APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1986, p. 340–344
0099-2240/86/080340-05$02.00/0
Copyright © 1986, American Society for Microbiology
Vol. 52, No. 2

Nitrogen Limitation in Natural Populations of Cyanobacteria (*Spirulina* and *Oscillatoria* spp.) and Its Effect on Macromolecular Synthesis

JAAP VAN RIJN* AND MOSHE SHILO

The Division of Microbial and Molecular Ecology, Institute of Life Sciences, The Hebrew University of Jerusalem, Jerusalem, Israel

Received 20 November 1985/Accepted 8 April 1986

Natural populations of the cyanobacteria *Spirulina* species and *Oscillatoria* species obtained from Israeli fishponds were limited in growth by nitrogen availability in summer. Physiological indicators for nitrogen limitation, such as phycocyanin, chlorophyll *a*, and carbohydrate content, did not show clear evidence for nitrogen limited growth, since these organisms are capable of vertical migration from and to the nitrogen-rich bottom. By means of ^{14}C labeling of the cells under simulated pond conditions followed by cell fractionation into macromolecular compounds, we found that carbohydrates synthesized at the lighted surface were partially utilized for dark protein synthesis at the bottom of these ponds.

A number of planktonic, gas-vacuolated cyanobacteria have been shown to carry out a diel vertical migration in freshwater bodies (5, 8, 22). The buoyancy regulation underlying this vertical migration is influenced by several environmental factors, of which light and nutrient availability are the most important (10). A change in these environmental factors causes the cyanobacterial cells to float up or sink down, a phenomenon that can be explained by two proposed mechanisms: (i) gas vesicle synthesis and collapse (4, 23) and (ii) fluctuations in the amount of (high-density) cell components (Oliver et al., Abstr. 4th International Symposium on Photosynthetic Prokaryotes, Bombannes, France, abstr. no. C35, 1982). The latter mechanism was shown to operate in natural populations of cyanobacteria in Israeli fishponds (22). Diel vertical migration of these cyanobacteria was correlated with an increase in ballast due to carbohydrate accumulation at the surface of these ponds and a decrease in ballast at the dark-water–sediment interface.

The role of nutrient availability on buoyancy regulation in these cyanobacteria was suggested by the observed density decrease in the cells as a result of an ammonia-enhanced carbohydrate degradation at the bottom of the ponds (22). In the present work we examined the flow of photosynthetically fixed carbon into macromolecular fractions of the cyanobacteria under simulated pond conditions to discern whether excess carbon stored in the light was used for protein synthesis in the dark.

Photosynthetically fixed carbon was traced for this purpose within different macromolecules by the method first described by Roberts et al. (16) and later modified by Konopka and Schnur (12). This method not only proves to be a convenient tool in determining the flow of photosynthetically fixed CO_2 into the different macromolecules, but also serves as a reliable indicator of nitrogen limitation in natural populations of cyanobacteria (9).

Our results indicate that low ambient nitrogen concentrations limited growth of these natural populations and confirmed that carbohydrates synthesized at the nitrogen-deficient, lighted surface were later partially utilized in protein synthesis at the dark, nitrogen-rich bottom.

* Corresponding author.

MATERIALS AND METHODS

Source of the cyanobacteria. Cyanobacteria were collected from a fishpond in the Jordan Valley, Israel, during midsummer, when *Spirulina* and *Oscillatoria* species dominated the photosynthetic community in the pond. Other photosynthetic organisms never exceeded 2% of the total photosynthetic community (by cell volume).

Field experiments. Ammonia enrichment (N^+) of natural cyanobacterial populations was carried out in polyethylene enclosures (diameter, 1 m; maximum capacity, 600 liters) which were placed in the fishpond. Ammonia (NH_4Cl; final concentration, 5 mg liter^{-1}) was periodically added to these enclosures. At each sampling period the water column in the enclosures was thoroughly stirred, samples were collected in polyethylene bottles, and analyses in duplicate were conducted within 1 h. Cyanobacterial suspensions, incubated in dialysis bags placed vertically, served as a control (N^0).

