Vol. 59: 271–284, 1990 | MARINE ECOLOGY PROGRESS SERIES
Mar. Ecol. Prog. Ser. | Published January 25

Incorporation and release of phosphorus by planktonic bacteria and phagotrophic flagellates

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ABSTRACT: Phosphorus binding by heterotrophic bacteria was studied in mixed assemblages of lakewater bacteria in continuous culture under C- and P-limitation and with different inocula (winter, summer). C:P ratios of the cultures varied from 80 to 100:1 under P-limitation and from 30 to 60:1 under C-limitation. Resulting estimates of bacterioplankton P-content suggest that bacteria can be regarded as a significant P-pool. This was supported by measurements of the P-content of bacterial communities in Lake Constance which showed that during summer on average 62 % of the particulate and 40 % of the total phosphorus in the euphotic zone was bound by bacteria. The partitioning of grazing to the regeneration of the bacterially bound phosphorus was evaluated in culture experiments with mixed bacterial populations and isolated strains of heterotrophic nanoflagellates under batch and 2-stage continuous culture conditions. P-release due to grazing was most efficient when bacteria were C-limited, leading to the regeneration of 50 to 90 % of the bacterial P. With P-limited bacteria, regeneration efficiency was highly variable and could be reduced to less than 20 %, depending on the kind of bacterial population. P-release in the absence of grazers occurred in a variable amount only from bacteria under carbon-starvation, parallel to a loss of biomass.

INTRODUCTION

It is generally assumed that most of the primary production in pelagic ecosystems is sustained by a continuous and rapid recycling of the growth-limiting inorganic nutrients. In the traditional view, bacteria have been considered the major agents effecting the rapid remineralisation of nutrients. However, doubts on this functional concept of the role of bacteria have been raised and supported by recent studies. Experimental and field results suggest that bacteria may act at least temporarily as sinks rather than as sources of nutrients.

The extent to which bacteria contribute to inorganic nutrient regeneration depends primarily on (1) the carbon:nutrient content of the organic substrates used by bacteria, (2) the efficiency of bacteria in converting these substrates into bacterial biomass, and (3) the carbon:nutrient ratio of the bacterial biomass.

Although our knowledge of the nature of the available organic substrates is still insufficient, it seems probable that at least in the pelagic environment they are constituted mainly by mineral-poor carbon compounds. In many natural waters the C:P ratio of dissolved organic matter (DOM) is > 500 (e.g. Lake Constance; epilimnion: 1000 to 2000). As a consequence the required amounts of N and P have to be taken mainly from the dissolved inorganic pools. Bacteria have been shown to be superior competitors to algae in the uptake of inorganic N (Wheeler & Kirchman 1986, Fuhrman et al. 1988) and P (Currie & Kalff 1984).

The view of bacteria acting as nutrient sinks in lakes and oceans is further supported by current estimates of bacterial biomass, which may often reach the same level as phytoplankton biomass (Simon & Tilzer 1987, Cho & Azam 1988). Also, the existing data on C:P (see Table 6) and C:N ratios (Nagata 1986, Lee & Fuhrman 1987) of bacteria suggest a high nutrient-binding capacity compared to algae.

Under these circumstances, algal production is expected to decrease in the presence of bacteria under nutrient-deficient situations, which is in agreement with experimental evidence (e.g. Bratbak & Thingstad 1985, Güde 1985). Hence, all processes favouring a reduction of bacterial nutrient-binding capacity should stimulate algal production. Of these processes, grazing appears to be one of the most efficient, because it results in a reduction of bacterial

0171-8630/90/0059/0271/$ 03.00

biomass. This is combined with a strong net regeneration of nutrients because grazers excrete nutrients which they receive in excess of their growth demands, as was already emphazised by Johannes (1965) and has been confirmed experimentally during recent years (e.g. Sherr et al. 1983, Goldman & Caron 1985, Güde 1985).

The objective of our study was to obtain an estimate of the bacterial P-pool of Lake Constance (FR Germany) as a model of a pelagic ecosystem and the potential P-recycling by heterotrophic nanoflagellates (HNF), probably the most important bacterivores in pelagic environments (Fenchel 1982, Sherr & Sherr 1984). The experimental approach for this purpose was to measure C:P ratios in mixed populations of lakewater bacteria grown under defined limitation conditions (C and P) and from different inocula (winter and summer plankton). Furthermore, the P-content of the natural bacterial community was estimated directly by determining particulate phosphorus in different size fractions.

To evaluate the regeneration of this bacterially bound phosphorus, we compared the net P-release of mixed bacterial populations in the absence and presence of bacterivorous flagellates in batch and continuous culture conditions.

The results strongly support the view of bacteria acting as P-sinks and of bacterivorous protozoa as efficient P-remineralizers. However, it also became obvious that the net regeneration efficiency seems to be largely dependent on the nutritional status and composition of the bacterial prey.

MATERIALS AND METHODS

Cultivation of bacteria. All culture experiments were carried out in a modified Chu 12 medium (Müller 1972) with the following constituents per liter: 16 mg $NaHCO_3$, 43 mg $Ca(NO_3)_2 \cdot 4H_2O$, 75 mg $MgSO_4$, 5 mg KCl and 2 ml of trace solution. Glucose was added to all cultures as the single carbon source at a concentration of 20 mg l^{-1}. Orthophosphate was added at 50 µg P l^{-1} (P-limitation) or 200 µg P l^{-1} (C-limitation). The media were sterilized by filtration through a 0.2 µm membrane filter. The inocula came from a pelagic sample station at 2 m depth in Lake Constance. For the first set of experiments a water sample was taken from the lake in January and a 2 ml subsample, 1 µm-Nuclepore prefiltered to exclude eucaryotes, served as inoculum for each culture (C- and P-limited). For the second set of experiments at the end of May the cultures were inoculated slightly differently. To assure protozoan-free inocula, and for a preselection towards species adjusted to low P-concentrations, 1 µm-filtered subsamples were pre-cultivated on P-deficient (2 µg P l^{-1}) Chu 12 medium supplemented with glucose (20 mg l^{-1}). After 5 d a 1 ml subsample from a definitely protozoan-free culture served as inoculum for the P-limited chemostat. After continuous growth for about 7 generations the C-limited chemostat was inoculated with 1 ml suspension from the P-limited chemostat. Each continuous culture system consisted of a 20 l medium reservoir, a 5 l culture vessel with a working volume of 3 l and an outflow vessel. Aeration and stirring of the cultures was provided by bubbling with sterile air. The chemostats were run at a dilution rate of 1 d^{-1} at 20 °C.

Analyses. Bacteria and HNF were counted by epifluorescence microscopy on formalin-fixed (1.5 % [w/v] final concentration) and diamidinophenylindole (DAPI)-stained samples (Porter & Feig 1980).

