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Inorganic Phosphorus Stimulation of Bacterioplankton Production in a Meso-Eutrophic Lake†

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Experiments were conducted to determine whether production of heterotrophic bacterioplankton in a small meso-eutrophic lake was influenced by the dissolved inorganic phosphorus (DIP) supply. DIP may indirectly limit bacterial production by limiting phytoplankton, which in turn may limit the carbon available to bacteria. Direct DIP limitation of bacteria occurs where the availability of DIP for bacteria is insufficient to maintain growth. This work examined direct DIP limitation of bacteria by removing phytoplankton and incubating flasks with or without added P in the dark. Bacterial production was measured via the rate of incorporation of [^{3}H]thymidine ([^{3}H]TdR) into DNA. Bacterial abundance was followed with epifluorescent direct counts. Rates of [^{3}H]TdR incorporation were significantly greater in flasks with added DIP, and changes in cell abundances generally paralleled increases in [^{3}H]TdR incorporation. Even very small additions of P (0.05 μM) were sufficient to stimulate production. DIP addition to whole lakewater also stimulated [^{3}H]TdR incorporation relative to that in zero-addition controls, but there was not a concurrent increase in bacterial cell numbers. The stimulation of [^{3}H]TdR incorporation after DIP addition to whole lakewater was significantly less than the stimulation due to DIP addition to 1-μm-pore-size-filtered lakewater. In this study, addition of DIP caused as much as an eightfold stimulation of [^{3}H]TdR incorporation.

Recent attention has focused on planktonic heterotrophic bacteria in fresh and salt waters because of a reevaluation of energy flow through these ecosystems. The extent of carbon flow through bacterioplankton had been largely overlooked, because bacterial biomass had been underestimated previously (11, 12). A literature review by Cole et al. (2) revealed that bacterial production comprises a significant part of secondary production and averages about 20% of planktonic primary production. The term "microbial loop" has been used to describe carbon flow through a dissolved organic carbon (DOC)-bacterium-protozoan food chain rather than through the classical phytoplankton-zooplankton-predator food chain (1). However, there has been debate about the extent to which microorganisms are links or sinks in carbon flow (5).

Since a large portion of carbon in aquatic ecosystems is now thought to flow through bacteria, factors controlling bacterioplankton production are relevant to ecosystem studies. It is not yet clear to what extent either organic carbon or inorganic nutrients may limit bacterioplankton production. While availability of organic carbon has traditionally been thought of as a key factor which limits bacterial growth, the high phosphorus requirement of bacteria relative to that of phytoplankton (15) and the percentage of uptake, 72 to 98%, traceable to bacteria (4) suggest that the dissolved inorganic phosphorus (DIP) supply could also limit bacterial production.

Heterotrophic bacterial production may be directly or indirectly limited by DIP. It is well established that phytoplankton are directly phosphorus limited in many fresh waters (13). Direct DIP limitation of phytoplankton could lead to indirect DIP limitation of bacteria where phytoplankton carbon is necessary for bacterial growth. Alternatively, DIP could directly limit bacteria where insufficient DIP is present to maintain bacterial growth.

To test the hypothesis that bacterioplankton production is directly DIP limited, inorganic phosphorus was added to 1-μm-pore-size-filtered lakewater and bacterial abundance and production were measured over 96 h. Removal of phytoplankton, microzooplankton, and macrozooplankton permits examination of direct DIP limitation and eliminates confounding effects such as grazing and nutrient regeneration. The rate of tritiated thymidine incorporation into DNA and rate of change in the cell numbers were measured to follow the response of bacteria to DIP additions.