The photosynthetic activity in the field was determined by incubation of the cyanobacterial suspension at a depth of 10 cm in the pond under a light intensity of 400 microeinsteins m^{-2} s^{-1}. Two transparent bottles and one dark bottle (Pyrex; volume, 135 ml) of each suspension (N^+ and N^0) were incubated for 1 h in the presence of 5 μCi of sodium bicarbonate (1.5 μg μCi^{-1}; Radiochemical Centre, Amersham, United Kingdom). The cell material was collected on GFC filters (Whatman, United Kingdom), which were dried in the presence of HCl fumes; the radioactivity on them was measured with a scintillation counter (LS 2800; Beckman Instruments, Inc., Fullerton, Calif.) after immersion in Insta-Gel-2 (Packard Instrument Co., Inc., Rockville, Md.). Counting efficiencies were determined in relation to an external standard (Beckman; ^{14}C).

Laboratory incubation. Cyanobacteria collected from the fishpond were passed through a 200-μm-mesh plankton net, filtered on GFC filters, washed with and suspended in filtered pond water (10-μm filter; Millipore, Tamar, Israel).

TABLE 1. Effect of ammonia addition on growth and composition in natural populations of cyanobacteria in the fishpond

Time (h)	Treatment	Amt of:				No. of trichomes (10^3) per ml	Assimilation no. (mg of C mg of Chl a^{-1} h^{-1})[a]
		Chl a[a]	Phycocyanin	Proteins	Carbohydrates		
0		0.52	0.60	44.2	20.6	4.7	
24	N^+	0.85	2.00	70.1	18.3	8.2	10.15
	N^0	0.42	0.53	45.0	20.1	4.7	10.70
48	N^+	0.94				9.2	
	N^0	0.49				4.7	
72	N^+	0.80	1.90	75.6	18.3	9.9	
	N^0	0.55	0.69	62.4	25.3	5.5	

[a] Chl a, Chlorophyll a.

Incorporation of ^{14}C was estimated on duplicate 50-ml cell suspensions in 250-ml Erlenmeyer flasks, each supplemented with 2 μCi of $NaH^{14}CO_3$. The flasks were illuminated by a 150-W slide projector (Prado Universal, Federal Republic of Germany) for 1 h at 27°C, after which the cells were fractionated into the different cell constituents. Light intensity was controlled by choice of the distance between the flasks and the light source.

Cell fractionation. The method of Roberts et al. (16) as modified by Konopka and Schnur (12) was used to fractionate the cells into polysaccharides, proteins, and low-molecular-weight compounds.

Other measurements. Protein was determined by the method of Lowry et al. (15) and calibrated with bovine serum albumin. Phycocyanin was determined by the method of Bennet and Bogorad (1). Total carbohydrates were determined by the method of Hassid and Abraham (6) by using D-glucose for calibration. Chlorophyll a was measured as described by Strickland and Parsons (19). Light intensity was measured with a Licor quantum sensor, sensitive to radiation between 400 and 700 nm. Ammonia was determined by the method of Solórzano (18). Cells were counted in 0.01-ml samples under 10- by 10-mm cover slips.

RESULTS

Determination of growth and composition of cyanobacteria in the pond without additions (N^0) and with supplemental ammonia (N^+) over a 3-day period are shown in Table 1. Whereas trichome number and cell components other than carbohydrates approximately doubled in the N^+ treatment within the first 24 h, N^0 suspensions showed only a slight increase in the parameters examined. The observed increase in the N^+ treatment continued, although to a lesser degree, over the next 48 h. It can be calculated from Table 1 that although the phycocyanin content per trichome rose considerably upon ammonia addition, the carbohydrate concentration was higher in the N^0 cells. Chlorophyll a concentrations in the cells were similar under both conditions, whereas protein concentration per trichome was lower in the N^0 suspension. The photosynthetic activity per unit of chlorophyll a (assimilation number) after 24 h of incubation did not