Soluble reactive phosphorus (SRP) was determined by the molybdenum reagent method (Vogler 1965). Total dissolved phosphorus (TDP) was analyzed after wet oxidation of a 0.2 µm culture filtrate by autoclaving for 1 h at 120 °C in the presence of potassium peroxodisulfate (0.15 % (w/v) final concentration). Particulate phosphorus was calculated as the difference of medium-P to TDP and spot-checked by direct determination after collection of bacteria on 0.2 µm membrane filters (Schleicher and Schüll) and subsequent oxidation with H_2O_2 and H_2SO_4 (Schmid & Ambühl 1965). After digestion of organic and particulate phosphorus, SRP was measured as described above.

For measuring bacterial carbon content, subsamples (30 to 100 ml) were filtered on a GF/F Whatman filter (Lee & Fuhrman 1987). The filters were dried and analyzed for organic carbon in a CHN-analyzer (Perkin Elmer). Data were corrected for biovolumes of bacteria passing the filter (generally < 30 %). Bacterial cell volumes in the GF/F-filtrates and in the cultures were measured with an image analyzer (Krambeck et al. 1981). Blanks were made with 0.2 µm-filtered culture fluid to correct for organic solubles in the cultures.

For determining viable cell counts, samples from the cultures were occasionally spread on CPS-agar to count colony forming units (CFU) after 1 wk incubation at 20 °C.

Alkaline phosphatase activity (APA) in the bacterial cultures was measured according to Chrost & Overbeck (1987), using methyl-umbelliferyl-phosphate as substrate.

Field measurements. Analyses of dissolved and particulate P in the epilimnion of Lake Constance were regularly (generally weekly) performed from October 1987 to December 1988. For measuring the P-content in the epilimnic bacterial community of Lake Constance, water samples were taken from a pelagic site at 2 m depth. The sample was filtered through a 1 µm Nuclepore filter (47 mm) to separate bacteria from other planktonic organisms. For the determination of particu-

late phosphorus, 0.5 to 1 l of the 1 µm filtrate was filtered on to a 0.2 µm membrane filter (Schleicher and Schüll) and analyzed as described above. Bacterial cell numbers in the water samples were determined as described above.

Grazing experiments. ***Batch culture:*** The mixed bacterial cultures from the chemostats were used as prey for all grazing experiments. Two species of heterotrophic bacterivorous nanoflagellates, isolated by enrichment and serial dilution from Lake Constance and belonging to the genera *Monas* and *Bodo*, were used as predators. For grazing experiments under batch culture conditions 400 ml bacterial suspension was taken from a chemostat and transferred to a batch vessel. The batch cultures were provided with oxygen and kept in suspension by a magnetic stirring system and maintained at a temperature of 20 °C. After inoculation with flagellates (pre-grown on the same bacteria), population development of bacteria and HNF and concentrations of SRP and TDP were monitored over a period of about a week.

Continuous culture: For grazing experiments under continuous culture conditions two 450 ml culture vessels were connected as second stages with each bacterial chemostat. Bacterial suspension was transported with peristaltic pumps from the first stage (bacteria) to the second stage (bacteria + flagellates). Culture fluid from the first stage, not used for the smaller second stages, was transported to an outlet vessel. To calculate grazing and P-regeneration rates, concentrations of bacteria, HNF, SRP and TDP were measured in both stages over a time course of 2 to 3 wk as described above.

Calculations. ***Batch culture:*** For the data analyses the typical growth curve of the flagellates in batch culture was divided by visual inspection into the 2 most pronounced growth phases. Growth Phase 1, including roughly the lag and the exponential phase, was defined from the inoculation with HNF until the point where the bacterial prey was reduced to a low level, beyond which grazing was probably strongly reduced. The remaining course of an experiment was defined as Growth Phase 2, approximately corresponding to the stationary phase of the flagellates.

The percentage of bacterial phosphorus released was calculated from the increase of dissolved P in the cultures during the course of the experiments, according to:

$$F_P\ (\%) = (TDP_1 - TDP_0) \times P_B^{-1} \times 100$$

where TDP_0 and TDP_1 = concentrations of total dissolved phosphorus (µg P l^{-1}) at the beginning and at a time point of the experiment; P_B = bacterial bound phosphorus (µg P l^{-1}) at the beginning of the experiment.

P-ingestion by the flagellates occurred nearly exclusively during the exponential growth phase and was determined by the expression:

$$I_P = Q_P \times (B_0 - B_1)$$

where B_0 and B_1 = bacterial concentrations (cells ml^{-1}) at the beginning of the experiment and at the time point with the lowest bacterial cell number, generally at the end of Growth Phase 1; Q_P = phosphorus cell quota for the bacterial prey at the beginning of the experiments (pg P $cell^{-1}$).

Net-P-excretion was calculated from the increase of total dissolved phosphorus:

$$E_P = TDP_1 - TDP_0$$

where TDP_0 and TDP_1 = concentrations of total dissolved phosphorus at the beginning and at a time point during the course of the experiment. The P-regeneration efficiency (%) was calculated by the expression:

$$R_P = (E_P \times I_P^{-1}) \times 100$$

For the maximum P-regeneration, R_P was calculated for the highest measured TDP-concentration during the course of the experiments.

Continuous culture: At given steady-state conditions the specific growth rate of the flagellates (µ) equals the dilution rate (D) of the second chemostat stage (Curds & Cockburn 1971).

Assuming complete mixing and negligible death and growth of the bacteria in the second stage, the protozoan ingestion rate I_B (bact. × HNF^{-1} × h^{-1}) can be determined by the expression:

$$I_B = D \times (B_1 - B_2) \times N^{-1}$$

where B_1 and B_2 = bacterial concentrations (cells ml^{-1}) in the first and the second chemostat stage, respectively; N = number of flagellates (cells ml^{-1}) in the second stage.

P-ingestion (µg P l^{-1} d^{-1}), was calculated by the expression:

$$I_P = (B_1 - B_2) \times Q_P \times D$$

where Q_P = phosphorus cell quota of the bacteria (pg P $cell^{-1}$); D = dilution rate (d^{-1}).

Net-P-excretion (µg P l^{-1} d^{-1}) was calculated according to:

$$E_P = (TDP_1 - TDP_2) \times D$$

where TDP_1 and TDP_2 = concentrations of total dissolved phosphorus in Stages 1 and 2, respectively.

Estimates of the P-regeneration efficiency (%) were made from the following equation:

$$R_p = (E_P \times I_P^{-1}) \times 100$$

RESULTS

Bacterial phosphorus binding

Culture experiments

The chemostats were run over a period of 8 to 10 wk to monitor population development and bacterially bound P. Under P-limitation bacterial populations exhibited high APA-activity (1.5 to 2.5 μM PO_4^{3-} h^{-1}) and SRP-concentrations generally below the detection limit (< 1 μg P l^{-1}). The C-limited cultures showed rather high SRP-concentrations and no detectable APA-activity. Plate counts and microscopical observations indicated that different bacterial populations developed in the C- and P-limited cultures and from winter and summer inoculum.

