Succession of bacterial community composition over two consecutive years in two aquatic systems: a natural lake and a lake-reservoir

Delphine Boucher, Ludwig Jardillier & Didier Debroas

Laboratoire de Biologie des Protistes UMR CNRS 6023, Université Blaise Pascal, Aubière, France

Correspondence: Didier Debroas, Laboratoire de Biologie des Protistes UMR CNRS 6023, Université Blaise Pascal, 63177 Aubière, France. Tel.: +0473407837; fax: +0473407837; e-mail: didier.debroas@free.fr

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Abstract

The succession in bacterial community composition was studied over two years in the epilimnion and hypolimnion of two freshwater systems: a natural lake (Pavin Lake) and a lake-reservoir (Sep Reservoir). The bacterial community composition was determined by cloning-sequencing of 16S rRNA and by terminal restriction fragment length polymorphism. Despite large hydrogeological differences, in the Sep Reservoir and Pavin Lake the dominant bacteria were from the same taxonomic divisions, particularly *Actinobacteria* and *Betaproteobacteria*. In both ecosystems, these major bacterial divisions showed temporal fluctuations that were much less marked than those occurring at a finer phylogenetic scale. Nutrient availability and mortality factors, the nature of which differed from one lake to another, covaried with the temporal variations in the bacterial community composition at all sampling depths, whereas factors related to seasonal forces (temperature and outflow for Sep Reservoir) seemed to account only for the variation of the hypolimnion bacterial community composition. No seasonal reproducibility in temporal evolution of bacterial community from one year to the next was observed.

Introduction

Heterotrophic bacteria are an important constituent of pelagic ecosystems, where they play a key role in the breakdown of organic matter and remineralization of nutrients. However, our knowledge of the extent and character of bacterial diversity has been limited by having to rely on the study of cultivated organisms: less than 1% of microorganisms can be cultivated using standard techniques (Amann *et al.*, 1995). The introduction of molecular tools applied to ecology (FISH, DGGE, T-RFLP, cloning-sequencing, etc.) has made a more precise study of the structure of the bacterial community possible (Giraffa & Neviani, 2001). Phylogenetic analyses have shown a broad diversity within the lacustrine pelagic bacterial plankton (Zwart *et al.*, 2002), which is often dominated by *Betaproteobacteria* (Methé *et al.*, 1998). More recently, Glöckner *et al.* (2000) demonstrated that the *Actinobacteria* class may be an important bacterioplankton group in freshwater ecosystems. However, as stressed by Zwart *et al.* (2002), lacustrine bacterioplankton diversity has not been studied to the same extent as marine ecosystems. According to those authors, a large percentage of Operational Taxonomic Units (OTUs) are common to many diverse freshwater habitats. However, some studies, such as those of Lindström (2000), Yannarell *et al.* (2003) and Urbach *et al.* (2001), have shown that each lake has a quite specific bacterial community composition (BCC). In the last study (Urbach *et al.*, 2001), the authors emphasized the unique character of the microbial community, which was mainly due to the absence of riverine influx. The importance of the inlet on the BCC was also underlined by Lindström & Bergström (2004), who showed that the contributions of allochthonous bacteria might depend on the hydraulic retention time. If allochthonous contributions have an influence on diversity, then the BCC of reservoirs with a short hydraulic retention time should differ from those of other ecosystems. However, compared with marine ecosystems and lakes with different trophic status and morphology, much less attention has been paid to the role of pelagic bacteria diversity in lake-reservoirs (Mašín *et al.*, 2003; Jardillier *et al.*, 2004).

In addition, although the molecular methods have been available for more than a decade, surprisingly few studies have been published on the spatio-temporal dynamics of

bacterioplankton communities. Some authors have studied spatial variation at single sampling occasions in BCC (Casamayor *et al.*, 2000; Humayoun *et al.*, 2003), whereas others have focused on the temporal dynamics of the bacterioplankton (Höfle *et al.*, 1999; Crump *et al.*, 2003) and observed more or less swift changes of this community. However, to our knowledge, except for Lindström (1998), Yannarell *et al.* (2003) and Zwisler *et al.* (2003), none of the temporal studies was conducted for longer than 1 year. Therefore, unlike phytoplankton and zooplankton successions, which have been modelled using notably the PEG model (Sommer *et al.*, 1986), long-term successions of bacterial populations remain imperfectly known.

In this work, we studied the dynamics of the bacterial communities during 2 consecutive years in two different ecosystems: a natural lake (Pavin Lake) and a lake-reservoir (Sep Reservoir). The two sites studied were characterized by different drainage basin surface areas and hydraulic retention times. We set out to determine: (1) whether the phylogenetic composition of the bacterioplankton of a lake-reservoir differs from that of a hydrologically more stable ecosystem; (2) how the BCC evolves in the long term, and (3) whether patterns were repeatable in 2 consecutive years.

Here, we studied the bacterial planktonic populations by cloning-sequencing of the 16S rDNA and by T-RFLP. We also compared the T-RFs obtained from clones with the T-RFs obtained from the environmental T-RFLP profiles. This comparison then enabled us to identify the different phylotypes in the T-RFLP analysis and to follow them throughout the study period.

Materials and methods

Study sites and sampling

The study was conducted in two lakes located in the Massif Central, France, and classified as oligomesotrophic according to the studies of Dévaux (1977), Tadonléké *et al.* (2000) and the standards of OECD (1982) based on concentration of chlorophyll *a*.

Sep Reservoir, lying at an altitude of 500 m (46°2′N and 3°1′E), was built in 1994. With an area of 33 ha, a mean depth of 14 m (max. depth 37 m) and a water volume of 4.7 mm^3, this reservoir has a catchment of 27 km^2 and a mean residence time of 220 days. Sep Reservoir was built to meet the irrigation requirements of farmers situated further down the valley. The reservoir's management therefore differs from 1 year to the next, and the reservoir was completely emptied at the end of 2001. The dam is 41 m high and the water is drawn off by a sluice at the bottom of the dam.

Pavin Lake, situated at an altitude of 1197 m (45°29′N and 2°56′E), is a typical crater mountain lake characterized by a maximum depth of 92 m, a mean residence time of 10 years for the mixolimnion, and a small surface (44 ha) and catchment area (50 ha). There is therefore a very low inflow and outflow of water from Pavin Lake.

Sampling was carried out at a permanent station situated at the deepest zone for the two lakes. Water samples from the epilimnion (1 and 5 m below the surface, respectively, for Sep Reservoir and Pavin Lake) and from the hypolimnion (1 m above the sediments and 30 m below the surface, respectively, for Sep Reservoir and Pavin Lake) were collected with a Van Dorn bottle. For the 2 years of the study (2001–2002), the samples were taken every 2 weeks from April to August and monthly from September to November for Sep Reservoir. For Pavin Lake, the samples were taken monthly in 2001 and from September to November in 2002, and every 2 weeks from April to August in 2002.

Sample preservation

Samples were collected and fixed immediately with a final concentration of 4% formaldehyde for total bacteria and 1% glutaraldehyde for flagellates. The metazooplankton was fixed in a sucrose/formaldehyde solution (final concentration 6% and 4%, respectively) (Prepas, 1978). For nucleic acid extraction, the water was prefiltered through a 5 μm polycarbonate pore size filter (Millipore, Molsheim, France) 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 micro-centrifuge tubes (Eppendorf) and were then frozen at -80 °C until nucleic acid extraction.

Biotic and abiotic variable measurements

The water temperature and dissolved oxygen were determined with a multiparameter probe (YSI GRANT 3800). Chemical analyses, namely ammonium (NH_4-N), nitrates (NO_3-N) and orthophosphate (PO_4-P) were performed using standard methods (American Public Health Association, 1992). Chlorophyll *a* concentrations were obtained by spectrophotometry (Strickland & Parson, 1972). Outflows from Sep Reservoir were measured by pressure captors situated on the drain pipe, whereas inflows were obtained by measuring both the variations in the water volume of the lake and the influence of evapo-perspiration and outflows.

Counts of prokaryotes and protists

For determining total prokaryotic abundance, 1–6 mL samples were filtered on 0.2 μm black polycarbonate filters (25 mm, Millipore), stained with 1 $\mu g\,L^{-1}$ (final concentration) of 4,6-diamidino-2-phenylindole (DAPI). Up to

400–800 bacterial cells were counted under an epifluorescence microscope (Porter & Feig, 1980). After being stained with primulin (final concentration of 200 μg mL^{-1}) (Caron, 1983), flagellates were filtered (5–10 mL samples) through a black polycarbonate membrane with a 0.8 μm pore size (Nuclepore, Whatman, Brentford, UK) and counted by means of epifluorescence microscopy. A total of 200–300 flagellates were counted per filter. The metazoan zooplankton was counted under a binocular microscope (Wild M3 Z, Heerbrugg, Switzerland) 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 (Thouvenot *et al.*, 2000).

DNA extraction

Genomic DNA extraction was conducted according to Ausubel *et al.* (1987). The filters were covered with TE buffer (1 × Tris and EDTA) and a lysozyme solution (final concentration, 250 μg mL^{-1}) and incubated at 37 °C for 30 min. Sodium dodecyl sulfate (10%) and proteinase K (final concentration, 100 μg mL^{-1}) were then added and the filters were incubated at 37 °C for at least 60 min. A CTAB solution (final concentration, 1% in a 0.7 M NaCl solution) was added and samples were incubated at 65 °C for 10 min. Nucleic acids were extracted with chloroform-isoamyl alcohol (24 : 1); the aqueous phase containing nucleic acids was kept and purified by adding phenol-chloroform-isoamyl alcohol (25 : 24 : 1). After adding isopropanol (0.6 vol.), the nucleic acids were precipitated at −20 °C for 12 h. After centrifugation, the DNA pellet was ethanol rinsed and resuspended in 50 μL of TE buffer. DNA yield was quantified by Fluorescence Assay (DNA quantitation Kit; Sigma, St Louis, MO) and nucleic acid extracts were stored at −20 °C until analysis.

