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Relative importance of nutrients and mortality factors on prokaryotic community composition in two lakes of different trophic status: Microcosm experiments

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Abstract

The effect of nutrient resources (N and P enrichment) and of different grazing communities on the prokaryotic community composition (PCC) was investigated in two freshwater ecosystems: Sep reservoir (oligomesotrophic) and lake Aydat (eutrophic). An experimental approach using microcosms was chosen, that allowed control of both predation levels, by size fractionation of predators, and resources, by nutrient amendments. Changes in PCC were monitored by fluorescent in situ hybridization (FISH) and terminal-restriction fragment length polymorphism (T-RFLP). The main mortality agents were (i) heterotrophic nanoflagellates and virus-like particles in Aydat and (ii) cladocerans in Sep. All the nutritional elements assayed (N-NO_3, P-PO_4 and N-NH_4) together with prokaryotic production (PP) always accounted for a significant part of the variations in PCC. Overall, prokaryotic diversity was mainly explained by resources in Sep, by a comparable contribution of resources and mortality factors in lake Aydat and, to a lesser extent, by the combined action of both.
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1. Introduction

Prokaryotes are mainly responsible for the recycling of nutrients and the decomposition of organic matter in the pelagic zone of freshwater ecosystems [1]. Because of its importance, the diversity and distribution of planktonic prokaryotes have thus attracted considerable attention with the development of molecular techniques, which have progressively identified the dominant eubacterial and archaeal groups in aquatic environments [2,3]. Some studies have also indicated variations in the prokaryotic community composition (PCC) with time, e.g., according to the productivity of the ecosystems [4]. Temperature, resources (bottom-up control) predation and viral lysis (top-down control) [5–7] have been demonstrated to be the main known processes able to control the spatial and/or temporal dynamics of prokaryotes. Among the resources, inorganic nutrients, but also the labile organic substrates produced by phytoplankton [8], are potential prokaryotes growth limiting factor. In whole lake and mesocosm experiments, Eubacteria have responded strongly to the addition of nitrogen and phosphorus, with or without carbon

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addition, challenging the assumption of a strict control of eubacterial growth by labile carbon [9]. Among the mortality agents able to control prokaryote abundance, heterotrophic and mixotrophic nanoflagellates, and ciliates are most often cited [10]. In certain lakes, cladocerans can also make a large contribution to the regulation of eubacterial populations [9,11,12]. The abundance of those predators, and, by that way, the importance of their impact, evolves spatially and temporally. At last, recent studies have shown that viruses can also play an important role in the control of these populations [13]. Fuhrman [7] noted that estimates of the virus contribution to prokaryotic mortality in aerobic waters range between about 10% and 50%.

Much research on the microbial trophic web has helped to determine the main factors controlling total prokaryotic abundance (DAPI-stained cells) and have led to the construction of models [14]. Bottom-up control (food supply) seems to be more important in regulating total prokaryotic abundances in oligotrophic systems, while top-down control (predation and viral lysis) seems to be more important in eutrophic systems [6]. However, current knowledge of the main factors controlling prokaryotic diversity is on the whole less advanced. Various seasonal studies or experiments in aquatic environments have shown that carbon resources and mineral nutrients influence prokaryotic community composition [1,15–17]. Many studies have shown that predation by protists and zooplankton can bring about changes in the BCC of freshwater ecosystems [e.g. [25]]. Prokaryotic morphotype may be the dominant criterion in selection by predators [20]. Other variables may be involved, such as the concentration of cells [21], their digestibility [12,22], their motility [23], their swimming speed [24], and the properties of prokaryotic membranes [25]. Thus, among the main variables able to structure eubacterial populations, the impact of predation has received much attention, and only a few studies have considered resources and predation concomitantly [16,26].

In the work reported here, we sought answers to the following questions. (i) What is the impact of mortality factors (predators and virus-like particles) and resources on prokaryotic communities (Eubacteria and Archaea)? (ii) What is the relative importance of each of these two variables? (iii) Does this relative importance depend on trophic status? To address these questions microcosm experiments were conducted in two freshwater ecosystems of different trophic status: one oligomesotrophic, Sep Reservoir (hereafter Sep), and one eutrophic, Lake Aydat (hereafter Aydat).

2. Materials and methods

2.1. Study sites

This study has been conducted in two lakes of different trophic status located in the Massif Central (France). The oligo-mesotrophic Sep reservoir, lying at an altitude of 500 m, was built in 1994 to irrigate croplands. It has an area of 33 ha, a mean depth of 14 m (max. depth 37 m), a volume of 4.7 mm^3 and a theoretical retention time of 220 days. The eutrophic lake Aydat, of volcanic origin, presents a larger area than the Sep reservoir, of 60.3 ha, but a lower maximum depth, of 15.5 m. It is located at 825 m altitude.

2.2. Experimental design

Two experiments, conducted in microcosms, were carried out on 29 May 2002 in the Sep reservoir and 23 July 2002 in lake Aydat. An experimental approach using microcosms was chosen, that allowed control of both predation levels, by graded elimination of predators, and resources, by nutrient enrichment. Nutrients enrichment and differential filtrations were crossed: for each level of filtration, there were two corresponding levels of nutrient, with (NP(+)) or without (NP(–)) enrichment of mineral nutrients. Each treatment was realized in 3 different microcosms (3 replicates) (Table 1).

Water samples were collected with a Van Dorn bottle and were homogenized in a basin. Microcosms were incubated the same day during 48 h in the epilimnion,

Table 1
Experimental design of microcosms experiments

		<1.2 μm Prokaryotes + viruses	<10 μm Prokaryotes + flagellates + viruses	UNF all planktonic communities
NP(−)	without enrichment of mineral nutrients	3 Microcosms of 2 l	3 Microcosms of 2 l	3 Microcosms of 4 l
NP(+)	enrichments with NH_4Cl, $NaNO_3$ and KH_2PO_4	3 Microcosms of 2 l	3 Microcosms of 2 l	3 Microcosms of 4 l

Predation and nutrients level were crossed: for each level of filtration, there were two corresponding levels of nutrient. Nutrients enrichment, of NH_4Cl, $NaNO_3$ and KH_2PO_4, were performed in order to reach eutrophic and hyper-eutrophic level for Sep reservoir and Aydat lake, respectively. Filtrations through 1.2 μm (<1.2 μm treatments) retrieved all eukaryotes and through 10 μm (<10 μm treatments) screened out large protists and zooplankton. Two litres polycarbonate bottles were used for <1.2 and <10 μm treatments and 4 l for UNF treatments. Each treatment was realised in 3 different microcosms (3 replicates). All these microcosms were incubated 48 h and samples were taken at $t = 0$, 24 and 48.

at 1 m depth in both ecosystem. Samples were taken at $t = 0$, 24 and 48. All steps and all samples were processed in sterilized Duran Schott glasses and autoclaved polycarbonate bottles (Nalgène) were used as microcosms.

Bottles of 2 l were filled with water serially filtered through 5 and 1.2 μm filters (<1.2 μm treatment) to retrieve all eukaryotes, or 10 μm polycarbonate filters (<10 μm treatment) to screen out large protists and zooplankton. All these filtrations were processed under a vacuum pressure not exceeding 100 mm Hg. Bottles of 4 l were filled with unfiltered water (UNF).