MATERIALS AND METHODS

Location and sampling. Calder Lake is a small (3.9-ha) meso-eutrophic, dimictic lake located at the Fordham University Biological Field Station in southern New York State. Its mean and maximum depths are 2.8 and 6.7 m, respectively. Secchi depth, which corresponds to the depth at which the light is approximately 10% of surface light, varied between 3.0 and 6.1 m in 1987 and 1988; during the summer periods, turbidity is higher. The fetch, the distance over which the wind has blown uninterrupted by land, is 337 m in this lake. The DIP concentration ranges from <0.03 to 0.84 μM (<1.0 to 26 μg of P liter^{-1}), and the total P concentration ranges from 0.12 to 2.74 μM (3.7 to 85 μg of P liter^{-1}) (16). The experiments were carried out from autumn 1988 to autumn 1989. Calder Lake water was collected by peristaltic pump from the epilimnion (~5 cm below the surface) at the deepest part of the lake, except in February and December 1989, when ice cover (~5 cm thick) rendered the sampling station inaccessible. In these two cases, water was collected near the outflow point, a small dam about 20 m from the sampling station.

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Experimental design. Water (15 liters) was collected into a translucent 20-liter (ca. 5-gal) polyethylene bottle with a stir bar and plugged with a sterile bung. The water was acclimated for 24 h in an incubator at 15°C on a 12-h light: 12-h dark cycle. This was done to control for temperature and light, since the experiments were done at different times of the year. Following acclimation, 0.6 liter of water was filtered through a 1-μm-pore-size Nuclepore filter and added to 5.4 liters of water previously filtered through 0.45-μm-pore-size (cellulose acetate) filters. Filtration of 90% of the water through 0.45-μm-pore-size filters was done to remove virtually all eukaryotes; 10% of the water was filtered through 1-μm-pore-size filters to remove most eukaryotes but retain a substantial bacterial inoculum. After vigorous shaking, the mixture was poured into six 1-liter flasks; three flasks were controls (no DIP addition); another three flasks received a stock solution of KH_2PO_4 to increase the ambient DIP concentration by 5 μM. Stimulation by addition of potassium was unlikely, since lakewater concentrations of potassium range from 20 to 50 μM.

Following DIP addition, the flasks were incubated at 15°C for 96 h in the dark on a shaker at 100 rpm. To measure bacterial production, incubations of subsamples with [*methyl*-^{3}H]thymidine ([^{3}H]TdR; New England Nuclear) were carried out every 24 h. The first incubation was done within ~4 h of DIP addition. Samples for cell counts were taken every 48 h to measure changes in cell abundances. Samples for measurement of DIP were taken every 24 h, and samples for DOC measurement were taken every 48 h. These procedures were carried out at each sampling date and are referred to as the basic experiments.

Additional experiments. In July and August 1989, two additional experiments were done concurrent with the basic DIP experiments. In July, a range of phosphate concentrations, +0.05, +0.10, +0.50, +1.0, and +5.0 μM, was used to examine the response of bacteria to various concentrations of DIP; the experiment was conducted for 72 h. The additional August experiment was the same in all respects as the basic experiments including phosphate treatment, except that in six flasks the lakewater was filtered only through 152-μm-mesh-size Nitex to exclude the largest zooplankton. Three of these flasks then received DIP, and the six flasks were incubated on a 12-h light: 12-h dark cycle for 96 h. The intent of this manipulation was to test for interacting effects between DIP treatment and the presence of other organisms (phytoplankton, flagellates, and small zooplankton).

Bacterial production. Bacterial production was measured via the rate of incorporation of [^{3}H]TdR into DNA (6). Aliquots (10 ml) were removed from the flasks and incubated with 40 μCi of [^{3}H]TdR (final concentration = 50 nM) for 1 h in a 15°C water bath. One aliquot per flask was a zero-time control; 2 ml of 5% formalin was added immediately after the isotope was added. The other two samples were killed after 1 h to stop further incorporation. The zero-time controls always had less than 20% of the incorporation in the other samples. Time course measurements indicated that incorporation of [^{3}H]TdR was linear for over 1 h. Following incubation, the samples were filtered at ~340 mm Hg onto 47-mm, 0.2-μm-pore-size Nuclepore filters, rinsed with ice-cold 5% trichloroacetic acid, and frozen for subsequent DNA extraction (6). Radioactivity was determined in a Beckman LS1801, and counting efficiency was determined from the relationship between the H number and the counting efficiency (9).