Clone library construction

Environmental DNA extracted from the 21 August 2002 sampling from Sep Reservoir and from the 28 August 2002 sampling from Pavin Lake was used to construct the 16S rRNA gene clone libraries. The general bacterial primers 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′; mostly *Eubacteria*: Lane, 1991) and 1492r (5′-GGT TAC CTT GTT ACG ACT T-3′ mostly *Eubacteria* and *Archaea*: Lane, 1991) were used in PCR amplifications. PCR reactions were performed with an MJ Research PTC 200 thermal cycler (MJ Research, Waltham, MA) using the following program: initial denaturation at 95 °C for 5 min; 30 standard cycles of denaturation (at 95 °C for 1 min), annealing (at 55 °C for 1 min) and extension (at 72 °C for 1 min) and a final extension at 72 °C for 5 min. A clone library was constructed for each lake using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA) with the pCR vector 2.1 according to manufacturer's instructions. Around 60 clones from each library were picked. The presence of the 16S rDNA insert in positive colonies was checked by PCR amplification using flanking vector primers (M13f and M13r). Expected-size amplicons were subsequently digested with restriction enzyme *Hae*III and the resulting restriction fragment length polymorphism (RFLP) products were separated by electrophoresis in a 2.5% low-melting-point agarose gel (NuSieve®, Cambrex Corp., East Rutherford, NJ) at 60 mV for about 3 h. Clones that produced the same RFLP pattern were grouped together and considered members of the same operational taxonomic unit (OTU). At least one clone of each OTU was selected for sequencing using primers 27f and 517f (5′-GCC AGC CGC GGT AA-3′). Sequencing reactions were performed by MWG (http://www.mwg-biotech.com).

Phylogenetic affiliations were designated based on the closest sequences retrieved from GenBank using the BLAST search tool (Altschul *et al.*, 1997) and from the Ribosomal Database Project (Maidak *et al.*, 1994). The sequences were aligned with complete sequences of an ARB database using the latter's automatic alignment tool (http://www.arb-home.de) (Ludwig *et al.*, 2004). The resulting alignment was checked and corrected manually, taking the secondary structure of the rRNA molecule into consideration. Sequences were inserted into an optimized tree according to the maximum parsimony criteria without allowing changes of the existing tree topology. This was done by using a special tool in the ARB software. The resulting tree was pruned to retain the closest relatives, sequences representative of prokaryotic evolutionary and our clones. Six chimeras were detected by constructing alternative phylogenetic trees using 300 bp pieces from 5′ and 3′ ends, and by the CHECK_CHIMERA program of RDP. Chimeras were removed from the data set. Twenty-two sequences for Sep Reservoir and 28 for Pavin Lake were deposited in GenBank with accession numbers AY750283 to AY752132.

T-RFLP analysis

16S rRNA genes from environmental samples and clones were amplified as above, except that fluorescently labeled forward primer 27f-FAM (6-carboxylfluorescein) (labeled at the 5′-end with fluorescent sequencing dye; MWG Biotech, Ebersberg, Germany) was used. PCR products were purified using the Qiaquick PCR purification Kit (Qiagen, Courtaboeuf, France), visualized on 1% agarose gels and quantified (DNA quantitation Kit; Sigma). Enzymatic digestions were performed separately for each restriction enzyme used by incubating 100 ng of PCR products with 20 U of *Msp*I, *Rsa*I (Sigma) or *Hha*I (Qbiogene, Montreal, Canada) at 37 °C overnight. The samples were desalted with Microcon

columns (Amicon, Millipore). The Terminal Restriction Fragments (T-RFs) were separated on an automated sequencer (ABI 3700). Terminal restriction fragment sizes between 50 and 850 bp with peak area of >50 fluorescence units were determined using Genescan analytical software (Freiberg, Germany). Samples were analyzed in triplicate and a peak was kept if it was occurred in at least two profiles. To account for small differences in the running time among samples, we considered fragments from different profiles with less than one base difference to be the same length. A total of 92 environmental samples were obtained for two sites over 2 years. These T-RFLP profiles were compared and T-RFs that differed by less than 1 bp in different samples were considered identical. The resulting values were rounded to the nearest integer. A program in VISUAL BASIC for EXCEL was developed to automate these procedures. The results were then expressed either in terms of presence or absence or as a relative percentage area compared to the total area. The relative abundance of T-RFs was determined by calculating the ratio between the peak area of each peak and the total peak area of all peaks within one sample. Ratios were converted to percentages.

*Msp*I generated the largest average number of T-RFs irrespective of the depth or the ecosystem considered (average number of T-RFs per sample: 92 for Pavin Lake and 91 for Sep Reservoir). The restriction enzymes *Rsa*I and *Hha*I then followed in decreasing order (average number of T-RFs per sample: 66 and 48, respectively). Hereafter we present only the data obtained using *Msp*I, which was the most discriminating restriction enzyme.

T-RF identification

To determine the spatio-temporal changes in the sequences, we have compared the T-RFs obtained from the clones and the T-RFs obtained from the environmental DNA. A clone was present at a given date only if the three T-RFs generated by the three restriction enzymes *Msp*I, *Rsa*I and *Hha*I were present in the three T-RFLP profiles generated from the environmental DNA.

Statistical analysis

All the calculations were done using only the results obtained from the most discriminating restriction enzyme, i.e. *Msp*I.

The dissimilarity (D) index was calculated to estimate temporal changes of BCC. This is given by $D(t1,t2) = 1/2\Sigma|x_{t1} - x_{t2}|$, where $\Sigma x_{t1} = \Sigma x_{t2} = 100$, and x_{t1} and x_{t2} indicate the percentages of the peak area at two consecutives dates. The D value ranges from 0 to 100. Then we considered that x could be, at a given date, a relative area of either: (1) a T-RF, (2) a bacterial division (*Actinobacteria*, *Proteobacteria*, *Bacteroidetes*, *Verrucomicrobia* and *Cyanobacteria*), or (3) an identified T-RF. This enabled us to calculate the different dissimilarity indices of T-RFs, bacterial division and identified T-RF from one given date to another.

A *t*-test was performed to compare the number of T-RFs from 2001 to 2002 on both sites, and the dissimilarity indices from the two ecosystems.

To explain the variation of BCC measured by T-RFLP and expressed by presence–absence or percentage of area (>2%), Canonical Correspondence analysis (CCA) was used (Ter Braak, 1986). Forward selection was used to select the environmental variables that explained a significant part of changes in BCC ($P < 0.05$). We have tested the following variables: NH_4-N, NO_3-N, PO_4-P, temperature, dissolved oxygen, chlorophyll *a*, pigmented flagellates (PF), heterotrophic nanoflagellates (HNF), and zooplankton. For Sep Reservoir, inflow and outflow were also tested in this analysis. However, with the exception of temperature in the hypolimnion, no variables explained significantly the variations of BCC in Pavin Lake. In this case, the percentages explained by the three strongest variables are presented. We do not present the biplots (dates and variables) from CCA but only the temporal evolution of variables which explain an important part of changes of BCC. These statistics were computed with R software using the Vegan package for the CCA and related methods (http://cran.r-project.org/).

Results

Main physical, chemical and biological characteristics of the study sites

For Pavin Lake the water stratification period extended from mid-June to late October in 2001 and from early May to late September in 2002 (Fig. 1A). For Sep Reservoir, this period extended from early May to late August in 2001 and from mid-April to mid-August in 2002 (Fig. 1B). Sep Reservoir was created to meet agricultural needs, and so is partially or totally drained every year, for example at the end of 2001 (Fig. 1B). The main physical, chemical and biological characteristics of the lakes are listed in Table 1.

Sep Reservoir

Phylogenetic composition of bacterioplankton

The phylogenetic analysis of the sequences obtained from the clone library showed that most of the OTUs belonged to *Actinobacteria* (40.9%) (Fig. 2; Table 2). *Bacteroidetes* was the most abundant bacterial division after *Actinobacteria* (22.7% of the OTUs), followed in decreasing order by *Betaproteobacteria* (18.2%), *Verrucomicrobia* (13.6%) and *Alphaproteobacteria* (4.6%). Only 9.1% of the OTUs of the library had as closest relatives, species already characterized

