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Bottom-up and top-down control of bacterial community composition in the euphotic zone of a reservoir

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ABSTRACT: Temporal changes in the bacterial community composition (BCC) and the impact of resources and predation on this community composition have been studied in the euphotic zone of the Sep reservoir (France), using terminal-restriction fragment length polymorphism (T-RFLP) and fluorescent *in situ* hybridization (FISH) for Eubacteria (EUB338) and eubacterial subgroups. Net growth and grazing rates of the various subgroups were computed from experiments conducted in dialysis bags in the presence or absence of predators. There was a significant difference between the grazing rates of different bacterial groups and subclasses, the alpha-proteobacteria (ALF1b) were subjected to the highest grazing rate (max. 1.76 d^{-1}). In contrast, the beta-proteobacteria (BET42a) seemed to be little consumed by bacterivorous organisms, and predation led to large variations in the grazing rates of *Cytophaga–Flavobacterium* (CF319a). The different composition of bacterial consumers (*Dinobryon* sp. and Cladocera) during the study could explain the observed differences in grazing impact. The mean net growth rates of total bacteria, EUB338, BET42a ALF1b, and CF319a in dialysis bags without predators were 0.21, 0.69, 0.45, 0.50 and 0.84 d^{-1}, respectively. Various statistical analyses indicated that the BCC could depend on the organic matter excreted by the phytoplankton. There were also positive correlations not only between net growth and/or net production of ALF1b and CF319a with the primary production and biomass of the main phytoplankton groups, but also between phytoplankton biomass, primary production (PP) and some operational taxonomic units (OTUs). Among the physical variables, input and output of water in the reservoir seem to play a role in determining the BCC in this ecosystem. The results suggest that the BCC depends on the combined impact of dominant substrate sources and selective predation by bacterial consumers.

KEY WORDS: Bacterial community composition · Top-down · Bottom-up · Reservoir · Terminal-restriction fragment length polymorphism · Fluorescent *in situ* hybridization

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INTRODUCTION

Heterotrophic bacteria are an important constituent of pelagic ecosystems, where they can account for a large proportion of the plankton biomass (Cho & Azam 1988); they are also considered to be the most stable component of plankton communities in terms of abundance (Jürgens & Güde 1994). However, this concept of stability is based on the fact that, until the recent development of molecular ecology methods, bacteria were considered to be a homogeneous functional unit. Phylogenetic analyses have shown that there is a great diversity within the lacustrine pelagic bacterial plankton (Zwart et al. 2002), which is often dominated by the beta subclass of Proteobacteria (Methé et al. 1998). More recently, Glöckner et al. (2000) demonstated that the Actinobacteria class may be an important bacterioplankton group in freshwater ecosystems.

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Various studies have demonstrated seasonal and spatial variations in bacterial community composition (BCC) (Øvreås et al. 1997, Pernthaler et al. 1998, Höfle et al. 1999, Konopka et al. 1999). Changes in bacterial populations may depend not only on the biomass of microzooplankton and of certain phytoplankton groups (Cryptophyceae and Chrysophyceae), but also on nutrient availability, as shown by a comparative study of BCC in 5 lakes differing in trophic and humic status (Lindström 2000).

Predation is likely to control bacterial communities (Güde 1986, Šimek & Straškrabová 1992) and can be selective. Recently, various works demonstrated that bacterial filaments, which because of their morphology are not consumed by predators, may occur in situations with high protistan grazing pressure (Jürgens et al. 1999, Šimek et al. 1999), but some bacteria are able to switch from non-filamentous to filamentous growth (Hahn et al. 1999). Although all these studies indicated that predation has a strong impact on BCC, the influence of this factor on the composition of the bacterial community has mainly been studied for heterotrophic nanoflagellates (HNF) (Hahn & Höfle 2001); the direct or indirect impact by other bacterial consumers, such as Cladocera, on BCC has yet to be determined.

Resources also have an effect on BCC. For example, Höfle & Brettar (1995) reported higher biodiversity in the layer in which there was a large turnover of carbon. The higher proportion of the *Cytophaga–Flavobacter* group in the coastal zone than in other marine ecosystems may possibly reflect the terrestrial influence, since the bacteria of this group are known to degrade complex macromolecules, i.e. lignin and cellulose (Pinhassi et al. 1997). Furthermore, some experiments have demonstrated that nutrient addition (nitrogen, phosphorus, organic matter) has an impact on BCC (Fisher et al. 2000, Massana et al. 2001). Therfore the data currently available reflect the fact that the influence of resources on total bacterial production has been well studied (e.g. Cole et al. 1988), but the effect on specific populations has rarely been examined. Net growth rates and/or net production of the various bacterial groups have rarely been measured, and the existing data are from microcosm measurements (Jürgens et al. 1999) or from natural aggregates incubated in cylinders (Weiss et al. 1996).

Thus, although improved molecular methods in recent years have provided information on the composition of the bacterial community, the factors controlling the temporal changes in these populations in nature remain little known. As emphasized by Konopka et al. (1999), a serious challenge is to determine the environmental forces that cause bacterial population changes. This study examines the respective roles of biological, chemical and physical parameters on the structure of the bacterial community. In contrast to most *in situ* studies on the effect of predation on the BCC, which have been conducted in meso-eutrophic or eutrophic lakes (Šimek et al. 1999, 2001, 2003, Gasol et al. 2002), the ecosystem chosen, the Sep Reservoir (Massif Central, France) is oligo- to oligo-mesotrophic, and phosphorus is a limiting element (Tadonleke et al. 2000). Since first being flooded it has been characterised by a high concentration of organic matter (Richardot et al. 2000) and by control of bacterial abundance by the bacterivores Cladocera and *Dinobryon* sp. (Thouvenot et al. 1999b).

To study the BCC, we used T-RFLP (terminal restriction fragment length polymorphism), which can detect high numbers of OTUs (operational taxonomic units) (Moeseneder et al. 1999). As this technique cannot quantify bacterial groups or their growth parameters, these were determined by FISH (fluorescent *in situ* hybridization).

MATERIALS AND METHODS

Study site, sampling and experimental design. The Sep reservoir (Massif Central, France), lying at an altitude of 500 m (46° 2′ N, 3° 1′ E), 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 d. In 1995 and 1996, the reservoir was completely empty by the end of summer.

The study was performed from April to August 1999 in the epilimnion of the reservoir. Abiotic and biotic variables (plankton abundance and bacterial community composition) were measured every 2 wk at 1 m depth. Net growth rates and net production of subclasses and bacteria groups were measured during the same period (except on 15 July and 23 August) in dialysis bags. On 4 dates, corresponding to marked differences in the dominant bacterial predators, we studied the effects of predation on bacterial community structure in more detail.

Biotic and abiotic variable measurements. Water temperature and dissolved oxygen were determined with a multiparameter probe (YSI GRANT 3800), and total phosphorus and total nitrogen by the methods of APHA (1992). Dissolved organic matter (DOM) was determined in water prefiltered through prewashed (sterilised water) 0.2 µm pore size polycarbonate filters. Dissolved proteins (DPROT) were determined by the micro BCA Protein Assay Reagent Kit (Pierce) using bovine serum albumin (BSA) as standard. Dissolved free monosaccharides (DFCHO) were determined according to Burney & Sieburth (1977) and Johnson & Sieburth (1977) using glucose as a standard.

Total dissolved carbohydrate (TDCHO) concentrations were determined after hydrolysis with HCl (1 N; 100°C, 15 h). TDCHO minus DFCHO gave dissolved polysaccharides (DCCHO). DOM concentrations were calculated assuming that 1 mg of glucose is equivalent to 0.4 mg carbon, and 1 mg of BSA is equivalent to 0.5 mg carbon. Chlorophyll *a* concentrations were obtained by spectrophotometry (Strickland & Parsons 1968) and phytoplankton primary production was determined by incorporation of ^{14}C as described by Richardot et al. (2000).