After the filtration step, microcosms corresponding to NP(+) treatments were enriched with small volume (<10 ml) of mixed aqueous solutions of nitrogen (NH_4Cl and $NaNO_3$) and phosphorus (KH_2PO_4). The ratio $NH_4Cl/NaNO_3$ was attempt to be respected, around 1/10 and 2/3 in Sep reservoir and Aydat lake, respectively. Nutrients were added, one time at the beginning of the experiment ($t = 0$), in order to reach an eutrophic level [27] for experiment conducted in the Sep reservoir, multiplying by a factor of 27 phosphorus concentrations (PO_4^{2-}: 0.00 and 0.11 mg l^{-1} in NP(−) and NP(+), respectively). Nitrogen concentrations were multiplying by a factor of 3 (NO_3^{2-}: 1.26 and 3.63 mg l^{-1} in NP(−) and NP(+), respectively; NH_4^+: 0.08 and 0.40 mg l^{-1} in NP(−) and NP(+), respectively). In lake Aydat, nutrients were amended in order to reach an hyper-eutrophic level by increasing the initial concentration of phosphorus by an order of 12 times (PO_4^{2-}: 0.02 and 0.24 mg l^{-1} in NP(−) and NP(+), respectively). Nitrogen concentrations were multiplying by a factor of 3.7 (NO_3^{2-}: 0.03 and 0.12 mg l^{-1} in NP(−) and NP(+), respectively; NH_4^+: 0.02 and 0.08 mg l^{-1} in NP(−) and NP(+), respectively).

2.3. Sample preservation

Samples were collected and fixed immediately with a final concentration of 4% formaldehyde for total prokaryotes and virus-like particles, 2.5% mercuric chloride for ciliates and 50 v/v glutaraldehyde for flagellates. The metazooplankton was fixed in a sucrose/formaldehyde solution (6% and 4% final conc., respectively) [28]. For fluorescent in situ hybridization (FISH), samples fixed with formaldehyde (4% final conc.) were filtered (2–6 ml) on 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 (Millipore) to screen out larger eukaryotes and particulate matter. The <5 μm fraction was collected with white polycarbonate filters (diameter 25 mm, pore-size 0.2 μm). Air-dried filters were rolled and transferred to 2 ml microcentrifuge tubes (Eppendorf) and were then frozen at −80 °C until nucleic acid extraction.

2.4. Abiotic variable measurements

Water temperature and dissolved oxygen were determined with a multiparameter probe (YSI GRANT 3800). Chlorophyll *a* concentrations were obtained by spectrophotometry [29]. Phosphate (PO_4-P), ammonium (NH_4-N) and nitrate (NO_3-N) were analysed in laboratory in water samples using standard methods [30].

2.5. Abundance and community composition of prokaryotes

2.5.1. Prokaryote abundances

We filtered 1–5 ml samples on 0.2 μm black polycarbonate filters (25 mm, Millipore), stained by 1 μg l^{-1} (final conc.) of 4,6-diamidino-2-phenylindole (DAPI), and counted them under an epifluorescence microscope [31]. Between 400 and 800 cells were counted for a total of 20–40 microscopic fields.

2.5.2. Fluorescent in situ hybridization with group-specific rRNA oligonucleotides

The abundance of the eubacterial domain, of 3 different eubacterial classes and groups and of the archaeal domain was analysed by in situ hybridization with fluorescence oligonucleotide probes on membrane filters [32]. The oligonucleotide probes chosen targeted most Eubacteria (EUB338, GCTGCCTCCCGTAGGAGT) [33], the β- and α-subclasses of the class Proteobacteria (BET42a, GCCTTCCCACTTCGTTT – ALF1b, CGTTCGYTCTGAGCCAG) [34] and the *Cytophaga–flavobacterium* cluster of the *Cytophaga–Flavobacterium–Bacteroides* phylum (CF319a, TGGTCCGT GTCTCAGTATC) [35]. For the Archaeal domain, the ARCH915 probe (GTGCTCCCCCGCCAATTCCT) [36] was used. The probes were fluorescently labeled with the indocarbocyanine dye Cy3 (MWG-Biotech). 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 probe, 35% v/v for other probes) and sodium dodecyl sulfate (SDS) (1 μl of a 10% solution). For hybridization, we used 4 μl of the EUB338 probe (50 ng μl^{-1}), 2 μl for the ALF1b, BET42a, CF319a and ARCH915 probes (100 ng μl^{-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 conc. 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 conc. 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 1000×) equipped with

a filter for UV excitation (DAPI) and for green excitation (Cy3). From 10–40 fields were counted for each probe and sample [20]. As in Pernthaler et al. [37], DAPI images were recorded at exposure times of 1/60 to 1/8 s, and Cy3 images were recorded at exposure times of 1/4 to 1/2 s. Prokaryotic length (L) and width (W) were measured at $t = 0$, 24 and 48 using an image analysis system (Qwin–Leica), under Cy3 excitation. Among the various values suggested as upper prey size limits for HNF [25], we chose the limit of 2.4 μm defined by Jürgens et al. [20].

2.5.3. T-RFLP analysis

Genomic DNA extraction and T-RFLP analysis were conducted according to Liu et al. [38]. DNA yield was quantified by Fluorescence Assay (DNA quantitation Kit – SIGMA).

The primers used for amplification of eubacterial small subunit rDNA (ssu rDNA) were 27f-FAM (6-carboxylfluorescein) (5′-AGA GTT TGA TCC TGG CTC AG-3′; mostly Eubacteria [39]) labeled at the 5′-end with fluorescent sequencing dye (MWG Biotech, Germany) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′; mostly Eubacteria and Archaea [39]). PCR reactions were performed according to Jardillier et al. [17]. Products were purified using the Qiaquick PCR purification Kit (Qiagen), 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 or *Rsa*I (Gibco BRL) at 37 °C overnight. The samples were desalted with Microcon columns (Amicon). The number of T-RFs obtained was lower with the action of *Rsa*I than of *Msp*I, as in a previous study [17]. However, the general trend of results obtained with these two enzymes was similar. For these reasons, we show only results obtained with *Msp*I.

The T-RFs (terminal restriction fragments) were separated on an automated sequencer (PE ABI 310). Terminal restriction fragment size between 50 and 800 pb with peak area of >50 fluorescence units were determined using Genescan analytical software. The samples were analyzed in triplicates and a peak was kept if it was occurred in at least 2 profiles and if its relative area was higher than 2%. To account for small differences in run time among sample, we considered fragment from different profiles with less than 1 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.

2.6. Virus-like particles abundance

Analysis of virus-like particles was performed using a FACSCalibur (Becton Dikinson) flow cytometer (FCM) and the protocol defined by Marie et al. [40]. Briefly, samples were diluted in TE buffer, stained with Sybr Green I(×1/10,000) and heated 10 min at 75 °C. We checked this protocol was suited for the analysis of virus-like particles in freshwater ecosystems. List mode files obtained concomitantly to FCM analysis were analysed using CYTOWIN.

2.7. Protist and metazooplankton counts and estimates of the predation rates on the prokaryote communities

Abundances of different communities of protists were determined at each time of the experiments ($t = 0$, 24 and 48) contrary to metazooplankton which were only counted at $t = 0$ and 48, because of the high volume needed. Flagellates were counted on black polycarbonate filters of 0.8 μm pore size (Nuclepore) after primulin coloration [41] and ciliates and large-sized phytoplanktonic species by the method of Utermöhl [42]. The metazoan zooplankton, contained in samples of 1 l, was filtered through a 55 μm sieve. Zooplankton was counted in a combined plate chamber (GmbH).

