Microbial Ecology

# Effects of Viruses and Predators on Prokaryotic Community Composition

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*Received: 16 February 2005 / Accepted: 13 June 2005 / Online publication: 15 December 2005*

### Abstract

Dialysis bags were used to examine the impact of predation and viral lysis on prokaryotic community composition (PCC) over a 5-day experiment in the oligo-mesotrophic Lake Pavin (France). The impact of the different predator communities (protists and metazoans) of prokaryotes was estimated by water fractionation (<5 μm: treatment filtered on 5 μm, without ciliates and metazoans; UNF: unfiltered treatment with all planktonic communities). Enrichments of natural viruses (<1.2 μm: with a natural virus concentration; <1.2 μm V and VV: with enrichment leading to a double or triple concentration of viruses, respectively) were used to indirectly assess the control of virioplankton. Viral activity was estimated from the frequency of visibly infected cells (FVIC). PCC was determined by fluorescence *in situ* hybridization (FISH) and terminal restriction fragment length polymorphism (T-RFLP). In this study, PCC was affected by the eukaryote communities (especially flagellates), and viruses to a lesser extent. Cyanobacteria declined significantly during the experiment and were highly correlated with the FVIC. In addition, the 503-bp terminal restriction fragment (T-RF) disappeared in treatments with virus enrichments, suggesting possible viral-associated mortality processes, whereas the 506-bp T-RF was not affected in these treatments. On one hand, these results suggest a control of the PCC: first, by viral lysis of some dominant phylotypes and second, by interspecific competition between resistant strains for the uptake of substrates released by this lysis. The increase of Archaea may suggest that these cells benefit such resources. On the other hand, the disappearance and the stable proportion of some dominant phylotypes suggested a selection pressure due to the predatory activity on prokaryotes. In conclusion, prokaryotic abundance appears to be mainly controlled by flagellate protists, which also affected PCC, whereas viruses seemed to be essentially responsible for profound changes in PCC via direct and indirect actions.

### Introduction

In aquatic ecosystems, microbial populations play an essential role in the mineralization of the nutrients and as a trophic link between dissolved organic carbon and microbial grazers [2]. Thus, it is important to understand how this community evolves and which factors control its dynamics. Among the different factors that are responsible for planktonic prokaryote mortality, predation by protists [e.g., 16, 29] and, to a lesser degree, metazooplankton [e.g., 24] have been thoroughly investigated. The discovery of highly abundant viral particles in natural waters [4] is based on current research on the impact of viral infection in aquatic microbial ecology [35]. It is not known whether viruses in aquatic environments are an active and important component of the microbial food web in terms of their potential roles in regulating prokaryotic mortality, production, and community structure [59, 60].

The effects of predation on the prokaryotic community composition (PCC) reported by many authors [e.g., 16, 24] are generally described as preferential selection of certain cells among the whole community. The main factor of selection for intercept-feeding predators such as flagellates seems to be prey morphotype [16]. Filament formation among planktonic eubacteria is interpreted by many authors as a resistance mechanism to flagellate

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predatory activity [21]. However, predation may be driven by other prokaryotic parameters, such as abundance [8], motility [30], swimming speed [31], and wall properties [16]. Cladocerans are also important grazers and their grazing activity may also shape the PCC [19, 24]. In this case, the variation of eubacterial diversity could be due to cell digestibility for metazoans [22].

The generally good correlation between prokaryotic and viral concentrations reported for pelagic systems have been used to argue that virioplankton are dominated by bacteriophages (i.e., viruses of heterotrophic prokaryotes) [54]. The relationships between the viruses and their hosts are controlled by the fact that viruses have no active form of motility, so they must be abundant enough to be encountered via passive diffusion [43]. In the environment, viral abundance is typically 10- to 20-fold higher than prokaryotic abundance [59], and viral mortality of prokaryotes is thought to increase with host abundance in marine environments [54], in contrast to lakes [7] where such a pattern is not evident. Wommack and Colwell [59] hypothesized that the numbers of a specific virus within the virioplankton are expected to change dramatically over time and space, in coordination with changes in abundance of their hosts, as shown in mesocosms by Øvreås *et al.* [38] for algal and planktonic prokaryote communities. It was reported that eubacterial morphotypes show different infection frequencies [55] and that the release of organic matter due to cell lysis may provide niches for specific species [32]. Recently, Schwalbach *et al.* [43], based on studies of marine microcosms, suggested that viruses have mixed effects on different members of the eubacterial community, some phylotypes increasing or decreasing in abundance and other phylotypes showing no response to changes in viral abundance. Nevertheless, previous studies demonstrated the ability of prokaryotes to quickly acquire resistance to co-occurring viruses, especially under favorable growth conditions [53], which could be caused by a reduction in the number or a change in the structure of virus receptors at the surface of the cells or by lysogenic prokaryotes [59]. Various mechanisms may be responsible for controlling the PCC: (1) the paradigm of phages "killing the winner," where viruses are thought to help control the population size of prokaryotes that are successful competitors for resources [56]; (2) the release of organic matter that can favor certain prokaryote species; (3) acquisition by lysogeny, leading to an immunity of the infected cells against homologous phages; and (4) the horizontal transfer of transduction genes, causing a transfer of host-encoded morphologic and metabolic traits [56].

These various results do show that although the impact of predation on the PCC has been extensively studied, that of viruses still has to be accurately evaluated [56], especially in lakes. To our knowledge, only the study by Šimek *et al.* [45] has examined the effects of viral infection on freshwater PCC. In their study, they concluded that protistan grazing exerts the highest control of the eubacterial diversity, ahead of viral lysis.

To examine the impact of viral lysis and predators of prokaryotes on the PCC, we conducted an experiment in dialysis bags over 5 days in the euphotic zone of an oligomesotrophic lake. Predatory activity on prokaryotes and viral lysis were estimated by differential filtration and virus enrichment, respectively. PCC was studied using fluorescence *in situ* hybridization (FISH) and terminal restriction fragment length polymorphism methods (T-RFLP).

## Methods

*Study Site.* The study was conducted in Lake Pavin, located in the French Massif Central (45°29′N, 2°56′E). Lake Pavin (altitude, 1197 m) is an oligomesotrophic, meromictic, and dimictic lake with partial overturns. It is a typical, crater mountain lake characterized by a maximum depth of 92 m and a low surface area (44 ha) and catchment area (50 ha). During the study, the water column was thermally stratified with a clearly identified thermocline between 10 and 15 m.

