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ORIGINAL ARTICLE

Functional responses of prokaryotes and viruses to grazer effects and nutrient additions in freshwater microcosms

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For aquatic systems, there is little data on the interactions between viruses, prokaryotes, grazers and the availability of resources. We conducted a microcosm experiment using a size fractionation approach to manipulate grazers, with a purpose to examine the effects of inorganic and organic nutrients on viral and prokaryotic standing stocks and activities, and on prokaryotic community composition as assessed by fluorescent *in situ* hybridization (FISH) method. Experiments were performed during periods of severe phosphate (P)-limiting conditions in the oligotrophic Sep Reservoir (Massif Central, France). In the absence of nutrient addition, the presence of grazers in microcosms stimulated prokaryotic growth and viral proliferation, likely through nutrient and substrate enrichment. Addition of nutrients had a stronger effect on viral infection of prokaryotes than grazing. Addition of P led to the most pronounced increase in prokaryotic abundance, production and growth efficiency, thus providing direct evidence of P limitation of prokaryotes. Enhanced prokaryotic activity in P treatments also stimulated viral abundance and viral-induced lyses of prokaryotes. Changes in prokaryotic community composition due to nutrient additions were evident in the grazer-free samples. Prokaryotic populations hybridizing for the probes bacteria, β-Proteobacteria and α-Proteobacteria responded to nutrient enrichment with significant increases in their relative abundances, whereas cells hybridizing for Archaea and Cytophaga-Flavobacterium (now known as Bacteroidetes) probes failed to show any functional response. Cells hybridizing for the latter cluster increased towards the end of incubation period in the control samples (that is, without nutrient additions) with grazers present, suggesting the development of grazing resistant forms. From our nutrient enrichment microcosm experiments, we conclude that the presence of grazers is a stimulating factor for prokaryotic growth and viral proliferation in the plankton, probably through nutrient regeneration process.
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Introduction

Heterotrophic prokaryotes are the most important biological component involved in the transformation and mineralization of organic matter in aquatic systems. Although their role in the above processes has been generally recognized, the question of how resources (bottom-up) and predators (top-down) control the development of prokaryotic populations is still a matter of debate. Top-down control has received perhaps the most attention.

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Bacterivory by protists and cell lysis by viruses are major sources of mortality for prokaryotes. Protists are often considered to be the main predators of prokaryotes through their selective feeding strategy and often exert large influence on prokaryotic community composition, especially in waters in which the nutrient resources for prokaryotes are limited (Jürgens *et al.*, 2002; Šimek *et al.*, 2003). However, in recent years, studies pertaining to the top-down control of prokaryotic populations have evoked much interest among microbial ecologists with the finding of large number of viruses (10^8–10^{11} l^{-1}) in aquatic systems (see reviews of Sime-Ngando *et al.*, 2003; Weinbauer, 2004; Suttle, 2007).

Studies examining larger data sets have now revealed that viral lysis can be a significant source of prokaryotic mortality and is sometimes comparable to bacterivory by protists (Fuhrman and Noble,

1995; Pradeep Ram *et al.*, 2005). Viral lysis has a significant effect on the cycling of nutrients and organic matter (Noble and Fuhrman, 1999; Wilhelm and Suttle, 1999) and studies indicate that viral lysis of prokaryotes can increase prokaryotic production and respiration by reducing the carbon flow to bacterivorous protists (Middelboe *et al.*, 1996; Fuhrman, 1999), but we still lack comparative studies from different environments.

As phage infection and proliferation is host density dependent, phages limit competitive dominant prokaryotes for nutrient acquisition and allow less competitive populations to develop. This has been mathematically formalized in the 'killing the winner' hypothesis (Thingstad and Lignell, 1997). Protistan bacterivory and viral lysis likely influence prokaryotic abundance and community composition, perhaps in different fashions. Protistan bacterivory impacts both standing stock and the morphological and taxonomic structure of prokaryotic communities, whereas viral lysis can be considered as a mechanism potentially increasing species evenness in prokaryotic community.

As prokaryotes are probably the dominant hosts for viruses in aquatic systems, the availability of nutrients and the physiological state of the host cells can greatly influence viral abundance and production. Therefore, factors which influence prokaryotic production should also impact viral production. As most of the aquatic systems are either energy (carbon) or nutrient (nitrogen or phosphorus) limited, severe resource limitation (nutrients) can limit prokaryotic growth and activity. This can instigate viruses to change their infection strategy, for example, from lytic to lysogenic 'lifestyle', which has been studied experimentally (Wilson and Mann, 1997; Williamson and Paul, 2004). Thus the availability of nutrients could constrain viral replication and/or prophage induction and thereby limit the production of free viral particles in aquatic ecosystems. Nutrient enrichment bioassays have been applied to prokaryotes in a wide variety of aquatic environments to determine the effects of limiting nutrients on the growth and activity of prokaryotes, but simultaneous information from such assays on virus–host interactions are less well studied (Hornàk *et al.*, 2005; Jardillier *et al.*, 2005). Very little is known on the interplay of viruses and grazers on the activity and diversity of prokaryotes.

Previous studies in the oligotrophic Sep Reservoir (France) have suggested that prokaryotic abundance and activity are strongly limited by the availability of phosphate (P) in summer, as inferred from the measured concentration of potential substrates (Tadonléké *et al.*, 2000). In such situations, other processes producing dissolved organic matter (DOM), such as excretion and sloppy feeding by zooplankton, can stimulate total prokaryotic production (Richardot *et al.*, 2001). A recent study carried out in the Sep Reservoir has indicated the stimulation of viral lysis by bacterivory and the existence of synergy between grazers, prokaryotic and viral activities in oligotrophic conditions, through a cascading effect from grazer-mediated resource enrichment (Sime-Ngando and Pradeep Ram, 2005). However, the ambiguity behind synergy between grazing and viral lysis (Šimek *et al.*, 2001) led us to hypothesize that nutrient environment could be a significant factor driving these interactions, primarily in nutrient limited conditions.

In this study, we hypothesize that under nutrient limited conditions the regenerated nutrients (especially P in the Sep Reservoir) due to bacterivory could play a vital role in maintaining prokaryotic activity, thereby supporting viral stock and lytic production. To test the hypothesis microcosm experiments with nutrient enrichments were conducted using water samples collected from the Sep Reservoir during periods of strong P limitation (summer), in which grazing pressure (by size fractionation) and resource (by nutrient additions) effects on natural prokaryote communities were manipulated. The microcosm experiments coincided with the period of the year when protistan bacterivory was at its maximum (Pradeep Ram *et al.*, 2005). Since most of the aquatic systems are resource limited, the outcome of our experiments would be of great significance in elucidating the role of viruses in microbial food web.

