35	14.48	35.32	15.98	5.214	21.194	180.0	158.81
-5	15.8	6.37	24.36	17.99	12.6	36.96	15.98	5.714	21.694	160.0	138.31
-10	15.2	5.49	27.89	22.4	17.7	45.59	14.29	4.486	18.776	170.0	151.22
01OCT -0	13.2	2.75	15.47	12.72	78.67	94.14	4.65	3.894	8.544	.	.
-1	12.8	3.62	15.47	11.85	14.29	29.76	4.65	3.6	8.25	.	.
-3	12.6	2.75	13.71	10.96	9.44	23.15	4.65	3.527	8.177	.	.
-5	12.5	1.88	11.94	10.06	8.05	19.99	5.49	3.169	8.659	.	.
-10	10.8	1.88	8.42	6.54	5.62	14.04	6.33	5.699	12.029	.	.
22OCT -0	10	13.16	10.18	0	8.05	18.23	8.99	9.524	18.514	170.0	151.49
-1	10	10.43	10.18	0	7.7	17.88	2.95	2.95	5.9	180.0	174.10
-3	10	11.34	15.47	4.13	7.7	23.17	2.95	3.717	6.667	160.0	153.33
-5	10	9.53	17.23	7.7	6.4	23.63	3.81	7.778	11.588	160.0	148.41
-10	10	11.34	20.75	9.41	7.96	28.71	2.95	4.753	7.703	220.0	212.30

MADISON ARM 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
20MAY -0	18.7	130018.7	.	1137.4	.	1.02	2423.59	61.78	61.78	0
-1	39.9	360039.9	.	743.3	.	1.02	.	.	.	0
-3	15.1	690015.1	.	975.9	.	1.16	999.08	0	0	0
-5	15.9	510015.9	.	812.8	.	3.04	2440.17	0	0	0
-10	.	.	.	.	.	.	.	.	.	0
02JUN -0	49.2	190049.2	.	422	.	2.87	858.23	0	0	0
-1	50.5	150050.5	.	719.1	5.2	3.16	.	.	.	0
-3	40.9	250040.9	.	604.7	2.5	2.28	1439.65	0	0	0
-5	21	150021	.	425.9	0.6	1.81	484.15	0	0	0
-10	.	.	.	.	.	.	.	.	.	0
16JUN -0	39.1	160039.1	.	531.6	2.318	0.5	259.84	0	0	0
-1	34.2	130034.2	.	445.8	3.589	1.02	.	.	.	0
-3	34.9	190034.9	.	421.8	4.564	1.16	2312.51	361.35	0	0
-5	38.2	130038.2	.	472.2	4.086	0.4	2266.58	545.42	51.15	0
-10	21.9	110021.9	.	463.2	0.182	0.97	.	.	.	0
30JUN -0	31.9	190031.9	.	394	2.924	1.25	755.53	0	0	31.46
-1	45.9	190045.9	.	395.8	4.829	1.49	.	.	.	5.27
-3	54.5	130054.5	.	433.8	5.345	1.73	446.35	0	0	2.68
-5	61.1	130061.1	.	489.2	4.577	2.39	2530.44	1686.28	0	0
-10	49.7	120049.7	.	362.5	0.444	4.3	.	.	.	0
14JUL -0	105.4	120105.4	.	974.9	.	6.62	720.7	500.92	19.13	.
-1	85.9	180085.9	.	804.2	.	5.1	.	.	.	.
-3	95.2	180095.2	.	914.7	.	5.43	3052.64	2380.63	148.3	.
-5	84.6	150084.6	.	787.5	.	4.57	1442.26	1130.78	30.69	.
-10	32	160032	.	481.2	.	.	.	.	.	.
28JUL -0	93.4	150093.4	.	917.2	8.115	6.01	3059.75	2810.46	0	182.37
-1	101.5	190101.5	.	952.6	10.18	7.55	.	.	.	170.04
-3	91	150091	.	843.3	8.817	4.37	799.87	240.9	0	59.17
-5	89.4	140089.4	.	872.7	3.398	5.1	4323.71	3731.24	0	181.64
-10	52.3	130052.3	.	629.7	0.109	1.62	.	.	.	13.31
17AUG -0	63.4	190063.4	.	724.8	11.399	6.34	1351.83	770.87	0	21.45
-1	113.2	210113.2	.	840.6	18.055	13.44	.	.	.	0.21
-3	88.2	220088.2	.	974.6	13.766	4.02	1357.05	240.9	0	15.67
-5	55.5	230055.5	.	863.7	5.099	3.71	1353.06	0	0	16.5
-10	61	90061	.	691.6	0.232	2.63	.	.	.	0.2
09SEP -0	142.6	190142.6	.	1035.3	14.961	4.82	1093.29	880.42	880.42	11.02
-1	96.7	.	.	808.4	15.687	6.37	.	.	.	3.41
-3	62.1	180062.1	.	711.9	12.509	6.13	.	.	.	4.37
-5	80.6	160080.6	.	875.8	4.957	6.83	4729.2	2801.26	231.69	3.63
-10	126.5	170126.5	.	1057.2	0.32	6.83	.	.	.	0.23
01OCT -0	2487.3	.	.	13725	38.855	203.66	38615.1	38615.08	38615.08	914.67
-1	123.5	.	.	1183.6	13.842	15.14	.	.	.	300
-3	134.6	.	.	992.6	8.606	7.65	1905.69	919.04	919.04	26.12
-5	63	.	.	645.7	1.804	3.59	12644.16	1451.93	1451.93	8.56
-10	33.3	.	.	670.5	0.511	7.73	.	.	.	3.34
22OCT -0	19.4	170019.4	.	730.9	.	6.09	2296.38	0	0	.
-1	22	180022	.	771.3	.	4.78	.	.	.	.
-3	37.6	160037.6	.	984.5	.	5.27	1666.52	92.68	92.68	.
-5	31.5	160031.5	.	883.4	.	7.89	5884.82	84.95	84.95	.
-10	54.9	220054.9	.	874.5	.	6.42	.	.	.	.

MADISON ARM 1988

INTEGRATED 0-5 METERS 1ST 2 DAYS , 0-10 METERS THEREAFTER

DATE	TEMP AVG	SRP mg/m^2	TDP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
20MAY88	9.41	8.09	84.62	76.53	47.50	132.12	11.28	15.40	26.67	2.50	2468.33
02JUN88	10.27	14.67	33.63	18.96	40.59	74.22	10.34	9.79	20.13	0.97	949.87
16JUN88	14.45	31.59	111.47	79.88	48.90	160.36	209.35	22.45	231.80	1.39	1153.20
30JUN88	17.11	36.70	207.53	170.83	52.14	259.66	.	39.74	.	1.40	.
14JUL88	17.37	43.41	147.89	104.48	76.43	224.31	60.47	33.43	93.90	1.62	1521.10
28JUL88	19.39	30.59	226.36	195.77	106.85	333.20	86.68	29.47	116.15	1.48	1358.85
17AUG88	17.61	55.75	283.66	227.91	124.54	408.20	119.70	38.09	157.79	1.88	1722.21
09SEP88	15.64	57.98	237.46	179.48	147.08	384.54	156.84	48.70	205.53	1.72	1514.00
010CT88	12.16	23.59	121.20	97.62	121.88	243.08	53.64	39.74	93.38	.	.
220CT88	10.00	106.61	163.48	58.74	73.28	236.76	35.53	55.73	91.26	1.79	1693.74

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C g/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	FIXING % B.G
20MAY88	115.30	2610.30	.	4.45	.	7.40	8.57	0.09	0.09	0.00	1.08	1.0
02JUN88	203.15	1173.15	.	2.92	16.00	12.55	5.37	0.00	0.00	0.00	0.00	0.0
16JUN88	329.10	1714.10	.	4.59	30.43	7.93	8.44	1.45	0.05	0.00	17.17	0.6
30JUN88	531.90	1926.90	.	4.28	36.53	25.44	4.78	1.69	0.00	29.00	35.28	0.0
14JUL88	748.05	2363.05	.	7.48	.	.	10.15	7.83	0.43	.	77.14	4.2
28JUL88	824.60	2299.60	.	8.20	49.13	44.97	10.91	8.55	0.00	1133.60	78.34	0.0
17AUG88	724.65	2604.65	.	8.32	78.74	50.93	6.77	1.76	0.00	100.63	25.96	0.0
09SEP88	938.90	2687.50	.	8.86	74.18	65.21	14.56	9.20	2.78	32.65	63.23	19.1
010CT88	2001.85	.	.	14.56	64.99	171.73	75.33	61.67	61.67	997.89	81.87	81.8
220CT88	365.40	2150.40	.	8.77	.	64.42	13.50	0.32	0.32	.	2.37	2.3

DATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
20MAY88	3.30	19.76	2.43	1.90
02JUN88	1.37	15.81	5.00	0.67
16JUN88	7.34	10.69	6.73	0.71
30JUN88	.	7.42	10.20	1.08
14JUL88	2.16	10.53	9.79	0.77
28JUL88	3.80	6.90	7.72	0.96
17AUG88	2.83	6.38	5.82	0.68
09SEP88	3.54	6.99	6.38	0.84
010CT88	3.96	.	16.43	1.68
220CT88	0.86	9.08	4.99	0.52