At the same time of counts, microsphere ingestions were realized in order to estimate grazing rates. Thus, a stock solution of tracer particles (0.5 μm diameter) was prepared from concentrated solution of Fluoresbrite plain microspheres (Polysciences) well dispersed with bovine serum albumin (BSA). The concentration of microspheres was estimated by epifluorescence microscopy. A final concentration in the samples of microspheres of between 2% and 5% and 8% and 12% of the prokaryote abundance in the lake was used for measuring metazooplankton and protozoan ingestions, respectively [11]. The number of microspheres ingested by the metazooplankton, ciliates, microflagellates and colonial flagellates was determined under an epifluorescence microscope Leitz fluovert FU, filter A (UV light) and in transmitted light. The ingestion of tracer particles by metazooplankton was estimated by examining the entire alimentary tract at a magnification of ×125–250 [43].

The filtration [TF; μl individual (indiv.)$^{-1}$ h^{-1}], ingestion (TI; cell indiv. $^{-1}$ h^{-1}) and grazing rates (TGR; cell l^{-1} h^{-1}) for each taxon were calculated as follows:

$$\mathrm{TF} = (M_t - M_0)/M \times T \quad \text{and}$$
$$\mathrm{TI} = \mathrm{TF} \times (B + \text{microspheres}),$$

$$\mathrm{TGR} = \mathrm{TI} \times \text{abundance of the taxon}(\mathrm{l}^{-1}),$$

where M_t is the number of microspheres ingested per individual (microspheres indiv.$^{-1}$) at incubation time t, M_0 is the number of microspheres ingested per individual (microspheres indiv.$^{-1}$) at incubation time 0 (background noise), M is the concentration of microspheres during incubation (microspheres μl^{-1}), T is the incuba-

tion time (h) and *B* is the prokaryotic concentration during incubation (cell μl^{-1}). The detailed protocol, used in this study, is described in the paper of Thouvenot et al. [11].

2.8. Prokaryotic secondary production

Prokaryotic production (PP) was determined by [methyl-^{3}H]-thymidine ([^{3}H]-TdR) incorporation method as described in Richardot et al. [44]. PP was calculated from rates of [^{3}H]-TdR incorporation using the conversion factor 2.0×10^{18} cells mol^{-1} [45] and 20 fg carbon per prokaryotic cell.

2.9. Statistical analysis

Univariate analysis. To test the effects of enrichments (NP) and filtrations (FILTR) on the different parameters measured, we used a 3-way (nutrients × filtration × time) analysis of variance (ANOVA) with repeated measures (i.e. microcosms) following the model of Winer [46]. Each of the microcosms was observed under all levels of the factor time, but each microcosm was assigned to only one combination of factors NP and FILTR. The equality of the variances and the normality of the residuals were tested, respectively, by Shapiro-Wilk and Levene tests. Data were transformed following the Taylor procedure [47] when the assumptions of ANOVA were not satisfied. The effects of filtration were decomposed into two orthogonal contrasts in order to study the impact of the different planktonic communities on the virus-like particles abundances, the prokaryotic abundances and the prokaryotic production. Contrast C1 determine significative difference between treatments with (<10 μm and UNF) and without predators (<1.2 μm) and C2 between treatments containing predators (<10 μm vs. UNF). In order to determine eubacterial groups and sub-classes with the highest mortality in treatments <10 μm and UNF, the Scheffé test was used for pairwise comparisons of means after ANOVA one way (Figs. 2 and 4).

Multivariate analysis. Matrix of presence-absence of T-RFs (at $t = 0$ and 48) were treated by correspondence analysis (COA). Correlation analysis between the two first ordination axis and explanatory variables were performed to understand the main factors controlling the eubacterial community composition.

To evaluate top-down and bottom-up effects on the prokaryotic community composition (PCC) (determined by FISH abundances or presence-absence of TRFs with an area superior to 2%), we used multivariate analysis with variation partitioning (variation partitioning analysis or VPA), as described by Borcard et al. [48] and Muylaert et al. [16]. All explanatory variables were divided into two groups: variables related to bottom-up regulation (conc. of $N\text{-}NH_4$, $N\text{-}NO_3$, $P\text{-}PO_4$ and PP) and variables related to top-down regulation (grazing rates of flagellates, ciliates, rotifers, cladocerans and virus-like particles abundances). For each experiment, we selected only variables which independently explained a significant amount of the variation in PCC by the forward canonical correspondence analysis. Then, for the set of bottom-up and top-down variables separately, we generated a minimal set of explanatory variables explaining variation in the community composition. The VPA allowed to distinguish pure top-down and bottom-up effects on PCC and a part explained by these both effects named shared part.

These statistics were computed with *R* software using ADE package for COA analysis and Vegan package for the VPA and related methods (http://cran.r-project.org/).

3. Results

3.1. Predation activity and structure of planktonic communities and viral abundance in microcosms

3.1.1. Microcosms in Sep (Experiment 1)

The results presented here correspond to mean values of different incubation times ($t = 0$, 24 and 48) for each treatment. Among the different factors studied, only filtration had a significant effect (FILTR: $p < 0.05$) on the total grazing rate (Fig. 1). This effect could be explained by the strong predation activity on prokaryotes of the metazooplankton, which made up at least 76% (treatment NP(+), microcosms enriched with mineral nutrients) of total predation activity (Fig. 1). Among these organisms, *Ceriodaphnia* sp. and *Daphnia longispina*, the only cladocerans present in the experiment, made up on average in the two levels NP(−) (microcosms without enrichment of mineral nutrients) and NP(+) 78 and 76% of total least 76% (treatment NP(+), microcosms enriched with mineral nutrients) of total predation activity for abundances of 12 and 6 indiv. l^{-1}, respectively. Copepods, which were the most abundant (Table 2) (37 and 33 indiv. l^{-1} for the treatments UNF NP(−) and NP(+)), rotifers (8 and 7 indiv. l^{-1} for the treatments NP(−) and NP(+)) contributed only very little to the prokaryotic predation, i.e., no more than 0.2% of total predation activity.

The predation activity of the flagellates did not vary significantly with the different treatments (Fig. 1). The pigmented flagellates were slightly less abundant (between 7 and 60×10^3 cells l^{-1}, respectively, in the treatments <10 μm NP(−) and UNF NP(+)) than the heterotrophic flagellates (abundance ranging between 17 and 95×10^3 cells l^{-1}) (Table 2). However, most of the predation of this community was due to pigmented flagellates, which represented at least 81% of the total grazing rates in the microcosms in which the water was filtered on 10 μm, and reached 100% of that of the