Methods

Study site

For the microcosm experiments, triplicate water samples were collected at 1 m depth in the oligotrophic Sep Reservoir (46°N, 3°E French Massif Central; see Tadonléké *et al.*, 2000; Pradeep Ram *et al.*, 2005 for site description) on 11 September 2003 in the deepest central point of the reservoir, by filling 25 l Nalgene carboys which had been previously cleaned with 1.2 N HCl and rinsed three times with Milli-Q and lake water. The sampling period corresponded to the clear water phase with a euphotic depth of about 3 m (estimated from the Secchi depth), representing about the half of the whole water column of the lake during the sampling period (Pradeep Ram *et al.*, 2005). The initial characteristics of the experimental water samples are shown in Table 1.

Size fractionation

Immediately after sampling and for each replicate, half of the experimental microbial samples were filtered separately through Nuclepore Track Etch Membranes (0.8 μm pore size) under attenuated light conditions and low differential pressure (< 50 mm Hg) to yield the 'grazer-free' fraction which was confirmed by microscopic examination. The second halves of the experimental samples (that is, < 200 μm) with grazers present were considered

Table 1 Mean (s.d. for triplicates) surface water characteristics of the Sep Reservoir during the experimental study

Parameters (units)	*Values*
Temperature (°C)	19.0 ± 0.1[a]
Dissolved oxygen (mg l^{-1})	6.4 ± 0.1[a]
NH_4–N (mg l^{-1})	0.05 ± 0.003
NO_2–N (mg l^{-1})	0.02 ± 0.001
NO_3–N (mg l^{-1})	0.38 ± 0.02
PO_4–P (mg l^{-1})	0.002 ± 0.0004
Total phosphorous (mg l^{-1})	0.13 ± 0.01
Chlorophyll *a* (μg l^{-1})	14.1 ± 0.46
Prokaryotic total abundance ($\times 10^6$ cells ml^{-1})	3.7 ± 0.5
Bacteria (%)	41.9 ± 5.3[b]
β-Proteobacteria (%)	26.3 ± 3.2[b]
α-Proteobacteria (%)	2.9 ± 0.5[b]
Cytophage-Flavobacterium (%)	3.2 ± 0.5[b]
Archaea (%)	0.8 ± 0.1[b]
Prokaryotic production (pmol l^{-1} h^{-1})	28.5 ± 2.8
Viral abundance ($\times 10^7$ ml^{-1})	3.7 ± 0.6
Frequency of infected bacterial cells (%)	7.4 ± 0.5

[a]Mean value for euphotic depth (that is, 0–3 m, Pradeep Ram *et al.*, 2005).
[b]Percentage of total prokaryotic abundance.

as 'grazer fraction'. Preliminary tests clearly showed that bacterivory was significantly higher in the grazer fraction compared to the <5 μm filtered samples, unlike those in similar studies (Šimek *et al.*, 2003; Weinbauer *et al.*, 2003; Hornàk *et al.*, 2005).The grazer-free fraction and grazer fraction would hereafter be referred throughout in the text as <0.8 and <200 μm fraction, respectively.

Upon completion of filtration, both <0.8 and <200 μm experimental samples were acclimatized for 1 h in the dark in *in situ* conditions and then equally distributed in 5-l capacity polycarbonate bottles previously washed with 1.2 N HCl and rinsed three times with Milli-Q water and with the appropriate experimental samples. Each set of bottles (both <0.8 and <200 μm fractions) received organic and inorganic nutrients, individually and in combinations, including carbon (glucose 125 μM C), nitrate ($NaNO_3$ 25 μM N) and phosphate (NaH_2PO_4 5 μM P) in four different treatments, namely, C, P, NP and CNP. Nutrients were added to reach up to their maximum concentrations known from the annual range recorded previously in the euphotic layer of the Sep Reservoir (Tadonléké *et al.*, 2002; Pradeep Ram *et al.*, 2005). One set of bottles which received no nutrient addition served as unaltered control (U). All treatments were prepared in triplicates (5 treatments × 3 replicates). The experimental samples were incubated in the dark at *in situ* temperature (18 ± 1 °C) conditions. Subsamples were taken from each replicates at 0, 24, 48 and 72 h time period and analysed for prokaryotic and viral abundance, activities and prokaryotic community composition (PCC). PCC analysis was not done in NP treatments. Precautionary measures were taken to avoid contamination of the experimental samples. All containers, filtering devices, glassware and tubing coming in contact with the experimental samples were acid-washed (10% hydrochloric acid) and thoroughly rinsed with de-ionized water prior to use.

Physicochemical analysis

The water temperature and dissolved oxygen were measured *in situ* with a WTW OXI 197 multiparametric probe. Samples for initial nutrient concentrations, namely dissolved ammonium (NH_4–N), nitrate (NO_3–N) and nitrite (NO_2–N) nitrogen, soluble orthophosphate (PO_4–P) and total phosphorus (TP) were analysed spectrophotometrically using standard methods (APHA, 1985). The initial chlorophyll *a* concentration (chl) was determined spectrophotometrically from samples (500 ml) collected on Whatman GF/F filters. Pigments were extracted in 90% acetone overnight in the dark at 4 °C, and concentrations were calculated from SCOR-UNESCO (1966) equations. Nutrients and chlorophyll concentrations were analysed in triplicates.

Prokaryotic and viral abundances

For prokaryotic (PA) and viral abundances (VA), water samples were fixed with 0.02 μm filtered buffered alkaline formalin (final concentration 2% v/v). Immediately after sampling, subsamples (1–2 ml) were filtered (<15 kPa vacuum) through Anodisc filters (0.02 μm pore size, Whatman), using cellulose acetate backing filters (1.2 μm pore size). Following staining with SYBR Green I fluorochrome (Molecular Probes, Leiden, The Netherlands) (final dilution, 2.5×10^{-3}-fold) as described by Noble and Fuhrman (1998), filters were air dried on absorbent paper and mounted between a slide and glass cover slip with a special antifading mountant. The mounting medium Citifluor (Citifluor, London, UK) was amended with approximately 20% (v/v) of Vecta Shield (Vector Laboratories, Peterborough, UK). This modification significantly reduced fading of the fluorochrome and gave highly stable fluorescence. When not analysed immediately, slides were stored at −20 °C until counting, using an epifluorescence microscope (LEICA DC 300F model). Prokaryotes were distinguished from virus-like particles on the basis of their relative size and brightness. A blank was routinely examined to control for contamination of the equipment and reagents.

