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Seasonal variation of phosphorus limitation of bacterial growth in a small lake

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Abstract

A series of bioassay experiments were performed from spring to autumn in a small dimictic lake (Deep Pond, Massachusetts) to examine the potential for bacterial growth limitation by organic carbon (glucose), inorganic nutrients (ammonium or phosphate), or both. The experiments demonstrated that phosphorus was the primary element limiting bacterial growth in Deep Pond during a large part of the summer. Significant increases (relative to controls) in bacterial cell volumes, protein production rates, and abundances were observed during 24-h incubations for samples amended with phosphate alone. Organic carbon was near colimitation for most of the samples, however, and dramatic increases in bacterial abundance and rates of protein production were obtained only when both substances (phosphate and glucose) were added together. There was no evidence for nitrogen limitation of bacterial growth during the study. Temperature was not an important determinant for bacterial production rates above 12°C, but below 12°C temperature acted to mute the effect of nutrient and organic carbon additions on production rates. Bacterial growth was not significantly increased by the addition of any combination of glucose, ammonium, or phosphate below 12°C. A significant, albeit complex, effect of the microbial community on the bacterial response to nutrient/carbon enrichment was apparent in the samples. Substrate/nutrient supply and biomass removal by bacterivores both appeared to play a role in the outcome of the experiments.

Pelagic heterotrophic bacteria play a fundamental role in aquatic ecosystems as regulators of the cycling of carbon and important nutrients such as nitrogen and phosphorus. Bacteria are the major consumers of dissolved organic matter (DOM) in the plankton. As such, they play vital roles in the recovery of organic matter, from detritus to living biomass, or in its remineralization back into inorganic compounds (Ducklow and Carlson 1992; Shiah and Ducklow 1994).

Bacterial growth and biomass in aquatic ecosystems are regulated by a number of factors, such as temperature (Shiah and Ducklow 1994), predation (Caron 1991), substrate supply (organic and inorganic nutrients) (Cole et al. 1988; Rivkin and Anderson 1997), and viral infections (Proctor and Fuhrman 1992). The relatively low variability of bacterial abundance in aquatic ecosystems has given rise to the speculation that bacterial abundance is rather tightly regulated by the different gain and loss factors operating on bacteria (Sanders et al. 1992).

Traditionally, organic carbon (for energy) has been considered the main factor limiting heterotrophic bacterial growth in pelagic environments. More recently, however, several investigators have demonstrated that bacterial growth in aquatic ecosystems can be limited by the availability of nitrogen and phosphorus rather than the supply of organic carbon (Morris and Lewis 1992; Elser et al. 1995; Pomeroy et al. 1995). Bacteria possess low carbon : nitrogen and carbon : phosphorus ratios (relative to most eukaryotic organisms) and apparently have a limited ability to change their cellular stoichiometry (Redfield et al. 1963; Goldman et al. 1987; Caron 1991). Therefore, bacterial nitrogen and phosphorus demand is high relative to the demand for carbon.

Nitrogen or phosphorus deficiency in the organic substrates utilized by bacteria for growth can lead to bacterial growth limitation by these elements. These conditions (i.e., nitrogen or phosphorus limitation) can create situations where heterotrophic prokaryotes will compete with phytoplankton for dissolved inorganic nutrients available in the surrounding water. Indeed, laboratory experiments have indicated that bacteria can compete effectively with primary producers for NO_3^-, NH_4^+ or PO_4^{-3} under these circumstances (Currie and Kalff 1984; Bratbak and Thingstad 1985; Suttle et al. 1990). Given this ability, one recent survey indicated that the number of field studies demonstrating the uptake of inorganic nitrogen or phosphorus by bacteria has increased as the ecological consequences of this behavior have been recognized by the scientific community (Kirchman 1994). More studies are required to determine the spatiotemporal breadth of this phenomenon, the nature of the limitation (nitrogen vs. phosphorus), and its consequences for bacterial nutrient cycling in various aquatic ecosystems.

We performed a series of experiments from spring to autumn in a small dimictic lake (Deep Pond) to examine the potential for bacterial growth limitation by organic carbon, inorganic nutrients (nitrogen or phosphorus), or both. Nineteen experiments were performed during the period April–October 1997, during which we determined bacterial abundance, bacterial production, chlorophyll *a* (Chl *a*),

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nanoflagellate abundance, and phosphate and ammonium concentrations in the lake. Bioassays were performed on each of these dates to determine the effects of ammonium, phosphate, or glucose additions alone or in combination on bacterial cell growth and rates of protein synthesis (leucine incorporation). Our studies demonstrated that growth of the bacterial assemblage in this small lake was limited by phosphorus during much of the stratified period.

Materials and methods

Study site—Deep Pond is located in the town of East Falmouth, Cape Cod, Massachusetts. It is a small oligotrophic dimictic seepage lake with a maximum depth of approximately 15 m and an area of approximately 2 ha. The drainage basin includes mixed conifer and deciduous forest and some residential properties. The lake is ice covered at some period during January and February. During our investigation (April–October 1997), a thermocline at about 5 m depth formed in the middle of May, and the lake was stratified until fall turnover in October.

Water sampling and experimental setup—On each sampling date, temperature was measured using a YSI Model 33 SCT meter at 1-m increments from the surface to 10 m depth at the deepest part of the lake (≈15 m). Water (8 liters) was collected in the morning (0700–0800 h local time) from the top 0.5 m, sieved through a 64-μm nylon net to remove mesozooplankton and large microzooplankton, and placed into a 10-liter polycarbonate bottle. The relatively rare zooplankton larger than 64 μm were removed because their low abundance might interfere in a random way with the availability of inorganic nutrients and carbon in the experimental containers. The sampling bottle and netting were acid washed before each use. Water was transported to the lab and the incubations were started within 1 h of sampling. Subsamples were removed for determinations of initial bacterial abundance, bacterial production, Chl *a*, nanoflagellate abundance, and ammonium and phosphate concentrations. Subsamples (200 ml) of lake water were transferred into acid-washed 250-ml polycarbonate bottles, and inorganic nitrogen (20 μmol L^{-1} as NH_4Cl), inorganic phosphorus (2 μmol L^{-1} as NaH_2PO_4), or glucose (100 μmol L^{-1} C) were added alone or in various combinations (no addition [control], +N, +P, +C, +CP, +CN, +NP, +CNP). All treatments were performed in triplicate for a total of 24 bottles.

The bottles were placed in an incubator at a light intensity of 35 μE m^{-2} s^{-1} photosynthetically active radiation (PAR), measured with a spherical light sensor (QSL-100 light meter, Biospherical Instruments). This light level is low and, it can be expected that the phytoplankton were light limited during the incubations. A low light level was chosen to avoid a dramatic increase in phytoplankton growth as a response to the nutrient amendments. We reasoned that this approach would avoid a large change in dissolved organic carbon (DOC) supply from phytoplankton (which might elicit a response from the bacteria), and it would minimize the competition of the algae with the bacteria for inorganic nutrients. The light cycle was 12 : 12 h light : dark and the temperature in the incubator was set to the in situ surface water temperature in the lake on each sampling date. Subsamples were removed after 12 h of incubation, and bacterial productions and abundances were determined. Subsamples were removed again after 24 h of incubation and processed for determinations of bacterial abundance, bacterial production, Chl *a*, and nanoflagellate abundance.

One experiment was conducted to examine the possibility of different responses of the bacterial assemblage (or other microbial populations present in the samples) over a diel cycle. For this experiment, water was collected in the morning and evening (2000 h) of the same day (8 July). Initial assemblage abundances were determined, and subsamples were incubated with inorganic phosphorus, glucose + inorganic phosphorus, or no added nutrients. Bacterial production was measured at 12 and 24 h according to the protocol given below.