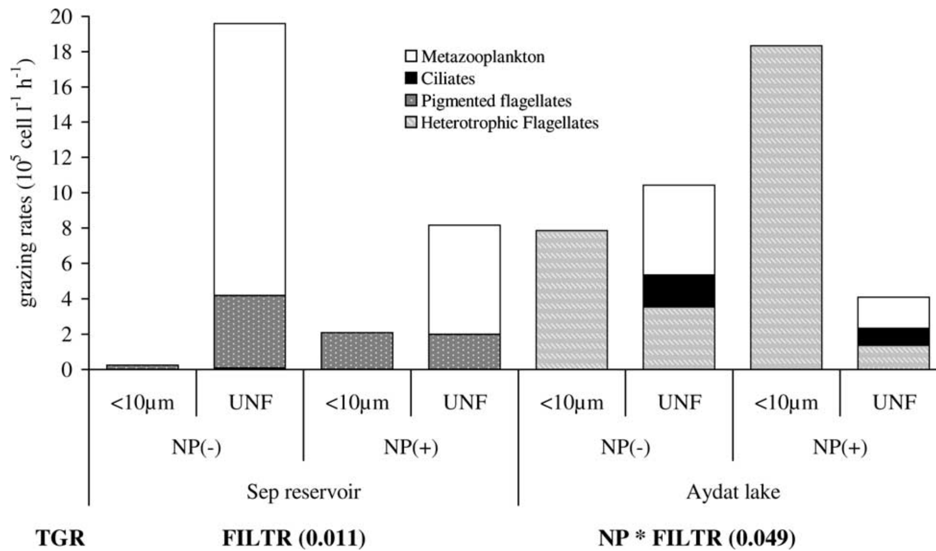

Fig. 1. Average grazing rates (10^5 cell l^{-1} h^{-1}), estimated by using the method of microspheres ingestion, for the whole study (t = 0, 24 and 48) in Sep and Aydat in the different treatments (<10 µm: filtered through 10 µm, UNF: unfiltered; NP(−): no nutrient addition, NP(+): nutrient addition). Values under histograms are probability obtained by an ANOVA analysis ($p < 0.05$) for significant effects of nutrient addition (NP) and/or filtration (FILTR). The interaction between both effects correspond to NP × FILTR.

Table 2
Average abundances of planktonic communities and mean prokaryotic production (incorporation of [^{3}H]-TdR) for the whole study (t = 0, 24 and 48), in Sep reservoir and Aydat lake, in the different treatment (<1.2 µm: filtered through 1.2 µm, <10 µm: filtered through 10 µm, UNF: unfiltered)

			Heterotrophic flagellates (10^3 cell l^{-1})	Pigmented flagellates (10^3 cell l^{-1})	Ciliates (cell l^{-1})	Copepods (indiv. l^{-1})	Clodocerans (indiv. l^{-1})	Rotifers (indiv. l^{-1})	Virus-like particles (10^6 ml^{-1})	Prokaryotic production (µg C l^{-1} h^{-1})
Sep reservoir	NP (−)	<1.2 µm	–	–	–	–	–	–	7.7	0.129
		<10 µm	17	7	–	–	–	–	5.5	0.125
		UNF	61	59	–	37	20	8	8.1	0.123
	NP (+)	<1.2 µm	–	–	–	–	–	–	6.7	0.200
		<10 µm	72	45	–	–	–	–	5.1	0.212
		UNF	95	60	–	33	15	7	6.3	0.268
Aydat lake	NP (−)	<1.2 µm	–	–	–	–	–	–	9.3	0.019
		<10 µm	587	8	–	–	–	–	11.6	0.065
		UNF	492	4	626	15	22	160	6.0	0.055
	NP (+)	<1.2 µm	–	–	–	–	–	–	9.3	0.061
		<10 µm	628	14	–	–	–	–	9.3	0.130
		UNF	400	4	407	17	17	149	8.0	0.150

Abbreviations as in Table 1.

flagellates in the UNF treatments. Among this community, undetermined flagellates of size of 5–10 µm were responsible, on average, of 82% of protist flagellates predation activity.

The viral abundance did not seem to depend on nutrient enrichment (NP: $p > 0.05$) and/or on the presence or absence of the different communities of planktonic organisms (C1 and C2: $p > 0.05$) (Tables 2 and 3).

3.1.2. Microcosms in Aydat (Experiment 2)

In this eutrophic ecosystem, especially during the experiment, predation on prokaryotes in treatments UNF (Fig. 1) was distributed equally between metazooplankton and protists, flagellates and ciliates being responsible for respectively 49% and 48% of total predation in the treatments NP(−) and NP(+). Total predation activity on prokaryotes showed no significant difference (Fig. 1) according to the different treatments. The predation activity of the ciliates was mainly due to vorticellids of average size about 50 µm (mean abundance 206 cells l^{-1}), which represented at least 86% (treatment UNF NP(−)) of the ciliate predation. Copepods and cladocerans were present at similar abundances (Table 2), respectively, 15 and 22 indiv. l^{-1}in treatment NP(−) and 17 indiv. l^{-1} in treatment NP(+). Rotifers were the most abundant zooplanktonic organ-

Table 3
Effects of nutrient addition and filtration effect on abundances of virus-like particles, total bacteria, EUB338, ARCH915, ALF1b, BET42a, CF319a and prokaryotic production

		NP	FILTR		NP × FILTR
			C1	C2	
Sep reservoir	Virus-like particle	0.222	0.383	0.157	0.785
	Total bacteria	0.315	0.002	0.662	0.321
	EUB338	0.000	0.000	0.000	0.003
	ARCH915	0.000	0.624	0.325	0.087
	ALF1b	0.000	0.000	0.054	0.000
	BET42a	0.000	0.000	0.000	0.281
	CF319a	0.105	0.002	0.000	0.012
	Prokaryotic production	0.000	0.152	0.069	0.037
Aydat lake	Virus-like particle	0.772	0.221	0.000	0.007
	Total bacteria	0.004	0.000	0.000	0.019
	EUB338	0.000	0.497	0.000	0.001
	ARCH915	0.000	0.000	0.234	0.080
	ALF1b	0.000	0.000	0.000	0.001
	BET42a	0.000	0.000	0.000	0.000
	CF319a	0.000	0.000	0.000	0.000
	Prokaryotic production	0.003	0.010	0.846	0.512

Values in the table are the probability obtained by an ANOVA analysis. The enrichment effect is noted NP. The filtration effect (FILTR) was decomposed into two orthogonal contrasts C1 (<1.2 μm vs. (<10 μm – UNF)) and C2 (<10 μm vs. UNF). The interaction between both effects is noted NP × FILTR. Abbreviations as in Table 2.

isms in the eutrophic lake (160 and 149 indiv. l^{-1}). Only cladocerans consumed prokaryotes efficiently, *Ceriodaphnia* sp. and *Daphnia longispina* being responsible on average, respectively, 46% and 50%, of the total predation activity on prokaryotes in treatments NP(−) and NP(+).

In contrast to Experiment 1, pigmented flagellates were not very abundant (Table 2) (abundances: 4 and 14×10^3 cells l^{-1}, respectively, in treatments UNF NP(−)/(+) and <10 μm NP(+)). The abundance of heterotrophic flagellates ranged between 400 (UNF NP(+)) and 628×10^3 cells l^{-1} (<10 μm NP(+)). The latter contributed strongly to predation activity, which was essentially due to choanoflagellates (on average 107 and 131×10^3 cells l^{-1}, respectively, in treatments <10 μm and UNF), and flagellates of size between 1 and 10 μm (average 365 and 249×10^3 cells l^{-1}, respectively, in treatments <10 μ m and UNF). The choanoflagellates were the main prokaryotes grazers in the treatment <10 μm, making up 69% of predation activity on prokaryotes (Fig. 1) on average for the two levels NP(−) and NP(+). Indeterminate flagellates of 1–10 μm were responsible for 58% of the flagellate predation activity in the UNF treatments. Filtration had a significant effect (Fig. 1, FILTR: $p < 0.05$) on the predation activity of the flagellates, which decreased in the UNF treatments, whereas the total abundances varied little. Only unidentified heterotrophs of 1–10 μm displayed wide variations in average abundance between treatments <10 μm (365×10^3 cells l^{-1}) and UNF (249×10^3 cells l^{-1}).