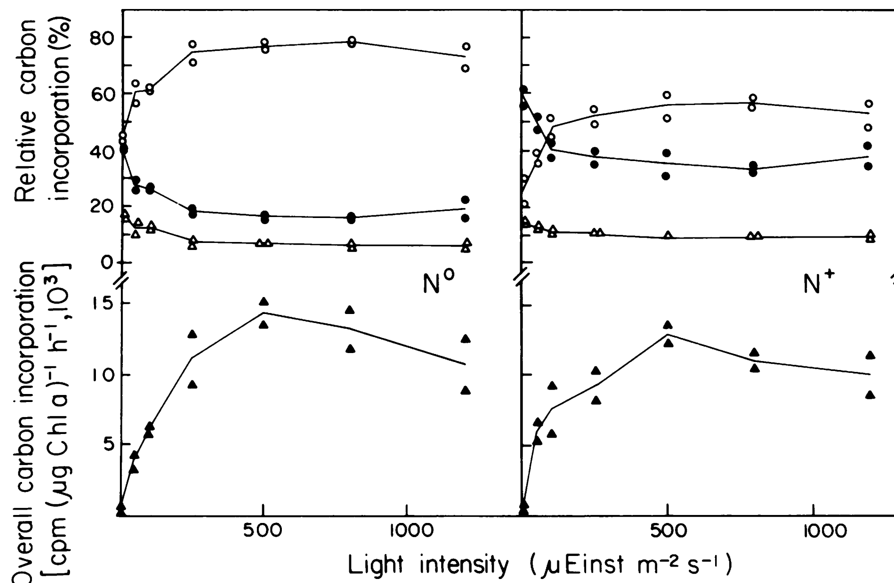

FIG. 1. Effect of light intensity on the relative incorporation of *Spirulina* and *Oscillatoria* species (5:1, by trichome number) into proteins (●), polysaccharides (○), and low-molecular-weight compounds (△) without (N^0) and with (N^+) supplemental NH_4Cl (final concentration, 5 mg liter^{-1}). Also shown is overall incorporation (▲) without (N^0) and with (N^+) supplemental NH_4Cl. $NaH^{14}CO_3$ incorporation was conducted for 1 h. The initial chlorophyll a concentration of both suspensions was 607 μg liter^{-1}.

differ significantly (6%) between the two treatments. This observation, in contrast to the effect of ammonia addition on growth, led us to examine the distribution of photosynthetically fixed carbon in several target macromolecular fractions.

Carbon incorporation was followed in $NaH^{14}CO_3$-supplemented cyanobacterial suspensions incubated for 1 h with and without ammonia over a range of light intensities. In the N^0 treatment (Fig. 1), most of the labeled carbon was incorporated into the polysaccharide fraction over the entire range of applied illumination (0 to 1,200 microeinsteins m^{-2} s^{-1}). Maximum relative incorporation into the polysaccharide fraction (about 75%) took place at light intensities of 250 microeinsteins m^{-2} s^{-1} and higher. Relative incorporation into proteins was highest at low light intensities and decreased with increasing light intensities. Relative incorporation into low-molecular-weight compounds showed the same trend as with proteins, but total incorporation was considerably lower.

The pattern of relative ^{14}C incorporation in the N^+ cyanobacterial suspension (Fig. 1) shows that, at light intensities lower than 100 microeinsteins m^{-2} s^{-1}, relatively more carbon is incorporated into the protein fraction, whereas at higher light intensities more carbon is fixed into the polysaccharide fraction. In this N^+ treatment, relative incorporation into protein is considerably higher (about 35%) over the range of light intensities examined, whereas polysaccharide incorporation is lower (about 55%) than in the cyanobacterial suspension not supplemented with ammonia. Figure 1 also shows the overall carbon incorporation per microgram of chlorophyll *a* for N^0 and N^+ cells. Again, no significant difference (9%) in overall photosynthetic activity was evident between the two treatments.