Two experiments were conducted to compare the responses of the bacterial community to inorganic and organic enrichment in the presence and absence of bacterial grazers and phytoplankton. Previous studies (Morris and Lewis 1992; Berman et al. 1994; Chrzanowski et al. 1995; Elser at al. 1995; Schweitzer and Simon 1995; Rivkin and Anderson 1997) have typically used 0.8 μm–filtered water or dilution with 0.2 μm–filtered water to examine the response of aquatic bacterial assemblages to nutrient enrichment. Experiments were conducted on 26 June and 12 August using water filtered through 0.8-μm polycarbonate filters (Poretics). Subsamples of this filtrate were processed according to the same protocol used for the diel experiment (i.e., controls, inorganic P enrichment, glucose + inorganic P enrichment). The experiments were performed together with experiments using <64 μm–filtered water (whole water) on those dates.

Chlorophyll a—Water samples (50 ml) were filtered onto Whatman GF/F glass fiber filters at the beginning of each experiment (four replicates) and from each replicate following the 24-h incubation period. Chl *a* was extracted in 90% acetone (12 h) in the dark at room temperature, and fluorescence was measured using a Turner 10 AU fluorometer calibrated with a Chl *a* standard (Sigma).

Nutrient analyses—The filtrates from the Chl *a* filtrations (50 ml) were frozen in 50-ml plastic test tubes and later analyzed for phosphate and ammonium concentrations. The filters had first been rinsed by letting 50 ml of Milli-Q water pass through them. Phosphate and ammonium concentrations were determined according to Parsons et al. (1984), using a Bausch & Lomb Spectronic 88 spectrophotometer and a 10-cm cell.

Cell enumeration—Samples for the enumeration of bacteria (t = 0, 12, and 24 h) and total nanoplankton (t = 0 and 24 h) were preserved with 0.2 μm–filtered formaldehyde (final concentration 2%). Bacterial abundance was determined using a Becton Dickinson FACScan flow cytometer (del Giorgio et al. 1996a). Subsamples (500 μl) were stained with 5 μl of a 10× diluted solution of SYTO 13 (Molecular Probes). Internal standards were 0.93-μm fluorescent beads (Fluoresbrite Microspheres, Polysciences). Final bead concentration in the samples was approximately 1,400 beads

μl^{-1}. The concentration of beads in the samples was determined by epifluorescence microscopy on each occasion when samples were analyzed by filtering 2 ml of a 5,000×-diluted bead stock solution onto 0.2-μm blackened polycarbonate filters. Selected bacterial samples were also counted microscopically after staining with 4′6′ diamidino-2-phenolindole (DAPI) (Porter and Feig 1980). Flow cytometric and microscopic counts were well correlated ($r^2 > 0.95$, $n = 25$), although the flow cytometric counts were on average 10% higher than the microscopic counts.

Nanoflagellates were enumerated from 5-ml samples filtered onto 0.8-μm black polycarbonate filters. The flagellates were stained using the fluorochrome DAPI (final concentration, 20 μg ml^{-1}) for 10 min and counted using a Zeiss IM 35 epifluorescence microscope at ×1,000. At least 200 cells were counted on each filter. Autotrophic and heterotrophic flagellates were not counted separately because many autotrophic nanoflagellates can be phagotrophic and, therefore, can contribute to bacterivory (Bird and Kalff 1987; Sanders and Porter 1988). The nanoflagellates were quantified at $t = 0$ for every experiment. We also quantified the number of nanoflagellates after 24 h in the control, +P, and +CNP treatments on five occasions from mid-May to mid-September in order to determine if enhanced bacterial growth would affect the abundance of flagellates during the incubations.

Bacterial production—Bacterial production was measured as bacterial uptake of ^{3}H-leucine (specific activity 150 Ci mmol^{-1}, Amersham Corp.), according to Smith and Azam (1992). One milliliter of ^{3}H-labeled leucine was diluted with 4 ml of nonradioactive L-leucine in the original vial. Five microliters of this diluted solution was added to each subsample to give a final leucine concentration of 20 nmol L^{-1} (each incubated subsample was 1.7 ml). These subsamples were incubated for 1 h in the dark in the same incubator used to incubate the enriched lake water samples. The incubations were terminated by adding tricarboxylic acid (TCA) to the samples (5% final concentration) and the bacterial cells were rinsed with 5% TCA and 80% ethanol by centrifuging (16,000 × g; 10 min) and aspirating the supernatant between centrifugations. Radioactivity in the samples was determined with a Beckman LS 5000TD liquid scintillation counter using Biofluor (DuPont) scintillation cocktail.

Bacterial carbon production was calculated using an intracellular isotope dilution of two and assuming a carbon to protein ratio of 0.86 (weight : weight) in bacterial protein (Simon and Azam 1989). During the summer period (8 July) when bacterial production was highest in the lake, we performed a test to see if the added amount of leucine saturated bacterial uptake. We added 2, 5, 10, 20, 50, and 100 nmol L^{-1} to triplicate samples of the controls, +P, and +CNP treatments at $t = 24$ h and incubated them as described above. Bacterial production was saturated at the 20 nmol L^{-1} addition in the samples where production was ≤2.5 μg C L^{-1} h^{-1}. However, activity in the +P and +CNP treatments was much higher after 24 h, and they were not saturated even at 100 nmol L^{-1}. Thus, the bacterial production calculations for values larger than approximately 2.5 μg C L^{-1} h^{-1} must be considered underestimations.

Bacterial biovolume—Bacterial biovolumes were determined on two occasions during the summer period (26 July and 12 August, days 207 and 224, respectively) at $t = 0$, 12, and 24 h for the 64- and 0.8 μm–filtered water in the control, +C, +N, +P, +CP, and +CNP treatments. One milliliter of the same samples that were used for the flow cytometric determination of bacterial abundance was filtered onto black 0.2-μm polycarbonate membranes (Poretics). Bacterial images were acquired with a Zeiss 4S Axioplan epifluorescence microscope equipped with Zeiss Neofluar 100×/1.30 oil immersion objective and an Optronics VI-470 CCD video camera system (Optronics Engineering). Digital black and white images in TIFF format (pixel size in the resulting image was 0.098 μm) were obtained with an ImageGrabber-24 (Neotech Ltd). Biovolume was measured using a neural network-based image analysis program (Labmicrobe) (Blackburn et al. 1998). The biovolume of at least 200 cells from each sample was measured. Overlapping cells were not analyzed.

Phosphatase activity—Qualitative information on bacterial utilization of organic phosphorus compounds was studied by examining the occurrence of phosphatase activity in the surface water of the lake on two occasions during the summer (16 June and 19 September). We used the ELF-Cytological Labeling Kit 1 (Molecular Probes), which employs a nonfluorescent substrate that becomes highly fluorescent and forms a water-insoluble product after cleavage by phosphatases. The fluorescent precipitate remains localized at the site of enzyme activity. This method has been used previously to study phosphatase activity in individual algal cells (González-Gil et al. 1998).

Two 5-ml lake water samples were filtered onto Transwell (Costar) tissue culture inserts with bottoms that were 0.4-μm polycarbonate filters. The ELF substrate was treated according to the instructions provided with the kit, and 200 μl of the resulting solution was added to each sample. The samples (in the Transwell inserts) were incubated for 1 h in the dark at room temperature in tissue culture plates. Following the incubations, the samples were filtered onto the Transwell membrane filters, and 200 μl of 10× diluted wash buffer (supplied with the ELF kit) was added and filtered through immediately. Some samples were stained with DAPI (10 μg L^{-1} final concentration) then incubated 10 min before the solution was drawn off by vacuum. The Transwell filters were cut out and mounted onto microscope slides. Negative controls consisted of developing buffer, added without any addition of ELF substrate. Samples were observed using a Zeiss Axiophot epifluorescence microscope at ×1,000 magnification with a Hoechst/DAPI filter set (excitation 365 ± 8 nm, emission >400 nm). Blue light excitation (excitation 470–490 nm, emission >500 nm) was used for the observation of cells containing Chl *a*. Photographic images were acquired using Kodak Ectachrome 200 slide film.