Prokaryotic production, respiration and growth efficiency

Prokaryotic production (PP) was determined by the incorporation of labelled thymidine (^{3}H-TdR) into bacterial DNA (Fuhrman and Azam, 1982). Experimental samples (10 ml each in triplicates for each experimental replicate) and 0.5 N NaOH formalin killed controls were inoculated with labelled thymidine (specific activity = 87 Ci $mmol^{-1}$, Amersham

Biosciences, Sarclay, France) at final concentration of 20 nmol in Pyrex tubes, and then incubated in *in situ* conditions and in the dark for 30 min. TdR incorporation was stopped by adding 5 N NaOH. Radioactive samples were filtered through 0.2 μm cellulose acetate filters (Sartorius, Goettingen, Germany), and rinsed twice with 3 ml ice-cold 5% trichloroacetic acid. Filters were placed in vials, allowed to dry and solubilized with 0.5 ml of ethyl acetate. After adding 5 ml of scintillation cocktail, radioactivity was counted with a liquid scintillation counter (Beckman, LS 6500). PP was calculated in moles of TdR incorporated into DNA.

Because >90% of prokaryotes in the Sep Reservoir are known as free-living cells (Jugnia *et al.*, 1999; Sime-Ngando and Pradeep Ram, 2005), prokaryote community respiration rates were estimated from oxygen consumption in <0.8 μm fractions as described previously (Pradeep Ram *et al.*, 2003, 2007). Water samples (control and treatment samples in triplicates) were carefully transferred to calibrated borosilicate glass bottles (300 ml capacity) by a sipper system to avoid the formation of air bubbles. Dissolved oxygen levels were measured by Winlker's method before (t0) and after the incubation (t24, t48 and t72) of the experimental water samples. Respiration rates were calculated as differences in O_2 concentration between initial and final replicate bottles, over the duration of the incubation.

Prokaryotic growth efficiency in the <0.8 μm fraction of control and nutrient treatments was derived as the slope of prokaryotic production versus the sum of prokaryotic production plus respiration and expressed as a percentage. For the calculation of growth efficiencies, prokaryote production and respiration rates were converted and expressed in terms of carbon using conversion factors of 1.67×10^{18} cells per mole of TdR and 20 fg C per cell (as determined previously by Jugnia *et al.*, 2000 for the Sep Reservoir prokaryotic communities) for production, and a respiratory quotient of 1 for respiration rates. Linear regression allowed us to use all data points for the calculation of prokaryotic growth efficiencies.

Phage infection

In formalin-fixed experimental samples, prokaryotic cells contained in 8 ml subsamples were collected on copper electron microscope grids (400-mesh, carbon-coated Formvar film) by centrifugation according to Sime-Ngando *et al.* (1996). Triplicate grids were prepared for each sample. Each grid was stained at room temperature (approximately 20 °C) for 30 s with uranyl acetate (2% w/w), rinsed twice with 0.02 μm-filtered distilled water and dried on a filter paper. Grids were examined using a JEOL 1200Ex transmission electron microscope operated at 80 kV at a magnification of ×20 000–60 000 to distinguish between prokaryotic cells with and without intracellular viruses. A prokaryote was considered infected when at least five viruses, identified by their shapes and sizes, were clearly visible inside the host cell. At least 500 prokaryote cells were inspected per grid to determine frequency of visibly infected cells (FVIC). Because mature phages are visible only late in the infection cycle, FVIC counts were converted to the frequency of infected cells (FIC) using the equation FIC = 9.524 FVIC−3.256 (Weinbauer *et al.*, 2002). Assuming steady state, that the infected and uninfected cells were grazed at the same rate, and that the latent period equalled the prokaryotic generation time, the FIC were converted to bacterial mortality (FMVL, as percentage of PP) using the equation: $FMVL = (FIC + 0.6FIC^2)/(1-1.2FIC$; Binder, 1999).

Fluorescence in situ *hybridization*

PCC was analysed by *in situ* hybridization using five different probes that were fluorescently labelled with the indocarbocyanine dye Cy3 (MWG-Biotech, Ebersberg, Germany). Two labelled oligonucleotides included the domain specific probes EUB338 for bacteria (Amann *et al.*, 1990), and ARCH915 for Archaea (Stahl and Amann, 1991). The group specific probes ALF1b and BETA42a (Manz *et al.*, 1992) were used to detect the α- and β-subclasses of *Proteobacteria*. Finally, the probe CF319a was used to detect members of *Cytophaga-Flavobacterium* cluster of the *Cytophaga-Flavobacterium-Bacteroides* phylum, actually known as Bacteroidetes (Ludwig and Klenk, 2001, Kirchman, 2002). Throughout the text, these targeted prokaryotic groups are designated as EUB, ARCH, ALF, BETA and CF respectively. They were analysed according to Glöckner *et al.* (1999).

Briefly, 4–6 ml of water samples were concentrated onto white polycarbonate filter (25 mm diameter, 0.2 μm pore-size, Millipore, St Quentin Yvelines, France) by applying a low vacuum. The prokaryotes on filters were subsequently fixed by overlying with 4% paraformaldehyde in phosphate-buffered saline (pH 7.2) and stored at −20 °C until analysis. For each subsample two filters were prepared, one for EUB and the second was cut into four sections prior to hybridization with the other labelled oligonucleotides. The hybridization buffer was composed of 180 μl of 5 M NaCl, 20 μl of 1 M Tris-HCl (pH 7.4), formamide (20% v/v for the ALF and ARCH probes, 35% v/v for other probes) and sodium dodecyl sulphate (SDS; 1 μl of a 10% solution). For hybridization, we used 2 μl of the ALF, BET, CF and ARCH probes (100 ng μl^{-1}) brought up to 80 μl with hybridization buffer. All hybridizations were performed at 46 °C for 90 min. Filters were rinsed with a Tris-HCl (1 mM), NaCl (250 mM final concentration for ARCH, 225 mM for ALF and 80 mM for other probes), and SDS (10% v/v) solution (15 min at 48 °C in the dark). Prokaryotes fixed on this filter were stained with DAPI (final concentration of 4 μg l^{-1}) for 15 min and were subsequently fixed between

slides with Citifluor oil. Hybridized cells were enumerated with a Leica epifluorescence microscope (magnification $\times 1250$) equipped with a filter for UV excitation (DAPI) and for green excitation (Cy3). About 10–40 fields were examined for each probe.

Statistical analyses
Response of prokaryotic and viral parameters to different nutrient treatments at different incubation time and other differences were tested by 1-way analysis of variance (ANOVA). A significant response was considered to be at $P<0.05$. Potential relationships among various measured variables were tested by Pearson's correlation analysis. All statistical analyses were performed using Minitab's software for Windows (Release 12, Minitab).