The abundance of virus-like particles showed no variation with nutrient level (Tables 2 and 3), but lower viral abundances in the UNF treatments resulted in significant differences between the treatments <10 μm and UNF (C2: $p < 0.05$).

3.2. Development of prokaryotic production and PCC during the experiments

3.2.1. Microcosms in Sep (Experiment 1)

The abundance of the prokaryotic community (DAPI-stained cells) depended on the absence or presence of other planktonic organisms (C1: $p < 0.05$) (Fig. 2 and Table 3), but not on the addition of nutrients, unlike prokaryotic production (Tables 2 and 3, NP: $p < 0.05$). However, the most accurate detailed analysis of the prokaryotic community composition (PCC) by the FISH method showed that the nutrients had an effect on all the targeted domains or groups except for CF319a. The abundance of ARCH915 did not vary significantly with filtration, and that of ALF1b did not vary between treatments <10 μm and UNF.

EUB338 and ARCH915 larger than 2.5 μm developed essentially in enriched treatments (NP(+)), and more markedly in treatment <1.2 μm.

Filtration, which allow to vary the predators quantitatively and qualitatively, showed significant variations in prokaryotic abundance between treatment <1.2 μm (samples filtered on 1.2 μm) and <10 μm or UNF. However, the amplitude of these variations could differ according to the group targeted. To compare these variations, we calculated the ratios $N_{<1.2\ \mu m}/N_{<10\ \mu m}$ ($N_{<1.2\ \mu m}$: abundance in treatment <1.2 μm; $N_{<10\ \mu m}$: abundance in treatment <10 μm) and $N_{<1.2\ \mu m}/N_{UNF}$ (N_{UNF}: abundance in treatment UNF) for the groups of Eubacteria studied (ALF1b, BET42a or CF319a).

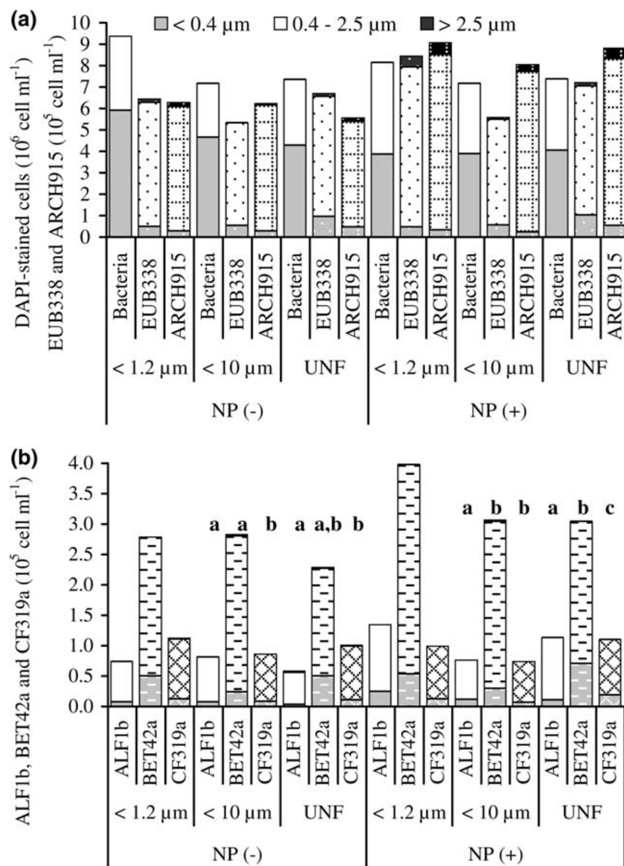

Fig. 2. Sep (Experiment 1). Average abundances (cells ml^{-1}) for the whole study ($t = 0$, 24 and 48) in the different treatments (<1.2 µm: filtered through 1.2 µm) of different size classes (<0.4, 0.4–2.5 and >2.5 µm): (a) DAPI-stained cells, EUB338 and ARCH915, (b) ALF1b, BET42a and CF319a. The Scheffé test was applied between the different eubacterial groups for each treatment. Groups in same treatment (<10 µm or UNF treatments) with different letters above histograms (a–c) were significantly different ($p < 0.05$). Abbreviations as in Fig. 1.

The ratios of different groups, for a given filtration, were treated by a 1-way ANOVA followed by a Scheffé test (Fig. 2). These ratios were always significantly different for the abundances of the different eubacterial groups studied and varied according to the treatment studied. Thus CF319a and ALF1b suffered the greatest mortality in the <10 µm treatments, in levels NP(−) and NP(+) respectively, against ALF1b and BET42a in the UNF treatments in the level NP(+).

The analysis of the matrix of presence/absence of T-RFs in the different treatments using correspondence analysis (Fig. 3(a)) showed discrimination on the first axis of Eubacteria composition according to the different filtrations. This differentiation could be associated by correlation analysis with the abundance of metazooplankton ($r = 0.63$, $p < 0.05$), nitrates ($r = 0.74$, $p < 0.05$) (one of the mineral nutrients added) and prokaryotic production ($r = -0.67$, $p < 0.05$). Axis 2 showed a marked difference in the structure of the Eubacteria between the treatment <1.2 µm NP(−) (without predator and without nutrient enrichment) and all the other samples.

The results of forward CCA showed that the variables that significantly accounted for the variations in abundance determined by the FISH method were, for the resources, the prokaryotic production and the concentrations of the 3 nutrients added in NP(+) treatments: nitrate, ammonium and orthophosphate. None of the variables linked to top-down regulation significantly accounted for the variation in abundance of cells marked by FISH or the diversity of the Eubacteria (presence/absence of T-RFs) at the threshold selected ($\alpha = 0.05$). The results of this analysis, which indicated that cladocerans followed by pigmented flagellates had the most marked effects, and those of the COA and ANOVA, led us to retain at least these two variables. This shows that the top-down type regulation controls had less impact than bottom-up (i.e. mineral nutrients) controls in this ecosystem. Mortality accounted for only 12.7% and 14.7% of the variations in PCC, while resources accounted for at least 40.2%.

3.2.2. Microcosms in Aydat (Experiment 2)

The total abundance of DAPI stained-cells (Fig. 4 and Table 3) depended on both nutrient level (NP: $p < 0.05$) and on the presence or absence of the different planktonic communities (C1 and C2: $p < 0.05$). The same was true for prokaryotic production (Tables 2 and 3), which was higher in the nutrient-enriched treatments and depended on the presence or absence of the different planktonic communities (C1: $p < 0.05$). Among the prokaryotes (DAPI-stained cells), the abundance of the different domains and groups studied evolved significantly in the enriched treatments (NP: $p < 0.05$). While the presence or absence of the different planktonic communities caused no variation in the abundance of EUB338, the community composition (treatment <10 µm and UNF) significantly modified their abundance (C2: $p < 0.05$). The abundance of ARCH915 varied significantly when other planktonic communities were present (C1: $p < 0.05$), but did not depend specifically on their composition. Among the Eubacteria, the abundances of ALF1b, BET42a and CF319a varied significantly in the presence or absence of the different planktonic communities (C1 and C2: $p < 0.05$). It is noteworthy that there was a strong interaction between the two factors studied, owing in most cases to an increase in cell abundance in the treatments UNF NP(+) where predation was weakest (Fig. 1).

As regards structure in size classes, EUB338, ARCH915 and ALF1b larger than 2.5 µm were more abundant in the enriched treatments, especially in the UNF treatments. No aggregates or flocs were observed by microscopy analysis, in DAPI- or FISH-staining.