Results

Temperature and lake stratification—Surface water temperature during this study varied between 6°C in the middle of April, when the water column was vertically well mixed,

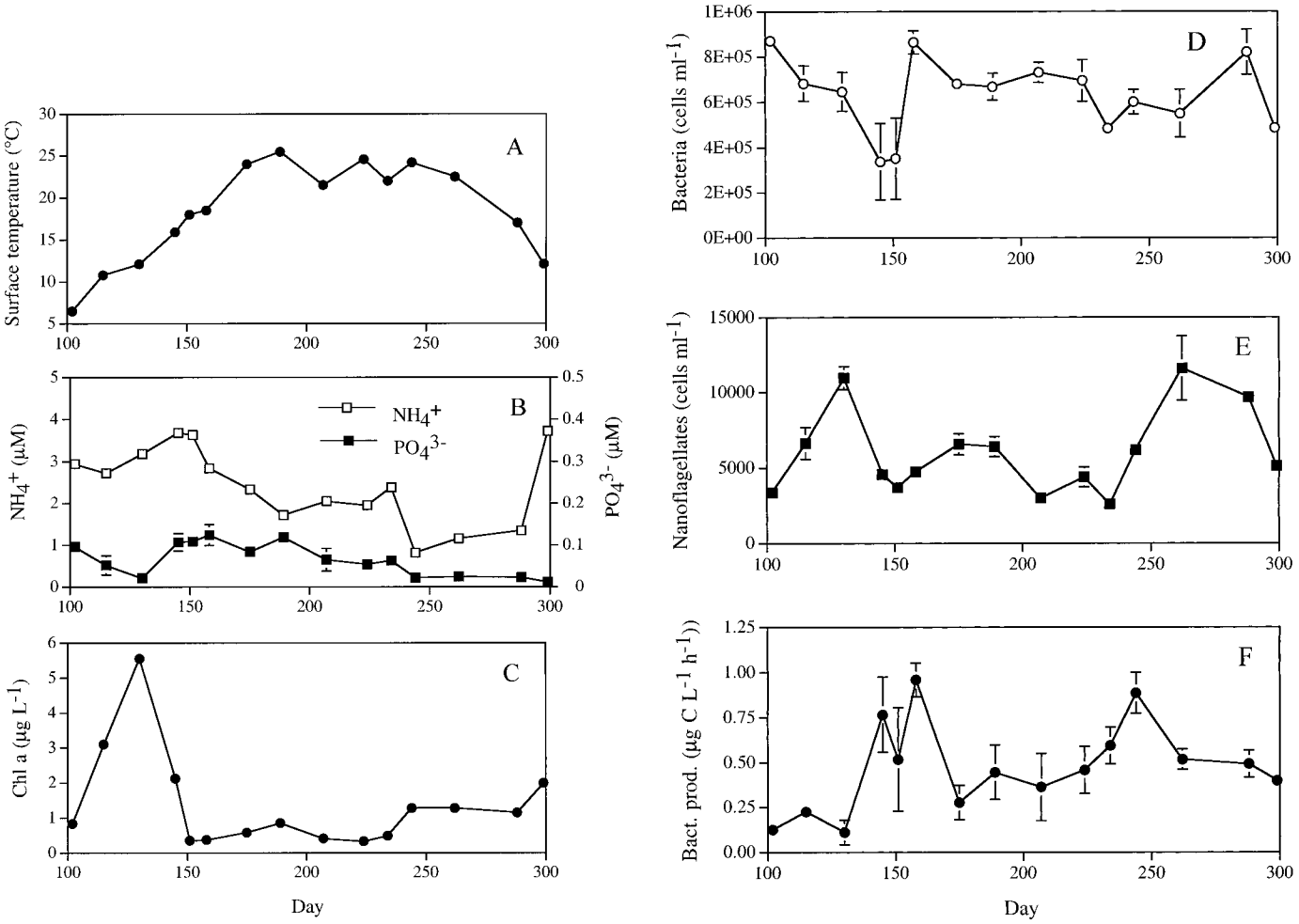

Fig. 1. (A) Surface water temperature (°C), (B) ammonium and phosphate concentration (μmol L^{-1}), (C) Chl *a* concentration (μg L^{-1}), (D) bacterial cell concentration (cells ml^{-1}), (E) nanoflagellate cell concentration (cells ml^{-1}), and (F) bacterial production (μg C L^{-1} h^{-1}) in Deep Pond 12 April to 26 October (days 102 to 299). All data (except temperature) are means ± SD of three replicates.

to a maximum of approximately 25°C near the end of June and beginning of July. The temperature remained above 20°C until mid-September, then decreased to approximately 12°C when the fall overturn took place near the end of October (Figs. 1A, 2). The seasonal thermocline was first detected at a depth of 4–5 m near the end of May, and this thermocline became stronger until a maximum temperature difference of 15°C was observed between surface water and hypolimnetic water at the beginning of July (Fig. 2). Two heavy rainfalls (>50 mm) associated with strong winds caused significant temperature decreases (2–3°C) in the epilimnion on 26 July and 22 August (days 207 and 234, respectively).

Seasonal variation of nutrients and Chl a—The concentration of phosphate in surface waters ranged from the detection limit (0.05 μmol L^{-1}) to 0.1 μmol L^{-1} during the study period (Fig. 1B). Phosphate decreased during the spring at a time that corresponded to the period of the spring phytoplankton bloom. Chl *a* concentration during the bloom reached 5.5 μg L^{-1} (Fig. 1C), then decreased (<1 μg L^{-1}) as the thermocline became strongly established during the summer. Phosphate concentration remained at levels near 0.1 μmol L^{-1} during much of the period of stratification, then decreased again in the autumn at a time that coincided with a less dramatic increase in the concentration of Chl *a* (2 μg L^{-1}). Ammonium concentrations in surface waters decreased during the summer and into early autumn but remained above 1 μmol L^{-1} for the entire period (Fig. 1B). The ammonium and phosphate concentrations resulted in N : P ratios for these constituents that varied between 14 and 300, with an average of 62, which is well above the Redfield ratio of 16.

Seasonal variation of bacterial and nanoflagellate abundances and bacterial production—Bacterial abundances varied between 3.5×10^5 and 8.7×10^5 cells ml^{-1}, with peak numbers occurring early in the spring, during the summer (early June to mid-August; days 158–224), and in mid-October (day 288) (Fig. 1D). Minimal bacterial abundances were observed at the end of May (days 145 and 151), coinciding with the end of the spring Chl *a* maximum, and just

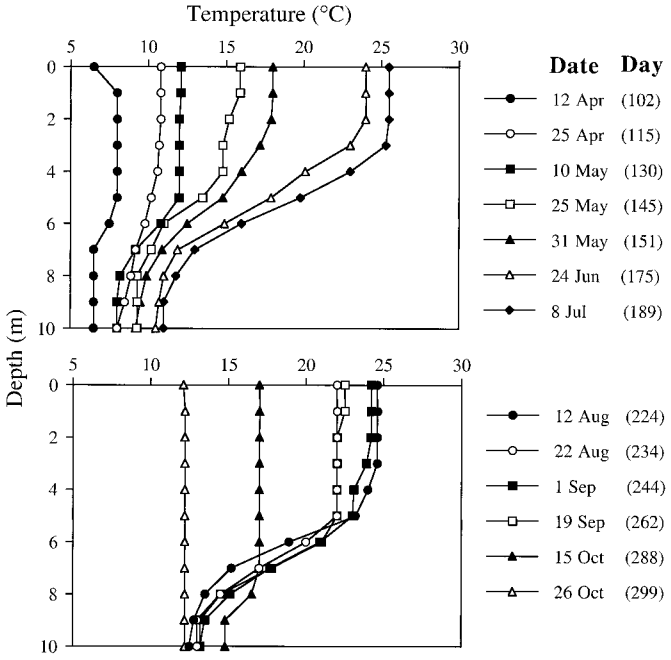

Fig. 2. Depth profiles of temperature in Deep Pond 12 April to 26 October (days 102 to 299).

after the peak in nanoflagellate abundance (Fig. 1E). On a yearly basis, there was no significant correlation between bacterial abundance or production and Chl *a* concentration.