*Experimental Design.* We conducted the study from the 11th to the 15th of June 2001, using 2-L dialysis bags with a cutoff of 12–14 kDa (Merck Eurolab, Strasbourg, France). Bags were prepared according to the protocol of Herndl *et al.* [17]. Different water samples collected at 1 m depth with a Van Dorn bottle were homogenized in a basin. The dialysis bags were then filled with unfiltered (UNF treatment) or filtered water. For these last treatments, corresponding to predator-free experiments, water samples were filtered successively through 5- and 1.2-μm pore-size filters (polycarbonate, Millipore, Bedford, MA, USA) to obtain <5-μm treatments (containing protists, viruses, and prokaryotes) and <1.2-μm treatments (without prokaryote predators), respectively. The latter treatments were enriched with natural virus-like particles (VLPs; see below), leading to 1-fold enrichment (treatment <1.2 μm V) and 2-fold enrichment (treatment <1.2 μm VV). All steps and all samples were processed in sterilized Duran Schott glasses. Each treatment was reproduced in triplicate and incubated *in situ* at 1 m depth immediately after being filled. Water samples were incubated for 96 h and homogenized daily to avoid significant prokaryotic growth on the surface of the dialysis bag [42]. Samples were taken every 24 h for determination of total abundance of prokaryotes, picocyanobacteria, flagellates, and viruses, at $t$ = 0, 24, 48, 72, and 96 h. To estimate the prokaryote community composition, bags were sampled

at each time point except at $t$ = 72 h for FISH analysis and at $t$ = 0 and 72 h for T-RFLP analysis.

*Virus Concentration.* Viral concentrates were obtained through a process of prefiltration to remove prokaryotes and protists and ultrafiltration to effectively concentrate virioplankton. Lake water (20–60 L, 1 m depth) was thus prefiltered onto a 3-μm membrane filter (Millipore TSTP 142 50). Prokaryotes were then removed by tangential flow filtration with a 0.22-μm filter. Viruses remaining in the prefiltered water were concentrated to a final volume of approximately 500 mL by using a spiral-wound ultrafiltration cartridge filter (Millipore) with a 30-kDa cutoff, according to the method of Suttle *et al.* [49].

*Sample Preservation.* Samples were collected and fixed immediately with a final concentration of 4% formaldehyde for total prokaryotes, 2% formaldehyde for viruses, and 1% glutaraldehyde for flagellates. For *in situ* hybridization (FISH), samples fixed with formaldehyde (4% final concentration) were filtered (2 to 6 mL) on a white 0.2-μm pore-size filter (25 mm, polycarbonate, Millipore) 4 h after sampling and then frozen at −20°C. For nucleic acid extraction, the water was prefiltered through a 5-μm polycarbonate pore-size filter to screen out larger eukaryotes and particulate matter, and then approximately 100 mL was filtered through a 0.2-μm polycarbonate pore-size filter (Millipore) [3]. Air-dried filters were rolled and transferred to 2-mL microcentrifuge tubes (Eppendorf, Wilmington, DE, USA) and were then frozen at −20°C until nucleic acid extraction.

*Counts of Protists.* After being stained with primulin (final concentration of 200 μg $mL^{-1}$) [9], flagellates were filtered through a black polycarbonate membrane of 0.8 μm pore size (Nuclepore, Boston, MA, USA). Both preparations were made within 24 h of sampling and were stored at −25°C to minimize autofluorescence fading. Counts were done under an Olympus HBS epifluorescence microscope equipped with an epifluorescent HB2-RFL light source and an HBO 100-W mercury lamp. Large-sized phytoplanktonic species were counted by Utermöhl's [52] method using a Wild M40 inverted microscope (Leitz, Grand Rapids, MI, USA).

*Counts of Viruses.* VLPs were counted by epifluorescence microscopy and the fluorochrome YoPro-1, which provides reliable counts of free viruses in aquatic systems [5]. The filters were transferred to glass slides, covered with single drops of a solution containing 50% glycerol, 50% phosphate-buffered saline (0.05 M $Na_2HPO_4$, 0.85% NaCl, pH 7.5), and 0.1% phenylenediamine (made fresh daily from a frozen 10% aqueous stock solution; Sigma, St. Louis, MO, USA) on 25-mm square coverslips. All working solutions were filter-sterilized immediately before use by using Anotop 10 units (Whatman, Maidstone, UK) equipped with 0.02-μm pore-sized inorganic membranes and sterile syringes. VLPs were counted using an Olympus HB2 microscope equipped with a 100/1.25 Neofluar objective lens and a wide blue filter set. The size, the distinctive shape, and very much brighter fluorescence of prokaryotes clearly distinguished these particles from viruses.

*Abundance and Community Composition of Prokaryotes.* For determining total prokaryotic abundance, 1- to 6-mL samples were filtered on 0.2-μm black polycarbonate filters (25 mm, Millipore), stained with 1 μg $L^{-1}$ (final concentration) of 4′,6-diamidino-2-phenylindole (DAPI). Cells were counted under an epifluorescence microscope. Prokaryotic cells (400–800) were counted in 20–40 microscopic fields. Heterotrophic and autotrophic prokaryotes (picocyanobacteria) were counted separately by using a green filter that allows pigmented cells to be distinguished from nonpigmented cells.