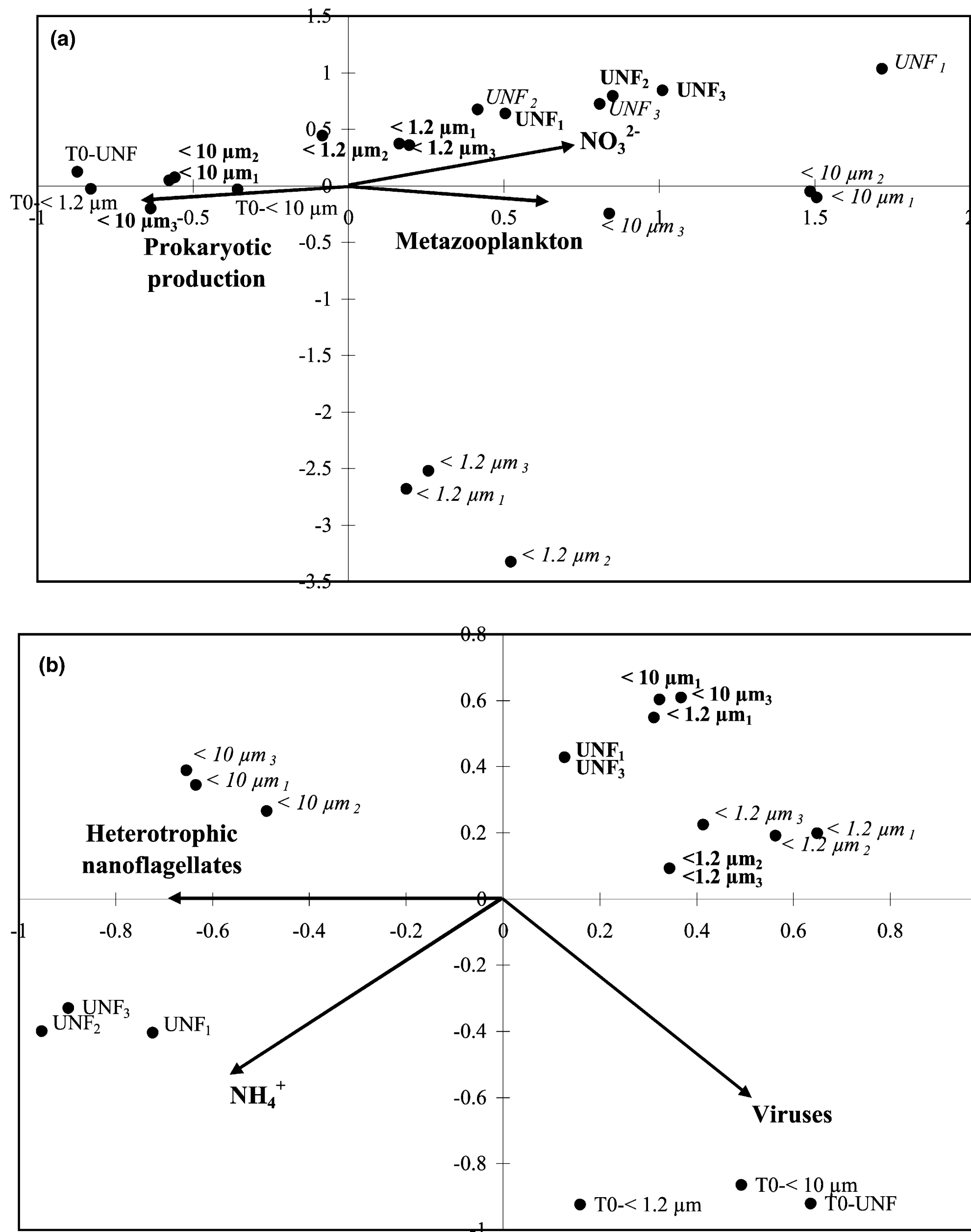

Fig. 3. Results of the correspondence analysis performed on the T-RFs (presence/absence) obtained from T-RFLP analysis of 16S rDNA digestion by *Msp*I from the sampling of Sep (a) and Aydat (b). Points represent samples. Abbreviations correspond to samples taken at $t = 48$ (NP(−): in italic, NP(+): bold type), except the 3 samples noted T0 ($t = 0$). Arrows represent correlation coefficients between explanatory variables and the first two ordination axes. The percentage of inertia explained by Axes 1 and 2 were 15.1% and 12.4% for Sep and 14.6% and 13.6% for Aydat, respectively. Abbreviations as in Fig. 2.

The different filtrations caused modifications in the composition and abundance of planktonic communities, which induced different amplitudes in the variations in abundance among the eubacterial groups studied (ALF1b, BET42a, CF319a) (Fig. 4); the statistical analysis was identical to that carried out previously for Sep. Thus, except for the treatment UNF NP(+), the strong reductions in abundance compared with treatment <1.2 µm were those of ALF1b and BET42a.

Factorial analysis applied to the observations made on the microcosms of Aydat (Fig. 3(b)) discriminates nutrient levels, with or without enrichment of mineral nutrients, according to Axis 1, except for the treatment <1.2 µm NP(−). The abundance of heterotrophic flagellates ($r = -0.69$, $p < 0.05$) and the ammonium concentration ($r = -0.58$, $p < 0.05$) are significantly correlated on this Axis. Axis 2 shows another samples distribution, those taken at $t = 0$ being isolated from the other