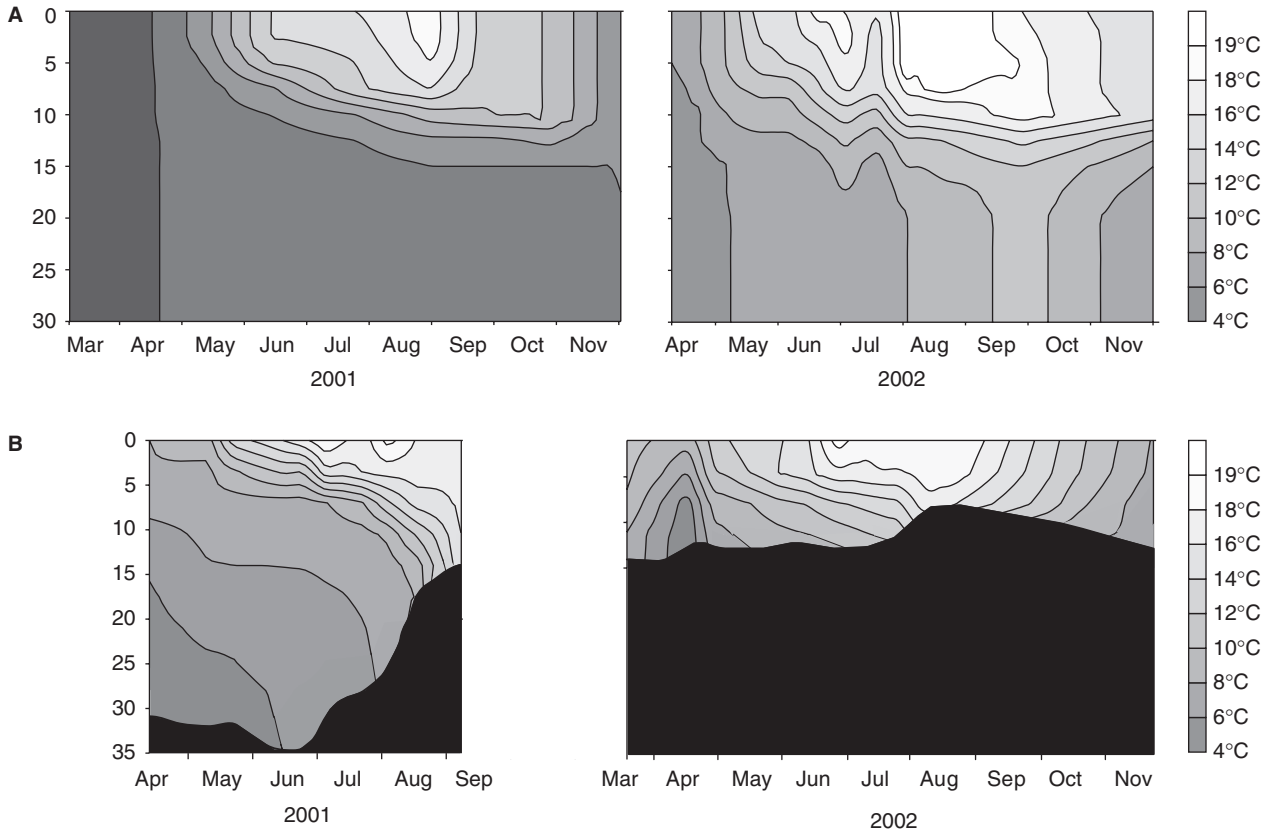

Fig. 1. Isobathymetric maps of Pavin Lake (A) and Sep Reservoir (B) in 2001 and 2002.

by techniques of culture, and these showed only a low similarity (85.6% and 89.2%). The obtained phylogenetic tree also showed that clones S4.22, S4.27, S10.6, S10.15 and S10.18 were distinctly grouped within *Actinobacteria*, clustered along with lake clones FukuN30 and Sta2-30 (Fig. 2; Table 2). The analysis of sequences from the library showed that 63.6% of sequences (66.7% of clones) could be associated with OTUs of aquatic origin.

Dynamics of the bacterial community composition

The analysis of the T-RFLP profiles for Sep Reservoir gave a total of 484 T-RFs detected by *Msp*I. The number of T-RFs per sample for *Msp*I ranged between 30 and 140 irrespective of depth (Fig. 3). The largest number of T-RFs was detected in the euphotic zone in 2001 (average 111 vs. 100 for the hypolimnion), while in 2002, the hypolimnion presented the largest number of T-RFs

Table 1. Environmental parameters measured in the Sep lake-reservoir and in Pavin Lake in 2001 and 2002

	Sep Reservoir		Pavin Lake	
	Epilimnion	Hypolimnion	Epilimnion	Hypolimnion
NH_4-N (mg N L^{-1})	0.08 (0.00–0.27)	0.14 (0.00–0.41)	0.05 (0.00–0.06)	0.26 (0.00–3.97)
NO_3-N (mg N L^{-1})	1.09 (0.08–1.68)	1.16 (0.10–2.96)	0.03 (0.00–0.17)	0.04 (0.00–0.18)
PO_4-P (mg P L^{-1})	0.02 (0.00–0.11)	0.02 (0.00–0.10)	0.02 (0.00–0.12)	0.02 (0.00–0.12)
Chl *a* (µg L^{-1})	8.20 (0.87–25.10)	3.77 (0.78–19.49)	1.27 (0.00–2.46)	1.77 (0.00–3.79)
Bacteria (10^6 cells mL^{-1})	8.62 (4.42–17.46)	7.45 (1.68–20.22)	3.82 (2.35–5.94)	1.15 (0.53–2.95)
Pigmented flagellates	24.59 (0.17–141.78)	8.80 (0.45–71.85)	0.34 (0.01–1.21)	0.07 (0.00–0.29)
Heterotrophic nanoflagellates (10^6 cells mL^{-1})	45.83 (5.32–210.49)	62.03 (3.71–180.60)	0.85 (0.14–5.62)	0.22 (0.05–1.30)
Zooplankton (ind L^{-1})	121.18 (10.27–730.13)*		30.06 (2.39–106.42)*	
Inflow ($L s^{-1}$)	304.0 (27.0–1137.1)		ND	
Outflow ($L s^{-1}$)	164.8 (8.1–1137.1)		ND	

*Water column. ND, not determined.

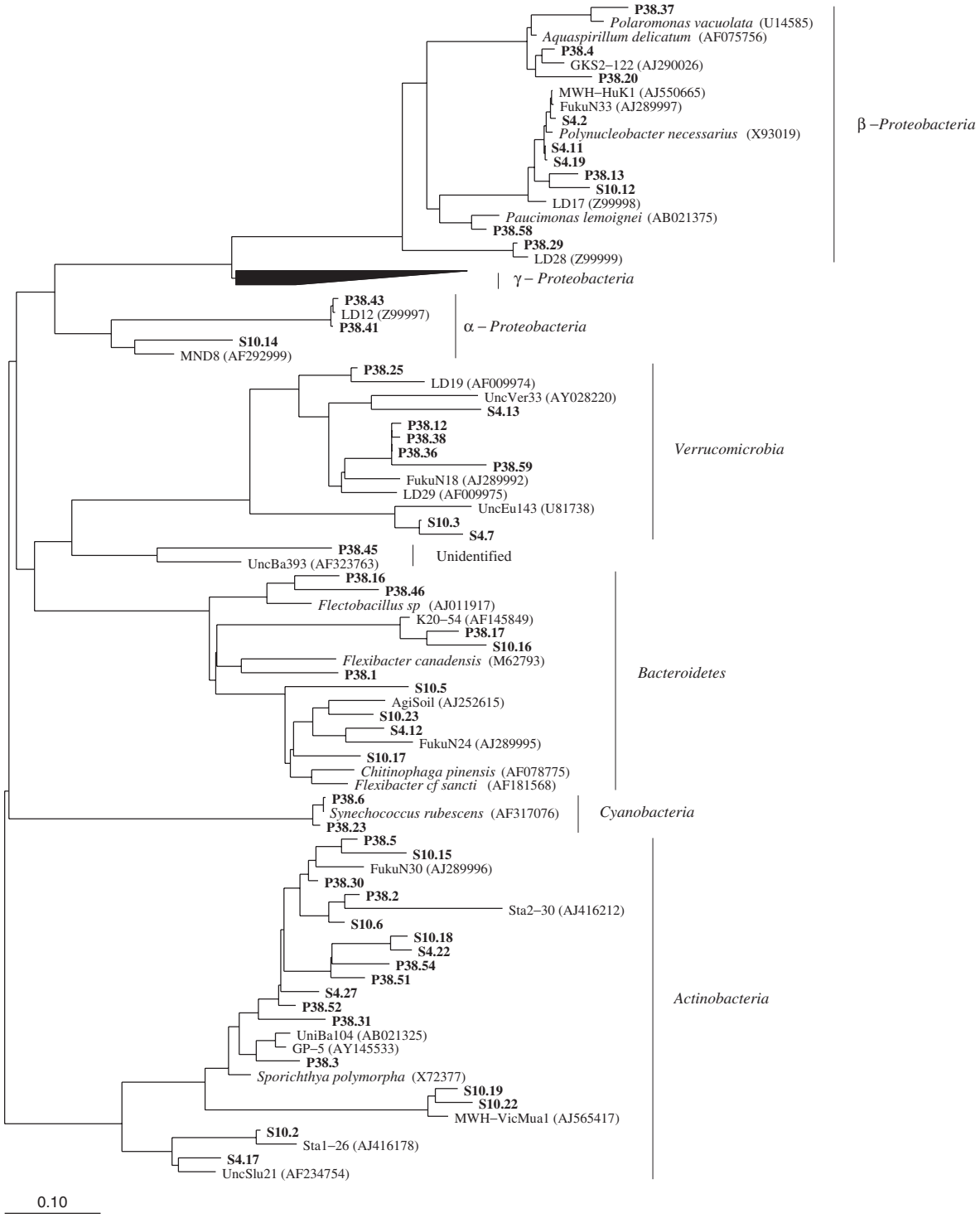

Fig. 2. Phylogenetic tree of the bacterial 16S rRNA gene sequences showing the placement of environmental clones of Sep Reservoir (S) and Pavin Lake (P). Sequences were inserted into an optimized tree according to the maximum parsimony criteria using ARB software.

(average 90 vs. 72 for the epilimnion). Overall, we observed a significant decrease in the number of T-RFs from 2001 (average 105) to 2002 (average 81) (*t*-test, $P < 0.05$). In all, 63.8% of the T-RFs were seen in both years, and 20.3% and 15.9% of the T-RFs were restricted, respectively, to 2001 and 2002.