*FISH with Group-specific Ribosomal RNA Oligonucleotides.* The abundance of three different classes and groups of the eubacterial domain and of the archaeal domain was analyzed by *in situ* hybridization with fluorescence oligonucleotide probing on membrane filters [14]. The oligonucleotide probes chosen targeted most cells of the beta and alpha subclasses of the class Proteobacteria (BET42a, 5′-GCCTTCCCACTTCGTTT-3′; ALF1b, 5′-CGTTCGYTCTGAGCCAG-3′) [26] and the *Cytophaga–Flavobacterium* cluster of the *Cytophaga–Flavobacterium–Bacteroides* phylum (CF319a, 5′-TGGTCCGTGTCTCA-GTATC-3′) [27]. For the archaeal domain, the ARCH915 probe (5′-GTGCTCCCCCGCCAATTCCT-3′) [47] was used. The probes were fluorescently labeled with the indocarbocyanine dye Cy3 (MWG Biotech, Germany). 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 ALF1b and ARCH915 probes, 35% v/v for other probes), and sodium dodecyl sulfate (SDS; 1 μL of a 10% solution). For hybridization, we used 2 μL of the ALF1b, BET42a, CF319a, and ARCH915 probes (100 ng $\mu L^{-1}$) brought up to 80 μL with hybridization buffer (unlabelled GAM42a was not used as a competitor to BET42a). Hybridization was conducted in moist conditions at 46°C for 90 min. Filters were rinsed with a Tris–HCl (1 mM), NaCl (250 mM final concentration for ARCH915, 225 mM for ALF1b, 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 1 μg $L^{-1}$) for 15 min and were subsequently fixed between slides with Citifluor oil. Slides were inspected with an inverted Leica epifluorescence microscope (magnification 1200×; Cambridge, UK) equipped with a filter for UV excitation (DAPI) and for green excitation (Cy3). From 10 to 40 fields were examined for each probe [21]. As in Pernthaler *et al.* [39], DAPI images were recorded at exposure times of 1/60 to 1/8 s and Cy3 images were recorded at exposure times of 14 to 12 s with a 3CCD Sony Camera (DXC 950P). Prokaryote length (L) and width (W) were measured at $t = 0$, 24, and 48 h using an image analysis system (Qwin, Leica) with a Cy3 filter. Among the various values suggested as upper prey size limits for Heterotrophic Nanoflagellates (HNF) [16], we chose the limit of 2.4 μm defined by Jürgens *et al.* [21]. Hereafter, ARCH915, ALF1b, BET42a, and CF319a mean the abundance of cells hybridizing with the correspondent oligonucleotide probes.

*T-RFLP Analysis.* PCC was monitored by T-RFLP and changes of diversity between treatments were assessed by T-RF presence/absence and relative peak areas. In this study, T-RF relative areas presented coefficients of variation not exceeding 1% on multiple runs (three) for each sample.

Genomic DNA extraction and T-RFLP analysis were conducted according to Liu *et al.* [25]. Cells were released from the filter surface using TE buffer. The use of a lysozyme solution, followed by incubation with proteinase K and SDS, led to the precipitation of polysaccharides and residual proteins and to cell wall destruction, respectively. The precipitate of proteins and lipids was removed by extraction with chloroform/isoamyl alcohol followed by extraction with phenol/chloroform/isoamyl alcohol. The supernatant was treated with isopropanol and centrifuged. The pellet was washed with ice-cold ethanol, centrifuged, dried under vacuum, and redissolved in TE buffer. DNA yield was quantified by fluorescence assay (DNA Quantitation Kit, Sigma).

The primers used for amplification of eubacterial small subunit rDNA were 27f-FAM (6-carboxylfluorescein) [5′-AGA GTT TGA TCC TGG CTC AG-3′; mostly Eubacteria: Lane [23], labeled at the 5′- end with fluorescent sequencing dye (MWG Biotech)] and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′; mostly Eubacteria and Archaea: Lane [23]). Polymerase chain reactions (PCRs) were performed according to Jardillier *et al.* [19]. Products were purified using the Qiaquick PCR purification Kit (Qiagen, Valencia, CA, USA), visualized on 1% agarose gels, and quantified (DNA Quantitation Kit, Sigma). Enzymatic digestions were performed by incubating 100 ng of PCR products with 20 U of *Msp*I (Gibco BRL, Grand Island, NY, USA) at 37°C overnight. The samples were desalted with Microcon columns (Amicon, Beverley, MA, USA). The T-RFs were separated on an automated sequencer (PE ABI 310). Terminal restriction fragment size between 50 and 850 bp with peak area of >50 fluorescence units were determined using GeneScan analytical software. Each sample was analyzed three times and a peak was kept if it occurred in at least two profiles. To account for small differences in the running time among samples, we considered fragments with less than one base difference to be the same length. The resulting values were rounded up or down to the nearest integer. A program in Visual Basic for Excel was developed to automate these procedures.

*Phage-Infected Prokaryotes.* Prokaryotes contained in triplicate aliquots of 8-mL formalin-fixed samples, were harvested by ultracentrifugation onto 400-mesh electron microscope grids with carbon-coated Formvar film, by using a Centrikon TST 41.14 Swing-Out-Rotor run at 70,000 × *g* for 30 min at 4°C [44]. Each grid was then stained for 30 s with uranyl acetate (2% w/w) and examined at 40,000× magnification by using a JEOL 1200EX transmission electron microscope to distinguish between virus-infected and uninfected prokaryotes. A prokaryote was considered infected when at least five phages, identified by shape and size, were clearly visible inside the host cell. At least 600 prokaryotic cells were inspected per sample. For the three grids analyzed per sample, standard deviations were always <20%. Although this is pseudoreplication, it still provides an estimate of within-sample variations caused by measurement error. At confidence limits of 95%, precision for our frequency of visibly infected cells (FVIC) range from 45 to 55%. For more details, refer to Bettarel *et al.* [7].

*Prokaryotic Production.* Prokaryotic production (PP) was determined by the [*methyl*-$^3$H]thymidine ([$^3$H]-TdR) incorporation method described by Richardot *et al.* [42]. PP was calculated from rates of [$^3$H]TdR incorporation using the conversion factor of $2.0 \times 10^{18}$ cells $mol^{-1}$ [12].

*Statistical Analysis.* Relationships between the different quantitative variables were determined by Pearson's correlation coefficient. We used one-way analyses of variance (ANOVA) to test for significant differences across the time course of the experiment in each treatment and two-way ANOVA to test the effects of treatments and incubation time for the various parameters measured. Matrices of T-RF presence/absence (at $t = 0$ and 48 h) were analyzed by correspondence analysis (COA). Statistical analyses were computed with *R* software using an ADE package for COA analysis (http://cran.r-project.org/).

## Results

*Prokaryotic Community Abundance and Production.* The abundance of the heterotrophic prokaryotic community (Fig. 1A), determined by DAPI staining minus autotrophic cells, increased significantly (one-way ANOVA, $P < 0.05$) in all treatments over the time course of the experiment, except for the <5-μm treatment. In the <1.2 μm V and UNF treatments, the variation leading to an increase after 96 h of incubation, although significant (one-way ANOVA, $P < 0.05$), was less marked.

Unlike heterotrophic prokaryotes, the abundance of cyanobacteria (Fig. 1C) decreased significantly (one-way ANOVA, $P < 0.05$) in all treatments, over the first 72 h of the experiment, with the strongest decrease in <1.2-μm treatments (with and without virus enrichment).