Sample preservation. Samples were collected and fixed immediately with a final concentration of 4% formaldehyde for total bacteria, 1% glutaraldehyde for flagellates and in Lugol's iodine for algae. The metazooplankton was fixed in a sucrose/formaldehyde solution (final concentration 6 and 4% respectively) (Prepas 1978). For *in situ* hybridization (FISH), samples fixed with formaldehyde (4% final conc.) 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 (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) (Bej 1995). Air-dried filters were rolled and transferred to 2 ml microcentrifuge tubes (Eppendorf), and were then frozen at –20°C until nucleic acid extraction.

Protist and metazooplankton counts. The protists were counted in samples collected from 1 m (unfiltered water) and at the start of the experiment (time, $t = 0$). After staining with primulin (final conc. 200 µg ml^{-1}) (Caron 1983) flagellates were recovered on black polycarbonate filters of 0.8 µm pore-size (Nuclepore). Both preparations were made within 24 h of sampling and were stored at –25°C to minimise losses of autofluorescence. Counts were made under an Olympus HBS epifluorescence microscope equipped with an epifluorescent HB2-RFL light source and an HBO-100 W mercury lamp. Large-sized phytoplankton were counted by Utermöhl's (1958) method using a Leitz (Wild M40) inverted microscope. The biovolume and biomass were calculated according to Thouvenot et al. (1999 a,b). The metazoan zooplankton was collected from the water column at $t = 0$ by 3 vertical hauls from the bottom to the surface with a Juday-type net of 55 µm mesh size and from the 3 unfiltered dialysis bags incubated for 1 d, and filtered through a 55 µm sieve. The metazoan zooplankton was counted under a binocular microscope (Wild M3 Z) in a Dolfuss chamber. To prevent the plankton from moving about or drying out, a few drops of 10% alcohol glycerine solution were added. Animals were made more visible by staining with a few drops of rose Bengal. If the density of animals in a sample was too high, a subsample was taken with a Motoda box. Rotifers were counted in a Sedgewick-Rafter cell, after subsampling (Thouvenot et al. 2000).

Bacterial abundance and bacterial community composition. ***Total bacteria:*** We filtered 1 to 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 (Porter & Feig 1980); 400 to 800 bacterial cells were counted in 20 to 40 microscopic fields.

Fluorescent in situ hybridization with group-specific rRNA oligonucleotides: The abundance of 4 different classes and groups was analysed by *in situ* hybridization with fluorescence oligonucleotide probes on membrane filters (Glöckner et al. 1996, Snaidr et al. 1997). The oligonucleotide probes chosen targeted most Eubacteria (EUB338, GCTGCCTCCCGTAGGAGT) (Amann et al. 1990), the beta- and alpha-subclasses of the class Proteobacteria (BET42a, GCCTTCCCACTTCGTTT—ALF1b, CGTTCGYTCTGAG CCAG) (Manz et al. 1992) and the *Cytophaga–Flavobacterium* cluster of the *Cytophaga–Flavobacterium–Bacteroides* phylum (CF319a, TGGTCCGTGTCTCAG TATC) (Manz et al. 1996). The probes were fluorescently labeled with the indocarbocyanine dye Cy3 (MWG-Biotech). The hybridization buffer was composed of 180 µl 5 M NaCl, 20 µl 1 M Tris-HCl pH 7.4, formamide (20% v/v for the ALF1b 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 of the BET42a, ALF1b and CF319a 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 Tris-HCl (1 mM), NaCl (5 M final conc. for ALF1b and 80 mM for the other probes) and SDS (10% v/v) solution (15 min at 48°C in the dark). Bacteria 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 to 40 fields were counted for each probe and sample (Jürgens et al. 1999). As in Pernthaler et al. (1997), 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. Bacterial length (L) and width (W) were measured at $t = 0$ using an image-analysis system (Qwin, Leica), under Cy3 excitation. Among the various values suggested as upper prey-size limits for HNF

(Hahn & Höfle 2001), we chose the limit of 2.4 µm defined by Jürgens et al. (1999).

T-RFLP analysis: Genomic DNA extraction and T-RFLP analysis were conducted according to Ausubel et al. (1987) and Liu et al. (1997). Genomic DNA was purified by using a Qiagen spin-column and 5 µl of total DNA were electrophoresed on a 2% (w/v) agarose electrophoresis gel to check for DNA integrity.

Each 50 µl PCR reaction consisted of 2 mM $MgCl_2$, 200 µM of each dNTP, 0.85 U of *TaqII* DNA polymerase (Eurobio), 0.2 µM of each oligonucleotide primer, 10 to 50 ng of DNA template and the PCR buffer supplied with the enzyme. The primers used for amplification of bacterial small subunit rDNA (ssu rDNA) were 27f-FAM (6-carboxyfluorescein) labeled at the 5′-end with fluorescent sequencing dyes (MWG Biotech) and 1492r. The sequences of 27f and 1492r were as follows: 27F, 5′-AGA GTT TGA TCC TGG CTC AG-3′ (mostly Eubacteria, Lane 1991); 1492R, 5′-GGT TAC CTT GTT ACG ACT T-3′ (mostly Eubacteria and Archaebacteria, Lane 1991). PCR reactions were performed in a Perkin Elmer Model 2400 thermal cycler, with denaturation at 95°C for 1.5 min, annealing at 55°C for 1.5 min and extension at 72°C for 1.5 min for a total of 30 cycles. Products were purified using the Qiaquick PCR purification Kit (Qiagen) and visualized on 1% agarose gels.

Enzymatic digestions were performed by incubating PCR products with 5 U of *Rsa*I or *Msp*I (Gibco BRL) for 5 h. The T-RFs (terminal restriction fragments) were separated on an automated sequencer (PE ABI 310). The resolution is ~1 bp for fragments up to 500 bp and ~5 bp for fragments up to 1000 bp. Lengths of the T-RFs were determined using GeneScan 211 software (ABI). 5′-FAM-labeled fragments were detected at a wavelength of 532 nm. The relative contributions of the different OTUs were computed by using peak-area thresholds of 500 fluorescence units.

Bacterial grazing and net growth rate. Temporal changes in the density of total bacteria, EUB338, BET42a, ALF1b and CF319a were determined from counts conducted on unfiltered water at $t = 0$. Net growth rates were measured on all dates except 15 July, and the grazing rates were determined on 28 April, 26 May, 29 June and 10 August. These dates corresponded to changes in the main bacterial predators whose activity was determined in the same ecosystem by Thouvenot et al. (1999a).

Dialysis bags (12 000 to 14 000 Da, Merck Eurolab) were used to study bacterial net growth and grazing rates. They were autoclaved before use and rinsed and stored at 4°C in 50% ethanol after experiments (Herndl et al. 1993). Different water samples, collected from 1 m depth with a Van Dorn bottle, were homogenized in a basin. Water samples for predator-free experiments were mixed and serially filtered through 5 and 1.2 µm pore-size filters (polycarbonate, Millipore). All steps and all samples were processed in sterilized Duran Schott glasses. Dialysis bags were filled with 2 l of water for the unfiltered treatment and with 0.5 l for the 1.2 µm treatment. Each treatment was conducted in triplicate and incubated *in situ* at 1 m immediately after the bags had been filled. Water samples were incubated for 1 d to avoid significant bacterial growth on the surface of the dialysis bag (Herndl & Malacic 1987, Richardot et al. 2000).