Table 2. Clones of the Pavin Lake (P) and Sep Reservoir (S) libraries, their accession numbers, T-RF, percent similarity, closest relatives, phylogenetic affiliations and sources of the closest relatives

OTU	Accession no.	No. of clones	T-RF *Msp* I (bp)	T-RF *Rsa* I (bp)	T-RF *Hha* I (bp)	Similarity (%)	Closest relative (accession no.)	Phylogenetic affiliation	Source
P38.2	AY752084	2	164	473	686	98.9	Sta2-30 (AJ416212)	*Actinobacteria*	FW
P38.3	AY752085	1	180	472	378	92.1	UniBa104 (AB021325)	*Actinobacteria*	Environmental samples
P38.5	AY752087	4	148	201	693	93.5	FukuN30 (AJ289996)	*Actinobacteria*	FW
P38.30	AY752097	1	158	472	685	95.3	FukuN30 (AJ289996)	*Actinobacteria*	FW
P38.31	AY752098	3	180	472	379	90.4	GP-5 (AY145533)	*Actinobacteria*	FW
P38.51	AY752106	1	156	472	685	93.4	FukuN30 (AJ289996)	*Actinobacteria*	FW
P38.52	AY752107	1	158	469	685	95.0	FukuN30 (AJ289996)	*Actinobacteria*	FW
P38.54	AY752108	1	158	472	685	90.3	Sta2-30 (AJ416212)	*Actinobacteria*	FW
P38.41	AY752102	1	461	443	530	99.9	LD12 (Z99997)	*α-proteobacteria*	FW
P38.43	AY752103	2	461	442	530	99.6	LD12 (Z99997)	*α-proteobacteria*	FW
P38.1	AY752083	1	558	330	111	84.3	*Flexibacter canadensis* (M62793)	*Bacteroidetes*	Soil
P38.16	AY752091	4	492	476	97	88.8	*Flectobacillus sp* (AJ011917)	*Bacteroidetes*	FW
P38.17	AY752092	1	97	322	102	96.1	K20-54 (AF145849)	*Bacteroidetes*	Soil
P38.46	AY752105	1	106	490	111	84.6	*Flectobacillus sp* (AJ011917)	*Bacteroidetes*	FW
P38.4	AY752086	1	503	442	220	97.4	GKS2-122 (AJ290026)	*β-proteobacteria*	FW
P38.13	AY752090	2	503	ND	220	94.2	LD17 (Z99998)	*β-proteobacteria*	FW
P38.20	AY752093	2	506	138	173	91.8	*Aquaspirillum delicatum* (AF078756)	*β-proteobacteria*	FW
P38.29	AY752096	1	504	487	587	99.9	LD28 (Z99999)	*β-proteobacteria*	FW
P38.37	AY752100	1	503	444	221	96.2	*Polaromonas vacuolata* (U14585)	*β-proteobacteria*	Antarctic sea water
P38.58	AY752109	1	509	494	595	97.1	*Paucimonas lemoignei* (AB021375)	*β-proteobacteria*	Soil
P38.6	AY752088	2	504	441	357	99.8	*Synechococcus rubescens* (AF317076)	*Cyanobacteria*	FW
P38.23	AY752094	1	504	441	358	98.9	*Synechococcus rubescens* (AF317076)	*Cyanobacteria*	FW
P38.12	AY752089	3	145	852	> 900	91.7	FukuN18 (AJ289992)	*Verrucomicrobia*	FW
P38.25	AY752095	1	84	873	247	99.6	LD19 (AF009974)	*Verrucomicrobia*	FW
P38.36	AY752099	1	143	848	> 900	92.4	FukuN18 (AJ289992)	*Verrucomicrobia*	FW
P38.38	AY752101	2	146	846	> 900	91.5	LD29 (AF009975)	*Verrucomicrobia*	FW
P38.59	AY752110	2	146	70	> 900	85.7	FukuN18 (AJ289992)	*Verrucomicrobia*	FW
P38.45	AY752104	1	162	496	229	75.5	UncBa393 (AF323763)	Unknown	Environmental samples
S4.17	AY752116	1	83	474	388	92.2	UncSlu21 (AF234754)	*Actinobacteria*	Environmental samples
S4.22	AY752118	1	297	472	687	95.7	Sta2-30 (AJ416212)	*Actinobacteria*	FW
S4.27	AY752119	1	158	470	405	92.9	FukuN30 (AJ289996)	*Actinobacteria*	FW
S10.2	AY752120	2	97	475	388	99.1	Sta1-26 (AJ416178)	*Actinobacteria*	FW
S10.6	AY752123	1	158	472	686	94.8	Sta2-30 (AJ416212)	*Actinobacteria*	FW
S10.15	AY752126	1	97	502	705	89.1	FukuN30 (AJ289996)	*Actinobacteria*	FW
S10.18	AY752129	1	296	472	685	94.6	Sta2-30 (AJ416212)	*Actinobacteria*	FW
S10.19	AY752130	1	182	474	388	95.4	MWH-VicMua1 (AJ565417)	*Actinobacteria*	FW
S10.22	AY752131	2	158	471	388	94.4	MWH-VicMua1 (AJ565417)	*Actinobacteria*	FW
S10.14	AY752125	1	131	450	537	88.7	MND8 (AF292999)	*α-proteobacteria*	FW sediments
S4.12	AY752114	2	107	131	> 900	90.8	FukuN24 (AJ289995)	*Bacteroidetes*	FW
S10.5	AY752122	2	107	132	82	85.6	*Chitinophaga pinensis* (AF078775)	*Bacteroidetes*	Environmental samples
S10.16	AY752127	1	99	325	105	91.8	K20-54 (AF145849)	*Bacteroidetes*	Soil

Table 2. Continued.

OTU	Accession no.	No. of clones	T-RF *Msp* I (bp)	T-RF *Rsa* I (bp)	T-RF *Hha* I (bp)	Similarity (%)	Closest relative (accession no.)	Phylogenetic affiliation	Source
S10.17	AY752128	1	107	132	82	89.2	*Flexibacter cf. sancti* (AF181568)	*Bacteroidetes*	Environmental samples
S10.23	AY752132	1	109	134	234	92.4	AgiSoil (AJ252615)	*Bacteroidetes*	Soil
S4.2	AY752111	4	461	138	225	99.6	MWH-HuK1 (AJ550665)	β-*proteobacteria*	FW
S4.11	AY752113	2	462	137	225	99.5	MWH-HuK1 (AJ550665)	β-*proteobacteria*	FW
S4.19	AY752117	1	463	86	225	99.3	FukuN33 (AJ289997)	β-*proteobacteria*	FW
S10.12	AY752124	1	503	445	221	94.6	MWH-HuK1 (AJ550665)	β-*proteobacteria*	FW
S4.7	AY752112	1	136	> 900	221	88.5	UncEu143 (U81738)	*Verrucomicrobia*	Environmental samples
S4.13	AY752115	4	294	491	113	85.8	UncVer33 (AY028220)	*Verrucomicrobia*	Arctic Ocean
S10.3	AY752121	4	138	> 900	222	86.9	UncEu143 (U81738)	*Verrucomicrobia*	Environmental samples

T-RF, terminal restriction fragment in bp, obtained using fluorescent forward primer (27F-FAM) and *Msp*I, *Rsa*I and *Hha*I restriction enzymes.

Of 484 detectable T-RFs, we present only the T-RFs that had a percentage area greater than 2% (102 T-RFs over the entire study period), and which we consider to be dominant (Fig. 3). For each sampling date, few T-RFs represented a high percentage of the total area at either depth. For the 2 years of study, the percentage of area of dominant T-RFs appeared to be stable over time, averaging 69.3% in 2001 and 72.8% in 2002 for the epilimnion (Fig. 3A), and 63.8% in 2001 and 69.4% in 2002 for the hypolimnion (Fig. 3B).

Marked variations in the dominant T-RFs were observed according to the environmental T-RFLP profiles. Some dominant T-RFs occurred on fewer than three dates for each depth (T-RFs 84, 99, 136, 171, 541). Others were present on one or more successive dates several times during the study period (T-RFs 57, 97, 138, 492), and some T-RFs could be considered to be recurrent (T-RFs 167, 503). However, no T-RF was present in all samples. The percentage areas of the dominant T-RFs fluctuated markedly over time and could reach high values (T-RF 457 was 34.5% in 2001 and 30.8% in 2002 for the epilimnion; T-RF 51 was 18.0% in 2001 and T-RF 53 was 18.3% in 2002 for the hypolimnion).

The data obtained with the three restriction enzymes showed T-RFs to be associated with all OTUs determined by cloning-sequencing except for one, the percentage area of which was below 2%. Among the dominant T-RFs, the identified T-RFs represented on average 34.3% and 28.1% of the total area in 2002, respectively, in the epilimnion and the hypolimnion. These percentages were lower (24.1% for the epilimnion and 7.2% for the hypolimnion) in 2001 than in 2002, the year the sample for the clone library was collected (Fig. 3). Thus the T-RFs 84, 97, 99, 136, 138 and 503 were, respectively, associated ($\pm$ 1 bp) with clones S4.17 (*Actinobacteria*), S10.2 and S10.15 (*Actinobacteria*), S10.16 (*Bacteroidetes*), S4.7 (*Verrucomicrobia*), S10.3 (*Verrucomicrobia*) and S10.12 (*Betaproteobacteria*). From these identifications, we could see that among the identified populations, *Betaproteobacteria* represented the main bacterial division of the euphotic zone in both years, followed by the *Actinobacteria* (Figs 3A and 4A). OTUs associated with these divisions were present for the euphotic zone throughout the study period with the exception of one single date (17 April 2002), when only four dominant T-RFs were detected. In contrast, the ratio of these two divisions was reversed in the hypolimnion in 2002 such that *Actinobacteria* were more plentiful (Figs 3B and 4B). A similar analysis could not be accomplished for 2001 because too few T-RFs were identified. In both years, the *Bacteroidetes* and the *Verrucomicrobia* were detected in smaller amounts than the above two divisions. The relative sizes of the divisions determined by the area of the T-RFs (Fig. 3) or by the abundance of clones were similar at the date of cloning. *Actinobacteria* represented the main bacterial division in Sep Reservoir (30.6%), followed by *Verrucomicrobia*