Compared to the natural assemblage the bacterial populations in the chemostats had drastically changed. The percentage of bacteria, capable of growing on CPS-agar plates increased from ca 0.1 % in the lake to between 80 and 100 % in the cultures. The mean cell volume in the different cultures varied between 0.11 and 0.26 μm^3 (Table 1), whereas in the lake it is in the range of 0.03 to 0.1 μm^3 (Simon 1987). The species diversity decreased to a few species and generally one colony type dominated by more than 70 %. A real steady state was not attained over the whole culturing period. However steady state-like phases occurred regularly, lasting between 1 and about 2 wk (7 to 14 generation times), then interrupted by shifts of cell number and biomass. The variation of cell number over the whole culture period was especially high in the P-limited cultures (Table 1).

The bacterial P-content was rather similar in all cultures (3 to 4 fg P $cell^{-1}$), except the C-limited culture from the winter inoculum (Table 1). Those bacteria were much smaller and had a lower Q_P value (1.3 fg P $cell^{-1}$). C:P ratios of the P-limited cultures varied only within a narrow range, with slightly higher values in the summer cultures (Table 1). The C-limited bacteria had lower and more variable C:P ratios, with obvious differences between summer and winter inocula. the larger bacteria from the summer inoculum had a significantly lower C:P ratio than the bacteria from the winter inoculum (Table 1).

Field results

Bacteria from winter and summer inocula came from very different field situations in Lake Constance, as shown in Table 2. The winter plankton was characterized by very low primary production, and low bacterial and HNF numbers. Due to the mixing of the whole water layer the SRP and TDP concentrations had reached the highest values of the year. The bacteria from the summer inoculum came from a situation at the end of May where primary production had reached maximum values shortly before the clear-water phase. Bacterial and protozoan populations had also high densities whereas dissolved phosphorus was already reduced to very low levels.

The separation of bacteria from other seston particles, for the determination of bacterially bound P, could be achieved quite successfully by the 1 μm filtration. Microscopical examinations revealed that virtually no non-bacterial particles (eucaryotes, cyanobacteria, detritus, inorganic particles) were present in the < 1 μm fraction. The percentage of bacteria retained on the filter varied between 10 % in summer and up to 30 % in spring. The values of bacterial P (BP) (Fig. 1) are not corrected for these losses so they can be seen as minimum estimates for the bacterial P-fraction.

The concentration of total particulate phosphorus (TPP) increased with the development of the spring bloom at the end of April, remained on a high level until the decline during the clear-water phase at the end of May and increased again with irregular fluctuations during summer until the winter mixing (Fig. 1).

Table 1. Ranges of C:P ratios and mean cell volume, average value and standard deviation of cellular P-content (Q_P) and cell number (N_B) of the mixed bacterial cultures in continuous cultivation

Culture	C:P ratio (molar)	Cell volume (μm^3)	Q_P (fg P $cell^{-1}$)	N_B (cells ml^{-1})
P-limited winter inoculum	80:1	0.20–0.23	3.5 ± 0.7	14.2 ± 3.5
C-limited winter inoculum	56–61:1	0.11–0.12	1.3 ± 0.2	97.7 ± 8.1
P-limited summer inoculum	81–96:1	0.21–0.26	3.0 ± 0.8	17.2 ± 5.1
C-limited summer inoculum	34- 41:1	0.17–0.23	3.9 ± 0.4	48.2 ± 5.9

Table 2. Field situation in Lake Constance during sampling of winter inoculum (January) and summer inoculum (May) for the chemostat cultures

	Primary production ($\mu g\ C\ l^{-1}\ h^{-1}$)	Bacteria ($10^6\ l^{-1}$)	HNF ($10^3\ l^{-1}$)	SRP ($\mu g\ P\ l^{-1}$)	TDP ($\mu g\ P\ l^{-1}$)
January	< 1	1.3	< 1	41	46
May	31	9.0	11.5	< 1	5

Concentrations of BP increased 2 wk after the peak of TPP. From June to October concentrations of TPP varied between 4.8 and 27.1 $\mu g\ P\ l^{-1}$ and of BP between 3.1 and 20.1 $\mu g\ P\ l^{-1}$. Because of fairly equal seasonal variations of TPP and BP the contribution of the bacterial fraction to the total particulate phosphorus was very high throughout this time, on average 62 % (range 41 to 96).

Concentration of total P (total dissolved P + total particulate P) declined after the spring bloom from about 50 to nearly 10 $\mu g\ P\ l^{-1}$ in summer (Fig. 1). Dissolved P during summer consisted mainly of organic P (mostly in the range 3 to 6 $\mu g\ P\ l^{-1}$) because SRP was generally below the detection limit (< 1 $\mu g\ P\ l^{-1}$; data not shown). Because of low dissolved P concentrations, the contribution of bacterial P to total P is also significant during that time of the year. Between June and October on average 40 % (range 20 to 66) of the total P consisted of bacterially bound phosphorus. Therefore, the bacterioplankton can be regarded as the most important P-pool during the summer P-depletion.

Phosphorus regeneration

Without grazing

In our experiments, designed to evaluate net-regeneration of bacterially bound phosphorus, the bacteria had a defined 'life history', determined by the limitation during continuous cultivation. When these bacteria were transferred from the chemostat to a batch vessel their growth slowed down or stopped because of substrate exhaustion. This is evident from the grazer-free control experiments (Figs. 2B to 5B). Cell numbers stayed more constant in the P-limited cultures (Figs. 2B and 4B), presumably due to supply of excess glucose, enabling some maintenance metabolism. Bacteria growing under C-limitation sometimes showed a slight increase of cell numbers, but this was always accompanied by a decrease of mean cell size and optical density (Fig. 3B), and thus was probably caused by starving divisions after the exhaustion of the substrate glucose. The loss of biomass, induced by C-starvation, was quite different in the populations derived from winter or summer inocula. With C-limited bacteria from the winter inoculum a significant biomass decrease occurred only at the end of the experiments (Fig. 3B) whereas with bacteria from the summer inoculum a more rapid and pronounced, but highly variable, decrease of cell numbers and cell size was observed (Figs. 5B and 6). The loss of biomass during carbon-starvation was generally followed by a release of phosphorus. In the extreme example shown in Fig. 5B the bacterial biomass dropped down to about 20 % of the initial value, paralleled by a large amount of released P. But generally the biomass decrease and the resulting P-release was lower, as shown in Fig. 6, where the C-limited bacteria from the summer inoculum were maintained for 2 wk in starvation and then inoculated with HNF (arrow). Here P-release started later and remained on a plateau between 20 and 25 % until the flagellates were inoculated. In all experiments with P-limited bacteria no significant P-release was detected, which is in agreement with the constant biomass over the course of the experiments (Figs. 2B and 4B).