Cell enumeration. Cell numbers were measured by the acridine orange direct count technique (7). Cells were counted under an Olympus BH2 microscope at a magnification of ×1,250. At least eight fields or 500 cells were counted for each filter. One filter per flask (three filters per treatment) was counted.

Chemical measurements. DIP in lakewater and experimental flasks was measured by the acid molybdate method (14). DOC was measured with an automatic carbon analyzer (ASTRO 2001).

Statistical analysis. To determine the effect of DIP addition, cell counts and [^{3}H]TdR incorporation at 96 h from added-P and control flasks were compared by *t* tests. *t* tests were also done on production rates calculated from changes in cell abundances and from [^{3}H]TdR incorporation into DNA, in order to compare the results of the two methods.

To examine differences among experiments, two-way analysis of variance was done with the DIP treatment (5 μM P versus no P added) and month (experiments carried out in 5 different months) as the main effects. Dependent variables were [^{3}H]TdR incorporation data at 72 h and cell count data at 96 h. For the dose-response experiment done in July, *t* tests were done with the [^{3}H]TdR data from 72 h to compare the control (no DIP added) with each of the five treatments (0.05, 0.10, 0.50, 1.0, and 5.0 μM P added). The results from the August whole-lakewater experiment and the basic August experiment were compared by model I analysis of variance with the data from 96 h only.

RESULTS

Effects of DIP additions and seasonal variation. Incorporation of [^{3}H]TdR into DNA was significantly greater at 96 h in experimental flasks with DIP added (5 μM P) than in control flasks with no added P ($P < 0.05$) in all of the five basic experiments (Fig. 1A shows the May experiment results as an example). The [^{3}H]TdR incorporation rate was three to eight times greater in the treatments (5 μM P added) compared with that in the controls (no P added) (Fig. 2A). The final production rates in the +5 μM P treatments were lowest in December and highest in August (Fig. 2A).

Cell numbers at 96 h were significantly greater ($P < 0.05$) in the treatments (5 μM P added) than in the controls (no P addition) in three of five experiments (May, July, and December; Fig. 1B and 2B). At 96 h, the cell numbers were 1.3 to 1.9 times greater in the added-DIP treatments than in the controls (Fig. 2B).

The magnitude of the difference between the controls and added-DIP treatments varied considerably, and analysis of variance for the [^{3}H]TdR data and cell density data showed that the responses to DIP addition were significantly different among months ($P < 0.05$).

To examine whether the initial DIP concentration influenced variation in growth in the control flasks among months, mean disintegrations per minute per liter per hour at 72 h were plotted versus the corresponding initial DIP concentrations (Fig. 3A). The DIP concentrations of the experimental control flasks at 0 h are used as estimates of the ambient lakewater DIP concentrations at the time of sampling. Concentrations ranged from below the limit of detection (<0.05 μM PO_4-P) in August and December to 0.11, 0.30, and 1.33 μM PO_4-P in July, May, and February, respectively. Rates of [^{3}H]TdR incorporation were significantly related to DIP concentration (Fig. 3A; regression analysis, $P < 0.01$, $r^2 = 0.42$).

Dose-response experiment. The dose-response experiment demonstrates that very small additions of DIP stimulate bacterial production (Fig. 3B). The [^{3}H]TdR data for the