MID LAKE 1988

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
20MAY -0	9.5	2.58	8.66	6.08	6.81	15.47	3.47	2.162	5.63	0.37	364.37
-1	9.2	2.58	10.38	7.8	7.23	17.61	19.89	3.244	23.13	0.8	776.87
-3	8.2	2.58	12.11	9.53	7.92	20.03	0.35	.	.	.	.
-5	8.2	4.35	.	.	6.4	.	0.35.	.	.	.	.
-10	7.3	14.95	17.27	2.32	7.51	24.78	26.15	10.89	37.04	0.15	112.96
-15	5.3	4.35	10.38	6.03	7.71	18.09	1.13	8.65	9.78	0.1	90.22
02JUN -0	12	2.58	6.55	3.97	4.49	11.04	15.2	2.162	17.36	0.15	132.64
-1	12	2.58	10.02	7.44	4.37	14.39	10.51	1.815	12.33	0.16	147.68
-3	12	4.35	10.02	5.67	3.37	13.39	11.29	2.162	13.45	0.12	106.55
-5	12	4.35	10.02	5.67	4.81	14.83	12.08	2.162	14.24	0.19	175.76
-10	11.8	4.35	10.02	5.67	3.03	13.05	15.2	2.541	17.74	0.15	132.26
-15	9.2	9.65	11.75	2.1	4.62	16.37	29.28	5.766	35.05	0.18	144.95
16JUN -0	17	2.32	6.55	4.23	3.27	9.77	8.87	1.76	10.63	0.13	119.37
-1	16.5	1.4	6.13	5.15	4.69	11.24	13.83	1.762	15.59	0.13	114.41
-3	16	1.4	8.23	6.89	4.3	12.59	39.45	2.17	41.62	0.13	88.38
-5	14.9	2.32	8.29	5.97	6.15	14.44	8.04	1.76	9.80	.	.
-10	13.2	4.18	15.22	11.04	4.62	19.84	8.87	1.762	10.63	.	.
-15	9.8	9.74	23.88	14.14	4.62	28.5	69.2	6.872	76.07	0.13	53.93
-20	6.9	32.94	27.35	-5.59	4.56	31.91	165.89	10.205	176.10	0.18	3.90
30JUN -0	18.2	0.47	18.05	17.58	5.16	23.21	49.37	1.76	51.13	0.1	48.87
-1	19.4	1.4	33.76	32.36	5.41	39.17	3.91	1.762	5.67	0.12	114.33
-3	19	6.03	45.99	39.96	6.15	52.14	3.91	1.809	5.72	0.11	104.28
-5	18.8	2.32	40.75	38.43	6.4	47.15	4.74	1.76	6.50	0.14	133.50
-10	17.2	1.4	28.53	27.13	9.53	38.06	10.52	2.467	12.99	0.1	87.01
-15	10.5	37.57	40.75	3.18	14.55	55.3	215.48	10.851	226.33	.	.
-20	8.5	27.37	44.24	16.87	16.37	60.61	119.61	15.484	135.09	0.23	94.91
14JUL -0	19.2	3.65	10.04	6.39	10.33	20.37	3.29	2.499	5.79	0.17	164.21
-1	19.1	0.91	10.04	9.13	11.01	21.05	0.75	2.184	2.93	0.17	167.07
-3	18.9	2.74	11.77	9.03	11.53	23.3	2.44	2.562	5.00	0.25	245.00
[illegible]	18.6	2.74	22.12	19.38	11.7	33.82	2.44	2.464	4.90	0.17	165.10
[illegible]	18.2	3.65	15.22	11.57	7.58	22.8	4.14	2.499	6.64	0.18	173.36
[illegible]	11.5	15.53	29.02	13.49	6.55	35.57	65.95	13.176	79.13	0.24	160.87
28JUL -0	20.5	0.91	13.49	12.58	10.33	23.82	2.44	1.83	4.27	0.14	135.73
-1	20.5	1.83	8.32	6.49	8.44	16.76	88.81	2.464	91.27	0.2	108.73
-3	20	0.91	16.94	16.03	12.39	29.33	4.14	2.856	7.00	0.2	193.00
-5	19.8	1.83	13.49	11.66	14.44	27.93	2.44	2.196	4.64	0.15	145.36
-10	17.9	1.83	18.67	16.84	5.7	24.37	8.37	2.499	10.87	0.09	79.13
-15	13.2	6.39	20.39	14	5.18	25.57	29.54	18.928	48.47	0.09	41.53
17AUG -0	18.9	1.95	15.25	13.3	13.29	28.54	4.17	3.704	7.87	0.13	122.13
-1	18.9	1.06	39.54	38.48	13.63	53.17	4.17	2.607	6.78	0.14	133.22
-3	18.6	1.06	27.4	26.34	11.4	38.8	3.33	2.667	6.00	.	.
-5	18.5	10.8	22.19	11.39	11.23	33.42	58.13	5.234	63.36	0.15	86.64
-10	18.5	28.49	41.28	12.79	10.54	51.82	123.9	5.253	129.15	0.17	40.85
-15	16.2	10.8	22.19	11.39	6.41	28.6	56.45	48.643	105.09	0.21	104.91
-18	12.7	26.72	39.54	12.82	9.16	48.7	128.12	3.704	131.82	0.76	628.18

MIO LAKE 1988

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TOP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
09SEP -0	16.5	5.49	15.55	10.06	11.35	26.9	8.39	2.627	11.02	0.23	218.98
-1	16.5	3.72	15.55	11.83	11.71	27.26	6.7	2.619	9.32	.	.
-3	16.5	4.6	15.55	10.95	11.08	26.63	6.7	2.593	9.29	0.15	140.71
-5	16.5	3.72	15.55	11.83	10.19	25.74	8.39	2.607	11.00	0.24	229.00
-10	16.5	4.6	15.55	10.95	9.2	24.75	10.92	2.667	13.59	0.28	266.41
-15	16.2	8.14	19.07	10.93	7.15	26.22	38.74	2.617	41.36	0.22	178.64
-18	13.5	123.18	170.66	47.48	74.04	244.7	587.65	2.627	590.28	0.32	-270.28
01OCT -0	14	7.97	17.23	9.26	10.13	27.36	30.68	6.05	36.73	0.19	153.27
-1	13.2	7.97	17.23	9.26	10.65	27.88	24.8	6.018	30.82	0.19	159.18
-3	12.8	7.97	22.51	14.54	14.98	37.49	.	6.12	.	0.21	.
-5	12.6	8.84	18.99	10.15	10.39	29.38	31.52	6.349	37.87	0.21	172.13
-10	12.4	7.1	20.75	13.65	8.74	29.49	34.03	6.338	40.37	0.22	179.63
-15	12.2	6.23	20.75	14.52	7.88	28.63	30.68	6.412	37.09	0.21	172.91
22OCT -0	10.8	9.53	29.56	20.03	9.51	39.07	21.95	4.457	26.41	0.16	133.59
-1	10.8	12.25	18.99	6.74	11.57	30.56	20.22	4.445	24.67	0.22	195.34
-3	10.8	13.16	18.99	5.83	10.54	29.53	18.49	4.387	22.88	0.2	177.12
-5	10.8	6.36	11.94	5.58	8.48	20.42	22.72	5.926	28.65	0.2	171.35
-10	10.8	6.36	11.94	5.58	11.74	23.68	20.19	5.958	26.15	0.21	183.85
-15	10.8	7.25	11.94	4.69	7.79	19.73	20.19	6.094	26.28	0.19	163.72
04NOV -0	8.2	6.85	15.55	8.7	10.67	26.22	15.25	5.454	20.70	0.21	189.30
-1	8.2	5.91	19.07	13.16	12.9	31.97	16.18	8.774	24.95	0.19	165.05
-3	8.2	5.91	20.84	14.93	8.61	29.45	15.25	3.512	18.76	0.2	181.24
-5	8.2	4.05	20.84	16.79	10.84	31.68	15.25	3.228	18.48	0.2	181.52
-10	8.2	4.98	13.79	8.81	8.27	22.06	16.18	3.654	19.83	0.2	180.17
-15	8.2	5.91	12.02	6.11	8.96	20.98	15.25	2.812	18.06	0.2	181.94