The abundance of total nanoflagellates increased from approximately 3,000 cells ml^{-1} in April to more than 10,000 cells ml^{-1} in the middle of May (day 130; Fig. 1E), coinciding with the Chl *a* maximum during this period. Then nanoflagellate abundance decreased again and oscillated between 2,500 and 6,500 cells ml^{-1} during the summer. A second maximum of 11,500 cells ml^{-1} occurred during September and October (days 262 and 288), associated with the second smaller increase in Chl *a* and the fall overturn.

Bacterial production was approximately 0.1–0.2 μg C L^{-1} h^{-1} during April and the beginning of May then increased fourfold to 0.8 μg C L^{-1} h^{-1} at the end of May following the Chl *a* peak (Fig. 1F). Bacterial production then remained at approximately 0.5 μg C L^{-1} h^{-1} during the summer until a second maximum was observed near the end of August and beginning of September (0.5–0.8 μg C L^{-1} h^{-1}). This latter peak coincided with the small increase in Chl *a* concentration that took place at that time, and it preceded the peak in nanoflagellate numbers.

In general, bacterial production remained below 0.4 μg C L^{-1} h^{-1} at temperatures <12°C, whereas it varied between 0.3 and 1.0 μg C L^{-1} h^{-1} over the temperature range 15.9–25.5°C. There was only a poor correlation ($P = 0.09$, *F*-test) between bacterial production and temperature at time zero during this seasonal study (Fig. 3A). However, there was a significant exponential relationship between temperature and bacterial production ($P = 0.0001$, *F*-test) in the +CNP treatments at the end of the 24-h incubations (Fig. 3B); that is, bacterial production was highly responsive to temperature when the samples were augmented with organic carbon and inorganic nitrogen and phosphorus.

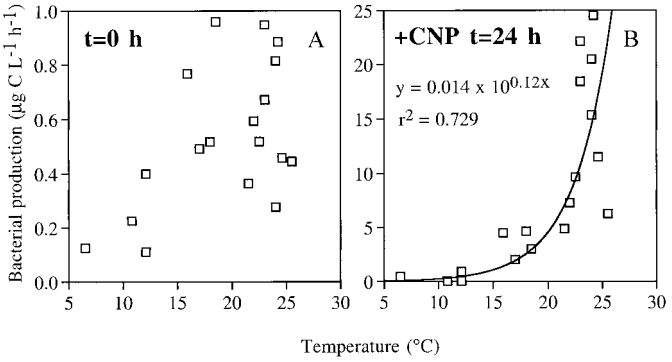

Fig. 3. Relationships between bacterial production and temperature for (A) measurements made at the time that lake water was collected (time = 0) and (B) measurements performed 24 h after the addition of carbon, nitrogen, and phosphorus to samples (+CNP).

Phytoplankton and nanoflagellate response to nutrient additions—Chl *a* increased by approximately 0.2 μg L^{-1} in the controls and the treatments supplied with nutrients for all experiments. Our objective in the use of a low light level was to maintain the phytoplankton assemblage in good condition, but not to stimulate the assemblage (to prevent dramatic changes in DOC production or nutrient uptake among the treatments). Because chlorophyll changed similarly in all experimental treatments, we conclude that these changes were probably a result of a physiological response by the phytoplankton to the low light level employed during the incubations and that nutrient additions did not appreciably affect phytoplankton growth.

The number of nanoflagellates did not increase significantly ($P > 0.05$, analysis of variance [ANOVA]) during the 24-h incubations in the control, +P, or +CNP treatments, except on 26 August (day 238), when the nanoflagellate abundance was significantly higher ($P = 0.0001$, ANOVA) in the +CNP treatment (Table 1). Assuming that the grazing rate for the nanoflagellates was similar among the treatments, we infer that grazing on the bacteria did not differ significantly between the different treatments during the 24-h incubation in most of our experiments.

Bacterial response to nutrient additions (abundance and production)—The +CNP treatment was performed to provide a positive control to observe maximal growth of the bacteria under nonlimiting nutritional conditions. As mentioned above, there were no increases in bacterial abundance or bacterial production in this treatment during the spring and autumn experiments when water temperature was below 12°C. However, bacterial abundance approximately doubled, and production increased twofold to 30-fold in the +CNP treatments when water temperature exceeded 12°C (the end of May to the end of September), compared to abundances and production in the unamended water samples (Fig. 4).

The addition of organic carbon (+C), inorganic nitrogen (+N), or carbon and nitrogen together (+CN) did not cause increases (relative to controls) in bacterial abundances or production values during the 24-h incubations. In contrast, the addition of inorganic phosphorus alone (+P) and, espe-

Table 1. Nanoflagellate abundance at $t=0$ and $t=24$ h in controls, and treatments with P and CNP additions for five of the experiments (mean±SD, $n=3$).

	10 May	24 June	26 July	26 August	19 September
$t=0$	10,957±771	6,563±692	3,008±148	3,922±62	11,599±2,142
$t=24$					
Control	9,753±393	5,797±323	3,545±314	3,380±108	9,222±1,367
+P	9,887±1,742	5,528±340	3,114±326	3,263±203	9,041±606
+CNP	11,302±1,086	5,272±335	2,846±59	11,580±726	10,160±1,645

cially, the addition of organic carbon and inorganic phosphorus together (+CP) resulted in increases in both bacterial abundances and production values in most of the water samples collected between mid-May and mid-September (Fig. 4).

The range of outcomes in these nutrient addition experiments are exemplified at three different times of the year in Fig. 5. There were no increases in bacterial production in any of the treatments during the experiment conducted on 25 April (day 115), when the water temperature was 10.8°C (Fig. 5A). In contrast, bacterial production increased significantly in all P treatments in the experiment conducted on 25 May (day 145), when surface water temperature was 15.9°C (Fig. 5B). No increase in production was observed in the C or N treatments (alone or in combination). A similar pattern was observed 1 month later (24 June, day 175), when the water temperature was 24.0°C (Fig. 5C). The response to enrichments, however, was 20% greater for the P treatment and seven times greater for the +CP treatment on 24 June compared to responses in these treatments on 25 May (compare Fig. 5B,C).

Responses of bacterial abundance and production to nutrient/carbon additions were quite variable in individual experiments when examined for the entire seasonal study (Table 2). However, median responses to the various treatments were consistent, with a slight increase in bacterial abundance and production in control treatments after 24 h, a considerably higher increase in the +P and +NP treatments, and an even greater response in the +CP and +CNP treatments. The responses in the +C, +N, and +CN treatments were either the same or lower than in the control treatments.