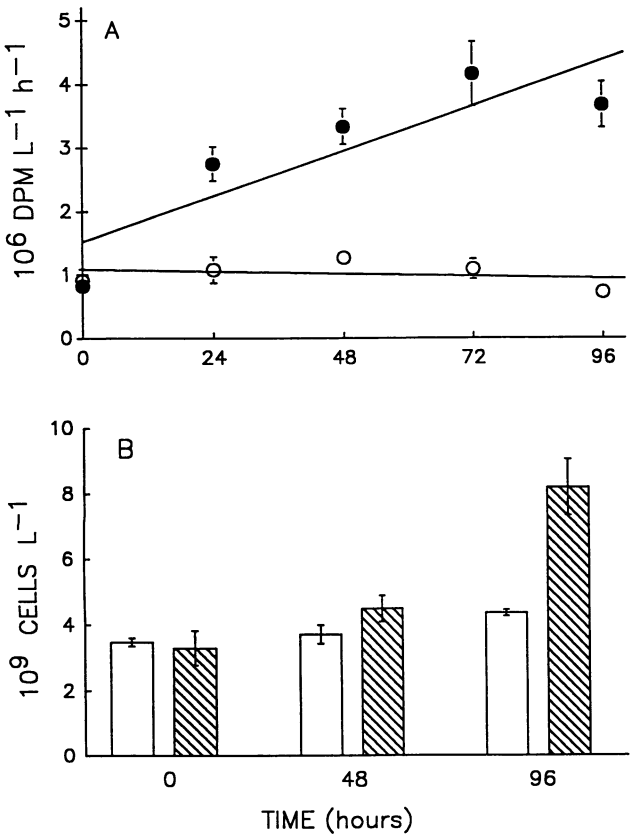

FIG. 1. (A) [^{3}H]TdR incorporation rates during the course of the May experiment. Open circles indicate the control (no P added); closed circles signify treatment (5 μM P added). Error bars show ±1 standard error; linear regression, n = 3. (B) Bacterial cell densities during the course of the May experiment. Open bars indicate the control (no P added); hatched bars represent treatment (5 μM P added). Error bars show ±1 standard error; n = 3.

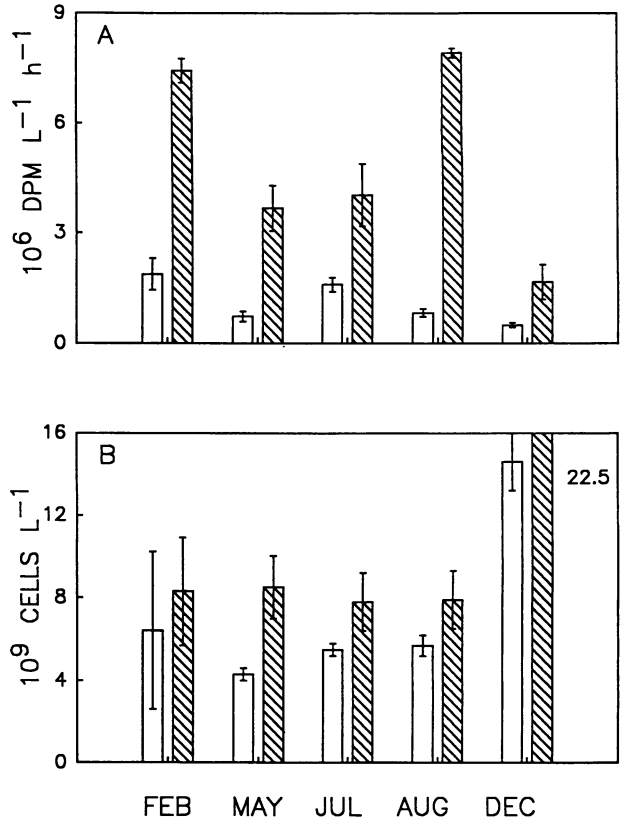

FIG. 2. [^{3}H]TdR incorporation rates (A) and bacterial cell densities (B) at the end (96 h) of each of the five basic experiments. Open bars indicate the control (no P added); hatched bars represent treatment (5 μM P added). Error bars show ±1 standard error; n = 3.

series of DIP additions done in July indicate that even a small amount of phosphate stimulated bacterioplankton production in these experiments. The lowest-concentration addition, 0.05 μM P, increased incorporation of [^{3}H]TdR to the same extent that was observed with the highest-concentration addition. Incorporation in the treatments (0.05, 0.10, and 5 μM P added) was significantly different from that in the controls ($P < 0.05$). Incorporation in the intermediate additions (0.5 and 1.0 μM) was not significantly different from that in the controls (no P added), probably because of the high variance in these observations (Fig. 3B).