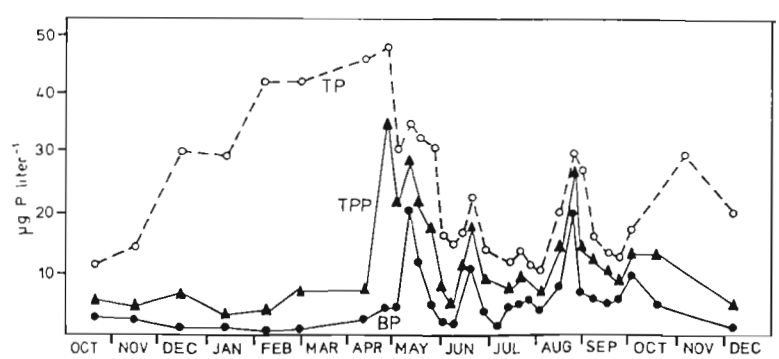

Fig. 1. Development of total P (TP; ○—○), total particulate P (TPP; ▲—▲) and bacterial P (BP; ●—●) in Lake Constance (pelagial, 2 m depth) from October 1987 to December 1988

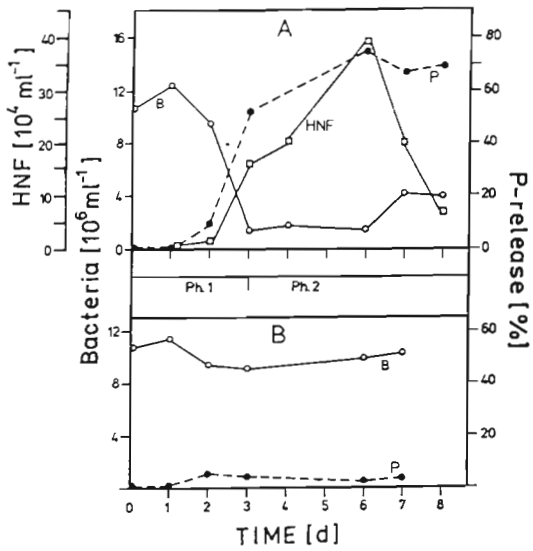

Fig. 2. P-regeneration experiment with P-limited bacteria (winter inocolum) in batch culture. (A) With HNF (*Monas* sp.); (B) without grazers. Development of bacteria (B; ○—○), HNF (□—□) and P-release (P; ●---●). Separation in exponential (Ph. 1) and stationary (Ph. 2) growth phase of HNF. Time zero indicates when bacteria were transferred from the chemostat to the batch vessel

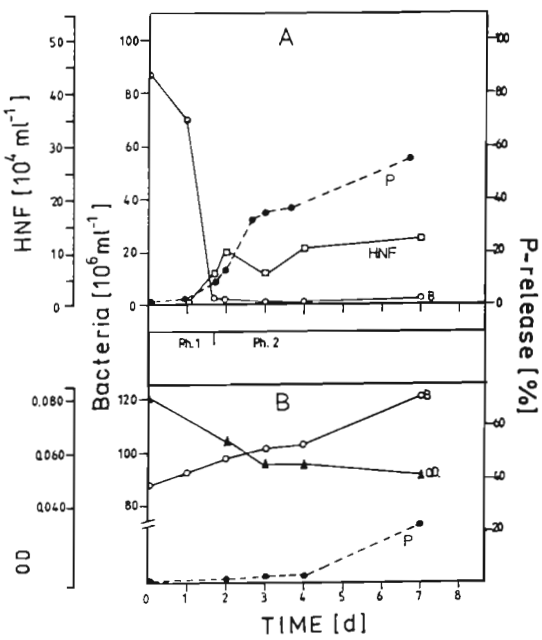

Fig. 3. P-regeneration experiment with C-limited bacteria (winter inoculum) in batch culture. (A) With HNF (*Bodo* sp.); (B) without grazers. Details as in Fig. 2; in B, course of the optical density (OD; ▲—▲) is also shown

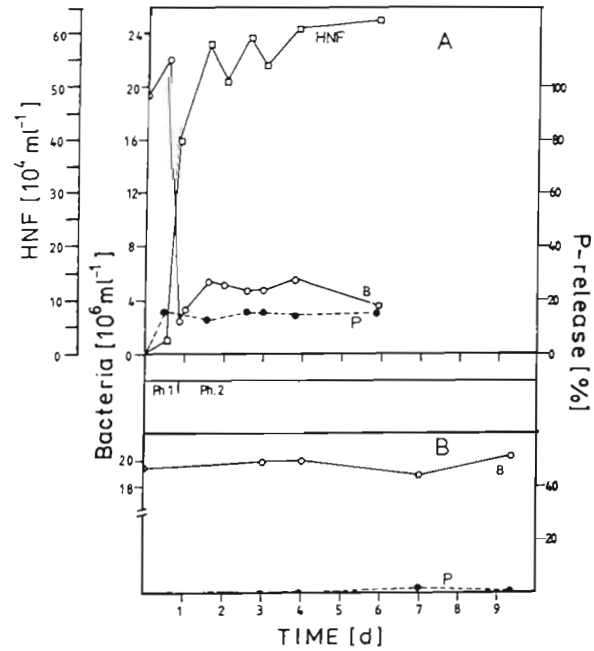

Fig. 4. P-regeneration experiment with P-limited bacteria (summer inoculum) in batch culture. (A) With HNF (*Monas* sp.); (B) without grazers. Details as in Fig. 2

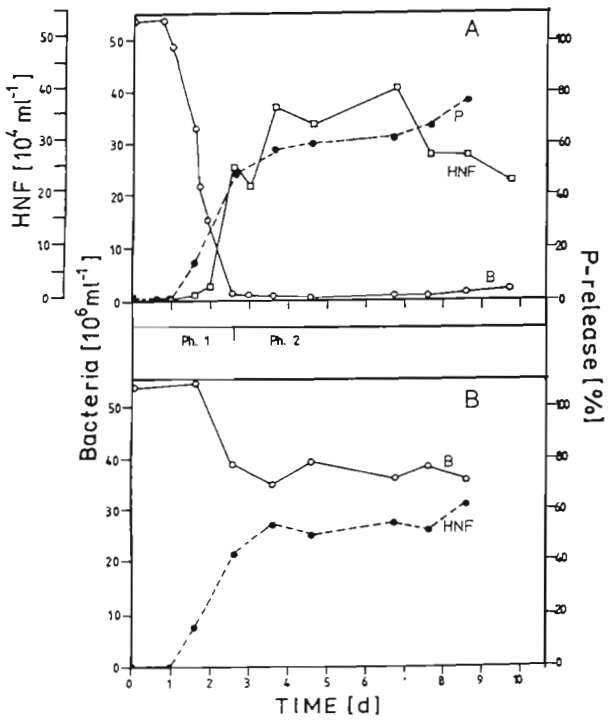

Fig. 5. P-regeneration experiment with C-limited bacteria (summer inoculum) in batch culture. (A) With HNF (*Bodo* sp.); (B) without grazers. Details as in Fig. 2