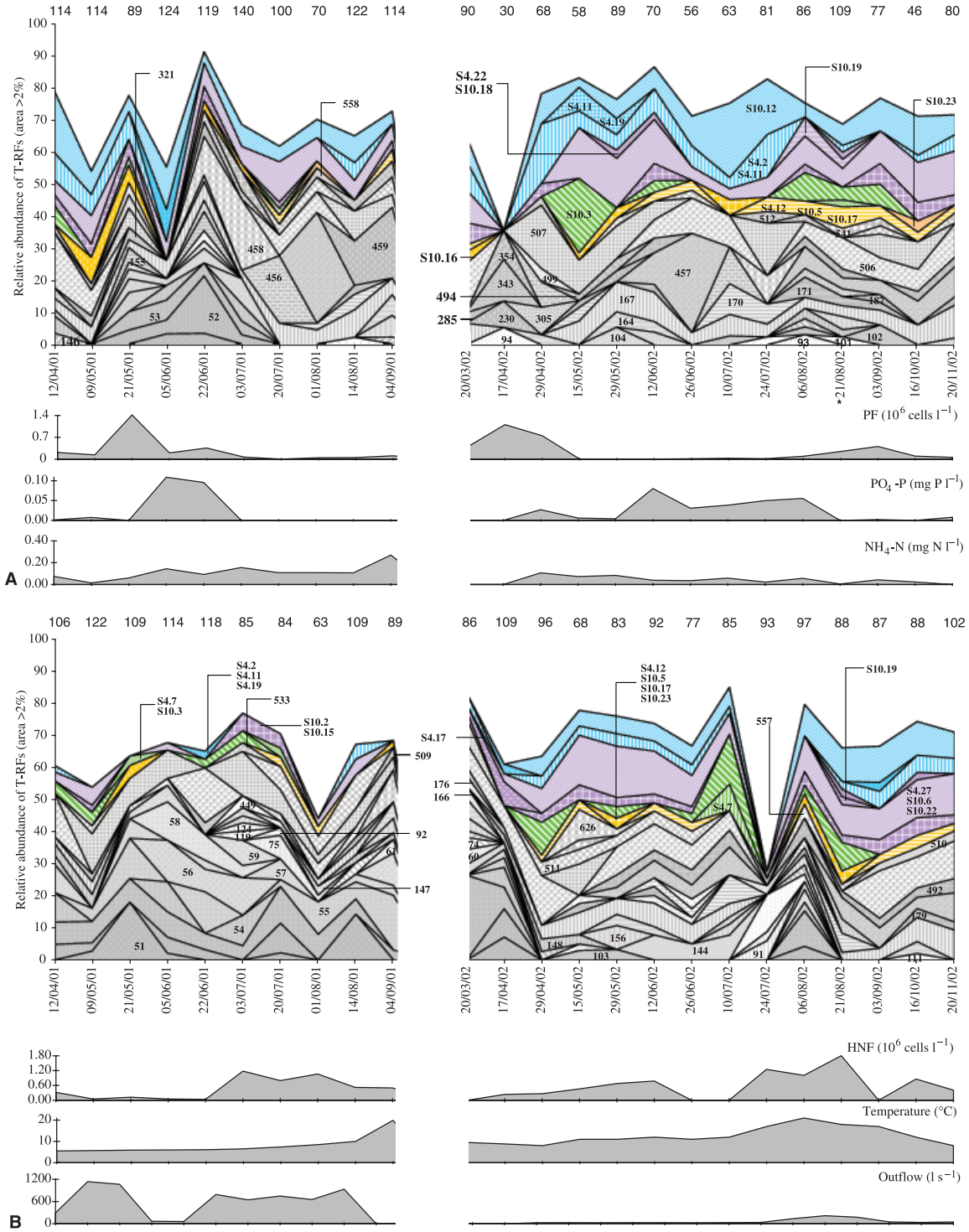

Fig. 3. Seasonal variation, for the Sep Reservoir epilimion (A) and hypolimnion (B), in number and relative abundance of the various operational taxonomic units (OTUs) detected by T-RFLP analysis of 16S rDNA digestion by *Msp*I, representing more than 2% of the total area (format date: day/month/year). Blue, green, purple and yellow areas represent, respectively, *Betaproteobacteria*, *Verrucomicrobia*, *Actinobacteria* and *Bacteroidetes*. The numbers above the profiles correspond to the total numbers of OTUs detected by *Msp*I in individual samples. Numbers on the T-RFLP profiles correspond to the length of T-RFs in bp (the lengths of the T-RFs corresponding to the clones are presented in Table 2). An asterisk indicates the date of cloning. The environmental variables presented in this figure are those which statistically best explain the variations in the dominant T-RFs, according to the CCA (Table 4).

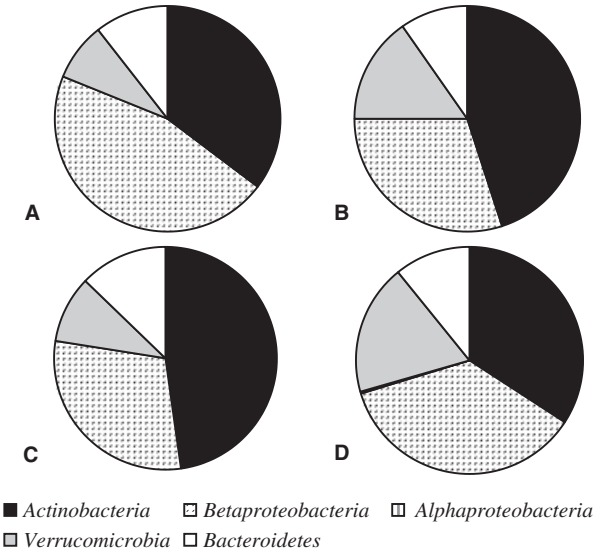

Fig. 4. Percentage of T-RFs associated with the different bacterial divisions in Sep Reservoir epilimnion (A) and hypolimnion (B) and in Pavin Lake epilimnion (C) and hypolimnion (D).

(25%), *Betaproteobacteria* (22.2%), *Bacteroidetes* (16.3%) and *Alphaproteobacteria* (2.8%) (Table 2). However, when considered at a finer phylogenetic scale, our results demonstrate strong temporal variations in bacterial communities (from 51.1 to 65.9) that were far greater than when T-RFs and identified T-RFs were grouped into these major phylogenetic divisions (31.0 ± 17.8 for the epilimnion and 45.5 ± 32.5 for the hypolimnion) (Table 3).

Analysis of the CCA showed that the PF, followed by the zooplankton and nutrients (PO_4-P, NH_4-N), were related to the presence or absence of the dominant populations in the epilimnion (Table 4). In the hypolimnion, outflow seemed to be the main factor associated with variations in BCC, followed by HNF, temperature and NH_4-N. The CCA performed on the areas of the dominant T-RFs gave similar results; PF, NH_4-N, PO_4-P, HNF for the epilimnion and outflow, temperature, HNF, NO_3-N for the hypolimnion were related to the temporal evolution of the BCC in Sep Reservoir. More particularly, we note that certain dates or periods were associated with a particular parameter. So, for the epilimnion, the elevated abundances in the PF corresponded to important changes in the structure of the dominant T-RFs (Fig. 3A). Results of CCA (graphs not given) showed that outflow and temperature explained the strong interannual variation of the dominant populations of the hypolimnion. Indeed, outflow was more important in 2001, and temperature was more important in 2002 (Fig. 3B). The elevated abundance of HNF corresponded to dates where changes in the area and in the presence/absence of the dominant T-RFs were observed.

Pavin Lake

Phylogenetic composition of bacterioplankton

Most of the OTUs and clones belonged to *Actinobacteria* (28.5% of the OTUs and 31.1% of clones) (Fig. 2; Table 2). However, unlike in Sep Reservoir, for this site *Betaproteobacteria* represented the second most abundant division in terms of OTUs (21.4%), followed by *Verrucomicrobia* (17.9%), *Bacteroidetes* (14.3%), *Alphaproteobacteria* (7.1%) and *Cyanobacteria* (7.1%). Using the percentage of the clone, *Betaproteobacteria* and *Verrucomicrobia* were the most abundant divisions (18.6% each) after *Actinobacteria*. *Bacteroidetes* (16.3% of clones), *Cyanobacteria* (9.3% of clones) and *Alphaproteobacteria* (7% of clones) then followed in decreasing order. In the Pavin Lake library, 28.6% of the OTUs had a 84.3%–99.8% similarity with species already characterized by culture techniques. The closest similarity was recorded between P38.6 and *Synechococcus rubescens*. Phylogenetic analysis showed that four clones from the library (P38.12, P38.36, P38.38 and P38.59) formed an isolated clade within *Verrucomicrobia* (Fig. 2). It also appears that clones P38.2, P38.5, P38.30, P38.51, P38.52 and P38.54 affiliated to *Actinobacteria* were a part of the same cluster that included clones S4.22, S4.27, S10.6, S10.15 and S10.18 of Sep Reservoir. Similarity values among clone library sequences belonging to this cluster ranged from 82.3% (S4.22 and S10.15) to 98.3% (S4.22 and S10.18), but were only 90.7% similar to the closest identified relative, *Sporichthya polymorpha*. Although some similarities were observed between Pavin Lake and Sep Reservoir because the same bacterial divisions occurred in both sites, at a finer phylogenetic scale the clone libraries appeared to differ. Indeed, the Pavin Lake library had, for example, no Sta1-26 (*Actinobacteria*), MWH-VicMua1 (*Actinobacteria*), FukuN24 (*Bacteroidetes*), FukuN33 (*Betaproteobacteria*) or MWH-Huk1 (*Betaproteobacteria*). Sep Reservoir library, in turn, had no LD12 (*Alphaproteobacteria*), Flectobacillus (*Bacteroidetes*), LD28 (*Betaproteobacteria*), LD19 (*Verrucomicrobia*) or *Cyanobacteria*. Moreover, the sequence analysis from the library of Pavin Lake showed a clear difference between the two ecosystems, 82.1% of the OTUs (88.9% of clones) being associated with OTUs of aquatic origin. More precisely, 50% of *Bacteroidetes* OTUs (71.4% of the clones) and 100% of *Verrucomicrobia* OTUs and clones of Pavin Lake could be associated with species of aquatic origin, vs. 20% of *Bacteroidetes* OTUs (28.6% of the clones) and 33% of *Verrucomicrobia* OTUs (44% of the clones) from Sep Reservoir.