Whole-lakewater experiment. In the August experiment with whole lakewater (large zooplankton removed but phytoplankton and microzooplankton included), [^{3}H]TdR incorporation rates were significantly increased by addition of DIP ($P < 0.05$; Fig. 4A). However, addition of DIP did not cause a significant increase in bacterial cell numbers in either whole lakewater ($P > 0.05$; Fig. 4B) or 1-μm-pore-size-filtered lakewater ($P > 0.05$; Fig. 2B).

While the additions of DIP increased [^{3}H]TdR incorporation relative to that in zero additions in both whole and 1-μm-pore-size-filtered lakewater, DIP also influenced the rates of production in whole versus filtered lakewater. With no added DIP, [^{3}H]TdR incorporation was greater in whole than filtered lakewater, but in the presence of 5 μM DIP, production in the filtered lakewater was greater than in whole lakewater (Table 1).

Independent measures of bacterioplankton production. To determine whether [^{3}H]TdR incorporation and cell dynamics were responding similarly to DIP additions, bacterial production was calculated from both the change in cell numbers and [^{3}H]TdR incorporation (assuming 2×10^9 cells produced per nmol of [^{3}H]TdR incorporated [10]). The two estimates are not significantly different in 8 of 12 possible comparisons. The agreement is good with the exception of the December and the August whole-lakewater values (Table 2).

DIP and DOC dynamics. There were no trends in DIP or DOC concentrations over 96 h in any of the five basic experiments. In the dose-response experiment, however, DIP decreased about 40% in the 0.05, 0.1, and 0.50 μM P treatments.

DISCUSSION

Addition of DIP significantly stimulated bacterial production, with strong responses to DIP addition in all five experiments. Addition of KH_2PO_4 caused three- to eightfold increases in the rate of incorporation of [^{3}H]TdR into DNA. The cell numbers at 96 h were significantly greater in DIP-treated flasks compared with those in controls in three of the five basic experiments. The results of the July dose-response experiment indicate that addition of as little as 0.05

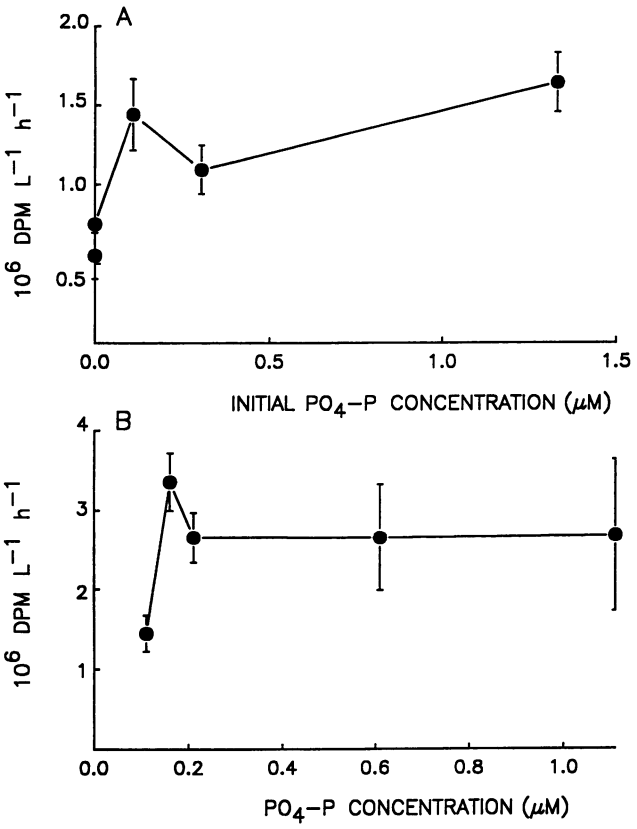

FIG. 3. [^{3}H]TdR incorporation rates at 72 h as a function of initial inorganic phosphorus concentrations in the control flasks for each of the five basic experiments (A) and as a function of inorganic phosphorus concentration in the dose-response experiment (B). Error bars show ±1 standard error; n = 3.