MID LAKE 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
20MAY -0	46.3	416.3	.	722	.	1.69	373.62	0	0	0
-1	52.2	852.2	.	504.4	.	1.34	.	.	.	0
-3	46.5	.	.	920.1	.	1.93	1261.26	0	0	0
-5	49.3	.	.	640.7	.	1.69	1891.47	0	0	0
-10	48.1	198.1	.	693.4	.	2.87	950.85	0	0	0
-15	42.9	142.9	.	657.7	.	1.75	.	.	.	.
02JUN -0	15.9	165.9	1.838	434.2	2.042	1.16	.	.	.	0
-1	17.7	177.7	.	230.7	.	.	.	.	.	0
-3	20.4	140.4	1.780	296	2.085	1.22	554.78	0	0	0
-5	22.8	212.8	1.907	380.2	0.992	1.28	2511.11	0	0	0
-10	14.8	164.8	.	181.4	0.036	0.52	138.13	35.23	19.23	0
-15	28.5	208.5	.	414.9	0.006	.	.	.	.	0
16JUN -0	33.6	163.6	1.858	506.5	1.555	0.26	1855.62	388.04	117.74	0
-1	33.5	163.5	.	479.3	2.48	1.63	.	.	.	0
-3	35.4	165.4	1.934	411.5	3.268	1.39	1790.87	578.15	0	0
-5	44.7	.	1.943	450.5	3.435	3.13	1923.59	0	0	0
-10	36	.	.	373.5	0.457	2.06	2319.62	0	0	0
-15	11.8	141.8	.	521.3	0.04	0.78	.	.	.	0
-20	18.3	198.3	.	432.5	.	0.07	.	.	.	.
30JUN -0	66	166	1.877	529.7	3.653	2.86	1874.49	1204.48	0	42.089
-1	79.9	199.9	.	575.7	10.988	2.95	.	.	.	0
-3	96	206	1.876	792.9	12.75	2.55	798.74	0	0	90.53
-5	83.2	223.2	1.828	723.7	8.633	2.98	524.35	230.16	69.56	24.657
-10	65	165	1.984	511.9	0.741	2.55	763.35	12.79	12.79	0
-15	38.5	.	.	390.6	.	1.16	.	.	.	0
-20	111	341	.	905.9	.	1.96	.	.	.	.
14JUL -0	160.5	330.5	2.211	1436.1	3.474	1.39	927.94	724.22	81.83	5.685
-1	155.8	325.8	.	1409.7	10.757	11.05	.	.	.	23.431
-3	170.5	420.5	2.529	1505.7	14.248	7.65	1398.28	802.99	0	66.416
-5	171.1	341.1	2.211	1354.9	9.124	10.31	2228.93	1284.78	0	30.63
-10	70.7	250.7	.	733.4	0.364	12.77	1876.33	1846.87	0	0
-15	21.3	261.3	.	475.8	0.495	5.06	.	.	.	0
28JUL -0	106.9	246.9	2.289	929	9.226	9.78	2703.82	2584.91	1534	180.305
-1	85.4	285.4	.	854.8	13.372	5.72	.	.	.	594.992
-3	129	329	2.098	1255.3	8.012	8.22	3081.33	2625.83	0	120.406
-5	161.8	311.8	2.254	1461.8	4.619	14.37	2969.58	1930.75	0	120.563
-10	29.1	119.1	2.188	536.9	0.1	2.11	63.88	54.06	0	875.37
-15	23	113	.	461	0.16	0.99	.	.	.	.
17AUG -0	155.3	285.3	4.632	1366.1	16.169	25.95	6249.55	5861.82	0	31.086
-1	96.2	236.2	.	964.9	13.147	6.96	.	.	.	17.234
-3	117.1	.	5.772	1138	12.332	4.95	889.17	80.3	0	20.38
-5	96.4	246.4	5.994	971.3	8.149	7.26	1521.28	563.78	563.78	10.436
-10	89.9	259.9	.	947.7	0.535	4.95	1463.95	883.29	0	0
-15	31.7	241.7	.	432.1	0.031	3.09	.	.	.	0
-18	27.1	787.1	.	525.7	.	0.62	.	.	.	.

MID LAKE 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
09SEP -0	74.3	304.3	5.697	838.4	9.808	6.33	1857.87	775.42	216.24	3.827
-1	123.7	.	.	951.5	11.771	4.86	.	.	.	3.811
-3	69.6	219.6	5.145	790.6	12.306	6.21	1244.63	508.39	508.39	6.225
-5	59.1	299.1	3.808	721.7	5.747	4.94	4885.56	1885.34	287.39	5.509
-10	55.7	335.7	.	767.8	0.507	3.42	997.2	420.56	211.33	0
-15	13.2	233.2	.	551.8	0.148	1.74	.	.	.	0
-18	622.6	942.6	.	5018.4	.	.	.	.	.	.
01OCT -0	133.5	323.5	3.049	1081.8	4.862	7.19	3553.74	2983.01	2983.01	13.634
-1	96.6	286.6	.	785.8	7.715	6.13	.	.	.	10.497
-3	117.1	327.1	2.121	943	10.952	7.89	2687.81	1989.37	1989.37	0
-5	76.4	286.4	4.052	818.3	2.688	6.29	3202.49	1601.07	1601.07	16.907
-10	53.5	273.5	.	711.5	0.342	2.85	364.52	169.91	169.91	0
-15	45.8	255.8	.	590.2	0.195	2.03	.	.	.	0
22OCT -0	49	209	3.950	856	9.828	8.1	13184.87	0	0	27.277
-1	45	265	.	759.2	10.345	7.32	.	.	.	13.55
-3	56.2	256.2	3.328	761.9	6.83	8.79	14317.29	1119.84	1119.84	7.995
-5	50.9	250.9	4.125	1109.1	3.109	5.47	2113.45	316.64	316.64	6.87
-10	18.5	228.5	.	549.7	0.316	4.73	4961.35	1868.97	1868.97	4.659
-15	28.5	218.5	.	654.3	0.036	5.55	.	.	.	3.916
04NOV -0	60.6	270.6	2.562	756.9	.	13.29	2300.59	216.24	216.24	.
-1	66.6	256.6	.	1023.9	.	6.66	.	.	.	.
-3	77.9	277.9	3.800	858.9	.	6.33	6972.3	795.47	795.47	.
-5	75.4	275.4	2.244	704	.	8.01	4182.81	641.01	641.01	.
-10	67.3	267.3	.	807.5	.	10.64	6999.1	1729.96	1729.96	.
-15	52.8	252.8	.	721.8	.	5.31	.	.	.	.

MID LAKE 1988

INTEGRATED 0-10 METERS

DATE	TEMP AVG	SRP mg/m^2	TDP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
20MAY88	8.21	62.92	134.84	65.75	71.27	211.02	98.87	66.31	285.17	4.86	4574.83
02JUN88	11.95	39.96	98.46	58.50	39.95	138.42	126.23	22.05	148.27	1.60	1446.73
16JUN88	15.05	24.63	96.74	72.12	50.32	147.07	154.40	18.43	172.82	1.30	938.34
30JUN88	18.43	26.02	365.60	339.58	69.22	434.82	81.26	19.47	100.73	1.19	1089.27
14JUL88	18.67	27.39	159.09	131.71	104.64	263.73	26.54	24.52	51.06	1.89	1833.94
28JUL88	19.48	16.00	147.00	131.00	107.40	254.39	172.18	24.26	196.44	1.52	1323.56
17AUG88	18.61	113.71	302.60	188.89	115.55	418.15	528.21	42.55	570.75	1.52	886.10
09SEP88	16.50	42.05	155.50	113.46	104.07	259.57	84.31	26.22	110.53	2.26	2147.79
010CT88	12.79	80.57	197.82	117.25	109.22	307.04	304.26	62.36	366.74	2.09	1698.26
220CT88	10.80	87.62	152.89	65.26	102.22	255.11	208.28	53.31	261.59	2.04	1773.41
04NOV88	8.20	50.74	185.48	134.74	100.52	286.00	156.22	43.35	199.57	1.99	1790.44

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C g/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	FIXIN % B.
20MAY88	487.25	5360.60	.	6.93	.	19.81	12.71	0.00	0.00	0.00	0.00	0.0
02JUN88	192.10	1787.10	18.65	2.94	11.84	10.57	11.35	0.09	0.05	0.00	0.78	0.4
16JUN88	384.30	1650.25	19.28	4.31	24.20	21.46	19.79	2.03	0.18	0.00	10.24	0.8
30JUN88	798.55	1988.55	18.47	6.53	75.88	27.76	8.55	2.64	0.28	288.40	30.92	3.2
14JUL88	1430.55	3315.55	20.54	12.42	79.21	100.58	16.22	11.44	0.08	278.03	70.57	0.5
28JUL88	1078.60	2598.60	22.04	10.72	57.11	85.48	22.31	17.33	2.30	3833.85	77.69	10.3
17AUG88	1018.30	2491.70	57.34	10.18	82.33	71.10	20.58	13.17	1.97	118.68	64.01	9.5
09SEP88	708.00	2891.55	44.26	7.87	68.55	48.72	25.49	10.08	3.13	39.36	39.56	12.2
010CT88	847.00	2932.00	34.19	8.25	46.17	57.71	24.17	15.48	15.48	81.74	64.03	64.0
220CT88	428.80	2463.80	39.00	8.35	45.76	63.58	75.37	8.58	8.58	85.65	11.38	11.3
04NOV88	718.15	2708.15	26.81	8.11	.	83.93	53.02	8.88	8.88	.	16.75	16.7

DATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
20MAY88	4.53	25.40	6.84	1.05
02JUN88	3.71	12.91	4.81	0.55
16JUN88	7.02	11.22	7.64	0.75
30JUN88	3.87	4.57	11.54	0.75
14JUL88	1.86	12.57	13.67	0.90
28JUL88	12.28	10.22	10.04	1.52
17AUG88	5.02	5.96	8.81	0.37
09SEP88	2.63	11.14	6.80	0.62
010CT88	4.55	9.55	7.76	0.77
220CT88	2.99	9.66	4.19	0.61
04NOV88	3.93	9.47	7.14	0.85