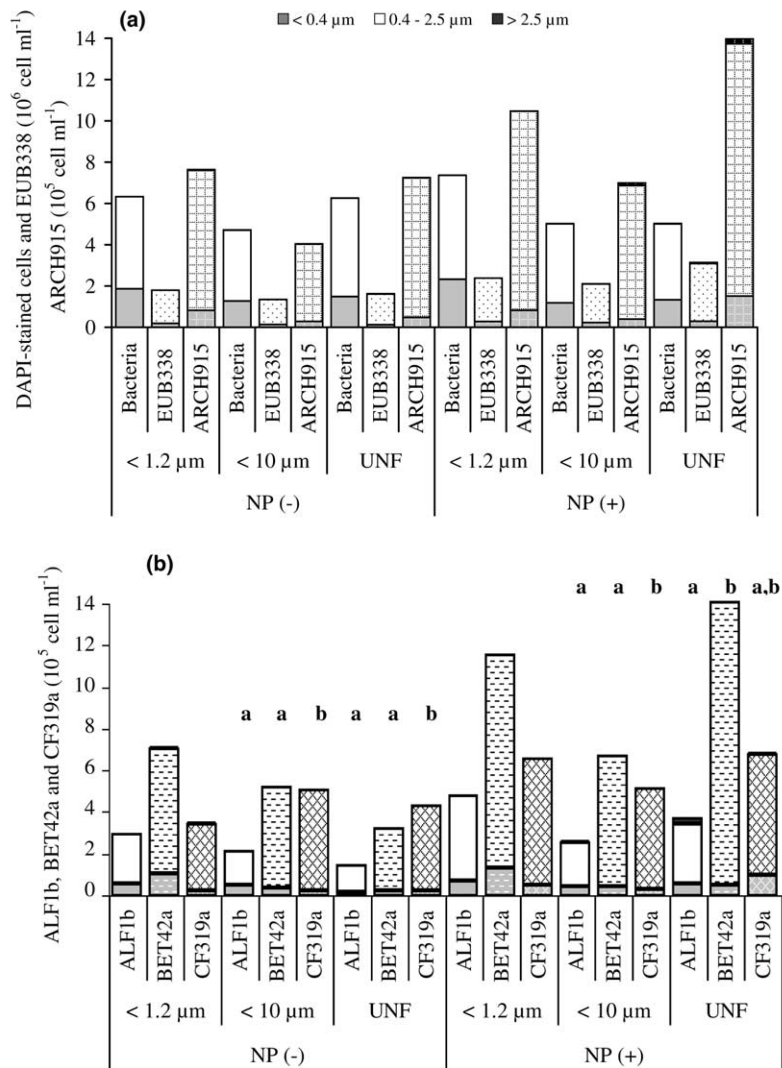

Fig. 4. Aydat (Experiment 2). Average abundances (cells ml^{-1}) for the whole study (t = 0, 24 and 48) in the different treatments of different size classes (<0.4, 0.4–2.5 and >2.5 µm): (a) DAPI-stained cells, EUB338 and ARCH915, (b) ALF1b, BET42a and CF319a. The Scheffé test was applied between the different eubacterial groups for each treatment. Groups in same treatment (<10 µm or UNF treatments) with different letters above histograms (a–c) were significantly different ($p < 0.05$). Abbreviations as in Fig. 2.

samples (except for the treatment UNF NP(−)). This axis is significantly correlated with viral abundance ($r = -0.60$, $p < 0.05$) and ammonium concentration ($r = -0.55$, $p < 0.05$).

Forward CCA analysis shows that the variations of PCC were significantly explained by the concentrations of NO_3^{2-}, NH_4^+, PO_4^{2-}, and the prokaryotic production. The factors of mortality were represented by the grazing rates of the heterotrophic flagellates, ciliates, cladocerans, and the abundance of virus-like particles for the analysis performed with T-RFs and with the same variables plus rotifers for the analysis performed with FISH abundances. The two methods of control account similarly for the variations in abundance of the cells marked by the FISH method. Lastly, 42.2% of the distribution of T-RFs was explained by bottom-up effects and 30.6% by mortality factors.

4. Discussion

We chose an experimental approach using microcosms that allowed control of both predation levels, by graded elimination of predators, and resources, by nutrient enrichment. Although microcosm experiments introduce some bias into the development of prokaryotic communities compared with those occurring naturally in the field, owing to confinement and handling effects, these experimental tools are very useful for investigating how environmental processes such as nutrient addition and mortality factors induce temporal variations in prokaryotic community structure, diversity and activity [49]. In addition, the relatively short incubation time of 48 h coupled with the volume chosen, depending on the planktonic communities they contain (i.e. 2 or 4 l), was likely to limit this confinement effect.

This short incubation time was sufficient to obtain significant changes in abundance and prokaryotic production, as observed by Fonnes Flaten et al. [50] in marine experiments with a similar incubation time. The Eubacteria community composition was determined using oligonucleotide probes targeting the main groups of Eubacteria generally encountered in freshwater ecosystems [2,32]. We registered low abundance of EUB338 in the Sep reservoir. This result has already been observed in the same ecosystem in a previous study [17], where in the same period of the year, abundance of EUB338 reached their lowest values. However, we did not take into account the class Actinobacteria, reported as a potentially important eubacterial planktonic group in freshwater ecosystems by Glöckner et al. [3]. The FISH method has also the disadvantage, in our case, of using group probes and not more specific probes. The *Cytophaga–Flavobacteria–Bacteroides*, β- and α-Proteobacteria are broad taxonomic groups. However, the observations of Horner-Devine et al. [51] suggest the existence of significant patterns, along a gradient of productivity, in richness at this taxonomic scale. In addition, these variations of structure observed in this study by the FISH method, even without taking account for Actinobacteria, are also visible in the two factorial analyses which distinguish the species richness (T-RFs) between the different treatments (Fig. 3).

4.1. Principal mortality factors in each experiment

In this study we took into account the main mortality agents, namely protists, metazoan zooplankton (Fig. 1 and Table 2) and virus-like particles (Table 2). However, it is to be noted that in the course of these two experiments, only viral abundance, and not viral lysis, was quantified. As is generally observed in aquatic ecosystems [13], the viral community was more abundant in the eutrophic ecosystem. However, during our experiments and thus for one date, this community was not stimulated by a greater prokaryotic production in the enriched treatments, in contrast to the observations made by Thingstad [14] and Weinbauer et al. [13] made during longer experiences. Thus, in our case, the short time of our incubation was not enough long to show measurable responses.

In Sep (Experiment 1), predation activity was greater in the experimental situations where the whole planktonic community was present (UNF) (Fig. 1). This difference is due to the high predator activity of the metazooplankton essentially due to the cladocerans *Daphnia longispina* and *Ceriodaphnia* sp., which are the main organisms controlling the abundance of the prokaryotic populations in this ecosystem [11,17]. Consistent with Thouvenot et al. [11], the main phagotrophic flagellates observed in these experiments were mixotrophic organisms. In the eutrophic lake (Experiment 2), the predation of the flagellate protists, essentially heterotrophic species, was significantly higher ($p < 0.05$) in the treatments <10 μm, whereas the total predation activity did not vary significantly between the treatments <10 μm and UNF. In the UNF treatments, the protists were also responsible for about half the total predation. These results, like those of Šimek et al. [19], show a strong control of the prokaryotic community through predation by flagellate protists in the eutrophic lake.

4.2. Impact of mortality factors and resources on PCC

Measurements of predation activity on prokaryotes and statistical analysis show that main predators, described above, and also virus-like particles, play a role in the control of the PCC (Figs. 3 and 5). However, the variation partitioning analysis emphasizes the lower impact of mortality factors on PCC in Sep than in the eutrophic lake.

In Aydat, except for CF319a, we found marked decreases in the abundance of the different groups in the presence of only heterotrophic flagellates as predators

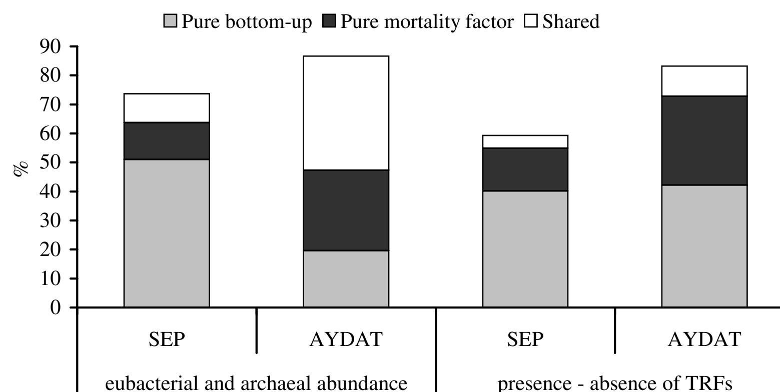

Fig. 5. Results of variation partitioning analysis for Sep and Aydat at the incubation time of 48 h. For each lake, the total variation explained in eubacterial and archaeal abundance (abundance of EUB 338, ARCH915, ALF1b, BET42a and CF319a) and diversity versus presence/absence of T-RFs (16S rDNA digestion by *Msp*I, with area >2%) is partitioned between bottom-up variation and pure mortality factor variation.

than in the absence of potential predators. Thus, the abundance of the groups most affected by predation, i.e., the two groups of Proteobacteria ALF1b and BET42a, remained the same from one treatment to another, except for the enriched treatment with all potential predators where an increase in the abundance of the Eubacteria and *Archaea* was recorded, probably owing to a reduction in predation (Fig. 1). The similarity of mortality of these eubacterial groups between the different predatory regimes and the results of COA analysis suggest a strong impact on PCC exerted by heterotrophic flagellates, according to various studies that have shown the impact of these predators on the diversity of eubacterial communities [18–20]. The selectivity by HNF can be explained by size, chemical properties of cell membranes or motility [52]. However, the PCC in all treatments may depend on other organisms and particularly virus-like particles as shown by significant correlation with Axis 2 of the COA (Fig. 3(b)) and the VPA analysis. Through both their lytic and lysogenic activities [53], viruses can cause modifications to the structure of the eubacterial community as shown by Schwalbach et al. [49]. Unlike this eutrophic ecosystem, virus-like particles seem not to have exerted such a role in Sep, since there were no significant differences of virus-like particle abundance between treatments (Table 3) and no relation with the T-RFs distribution (Fig. 3(a)). Thus, in Sep, the main mortality factor of PCC regulation was certainly predators.