Results

In situ *characteristics of the experimental samples*
Initial physico-chemical and biological characteristics of the experimental samples are listed in Table 1. For all these variables, the within-sample variability was judged satisfactory, with coefficients of variation generally $\leqslant 20\%$. Water temperature remained constant at about 19 °C throughout the euphotic layer and oxygenic conditions prevailed with dissolved oxygen content at about $6\,mg\,l^{-1}$. Inorganic nutrient concentrations were low, except for nitrate ($0.38\,mg\,l^{-1}$). The very low concentration of dissolved inorganic phosphate (P, $0.002\,mg\,l^{-1}$) compared to inorganic nitrogen and the related high N:P atomic ratios (>200) indicated severe P limiting conditions, typical of the Sep Reservoir during summer (Tadonléké *et al.*, 2002). Chlorophyll a pigment was about half the median value known from the annual range (that is, 1–43 $\mu g\,l^{-1}$) recorded in the euphotic layer during the study year (Pradeep Ram *et al.*, 2005). PCC was dominated by EUB which constituted 42% of the total abundance of $3.7 \pm 0.5 \times 10^6$ cells ml^{-1}, at a total thymidine incorporation rate of 28.5 ± 2.8 pmol $l^{-1}\,h^{-1}$. Within the EUB, the BETA subclass was the dominant group (63% of EUB). Cells hybridizing with ALF, CF and ARCH probes were $<4\%$ of the total prokaryotic abundance.

Effects of filtration
Size fractionation of the experimental samples by filtration generally lowered prokaryotic and viral variables under study but differences between 0.8 and $<200\,\mu m$ fractions were not statistically different (ANOVA, $P>0.05$). The initial levels of prokaryotic and viral variables are shown in Table 2, for comparison between grazer-free and grazer fractions. VA at the start of the experiment was roughly an order of magnitude higher than the abundance of prokaryotes (PA). Analysis of PCC based on fluorescent *in situ* hybridisation (FISH) with group specific probes did not show any significant differences between <0.8 and $<200\,\mu m$ fractions at t0. Thus at t0, the experimental manipulation yielded no marked bias apparent at our level on the variable under study, including the probed prokaryotic groups present in the experimental samples.

Table 2 Mean (s.d. for triplicates) initial characteristics of prokaryotic and viral variables at the start of the experiment

Parameters (units)	*$<200\,\mu m$ fraction*	*$<0.8\,\mu m$ fraction*
Prokaryotic abundance ($\times 10^6$ cells ml^{-1})	3.7 ± 0.5	3.2 ± 0.3
Prokaryotic production (pmol $l^{-1}\,h^{-1}$)	28.5 ± 2.8	21.8 ± 2.1
Viral abundance ($\times 10^7\,ml^{-1}$)	3.7 ± 0.6	3.3 ± 0.5
Frequency of infected bacterial cells (%)	7.4 ± 0.5	5.4 ± 0.7

In our time course experiments and for the control treatments (that is, without nutrient addition), the $<200\,\mu m$ fraction showed an increasing trend from t0 to t48 for PA and VA and from t0 to t72 for FIC, reaching the highest abundances of prokaryotes ($7.3 \pm 0.6 \times 10^6$ cells ml^{-1}) and viruses ($6.0 \pm 0.5 \times 10^7$ viruses ml^{-1}), and of viral infection ($11.7 \pm 0.6\%$) as well, compared to $<0.8\,\mu m$ fraction (Figures 1a and c, and Figures 2a–d).

Effects of nutrient addition
Prokaryotic abundance, production and growth efficiency. The results presented here correspond to mean values (for triplicates) of different incubation times (t0, t24, t48 and t72). Nutrient addition grossly led to significant (ANOVA, $P<0.05$) increases in both the abundance (PA) and production (PP) of prokaryotes, compared to the control (that is, with no nutrient addition). Regardless of the type of nutrients, both variables indeed increased from t0 to t48 in the $<0.8\,\mu m$ fraction, and from t0 to t24 (but longer to t48 for PP in CNP treatment) in the $<200\,\mu m$ fraction (Figure 1). The maximum growth rates were observed in treatments with P addition, namely, in the P treatments for PA in both fractions and for PP in the $<0.8\,\mu m$ fraction, and in CNP treatment for PP in the $<200\,\mu m$ fraction. Between treatment comparisons showed that the latter increase (that is, of $<200\,\mu m$ filtered PP in CNP treatment) was significant (ANOVA, $P<0.05$), contrasting with the former increases in P treatments which were not significantly different from those in C, NP and CNP treatments. Removing grazers in experimental samples (that is, $<0.8\,\mu m$ fraction) generally enhanced the effects of nutrient addition on prokaryotes, especially on PP (Figures 1b and d). Besides, the phosphorus-stimulated increases in PA