DAM STATION 1988

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
20MAY -0	8.3	4.35	10.38	6.03	6.33	16.71	0.35	2.16	2.51	0.13	127.49
-1	8.1	6.11	15.55	9.44	7.02	22.57	1.13	2.18	3.31	0.13	126.69
-3	7.8	4.35	12.11	7.76	8.06	20.17	2.69	2.52	5.21	0.22	214.79
-5	7.2	6.11	12.11	6	8.2	20.31	2.69	.	.	.	.
-10	6.9	4.35	12.11	7.76	8.75	20.86	19.11	2.52	21.63	0.18	158.37
-15	6	7.88	13.83	5.95	8.34	22.17	30.06	6.90	36.96	0.15	113.04
02JUN -0	12.8	2.58	8.29	5.71	4.43	12.72	7.38	1.80	9.18	.	.
-1	12.8	2.58	6.55	3.97	3.98	10.53	5.82	1.82	7.64	0.19	182.37
-3	12.8	4.35	6.55	2.2	5.2	11.75	7.38	1.80	9.18	0.14	130.82
-5	12.8	4.35	8.29	3.94	4.18	12.47	6.6	1.80	8.40	0.19	181.60
-10	12.2	2.58	16.95	14.37	2.84	19.79	12.08	2.18	14.26	0.21	195.74
-15	6.8	9.65	16.95	7.3	5.39	22.34	24.58	7.21	31.79	0.19	158.21
-20	6	13.18	13.49	0.31	5.77	19.26	35.53	10.09	45.62	0.17	124.38
16JUN -0	17	1.4	11.06	9.66	4.46	15.52	12.18	1.76	13.94	0.11	96.06
-1	16.8	2.32	18.05	15.73	5.91	23.96	.	2.17	.	0.12	.
-3	16	4.18	21.54	17.36	5.32	26.86	.	2.11	.	0.14	.
-5	15.9	4.18	32.02	27.84	10.46	42.48	4.74	3.52	8.26	0.16	151.74
-10	13.2	2.32	32.02	29.7	4.92	36.94	5.56	2.17	7.73	0.12	112.27
-15	9.6	13.45	32.02	18.57	4	36.02	20.44	8.09	28.53	0.13	101.47
-20	7.9	31.08	26.78	-4.3	4.79	31.57	74.16	12.33	86.49	0.2	113.51
30JUN -0	19.9	9.74	26.78	17.04	7.14	33.92	6.39	2.11	8.50	0.1	91.50
-1	19.8	4.18	14.56	10.38	7.14	21.7	71.68	2.17	73.85	0.17	96.15
-3	19.2	1.4	21.54	20.14	7.06	28.6	3.91	2.46	6.37	0.11	103.63
-5	19.1	3.25	32.02	28.77	5.99	38.01	36.14	2.11	38.25	0.12	81.75
-10	17	9.74	39	29.26	5.74	44.74	27.05	2.17	29.22	0.11	80.78
-15	10.8	10.67	39	28.33	6.48	45.48	35.32	8.80	44.12	0.13	85.88
-20	9	17.17	45.99	28.82	8.29	54.28	54.32	13.39	67.71	0.17	102.29
14JUL -0	.	.	.	.	.	.	.	.	.	.	.
-1	.	.	.	.	.	.	.	.	.	.	.
-3	.	.	.	.	.	.	.	.	.	.	.
-5	.	.	.	.	.	.	.	.	.	.	.
-10	.	.	.	.	.	.	.	.	.	.	.
-15	.	.	.	.	.	.	.	.	.	.	.
28JUL -0	19.2	1.83	15.22	13.39	11.53	26.75	2.44	2.11	4.55	0.13	125.45
-1	19.2	2.74	15.22	12.48	9.98	25.2	2.44	1.79	4.23	0.12	115.77
-3	19.1	3.65	10.04	6.39	12.73	22.77	6.68	1.83	8.51	0.18	171.49
-5	19.1	1.83	8.32	6.49	15.47	23.79	9.22	1.79	11.01	0.15	139.00
-10	17.9	1.83	8.32	6.49	6.73	15.05	6.68	2.55	9.23	0.15	140.77
-15	13.9	4.57	10.04	5.47	6.9	16.94	18.53	13.02	31.55	0.15	118.45
-20	10.2	9.13	16.94	7.81	15.13	32.07	53.25	24.63	77.88	0.19	112.12
17AUG -0	19	1.95	8.3	6.35	12.26	20.56	2.48	2.61	5.09	0.2	194.91
-1	19	1.95	10.04	8.09	10.37	20.41	2.48	3.05	5.53	0.27	264.47
-3	19	1.95	10.04	8.09	13.8	23.84	3.33	2.62	5.95	0.18	174.05
-5	19	1.95	11.78	9.83	10.19	21.97	4.17	2.63	6.80	0.19	183.20
-10	18.9	1.95	11.78	9.83	8.82	20.6	3.33	2.62	5.95	0.22	214.05
-15	15.8	16.99	23.92	6.93	6.59	30.51	95.23	2.59	97.82	0.19	92.18
-19	12.8	42.65	79.46	36.81	12.26	91.72	216.65	15.64	232.29	0.68	447.71

DAM STATION 1988

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
09SEP -0	16.2	7.26	15.55	8.29	11.6	27.15	20.19	3.37	23.56	0.2	176.44
-1	16.2	6.37	17.31	10.94	11.17	28.48	21.88	3.33	25.21	0.19	164.79
-3	16.2	5.49	158.32	152.83	10.91	169.23	18.51	3.35	21.86	0.17	148.14
-5	16.2	5.49	20.83	15.34	9.44	30.27	20.19	3.43	23.62	0.15	126.38
-10	16.2	6.37	24.36	17.99	9.52	33.88	20.19	2.99	23.18	0.17	146.82
-15	16.1	7.26	20.84	13.58	8.74	29.58	22.72	2.63	25.35	0.16	134.65
-18	15.5	81.59	73.72	-7.87	14.29	88.01	409.74	2.62	412.36	0.27	-142.36
01OCT -0	13.2	15.79	33.08	17.29	7.62	40.7	93.65	8.19	101.84	0.28	178.16
-1	13.1	15.79	27.8	12.01	7.7	35.5	96.17	7.79	103.96	0.28	176.04
-3	12.8	15.79	.	.	7.27	.	91.13	8.28	99.41	.	.
-5	12.6	15.79	27.8	12.01	8.05	35.85	90.29	8.11	98.40	0.36	261.60
-10	12.5	15.79	27.8	12.01	7.96	35.76	91.97	8.10	100.07	0.26	159.93
-15	12.5	15.79	24.27	8.48	7.7	31.97	96.17	8.19	104.36	0.31	205.64
22OCT -0	10.6	7.92	17.12	9.2	9.85	26.97	22.69	6.57	29.26	0.23	200.74
-1	10.6	7.92	20.51	12.59	9.86	30.37	22.69	4.87	27.56	0.22	192.44
-3	10.6	7.01	15.43	8.42	9.16	24.59	22.69	4.58	27.27	0.2	172.73
-5	10.6	7.92	18.82	10.9	8.99	27.81	21.83	5.97	27.80	0.21	182.20
-10	10.6	7.92	20.51	12.59	8.48	28.99	19.27	4.59	23.86	0.36	336.14
-15	10.6	7.92	25.59	17.67	9.85	35.44	22.69	5.75	28.44	0.21	181.56
04NOV -0	8.5	6.85	20.84	13.99	9.26	30.1	30.19	6.55	36.74	0.28	243.27
-1	8.5	5.91	22.56	16.65	9.61	32.17	28.32	5.48	33.80	0.28	246.20
-3	8.5	5.91	22.56	16.65	9.26	31.82	30.19	5.27	35.46	0.27	234.54
-5	8.5	6.85	22.56	15.71	9.09	31.65	32.06	4.66	36.72	0.22	183.28
-10	8.5	6.85	19.07	12.22	9.44	28.51	28.32	5.12	33.44	0.23	196.56
-15	8.5	5.91	17.31	11.4	8.83	26.14	28.32	4.22	32.54	0.21	177.46