Bacterial net growth rates (r) with bacterial predators (r_b, d^{-1}) and without predators (r, d^{-1}) were calculated from the difference in abundance from Day 0 to Day 1 (t = 24 h), assuming exponential growth in the dialysis bags. The absence of predators in the predator-free bacterial dialysis bags (<1.2 µm) was verified by microscopic examination. We used the equations:

$$r_b = (\ln N_{b_t} - \ln N_{b_0}) / t$$

and

$$r = (\ln N_t - \ln N_0) / t$$

where N_0 and N_t are bacterial abundance (N_{b_0}, N_{b_t} = dialysis bags with predators, N_0, N_t = bags without) at the beginning and the end of the experiment. Grazing rates (g) are the differences between the treatment with and that without predators: $g = r - r_b$ (Del Giorgio et al. 1996). The total grazing rate activity was computed as:

$$\text{GR (bacteria ml}^{-1}\text{ d}^{-1}) = g \times N_b 0$$

Bacterial net production (P, bacteria ml^{-1} d^{-1}) is the product of abundance at $t = 0$ and the net growth rate:

$$P = N_0 \times r$$

Statistical analysis. The relations between the different quantitative variables were determined by Pearson's correlation coefficient (see Table 3). We used 1-way analyses of variance (ANOVA) to test for differences in bacteria size and grazing between the groups (BET42a, ALF1b and CF319a) on any given date. The equality of the variances and the normality of the residuals were tested beforehand. The Scheffé test was used for pairwise comparisons of means. The independence between the size classes defined for each sampling date and eubacterial subgroups (BET42a, ALF1b and CF319a) was tested by χ^2 analysis. Co-inertia analysis was performed to reveal the relationships between the bacterial community composition (OTUs) and environmental data. This is an extension of the initial approach analysis described by Tucker (1958), also known as the first step of a partial least-square regression (PLS). The first matrix was the environmental array (X), and the second matrix (Y) represented the presence or absence of each OTU (coded by 1 or 0) in each sample. Co-inertia analysis is a 2-table ordination method. The X

matrix was analysed by principal components analysis (PCA) and the *Y* matrix by correspondence analysis (COA). The simultaneous analysis of both matrices consisted of calculating the maximum covariance between the environmental axis (PCA of Matrix *X*) and the 'taxa' axis (COA of Matrix *Y*) (Dolédec & Chessel 1994). ADE 4.0 (ecological data analysis, http://pbil.univ-lyon1.fr/ADE-4/ADE-4.html) (Thioulouse et al. 1997) was used to perform this ordination. This analysis is a 2-table ordination technique method similar to the canonical correspondence analysis (CCA) of Ter Braak (1987). However, co-inertia analysis enables matrices with similar (even low) as well as different numbers of environmental variables, species, and/or samples to be connected. In CCA, the number of species and the number of environmental variables must be much lower than the number of samples.

RESULTS

Main physico-chemical and biological characteristics of study site

The reservoir was thermally stratified throughout the study period; mean temperature and dissolved oxygen at 1 m were 18.5°C and 8.7 mg l^{-1}, respectively. The average dissolved protein (DPROT), monosaccharide (DFCHO) and polysaccharide (DCCHO) concentrations were 5.2, 2.1 and 1.0 mg l^{-1}, respectively (Table 1). The phytoplankton (mean annual biomass 118.9 ± 176.1 µg C l^{-1}) was dominated by chrysophytes (84.7 ± 172.9 µg C l^{-1}) (Fig. 1A). Among this taxon, the biomass of Chrysophyceae, mainly consisting of the species *Dinobryon* sp. (>92%), reached highest values on 28 April and 11 May and then strongly declined from 26 May; its biomass on 10 August was low (35.0 µg C l^{-1}). Average chlorophyll *a* (chl *a*) concentration was 5.1 µg l^{-1}, reaching a maximum value on 28 April (17.4 µg l^{-1}), when there was a bloom of *Dinobryon* sp. The abundance of heterotrophic nanoflagellates (HNF) varied from 0.04 to 1.41 × 10^3 cells ml^{-1} (mean: 0.50 × 10^3 cells ml^{-1}). This abundance was relatively stable in spring, then fell in mid-July (0.04 × 10^3 cells ml^{-1}) and increased strongly from the end of July to early August (1.41 × 10^3 cells ml^{-1} on 10 August) (Fig. 1B). Chrysomonadines and *Kathablepharis* sp. accounted on average for 94.8% of the total biomass of HNF. The average metazoan zooplankton biomass determined for the water column was 37.2 g dry wt l^{-1}. In the epilimnion of the reservoir, cladocerans (*Daphnia longispina*, *Ceriodaphnia quadrangula* and *Bosmina longirostris*) reached their highest biomass from 26 May to 29 June (Fig. 1C); from 15 July to 10 August their biomass declined.

Temporal changes in structure of bacterial community

Average total bacterial abundance (of 2.8 ± 0.8 × 10^6 cells ml^{-1}) increased strongly in May, reaching 4.1 × 10^6 cells ml^{-1} on 14 June before stabilising at a value close to 2.7 ± 0.2 × 10^6 cells ml^{-1} by the end of the study (Fig. 2A).

The bacteria hybridized with EUB338 (average 1.3 ± 0.5 × 10^6 cells ml^{-1}) accounted for between 26.2 and 62.8% of total bacterial abundance (Fig. 2C). The beta subclass (BET42a) of Proteobacteria, which accounted on average for 14.2% of total bacteria (mean concentration 0.4 ± 0.2 × 10^6 cells ml^{-1}), was the dominant group. The alpha subclass (ALF1b) of Proteobacteria and the *Cytophaga–Flavobacterium* group (CF319a) accounted for 5.9% (mean concentration 0.2 ± 0.03 × 10^6 cells ml^{-1}) and 4.1% (mean 0.1 ± 0.04 × 10^6 cells ml^{-1}) of total abundance, respectively. The abundance of BET42a was relatively high until the end of May (mean 0.55 ± 0.17 × 10^6 cells ml^{-1}) but fell to an average value of 0.25 ± 0.09 × 10^6 cells ml^{-1} from 14 June onward (Fig. 2B). Changes in the abundance of ALF1b were characterised by a maximum on 10 August of 0.22 ± 0.02 × 10^6 cells ml^{-1} and by 2 peaks of abundance for CF319a: on 26 May (0.14 × 10^6 cells ml^{-1}) and 10 August (0.17 × 10^6 cells ml^{-1}).

During this study, the mean size of bacteria was 0.90 ± 1.15 µm. For the 4 series of samples used in the

Table 1. Environmental parameters measured in Sep reservoir from April to August 1999. DCCHO, DFCHO, DPROT: dissolved polysaccharides, monosaccharides and proteins, respectively

Parameter	Mean ± SD
Hydrology	
Inflow (l s^{-1})	91.6 ± 78.5
Outflow (l s^{-1})	311.1 ± 220.3
Epilimnion	
Temperature (°C)	18.5 ± 4.5
Oxygen (mg l^{-1})	8.7 ± 0.8
pH	7.2 ± 0.8
N-NH_4 (mg N l^{-1})	0.02 ± 0.01
N-NO_3 (mg N l^{-1})	1.2 ± 0.6
P-PO_4 (mg P l^{-1})	0.01 ± 0.02
DCCHO (mg l^{-1})	1.0 ± 0.9
DFCHO (mg l^{-1})	2.1 ± 1.0
DPROT (mg l^{-1})	5.2 ± 0.8
Chl *a* (µg l^{-1})	5.1 ± 4.9
Primary production (µg C l^{-1} h^{-1})	41.6 ± 35.6
Water column	
Zooplankton (µg dry wt l^{-1})	37.2 ± 23.4
Cladocerans (%)	27.5 ± 15.9
Rotifers (%)	17.5 ± 12.1
Copepods (%)	55.0 ± 11.2