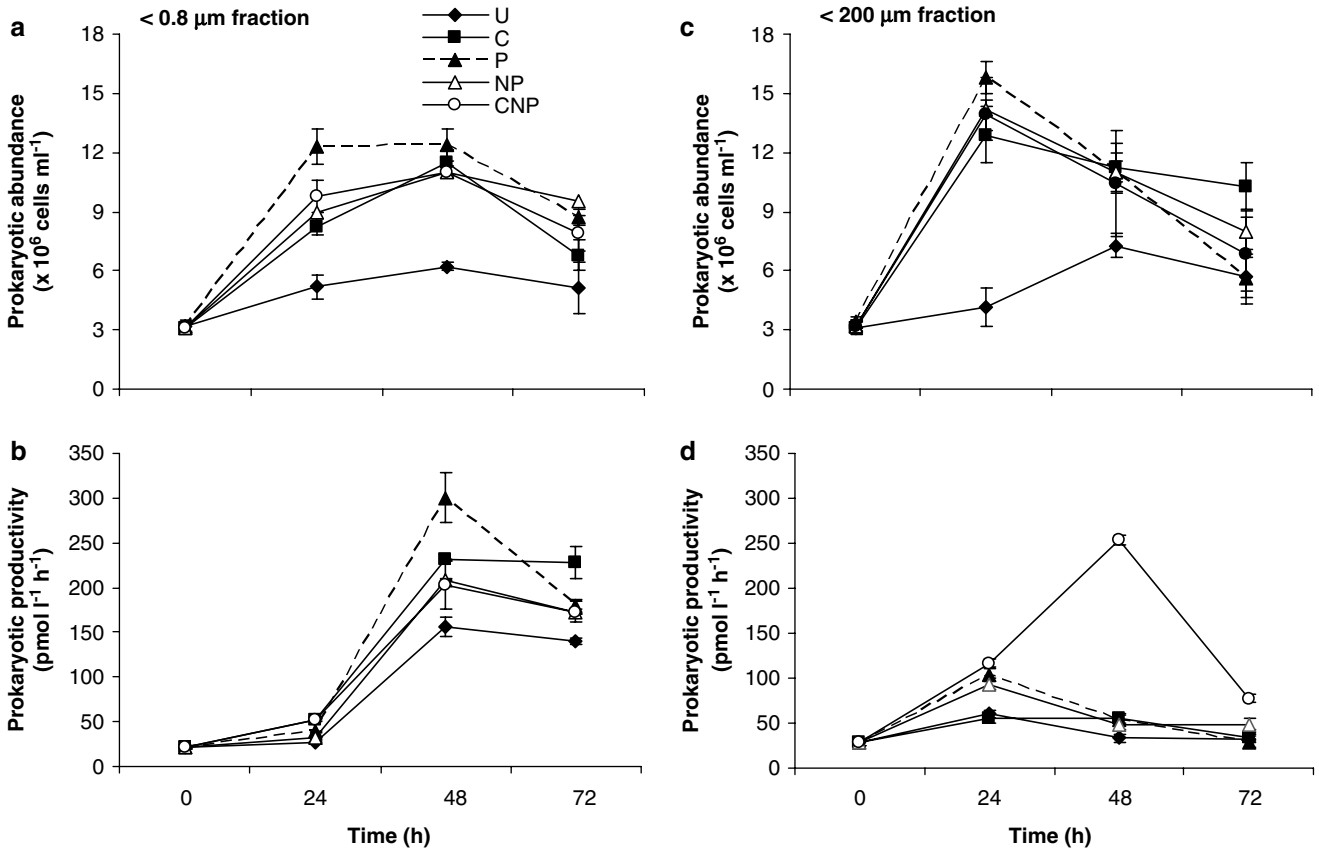

Figure 1 Time course changes in prokaryotic abundance and productivity in the <0.8 (**a**, **b**) and $<200\,\mu m$ (**c**, **d**) fractions of experimental samples with different nutrient additions. C, organic carbon; N, nitrogen; P, phosphorous; U, unamended. Values are means for triplicate samples, and vertical bars show $\pm$ s.d.

and PP were attested by growth efficiencies of prokaryotes (calculated from time integrated production and respiration rates) which were significantly (ANOVA, $P<0.005$) higher in P treatments ($36\pm5\%$) than in controls ($18\pm6\%$) without nutrient addition. Growth efficiencies in other treatments (that is, C, NP and CNP) were also higher (ANOVA, $P<0.05$) than in controls (Figure 3).

Viral abundance and infection. Like prokaryotic parameters, nutrient addition also evoked marked increases in viral parameters compared to the controls in both experimental fractions, this being more marked for FIC than for VA (Figure 2). Incubation of water samples with P alone produced a statistically significant increase in VA ($P<0.001$), compared to all treatments in both fractions (Figures 2a and c). The increase in viruses corresponded to similar increase in PA in the same samples. The maximum VA observed in the P treatment of <0.8 and $<200\,\mu m$ fractions were at $10.9\pm0.9\times10^7$ viruses per ml (t24) and $9.4\pm0.9\times10^7$ viruses per ml (t48), with net increase (compared to t0) of 291% and 234%, respectively. FIC was increased from t0 to t48 in all treatments of the $<0.8\,\mu m$ fraction (Figures 2b and d). FIC was typically highest in P treatment of the $<0.8\,\mu m$ fraction (25.8% at t48), with significantly higher values (ANOVA, $P<0.05$) than in other treatments. No such differences between treatments were observed in the $<200\,\mu m$ fraction, although nutrient addition also stimulated FIC compared to control. FIC was correlated to PP ($P<0.001$) in the $<0.8\,\mu m$ fraction only.

Prokaryotic community composition. Detailed analysis of PCC by the FISH method showed that the initially dominant EUB, primarily the BETA subclass (Table 1), remained dominant in all treatments throughout the experimental incubations (Figure 4). For control treatments without nutrient addition, few consistent trends were noted: in contrast to EUB, the relative abundance of ALF increased twofold from t0 and t48 in the $<0.8\,\mu m$ fraction, while CF regularly increased during the incubation time from 3% to 12% (of total VA) in the $<200\,\mu m$ fraction. Addition of nutrients resulted in more bacterial growth stimulation, primarily for BETA and ALF, this being apparently more marked when P was added. Addition of carbon alone doubled the percentage of ALF within the 72 h of incubation but in the $<0.8\,\mu m$ fraction only, somewhat similar to the observation made in the related control treat-