DAM STATION 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
20MAY -0	42.1	172.1	.	469.3	.	1.81	.	.	.	.
-1	46.6	176.6	.	488.4	.	3.04	.	.	.	.
-3	63.1	283.1	.	677.6	.	2.28	.	.	.	.
-5	50	.	.	646.5	.	2.57	.	.	.	.
-10	65.5	245.5	.	499.9	.	1.22	.	.	.	.
-15	51.3	201.3	.	763.2	.	.	.	.	.	.
02JUN -0	28.8	.	.	563.1	3.431	2.57	149.02	42.56	0	0
-1	8.7	198.7	.	411.8	3.766	2.51	.	.	.	0
-3	51.7	191.7	.	579.2	1.681	2.51	144.415	0	0	0
-5	99.4	289.4	.	654.1	0.657	1.93	.	.	.	0
-10	32.8	242.8	.	614.4	0.058	0.81	119.88	0	0	0
-15	36.2	226.2	.	481.8	.	1.1	.	.	.	0
-20	44.2	214.2	.	535.4	.	1.04	.	.	.	.
16JUN -0	45.5	155.5	.	415.1	.	2.16	355.21	43.47	43.47	.
-1	39.9	159.9	.	630.5	.	2.04	.	.	.	.
-3	41.4	181.4	.	566.3	.	1.57	1168.73	449.83	56.37	.
-5	42	202	.	454.4	.	1.4	973.82	344.93	344.93	.
-10	22	142	.	383.5	.	0.75	2391.22	0	0	.
-15	16.4	146.4	.	266	.	0.39	.	.	.	.
-20	16.9	216.9	.	266.6	.	0.63	.	.	.	.
30JUN -0	79	179	.	677.4	7.367	0.87	1747.09	8.69	8.69	0
-1	89.4	259.4	.	662.6	11.214	2.36	.	.	.	3.081
-3	87.8	197.8	.	766.9	11.189	3.22	1219.37	790.2	388.71	14.684
-5	78.9	198.9	.	708.3	7.851	3.59	381.19	30.69	30.69	0
-10	69.3	179.3	.	525.4	0.904	1.51	1333.47	5.11	5.11	0
-15	53.1	183.1	.	441	.	1.34	.	.	.	0
-20	66.8	236.8	.	585.6	.	2.22	.	.	.	.
14JUL -0	.	.	.	.	.	.	.	.	.	.
-1	.	.	.	.	.	.	.	.	.	.
-3	.	.	.	.	.	.	.	.	.	.
-5	.	.	.	.	.	.	.	.	.	.
-10	.	.	.	.	.	.	.	.	.	.
-15	.	.	.	.	.	.	.	.	.	.
28JUL -0	201.6	331.6	.	1208.2	.	7.97	2907.23	2678.76	69.05	.
-1	169.4	289.4	.	1323.9	.	12.81	.	.	.	.
-3	185.5	365.5	.	1453.9	.	9.28	2991.82	2971.06	0	.
-5	181.8	331.8	.	1476.5	.	12.89	3008.55	2955	0	.
-10	81.3	231.3	.	735.2	.	3.5	205.25	0	0	.
-15	45.6	195.6	.	417	.	2.48	.	.	.	.
-20	108.1	298.1	.	1084.3	.	3.18	.	.	.	.
17AUG -0	83.7	283.7	.	939.4	8.431	2.48	2051.36	1124.7	447.93	4.351
-1	98.7	368.7	.	1023.5	13.472	3.71	.	.	.	20.165
-3	125.7	305.7	.	1131.6	12.925	4.64	815.93	200.75	0	12.978
-5	68.3	258.3	.	872.7	6.748	1.86	570.22	54.06	54.06	0
-10	79.5	299.5	.	1386.6	0.869	4.48	1023.53	802.99	0	0
-15	.	.	.	609.8	.	0.78	.	.	.	0
-19	51	731	.	729.4	.	1.7	.	.	.	.

DAM STATION 1988

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	B.G.BIO (µg/l)	C2H4/ l*h
09SEP -0	21.4	221.4	.	672.4	.	4.65	2731.95	123.57	123.57	.
-1	33.6	223.6	.	828	.	4.16	.	.	.	.
-3	44.1	214.1	.	868.2	.	4	2550.9	1204.48	0	.
-5	55.2	205.2	.	813.5	.	4.32	1296.7	849.53	0	.
-10	38.5	208.5	.	630.7	.	4.53	1650.94	216.24	216.24	.
-15	55.4	215.4	.	677.9	.	4.49	.	.	.	.
-18	41.4	311.4	.	602.4	.	1.05	.	.	.	.
01OCT -0	45.6	325.6	.	734.1	.	3.09	818.28	0	0	.
-1	46.2	326.2	.	492.2	.	3.04	.	.	.	.
-3	41.2	.	.	656.5	.	3.12	1479.19	293.47	293.47	.
-5	24.2	384.2	.	569.4	.	2.43	542.66	502	502	.
-10	36.6	296.6	.	598.6	.	2.11	1112.93	1081.02	278	.
-15	16.7	326.7	.	599.5	.	2.64	.	.	.	.
22OCT -0	61.7	291.7	.	755	15.5	6.99	3178.05	0	0	12.706
-1	44.4	264.4	.	715.3	6.934	5.39	.	.	.	7.857
-3	22.8	222.8	.	520.2	5.334	5.23	1658.35	285.75	285.75	10.988
-5	17.5	227.5	.	520.3	1.293	6.54	1936.89	278.03	278.03	29.866
-10	21.1	381.1	.	660.9	0.087	6.09	2793.38	0	0	19.771
-15	13.2	223.2	.	572.5	0.233	6.38	.	.	.	12.902
04NOV -0	56.3	336.3	.	646.6	.	5.55	6555.766	656.46	0	.
-1	63.2	343.2	.	762.7	.	5.88	.	.	.	.
-3	52.4	322.4	.	709	.	6.78	4283.77	139.01	139.01	.
-5	49.9	269.9	.	620.9	.	5.8	11757.49	38.62	38.62	.
-10	64.3	294.3	.	771.2	.	5.64	2368.77	0	0	.
-15	55.3	265.3	.	861.6	.	6.39	.	.	.	.

DAM STATION 1988

INTEGRATED 0-10 METERS

DATE	TEMP AVG	SRP mg/m^2	TDP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
20MAY88	7.45	52.30	125.40	73.10	80.39	205.79	64.44	24.53	105.39	1.88	1774.61
02JUN88	12.64	35.54	98.46	62.93	40.32	138.78	80.48	18.98	99.46	1.85	1751.31
16JUN88	15.40	32.97	267.81	234.84	70.65	338.45	.	26.12	.	1.38	.
30JUN88	18.71	49.67	287.88	238.22	63.72	351.60	312.65	22.06	334.71	1.22	885.29
14JUL88	.	.	.	.	.	.	.	.	.	.	.
28JUL88	18.80	23.31	100.44	77.14	117.17	217.61	67.21	20.01	87.22	1.51	1417.78
17AUG88	18.97	19.50	109.97	90.47	107.00	216.97	34.54	26.85	61.39	2.08	2018.61
09SEP88	16.20	59.31	484.19	424.88	101.22	585.40	201.08	32.87	233.94	1.68	1441.06
010CT88	12.73	157.90	280.64	122.74	77.98	359.83	919.28	80.97	1000.25	3.11	2106.21
220CT88	10.60	77.38	187.33	109.95	90.70	278.03	215.34	52.12	267.46	2.48	2212.54
04NOV88	8.50	65.21	216.02	150.81	92.98	309.00	300.97	51.15	352.11	2.45	2092.89

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C mg/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	FIXING % B.G
20MAY88	555.90	2484.15	.	5834.95	.	22.07	.	.	.	.	.	
02JUN88	560.75	2400.70	.	5883.00	13.17	18.85	1.37	0.06	0.00	0.00	4.68	0.0
16JUN88	367.40	1742.40	.	4835.05	.	14.06	12.84	2.40	1.41	.	18.67	11.0
30JUN88	798.60	2018.60	.	6658.95	72.62	26.76	10.34	2.11	1.11	33.99	20.40	10.6
14JUL88	.	.	.	.	.	.	.	.	.	.	.	
28JUL88	1565.45	3070.45	.	12503.50	.	95.63	22.88	21.79	0.10	.	95.21	0.4
17AUG88	879.10	2959.10	.	10789.10	76.06	33.80	9.67	4.39	0.86	58.38	45.35	8.9
09SEP88	438.75	2113.75	.	7738.60	.	43.01	19.14	6.71	0.73	.	35.06	3.7
010CT88	350.70	3448.70	.	5907.75	.	26.13	9.61	5.19	3.19	.	54.06	33.1
220CT88	257.05	2737.05	.	5964.15	33.56	60.16	22.68	1.69	1.69	194.07	7.44	7.4
04NOV88	563.15	3008.15	.	6986.50	.	59.56	67.62	1.47	0.48	.	2.17	0.7

DATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
20MAY88	2.02	12.07	6.92	0.47
02JUN88	2.80	17.30	13.91	0.53
16JUN88	.	5.15	5.20	0.79
30JUN88	6.74	5.74	12.53	0.44
14JUL88	.	.	.	.
28JUL88	3.74	14.11	13.36	0.86
17AUG88	3.15	13.64	8.22	1.38
09SEP88	3.94	3.61	4.33	0.55
010CT88	6.33	9.58	4.50	0.51
220CT88	3.46	9.84	2.83	0.67
04NOV88	5.40	9.74	6.06	0.78