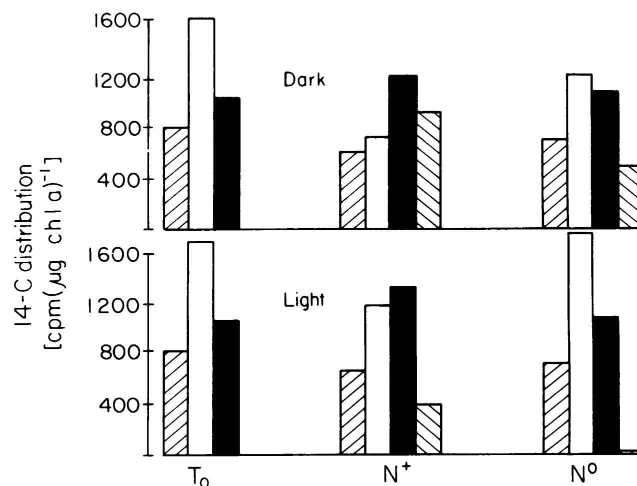

FIG. 3. Distribution of ^{14}C into subcellular fractions of *Spirulina* and *Oscillatoria* species (5:1, by trichome number). The cells were preincubated in the light under 1,400 microeinsteins m^{-2} s^{-1} for 1 h, washed twice in filtered pond water (T_0, initial counts), and incubated for 1.5 h in the dark or in the light (20 microeinsteins m^{-2} s^{-1}) with (N^+; final concentration, 5 mg $liter^{-1}$) or without (N^0) supplemental NH_4Cl. The initial chlorophyll *a* concentration of the suspensions was 547 µg $liter^{-1}$. Symbols: □, low-molecular-weight fraction; □, carbohydrates; ■, protein, □, rest fraction.

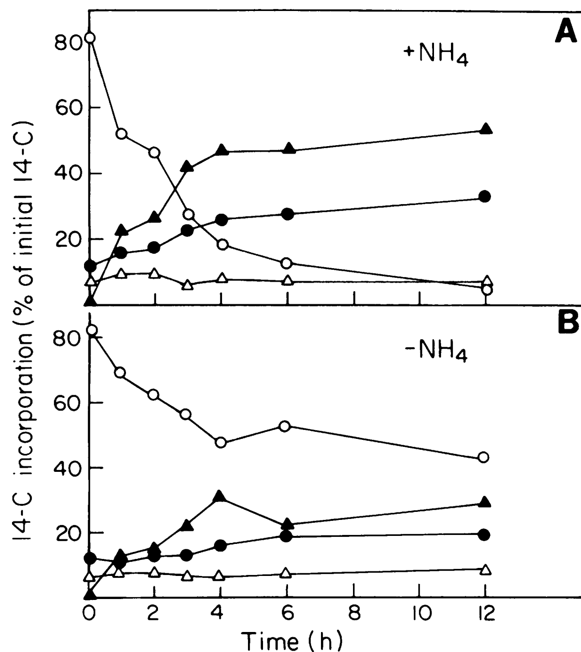

FIG. 2. Time course of ^{14}C distribution into subcellular fractions in the dark as a percentage of the overall initial ^{14}C with and without supplemental NH_4Cl in a natural population of *Spirulina* and *Oscillatoria* species (4:1, by trichome number). Cells were preincubated with $NaH^{14}CO_3$ for 1.5 h under 1,400 microeinsteins m^{-2} s^{-1}. The NH_4Cl concentration during dark incubation was periodically adjusted to 5 mg $liter^{-1}$. The initial chlorophyll *a* concentration of the suspensions was 406 µg $liter^{-1}$. Symbols: ●, proteins; ○, polysaccharides; △, low-molecular-weight compounds; ▲, rest fraction. The rest fraction represents the difference between overall initial radioactivity and the total radioactivity measured at each sampling time.

In another experiment (Fig. 2), the flow of ^{14}C among macromolecules in the dark was examined over time after preincubation with $NaH^{14}CO_3$ in the light. Incubation in the dark in the presence of ammonia led to a sharp decrease of label in the polysaccharide fraction from an initial 81 to 5% after 12 h. Radioactivity in the protein fraction increased from 12 to 33% over this period, whereas the amount of label in the low-molecular-weight fraction hardly changed. The remaining fraction, representing the difference between overall initial radioactivity and total radioactivity measured at each sampling time, increased substantially, up to 54% after 12 h. The amount of label in the polysaccharide fraction of N^0 cells (Fig. 2B) decreased at a rate slower than that of N^+ cells over the 12-h period, whereas label in the protein fraction increased from 12 to 19%. In the low-molecular-weight fraction the amount of label remained low during this period, whereas that in the remaining fraction increased moderately relative to N^+. In a similar experiment (data not shown), cells preincubated with $NaH^{14}CO_3$ in the light were incubated in the dark (N^0) with added L-methionine D,L-sulfoximine (final concentration, 1 mM), an irreversible inhibitor of glutamine synthetase (17). After 12 h of incubation, cells thus treated showed no increase of label in the protein fraction.