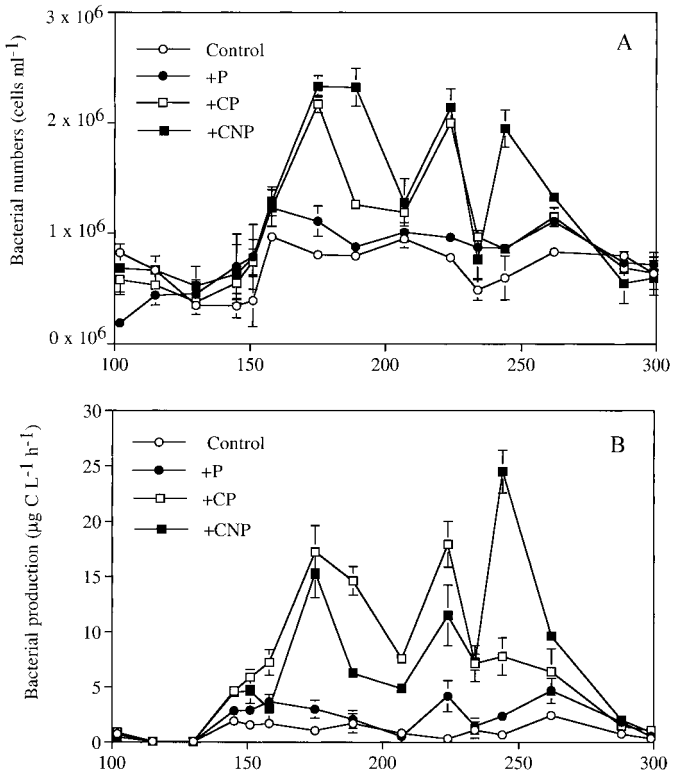

Fig. 4. Bacterial abundance (cells ml^{-1}) (A) at t = 24 h for control, +P, +CP, and +CNP treatments and (B) bacterial production (μg C L^{-1} h^{-1}) at t = 24 h for control, +P, +CP, and +CNP treatments (mean ± SD, n = 3). Results from +NP treatments were similar to the +P treatments and have been omitted. Results from the +C, +N, and +CN treatments were never significantly greater than the controls and have also been omitted.

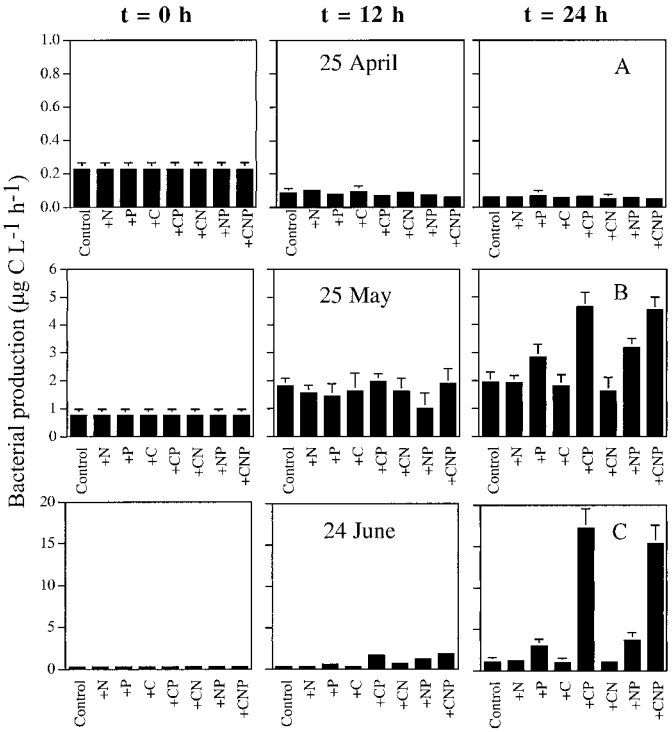

Fig. 5. Examples of the response of bacterial production (μg C L^{-1} h^{-1}) to nutrient and carbon additions during experiments conducted at different times of the year. (A) 25 April, water temperature 10.8°C; (B) 25 May, 15.9°C; (C) 24 June, 24°C (mean ± SD, n = 3).

Table 2. Changes in bacterial abundance and production among different nutrient treatments at $t=24$ h (expressed as a percentage of the mean values at $t=0$).

	Control	+N	+P	+C	+CP	+CN	+NP	+CNP
Bacterial numbers								
Whole period								
Range	−45.8–51.6	−47.7–72.7	−77.1–103.9	−40.6–111.7	−29.6–168.6	−59.0–82.1	−41.9–88.7	−31.2–225.5
Median	11.6	4.0	27.1	−13.8	37.9	−0.2	24.8	57.3
25 May–19 Sept								
Range	−0.3–30.1	−25.6–72.7	31.4–124.5	−29.3–136.5	42.3–219.1	−17.1–103.4	24.8–93.1	49.3–247.3
Median	12.2	12.6	53.8	15.0	94.2	26.3	44.3	131.6
Bacterial production								
Whole period								
Range	−72.6–496.0	−72.6–557.1	−69.5–984.8	−74.8–607.1	−70.8–6,117	−77.9–334.9	−75.2–1,228	−78.3–5,440
Median	86.0	108.6	273.2	66.9	779.4	85.8	281.9	799.8
25 May–19 Sept								
Range	−30.4–370.7	31.0–393.3	41.87–984.8	33.6–368.2	504.3–6,117	−51.9–332.6	21.3–1,228	215.4–5,440
Median	137.9	140.8	323.8	107.6	1,120	151.1	540.8	1,274

Water was collected in the morning and evening on one sampling date during the summer (24 June, day 175) to examine how the nutrient status or DOC supply might change over the diel cycle and how that might affect the bacterial responses to nutrient additions. The responses of bacterial production or bacterial abundance were very similar for samples collected in the morning and evening (Table 3), suggesting that the morning sampling performed during this study provided samples that were representative of the response of the bacterial assemblage to nutrient amendment.

The bacterial responses to nutrient additions were different in the presence and absence of phytoplankton and protistan grazers. On two occasions, the whole water and 0.8-μm filtrate exemplify these differences (26 July and 12 August, days 207 and 224; Table 4). In general, the bacterial production increased more in the +P treatment using 0.8-μm filtrate than in the whole water (64-μm filtrate). In the +CP treatment, the bacterial production increased even more in the 0.8-μm filtrate than in the in the whole water.

Table 3. Bacterial abundance and production at $t=0$ and $t=24$ h for two experiments started on the same day (24 June, day 175) with water collected in the morning and evening.

	Control	SD	+P	SD	+CP	SD
Bacterial abundance (cells $ml^{-1}\times10^6$)						
Morning						
$t=0$	0.78	0.04	0.78	0.04	0.78	0.04
$t=24$	0.70	0.05	1.07	0.15	1.27	0.05
Evening						
$t=0$	0.68	0.03	0.68	0.03	0.68	0.03
$t=24$	0.81	0.03	1.11	0.14	2.17	0.08
Bacterial production (μg C L^{-1} h^{-1})						
Morning						
$t=0$	0.40	0.04	0.40	0.04	0.40	0.04
$t=24$	0.66	0.27	3.32	0.28	17.73	1.54
Evening						
$t=0$	0.28	0.10	0.28	0.10	0.28	0.10
$t=24$	1.06	0.52	3.01	0.81	17.22	2.38

Bacterial response to nutrient additions (cell volume and turnover time)—Bacterial cell volumes in the whole-water fractions (64-μm filtrate) were similar ($\approx$0.070 μm^3) at the beginning of the experiments on the 2 d when these measurements were performed (26 July and 12 August; days 207 and 224; Table 4). Bacterial biovolumes in the controls on both dates decreased to approximately 0.040 μm^3 during the 24-h incubations, whereas average cell volumes in the treatments where P was added were significantly higher than in the controls at the end of the incubation period. Biovolumes in the +P treatment on 26 July remained close to the initial value after 24 h of incubation (0.065 μm^3), but all other treatments receiving P had greater cell volumes on both dates. The largest increases in biovolumes were obtained in the +CP and +CNP treatments on 12 August (up to 0.125 μm^3 after 24 h of incubation; Table 4, Fig. 6). Bacterial biovolumes after 24 h in the treatments amended with N alone or C alone were similar to biovolumes in the controls. Changes in bacterial cell volumes in the 0.8-μm filtrates on these two dates showed the same pattern as changes in the whole water (Table 4). Biovolumes in the filtrates doubled or tripled in the +P, +CP, and +CNP treatments (except for the more modest increase observed in the +P treatment on 26 July).