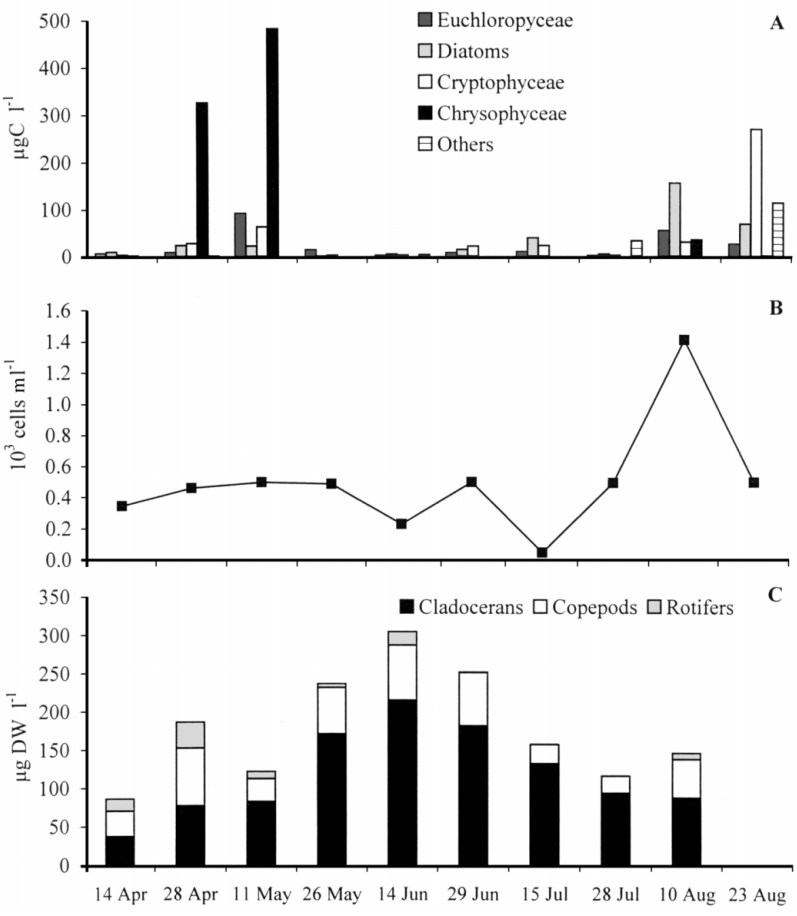

Fig. 1. Seasonal variation in (A) biomass of algae, (B) abundance of heterotrophic nanoflagellates, and (C) biomass of metazooplankton in the epilimnion during 1999

experiments on the impact of predation on the different bacterial groups, the mean size of BET42a, ALF1b and CF319a did not differ significantly (1-way ANOVA, df = 2) on any given date (Table 2). There was also no difference in the distribution of the various size classes studied (0 to 0.4, 0.4 to 2.5 and >2.5 µm) for the respective bacterial groups (BET42a, ALF1b and CF319a) between dates (χ^2 test, df = 4) (Table 2).

Digestion of amplification products by *Msp*I and *Rsa*I identified a total of 61 and 25 different operational taxonomic units (OTUs), respectively. The number of OTUs detected with *Msp*I decreased regularly from 14 April to 11 May and then increased sharply from 26 May (Fig. 3). They were positively correlated with the biomass of *Ceriodaphnia* sp. in the epilimnion and negatively with the amount of water entering the reservoir and with the biomass of Chrysophyceae (Table 3). The OTUs generated by *Rsa*I were linearly correlated with the DFCHO/TDCHO (DFCHO + DCCHO) ratio and with pH. There were temporal changes in dominance of individual OTUs (Fig. 3). For example, the 509 bp OTU, present on all dates, accounted on average for 29.4% (4.6 to 49.6%) of the total area and was dominant from 14 April to 14 June and from 28 July to 10 August, while the 458 bp OTU increased progressively from the end of May and became dominant on 15 July (40.9%). These temporal changes showed large seasonal variation. We used co-inertia analysis to examine the relationships between the OTUs identified (presence or absence) and environmental variables. Only the results obtained using *Msp*I, which identified the highest number of OTUs, are presented (Fig. 4). In this analysis, those environmental variables that have the higher coordinates on the 2 axes comprise the most important feature correlated with the OTU distribution. The OTUs with negative coordinates on Axis 1 were mainly related to temperature and PP. The OTUs with high positive coordinates on the same axis (470, 483, 305 and 608) were mainly associated with input of water into the reservoir and with high rotifer biomass. The distribution of the OTUs on the second axis depended on cladoceran biomass and output of water from the reservoir on the one hand, and on the biomass of the main phytoplankton groups and HNF on the other.

Changes in net bacterial production and bacterial grazing

The mean net growth rates (in dialysis bags without predators) of total bacteria EUB338, BET42a, ALF1b and CF319a were 0.21, 0.45, 0.50, 0.69 and 0.84 d^{-1}, respectively.

Total net bacterial production (Fig. 5B) increased from the end of April to mid-June (mean 1.07×10^6 cells ml^{-1} d^{-1}), reaching a maximum of 2.00×10^6 cells ml^{-1} d^{-1} on 14 June and then falling again on 29 June. Thereafter it remained low. The net production of EUB338 reached its highest value of 1.46×10^6 cells ml^{-1} d^{-1} on 26 May (mean: 0.79×10^6 cells ml^{-1} d^{-1}) and then decreased strongly during the summer. From April to June, the net production of BET42a was higher than that of ALF1b and CF319a (means of 0.180, 0.122 and 0.097×10^6 cells ml^{-1} d^{-1} for BET42a, ALF1b and CF319a, respectively) (Fig. 5C). The temporal changes in the net production of the different groups and subclasses studied were also characterised by a change from mid-June onward. As with the net production of EUB338, the net production of BET42a, ALF1b and the CF319a group decreased

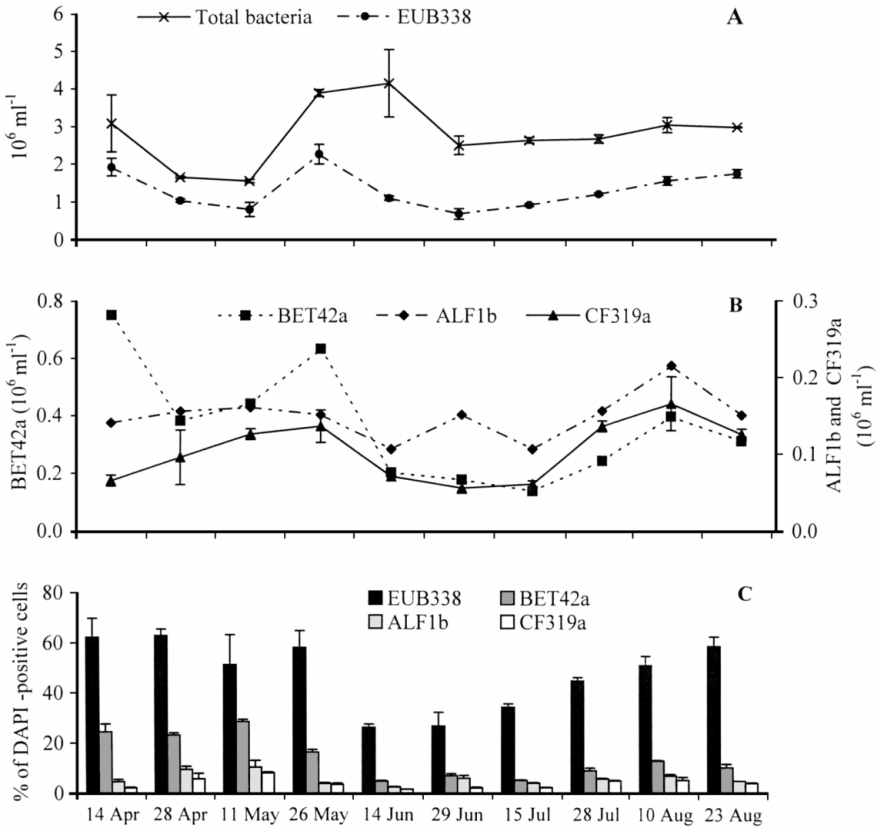

Fig. 2. Seasonal variation in (A) numerical abundance of total bacterial and bacteria hybridized with EUB338, (B) beta-proteobacteria (BET42a), alpha-proteobacteria (ALF1b) and *Cytophaga–Flavobacterium* (CF319a), and (C) in percentages abundance of EUB338, BET42a, ALF1b and CF319a