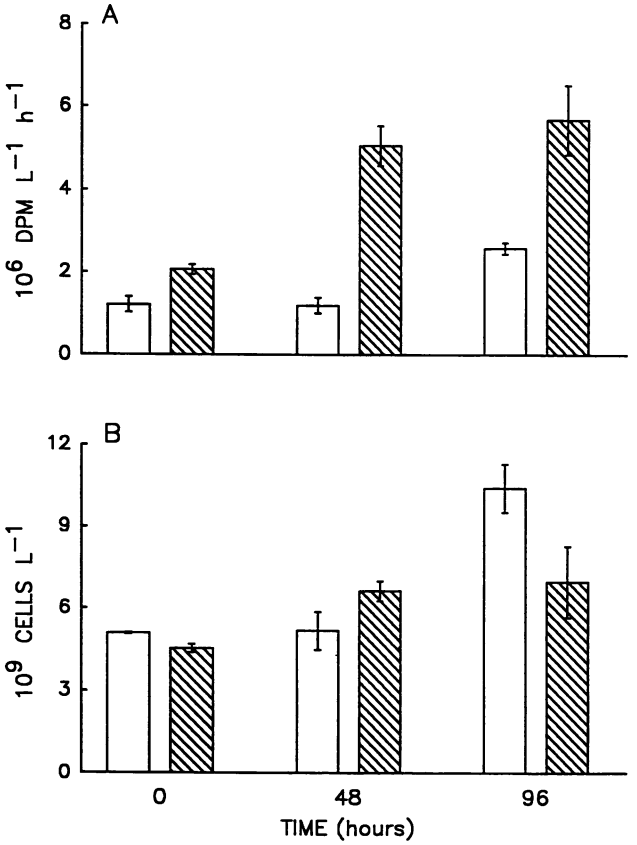

FIG. 4. [^{3}H]TdR incorporation rates (A) and bacterial cell densities (B) during the course of the August whole-lakewater experiment. Open bars indicate the control (no P added); hatched bars represent treatment (5 μM P added). Error bars show ±1 standard error; n = 3 for panel A; n = 2 for panel B.

μM P (~1.5 μg of P per liter) stimulates bacterial production to the same extent as addition of 5 μM P (Fig. 3B). Apparently, addition of 0.05 μmol of P is sufficient to meet the P demands of bacteria in this experiment. We can estimate P demand after converting [^{3}H]TdR incorporation to carbon production, assuming 20 fg of C per cell and assuming a 106:1 C/P molar ratio. Bacterial carbon production in the 0.05 μM treatment was 18.3 μg of C liter^{-1} day^{-1}. At this growth rate, bacteria would require about 0.04 μmol of P over the 3-day experiment. The total concentration available was 0.16 μM (0.11 + 0.05 added). Therefore, bacteria should deplete about 25% of P in the flasks. For this particular treatment, measured DIP concentrations declined by about 40%.

The fact that P additions greater than 0.05 μM P did not further stimulate [^{3}H]TdR incorporation suggests that secondary limiting factors are controlling bacterial production when excess P is available. We can estimate the amount of carbon and nitrogen required as bacteria use 0.04 μM P. Assuming a 106:16:1 C/N/P molar ratio and a 50% carbon growth efficiency, bacteria would require about 9.7 μM C and 1.3 μM N. If organic carbon availability limits bacterial production following addition of 0.05 μM P, then it implies that the available C pool is very small, only about 3% of the total DOC. Secondary N limitation is quite feasible in mid- to late summer, when NO_3 plus NH_4 in Calder Lake is less than 1.5 μM (16).