Growth rates and turnover times of the bacterial assemblages in the treatments on 26 July and 12 August were calculated using the total carbon content of the bacterial assemblage and the bacterial carbon production measurements. Carbon content of the bacterial cells was calculated using the biovolume measurements and a second-order polynomial fit to biovolume and carbon data (Simon and Azam 1989). Growth rates estimated in this manner indicated significant increases (up to two- to threefold) for controls, +C, and +N treatments after 24 h on the two dates. These increases were

Table 4. Bacterial abundance, production, and growth parameters (mean ± SD) for the two bioassay experiments in July and August performed with whole water and 0.8 μm-filtered water. Cell volumes (μm^3) were measured by image analysis; carbon content (fg cell^{-1}) was calculated using cell volume measurements and a second-order polynomial fit to biovolume and carbon data by Simon and Azam (1989); growth rates (h^{-1}) were calculated by dividing bacterial production with bacterial carbon concentration (carbon content per cell × cell number); and turnover time (h) is the inverse growth rate.

	Cell number (cells ml^{-1} × 10^6)	Volume (μm^3)	Carbon (fg cell^{-1})	Production (μg C L^{-1} h^{-1})	Growth rate (h^{-1})	Turnover time (h)
26 July (day 207)						
Whole water						
t=0	0.73±0.04	0.071±0.002	18.3±0.4	0.36±0.19	0.027	37.0
t=24 h						
Control	0.95±0.08	0.040±0.012	13.4±2.0	0.81±0.30	0.065	15.4
+P	1.01±0.08	0.065±0.012	17.3±1.8	0.52±0.20	0.030	33.3
+CP	1.19±0.06	0.077±0.004	19.2±0.7	7.59±0.34	0.333	3.0
+CNP	1.28±0.02	0.085±0.008	20.5±1.2	4.87±0.56	0.189	5.3
0.8-μm fraction						
t=0	0.60±0.05	0.039±0.008	13.2±1.3	0.07±0.02	0.009	111.0
t=24 h						
Control	1.07±0.16	0.053±0.003	15.5±0.5	0.25±0.08	0.015	66.7
+P	1.00±0.03	0.069±0.003	18.7±0.4	5.83±0.71	0.322	3.1
+CP	1.10±0.04	0.126±0.021	26.2±2.9	9.38±1.40	0.328	3.0
+CNP	1.33±0.29	0.116±0.006	24.9±0.8	8.87±0.82	0.322	3.1
12 August (day 224)						
Whole water						
t=0	0.69±0.09	0.070±0.003	18.2±0.5	0.46±0.13	0.037	27.0
t=24 h						
Control	0.78±0.02	0.039±0.004	13.4±0.6	0.32±0.08	0.031	32.2
+C	0.62±0.03	0.044±0.002	14.1±0.3	0.61±0.36	0.070	14.3
+N	0.68±0.00	0.036±0.008	12.8±1.4	0.60±0.05	0.069	14.5
+P	0.97±0.04	0.056±0.006	15.9±0.9	4.19±1.40	0.272	3.7
+CP	2.00±0.04	0.125±0.016	26.2±2.2	17.9±2.07	0.343	2.9
+CNP	2.14±0.17	0.125±0.004	26.1±0.5	11.5±2.74	0.206	4.9
0.8-μm fraction						
t=0	0.37±0.01	0.059±0.008	16.5±1.2	0.10±0.02	0.017	58.8
t=24 h						
Control	0.98±0.09	0.043±0.004	13.9±0.6	0.62±0.23	0.045	22.2
+C	1.06±0.10	—	—	0.36±0.10	—	—
+N	0.63±0.41	—	—	0.92±0.52	—	—
+P	1.10±0.56	0.117±0.014	25.0±2.0	12.5±1.08	0.398	2.5
+CP	2.48±0.02	0.115±0.013	24.8±1.8	16.2±0.79	0.264	3.8
+CNP	2.03±0.23	0.117±0.012	25.1±1.6	15.8±0.85	0.258	3.9

modest, however, relative to increases in the other treatments. Growth rates in the +P, +CP, and +CNP treatments yielded increases of 5.6- to 12-fold (whole water) and 15- to 36-fold (0.8-μm filtrate), with one exception: bacterial growth rate in whole water +P on 26 July did not increase significantly during the 24-h incubation.

The in situ (i.e., t = 0, whole water) turnover times of the bacterial assemblages determined from the growth rate data were approximately 30–40 h on 26 July and 12 August (Table 4). These turnover times were greatly decreased after 24 h of incubation in treatments amended with phosphate (with one exception, as noted above). Turnover times at the end of the incubations on these two occasions ranged from 2.5 to 5.3 h. The larger cell volumes of the bacteria in these treatments after 24 h were more than compensated by the very large increases in bacterial production, resulting in turnover times that were very short.

Alkaline phosphatase activity—Phosphatase activity was determined at t = 0 for samples collected on 24 June and 19 September (days 175 and 262). The ELF method used does not provide quantitative measurements of the phosphatase activity but indicates where the active enzymes are situated instead. Phosphatase activity could be detected on single bacterial cells (Fig. 6), indicating that the bacteria were producing phosphatases that where active in the lake. However, most of the activity was observed associated with detrital aggregates containing large numbers of bacteria and on phytoplankton colonies with attached bacteria (Fig. 6).