Table 2. Changes in distribution of length classes (0 to 0.4, 0.4 to 2.5 and >2.5 µm) and average length (µm) of bacteria hybridized with EUB338, BET42a, ALF1b, CF319a from 28 April to 10 August 1999. Abbreviations as in Fig. 2

Length (µm)	28 Apr	26 May	29 Jun	10 Aug
EUB338				
0 to 0.4	2.4	8.6	1.8	0.3
0.4 to 2.5	93.2	89.8	92.3	96.1
>2.5	4.4	1.6	6.0	3.6
Avg. length	1.12 ± 0.03	0.78 ± 0.07	1.15 ± 0.25	1.01 ± 0.04
BET42a				
0 to 0.4	4.9	7.0	0.9	0.5
0.4 to 2.5	95.1	93.0	98.1	99.5
>2.5	0.0	0.0	0.9	0.0
Avg. length	0.75 ± 0.02	0.69 ± 0.27	0.87 ± 0.14	0.8 ± 0.01
ALF1b				
0 to 0.4	4.3	2.4	0.0	1.0
0.4 to 2.5	93.6	96.8	99.0	95.2
>2.5	2.2	0.8	1.0	3.9
Avg. length	0.79 ± 0.07	0.83 ± 0.05	0.77 ± 0.06	0.87 ± 0.04
CF319a				
0 to 0.4	1.5	4.3	0.0	0.0
0.4 to 2.5	97.0	95.7	100.0	97.6
>2.5	1.5	0.0	0.0	2.4
Avg. length	0.7 ± 0.12	0.69 ± 0.04	0.97 ± 0.09	0.75 ± 0.06

strongly during the summer. The net growth rates and net bacterial productions (total bacteria, EUB338, BET42a, ALF1b, CF319a) were not correlated with temperature (Fig. 5A). The net growth of total bacteria was correlated with dissolved organic carbon determined from proteins and polysaccharides (Table 3). Bacterial net growth and/or net production of ALF1b were significantly and positively correlated with the biomass of Euchlorophyceae, diatoms and Cryptophyceae, with PP and the abundance of ALF1b. The same variables determined for BET42a were correlated with bacterial grazing, with net growth of BET42a only being correlated with Daphniidae. Net production of CF319a was significantly associated with PP, diatoms and output of water. Release of water was also associated with net growth of the same group.

The average bacterivorous activity, measured by bacterial grazing rates in the dialysis bags, was 2.44×10^6 cells ml^{-1} d^{-1}. The highest values were recorded in May and early June with a maximum of 5.86×10^6 cells ml^{-1} d^{-1} (Fig. 5D). Over the whole study period, we studied the impact of predation on different bacterial groups on 4 dates corresponding to the potential predation activity of different bacterial consumers. The net growth rates of the various bacterial groups in the absence of predators (r) differed significantly irrespective of the date of the experiment ($p < 0.05$) (Table 4). Negative grazing rates reflect the fact that the growth of the various bacterial groups in the treatments filtered through 1.2 µm was lower than recorded in the unfiltered treatments. With the exception of 28 April, when *Dinobryon* sp. was the main bacterial predator, there was a significant difference between the grazing rates of different bacterial groups and subclasses (1-way ANOVA). On 26 May, ALF1b and CF319a were preferentially grazed (the dominant predators were *Daphnia longispina* and *Ceriodaphnia quadrangula*), with no significant difference in grazing rates between them (1.76 and

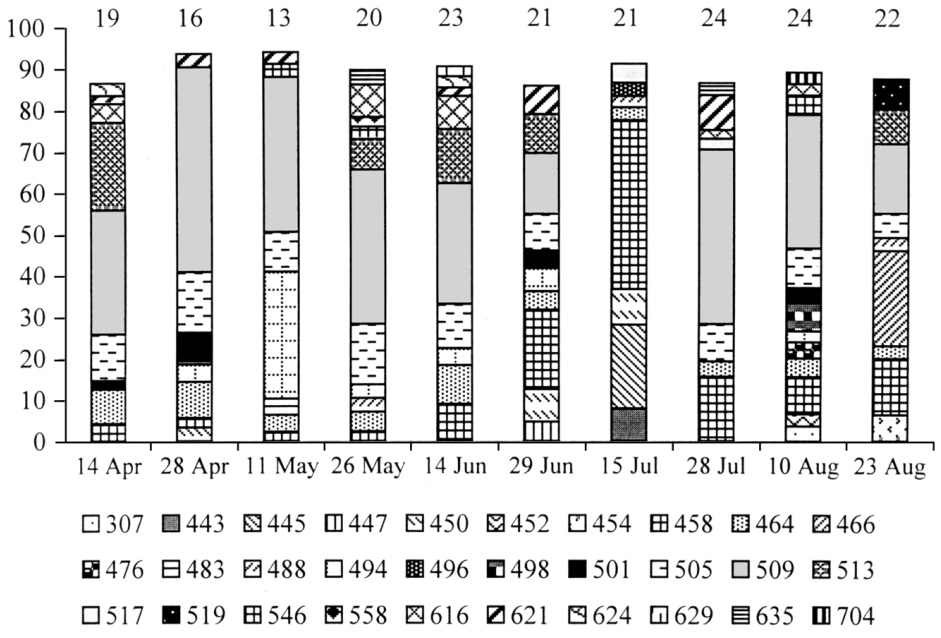

Fig. 3. Seasonal variation in number and relative abundance of the various operational taxonomic units (OTUs) detected by T-RFLP analysis of 16S rDNA digestion by *Msp*I, representing more than 2% of total area. Numbers above columns: numbers of detectable OTUs in individual samples

1.66 d^{-1}, respectively), but they were significantly higher than the grazing rates of BET42a ($p < 0.05$). On 29 June, ALF1b were still the bacteria subjected to the highest grazing rate (1.76 d^{-1}), a rate significantly higher than for 2 other groups and subclasses ($p < 0.05$). In August, the grazing rates of ALF1b and CF319a did not differ significantly.