The responses in the control flasks (no P added) appear to be related to the initial DIP concentration (Fig. 3A). Consistently, higher concentrations of lakewater DIP correspond with greater rates of [^{3}H]TdR incorporation in the controls ($P < 0.01$). This complements the results of the dose-response experiment (Fig. 3) and supports the hypothesis that bacterioplankton production is limited by DIP in Calder Lake.

In a similar set of experiments, Coveney and Wetzel (3) added DIP to whole lakewater and monitored changes in [^{3}H]TdR uptake and in cell numbers. In their experiment, the rate of [^{3}H]TdR uptake increased with the addition of DIP but cell numbers did not. Our August whole-lakewater experiment approximates their experiment. Addition of 5

TABLE 1. [^{3}H]TdR incorporation rates in the August whole-lakewater and filtered-lakewater experiments at 96 h

DIP treatment	[^{3}H]TdR incorporation (10^6 dpm liter^{-1} h^{-1}) in[a]:	
	Whole lakewater	Filtered lakewater
None added	2.6	0.8
5 μM P added	5.7	7.9

[a] Values for each row and each column are significantly different at $P <$ 0.05.

TABLE 2. Bacterial production as determined from cell number and [^{3}H]TdR incorporation

Treatment and mo	Bacterial production (10^9 cells liter^{-1} day^{-1}) determined by:			
	Cell no.		[^{3}H]TdR incorporation	
	Mean	SD	Mean	SD
No P added				
February	0.7	2.0	0.5	0.1
May	0.3	0.1	0.3	0.0
July	−0.2	0.3	0.3	0.1
August	0.8	0.3	0.2	0.0
December[a]	2.3	0.5	0.1	0.0
August[a,b]	2.6	0.8	0.5	0.1
5 μM P added				
February	1.8	0.9	1.8	0.1
May	2.1	0.9	1.0	0.1
July	0.8	0.5	0.8	0.1
August	1.6	0.5	1.9	0.1
December[a]	6.7	2.6	0.4	0.0
August[a,b]	0.2	0.5	1.5	0.3

[a] Values determined by the two methods in this row are significantly different at $P < 0.05$.
[b] Whole-lakewater experiment.

μM P significantly increased [^{3}H]TdR incorporation, but there was no concurrent increase in cell densities. The presence of grazers could explain high growth rates without concomitant increases in cell numbers.

When the August whole-lakewater experiment is analyzed in conjunction with the basic (bacterium-only) August experiment (Table 1 and Fig. 2A and 4A), the results demonstrate that the presence of phytoplankton and/or microzooplankton has a significant effect on bacterial production. For the zero P addition controls, [^{3}H]TdR incorporation rates at 96 h are significantly greater in the whole compared with those in the filtered lakewater. For the added-P treatments, the incorporation rates for the filtered lakewater are significantly greater than those for the whole lakewater.

Microzooplankton in whole lakewater both graze on bacteria and regenerate DIP. In the absence of added P, the incorporation rates may be much higher in the whole lakewater than in the filtered lakewater because of grazer regeneration of DIP. At high DIP concentrations (5 μM P added), the incorporation rates for the filtered lakewater are greater than those of the whole lakewater, possibly because of heavy grazing pressure in the whole lakewater. One could hypothesize that microzooplankton have a positive effect at low DIP concentrations (nutrient regeneration) and a negative effect at high concentrations (grazing).

It is clear that while DIP may limit bacterioplankton production during certain times of the year in some freshwater systems, temperature, organic substrates, and grazing also modulate bacterial production. Most ecosystem models still invoke heterotrophic bacteria primarily as remineralizers, converting dissolved organic matter into inorganic nutrients and replenishing the nutrient supply available to other organisms (8, 15). On the other hand, phytoplankton are viewed as the major depleters of inorganic nutrients in the epilimnion. In light of this classical picture of inorganic nutrient sources and sinks, demonstration of direct DIP limitation of bacterioplankton production may add another level of complexity to the established system links.

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