PP (Fig. 1B) increased significantly in all treatments (one-way ANOVA, $P < 0.05$) after 96 h of incubation. Prokaryotic community abundance was significantly correlated with PP ($r = 0.57$, $P < 0.05$).

### Prokaryotic Community Composition

*FISH Results.* The abundance of ALF1b, BET42a, and CF319a (Fig. 2A) showed marked changes over the incubation period.

First, the total abundance of the cells that hybridized with the three eubacterial probes generally increased

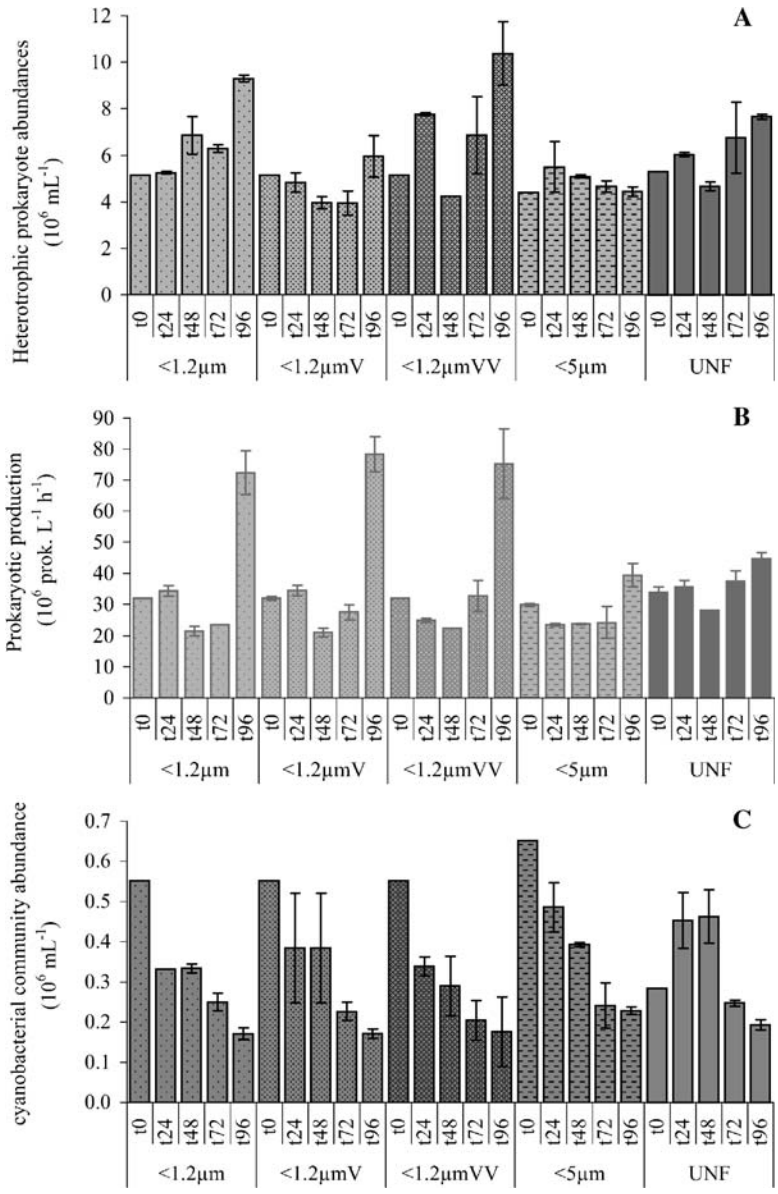


**Figure 1.** Time course changes over 96 h in the DAPI-stained cell abundance ($10^6$ mL$^{-1}$ ± SD) less autotroph cells (A), in the prokaryotic production ($10^6$ prok. L$^{-1}$ h$^{-1}$ ± SD) (B), in the cyanobacterial community abundance ($10^6$ mL$^{-1}$ ± SD) (C), in the <1.2 μm, <1.2 μm V (enriched by 1-fold with the initial concentration of virus), <1.2 μm VV (enriched by 2-fold with the initial concentration of virus), <5 μm, and UNF (unfiltered) treatments.

significantly (one-way ANOVA, $P < 0.05$) from the beginning to the end of the experiment, with the exception of the <1.2 μm VV. Across the virus gradient (from <1.2 μm, <1.2 μm V, to <1.2 μm VV treatments) this total abundance diminished.

Second, a strong variation of the relative abundance in the three eubacterial probes was observed. Thus, at the beginning of the experiment, BET42a was the dominant group studied, representing 44% of all cells hybridizing with the cells targeted by the three eubacterial probes, ALF1b and CF319a being similar in relative abundance (27 and 29% of total abundance, respectively). After 96 h of incubation, ALF1b (49 ± 2%, the error is the SD) and CF319a (58 ± 3%) became the dominant groups for <5 μm and <1.2 μm treatments, respectively. In the other treatments, BET42a was the major group of the three eubacterial groups studied, representing on average 59 ± 12% in the <1.2 μm, <1.2 μm VV, and UNF treatments.

The abundance of cells hybridizing with ARCH915 increased significantly (one-way ANOVA, $P < 0.05$) over the whole incubation period, except in the <1.2 μm and UNF treatments. The abundance of ARCH915 at $t = 96$ h increased from the <1.2 μm treatment to <1.2 μm VV (Fig. 2B).

Prokaryotes larger than 2.5 μm in length represented no more than 6% of the cells, and the size structure of the prokaryotic community did not differ significantly between the treatments (results not shown).

*T-RFLP Results.* The Shannon index demonstrated a slight decrease through the virus gradient from 4 ± 0.5 to 3 ± 1. Similarly, the total number of T-RFs decreased, from 69 ± 1 to 48 ± 17. Overall, the diversity, estimated by the total number of T-RFs and the Shannon index, was similar in treatments with natural virus concentrations.

Dominant T-RFs (with an area of greater than 5% for at least once, at $t = 0$ or 72 h in the different treatments) showed different relative areas among the different treatments after 72 h of incubation (Fig. 3). However, the sum of the relative areas of dominant T-RFs exceeded one third of total relative area after 72 h and reached 77% in the <1.2 μm VV treatment. Whereas the 100-bp T-RF was recorded only in the <1.2 μm V and <1.2 μm VV treatments, the 495-bp T-RFs were observed only in the presence of eukaryote predators. The 99-bp T-RF became dominant in all treatments after 72 h of incubation. Although the 507-bp T-RF was not present or was below the detection limit at the beginning of the experiment, it was considerable after 3 days of incubation in all treatments except the <1.2 μm treatment. The 97-bp T-RF was present in all treatments, even in the <1.2 μm VV treatment.