In this oligomesotrophic lake, the mixotrophic pigmented flagellates, undetermined of size of 5–10 μm, exerted a stronger predation on CF319a in the non-enriched treatments and on the ALF1b in the enriched treatments. The variations in mortality according to the enrichment may occur partly because the mixotrophic flagellates can respond to changes in food availability by changing their physiology in some way, perhaps by adjusting digestive enzymes, as hypothesized by Selph et al. [54] for chrysomonad flagellates.

Metazooplankton may play a role in the control of PCC in these lakes, more particularly in Sep where eubacterial diversity is related to these organisms (Fig. 3(a)), as already observed in a few other freshwater ecosystems [12,16]. Unlike Aydat, in experiment 1, the mortality of proteobacteria with the predation activities. These results may be due to the predation activity of *Daphnia longispina* and *Ceriodaphnia* sp. The highest mortality of ALF1b, in the absence of N and P enrichment, thus confirms the results obtained earlier in Sep [17] (Fig. 2). Also, despite strong predation in presence of cladocerans, the abundance of EUB338 and certain groups (CF319a and ALF1b in NP(+)) was higher in these treatments than in those with only flagellates as predators. As the prokaryotes in oligotrophic ecosystems can be limited by both nutrient and carbon [1], it may be that the Eubacteria of Sep were stimulated by phytoplanktonic excretion and/or by sloppy feeding induced by the cladocerans [9]. Thus, the action of metazooplankton on PCC may therefore have two origins: (i) the difference of mortality in the presence of these organisms may be due to different capacities of digestion for different eubacterial groups [22], (ii) to unselective removal of larger and potentially more active cells frequently affiliated to different phylogenetic groups, as suggested by Langenheder and Jürgens [12] or (iii) a modification of the PCC, as a result of a variation in the quality of the organic matter induced by phytoplanktonic excretion and sloppy feeding [44]. The latter is a case of interaction between a top-down factor and a bottom-up factor corresponding probably to a shared part in VPA analysis.

The activity of predation and/or viral lysis seems to have only a weak effect on structuring into size classes. We observed only a slightly greater development of EUB338 and ARCH915 in size class >2.5 μm in the treatments with no predators (Experiment 1). In the second experiment this size class became significantly more abundant in the enriched treatments, but when the predation was weaker (ALF1b in UNF NP(+)). Consistent with an earlier study at Sep [17] and with the study of Wu et al. [55], we thus observed no appearance of filamentous prokaryotes that might represent a strategy of resistance to predation by flagellate protists [20]. In a same way, we didn't observed appearance or development of flocs and/or aggregates, as already shown by Langenheder and Jürgens [12] in one of the 3 eutrophic ponds studied. Also, the increase in the abundance of size class >2.5 μm suggests an increase in metabolic activity as shown by the increased prokaryotic production (Tables 2 and 3). This production was strongly stimulated by the N and P enrichments in both the ecosystems studied (Tables 2 and 3), as observed by Fisher et al. [1]. Various studies also reported that the larger eubacterial size fractions had the highest specific growth and production rates [26].

These observations and the VPA analysis highlight the important role of nutrients in the control of PCC in both ecosystems. With the exception of CF319a in Sep, N and P enrichment increased the abundances of EUB338, ARCH915 and eubacterial groups. The finding that CF319a were not stimulated by nutrient enrichment may result from the fact that this eubacterial group is most often associated with activities of breakdown of complex molecules [56]. In addition, the diversity of Eubacteria (distribution of different T-RFs) was linked to nitrates in Sep, and to ammonium in Aydat (Fig. 3) and the set of nutrients assayed ($P\text{-}PO_4$ $N\text{-}NO_3$, and $N\text{-}NH_4$) together with PP always accounted for a significant part of the PCC variations (Fig. 5). This link with PP shows a modification to the structure of the Eubacteria in favour of changes induced by resources [57],

and also underlines the great importance of bottom-up control in lake ecosystems. Likewise, two freshwater lake studies, those of Fisher et al. [1], in a comparative lake study and in mesocosm experiments ,respectively, showed that N and P were responsible for the variation in the eubacterial community composition. Finally, in a lake experiment Gasol et al. [26] showed that the main different eubacterial groups had different patterns of response to the resources.

4.3. Relative importance of bottom-up and top-down factors on PCC

According to the model of Sanders et al. [6], who did not take diversity into account, prokaryotes are mainly controlled by resources in nutrient-poor ecosystems and by predation and viral lysis in eutrophic ecosystems (Fig. 5). In the model of Thingstad [14], viruses and predators control the diversity of the steady-state prokaryotic community. However, this scheme cannot be fully applied to PCC in this study as shown by the variation partitioning analysis which is a powerful tool to discern the relative importance of two factors on the structure of a community [16,48]. This study therefore confirms the impact of predation on PCC but show also that virus-like particle impact on PCC may be preponderant only in eutrophic ecosystems, as has been reported elsewhere (for review see [13]). It especially emphasizes the role of resources, which may control PCC via N and P enrichments or phytoplanktonic excretion and sloppy feeding, and the importance of which has already been suggested by several authors in seasonal studies [16,17,58,59]. Thus, in Sep, resources explained a large part of the diversity of prokaryotes, 51% and 40% for the prokaryotic domain and groups and T-RFs, respectively, whereas in the eutrophic lake the resources and mortality factors made comparable contributions. In the latter ecosystem, the analysis shows that the residual part was small and that the shared part could be comparable in size to the two factors studied, when the variable to be accounted for was the diversity of Eubacteria (T-RFs). The results of Gasol et al. [26] seem also to indicate the existence of a complex interaction between bottom-up and top-down factors in planktonic prokaryotes. The nature of this link between mortality and resources is still not clear. It might, however, be the resultant, in this eutrophic lake, of modifications of diversity generated by the organic matter produced, for example, by cell disruption by viral lysis (viral loop).

5. Conclusions

This study clearly shows that in both ecosystems studied, resources constitute the main factor of control of PCC, and to a lesser extent, the mortality factors and combined action of these two factors. Our results suggest that virus-like particles may play an important role in the control of the PCC mainly in eutrophic ecosystems. Resources and mortality factors seem also to have an impact on the richness and diversity of Eubacteria.

However, these experiments do not allow the relative importance of viral lysis and predation to be estimated, and the full impact on the PCC of these two mortality factors remains to be determined.

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