1989 DATA

GRAYLING ARM 1989

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (µg/l)	DON (g/l)
21MAY -0	9.2	11.18	26.45	15.27	22.16	48.61	4.97	6.68	11.65	229.8	218.15
-1	9.1	9.45	28.37	18.92	22.48	50.85	4.97	6.72	11.69	166.8	155.11
-3	8.9	8.59	28.37	19.78	23.19	51.56	3.34	6.68	10.02	219.3	209.28
-5	7.7	12.91	28.37	15.46	30.60	58.97	9.86	12.33	22.19	178.0	155.81
04JUN -0	11.6	9.45	24.53	15.07	17.54	42.07	4.16	6.68	10.84	235.0	224.16
-1	10.8	7.72	24.53	16.80	17.06	41.59	1.71	21.29	23	176.1	153.1
-3	10.0	8.59	24.53	15.94	15.71	40.24	1.71	6.68	8.39	149.7	141.31
-5	9.4	12.04	28.37	16.33	11.88	40.25	1.71	25.77	27.48	98.0	70.52
13JUN -0	15.2	7.72	26.45	18.72	7.66	34.11	1.71	5.56	7.27	245.4	238.13
-1	14.6	7.72	22.60	14.88	7.19	29.79	3.34	48.18	51.52	135.2	83.68
-3	14.0	8.59	22.60	14.02	7.66	30.26	4.16	6.68	10.84	273.3	262.46
-5	10.1	11.18	26.45	15.27	12.68	39.13	3.34	12.33	15.67	155.7	140.03
29JUN -0	15.4	5.13	24.53	19.39	61.51	86.04	3.34	6.68	10.02	189.7	179.68
-1	15.2	5.13	24.53	19.39	14.05	38.58	2.53	6.72	9.25	200.3	191.05
-3	15.2	5.13	24.53	19.39	13.74	38.27	4.16	.	.	125.9	.
-5	15.2	6.86	22.60	15.74	11.19	33.79	4.16	35.61	39.77	120.1	80.33
19JUL -0	20.6	3.40	30.29	26.89	14.03	44.32	2.53	30.25	32.78	155.7	122.92
-1	19.2	3.40	30.29	26.89	8.46	38.75	2.53	6.68	9.21	144.5	135.29
-3	18.6	4.27	26.45	22.18	7.03	33.48	4.97	6.72	11.69	172.4	160.71
-5	18.1	6.86	24.53	17.67	6.31	30.84	6.60	22.62	29.22	245.4	216.18
03AUG -0	19.2	8.59	30.29	21.70	21.04	51.33	4.97	.	.	163.1	.
-1	19.2	7.72	30.29	22.57	20.72	51.01	4.16	73.45	77.61	146.2	68.59
-3	19.2	7.72	30.29	22.57	19.29	49.58	4.97	21.29	26.26	213.3	187.04
-5	19.1	7.72	30.29	22.57	21.20	51.49	4.97	17.81	22.78	137.5	114.72
23AUG -0	16.8	10.32	37.98	27.66	28.55	66.53	8.23	35.86	44.09	179.8	135.71
-1	16.8	9.45	37.98	28.53	27.43	65.41	8.23	33.39	41.62	330.7	289.08
-3	16.7	9.45	37.98	28.53	23.61	61.59	11.49	29.13	40.62	337.8	297.18
-5	16.6	11.18	39.90	28.72	18.67	58.57	15.57	36.72	52.29	318.5	266.21
07SEP -0	15.0	7.72	30.29	22.57	40.33	70.62	5.79	35.86	41.65	315.5	273.85
-1	14.8	6.86	28.37	21.51	39.54	67.91	5.79	64.54	70.33	330.7	260.37
-3	13.8	6.86	28.37	21.52	31.41	59.78	5.79	30.25	36.04	257.9	221.86
-5	13.3	6.86	32.21	25.35	29.50	61.71	8.23	28.93	37.16	236.7	199.54
20SEP -0	.	13.77	41.82	28.05	22.97	64.79	22.90	48.18	71.08	349.2	278.12
-1	.	14.64	43.74	29.11	19.63	63.37	19.64	51.19	70.83	302.9	232.07
-3	.	13.77	28.05	41.82	18.35	46.4	19.64	50.42	70.06	265.3	195.24
-5	.	16.36	41.82	25.46	20.42	62.24	23.72	53.42	77.14	367.3	290.16
08OCT -0	10.1	21.55	51.43	29.89	131.11	182.54	24.53	80.68	105.21	441.9	336.69
-1	9.0	24.14	57.20	33.06	21.38	78.58	32.68	91.25	123.93	335.9	211.97
-3	8.8	25.87	57.20	31.33	11.19	68.39	35.94	93.00	128.94	308.1	179.16
-5	8.6	27.59	61.04	33.45	14.85	75.89	32.68	93.48	126.16	278.1	151.94
10NOV -0	1.0	12.04	36.06	24.01	23.13	59.19	3.34	15.69	19.03	190.9	171.87
-1	0.9	12.91	39.90	26.99	24.57	64.47	5.79	10.02	15.81	106.5	90.69
-3	0.9	12.91	39.90	26.99	22.97	62.87	6.60	10.08	16.68	196.6	179.92
-5	0.9	12.04	39.90	27.86	22.34	62.24	4.97	10.02	14.99	212.6	197.61

GRAYLING ARM 1989

DATE DEPTH	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR (µgC/l*h)	CHL a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
21MAY -0	164.3	394.1	3.99	1167	.	5.37	3240.3	0	0	0
-1	173.2	340	3.44	1072	.	4.98	3086.5	0	0	0
-3	150.6	369.9	2.78	927	.	4.41	920.2	0	0	0
-5	156.1	334.1	3.91	1014	.	5.24	903.7	0	0	0
04JUN -0	153.5	388.5	3.33	698	6.00	2.83	3452.8	0	0	1.16
-1	127.4	303.5	4.16	764	10.08	3.18	2626.8	0	0	3.33
-3	131.5	281.2	3.86	816	5.23	3.27	1045.0	0	0	4.1
-5	120.2	218.2	2.78	522	0.59	2.33	627.4	0	0	1.77
13JUN -0	54.0	299.4	3.97	600	4.71	2.75	2632.4	129.3	129.3	9.68
-1	68.7	203.9	4.92	620	7.33	2.90	338.4	22.3	22.3	16.46
-3	75.7	349	3.98	595	5.68	2.45	391.1	44.7	44.7	17.78
-5	71.3	227	3.80	625	2.19	2.94	431.7	0	0	0.76
29JUN -0	992.6	1182.3	3.25	5203	134.75	94.52	46127.8	45560.2	45560.2	2582.8
-1	268.3	468.6	2.95	1366	53.59	15.45	4050.1	2444.8	2444.8	510.26
-3	167.2	293.1	3.22	1137	16.94	8.43	8138.0	5278	5278	256.41
-5	117.3	237.4	3.90	649	1.61	4.54	2789.3	860.2	860.2	45.55
19JUL -0	195.6	351.3	3.61	1098	20.48	8.70	12060.4	11852.9	3997.7	60.24
-1	120.0	264.5	2.68	699	16.98	3.54	3596.6	2956.9	2171.4	69.48
-3	67.3	239.7	3.49	451	14.01	1.95	2763.8	1671.7	1671.7	19.3
-5	35.3	280.7	3.07	496	3.89	2.07	4615.3	4528.8	77.5	3.39
03AUG -0	181.6	344.7	6.37	1330	23.85	11.89	13561.5	12999.2	10119	209.74
-1	161.9	308.1	6.23	1125	35.27	13.86	11441.6	5430.7	5430.7	207.94
-3	218.0	431.3	6.38	1257	12.01	11.80	8125.4	7956	3452.4	144.52
-5	162.2	299.7	10.37	1219	1.27	15.13	6460.6	5490.1	5490.1	85.47
23AUG -0	354.1	533.9	9.15	1941	21.77	26.22	14182.4	13964.7	13964.7	.
-1	275.0	605.7	8.29	1617	67.07	19.25	12562.1	11389	11389	.
-3	265.8	603.6	7.40	1440	7.02	19.25	14865.2	13611.7	12040.7	.
-5	224.8	543.3	8.26	1112	0.24	12.75	8101.9	7215.1	7215.1	.
07SEP -0	513.4	828.9	6.89	2612	14.61	33.35	12900.8	12273.4	12273.4	200.9
-1	404.3	735	9.49	2143	2.56	36.20	16984.2	16473.2	16473.2	130.8
-3	406.7	664.6	10.52	2147	0.73	27.64	11532.7	11171.8	11171.8	45.61
-5	305.2	541.9	8.94	1672	0.13	22.57	16707.3	16468	16468	25.38
20SEP -0	171.7	520.9	11.37	1099	3.98	5.68	3968.5	3633.4	3633.4	36.79
-1	181.8	484.7	9.67	1049	17.24	8.04	2841.8	2505.9	2505.9	29.15
-3	170.9	436.2	10.44	881	1.75	4.59	6884.4	2958.5	2958.5	24.19
-5	108.3	475.6	7.30	798	0.05	2.75	1140.5	850.8	850.8	0
08OCT -0	284.0	725.9	58.76	15285	129.88	275.20	98800.9	98373.7	98373.7	1144.65
-1	93.0	428.9	73.40	826	12.85	5.81	6931.0	1365.4	1365.4	27.31
-3	72.0	380.1	27.78	550	1.17	2.33	226.0	0	0	14.37
-5	82.3	360.4	9.27	671	0.18	1.84	190.7	0	0	0
10NOV -0	162.4	353.3	10.41	1087	4.57	13.39	1818.6	0	0	0
-1	173.5	280	24.49	1217	17.44	12.28	2319.1	0	0	0
-3	165.3	361.9	9.62	1182	2.50	11.33	2187.5	0	0	0
-5	169.0	381.6	13.14	1224	0.30	10.69	1777.8	0	0	0