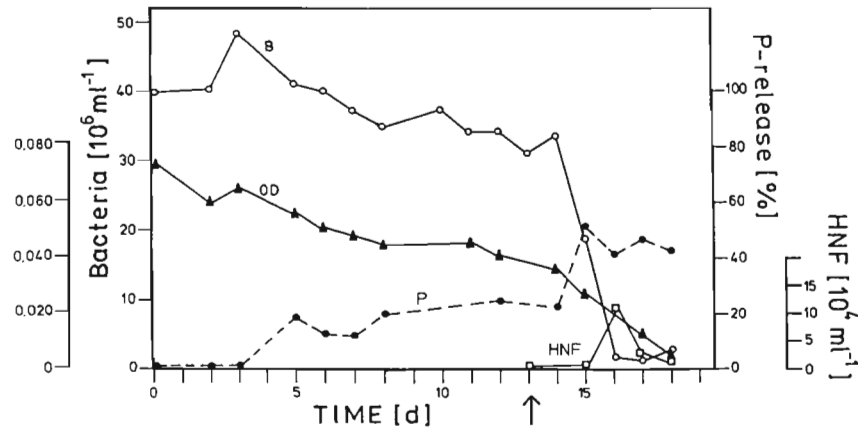

Fig. 6. P-regeneration experiment with C-limited bacteria (summer inoculum) in batch culture. HNF were inoculated after 13 d in batch culture (arrow). Details as in Fig. 2; course of the optical density (OD; ▲—▲) is also shown

With grazing

Heterotrophic nanoflagellates were inoculated immediately after transferring the bacteria from the chemostat to the batch vessel. The exponential growth phase of the flagellates (Phase 1) started after a lag phase of generally about 1 d and resulted in a rapid decline of bacteria (Figs. 2A to 5A). The remaining abundance of bacteria at the end of exponential HNF growth was generally below 1×10^6 ml^{-1} in the C-limited cultures but 2 to 4×10^6 ml^{-1} with P-limited bacteria. After depletion of the bacterial prey the flagellates reached the stationary phase (Phase 2) with subsequent irregular fluctuations. With both HNF species a development of small starving protist cells was frequently observed, resulting in sometimes still-increasing HNF numbers during Phase 2.

With the appearance of HNF in the cultures, the concentration of total dissolved phosphorus increased. Except for the experiments with P-limited bacteria from the summer inoculum the amount of released P increased throughout all growth phases of the flagellates until the end of an experiment. As shown in Figs. 2A to 5A, net P-release due to grazing was high in the experiments with C-limited bacteria (from both inocula) and with P-limited bacteria from the winter inoculum. During these experiments 50 to 90% of bacterially bound phosphorus was released as DOP and SRP. In contrast to these results the P-release from P-limited bacteria of the summer inoculum was only in the region of 20% (Fig. 4A).

There were no significant differences in the amount of released P between the 2 predators *Monas* sp. and *Bodo* sp. Only the time period of P-regeneration sometimes showed differences. Regeneration efficiency with *Bodo* was generally lower than with *Monas* during exponential growth; P-release occurred later in the stationary phase (Table 3).

Overall, the maximum P-regeneration efficiency depended more on limitation and origin of the bacterial prey than on the HNF-species used as predators.

Most of the excreted P consisted of SRP. In experiments with P-limited bacteria the proportion of SRP to total released phosphorus was at the end of the experi-

Table 3. Summarized results from the batch grazing experiments. P-release during HNF Growth Phase I and II and maximum P-regeneration efficiency R_P

Bacteria	HNF	P-release (%)		R_P (%)
		I	II	
P-limited winter inoculum	*Monas*	31–60	63–80	63–91
	Bodo	17–22	53–56	57–59
C-limited winter inoculum	*Monas*	34–50	67–83	71–84
	Bodo	13–38	65–87	68–89
P-limited summer inoculum	*Monas*	6–16	14–29	18–37
	Bodo	11–13	13–14	19–22
C-limited summer inoculum	*Monas*	27–32	51–66	52–70
	Bodo	50	77	79

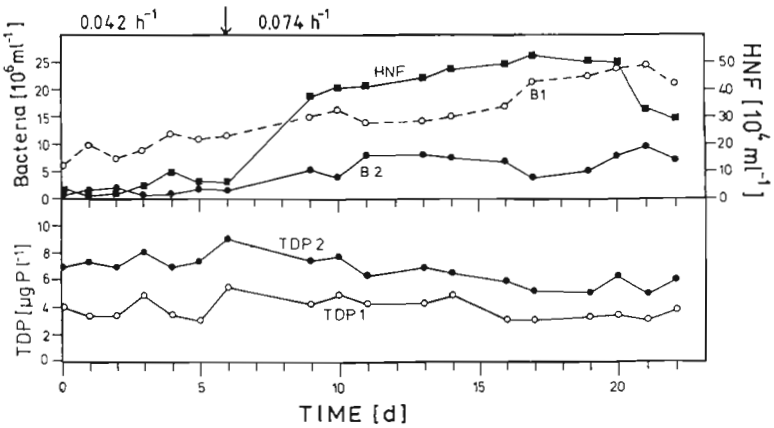

Fig. 7 Grazing experiment in 2-stage continuous cultivation with P-limited bacteria (summer inoculum) and *Monas* sp. Concentrations of bacteria in Stage 1 (B1; ···) and Stage 2 (B2; •—•), HNF in Stage 2 (▪—▪), total dissolved P (TDP) in Stage 1 (○—○) and Stage 2 (•—•) at dilution rates of 0.042 h^{-1} (Days 0 to 6) and 0.074 h^{-1} (Days 7 to 22)

ments in the range 32 to 91 % (mean 59 %), with C-limited bacteria in the range 69 to 100 % (mean 82 %). The percentage of SRP from TDP varied during the course of the experiment but generally increased with time (data not shown).

Grazing experiments in continuous cultivation

Continuous cultures were run only with the bacteria from the summer inoculum as prey. Because of the fluctuations already occurring in the first (bacterial) stage, no real steady state conditions could be expected for the second stages. However, as shown for the experiments with *Monas* in Figs. 7 and 8, fairly stable densities and hence steady state-like conditions were observed for a given dilution rate, at least over a period of several days (corresponding to > 4 volume changes). Parameters for HNF-growth and P-regeneration were calculated for such relatively stable phases during the chemostat runs with a variation of the bacterial densities and P-concentrations of less than 10 %, and are listed in Table 4 (*Monas*) and Table 5 (*Bodo*).

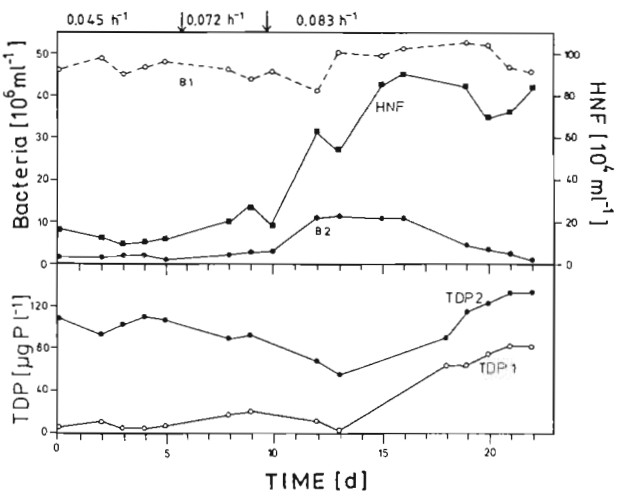

Fig. 8. Same as Fig. 7 but with C-limited bacteria (summer inoculum) and *Monas* sp. at dilution rates of 0.045 h^{-1} (Days 0 to 6), 0.072 h^{-1} (Days 7 to 10) and 0.083 h^{-1} (Days 11 to 22)

Monas sp. showed a characteristic growth pattern in the chemostat experiments. The HNF-concentration always increased dramatically with increasing dilution rate (Figs. 7 and 8) whereas with *Bodo* sp. more irregular fluctuations of cell numbers, irrespective of the dilution rate, occurred (not shown).