Dynamics of the bacterial community composition

In the 2 years of the study, 436 T-RFs were detected for Pavin Lake. The number of T-RFs per sample ranged between 69

and 133. Both years, the hypolimnion presented the maximum T-RFs (2001 average was 89 compared to 81 for the epilimnion; 2002 average was 107 compared to 87 for the epilimnion) (Fig. 5). Interannual variations in the richness were observed but, unlike in Sep Reservoir, year 2002 showed an increase in the number of T-RFs (average 97) over the year 2001 (average 85). The percentage of common T-RFs in the 2 years was similar to that of Sep Reservoir (62.8%); about 5.5% of the T-RFs were restricted in 2001 compared to 31.7% in 2002. Only one T-RF (T-RF 97) seemed to be present all the time at both depths.

Of 436 T-RFs detected, 90 were considered dominant. As in Sep Reservoir, the percentage area of dominant T-RFs in Pavin Lake showed some stability in time: on average, 76.8% and 71.1% in in 2001 and 2002, respectively, for the epilimnion (Fig. 5A), and 74.5% and 70.7% in 2001 and 2002, respectively, for the hypolimnion (Fig. 5B). Marked variations in the dominant T-RFs were also observed according to the environmental T-RFLP profiles. As in Sep Reservoir, some T-RFs in Pavin Lake showed an area greater than 2% on only a few dates (T-RFs 84, 142, 173). Others were observed over short periods (T-RFs 106, 148, 176) or were present regularly (T-RFs 156, 164) in both years and at both depths. Of the 24 samplings from Pavin Lake, only two T-RFs (T-RFs 97, 506) were present more than 21 times among the dominant T-RFs. Some dominant T-RFs were also restricted to one depth (T-RF 110, 143, 179 for the epilimnion and T-RF 150 for the hypolimnion). The fluctuations in the percentages of the T-RFs areas were moderately large, although the environmental profiles seemed more stable than those of Sep Reservoir. The areas of the dominant T-RFs could be large. Indeed, T-RF 156 was 23.3% in 2001 and T-RF 158 was 22.6% in 2002 for the epilimnion; T-RF 158 was 15.4% in 2001 and T-RF 152 was 16.9% in 2002 for the hypolimnion.

The data obtained with the three restriction enzymes showed that of 28 OTUs, 23 were associated with dominant T-RFs (Fig. 5), and 26 with total T-RFs present at the date of the cloning. Among these T-RFs, those that were identified presented an area percentage greater than that of Sep Reservoir: they represented on average 47.2% of the total area in 2001 and 48.2% in 2002 for the epilimnion and 35.8% in 2001 and 28.9% in 2002 for the hypolimnion. The T-RFs 84, 97, 106, 143, 148, 156, 164, 179 and 506 were associated (± 1 bp), respectively, with clones P38.25 (*Verrucomicrobia*), P38.17 (*Bacteroidetes*), P38.46 (*Verrucomicrobia*), P38.36 (*Verrucomicrobia*), P38.5 (*Actinobacteria*), P38.51 (*Actinobacteria*), P38.2 (*Actinobacteria*), P38.3 and P38.31 (*Actinobacteria*), P38.20 (*Betaproteobacteria*). The environmental T-RFLP profiles and the results of the cloning (Table 2) showed that *Actinobacteria* dominated the BCC at the date of the cloning, followed by *Betaproteobacteria* and *Verrucomicrobia*. Unlike Sep Reservoir, *Actinobacteria* represented the main bacterial division for the epilimnion of Pavin Lake identified during the 2 years of study, followed by *Betaproteobacteria* (Figs 5A and 4C). In the hypolimnion, *Actinobacteria* were the most abundant identified division in 2001, whereas *Betaproteobacteria* predominated in 2002 (Fig. 5B). For both depths there then followed, in smaller amounts in both years, the *Bacteroidetes* and the *Verrucomicrobia* (Fig. 4D). The analysis of the environmental T-RFLP profiles clearly revealed the successions of bacterial populations. As in Sep Reservoir, the dissimilarity values for Pavin Lake showed that changes among the T-RFs and identified T-RFs were greater than those occurring to the bacterial divisions (Table 3).

As in Sep Reservoir, the presence or the absence of the dominant populations in the epilimnion of Pavin Lake seemed to be the consequence of potential predators (HNF) and nutrients (PO_4-P), but according to the CCA analysis, these factors did not significantly account for the distribution of the T-RFs (Table 4). For the hypolimnion, the temperature, the nutrients (PO_4-P) and the zooplankton seemed to be related to the successions of the dominant bacterial populations, although only the effect of the temperature was statistically significant (Table 4). When the CCA was performed from the areas of the dominant T-RFs, similar factors seemed to be related to the distribution of the dominant populations. Predators (HNF and zooplankton) and nutrients (NH_4-N) for the epilimnion, temperature (statistically significant), zooplankton and PO_4-P for the hypolimnion, were related to the temporal evolution of the dominant T-RFs in Pavin Lake (Table 4). For the epilimnion the elevated abundance of the HNF was associated with changes of the BCC (Fig. 5A). However, although the statistical analysis was able to separate the effect of each variable, it is clear that at a given date these variables did not have an isolated effect on the regulation of the BCC. For the hypolimnion, we observed that the temperature and the zooplankton were associated with changes in the dynamics of the dominant bacterial populations (Fig. 5B).

Discussion

Methodological aspects

The techniques of molecular ecology have allowed spectacular progress to be made in the determination of bacterial community structure. In this work, we chose T-RFLP (Liu *et al.*, 1997) as a fingerprinting method. This method is considered to have a high resolution and reproducible results (Moeseneder *et al.*, 1999), and offers the advantage of being semiquantitative (Braker *et al.*, 2001). The T-RFLP method is more sensitive than the DGGE, able to detect 1 fg of double-stranded DNA compared with 20 pg for DGGE (Moeseneder *et al.*, 1999). So whatever restriction enzymes

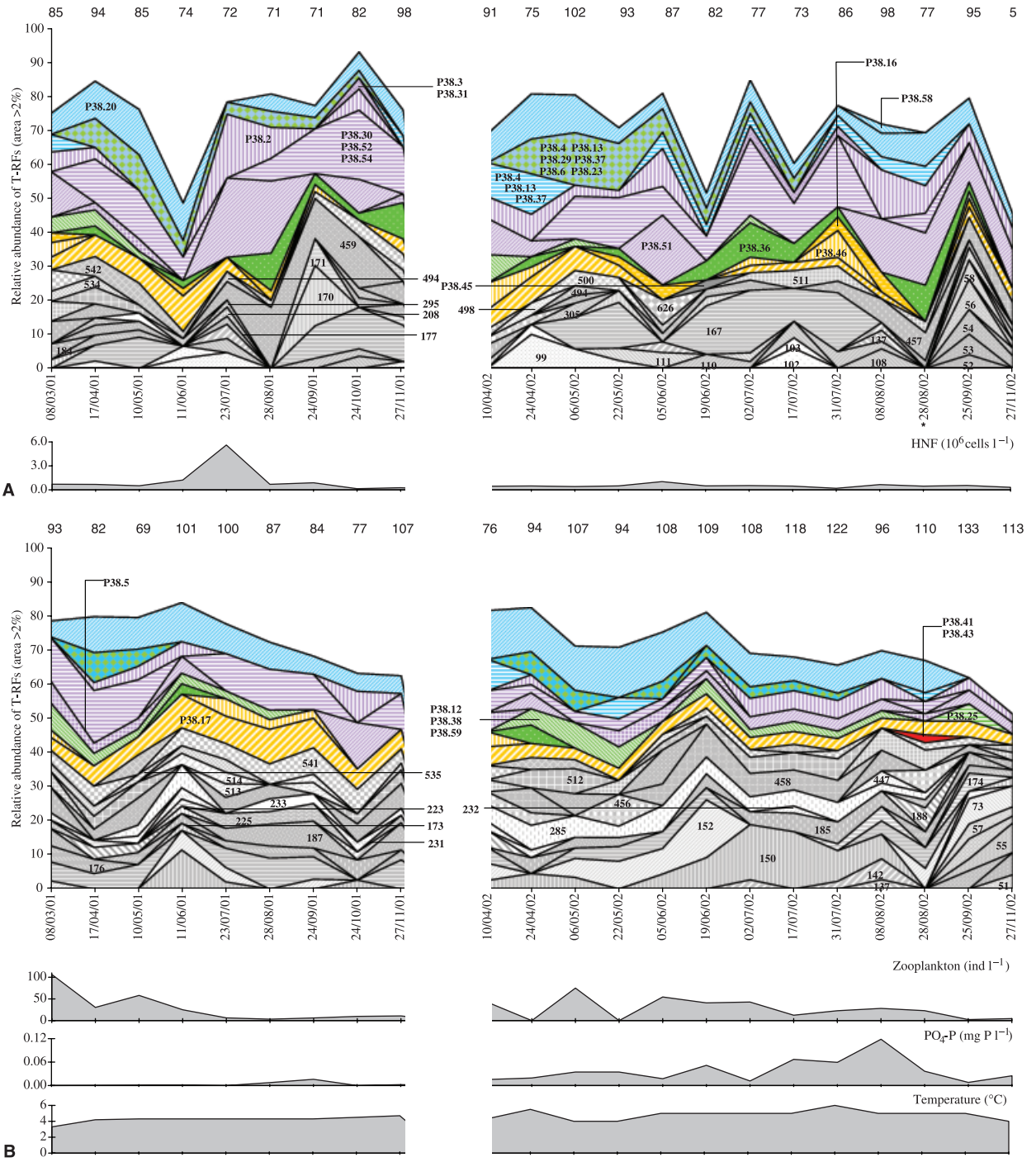

Fig. 5. Seasonal variation, for the Pavin Lake epilimion (A) and hypolimnion (B), in number and relative abundance of the various operational taxonomic units (OTUs) detected by T-RFLP analysis of 16S rDNA digestion by *Msp*I, representing more than 2% of the total area (format date: day/month/year). Blue, green, purple, red and yellow areas represent, respectively, *Betaproteobacteria*, *Verrucomicrobia*, *Actinobacteria*, *Alphaproteobacteria* and *Bacteroidetes*. The numbers above the profiles correspond to the total numbers of OTUs detected by *Msp*I in individual samples. Numbers on the T-RFLP profiles correspond to the length of T-RFs in bp (the lengths of the T-RFs corresponding to the clones are presented in Table 2). An asterisk indicates the date of cloning. The environmental variables presented in this figure are those which statistically best explain the variations in the dominant T-RFs, according to the CCA (Table 4).