Analysis of the matrix of presence/absence of T-RFs in the different treatments using correspondence analysis (Fig. 4) reflects the diversity between treatments caused by the difference in the eubacterial composition. Indeed, the T-RF compositions in treatments after 72 h of incubation were strongly different from those at the start

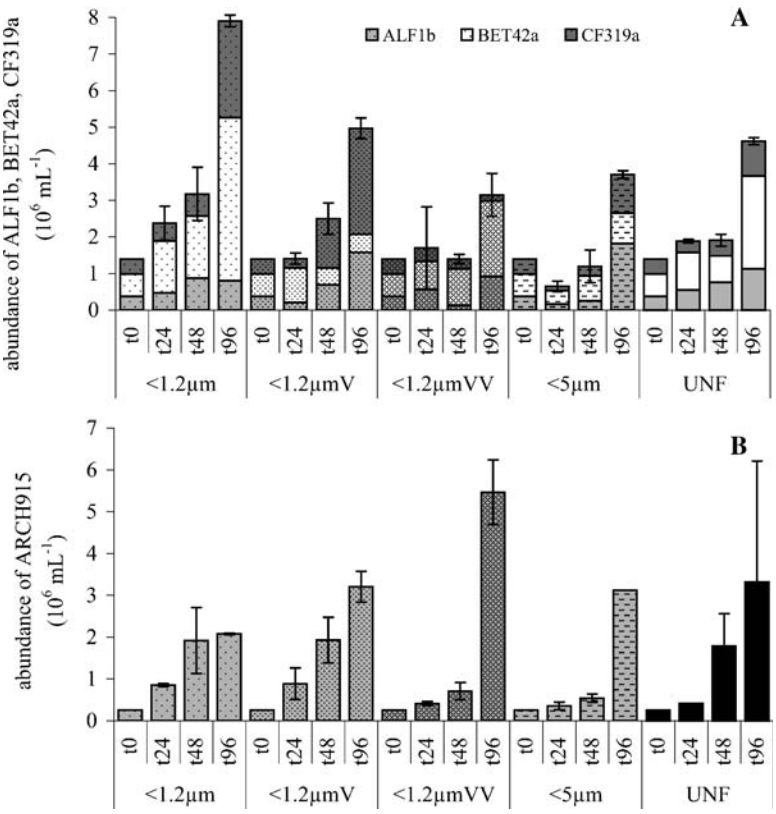


**Figure 2.** Time course changes over 96 h in the abundance ($10^6$ mL$^{-1}$ ± SD) of cells hybridizing with oligonucleotide probes: ALF1b (alpha-proteobacteria), BET42a (beta-proteobacteria), CF319a (*Cytophaga–Flavobacterium*) (A) and ARCH915 (Archaea) (B) in the <1.2 μm, <1.2 μm V, <1.2 μm VV, <5 μm, and UNF treatments over the whole experiment. Abbreviations as in Fig. 1.

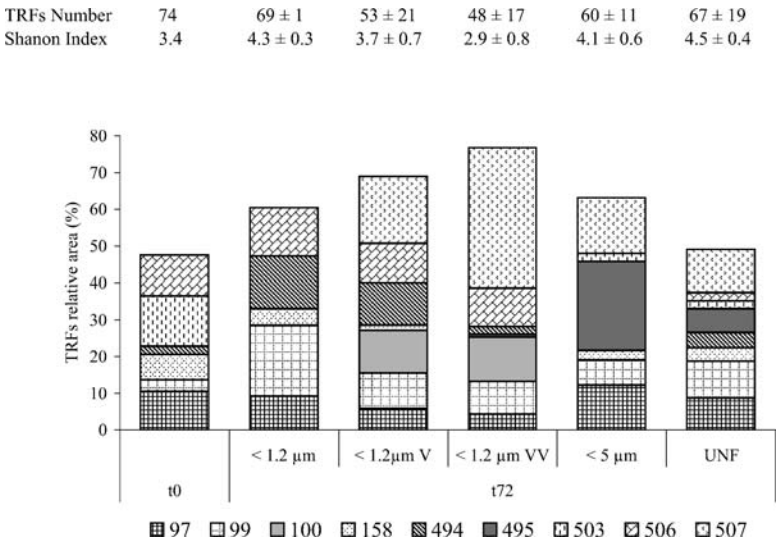


**Figure 3.** Relative area percentage of T-RFs detected from T-RFLP analysis of 16S rDNA digestion by *Msp*I, representing more than 5% in the <1.2 µm, <1.2 µm V, <1.2 µm VV, <5 µm, and UNF treatments after 72 h of incubation. *Numbers above histograms*: first line, the total number of T-RFs; second line, mean values of Shannon index calculated with all the TRFs. Abbreviations as in Fig. 1.

of the experiment. Axis 2 discriminated the various treatments on their T-RF composition, especially those with virus enrichment (<1.2 µm V and <1.2 µm VV treatments) from those with natural concentrations of viruses (<1.2 µm, <5 µm, and UNF treatments). This result suggested a greater impact of viruses, compared to that of flagellates and total planktonic community, on eubacterial diversity.

*Flagellate Abundance.* During the whole study, the flagellates were dominated in the UNF treatments by pigmented cells, *Rhodomonas* sp., and *Chrysidalis* sp. Their abundance was $23.1 \times 10^5$ cells $L^{-1}$ at $t = 0$ and reached $36.0 \times 10^5$ cells $L^{-1}$ at the final time. The heterotrophic cells were *Kathablepharis* sp. and unidentified cells. Their abundance remained stable [mean of $(6.8 \pm 1.3) \times 10^5$ cells $L^{-1}$] (Fig. 5A).

In <5 µm treatments, only flagellates smaller than 5 µm were present, namely, *Chrysidalis* sp. and unidentified cells, partly explaining the significant reduction in flagellate abundance. The abundance of pigmented flagellates became quantitatively dominant from 24 h of incubation onward (from $3.0 \times 10^4$ to $9.1 \times 10^4$ cells $L^{-1}$).