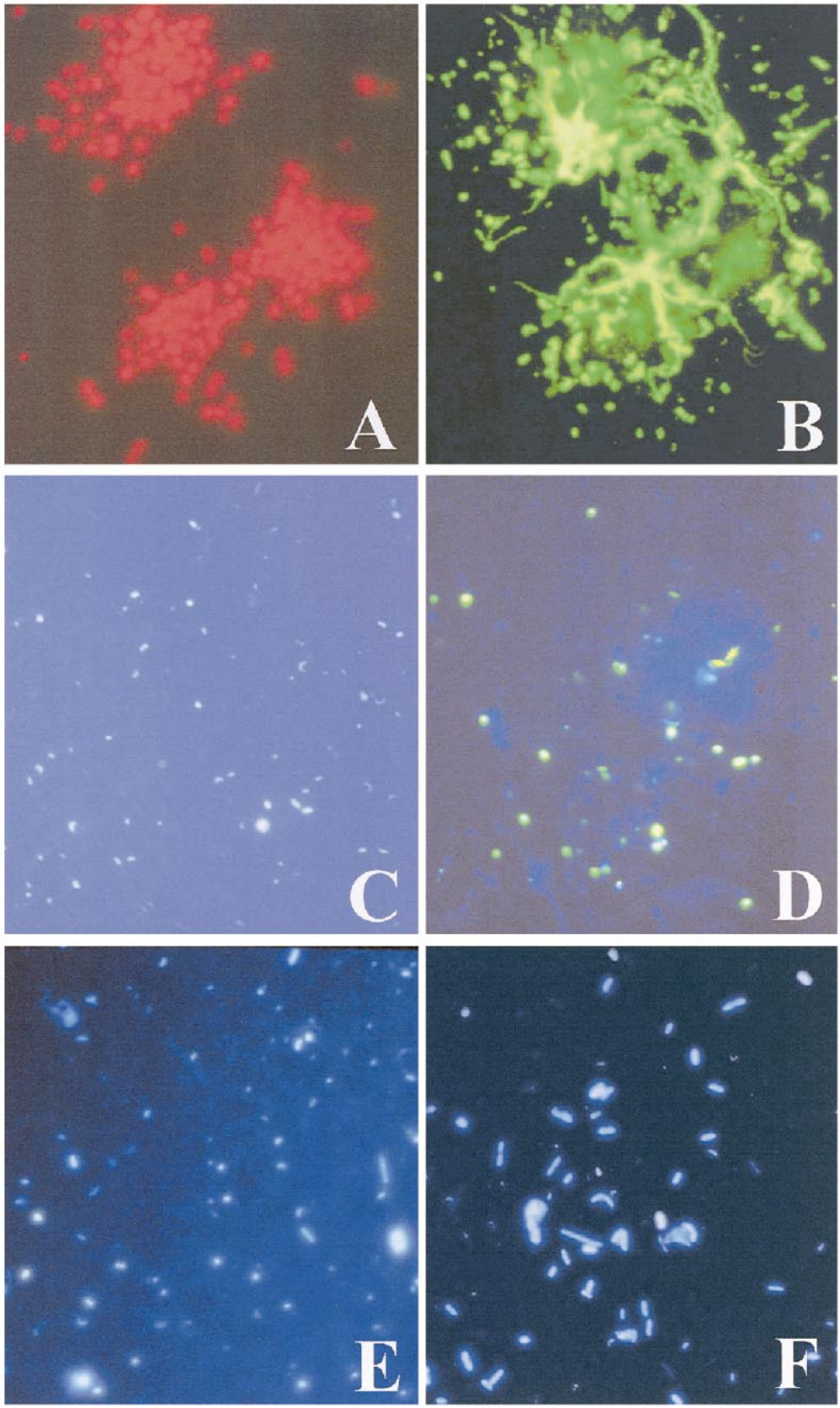

Fig. 6. Epifluorescent micrographs of (A) phytoplankton/bacteria aggregates in blue light excitation; (B) same picture as (A) in ultraviolet (UV) excitation, showing phosphatase activity by the fluorescent ELF product in the phytoplankton/bacteria aggregates; (C) DAPI-stained bacterial cells at $t = 0$ (UV excitation); (D) yellow fluorescence by the ELF product on single bacterial cells in the bacterial community at $t = 0$; (E) DAPI-stained bacterial cells in the +P treatment at $t = 24$ h; (F) DAPI-stained bacterial cells in the +CP treatment at $t = 24$ h. All images from 12 August.

Discussion

Bacterial growth limitation by inorganic nutrients and organic carbon—The bacterial community in Deep Pond was stimulated by the addition of phosphate during most of the summer period in this study (Fig. 4) despite the fact that phosphate concentrations remained between 0.05 and 0.1 μmol L^{-1} during this period. Since several kinetic experiments have shown that bacterial phosphate uptake can be saturated well below a phosphate concentration of 0.1 μmol L^{-1} (Currie and Kalff 1984; Chróst and Overbeck 1987; Cotner and Wetzel 1992; Thingstad et al. 1993), we speculate that the amount of phosphate may have been overestimated by the analytical method (SRP, soluble reactive phosphorus), either by the measurement of biologically unavailable complexes (DeHaan and Salonen 1990) or by a hydrolyzation of labile organic phosphorus compounds (Wetzel and Likens 1990). However, bacterial responses to phosphate additions have also been shown to take place at SRP concentrations around 0.1 μmol L^{-1} (Morris and Lewis 1992). The observed phosphatase activity using the ELF method (Fig. 6) also indicated that either the method used for phosphate analysis overestimated the biologically available inorganic phosphate or that concentrations of approximately 0.1 μmol L^{-1} of phosphate can induce phosphatase activity.

There was usually an increase in bacterial production in the control treatments of our experiments during the 24-h incubations (compared to initial production values). In light of the effects of C, N, and P additions to the samples, these increases imply that phosphate was increased slightly in the sampled water by the filtration process, by contamination during preparation of the treatments, or both, although we cannot rule out the possibility that the removal of bacterivorous mesozooplankton (*Daphnia* spp.) had an effect (Pace and Funke 1991). Also, an increased bacterial and/or a decreased algal competitive ability for inorganic nutrients at low light levels might have contributed to the increase in bacterial production in the controls. In any event, changes in bacterial production and cell number in the controls were modest compared to treatments amended with P (with or without N and C).

Dramatic stimulations of the bacterial assemblages in treatments amended with phosphate were apparent as increases in bacterial production, often after only 12 h of incubation, and also by increases in bacterial abundances after 24 h when compared to the controls (Fig. 4). This result is in agreement with several other studies that have demonstrated a stimulation of bacterioplankton production by the addition of phosphate to freshwater ecosystems (Toolan et al. 1991; Morris and Lewis 1992; Le et al. 1994; Chrzanowski et al. 1995; Elser et al. 1995). After the spring bloom, a significant amount of P settles out of the mixing layer, a process that might decrease the available P for bacterioplankton in freshwater systems (Birch and Spyridakis 1981).

In marine waters, stimulation of bacterial production has been observed with the addition of labile organic carbon (Wheeler and Kirchman 1986; Kirchman et al. 1990; Shiah and Ducklow 1994; Carlson and Ducklow 1996), but additions of phosphate, ammonium, or both also have been shown to stimulate bacterial growth in marine waters (Zweifel et al. 1993; Elser et al. 1995; Pomeroy et al. 1995; Cotner et al. 1997; Rivkin and Anderson 1997), sometimes coinciding with high phosphatase activity (Cotner and Wetzel 1992, Cotner et al. 1997). Collectively, these studies indicate that nitrogen, phosphorus, or both can play a fundamental role in regulating the growth of bacteria in plankton.

Both bacterial cell volumes and turnover times were strongly affected in some of the treatments on the two occasions when these properties were examined (Table 4). In particular, increases in cell volume and decreases in turnover time in the +CP and +CNP treatments (and in some cases in +P) were large compared to changes in the control, +C, +N, and +CN treatments. For the most part, increases in cell volume correlated well with increases in growth rate and, therefore, decreases in the turnover rates for these bacterial assemblages. However, growth rates increased disproportionally to bacterial biomass. As an example, growth rate increased 12-fold in whole water on 26 July, while bacterial biomass only doubled (+CP treatment). This result implies that observed increases were not simply due to differences in grazing rates among the treatments. It also indicates a lag between the response of bacterial growth rate to nutrient amendment and the subsequent increase in bacterial biomass (see below).

Bacterial turnover times in the lake were approximately 30–40 h in July/August on the two dates when bacterial biovolumes were measured. These rates are comparable to results for both oligotrophic lake systems and marine waters at comparable temperatures (Morris and Lewis 1992; Hoch and Kirchman 1993; Pomeroy et al. 1995; del Giorgio et al. 1996b).

Responses of the bacterial assemblages to the addition of organic carbon and inorganic nutrients indicated that, although phosphorus was the primary element limiting bacterial growth during much of the summer, many of the samples were close to conditions of colimitation by organic carbon availability. Phosphorus limitation was apparent in many of the samples investigated during the summer as significant increases in bacterial production (relative to controls) upon the addition of phosphate alone (Fig. 4B), but not ammonium or glucose (the latter two either separately or in combination). However, increases in bacterial production in the +P treatments were typically modest relative to increases observed in the treatments receiving both phosphate and glucose (+CP; Fig. 4B). This finding was somewhat surprising because it had been proposed that labile organic carbon compounds may accumulate during periods of severe nutrient limitation of bacterial growth to be used at a later time when the limiting nutrient becomes available (Cotner et al. 1997). Interestingly, this condition (limitation by phosphorus with near colimitation by organic carbon availability) persisted throughout the summer, but we never observed a situation in which carbon limitation was the primary factor limiting bacterial production.

Not surprisingly, the response of leucine uptake and protein production appeared to be faster than the division of bacterial cells in most experiments. Addition of the limiting element (in this case, phosphorus) gave a detectable response

in the rate of bacterial protein synthesis in less than 12 h. A longer period of time apparently was required for the bacterial cells to synthesize all needed cell components and divide. Similar results have been obtained previously (Berman et al. 1994). The latter study also observed that the volume of bacterial cells doubled before there was an increase in cell numbers.

There were notable differences in the bacterial growth response to nutrient additions at different times of the year in the present study. Some experiments exhibited no change in bacterial abundances, even though bacterial productions were stimulated by the addition of phosphate or phosphate + carbon. An increase in bacterial production (thymidine incorporation) without a concomitant increase in bacterial abundance when glucose or glucose + ammonium were added to natural water samples has been noted previously (Shiah and Ducklow 1994). Such results may have been due to a balance between bacterial growth and the loss of cells via viral lysis or grazing by bacterivorous protists in these experiments (Sanders et al. 1992; Fuhrman and Suttle 1993). However, it is also possible that an increase in bacterial abundance would have been detected if the incubations had lasted longer (i.e., perhaps there was a long lag between the shift-up in protein synthesis and that in cell division). Conversely, concurrent increases in bacterial abundance and production with phosphate or phosphate + carbon supplementation presumably indicated that mortality losses for the bacterial assemblage were low and that increases in the rates of bacterial protein synthesis and cell division were closely coupled (Morris and Lewis 1992; Rivkin and Anderson 1997).