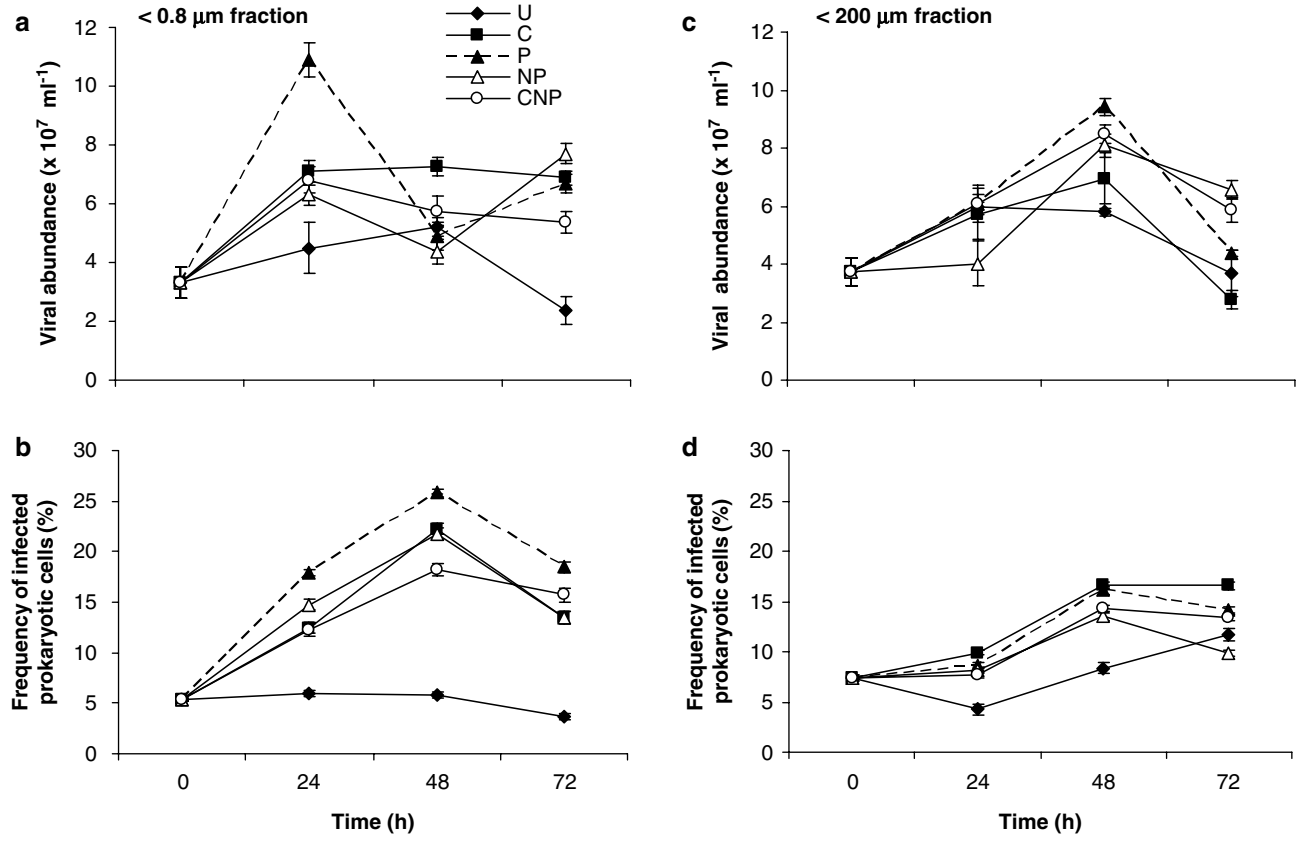

Figure 2 Time course changes in viral abundance and frequency of infected prokaryotic cells in the <0.8 (**a**, **b**) and $<200\,\mu m$ (**c**, **d**) fractions of experimental samples with different nutrient additions. C, organic carbon; N, nitrogen; P, phosphorous; U, unamended. Values are means for triplicate samples, and vertical bars show $\pm$ s.d.

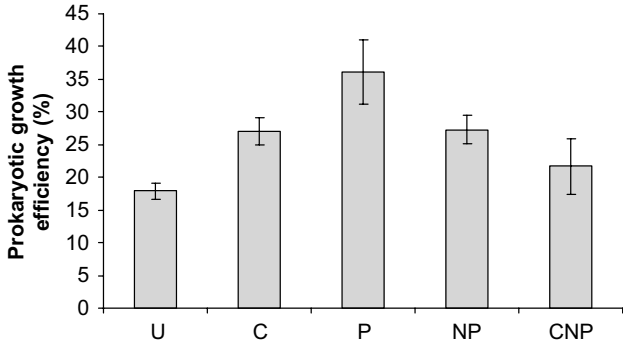

Figure 3 Time integrated measurement of prokaryotic growth efficiency in nutrient amended experimental samples. C, organic carbon; N, nitrogen; P, phosphorous; U, unamended. Values are means for triplicate samples, and vertical bars show $\pm$ s.d.

ment (Figures 4a and b). Similar increases in ALF were also noted in P treatment for the $<0.8\,\mu m$ fraction, and in CNP treatments for the two fractions. In addition, the percentages of BETA increased from t24 to t72 by about fivefold, in P treatment for the two fractions, and in CNP treatment but for the $<0.8\,\mu m$ fraction only. Similar but weaker increasing trends (that is, <1.5-fold) were also noted for EUB in the same experimental samples (Figures 4c, d and g).

Discussion

Experimental approach

We have chose an experimental approach using microcosms, which allowed the control of both predation levels (by size fractionation) and resource effects (nutrient enrichment) to study their influences on prokaryotes and viruses in an oligotrophic freshwater lake. Although microcosm experiments introduce some bias into the development of prokaryotic communities compared with naturally occurring communities, owing to confinement and handling effects, these experimental tools are useful for investigating how environmental processes induce temporal variations in prokaryotic structure, diversity and activity (Schwalbach *et al.*, 2004). In the present study, short incubation time (up to 72 h) coupled with the volume of microcosms (that is, 5 l), was likely to limit confinement effects. This short incubation time was realistic compared to the generation time of microorganisms, and considered sufficient to obtain significant changes in prokaryote and viral parameters, as observed in natural systems (Felip *et al.*, 1996; Vrede, 2005).