*Viral Abundance.* The enrichments doubled the concentration of VLPs at the initial time in the <1.2 µm V treatment [$(5.8 \pm 0.5) \times 10^7$ $mL^{-1}$ VLPs] and multiplied it almost 3-fold in <1.2 µm VV treatments ($8.4 \pm 1.1 \times 10^7$ $mL^{-1}$ VLPs). In these virus-amended treatments, the abundance of the VLPs decreased significantly (one-way

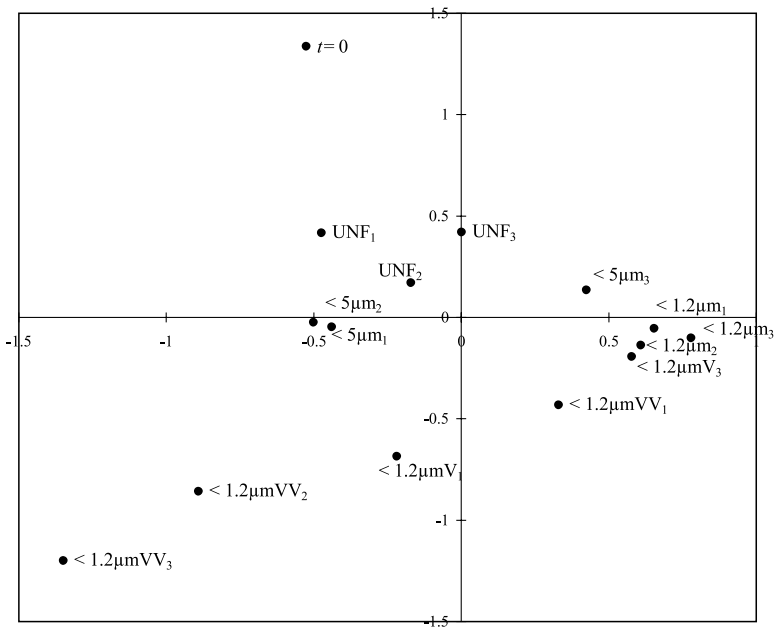


**Figure 4.** Results of the correspondence analysis performed on the T-RFs (presence–absence) detected from T-RFLP analysis of 16S rDNA digestion by *Msp*I in the <1.2 µm, <1.2 µm V, <1.2 µm VV, <5 µm, and UNF treatments after 72 h of incubation. The percentage of inertia explained by axes 1 and 2 are 17.2 and 15.4%, respectively. Abbreviations as in Fig. 1.

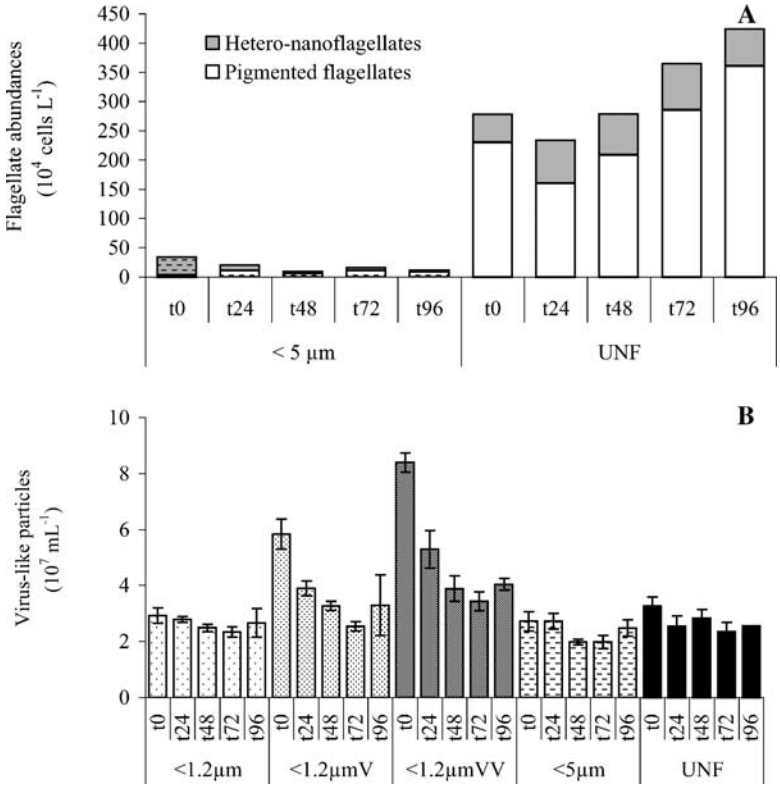


**Figure 5.** Time course changes over 96 h in the pigmented (A) and heterotrophic (B) flagellate abundance (cells $L^{-1}$ ± SD) in the UNF and <5 µm treatments, and in the abundance of VLPs ($10^7$ mL$^{-1}$ ± SD) (C) over the whole experiment in the <1.2 µm, <1.2 µm V, <1.2 µm VV, <5 µm, and UNF treatments. Abbreviations as in Fig. 1.

ANOVA, $P < 0.05$) during the experiment by about 50. This was also true from the beginning of the experiment up to 72 h of incubation (one-way ANOVA, $P < 0.05$). Contrary to the other treatments, viral abundance (Fig. 5B) in the <1.2 µm treatment did not vary significantly (one-way ANOVA, $P < 0.05$) between $t = 0$ and 72 h or even $t = 96$ h.

*Frequency of Visibly Infected Cells.* The FVIC (Fig. 6) decreased significantly during the incubation in all treatments, and varied significantly (two-way ANOVA: time × <1.2 µm treatments, with and without virus enrichment, $P < 0.05$) across the virus gradient (from <1.2 µm to 1.2 µm VV). FVIC was strongly and positively correlated with picocyanobacteria abundance and was negatively correlated with the abundance of other prokaryotes, except for the BET42a (Table 1).

## Discussion

*Methodological Aspects.* The incubation of microorganisms in microcosms remains an invaluable tool for answering specific questions, especially in microbial ecology [46].

Nevertheless, the filtration procedure used in this experiment not only may lead to shifts in dissolved organic matter (DOM) sources by enrichment *via* cell lysis, but may also eliminate carbon sources, such as autotrophic cells, predators, and viral activities. Thus, it may limit the growth of some prokaryotic populations.