The amount of P-regeneration was independent of the HNF species used but, as in the batch experiments, dependent on the limitation of the bacterial prey. TDP-concentrations always increased in the second stage but with P-limited bacteria the increase was in the range of only 2 to 4 µg P l^{-1}, resulting in P-regeneration efficiencies less than 10 % (Tables 4 and 5). With C-limited bacteria the TDP-increase in the second chemostat stage was in the range of 40 to about 110 µg P l^{-1} and calculated P-regeneration efficiencies ranged from 23 to 60 %, with higher values for lower growth rates and for *Monas* sp. (Tables 4 and 5).

Calculated grazing rates also decreased with higher growth rate for *Monas* sp. (Table 4) but not for *Bodo* sp. (Table 5). The range of bacterial ingestion rate for all experiments was 1.5 to 17.6 bact. HNF^{-1} h^{-1} for *Monas* and 2.9 to 8.1 bact. HNF^{-1} h^{-1} for *Bodo*.

The results from grazing experiments in 2-stage continuous cultures confirmed the results obtained in the batch experiments, where with the bacterial cultures from the summer inoculum high P-regeneration was also achieved only with C-limited bacteria as prey.

DISCUSSION

Bacterial P-binding

To understand the functional properties of bacteria in the cycling of inorganic nutrients it is important to know what fraction of N and P in the production zone of

Table 4. Results of 2-stage continuous culture experiments with *Monas* sp. and P- and C-limited bacteria

	P-limited bacteria		C-limited bacteria	
Specific growth rate μ (d^{-1}):	1.0	1.8	1.1	1.8
Bacteria in Stage 1 (10^6 bact. ml^{-1})	10.9 ± 1.4	15.3 ± 1.2	47.4 ± 1.7	45.2 ± 3.9
Bacteria in Stage 2 (10^6 bact. ml^{-1})	1.4 ± 0.6	6.7 ± 1.6	3.2 ± 0.6	6.9 ± 2.3
HNF in Stage 2 (10^4 HNF ml^{-1})	3.3 ± 1.1	43.5 ± 4.6	12.5 ± 1.5	55.4 ± 7.2
Ingestion rate (bact. HNF^{-1} h^{-1})	11.8 ± 2.3	1.5 ± 0.5	15.8 ± 2.1	5.3 ± 0.6
Total diss. P Stage 1 (µg P l^{-1})	4.5 ± 1.4	4.3 ± 0.7	7.4 ± 3.0	17.9 ± 14.8
Total diss. P Stage 2 (µg P l^{-1})	7.9 ± 0.9	6.8 ± 0.6	123.5 ± 15.7	84.9 ± 18.4
P-regeneration efficiency R_P (%)	9.1 ± 1.8	9.7 ± 2.4	60.3 ± 9.6	40.6 ± 3.4

marine and lake ecosystems is bound by bacterioplankton. The amount to which growth-limiting nutrients are tied up in bacterial biomass has strong implications for the overall productivity of the system as well as for the relevance of bacterial grazers as remineralizers.

In our studies we focused on the P-pool of pelagic bacteria, which we tried to assess in Lake Constance by a combined laboratory and field approach. The laboratory approach taken here was designed to examine the influence of the preculture conditions experienced by the bacterial inoculates (winter: P-excess; summer: P-deficiency) and of the actual growth limitation (P and C-limitation) on the bacterial P-content. Due to the different populations in the mixed cultures as a result of the different limitations and inocula, quite distinct nutritional requirements and species compositions had to be expected and were indeed observed. However, in spite of these differences, the observed C:P ratios fluctuated in a fairly narrow range (Table 1). As expected the bacteria always had a higher C:P ratio under P- than under C-limitation. The difference is especially obvious in the cultures of the summer inoculum, where the bacteria had a longer 'life history' with P-deficiency. A certain variability in the chemical composition of bacteria, depending on environmental conditions, is well documented (Tempest & Njeissel 1978). In the P-

Table 5. Results of continuous culture experiments with *Bodo* and P- and C-limited bacteria

	P-limited bacteria		C-limited bacteria	
Specific growth rate μ (d^{-1}):	1.1	2.3	1.0	2.2
Bacteria in Stage 1 (10^6 bact. ml^{-1})	16.7 ± 0.5	17.1 ± 1.3	52.3 ± 1.4	32.5 ± 1.0
Bacteria in Stage 2 (10^6 bact. ml^{-1})	4.6 ± 1.4	5.0 ± 0.5	2.7 ± 0.6	4.7 ± 0.6
HNF in Stage 2 (10^4 HNF ml^{-1})	21.7 ± 5.7	15.9 ± 4.1	42.2 ± 2.6	36.8 ± 6.5
Ingestion rate (bact. HNF^{-1} h^{-1})	2.9 ± 1.1	8.1 ± 2.7	5.0 ± 0.2	7.1 ± 2.0
Total diss. P Stage 1 (µg P l^{-1})	2.3 ± 0.1	3.0 ± 0.5	75.7 ± 3.4	93.0 ± 5.7
Total diss. P Stage 2 (µg P l^{-1})	5.0 ± 0.6	5.3 ± 1.7	141.3 ± 9.4	125.6 ± 3.8
P-regeneration efficiency R_P (%)	6.5 ± 1.6	6.8 ± 3.6	38.4 ± 6.6	22.9 ± 5.8

Table 6. Bacterial C:P ratios in the literature

Bacteria	Limitation	C:P ratio (molar)	Source
Aerobacter aerogenes	Mg^{2+} P	56–81:1 47–133:1	Dicks & Tempest (1966)
Bacillus subtilis	K^+ P	37–69:1 57–100:1	Tempest et al. (1968)
Pseudomonas aeruginosa	P	27–43:1	Fuhs et al. (1972)
Corynebacterium bovis		12–26:1	Chen (1974)
Arthrobacter globiformis	unlim.	47:1	Van Veen & Paul (1979)
Pseudomonas putida	C P	16:1 500:1	Bratbak (1985)
Mixed culture (marine)	C P	8:1 55:1	Bratbak (1985)
Escherichia coli	C	63:1	Heldal et al. (1985)
Vibrio natriegens	P	32–104:1	Nissen et al. (1987)
Bacterioplankton		7–76:1	Vadstein et al. (1988)
Mixed culture	C P	30–60:1 80–100:1	This study

limited cultures a selection in favour of those bacteria which can minimize their cellular P-content without reducing the growth rate is expected. The highest C:P ratios in the P-limited cultures were in the region of 100:1 (Table 1), which is in good agreement with data reported in the literature (Table 6). Very low C:P ratios can be found when bacteria are able to accumulate large amounts of surplus P, in the form of polyphosphate granula, as was demonstrated for several species (Kulaev & Vagabov 1983). But this capability is apparently limited to a few species which are extremely effective in accumulating surplus P. In the present study the observed C:P ratios for C-limited bacteria were in the range of 30 to 60:1 which agrees well with most literature data (Table 6). The lowest values (about 30:1) were found for the C-limited bacteria from the summer inoculum. Their preceeding P-limited precultivation could have been the reason for accumulating more surplus P than the C-limited bacteria from the winter inoculum.