Incubation of filtered (0.8-μm) and unfiltered water samples—The experiments in the present study were conducted primarily with ''complete'' microbial communities (i.e., all microbial species <64 μm). Therefore, the observed stimulations of bacterial growth by additions of organic carbon and inorganic nutrients were a combined result of the direct effects of organic/inorganic additions on the bacteria as well as indirect effects mediated by other microbial populations and processes in the food web (e.g., nutrients and DOM released by the grazing activity of phagotrophic protists, excretion by phytoplankton, bacterial mortality via viral lysis, and protistan bacterivory). Similar whole-water incubation approaches have been employed in mesotrophic lake water and in oligotrophic marine waters (Toolan et al. 1991; Cotner et al. 1997). These studies have demonstrated a significant stimulation of bacterial production by the addition of phosphate.

In contrast to this approach of using whole microbial communities, most experiments that studied bacterial growth limitation by inorganic nutrients generally involved either removal of most phytoplankton and bacterial grazers by filtration (Berman et al. 1994; Schweitzer and Simon 1995) or the dilution of these assemblages with filtered water (Morris and Lewis 1992; Chrzanowski et al. 1995; Elser et al. 1995; Rivkin and Anderson 1997). In this way, the bacterial community has been separated (or diluted) from most other organisms normally interacting with them. These methods have the advantage that bacterial losses by protistan grazing are minimized, and therefore bacterial growth rates can be determined directly from changes in abundance over the course of the incubations. However, reduction of the abundance of phytoplankton can reduce the competition between phytoplankton and bacteria for inorganic N or P and thereby possibly increase the growth response of the bacteria to a greater extent than would have been the case in a natural community. Prefiltration, dilution, or both can also affect the response of a bacterial assemblage to nutrient/organic additions in ways that are difficult to decipher. The removal of microbial consumers would affect the release of both utilizable organic substrates and inorganic nutrients released via the grazing activity of these populations, and the removal of phytoplankton could reduce the amount of labile organic material that would normally be released by these cells. The removal of those sources of dissolved substances could affect the nature and degree of nutrient/carbon limitation of bacterial growth in the samples.

In our experiments, the components considered to be the main interactors with bacteria (phytoplankton and microbial grazers) were present in the incubation bottles; thus, the responses of the bacterial communities to the amendments should have more closely reflected the nutritional status of the bacterial community in the lake. We expect that these responses were largely a consequence of the availability of organic and inorganic C, N, and P at the start of the experiments. However, the presence of phytoplankton and phagotrophic protists also could have caused responses in bacterial growth that were not directly related to the additions but, rather, to changes in the nutrient status of the former assemblages during the incubations. For example, release of the phytoplankton assemblage from severe nutrient (P) limitation could result in qualitative and quantitative changes in the assimilable DOC made available to the bacteria. We attempted to minimize these changes by incubating our samples under dim light conditions during the 24-h incubations. Similarly, significant increases in the abundance of micrograzers during the incubations (in response to increases in bacterial abundance) could have resulted in differences in the rate of removal of bacteria and the release of organic and inorganic materials in some of the treatments. We attempted to characterize the magnitude of this latter parameter by monitoring changes in nanoflagellate abundance during the experiments. We detected a significant change in nanoflagellate numbers during the 24-h incubation period only during one experiment. Thus, we assume that the grazing activity and the regeneration of inorganic nutrients did not change substantially during the 24-h incubations compared to the situation in the lake at the time of sampling.

Nevertheless, some significant differences were observed between the response of bacteria in the <0.8-μm filtrates and whole water (<64-μm filtrates) on the two dates when these size fractions were directly compared (Table 4). The most striking differences were observed in the +P treatment, in which production of the bacterial assemblages in the absence of most phytoplankton and grazers (<0.8-μm filtrate) showed a much greater response to phosphate additions than the bacterial assemblages in whole water. These differences were most probably an effect of the grazer removal of bacterial biomass in the whole water and not of competition

with phytoplankton, since the addition of P (2 μmol L^{-1}) could not have been assimilated by the low initial biomass of phytoplankton (0.5 μg L^{-1} as Chl *a*) during the 24-h incubation.

Large differences in the response of the bacterial assemblages in whole water and 0.8-μm filtrate were not apparent in the +CP treatments. That is, when labile organic carbon and phosphate were added, the presence of the entire microbial community did not constrain increases in bacterial production (relative to increases observed in the <0.8-μm filtrate) as it apparently did in the +P treatments (compare +P and +CP treatments in Table 4). We speculate that the addition of carbon together with phosphorus allowed more rapid growth of the bacterial assemblage in the +CP treatments (relative to their response in the +P treatments) and that the higher bacterial growth in the +CP treatments was not controlled by bacterivore grazing activity during the 24-h incubations. This hypothesis is consistent with our finding that the responses of the bacteria in the 0.8-μm filtrates were not as great in the +P treatments as they were in the +CP treatments, and it is consistent with our finding of near colimitation of bacterial growth by carbon in many of the samples (see previous section).

Effects of temperature—Bacterial abundance and production in aquatic ecosystems have been shown to covary with temperature (e.g., White et al. 1991; Hoch and Kirchman 1993), but the interplay between nutrient/substrate limitation and temperature limitation of bacterial growth is an intricate one. For example, Shia and Ducklow (1994) and Tibbles (1996) demonstrated that the enrichment of seawater samples with labile substrates had less effect on bacterial production than an increase in temperature. Furthermore, it has been demonstrated that specific growth rates of bacteria in the subarctic Pacific were enhanced not only by the addition of DOM but by increased temperature and that the response was faster when temperature increased than when DOM was added (Kirchman et al. 1993).

Bacterial production in Deep Pond was related to temperature in the sense that low production rates were recorded at low temperatures (<12°C) and higher production rates were obtained at higher water temperatures. However, there was no significant relationship between in situ bacterial production and temperature without nutrient or substrate additions (Fig. 3). The in situ bacterial production rates occurred over a wide range of values for water temperatures between 12 and 25°C (0.25 and 1.00 μg C L^{-1} h^{-1}) and were poorly correlated with temperature over this range. This finding is similar to that of Ochs et al. (1995), who found that bacterial production and growth rates in the epilimnion of an oligotrophic lake were not related to temperature above 14°C. They are also consistent with the observations of Scavia and Laird (1987) and Hoch and Kirchman (1993), who demonstrated that bacterial growth rates were correlated with temperatures below 10–12°C but that there was no correlation with temperatures above 10–12°C.

There was no response to any organic/nutrient amendment at temperatures below 12°C in Deep Pond, indicating that temperature had a dominant effect on bacterial growth at low temperature for the ranges of organic and inorganic compounds examined in this study. However, a significant correlation was observed between bacterial production and temperature for water samples ≥12°C when the samples were amended with C, N, and P and incubated for 24 h (Fig. 3B). An exponential relationship between bacterial growth and temperature at unlimited substrate supply is well known from laboratory experiments (e.g., Topiwala and Sinclair 1971). These observations imply that bacterial production rates in Deep Pond were weakly related to temperature over much of the summer but that temperature exerted a dominant effect at times of cold water. Nutritional status clearly played a dominant role in controlling bacterial growth during periods of warm water. Perhaps the temperature sets an upper bound for microbial productivity. This bound is low in colder waters and the nutrient (and carbon) concentrations are high relative to this bound. In the summer, however, the upper bound for microbial productivity increases and nutrients and organic carbon can become limiting.

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