DISCUSSION

Methodological aspects

T-RFLP was applied as fingerprint method to study the BCC in this reservoir. This method has the advantage of being a semi-quantitative (Liu et al. 1997),

Table 3. Relationships between bacterial abundance, operational taxonomic units (OTUs), bacterial growth, bacterial production, and biotic and abiotic variables from April to August 1999. Numbers in parentheses = r-values: $^{*}p < 0.05$, $^{**}p < 0.01$. DOM: dissolved organic matter; HNF: heterotrophic nanoflagellates; TDCHO: total dissolved organic matter; other abbreviations as in Fig. 2

Parameter	Variables showing significant correlation
Bacterial abundance (cells ml^{-1})	
EUB338	DOM polymerized (C-DPROT + C-DCCHO) (0.73*)—*Bosmina longirostris* (0.69*)
BET42a	Outflow (–0.65*)—Temp (–0.68*)—N-NO3 (0.74*)—pH (–0.65*)—*Ceriodaphnia quadrangula* (–0.80**)
ALF1b	PP (0.67*)—diatoms (0.68*)—HNF (0.87**)—*Daphnia longispina* (–0.73*)
CF319a	PP (0.67*)—DPROT (0.69*)—*D. longispina* (–0.81**)
OTUs	
*Msp*I	Inflow (–0.71*)—Chrysophytes (–0.77*)—*C. quadrangula* (0.69*)
*Rsa*I	DFCHO/TDCHO (0.68*)—pH (–0.68*)
Bacterial growth (d^{-1})	
Total bacteria	DOM (C-DPROT + C-TDCHO) (0.73*)
BET42a	*D. longispina* (0.73*)—total bacteria mortality (0.90**)
ALF1b	Euchlorophyceae (0.89**)—Cryptophyceae (0.84**)—HNF (0.75*)—ALF1b (0.80*)
CF319a	Outflow (–0.74*)
Bacterial production (10^6 ml^{-1} d^{-1})	
BET42a	Other phytoplanktonic taxa (–0.76*)—total bacteria mortality (0.75**)
ALF1b	PP (0.71*)—Euchlorophyceae (0.86**)—diatoms (0.78*)—Cryptophyceae (0.78*)—ALF (0.87**)

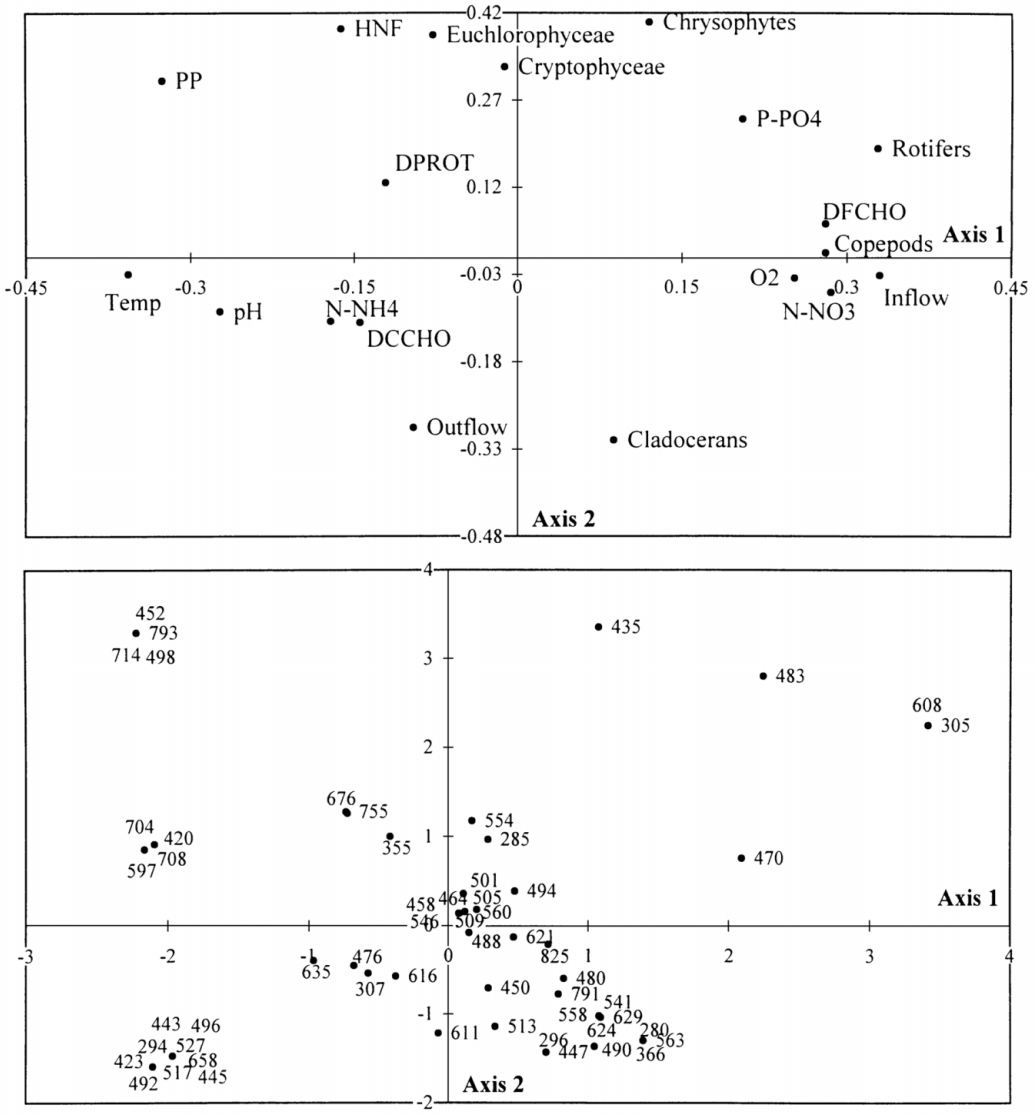

Fig. 4. Co-inertia analysis performed on the 19 environmental variables and OTUs obtained from T-RFLP analysis of 16S rDNA digestion by *Msp*I, that each represented more than 2% of the total area. Percentage of inertia explained by Axes 1 and 2 are 33 and 25%, respectively. Abbreviations as in Table 1

highly reproducible and robust technique that yields high-quality fingerprints (Osborn et al. 2000) with a level of resolution similar to that of denaturing gradient gel electrophoresis (Moeseneder et al. 1999). Because of its quantitative aspect, the FISH method can be used to measure not only morphological changes, but also abundance changes in dialysis bags; it is, currently, the only technique capable of determining the net production of target bacterial groups. Compared with methods based on the use of radioactive markers in this ecosystem, the measurement of net bacterial production over a period of 1 d in dialysis bags has the advantages firstly of integrating strong daily variations in bacterial production, and secondly of only taking into account the active fraction, which comprise but a small percentage of the total abundance (Jugnia et al. 2000). The filtration procedures of this method may lead to DOM enrichment by cell lysis. Mortality caused by predatory bacteria and phages, which can result in different mortality rates in different taxa, cannot be entirely excluded for the treatments without grazers. For this ecosystem, a model of the functioning of the microbial trophic network (Roué et al. 2001) suggested on the basis of experimental data (Thouvenot et al. 1999a,b) that bacterial mortality can be essentially explained by bacterivorous activity, as

already demonstrated for the epilimnic part of the water columnn of Lake Plußsee (Weinbauer & Höfle 1998). These data confirm that bacterial mortality caused by viruses can vary greatly from one lake ecosytem to another (Weinbauer et al. 2002).

Structure and dynamics of the bacterial groups

The relative abundance of the Eubacteria phylum, determined by FISH, can vary greatly between ecosystems, varying between 35% (Jürgens et al. 1999) and 100% of total bacterial abundance (Weiss et al. 1996). The mean value recorded in our study (45.3%; 26.2 to 62.8%) is slightly lower than that recorded in oligotrophic (52 to 55%) (Glöckner et al. 1996, Pernthaler et al. 1998) or mesotrophic (60%) (Glöckner et al. 1996) ecosystems. The results of most studies in freshwater environments show that the abundance of the beta-proteobacteria subclass is often higher than that of alpha-proteobacteria, which is itself higher than that of the *Cytophaga–Flavobacterium* group (Alfreider et al. 1996, Methé et al. 1998). The proportions we recorded in this study therefore agree with these observations, since BET42a was the most abundant subclass. However, other groups such as the Actinobacteria (Glöckner et al. 2000), which were not counted in this study, can dominate freshwater bacterioplankton.