According to these data it appears that bacteria have, as compared to algae, only a rather limited ability to change their P-content in response to environmental conditions. Reduction of the bacterial P-content is probably limited by the large proportion of non-replaceable P-containing cell compounds (e.g. nucleic acids). On the other hand, the small size of aquatic bacteria limits the space available for the accumulation of large amounts of excess P much more than for algae.

To demonstrate the significance of the bacterial C:P ratios for the lake ecosystem, we can transfer the experimentally derived values to the natural situation. Simon & Tilzer (1987) estimated bacterial carbon for Lake Constance and found values often exceeding 100 µg C l^{-1}, although these values were calculated with conservative conversion factors. With a mean bacterial carbon of 100 µg C l^{-1} and a mean C:P ratio of 80:1 we would expect to find 3.2 µg P l^{-1} in bacteria. This value would increase to 6.4 µg P l^{-1} with a C:P ratio of 40:1. During summer P-depletion these values correspond to roughly 30 to 60 % of the total P in the euphotic zone of Lake Constance.

These values were indeed found when we directly estimated the bacterial P-content by size-fractionation. As is shown in Fig. 1 bacteria constitute the most important pelagic P-pool in summer with generally more than 50 % of particulate P. Even when the total P (dissolved and particulate) is considered, the bacterial fraction represents more than 30 % during the summer P-depletion. Although the preconditions for size fractionation are comparatively favorable in Lake Constance, it has to be emphasized that the resulting P-content must be regarded as a minimum value due to the retention of 10 to 30 % of bacteria on the 1 µm filters.

Data from other aquatic ecosystems on the bacterial P-pool are scarce. The study of Vadstein et al. (1988) in a eutrophic Norwegian lake revealed similar results with a contribution of bacteria to total particulate phosphorus in the range 20 to 60 % (mean 30 %). Much lower P-binding capacities were estimated for bacteria of Lake Kinneret (Israel) by Berman (1985). However, due to a lower carbon conversion factor, here bacterial

biomass was also estimated to be one order of magnitude lower than in Lake Constance.

Because of similar low C:N ratios in bacteria (Lee & Fuhrman 1987), the contribution of the bacterial N-pool should be also high in N-limited marine environments. Especially for oligotrophic and mesotrophic aquatic ecosystems, where POC is often dominated by bacterial carbon (Azam et al. in press), we must expect to find a high proportion of the inorganic nutrients in the bacterioplankton.

P-regeneration

The demonstrated high P-binding capacity of bacterioplankton makes obvious that the recycling of phosphorus in Lake Constance cannot be understood without consideration of the role of bacteria. The understanding of the fate and dynamics of the bacterial P-pool is important especially during the summer P-depletion where bacteria constitute the major part of particulate P in the euphotic zone.

Possible mechanisms for the release of bacterial P to the environment are 'autolysis', excretion, starvation and grazing. Little is known on the extent of bacterial death by 'autolysis' in the natural environment, but up to now convincing evidence for the importance of this death factor is lacking (Güde 1986, Kjelleberg et al. 1987, Pace 1988). Considering again the high C:P ratios of natural DOM, a net excretion of P can be excluded at least for growing bacteria because in this case the input must always exceed the output. Therefore we concentrated our experimental approach on the role of bacterial starvation and of grazing for P-regeneration.

In the batch experiments without grazers (Figs. 2B to 5B) it became obvious that any net regeneration of P was strongly coupled to a loss of biomass. Because P-starved bacteria maintained their biomass during the course of the experiments, practically no P-release could be observed for these bacteria (Figs. 2B and 4A). By contrast, C-starved bacteria showed a more or less pronounced reduction of biomass caused by a decrease of cell density and by a diminution of cell size. C-starvation-induced phenomenona are well known and have been evaluated in several studies (reviewed by Morita 1982). The loss of biomass and the resulting P-release were much more pronounced with the C-limited bacteria from the summer inoculum (Figs. 5B and 6) but also highly variable. This variability may be explained by several hypotheses (e.g. bacterial populations with different amounts of reserve carbon and maintenance metabolism) which all, however, remain unproven.

The P-release by C-starved bacteria seems logical because there is no need to store excess P when the cellular biomass is reduced by maintenance metabolism. P bound to respired cell compounds (e.g. RNA, membrane) will then be excreted. Despite the fact that this starvation process can sometimes result in large amounts of finally released P (Fig. 5B), it could explain only regeneration cycles occurring on a time scale of some days or weeks. In contrast, only mechanisms being effective on a time scale of hours must be in action to explain the postulated rapid recycling rates in nature.

According to the results obtained here, grazing can be considered as a regeneration mechanism fulfilling, at least potentially, this condition. Without noticeable time lag, regeneration of P was observed coincident with the appearence of HNF in all batch experiments with C-limited bacteria and with P-limited bacteria from the winter inoculum (Figs. 2A, 3A and 5A). The observed net P-excretion occurred during all growth phases and led to a final net regeneration of 50 to 90 % of bacterial P (Table 3). The only exceptions were the experiments with bacteria from the summer inoculum grown under P-limitation because in this case regeneration efficiencies of only 20 % were observed (Fig. 4A). Similar results were obtained by Andersen et al. (1986) in batch experiments with an omnivorous flagellate and mixed populations of bacteria. The authors explained the low regeneration efficiencies by a more efficient P-conservation of the flagellates when growing on P-limited bacteria. However, in our study this explanation seems not to be sufficient because in the winter experiments a much higher P-regeneration was observed for identical HNF species grazing on P-limited bacteria with P-contents comparable to the summer bacteria (Table 1). Therefore, we assume a significant reuptake of released P by the high number of ungrazed bacteria observed in the P-limited summer experiments, rather than a significantly higher P-assimilation by the flagellates. The differences between C- and P-limited bacteria from the summer inoculum were also reflected by the results obtained from the chemostat experiments (Tables 4 and 5). With P-limited bacteria as prey, P-regeneration efficiency was less than 10 % and with C-limited bacteria up to 60 % (*Monas* sp.) and 38 % (*Bodo* sp.), respectively Thus, the regeneration efficiencies observed in the chemostats were generally lower than those of the batch experiments. This can be explained because only the regeneration during growth of HNF takes place in the chemostat, whereas the integrated regeneration over the exponential and stationary growth phase is covered in the batch experiments.