Finally, cell suspensions were preincubated with $NaH^{14}CO_3$ for 1 h under a light intensity of 1,400 microeinsteins m^{-2} s^{-1} and after washing were further incubated for 1.5 h under light (20 microeinsteins m^{-2} s^{-1}) and dark conditions, with and without ammonia. In the dark there was a large decrease of label in the polysaccharide fraction (more in N^+ than in N^0), whereas the label in the protein fraction increased only in N^+ cells (Fig. 3). As in Fig.

2, the remaining fraction was higher in N^+ cells. The light-dark comparison revealed that in N^0 cells polysaccharide-associated label decreased only in the dark. Its slight increase in the light presumably reflects polymerization of low-molecular-weight compounds, in which the ^{14}C count decreased, whereas the label in the protein fraction was stable. The remaining fraction of illuminated postincubation cells (N^0) was virtually zero, possibly indicating that respiration at the expense of stored polysaccharides was much lower in these cells than in those incubated in the dark. Among N^+ cells, loss of label in the polysaccharide fraction was smaller in the light than in the dark. Label in the protein fraction of the N^+ cells increased comparably in both light and dark postincubation. The remaining fraction of N^+ cells in the light was considerably lower than it was in the dark.

TABLE 2. C/N ratio (by weight) of a natural population of cyanobacteria (*Oscillatoria*, *Spirulina*, and *Microcystis* species) collected from the fishpond at the different times

Date (mo/day)	Time	C/N ratio
7/24	11:00	4.36 ± 0.06
7/24	18:30	4.72 ± 0.08
7/25	06:30	4.05 ± 0.22
8/1	11:00	4.27 ± 0.08
8/2	07:30	3.47 ± 0.05
8/7	11:00	4.39 ± 0.11
8/8	06:30	3.66 ± 0.06
8/8	12:00	4.81 ± 0.12

DISCUSSION

Ammonia addition to the water column of the fishponds resulted in increased growth of the cyanobacterial community (Table 1). Of the parameters examined in this bioassay experiment, in addition to the vigorous increase in cyanobacterial trichomes upon NH_4 addition, the phycocyanin content (per trichome or per unit of protein) and the carbohydrate content clearly differed from that of the cyanobacteria incubated in dialysis bags in the pond, which served as a control. Phycocyanin increased with the addition of ammonia but never exceeded 3% of the protein concentration, a value which is considered to be low in comparison with nutrient sufficient cultures of cyanobacteria (2, 12; W. Zevenboom, Ph.D. thesis, University of Amsterdam, 1980).

The carbohydrate/protein ratios were 0.42 (±0.02) for N^0 cells and 0.25 (±0.01) for N^+ cells (Table 1). Healy (7) and Konopka and Schnur (12) found ratios of ≤0.5 for nutrient-sufficient cultures of cyanobacteria and ≥1.0 for nutrient-limited cultures. By these criteria, all of the cyanobacteria examined in this study would fall in the nutrient-sufficient category, although our results indicate that cyanobacterial growth in these ponds was limited by nitrogen availability. The measurements in Table 1 were made on samples collected after mechanical stirring of the water column within the enclosures or the dialysis bags and therefore without reference to possible cyanobacterial heterogeneity at different depths. A determination of the carbohydrate/protein ratio of cyanobacteria collected from the surface layer of the fishpond yielded values as high as 1.3.

The ability of these organisms to migrate through the water column enables them to make contact with the nitrogen-rich water-sediment interface (22), and extreme nitrogen deficiency would not be expected. Under these conditions, in which the cyanobacterial cells are periodically exposed to ammonia, it was therefore of interest to examine the flow of carbon in the light in the absence of ammonia, as in the pond's top layers, and in the dark with ammonia, as at the water-sediment interface.