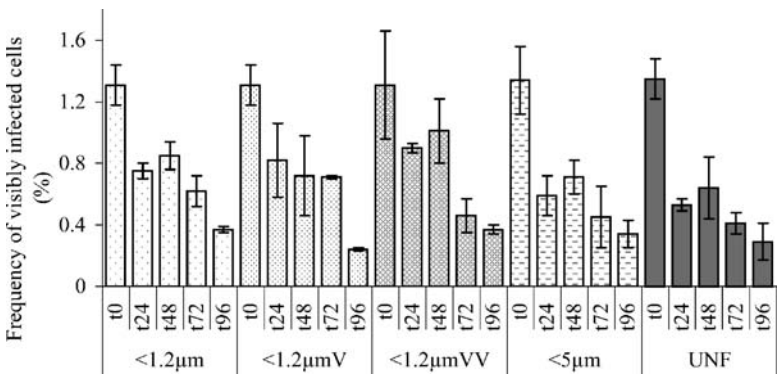


**Figure 6.** Time course changes over 96 h of the frequency of FVIC (% ± SD) over the whole experiment in the <1.2 µm, <1.2 µm V, <1.2 µm VV, <5 µm, and UNF treatments. Abbreviations as in Fig. 1.

**Table 1. Relationships between cells abundance, prokaryotic production, and mortalities over the 96 h of incubation**

| | *Variables that showed significant correlation with FVIC* |
|---|---|
| *Cell abundance ($mL^{-1}$)* | |
| DAPI-stained cells | −0.42* |
| ARCH915 | −0.69** |
| ALF1b + BET42a + CF319a | −0.65** |
| BET42a | NS |
| ALF1b | −0.66** |
| CF319a | −0.52* |
| Cyanobacteria | 0.72** |
| Prokaryotic production (prok. $L^{-1}$ $h^{-1}$) | −0.48* |
| Mortalities ($10^6$ prok. $ML^{-1}$ $d^{-1}$) | 0.51** |

Numbers are *r* values.
NS, Not significant.
*$P < 0.05$.
**$P < 0.01$.

Indeed, the prokaryotic production is highly dependent on DOM [42]. However, the absence of higher prokaryotic production, between $t = 0$ and $t = 72$ h, in treatments other than in the untreated control (UNF treatment) suggests that these different DOM biases have only a slight effect. These filtration procedures may also exclude particle-associated bacteria, thus constituting a bias for DNA analysis. However, prokaryotes attached to particles of less than 5 μm in size represented less than 2% of total heterotrophic prokaryote abundance in Lake Pavin in 2000 [10].

The virus concentration process using a Prepscale pump might destroy virus particles, but Noble *et al.* [37] indicated that this method led to intact and distinctly recognizable free virus particles. Moreover, the virus enrichments may introduce another source of DOM by adding high molecular weight molecules that co-concentrate with viruses. The viruses added may be a source of carbon for certain prokaryotes.

In this study, we used the FISH method, a well-established quantitative method, for the examination of bacterial diversity [1]. However, for methodological reasons, we did not take into account the class Actinobacteria, reported as a potentially important eubacterial planktonic group in freshwater ecosystems by Glöckner *et al.* [15]. Thus, to study changes of eubacterial diversity between treatments, T-RFLP was used. A number of studies have shown that the interpretation of the results of fingerprinting techniques such as T-RFLP is potentially biased by the PCR amplification [40, 50]. However, this molecular method is highly reproducible [43] and a recent report seems to show that it can yield results proportional to bacterial abundance [40]. The FISH method has the disadvantage, in our case, of using group probes and not more specific probes. The *Cytophaga–Flavobacteria–Bacteroides* and beta- and alpha-Proteobacteria are broad taxonomic groups. However, the observations of Horner-Devine *et al.* [18] suggest the existence of significant patterns at this taxonomic scale.

The high proteobacterial, eubacterial, and archaeal abundance observed after 96 h of incubation may result from confinement effects despite the use of dialysis bags, as was already demonstrated by Øvreås *et al.* [38]. Therefore, most of the discussion will be focused on data obtained at $t = 72$ h and before. Such an incubation time seems to be sufficient to observe shifts in PCC, since this had been the case for a period of 24 h [19].

*Impact of Viruses and Flagellate Predation on Prokaryotes.* One way of determining virus-mediated effects on the prokaryotic community is to increase the relative number of infectious particles in the sample, thereby increasing the number of potential encounters between viruses and potential hosts [37, 58]. In our experiments, the enrichment led to an increase of VLPs, after which these particles decreased during the incubation, similarly to the results reported by Noble *et al.* [37] in marine microcosms. However, contrary to Noble *et al.* [37], who observed an increase in infected cells after viral amendment, we were unable to observe an amendment-related change in FVIC at the beginning of the experiment. Meanwhile, there was a significant difference in FVIC ($P < 0.05$) along the virus gradient, suggesting that viral activity differs between these treatments. Thus, viral enrichment did not lead to a prokaryotic lysis proportional to the probability of contact rates between prokaryotes and viruses [36]. A first hypothesis is that the added viruses may be subjected to a high destruction rate, inactivation, after inoculation in the dialysis bags. On other hand, the virus may trigger prokaryotic production of extracellular enzymes, which are capable of degrading viral proteins and nucleic acids. A third possibility is that many of the viruses added in the enrichment may not have had a corresponding or sufficiently abundant host in the freshwater incubations [37, 48].

In the presence of higher heterotrophic flagellate abundance (UNF treatment), prokaryotic abundance was not lower. One hypothesis is that the predatory activity of flagellates may change between filtration treatments due to water fractionation, which leads to the removal of communities and thus to modifications of trophic relations and potential sources of resources [20]. On the other hand, the significant correlation between PP and prokaryotic community abundance demonstrates that this community seems to depend essentially on resources in this oligomesotrophic ecosystem.

*Effects of Treatments on Prokaryotic Community Composition.* To more accurately observe the effects of viruses and planktonic communities on the PCC (heterotrophic and autotrophic eubacteria), we used

T-RFLP analysis at $t = 0$ and $t = 72$ h before a potential confinement effect. The statistical analysis (Fig. 4) suggests that PCC was affected by eukaryotic communities and viruses. Indeed, this axis discriminates the different treatments, especially those with predators (i.e., <5 μm and UNF treatments) from those with virus amendment (<1.2 μm V and VV treatments). Virus-enriched treatments, at $t = 72$ h, were the most separated from $t = 0$ h. Relative cellular abundance of eubacterial groups also demonstrate modifications in the PCC, but the negative correlations between the decreasing FIVC and the increasing abundance of prokaryotes during our study seem to indicate that viruses did not strongly affect their abundance. These results seem to confirm the hypothesis that viral lysis is a significant factor for producing shifts in the composition of heterotrophic eubacteria and cyanobacteria and not their abundance [34].