There were some differences between the 2 HNF isolates with regard to P-excretion which, however, did not change the general patterns described above. In continuous culture, P-regeneration by *Monas* sp. was significantly higher than by *Bodo* sp. (Tables 4 and 5).

In batch cultures both showed excretion of P during exponential and stationary growth. But *Monas* exhibited more excretion during exponential and *Bodo* during stationary growth phase. Two different physiological mechanisms are probably relevant for the P-release during different growth phases, the sum of which results in the overall P-regeneration. During exponential and balanced growth the flagellates excrete the amount of P which exceeded their requirements (assuming no accumulation of surplus-P). During the stationary growth phase, P is also released due to biomass losses caused by starvation of the flagellates. Both mechanisms should also be relevant under natural conditions because the temporal and spatial patchiness of food supply for protozoa implies the occurence of both growth situations (Fenchel 1987).

These results are in agreement with the current view on the role of protozoa in the recycling of nutrients. HNF and also some species of ciliates are known to be voracious consumers of bacteria (e.g. Fenchel 1982, Sherr & Sherr 1987) and are currently believed to be an important factor in the recycling of mineral nutrients (Azam et al. 1983, Goldman & Caron 1985, Güde 1985). Nitrogen and phosphorus bound in bacterial biomass will be excreted by the bacterial grazers in the amount they exceed the requirements of the protozoa. This means that the excretion rate depends on the carbon: nutrient ratio of prey and predator and on the respiration rates and growth efficiencies of the predators. Because the knowledge on possible growth efficiencies is limited, the importance of this aspect cannot at present realistically be evaluated. However, the relatively low C:nutrient ratios of planktonic bacteria, already discussed, and demonstrated high weight-specific nutrient regeneration rates of protozoa (Johannes 1965, Goldman et al. 1985, Andersen et al. 1986) strongly support the role of protozoa as important remineralizers.

In summary, our experiments confirmed the strong potential for P-regeneration by phagotrophic flagellates and are in agreement with results obtained by Berman et al. (1987) and by Bloem et al. (1989) with non-P-limited bacteria as prey. Our results clearly demonstrate that the observed pattern for P-regeneration is apparently much more dependent on the quality and quantity of bacterial prey than on the flagellate species. However, in addition to the nutrient content, other properties of the bacterial prey must also control net regeneration efficiencies because different net regeneration of P was observed for bacterial populations with similar P-contents (Table 3).

Because of the demonstrated high bacterial P-contents we assume that grazing on bacteria must be considered as one of the most effective mechanisms for P-regeneration.

Ecological implications

The high P-binding capacity of bacteria as postulated from the culture experiments and verified by the field study gives obviously strong support to the conception of bacteria acting primarily as nutrient sinks. This role is consistent with the observation that usually more than 50% of P-uptake occurred in the bacterial size fraction of Lake Constance (Güde in press). On the other hand, the large bacterial P-pool can also be considered as an important nutrient reservoir to be potentially available for primary producers. An advantage of this reservoir is that sinking losses are almost negligible due to the small size of planktonic bacteria. The mobilization of the bacterial P-pool is obviously possible as has been demonstrated by the seasonal fluctuation in the course of bacterial P in Lake Constance (Fig. 1). Thus, bacterioplankton can be considered to some extent as a buffer contributing to the stabilization of the P-content in the euphotic zone by accumulation and release of phosphorus. However, as will be shown in the following, there remains some uncertainty especially with respect to the relevance of the possible mechanisms of P-release.

According to the experimental evidence presented here and by other studies, grazing can be regarded as one of the most effective mechanisms for net P-release. However, 2 problems result when the field situation is considered. First, the release can only be utilized for new primary production if it is a real net release, i.e. the reuptake by bacteria is smaller than the release. As is indicated by the results obtained from the P-limited summer cultures (Fig. 5A) and by the above-mentioned highly efficient uptake of ortho-P by bacteria (Currie & Kalff 1984, Berman 1985, Güde in press), reuptake must indeed be considered as important. Thus, the release due to grazing could only become effective under non-steady state conditions with temporarily separated sequences of uptake and release. Because of short-term fluctuations this condition could at least potentially be regarded as fulfilled, although its relevance for the P-regeneration has not yet been proven. Second, it has been demonstrated that there are longer periods (in the order of weeks) during which grazing cannot be regarded as an important regeneration mechanism because of low abundances of grazer organisms (Jürgens & Güde in press).

P-release due to C-starvation-induced loss of bacterial biomass has been demonstrated as an additional mechanism. As has been emphasized, however, it is difficult to explain the postulated fast recycling rates on the basis of this comparatively slow process. Thus, this process could only to a small extent cover the estimated

algal P-demands. Alternative loss factors for bacteria such as parasitism or lysis by phages may also lead to P-release from bacteria. However in spite of some circumstantial evidence (e.g. Bergh et al. 1989) our current knowledge is by far insufficient for a realistic evaluation of its importance in nature.

For an evaluation of the importance of grazing for the recycling of phosphorus we have also to consider the chemical composition of the released P. From our grazing experiments it became evident that the major fraction of released bacterial P consisted of SRP, which is assumed to be easily biologically available for algae and bacteria. But to a considerable extent DOP also contributed to the P-release and the DOP concentrations in Lake Constance and other aquatic environments are mostly significantly higher than SRP. These observations point to the potentially important role of DOP in P-fluxes into and out of bacteria and algae (Currie & Kalff 1984, Berman 1988, Chrost 1988). It has been suggested that P-hydrolyzing enzymes such as 5' nucleotidase (Ammermann & Azam 1985) and APA (Chrost & Overbeck 1987) are a main factor in the regeneration of P. Also in this concept the crucial question remains in which way and how much DOP is released from plankton organisms. Moreover, it is difficult to reconcile this view with the proven predominance of bacterial P-uptake in many field experiments, because there is no reason why the fate of inorganic P released from enzymatic hydrolysis of phosphoesters should differ from that already present in the water.

In summary, this study has shown that bacterioplankton bind high amounts of P due to their large biomass and due to comparatively low C:P ratios. This supports the view that bacterial growth may contribute to a decrease rather than to a stimulation of primary production in P-deficient situations. According to the experiments, regeneration of bacterial P is linked with losses of biomass either due to grazing or to starvation. Because grazing proved to be much more efficient it may substantially contribute to the necessary continuous supply of nutrients for primary producers. However, the results have also shown that the actual limitation conditions should be known for a realistic evaluation of regeneration efficiencies because generally the probability of net P-regeneration is reduced under P-limitation of bacteria. Therefore, a better knowledge of the actual limitation conditions of bacterioplankton is urgently needed.

Acknowledgements. We thank J. Meichle and E. Stöhr for technical assistance and M. Simon for critical comments on the manuscript. This study was supported by Deutsche Forschungsgemeinschaft within the joint collaborative research programme 'Cycling of matter in Lake Constance' (SFB 248).

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This article was submitted to the editor

Manuscript first received: April 4, 1989
Revised version accepted: October 20, 1989