GRAYLING ARM 1989 INTEGRATED 0-5 METERS

DATE	TEMP AVG	SRP mg/m^2	TOP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
21MAY89	8.7	49.86	140.89	91.04	121.78	262.67	26.48	39.11	65.59	0.98	916.11
04JUN89	10.3	45.53	126.49	80.95	77.66	204.15	9.78	74.41	84.18	0.78	694.87
13JUN89	13.4	43.80	118.78	74.99	42.62	161.39	17.53	100.74	118.27	1.03	909.54
29JUN89	15.2	27.38	120.72	93.30	90.50	211.22	17.95	91.36	107.68	0.77	728.13
19JUL89	19.0	22.20	138.01	115.81	40.08	178.09	21.60	61.21	82.81	0.88	802.00
03AUG89	19.2	39.04	151.45	112.42	101.38	252.83	23.63	207.29	230.52	0.86	625.98
23AUG89	16.7	49.42	191.82	142.41	121.31	313.13	55.01	163.00	218.01	1.58	1362.05
07SEP89	14.1	34.73	146.65	111.94	171.80	318.45	31.39	204.17	235.56	1.41	1170.74
20SEP89	.	72.75	184.44	166.79	98.05	282.49	103.91	255.14	359.05	1.53	1167.81
08OCT89	9.0	126.32	286.96	160.65	134.86	421.81	165.85	456.70	622.54	1.62	996.56
10NOV89	0.9	63.25	197.58	134.33	116.70	314.28	28.53	53.06	81.58	0.86	779.42

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C g/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	% FIX B.G
21MAY89	799.25	1780.95	16.63	5.06	.	24.22	8.99	0.00	0.00	0.00	0.00	0.0
04JUN89	651.05	1430.10	18.41	3.65	29.17	15.06	8.38	0.00	0.00	15.55	0.00	0.0
13JUN89	352.75	1380.55	21.13	3.05	26.90	13.57	3.04	0.19	0.19	65.85	6.17	6.1
29JUN89	1350.45	2117.65	16.39	7.57	183.25	91.84	48.20	37.86	37.86	2615.16	78.55	78.5
19JUL89	447.70	1332.50	15.87	3.00	67.62	15.63	21.57	18.23	8.68	176.33	84.54	40.2
03AUG89	931.85	1796.80	35.66	6.09	90.12	65.47	46.65	36.05	25.60	791.29	77.27	54.8
23AUG89	1345.95	2926.00	40.06	7.39	125.77	93.24	63.77	58.50	55.36	.	91.75	86.8
07SEP89	1981.75	3388.05	47.66	10.49	12.74	148.83	71.70	69.66	69.66	413.25	97.15	97.1
20SEP89	808.65	2335.50	48.37	4.68	31.40	26.83	21.16	12.34	12.34	110.50	58.34	58.3
08OCT89	507.80	2126.90	204.31	10.65	86.74	152.82	60.44	51.23	51.23	642.03	84.77	84.7
10NOV89	841.05	1702.05	74.32	5.96	33.75	58.46	10.54	0.00	0.00	0.00	0.00	0.0

DATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
21MAY89	1.32	6.78	6.56	0.78
04JUN89	1.85	7.01	8.38	1.63
13JUN89	2.70	8.55	8.28	2.30
29JUN89	3.93	10.03	14.92	3.34
19JUL89	3.73	7.48	11.17	2.76
03AUG89	5.91	7.11	9.19	5.31
23AUG89	4.41	9.34	11.10	3.30
07SEP89	6.78	10.64	11.54	5.88
20SEP89	4.94	8.27	8.25	3.51
08OCT89	4.93	5.04	3.77	3.62
10NOV89	1.29	5.42	7.21	0.84

MID LAKE 1989

DATE -DEPTH(m)	TEMP (deg C)	SRP (µg/l)	TDP (µg/l)	DOP (µg/l)	PART-P (µg/l)	TOT-P (µg/l)	NH4-N (µg/l)	NO3-N (µg/l)	DIN (µg/l)	TDN (mg/l)	DON (µg/l)
08JUN -0	17	4.27	28.37	24.1	30.3	58.67	2.53	10.08	12.61	0.09	77.39
-1	15	4.27	26.45	22.18	7.2	33.65	4.97	6.68	11.65	0.107	95.35
-3	13.1	10.32	24.53	14.21	9.5	34.03	0.9	6.72	7.62	0.088	80.38
-5	10	4.27	26.45	22.18	11	37.45	0.08	6.68	6.76	0.177	170.24
-10	9	4.27	24.53	20.26	6.9	31.43	4.97	6.72	11.69	0.086	74.31
-15	8	8.59	28.37	19.78	5.8	34.17	40.02	8.9	48.92	0.121	72.08
19JUL -0	22.6	3.4	26.45	23.05	11.5	37.95	0.08	14.57	14.65	0.121	106.35
-1	19.1	3.4	26.45	23.05	10.9	37.35	2.53	6.68	9.21	0.069	59.79
-3	18.4	3.4	24.53	21.13	17.8	42.33	0.08	6.72	6.80	0.073	66.20
-5	18.1	2.54	26.45	23.91	17.1	43.55	0.08	10.02	10.10	0.158	147.90
-10	14.8	6.86	32.21	25.35	22.6	54.81	9.05	10.08	19.13	0.092	72.87
-15	11.6	17.23	41.82	24.59	34.5	76.32	54.69	21.14	75.83	0.153	77.17
08OCT -0	11	6.86	32.21	25.35	50.2	82.41	18.83	8.96	27.79	0.248	220.21
-1	11	7.72	30.29	22.57	15.2	45.49	19.64	10.02	29.66	0.161	131.34
-3	10.9	8.59	32.21	23.62	9.6	41.81	21.27	8.96	30.23	0.115	84.77
-5	10.9	7.72	32.21	24.49	9.8	42.01	22.09	10.02	32.11	0.123	90.89
-10	10.9	6.86	32.21	25.35	13.2	45.41	21.27	8.96	30.23	0.104	73.77
-15	10.9	7.72	34.14	26.42	9	43.14	20.46	1.02	21.48	0.11	88.52

DATE -DEPTH(m)	PART-N (µg/l)	TOT-N (µg/l)	DOC (mg/l)	PART-C (µg/l)	PPR µgC/l*h	Chl a (µg/l)	PHYTBIO (µg/l)	B.G.BIO (µg/l)	FIXING B.G.BIO (µg/l)	nmol C2H4/ l*h
08JUN -0	37.6	127.6	3.348	488.5	4.45	1.3	296.3	0	0	3.08
-1	33.7	140.7	3.095	491.1	3.49	1.08	378.4	170.7	170.7	6.32
-3	58.3	146.3	3.442	487.6	2.94	1.62	412.5	0	0	3.28
-5	65.3	242.3	2.712	536.7	3.45	3.08	308.5	0	0	2.02
-10	52.1	138.1	5.822	499.8	0.3	2.68	777.7	0	0	0
-15	151.1	272.1	2.072	1422.9	0.14	1.76	2684.7	0	0	0
19JUL -0	552	673	3.302	3334.7	44.7	28.12	13423.1	13347.8	5056.2	257.51
-1	86.3	155.3	2.317	1056.6	13.41	4.33	3035.6	2657.2	38.8	21.09
-3	131.9	204.9	2.336	1209.5	20.3	7.78	5252.5	4763	835.4	23.05
-5	114.1	272.1	2.876	1004.3	12.63	9.48	2211.9	1809.1	499.9	10.45
-10	17.5	109.5	3.168	338.4	0.4	1.1	632.1	372.4	372.4	5.54
-15	13.9	166.9	2.138	333.1	0.21	0.27	336.6	186.2	186.2	0
08OCT -0	771.3	1019.3	90.27	3792.3	13.7	75.66	23213.3	22064.3	22064.3	112.47
-1	68.2	229.2	116.1	569.6	7.42	5.37	1864.5	1683.5	1683.5	29.28
-3	64.4	179.4	58.52	470.7	6.83	3.23	709.2	574.1	574.1	4.77
-5	91.1	214.1	9.92	607.9	4.22	8.13	2107.3	1675.8	1675.8	21.75
-10	102.5	206.5	10.82	581	0.46	6.82	7232	6790.3	2024.8	0
-15	85.8	195.8	19.9	601.9	0.21	.	1231.9	1039.6	1039.6	9.71

MID LAKE 1989

INTEGRATED 0-10 METERS

DATE	TEMP AVG	SRP mg/m^2	TDP mg/m^2	DOP mg/m^2	PP mg/m^2	TOT-P mg/m^2	NH4-N mg/m^2	NO3-N mg/m^2	DIN mg/m^2	TDN g/m^2	DON mg/m^2
08JUN89	11.61	54.80	256.82	202.02	100.70	357.52	23.22	68.68	91.91	1.22	1124.09
19JUL89	17.80	39.64	275.06	235.42	174.05	449.11	26.90	91.02	117.92	1.09	975.09
080CT89	10.92	76.36	319.22	242.86	134.40	453.62	211.91	94.90	306.81	1.29	979.20