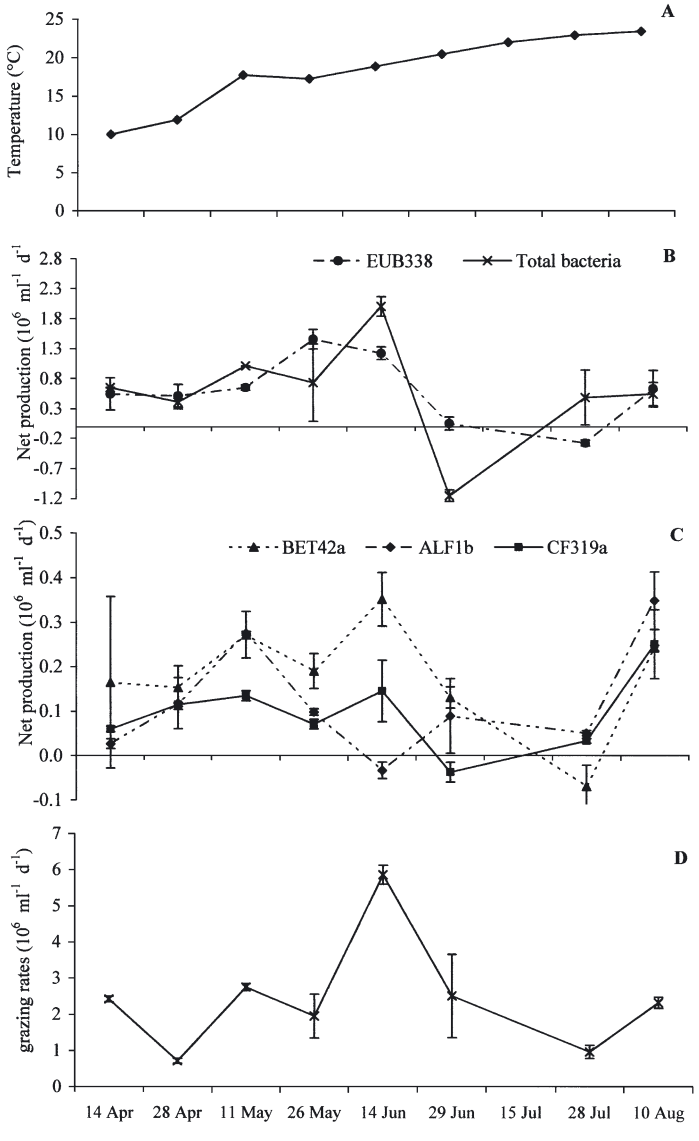

Fig. 5. Seasonal variation in (A) epilimnion temperature, (B) net production of total bacteria and bacteria hybridized with EUB338, (C) beta-proteobacteria (BET42a), alpha-proteobacteria (ALF1b) and *Cytophaga–Flavobacterium* (CF319a), and (D) total grazing rate

The temporal changes in the number of OTUs and also in the relative proportions of the restriction fragments showed that apparent stability of bacterial abundance can mask large changes in the BCC. Studies conducted over several years tend to show that bacterial communities are stable during any given season (Höfle & Brettar 1995). However, as in our study, there is seasonal variability in the structure of the bacterial community in marine (Lee & Fuhrman 1990, Rehnstam et al. 1993) and lacustrine (Lindström 1998, Pernthaler et al. 1998) environments. The temporal changes in the various areas in our study showed that a limited number of OTUs dominated the BCC at distinct periods. Using various techniques, including DGGE (denaturing gradient gel electrophoresis), 5S rRNA electrophoresis and DNA hybridization, other studies have also tended to show that the bacterioplankton is dominated by a relatively restricted number of taxa. For example, in coastal environments the number of dominant taxa can vary from 7 to 10 (Pinhassi et al. 1999) or from 8 to 14 (Pinhassi & Hagström 2000). The few studies that have been conducted in lakes have shown that 10 to 20 taxa dominated in a meromictic lake (Øvreås et al. 1997) and from 2 to 8 in the epilimnion of a eutrophic lake (Höfle et al. 1999).

Although various studies have described the structure of bacterial communities in various ecosystems, few have attempted to determine the factors controlling temporal changes. Factors such as temperature, organic and inorganic resources and predators help to control bacterial populations, as has been shown in many studies (Caron 1994, Felip et al. 1996, Pace and Cole 1996). Therefore, by focusing on whole-community responses (for example total bacterial production), such studies may fail to detect the effects of these factors on some bacterial groups.

Table 4. Net growth and mortality rates of alpha(ALF1b)- and beta(BET42a)-proteobacteria and *Cytophaga–Flavobacterium* (CF319a). Growth or mortality rates on same date with a different letter (a, b, c) are significatively different ($p < 0.05$)

Bacteria	Growth rate r (d^{-1})	ANOVA p	Scheffé *F*-test	Mortality g (d^{-1})	ANOVA p	Scheffé *F*-test
28 Apr						
BET42a	0.40 ± 0.13		a	0.66 ± 0.13		
ALF1b	0.74 ± 0.36	0.017	a,b	0.74 ± 0.29	0.620	
CF319a	1.15 ± 0.05		b	0.81 ± 0.32		
26 May						
BET42a	0.30 ± 0.06		b	0.95 ± 0.04		b
ALF1b	0.66 ± 0.05	0.001	a	1.76 ± 0.19	0.002	a
CF319a	0.51 ± 0.08		a	1.66 ± 0.21		a
29 Jun						
BET42a	0.73 ± 0.13		a	0.12 ± 0.14		b
ALF1b	0.60 ± 0.56	0.011	a	1.76 ± 0.06	0.000	a
CF319a	−0.61 ± 0.37		b	−0.33 ± 0.07		c
10 Aug						
BET42a	0.61 ± 0.02		b	−0.49 ± 0.15		b
ALF1b	1.58 ± 0.29	0.017	a	0.53 ± 0.04	0.001	a
CF319a	1.47 ± 0.46		a	0.95 ± 0.35		a

Top-down control of bacterial community composition

The highest bacterial mortalities were measured on 14 June, when the Cladocera reached their highest abundance and when the densities of HNF and phytoplankton were low. These results therefore support those of Thouvenot et al. (1999b), who showed that Cladocera were the main bacterial consumers in this ecosystem. *Daphnia longispina* and *Ceriodaphnia quadrangula* accounted on average for 72% of total bacterivore activity in the epilimnion. Among the flagellates, *Dinobryon* sp. (a mixotrophic flagellate) accounted for up to 75% of total predation at any given date in this ecosystem, whereas HNF, rotifers and the ciliates displayed low bacterivore activity (Thouvenot et al. 1999b). The heavy predation of metazooplankton on protozoans could explain the low abundance of HNF during a large part of our study, in agreement with the observations of Jürgens (1994) and Thouvenot et al. (1999a) in the same reservoir. Thus, the absence of resistant bacterial forms such as filaments could be due to the fact that HNF, which can lead to the appearance of these morphotypes (Sommaruga & Psenner 1995), are not the main bacterivore organisms in this ecosystem.

It seems likely that the bacterivore activity of *Dinobryon* sp. caused the great decrease in bacteria abundance recorded in April, which coincided with a period of low net production rates of the total bacteria. The dominance of Cladocera in the epilimnion (26 May to 29 June), which on the whole coincided with the highest net production rates (Fig. 5), was reflected by a decrease in total bacteria and EUB338. Thus, the dates on which the grazing of bacterial groups was studied (Table 4) coincided with a marked dominance of bacterial consumers in this ecosystem. If we use the filtration rates determined by Thouvenot et al. (1999b) at the same site and for the same species, we can calculate the relative impact of these various predators. As a percentage of total bacterivore activity, *Dinobryon* sp. accounted for 96.4% on 28 April, whereas Cladocera accounted for 86.3 and 89.2% of activity on 26 May and 29 June. On 10 August, both these organisms were the main predators on bacteria.