Table 3. Dissimilarity indices for T-RFs, bacterial divisions and identified T-RFs over the whole period (2001–2002)

	Sep Reservoir		Pavin Lake	
	Epilimnion	Hypolimnion	Epilimnion	Hypolimnion
Dissimilarity indice of T-RFs	63.5 ± 20.7	61.3 ± 23.1	56.5 ± 17.5	52.9 ± 21.6
Dissimilarity indice of bacterial divisions	31.0 ± 17.8	45.5 ± 32.5	23.9 ± 14.9	17.5 ± 11.2
Dissimilarity indice of identified T-RFs	51.1 ± 18.0	65.9 ± 33.6	50.1 ± 20.0	46.2 ± 27.3

The values correspond to means and standard deviations.

are used, the number of T-RFs per sample determined in this work is much greater than the number of bands (between nine and 35) usually obtained by DGGE (e.g. Lindström, 2000; Schauer *et al.*, 2000; Lindström & Bergström, 2004). The number of T-RFs can nevertheless be biased by the formation of pseudo-T-RFs (Egert & Friedrich, 2003) or by slightly different amounts of DNA per sample (Dunbar *et al.*, 2001). Unlike DGGE, the identity of OTUs detected by T-RFLP cannot be confirmed through direct sequencing. T-RFLP allows tentative identification of the species present by direct comparison with a database of sequences (Marsh, 1999). However, such identification can depend on the presence of the fluorescent sequencing dyes and on the purine fragment content (Kaplan & Kitts, 2003). We therefore chose to identify the T-RFs from clones characterized in the ecosystems studied (Moeseneder *et al.*, 2001a). T-RFLP bands can include many different phylotypes, but the use of several restriction enzymes can remedy this problem. More generally, all the fingerprinting techniques are PCR-based and so may be subject to potential PCR bias such as heteroduplex or chimera formation (Kopczynski *et al.*, 1994), the choice of the annealing temperature, the DNA quantity used (Schwalbach *et al.*, 2004) or the SSU rDNA copy number (Fogel *et al.*, 1999). However, as stressed by Sekigushi *et al.* (2002), the effects of the bias can be minimized when, as here, relative changes are studied within a single environment.

Diversity and bacterial community composition

The average number of T-RFs per sample obtained for the whole period in our study was of the same order of magnitude as that found by Moeseneder *et al.* (2001b) for equivalent restriction enzymes. However, although the bacterioplankton displays a broad diversity, the bacterial community seemed to be dominated by a small number of taxa. Overall, between four and 18 T-RFs account for 27.7% and 93.2% of the total area of the T-RFs. These results are similar to those observed by other authors using T-RFLP or other techniques such as DGGE (Höfle & Brettar, 1995; Pinhassi & Hagström, 2000; Jardillier *et al.*, 2004; Schwalbach *et al.*, 2004). No matter how deep they are, 8% of T-RFs (Sep Reservoir) and 13% of T-RFs (Pavin Lake) were present only one third of the time among the T-RFs considered to be dominant. Besides, nearly 36% (Sep Reservoir) and 27% (Pavin Lake) of these T-RFs appeared dominant only once. These data show that rare members of the bacterial community can undergo rapid temporal changes (Jaspers *et al.*, 2001). Even so, their presence

Table 4. Results of canonical correspondence analysis: percentage of variation in bacterial community composition explained by the different environmental variables in the two lakes

	Sep Reservoir				Pavin Lake			
	Epilimnion		Hypolimnion		Epilimnion		Hypolimnion	
	P–A	> 2%	P–A	> 2%	P–A	> 2%	P–A	> 2%
Zooplankton	12.9					10.8	12.0	15.4
PF	18.9	16.7						
HNF		10.9	12.7	10.6	19.4	11.1		
PO_4-P	11.6	11.8			14.0		14.0	12.2
NH_4-N	9.8	13.2	9.6			12.9		
NO_3-N				9.1				
Outflow			14.2	16.7	*	*	*	*
Temperature			10.7	11.8	14.1		16.0	16.6
Total inertia	4.2	4.0	4.9	4.1	4.6	3.5	3.2	2.9
Sum of constrained eigenvalues	2.4	2.2	2.8	2.4	1.9	2.0	1.5	1.4

*The outflow of Pavin Lake was not tested.

The percentages correspond to the analysis of the matrices containing presence–absence (P–A) and the areas of dominant T-RFs. The underlined values denote variables which significantly ($P < 0.05$) accounted for the variation of the BCC.

in ecosystems can still be functionally important, because they can make non-negligible contributions to biogeochemical processes (Ward, 1982).

Comparison between the T-RFs obtained from clones and the T-RFs obtained from the environmental T-RFLP profiles enabled us to identify the different phylotypes in the T-RFLP analysis. Also, at the date of the cloning, all sequences could be assigned to a T-RF. The proportions of the main bacterial divisions determined by cloning-sequencing corresponded to those determined from the T-RFs identified. It is likely that failure to identify certain T-RFs was due (1) to the strong fluctuations in the dominant T-RFs, which were particularly visible in the lake-reservoir, and (2) to the limited size of the two libraries, which did not cover the full diversity of the bacterial community. Thus, the number of OTUs determined by cloning-sequencing was lower than the number of T-RFs and the area percentage of the identified T-RFs was always less than 50% of the total area.

This long-term study shows that, even though the clone librairies of the two sites differed, *Betaproteobacteria* and *Actinobacteria* were the most abundant bacterial divisions in both sites. The predominance of *Betaproteobacteria* in lake ecosystems has already been demonstrated in many studies based on cloning-sequencing and/or FISH (e.g. Hiorns *et al.*, 1997; Pernthaler *et al.*, 1998; Zwisler *et al.*, 2003). In addition, the few reported studies conducted on lake-reservoirs provide evidence that *Betaproteobacteria* dominate the BCC, followed by the *Bacteroidetes* or *Alphaproteobacteria* (Mašín *et al.*, 2003; Jardillier *et al.*, 2004). Our results show that although *Betaproteobacteria* predominated among the bacterial community of the epilimnion during the 2 years of the Sep Reservoir study, *Actinobacteria* formed a particularly important group, being the second largest division after the *Betaproteobacteria* at Sep Reservoir, and dominating, for the 2 years, the BCC of the euphotic zone at Pavin Lake. Unlike *Betaproteobacteria*, bacteria of the cluster *Actinobacteria* are not detected by the traditional FISH method without helper probes (Glöckner *et al.*, 2000) or enzymatic amplification signal (CARD-FISH) (Sekar *et al.*, 2003). Moreover they have been neglected in many studies on the dynamics of BCC. Thus only a few studies have demonstrated the importance of this last group in lake ecosystems either at one time (Glöckner *et al.*, 2000; Urbach *et al.*, 2001; Zwart *et al.*, 2002; Warnecke *et al.*, 2004) or over a period (Zwisler *et al.*, 2003). Most of the sequences belonging to *Actinobacteria* in both our lakes were in cluster acI defined by Warnecke *et al.* (2004). This cluster is separated into three lineages of which at least two nearly complete sequences can be distinguished. Subcluster acI-A is consistent with cluster ACK-M1 described by (Zwart *et al.*, 2002), whereas subclusters acI-B and acI-C represent novel lineages. Part of the sequences from our two libraries is therefore distributed in subclusters acI-A and acI-B.

The *Bacteroidetes*, which can dominate the bacterioplankton (Zwart *et al.*, 1998; Glöckner *et al.*, 2000), only represented a small percentage of the BCC in our two ecosystems as determined from the environmental T-RFLP profiles. Also, in contrast to the above two divisions, we found, in agreement with other authors (Pernthaler *et al.*, 1998; Jaspers *et al.*, 2001), pronounced temporal changes in the *Bacteroidetes* in both lakes, except for the hypolimnion of Pavin Lake. *Bacteroidetes* are known to degrade polymeric substrates of a labile and rather recalcitrant nature and to comprise meso- and psychrophilic species (Kirchman, 2002). Moreover, in mesocosm experiments, where protein was added to stimulate phytoplankton bloom decay, increased enzymatic activities were found as the *Bacteroidetes* became dominant (Pinhassi *et al.*, 1999). Input of organic matter is particularly elevated in Sep Reservoir (Richardot *et al.*, 2000), and the high proportion of *Bacteroidetes* OTUs is also not surprising. Thus, a higher proportion of this division might have been expected at Sep Reservoir over time. Their ability to degrade organic matter is certainly not sufficient to explain their distribution in lake environments.