DATE	PART-N mg/m^2	TOT-N mg/m^2	DOC g/m^2	PART-C g/m^2	PPR mgC/m^2	CHL a mg/m^2	PHYTBIO g/m^2	B.G.BIO g/m^2	FIXING B.G.BIO g/m^2	µmol C2H4/ m^2*h	% B.G.	FIXIN % B.
08JUN89	544.75	1760.75	37.25	5.08	26.17	22.99	4.56	0.26	0.26	24.65	5.61	5.6
19JUL89	1112.35	2205.35	27.78	10.03	128.27	72.05	31.09	27.45	6.94	256.92	88.28	22.3
080CT89	1191.85	2477.85	398.09	7.27	47.56	97.85	41.28	37.55	25.63	185.82	90.96	62.1

OATE	DIN:SRP	TN:TP	PN:PP	NO3:SRP
08JUN89	1.68	4.92	5.41	1.25
19JUL89	2.97	4.91	6.39	2.30

BACTERIAL DATA

GRAYLING ARM 1988

DATE -DEPTH(m)	BPR pmol/ l/h	BAPC pmol/10^6 cell/h	BV x10^6 µm^3/l	BN x10^6 cell/ml
02JUN-0m	,	,	30.90	0.75
-3m	,	,	23.48	0.57
-5m	,	,	22.25	0.54
16JUN-0m	16.30	15.80	44.19	1.03
-3m	15.86	10.90	62.63	1.46
-5m	,	,	39.47	0.92
30JUN-0m	11.40	5.50	90.27	2.08
-3m	11.04	8.10	59.46	1.37
-5m	,	,	58.16	1.34
14JUL-0m	28.40	15.50	78.51	1.83
-3m	16.30	7.80	89.23	2.08
-5m	,	,	67.78	1.58
28JUL-0m	40.20	16.00	103.16	2.51
-3m	36.97	14.10	107.68	2.62
-5m	,	,	95.35	2.32
17AUG-0m	37.70	15.10	101.34	2.49
-3m	33.50	11.80	115.59	2.84
-5m	,	,	100.53	2.47
09SEP-0m	12.20	5.80	85.01	2.12
-3m	11.14	4.90	91.03	2.27
-5m	,	,	86.62	2.16
01OCT-0m	14.14	5.90	95.44	2.38
-3m	12.73	6.30	81.00	2.02
-5m	,	,	85.41	2.13
22OCT-0m	8.30	4.70	71.20	1.78
-3m	10.89	4.80	91.60	2.29
-5m	,	,	62.80	1.57

MADISON ARM 1988

DATE -DEPTH(m)	BPR pmol/ l/h	BAPC pmol/10^6 cell/h	BV x10^6 μm^3/l	BN x10^6 cell/ml
02JUN-0m	,	,	23.90	0.59
-3m	,	,	28.35	0.70
-5m	,	,	25.92	0.64
16JUN-0m	6.92	8.88	32.68	0.78
-3m	9.07	8.10	46.93	1.12
-5m	,	,	45.67	1.09
30JUN-0m	3.10	2.80	45.14	1.12
-3m	2.20	1.40	60.45	1.50
-5m	,	,	38.29	0.95
14JUL-0m	4.30	2.60	70.36	1.64
-3m	6.50	3.20	88.37	2.06
-5m	,	,	42.47	0.99
28JUL-0m	29.26	13.10	92.10	2.23
-3m	28.11	14.40	80.54	1.95
-5m	,	,	81.77	1.98
17AUG-0m	5.37	3.10	71.23	1.75
-3m	9.70	6.00	65.93	1.62
-5m	,	,	68.38	1.68
09SEP-0m	7.20	3.70	78.60	1.96
-3m	8.60	4.50	77.39	1.93
-5m	,	,	80.20	2.00
01OCT-0m	15.80	6.00	104.28	2.62
-3m	12.40	8.10	60.89	1.53
-5m	,	,	79.60	2.00
22OCT-0m	5.60	3.70	59.45	1.49
-3m	4.20	3.30	50.67	1.27
-5m	,	,	52.27	1.31

MIDLAKE 1988

DATE -DEPTH(m)	BPR pmol/ l/h	BAPC pmol/10^6 cell/h	BV x10^6 µm^3/l	BN x10^6 cell/ml
02JUN-0m	,	,	22.00	0.55
-3m	,	,	27.20	0.68
-5m	,	,	23.20	0.58
16JUN-0m	6.91	5.40	53.21	1.27
-3m	6.89	5.70	50.28	1.20
-5m	,	,	46.09	1.10
30JUN-0m	7.35	6.60	48.17	1.11
-3m	6.32	4.60	59.89	1.38
-5m	,	,	46.87	1.08
14JUL-0m	11.37	8.00	60.92	1.42
-3m	1.14	5.10	66.50	1.55
-5m	,	,	54.48	1.27
28JUL-0m	31.60	15.30	85.49	2.07
-3m	30.36	12.50	100.36	2.43
-5m	,	,	92.51	2.24
17AUG-0m	8.02	4.40	73.67	1.81
-3m	6.00	3.60	67.56	1.66
-5m	,	,	65.53	1.61
09SEP-0m	10.25	6.00	68.57	1.71
-3m	8.21	4.60	71.78	1.79
-5m	,	,	69.77	1.74
01OCT-0m	9.31	5.00	74.82	1.88
-3m	9.60	6.20	61.69	1.55
-5m	,	,	71.64	1.80
22OCT-0m	5.28	4.00	52.40	1.31
-3m	4.38	2.90	66.40	1.66
-5m	,	,	46.68	1.17

GRAYLING ARM 1989

DATE -DEPTH(m)	BPR pmol/ l/h	BAPC pmol/10^6 cell/h	BV x10^6 µm^3/l	BN x10^6 cell/ml
21MAY-0m	9.35	25.47	17.07	0.37
-1m	8.74	11.11	36.60	0.79
-3m	10.45	14.24	34.13	0.73
-5m	9.53	8.76	50.59	1.09
04JUN-0m	4.65	12.67	17.21	0.37
-1m	4.41	5.17	40.05	0.85
-3m	4.35	5.93	34.42	0.73
-5m	4.18	3.84	51.03	1.09
13JUN-0m	9.12	5.25	76.69	1.74
-1m	9.51	4.52	93.04	2.11
-3m	6.49	3.62	79.34	1.80
-5m	3.23	2.05	69.88	1.58
29JUN-0m	14.52	10.17	71.31	1.40
-1m	12.80	9.31	69.99	1.38
-3m	9.23	6.29	74.72	1.47
-5m	8.96	6.61	68.97	1.36
19JUL-0m	24.52	5.16	223.30	4.75
-1m	28.87	11.10	122.29	2.60
-3m	28.33	10.23	130.14	2.77
-5m	15.20	5.48	130.47	2.78
03AUG-0m	22.55	11.65	84.37	1.94
-1m	24.57	10.09	106.21	2.44
-3m	24.99	9.27	117.55	2.70
-5m	24.16	9.19	114.62	2.63
23AUG-0m	21.50	8.48	117.67	2.54
-1m	21.61	8.95	112.06	2.42
-3m	21.72	8.41	119.80	2.58
-5m	21.63	9.34	107.42	2.32
07SEP-0m	19.37	8.96	100.32	2.16
-1m	19.01	8.69	101.57	2.19
-3m	19.14	8.69	102.17	2.20
-5m	16.96	7.63	103.10	2.22
20SEP-0m	8.59	5.72	64.44	1.50
-1m	10.75	6.74	68.38	1.59
-3m	10.89	5.37	87.00	2.03
-5m	10.65	5.89	77.56	1.81
08OCT-0m	6.93	3.15	90.21	2.20
-1m	4.78	3.52	55.69	1.36
-3m	7.27	5.06	58.98	1.44
-5m	7.24	4.76	62.51	1.52
10NOV-0m	5.20	3.71	57.30	1.40
-1m	5.02	2.28	90.06	2.20
-3m	5.13	2.91	72.07	1.76
-5m	5.02	3.05	67.28	1.65

MIDLAKE 1989

DATE -DEPTH(m)	BPR pmol/ l/h	BAPC pmol/10^6 cell/h	BV x10^6 µm^3/l	BN x10^6 cell/ml
04JUN-0m	8.63	4.69	76.28	1.84
-1m	22.47	14.03	66.44	1.60
-3m	20.67	11.89	72.13	1.74
-5m	12.49	8.28	62.58	1.51
-10m	9.50	6.06	65.07	1.57
-15m	3.63	2.06	73.12	1.76
19JUL-0m	6.28	3.24	89.72	1.94
-1m	11.51	4.65	114.39	2.48
-3m	15.71	6.16	117.90	2.55
-5m	13.97	7.65	84.32	1.83
-10m	5.18	3.12	76.74	1.66
-15m	2.09	2.13	45.32	0.98
08OCT-0m	3.78	1.63	93.58	2.32
-1m	4.72	2.07	91.96	2.28
-3m	5.31	2.55	83.90	2.08
-5m	3.98	1.69	95.19	2.36
-10m	4.03	1.46	111.31	2.76
-15m	3.72	1.34	111.87	2.78