In all treatments, we did not observe a substantial proportion of filamentous prokaryotes (size larger than 2.5 μm). These prokaryotic morphotypes are thought to appear when HNF are the main prokaryote predators (for a review, see [16]) and have been shown to remain uninfected by mature phages [45]. The absence of filament development was probably due to the fact that there was no strain able to switch to that kind of morphotype [41] or because the protists that were present during this study are able to feed on filament forms [61].

Along the virus gradient, the sum of the abundance of cells hybridizing with the three eubacterial probes was less abundant after 48 h of incubation, suggesting an impact of the virus amendment on the abundance of eubacteria. In contrast to these organisms, Archaea had similar abundance in the <1.2 μm and <1.2 μm V treatments but were less abundant in the treatment with the highest virus amendment. This trend may suggest that most viruses in this experiment are not phages of archaeal cells. Indeed they seem to suffer viral lysis only when concentrations of VLPs are very high. However, by using a fingerprinting method (T-RFLP), Winter *et al.* [58] showed that viruses controlled the richness and specific phylotypes of this community in a marine environment.

The development of prokaryotic populations that were adapted to the specific experimental conditions, observed by changes in the relative abundance of the ALF1b, BET42a, and CF319a, was demonstrated by the T-RFLP results. Indeed, in our experiment and for the eubacterial community, the Shannon index and the total number of T-RFs decreased along the virus gradient, unlike the sum of the relative areas of the dominant T-RFs. A decrease in eubacterial richness and changes in the dominant T-RFs were also observed by Winter *et al.* [58] and Schwalbach *et al.* [43] during experiments conducted with various concentrations of viruses.

A sequencing study and phylogenetic analysis of rDNA 16S in this lake showed that the T-RF of 503 bp could be attributed to *Synechococcus* sp. (D. Boucher, pers. com.). In this ecosystem at this period, this T-RF, representing about 15% at $t = 0$, was the most dominant, and picocyanobacteria represented up to 9% of the total abundance of prokaryotes. Thus, the abundance of the picocyanobacterial community seems high enough in our experiment to allow sufficient contact rates with viruses. The FVIC was correlated with the abundance of picocyanobacteria, which is characterized by a large decrease over time. In addition, the 503-bp T-RF disappeared in treatments of the viral gradient. Thus, it seems likely that this potential host (i.e., picocyanobacteria) had a specific virus, which may have been the most abundant, and FVIC would have clearly reflected its infection. All these results, following the assumption of a host–parasite and not a prey–predator relation, seem to indicate that the picocyanobacterial community could be strongly infected and thereby constitute a potential host source for the viruses present during our experiment. Øvreås *et al.* [38] suggested that a significant proportion of viruses might be associated with algae and cyanobacteria [28] and that viruses play an important role in the termination of phytoplankton blooms [11].

The disappearance of the 503-bp T-RF in treatments containing only viruses and the decrease of the 97-bp T-RF along the virus gradient suggest that viruses infected some of the most abundant members of the prokaryotic community. However, all the dominant T-RFs did not seem to be susceptible to infection. Indeed, the 506-bp T-RF, the second most important T-RF at $t = 0$ h, did not show any significant variation between the treatments filtered through 1.2 μm, whereas the 100-bp T-RF, which was not detected at the beginning of the incubation because of the sensitivity of the T-RFLP method, appeared only in the virus-amended treatments. This lack of effect of virus concentration on some T-RFs could be due to host specificity of the ambient virus consortium. Moreover, both these T-RFs can define eubacterial populations that become resistant to viruses. Indeed, previous studies have demonstrated the ability of eubacteria to quickly acquire resistance to co-occurring viruses [53]. Nevertheless, some eubacteria can indirectly benefit from the presence of viruses though the use of highly labile dissolved organic carbon released from lysed cells [33]. Another hypothesis is that these eubacterial populations benefited from resources resulting from the added viruses by stimulating their growth. This *de novo* production may therefore explain the increase of the relative area of the 507- and 100-bp T-RFs in the treatments with virus enrichments.

Archaea, known to be metabolically active in the water column of ocean water [13] and whose abundance increased over the incubation, especially in the virus treatments, could be the main beneficiary of the release of lysis products.

These results, obtained in controlled conditions, suggest a control of the PCC by the direct or indirect effects of viruses, which could be structured by interspecific competition among resistant strains, although this conclusion has to be taken with care. According to Schwalbach *et al.* [43], who, similarly, used a fingerprinting method at only one date, competition aspects could predominate at time scales of 2 to 5 days.

The stable abundance of the 506-bp T-RFs in all the <1.2 μm treatments between $t = 0$ and $t = 72$ h, but which was strongly reduced or disappeared in the presence of eukaryotes (in <5 μm and UNF treatments) suggest that there are potentially some phylotypes in the natural environment that are not susceptible to virus infection, as argued by Winter *et al.* [58], but which are sensitive to predation [57] and more especially to the HNF activity in these experiments. A second hypothesis is that these phylotypes benefit from the DOM released by autotrophic cell activity, which was absent in the <1.2 μm treatments.

## Conclusions

Overall, these results seem to indicate that predation and viral lysis constitute two mortality factors that affect the PCC by favoring or discriminating certain populations. With the exception of the study of Šimek *et al.* [45], to our knowledge, this study is the only one that takes into account the impact of viruses on freshwater PCC by using molecular methods. Overall, viral infection seems to affect a few abundant susceptible phylotypes, whereas others could be resistant in contrast to the concept of "phages kill the winner". Hence, the preferential lysis of cells, such as cyanobacteria in our study, may lead to the release of organic compounds that probably stimulate the activity of certain heterotrophic eubacterial or archaeal cells. The modifications in the microbial population structure could therefore result at first from the viral lysis and then from interspecific competition for resources. However, according to the model of Middelboe *et al.* [34], the growth of new phylotypes could explain how the overall density of eubacteria in the system was mainly unaffected by phages, since resistant clones complemented the fluctuations caused by viral lysis. To confirm this hypothesis, studies are needed to accurately determine the diversity of viruses and of their potential hosts in the same time frame and in similar experimental conditions.

## Acknowledgments

We thank Christian Faye and Sébastien Specel for automated sequencer and GENESCAN analysis, Denis Sargos for his technical assistance, Ibrahima Anne, Karel Horňák, and Michal Mašín for their help in the field, and Marie Charpin and Jean-François Carrias for their personal communication of results for picocyanobacteria not shown in this study. This study was supported by contract R-095 SOMIVAL and a CNRS international project ("PICS").

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