Carbon incorporation into polysaccharides as compared with incorporation into proteins is expected to be high when (i) the rate of carbon assimilation exceeds the maximum rate of incorporation of at least one essential nutrient required for protein synthesis, or (ii) when protein synthesis is low due to the fact that one or more nutrients are present in suboptimal amounts. Both situations were observed in this study. Under high light intensities and correspondingly high overall carbon assimilation rates, with or without supplemental ammonia, the incorporation into the polysaccharide fraction exceeded the incorporation of carbon into the protein fraction (situation i). The effect of a limiting nutrient on the relative carbon incorporation (situation ii) was demonstrated by the increased incorporation of carbon into the protein fraction upon ammonia addition. This is consistent with the spurt of growth found in the bioassay experiment (Table 1), and it may therefore be concluded that cyanobacterial growth in these fishponds is nitrogen limited.

The fate of the excess carbon stored in cyanobacterial cells of the upper pond layers was examined in the experiments shown in Fig. 2 and 3. By following the carbon label into the various macromolecular fractions, we found that polysaccharide breakdown in the presence of ammonia was correlated with an increase in protein synthesis. Similar results were obtained in *Oscillatoria agardhii* Gomont (21) and *Anacystis nidulans* (14) and recently in the green alga *Dunaliella tertiolecta* (3). However, a substantial part of the radioactivity present at the start of our experiment was lost during the incubation period, probably indicating that respiration at the expense of carbohydrates is a major mechanism causing carbohydrate decrease in the dark.

The carbon flow, namely, the carbon enrichment in proteins divided by carbon loss in polysaccharides, in the dark with supplemental ammonia was 0.23 (at 12 h; Fig. 2) and 0.19 (at 1.5 h; Fig. 3). Only a limited part of the consumed polysaccharides, therefore, is used for synthesis of proteins. Presumably most of the lost polysaccharides are respired, as indicated by the steep increase in the remaining fraction in Fig. 2 and 3.

In *Microcystis aeruginosa*, another cyanobacterium which was shown to display polysaccharide-dependent buoyancy regulation, similar low rates of protein synthesis at the expense of polysaccharides were measured. The dark growth yield for *M. aeruginosa*, defined as grams "rest" produced per gram of polysaccharides consumed, where "rest" stands for dry weight minus polysaccharides, was 0.28. In contrast, an organism that hardly displays buoyancy regulation, *O. agardhii* Gomont, gave much higher growth yields, approximately 0.8 (J. G. Loogman, Ph.D. thesis, University of Amsterdam, 1982). To what extent a low growth yield favors changes in the buoyant density, as suggested by Kromkamp and Mur (13), depends among other factors on the rate of carbohydrate breakdown due to respiration, a rate which depends in turn on the availability of a limiting nutrient, as shown in this study. As a consequence, cyanobacteria occurring in an environment in which the limiting nutrient is seperated both in space and time will show the highest density changes.

We conclude that the cyanobacteria examined in this study display a mechanism providing them with an efficient

utilization of two essential growth factors. These spatially and temporally separated factors, light and ammonia, are utilized by the cyanobacteria by virtue of an unique combination of buoyancy regulation and ammonia assimilation in the dark with subsequent protein synthesis. Both processes are to some extent mediated by polysaccharide turnover in the cyanobacterial cells, as manifested by the observed diel fluctuations of the intracellular polysaccharide content. An example of these fluctuations is shown in Table 2, where the C/N ratios (by weight) on surface samples of a natural cyanobacterial population are given for three different days. The high daytime ratios again indicate that the cells are exposed to high carbon and low nitrogen levels by day, and the opposite circumstance holds for the night. The ecological significance of polysaccharide fluctuations in cyanobacteria has recently been stressed by various authors (Oliver et al., Abstr. 4th International Symposium on Photosynthetic Prokaryotes; 13, 20, 22), emphasizing the importance of research into biochemical aspects of polysaccharide regulation and transformation in the cyanobacteria.

ACKNOWLEDGMENTS

This work was supported by the German Ministry for Research and Technology (BMFT) in the framework of the joint German-Israeli research program on aquaculture (NCRD project GR 154/AQ10) and by the Wolfson Foundation.