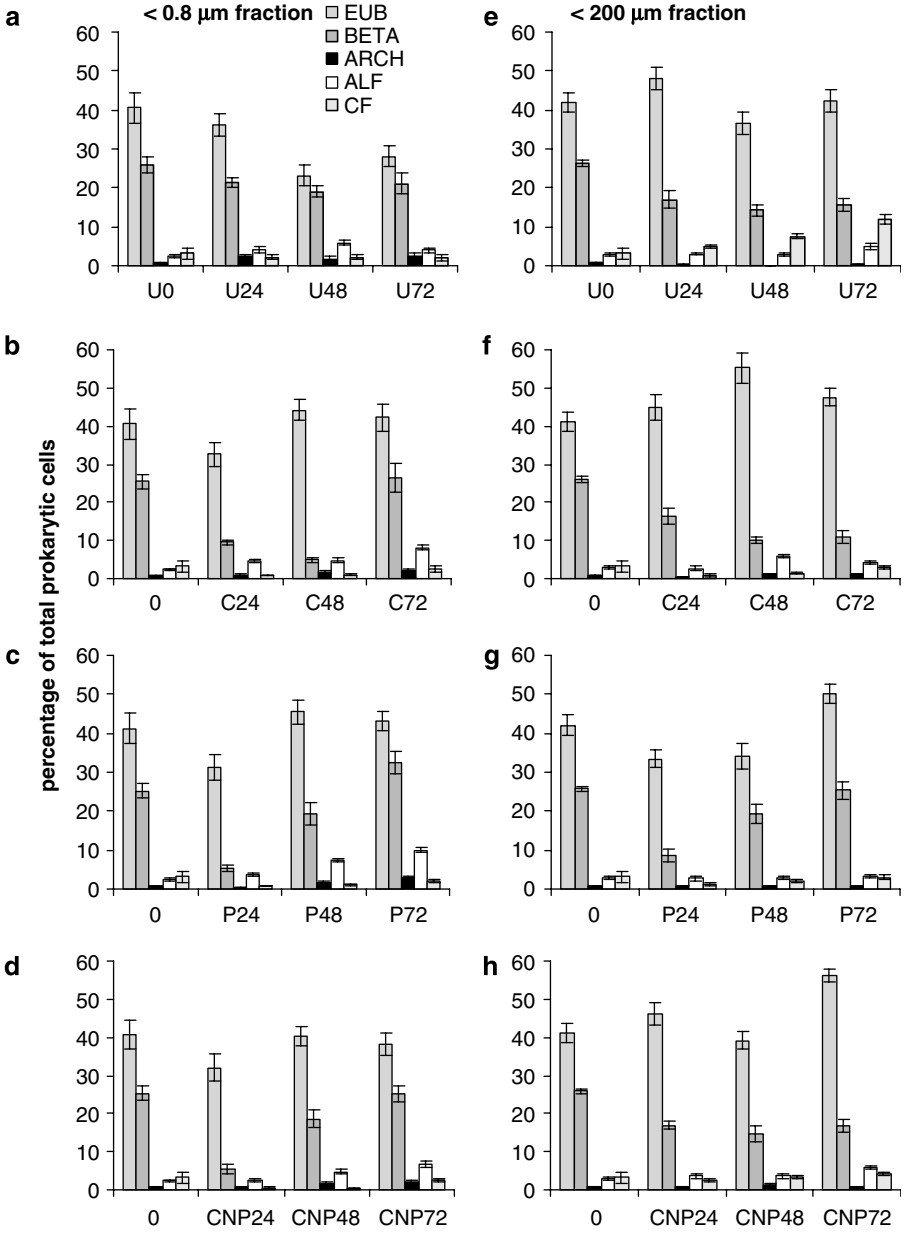

Figure 4 Time course changes in the relative abundances of FISH-targeted bacteria (EUB), α-proteobacteria (ALF), β-proteobacteria (BETA), the *Cytophaga-Flavobacterium* cluster (CF) and Archaea (ARCH) in the <0.8 (**a**, **b**, **c**, **d**) and <200 µm (**e**, **f**, **g**, **h**) fractions of experimental samples with different nutrient additions. C, organic carbon; N, nitrogen; P, phosphorous; U, unamended. Values are means for triplicate samples, and vertical bars show ± s.d.

Size fractionation by filtration of the water samples through 0.8 µm filters eliminated most of the grazers (98%, data not shown) and was dominated (>90% of total abundance) by free-living prokaryotes in the natural assemblages under study, as previously reported for the Sep Reservoir (Jugnia *et al.*, 1999; Sime-Ngando and Pradeep Ram, 2005). Previous studies in this reservoir have revealed that microzooplankton rather than nanoflagellate account for about 60% of seasonal predation on prokaryotes (Jardillier *et al.*, 2004; Pradeep Ram *et al.*, 2005). Our preliminary experiments using the water samples from Sep Reservoir have suggested that bacterivory was significantly higher in <200 µm fraction than in <5 µm fraction samples, in contrast from other studies (see Šimek *et al.*, 2001, 2003; Hornàk *et al.*, 2005).

Grazer stimulation of prokaryotic and viral activities

Based on the reasonable assumption that flagellates graze on both infected (Binder, 1999) and noninfected prokaryotic cells, it could be assumed that grazing pressure should result in reduced viral infection. However, in our control experimental samples without nutrient addition, the abundances

of prokaryotes (PA) and viruses (VA) and FIC increased more in the presence of grazers (that is, $<200\,\mu m$ fraction) than in their absence ($<0.8\,\mu m$ fraction), corroborating previous findings suggesting that grazers can stimulate bacteria and their main viruses. The grazer stimulation of viral proliferation was first reported by Šimek *et al.* (2001) and others in Rimov Reservoir (Weinbauer *et al.*, 2003, 2007). Similar conclusion was also evident from our earlier experimental studies indicating that increase in viral lytic production in two different lakes, that is, the Lake Bourget (Jacquet *et al.*, 2007) and the Sep Reservoir (Sime-Ngando and Pradeep Ram, 2005), can be related to the presence of bacterivores and their potential activity. We consider that grazing activity generates growth resources for prokaryotes, the main potential hosts for viruses, and assume a synergy between grazer, prokaryotic and viral activities in oligotrophic conditions at a short time scale, through a cascading effect from grazer-mediated resource enrichment (Sime-Ngando and Pradeep Ram, 2005). The present study thus corroborates this assumption and further provides evidence that the addition of conservative nutrients had stronger effects on prokaryotic production and the related viral infection than do grazing, because the stimulating effects of the added nutrients, primarily of P for the targeted communities, were apparently stronger in the absence than in the presence of grazers (Figures 1 and 2).

Effects of nutrient enrichment on prokaryotic and viral activities

Our experiment was carried out during a clear water phase, following the apparent exhaustion of phosphorous in the reservoir (Pradeep Ram *et al.*, 2005). The N:P ratio in the experimental samples (223:1) was very high, characteristic of P limiting conditions in the Sep Reservoir during the summer (Tadonléké *et al.*, 2002). Limitations of prokaryotic growth and activity in Sep Reservoir especially by P has always been inferred from the measured concentration of potential limiting substrates rather than by nutrient bioassay approach. Therefore in our study, nutrient treatments served as a case to investigate the effects of increased inorganic nutrient and carbon concentrations on prokaryote and viral activity.

Nutrient additions evoked positive responses in prokaryote and viral parameters with increase in their abundances and activities compared to unamended controls. Prokaryote abundance, productivity and growth efficiency increased with P additions, especially in grazer free fraction, thus providing direct evidence that P availability was a major limiting nutrient for prokaryotic activity in the Sep Reservoir during the studied period. We found that relieving nutrient stress (P limitation) was associated not only with an enhanced prokaryotic growth rate, but also stimulated viral abundance, production and viral-induced lyses of prokaryotes. As viral lysis is well known to increase growth rates of prokaryotes (for example, Weinbauer *et al.*, 2003), proportionately greater impacts of viruses on the growth rate estimates in the P treatments may have occurred in our experiment. Thus, nutrient enrichment had stronger effects on prokaryotic production and the related viral infection than do grazing in natural P-deficient samples.

During our experiment, a general stimulation of prokaryotic growth by P indeed resulted in higher virus induced prokaryote mortality (that is, 6%–43%), enhancing VA in the grazer free samples. Microcosm experiments using marine water samples from the Gulf of Mexico have indicated P to exert major influence on viral parameters, which occurs through increased host cell activity (Williamson and Paul, 2004). In the present study the mean growth efficiency of prokaryotes in natural samples (untreated control) was low ($18\pm6\%$). Additions of nutrients understandably increased this efficiency, especially in P-treated samples ($36\pm5\%$), which coincided with high VA and FIC. This could suggest that increased viral lysis could result in reduced flow of carbon to higher trophic levels (Fuhrman, 1999). As a consequence, large amount of organic carbon could be recycled within prokaryote-viral loop. Viruses can also have a control on the growth efficiency of their host by lysing the active cells but to what extent still is elusive. P limitation might be particularly important since phosphorous is a significant component of DNA, and virus particles comprise a large proportion of DNA (Wilson *et al.*, 1996); as a result viruses are more sensitive to P limitation. Viral molecules are potentially rich source of nutrients (P, N and organic carbon) in oligotrophic systems. However to what level do they serve as food source for grazers remains largely unknown (Gonzàlez and Suttle, 1993; Bettarel *et al.*, 2005).