When there was a significant difference between the grazing rates of different bacterial groups and subclasses, it was always ALF1b that were preferentially consumed. In contrast, BET42a seemed to be little consumed by bacterial consumers and predation led to large variations in the grazing rates of CF319a (Table 4). The succession of the main bacterial consumers could be the cause of these variations in grazing rates. Furthermore, the relation between the structure of the bacterial community and predation is also supported by the significant correlations between the changes in OTUs, bacterial abundance and the main bacterial consumers, i.e. Cladocera (Table 3).

The clear-water phase corresponded to a decrease in the abundance of protists and an increase in the metazoan zooplankton, and also in a change in the BCC. For example, the increase in the biomass of Cladocera in the epilimnion was associated with an increase in the number of OTUs, reflecting a change in the BCC. This number was significantly correlated with the abundance of *Ceriodaphnia quadrangula* ($r = 0.69$, $p < 0.05$) and, more precisely, the co-inertia analysis related the presence of specific OTUs to cladoceran biomass (Fig. 4). This period was also related to a decrease in the percentage hybridization of the various probes. A decrease in size of EUB338 on 26 May, which implies less rRNA, could partially explain this phenomenon. This decline could also indicate that there was a decrease in the number of active bacteria (whose detection depends on the number of ribosomes) in the environment. The significant correlation between HNF (which have a low bacterivore activity in this ecosystem) and the abundance of ALF1b ($r = 0.87$, $p < 0.01$) could only be the result of a covariation

between these organisms which are simultaneously subjected to predation by Cladocera ($r = -0.73$, $p < 0.05$). Moreover, the correlation between the abundance of ALF1b, their net growth rates and net production suggests that there is a bottom–up type control of these groups. Some OTUs were associated with the biomass of Chrysophytes (Fig. 4), but when *Dinobryon* sp. was the dominant predator (28 April), the groups and subclasses studied were subjected to the same predation pressure. However, it is possible that variations in community structure within the subclasses studied were undetected by the FISH method and could have caused this anomaly. To our knowledge, there is no published information on the impact of *Dinobryon* sp. on the structure of a bacterial community. Although selective predation by HNF has already been demonstrated in many studies (Hahn & Höfle 2001), only the works of Höfle et al. (1999) and Langenheder & Jürgens (2001) and this study have indicated that Cladocera have an impact on BCC.

Bacterial morphotype could be the dominant criterion in the selection by predators (Šimek & Chrzanowski 1992). Cladocera can ingest bacteria that are larger than 0.5 µm (Brendelberger & Geller 1985), and the HNF can consume bacteria whose size is less than 2.4 µm (Jürgens et al. 1999). However, in this study, bacteria in this size class (0.5 to 2.4 µm) represented at least 89.8% of total bacteria abundance (Table 2), and therefore this factor cannot be considered the main selection criterion. Furthermore, the various statistical analyses showed that there were no significant differences in mean size and in the distribution of the various size classes between the 3 eubacterial subgroups (BET42a, ALF1b and CF319a).

In the experiments of 26 May and 10 August, bacteria with the highest net growth rates were subjected to the highest predation. The preferential consumption of ALF1b when Cladocera were the dominant predators also coincided with the period when the net growth rates of these groups were highest. According to Langenheder & Jürgens (2001), preferential predation by Cladocera on the fast-growing portion of the BCC, which consists of relatively large cells (Jürgens 1994), could partly explain some of our experimental results. Grazing by Cladocera could lead to the accumulation of a relatively large pool of less metabolically active bacteria (Del Giorgio et al. 1996) and could explain the decrease in coverage of total bacteria by probes during the clear-water phase. However, the differential impact on the various groups and subclasses detected on 29 June cannot be explained by a preferential predation of active cells. Some bacterial species could either develop colonially, or could adhere to clumps of organic matter, and would therefore be more vulnerable to predation by Cladocera. We can hypothesize that this selectivity is related to the digestibility of bacteria (which may differ between bacterial groups) and/or the efficiency of the predators, as has already been speculated for certain algae (Jürgens & Güde 1994). There is also some evidence that not all bacteria are digested by zooplankton (King et al. 1991), and viable gut-passage might be another mechanism of surviving cladoceran grazing.

Bottom-up control of bacterial community

The specific mean net growth rate of ALF1b, 0.69 d^{-1}, is intermediate between that reported by Weiss et al. (1996), 0.54 d^{-1}, and that reported by Jürgens et al. (1999), 1.42 d^{-1}. The specific mean net growth rates of BET42a (0.54 d^{-1}) and of CF319a (0.84 d^{-1}) measured in this reservoir were lower than those measured in various *in vitro* experiments, where they were 0.64 to 1.56 d^{-1} (Weiss et al. 1996, Jürgens et al. 1999) and 2.71 d^{-1} (Jürgens et al. 1999), respectively. These growth rates could differ because of differences in the composition of the bacteria making up these large phylogenetic groups and/or because productivities and substrate levels differed.

Many studies have shown that total bacterial production is dependent on physical factors such as temperature (Hoch & Kirchman 1993) and/or the concentration of inorganic or organic nutrients (Cole et al. 1988). The significant relations between the bacterial net growth of total bacteria and the concentrations of polymerised DOM (C-TDCHO + C-DPROT) determined in this study tend to confirm the main role of organic matter supply in controlling the growth of bacteria in this lake (Richardot et al. 2000). The association between phytoplankton biomass, PP and certain OTUs (Fig. 4) plus the positive correlations between net growth and/or net production of ALF1b and CF319a on the one hand and the PP and biomass of the main phytoplankton groups on the other hand (Table 3) suggest that the BCC depends strongly on the organic matter excreted by specific phytoplankton groups. This control by phytoplankton excretion of the growth of specific bacterial groups, demonstrated here by 2 different methods (FISH and T-RFLP), confirms the works of Lindström (2000, 2001) who showed that the bacterial community composition, determined by DGGE, seemed to be strongly dependent on phytoplankton biomass, in particular on that of Chrysophyceae and Cryptophyceae. Other processes producing DOM, such as excretion and sloppy-feeding by Cladocera, that stimulate total bacterial production, in particular in this ecosystem (Richardot et al. 2001), could be involved in the control of some bacterial groups. Various factors tend to show that the filter-

feeding activity of these organisms could preferentially favour the development of certain bacterial groups, and especially BET42a, as shown by the positive correlation between the net growth parameters of BET42a (net production and/or net growth rates) and total bacterial grazing and/or *Daphnia longispina* biomass (Table 3). Among the physical variables, inflow and outflow of water in the reservoir also seemed to play a role in structuring the bacterial community composition in this ecosystem. The correlation between the number of OTUs and the water entering the reservoir suggests that the bacterial community composition could be influenced by various compounds coming from the catchment (Lindström 1998) and allochthonous bacteria (Lindström 2001). The net production and net growth rates of CF319a were negatively correlated with outflows from the reservoir. It is highly likely that, rather than there being a direct relationship between these 2 variables, the decrease in phytoplankton growth caused by the instability of the water column during the emptying of the reservoir (Tadonléké et al. 2000) caused a concomitant decrease in the growth of these bacterial groups, whose abundance is closely related to phytoplankton production.

This study has shown that in this reservoir BCC varies seasonally as a function of variations in the supply of organic matter (phytoplankton and primary production) of predation by Cladocera and of input and output of water and that grazing rates differ significantly between the different bacterial groups and subclasses, with grazing vulnerability varying as a function of season. The relative importance of these various factors in structuring bacterial community composition and the repercussions in terms of functioning of the microbial trophic network, i.e. biogeochemical cycles, are major challenges for future research.

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