LITERATURE CITED

1. **Bennet, A., and L. Bogorad.** 1973. Complementary chromatic adaptation in a filamentous blue-green alga. J. Cell Biol. **58**:419–435.
2. **Boussiba, S., and A. Richmond.** 1980. C-phycocyanin as a storage protein in the blue-green alga *Spirulina platensis*. Arch. Microbiol. **125**:143–147.
3. **Cuhel, R. L., P. B. Ortner, and D. R. S. Lean.** 1984. Night synthesis of proteins by algae. Limnol. Oceanogr. **29**:731–744.
4. **Dinsdale, M. T., and A. E. Walsby.** 1972. The interrelations of cell turgor pressure, gas vacuolation, and buoyancy in a blue-green alga. J. Exp. Bot. **23**:561–570.
5. **Ganf, G. C., and R. L. Oliver.** 1982. Vertical seperation of light and available nutrients as a factor causing replacement of green algae by blue-green algae in the plankton of a stratified lake. J. Ecol. **70**:829–844.
6. **Hassid, W. Z., and S. Abraham.** 1957. Chemical procedures for analysis of polysaccharides. I. Determination of glycogen and starch—determination of glycogen with the anthrone reagent. Methods Enzymol. **3**:35–36.
7. **Healy, F. P.** 1978. Physiological indicators of nutrient deficiency in algae. Mitt. Int. Ver. Theor. Angew. Limnol. **21**:34–41.
8. **Konopka, A.** 1982. Buoyancy regulation and vertical migration by *Oscillatoria rubescens* in Crooked Lake, Indiana. Br. Phycol. J. **17**:427–442.
9. **Konopka, A.** 1983. The effect of nutrient limitation and its interaction with light upon products of photosynthesis in *Merismopedia tenuissima* (Cyanophyceae) J. Phycol. **19**:403–409.
10. **Konopka, A.** 1984. Effect of light-nutrient interactions on buoyancy regulation by planktonic cyanobacteria. Proc. Symp. Microb. Ecol. **111**:42–48.
11. **Konopka, A., and M. Schnur.** 1980. Effect of light intensity on macromolecular synthesis in cyanobacteria. Microb. Ecol. **6**:291–301.
12. **Konopka, A., and M. Schnur.** 1981. Biochemical composition of photosynthetic carbon metabolism of nutrient limited cultures of *Merismopedia tenuissima* (Cyanophyceae). J. Phycol. **7**:118–122.
13. **Kromkamp, J. C., and L. R. Mur.** 1984. Buoyant density changes in the cyanobacterium *Microcystis aeruginosa* due to changes in the cellular carbohydrate content. FEMS Microbiol. Lett. **25**:105–109.
14. **Lehman, M., and G. Wober.** 1976. Accumulation, mobilization and turnover of glycogen in the blue-green bacterium *Anacystis nidulans*. Arch. Microbiol. **111**:93–97.
15. **Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**:265–275.
16. **Roberts, R. B., D. B. Cowie, P. H. Abelson, E. T. Bolton, and R. J. Britten.** 1955. Studies on biosynthesis in *Escherichia coli*. Publ. Carnegie Inst. **607**:1–52.
17. **Ronzio, B. A., W. B. Rowe, and A. Meister.** 1969. Studies on the mechanism of inhibition by glutamine synthetase by methionine sulfoximine. Biochemistry **8**:1066–1075.
18. **Solórzano, L.** 1969. Determination of ammonia in natural waters by the phenol-hypochlorite method. Limnol. Oceanogr. **14**:799–801.
19. **Strickland, J. D., and T. R. Parsons.** 1968. A practical handbook for seawater analysis. Bull. Fish. Res. Bd. Can., bulletin no. 167.
20. **Thomas, R. H., and A. E. Walsby.** 1984. Buoyancy regulation in a strain of *Microcystis*. J. Gen. Microbiol. **131**:799–809.
21. **van Liere, L., L. R. Mur, C. E. Gibson, and M. Herdman.** 1979. Growth and physiology of *Oscillatoria agardhii* Gomont cultivated in continuous culture with a light-dark cycle. Arch. Microbiol. **123**:315–318.
22. **van Rijn, J., and M. Shilo.** 1985. Carbohydrate fluctuations, gas vacuolation, and vertical migration of scum-forming cyanobacteria in fishponds. Limnol. Oceanogr. **30**:1219–1228.
23. **Walsby, A. E.** 1969. The permeability of blue-green algal gas vacuole membranes to gas. Proc. R. Soc. Lond. Ser. B. **173**:233–255.