When increases in phage abundances co-occurred with increases in prokaryote abundances in nutrient enriched samples, we consider that this was simply the result of nutrient stimulation of heterotrophic prokaryote growth. We believe that the tight coupling between viral lysis and prokaryotic growth caused the increase in viruses observed. Since viruses hijack the metabolism of the hosts, they are clearly but indirectly influenced by nutrient limitation, which can directly control the resource for viruses, that is, hosts (Weinbauer *et al.*, 2003). During our experiment, VA and FIC increased up to t24 or to t48 (more in $<0.8\,\mu m$ than in $<200\,\mu m$ fraction of nutrient-treated samples) and then declined subsequently towards the end of incubation period (Figure 2). The rapid response of viruses likely reflected high initial production rate at the expense of host cell growth following nutrient additions (Figure 1), and then declining with the availability of prokaryotic hosts.

Addition of nutrients can also cause induction of lysogens. However, this could be unlikely in the present scenario, as our microcosm experiments was performed at the time of the year which coincided with high abundance of protistan bacterivores ($11.9 \times 10^6\,l^{-1}$), increased protistan grazing (5.6×10^7 cells $l^{-1}\,h^{-1}$), and high percentage of lytic infection (Pradeep Ram *et al.*, 2005). The percentage of lysogeny has not been previously estimated in the Sep Reservoir. High prokaryotic standing stock and activity accompanied by high viral infection during the period of severe P limitation possibly suggest that nutrient bioassay approach ($<0.8\,\mu m$ sample fraction) served as a proxy to experimentally validate our hypothesis that regeneration of inorganic nutrients (especially P) due to the significant grazing by bacterivores is a stimulating factor for prokaryotic growth and viral proliferation. Moreover FIC was best related to PA ($r=0.44$, $P<0.05$) and PP ($r=0.81$, $P<0.001$) in the $<0.8\,\mu m$ fraction. Other different processes like sloppy feeding, excretion, egestion and release of faecal pellets from zooplankton could also lead to nutrient regeneration.

Effect of nutrient enrichment on prokaryotic community composition and the ecological implications

The prokaryotic community composition was determined using oligonucleotide probes targeting major groups of prokaryotes generally encountered in freshwater ecosystems (Glöckner *et al.*, 1996; Pernthaler *et al.*, 1998). The relative abundance of bacteria phylum, determined by FISH, can vary greatly between ecosystems, varying between 35% (Jürgens *et al.*, 2002) and 100% of the total prokaryotic abundance (Weiss *et al.*, 1996). The values recorded in our study (mean 45%; range 25–57%) were similar to those reported in the same ecosystem in previous studies (Jardillier *et al.*, 2004, 2005, Sime-Ngando and Pradeep Ram, 2005), but were slightly lower than those recorded in oligotrophic (52–55%) (Pernthaler *et al.*, 1998; Glöckner *et al.*, 1999) or mesotrophic systems (60%) (Glöckner *et al.*, 1999). This could suggest that other typical freshwater groups such as *Actinobacteria*, which were not taken into account in the studies conducted in the Sep Reservoir, could represent a potentially important cluster in this milieu.

The percentage of prokaryotes hybridizing for specific probes in various nutrient treatments was suggestive of their lifestyle. Prokaryotic populations hybridizing for the probes EUB, BETA and ALF responded to nutrient treatments, primarily to P (Figure 4). This implies a higher cell-specific activity of these groups of bacteria in the resource-enriched treatments, and suggests that they are likely good competitors for resources. Cells hybridizing for ARCH and CF probes failed to show response with nutrient addition. Although CF was found to be bad competitor for resources, interestingly they displayed protective mechanism against grazing and infection with their ability to form filamentous and flocs in the presence of grazers. CF increased with incubation time in the grazer enhanced fraction which received no nutrient additions (that is, $<200\,\mu m$ fraction controls, Figure 4e), thus validating our earlier finding in the Sep Reservoir (Sime-Ngando and Pradeep Ram, 2005). The ability of these bacteria to form such floc and filaments in response to strong predatory pressure has also been reported in mesotrophic Rimov Reservoir (Šimek *et al.*, 2001, 2003). The finding that CF was not stimulated by nutrient enrichment may result from the fact that this bacterial group is most often associated with activities of breakdown of complex molecules (Kirchman, 2002).

Among the targeted groups, the subclass BETA was quantitatively dominant, corroborating with the earlier findings in the Sep Reservoir (Jardillier *et al.*, 2004, 2005; Sime-Ngando and Pradeep Ram, 2005) and other freshwater systems (Glockner *et al.*, 2000; Hornàk *et al.*, 2005). These phylotypes probably harboured a higher rRNA-targeted content and contributed significantly to the faster growing and highly active fractions in prokaryotic community. The members of this phylotypes (BETA subclass) were likely preferentially lysed, as suggested by its significant correlation with VA ($P<0.01$) and FIC ($P<0.001$). Apparent changes in PCC were obvious with addition of nutrients, which in turn supported high viral density and production. Shifts in PCC due to nutrient addition can correspond to the changes in average cell carbon content and therefore can complicate calculation of growth efficiency. From our microcosm study it is evident that regeneration of nutrients (especially P) by grazers had strong impact on PCC, which could suggest possible life strategy of different prokaryotes in relation to the availability of nutrients, especially P. Such a significant relationship between P availability and growth response of relatively broad taxonomic groups of prokaryotes has been established in Rimov Reservoir (Šimek *et al.*, 2006).

In the present study we observed consumer-specific effects on PCC (against a background of BETA dominating in all the treatments) suggesting distinct vulnerabilities to the two sources of mortality, that is, grazers and viruses. Unlike synergy, antagonistic effects of viral lysis and protozoan grazing on prokaryotic abundance, production and diversity has not received enough attention in viral ecology. The findings of the present study provide evidence of existence of synergy between viruses, prokaryotes and grazers, especially in oligotrophic and nutrient limited systems. However, at community level the interactions of prokaryotes, viruses and grazers could be more complex than assumed. For example, in the $<200\,\mu m$ fraction, high PP (t48) in the CNP treatment did not result in high FIC, nor it altered the PCC (at least the dominant BETA

subgroup). This indicates some sort of chaotic behaviour in microbial biotopes (Arndt *et al.*, 2000; Becks *et al.*, 2005). However, such type of anomalies was not present in the $<0.8\,\mu m$ fraction. Although the pattern of 'deterministic chaos' has been well described by theoreticians through mathematical models (Vaynes and Pavlou, 1999; Kooi and Boer, 2003) in chemostat conditions, there is still lack of experimental evidence in real biological systems of microbial food web. Competition among predators for the same prey or host could result in synergistic or antagonistic effects, as both predators (viruses and protists) regenerate nutrients, thus fuelling the growth of their hosts. Such effects among coexisting populations (predator–prey systems) could lead to chaos, an aspect which so far has been less dealt with in aquatic ecosystems. So in the light of the above aspects it should be noted that the results of such a short-term experiments should be extrapolated to nature with care.

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