The *Verrucomicrobia* have not been extensively studied in lakes (Zwart *et al.*, 1998; Lindström *et al.*, 2004) but our study shows that their relative importance is equivalent to that of *Bacteroidetes*. Their physiology is still little known because it has not been possible to cultivate more than a small fraction of these bacteria. However, the preferential use of sugars for growth has been demonstrated for certain isolates from freshwater ecosystems (Hugenholtz *et al.*, 1998) and Lindström *et al.* (2004) found that a member of the *Verrucomicrobia* was favoured by increased phosphorus availability. However, no linear relation was found between the area of *Verrucomicrobia* and concentration of PO_4-P for Sep Reservoir and Pavin Lake (Sep Reservoir: $r = -0.02$, $n = 48$; Pavin Lake: $r = -0.06$, $n = 44$).

Alphaproteobacteria are commonly detected in lakes (Pernthaler *et al.*, 1998; Glöckner *et al.*, 2000; Zwisler *et al.*, 2003). We found them in one of our ecosystems, Sep Reservoir, where in a previous study (Jardillier *et al.*, 2004) they account for 5% of total active bacteria as determined by FISH. However, in our study, this division, which was detected by cloning-sequencing, did not appear in the T-RF dynamics as it represented less than 2% of the total area. The temporal variations of these bacteria may be associated with predation by cladocerans and/or protists (Jürgens *et al.*, 1999; Jardillier *et al.*, 2004), and with the breakdown of labile organic matter (Brachvogel *et al.*, 2001).

Our study therefore shows that despite marked hydrogeological differences between the two ecosystems studied, the diversity and structure of the bacterial community at the scale of general bacterial divisions were similar. Moreover, most of the OTUs belonged to clusters defined as specific to aquatic ecosystems (Table 2). These findings seem to conflict

with those of other authors who have reported that inflowing allochthonous bacteria can influence bacterioplanktonic composition in lakes (Lindström, 1998; Crump *et al*., 2003; Lindström & Bergström, 2004). For example, the unique microbiological features of Crater Lake, the characteristics of which are similar to those of Pavin Lake, may be the result of a lack of riverine influx (Urbach *et al*., 2001). Specifically, the two ecosystems studied differed in the percentage of OTUs and clones of non-aquatic origin, which were greater at Sep Reservoir. The differences between the two ecosystems were mainly reflected in the *Bacteroidetes* and *Verrucomicrobia*. This hypothesis of a higher percentage of non-aquatic clones in the reservoir seems reasonable considering the shorter residence time of the reservoir. However, in this study, similarities of OTUs with the closest relative were generally low (Table 2) and only one sequence had within 97% sequence similarity with another non-aquatic organism. These sequences could also belong to rare aquatic clones. The *Actinobacteria*, which are traditionally associated with terrestrial ecosystems (Rheims *et al*., 1999), belonged in both our lakes to cluster acI (Warnecke *et al*., 2004), which groups only with sequences from fresh and estuary water. These results thus tend to confirm that lake *Actinobacteria* form a specific clade despite the close physicochemical connection between terrestrial and aquatic ecosystems *via* influx from the catchment area.

Temporal variability of BCC

Although there was a common general BCC on the scale of the bacterial divisions of our two lakes, strong interannual variations in diversity (i.e. T-RFs) were observed; only 63% of the T-RFs were common to the 2 years at the two sites studied. Moderately marked annual temporal changes were superimposed on these interannual BCC variations. Whatever the ecosystem or the depth sampled in our study, the temporal evolution of the BCC, expressed as dissimilarity in T-RFs, supports the hypothesis of rapid changes in BCC. These dissimilarity indices were not significantly different between the two ecosystems (*t*-test, $P > 0.05$). Temporal changes have the potential to be very rapid, as was demonstrated in several mesocosm experiments that showed dramatic changes in the BCC on a daily to weekly basis (van Hannen *et al*., 1999; Schäfer *et al*., 2001). Likewise, certain temporal studies have also demonstrated rather rapid variations (Höfle *et al*., 1999; Jaspers *et al*., 2001; Yannarell *et al*., 2003). The studies conducting using the FISH method, which will target specific groups, show more gradual changes in the BCC (Pernthaler *et al*., 1998; Zwisler *et al*., 2003). However, in contrast to fingerprinting techniques, this type of method cannot be used to study the bacterial community on a finer phylogenetic scale. Work conducted using DGGE has also demonstrated gradual changes in BCC, some of which could be linked to seasonal trends (Lindström, 1998; Pinhassi & Hagström, 2000; Schauer *et al*., 2000; Zwisler *et al*., 2003). However, the apparent conflict between gradual or dramatic changes in BCC may derive from methodological differences. Jaspers *et al*. (2001) showed that rapid changes could be detected using a group-specific PCR DGGE, but that DGGE carried out with *Eubacteria* primers will only show weak changes. Moreover, the dissimilarity calculation carried out between major bacterial divisions showed temporal fluctuations that were much less marked than those occurring on a finer phylogenetic scale. Thus *Actinobacteria* and *Betaproteobacteria* presented variations that were less marked over time than those that occurred inside these two bacterial divisions (i.e. identified T-RFs). We can thus suppose, like Schauer *et al*. (2003), that there is a general stable structure of the bacterioplankton at the scale of the bacterial divisions that contrasts with a rapid succession of populations within each bacterial division.

Seasonal cycles in temperate lakes are driven by the basic physical parameters of light, temperature and wind, which control the dynamics of all biota via nutrient upwelling and primary production. Primary producers are directly connected by microbial food webs with bacterioplankton. In our 2-year study, irrespective of the level of BCC observation (T-RFs or bacterial divisions), we found no succession of the bacterial community which could be related to a specific season, unlike other authors (Pinhassi & Hagström, 2000; Schauer *et al*., 2000; Yannarell *et al*., 2003; Zwisler *et al*., 2003), who sometimes reported on relatively short periods, and so could not demonstrate reproducibility of the effect. Such results were to be expected for Sep Reservoir, mainly because of its short residence time and the importance of its catchment area. In fact, Lindström (1998) had already shown, for a lake ecosystem with a retention time similar to that of Sep Reservoir, that there were annual variations in BCC that were not reproducible from one year to the next. This may be a result of nutrient inflow from the catchment area, which may cause a decoupling between primary production and bacterial production (Richardot *et al*., 1999). More precisely, the changes in BCC in the hypolimnion are significantly associated with temperature changes and the outflow that varied greatly from 1 year to another. Conversely, seasonal reproducibility from 1 year to the next could be expected for Pavin Lake, as Yannarell *et al*. (2003) had demonstrated that physical parameters (temperature, thermal stratification) could explain trends in BCC to some extent. However, these variables only account for phases of stability and instability of BCC from one year to the next, and not a seasonal reproducibility of the BCC determined by ARISA. Such reproducibility was bound to be low because of the strong interannual variations; only 30–50% of the ARISA fragments were common to the 2 years of the study.

In our study, the CCA performed on our two lakes showed that among the different biophysico-chemical parameters studied, nutrients and potential predators (PF, HNF, zooplankton) accounted for the temporal variations in BCC at all sampling depths. Factors related to seasonal forces, temperature and outflow for Sep Reservoir seemed to account only for the variation of the hypolimnion BCC of both sites. More specifically, the influence of HNF, PF and zooplankton on bacterial abundance and composition was clearly established in these ecosystems (Thouvenot *et al.*, 1999; Jardillier *et al.*, 2004, 2005). Thus the prey-predator and host-parasite relations, or the existence of a multitude of ecological niches (Jaspers *et al.*, 2001), may be responsible for the rapid changes. Various experiments on the influence of bottom-up and top-down factors on BCC show changes in this community at time scales much finer than the sample timing used in this study. Thus the bioavailability of resources can affect the structure of the bacterial assembly on a relatively short time scale (Höfle *et al.*, 1999; Pinhassi *et al.*, 1999). The same is the case for top-down factors such as predation by flagellate protists and metazoa (Simek *et al.*, 1997; Jürgens *et al.*, 1999; Muylaert *et al.*, 2002), and viral lysis (Middelboe *et al.*, 2001). More specifically, the action of viruses, which control bacterial diversity through the 'kill the winner' process (Furhman & Suttle, 1993), must cause very rapid variations in BCC.

We also note that the analyses carried out at Pavin Lake do not significantly account for the changes in BCC, and that the part unexplained by CCA is substantial. Although nutrients and trophic interactions can help us to understand BCC trends, these trends may also be caused by the lack of a steady state among these communities, owing to their marked biodiversity (Hutchinson, 1961). The strong variations in the planktonic populations, like those we observed in this study, also point to the existence of a chaotic system (Sheffer *et al.*, 2003), but this could not be confirmed here because of insufficient data. However, such variations do not necessarily affect how the system operates. The bacterial populations can present a marked temporal variation profile despite constant environmental conditions and a functionally stable system. Fernandez *et al.* (1999) showed over a 605-day period that an extremely dynamic community of a reactor can maintain a stable ecosystem function.

Conclusion

This study shows that despite large hydrogeological differences, the two sites studied present similarities of the bacterial divisions: *Actinobacteria* and *Betaproteobacteria* are the most abundant divisions at the two sites. One of our starting hypotheses was that the hydric instability of the Sep lake-reservoir would generate marked spatio-temporal variations in BCC that we would not be able to observe at Pavin Lake. Yet at both sites, the BCC (i.e. T-RFs) displayed marked temporal changes that could be associated mainly with nutrient availability and mortality factors, the nature of which differed from one site to another. Factors related to seasonal forces, temperature and outflow for Sep Reservoir seemed to account only for the variation of the hypolimnion BCC of both sites. Moreover, no seasonal reproductibility in temporal evolution of bacterial community from 1 year to the next was observed. Although marked rapid spatio-temporal variations of the bacterioplankton have been found, it remains to be determined whether these variations are associated with functional changes or are linked to modifications at another level of organization (groups, specific clades, etc.). This information is of prime importance to devise a mechanistic model, analogous to the PEG model for phytoplankton and zooplankton, to predict ecological succession of bacterioplankton.

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