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MICROBIOLOGY OF AQUATIC SYSTEMS

A 2-Year Assessment of the Main Environmental Factors Driving the Free-Living Bacterial Community Structure in Lake Bourget (France)

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Abstract Despite the considerable attention that has been paid to bacterioplankton over recent decades, the dynamic of aquatic bacterial community structure is still poorly understood, and long-term studies are particularly lacking. Moreover, how the environment governs diversity patterns remains a key issue in aquatic microbial ecology. In this study, we used denaturing gradient gel electrophoresis of PCR-amplified partial 16S rRNA gene fragments and multivariable statistical approaches to explore the patterns of change in the free-living bacterial community in the mesotrophic and monomeromictic Lake Bourget (France). A monthly sampling was conducted over two consecutive years (2007 and 2008) and at two different depths characterizing the epi- and hypolimnion of the lake (2 and 50 m, respectively). Temporal shifts in the bacterial community structure followed different patterns according to depth, and no seasonal reproducibility was recorded from 1 year to the next. Our results showed that the bacterial community structure displayed lower diversity at 2 m (22 bands) compared to 50 m (32 bands) and that bacterial community structure dynamics followed dissimilar trends between the two depths. At 2 m, five shifts in the bacterial community structure occurred, with the temporal scale varying between 2 and 8 months whereas, at 50 m, four shifts in the bacterial community structure took place at 50 m, with the temporal scale fluctuating between 3 and 13 months. More than 60% of the bacterial community structure variance was explained by seven variables at 2 m against eight at 50 m. Nutrients (PO_4-P, NH_4-N and NO_3-N) and temperature were responsible for 49.6% of the variance at 2 m whereas these nutrients, with dissolved oxygen and chlorophyll *a* accounting for 59.6% of the variance at 50 m. Grazing by ciliates played also a critical role on the bacterial community structure at both depths. Our results suggest that the free-living bacterial community structure in the epi- and hypolimnion of Lake Bourget is mainly driven by combined, but differently weighted, top-down and bottom-up factors at 2 and 50 m.

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Introduction

Bacteria represent the most abundant group of planktonic organisms in freshwater lakes, and are centrally involved in biogeochemical cycling [13]. A large fraction of the primary production is processed through bacterial community activity, and on average, bacterial biomass synthesis consumes 30% of the photosynthetically derived carbon [7]. In lakes, multiple interacting factors and processes drive natural microbial communities and it has been shown that biotic interactions (i.e. food-web or predator–prey dynamics) as well as abiotic factors (resources) play a significant role in shaping bacterial communities [22, 28]. It has been suggested that bacterial growth is mainly top-down (grazer, viruses) controlled in nutrient-poor environments and bottom-up (resource) controlled in nutrient-rich environments [17]. While several studies have been carried out to evaluate the role of bottom-up and top-down regulation on biomass or production of the bacterial community, still little is known on the relative importance and combined effects of these regulating factors.

Over the past two decades, there has been an impressive expansion of research into the issue of the microbial diversity importance in the functioning of aquatic ecosystems. In order to obtain a better understanding of the growth dynamics, spatio-temporal variations, and controlling factors of the prokaryotic community, it is of great importance to elucidate the temporal dynamics of their structure. Molecular methods (based on fingerprintings, fluorescence in situ hybridization, or cloning/sequencing) have been made available to assess these dynamics, and it is now well accepted that polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE) which allows the separation of similar-length nucleic acid molecules, typically PCR products using specific bacterial primers, represents (1) an adequate means to characterize the bacterial composition and community structure (particularly for the analysis of a great number of samples) and (2) allows a reliable comparison between a variety of samples and ecosystems [11].

To date, several investigations dealing with the temporal evolution of the bacterial community structure and composition in lakes have been performed and different trends have been highlighted according to the studied lakes. While some studies describe a pronounced seasonal evolution of the bacterial community composition in lakes [51, 53, 54], others demonstrated a more gradual change and did not find any apparent connection with seasonality [1, 26, 27]. However, to the best of our knowledge, and except for Boucher et al. [1], none of the temporal studies published so far have been conducted at different depths and over a period of time longer than a year. Moreover, none of these studies demonstrated a statistically robust relation between the temporal evolution of bacterial community structure and associated limnology ecosystem processes. The role of "top-down" and "bottom-up" control on bacterial community structure dynamics is frequently proposed in the literature but such complex ecological questions may be addressed with robust statistical analysis as canonical correspondence analysis (CCA). Nevertheless, the use of this direct gradient analyze in combination with molecular fingerprinting is scarce in spite of the power of this method for this purpose [37, 38].

Lake Bourget (largest natural French lake) is a mesotrophic deep lake which exhibits clear summer stratification. Previous studies carried out in this lake, have mainly been performed to describe the spatial and temporal variations in the abundance of the bacterial community in relation with other biological compartments [8, 35]. In return, data on the composition and the dynamic structure of this community remained scarce [12]. Moreover, no information has been made available concerning the factors driving this structure yet.

Thus, in this study, we conducted a complete survey over a period of 2 years (2007–2008) in Lake Bourget by sampling in both the epi- and hypolimnion (2 and 50 m, respectively). We examined the temporal evolution of the free-living bacterial community structure at these two depths by using DGGE of the amplified V3 region of 16S rRNA gene. As a first objective, we sought to determine the temporal scales at which changes in bacterial community structure occur in epi- and hypolimnion in Lake Bourget by using multivariate nonparametric statistical methods. Secondly, we took a large set of environmental parameters including physico-chemical variables and biological parameters to investigate "top-down" and "bottom-up" control as potential driving forces for temporal shifts in bacterial community structure at both depths using multivariate statistical direct gradient ordination methods.

Material and Methods

Study Site and Sampling Strategy

Water samples were collected in Lake Bourget, situated in the western edge of the Alps (45°44′N; 05°51′W; 231 m altitude). It is an elongated and north–south-oriented lake (length 18 km; width 3.5 km; area 44×10^6 m^2; volume 3.5×10^9 m^3; maximum depth 147 m; mean depth 80 m; residence time 8.5 years). Lake Bourget is considered as mesotrophic and has been characterized by a recurrent bloom of the filamentous cyanobacterium *Planktothrix rubescens* since 1998 [19]. More details (including a map of this lake with its bathymetry) are available in Jacquet et al. [19]. Due to their different and contrasting environmental and biological parameters, as revealed by previous works [8, 35], sampling was carried out at 2 m (located in the upper epilimnion) and at 50 m (located in the upper hypolimnion), once a month from January 2007 to December 2008. We chose these two depths because of their differences and contrasting characteristics (physico-chemical and biological parameters) which exhibit highly reproducible seasonal patterns from 1 year to another [35]. A total of 2 l was collected using a Niskin bottle at the reference sampling station of the lake (referred to as point B) located above the deepest part of the ecosystem. Samples were put into sterile polycarbonate bottles and kept in the dark at 4°C until being processed immediately on return to the laboratory (i.e. within the next 3 h).

Physico-chemical Variables

The total organic carbon (TOC) and nutrient concentrations, i.e. total nitrogen (TN), dissolved ammonium (NH_4-N), dissolved nitrates (NO_3-N), total phosphorus (TP), and orthophosphates (PO_4-P) were measured at each sampling station and date, according to the standard French protocols

AFNOR (details available at http://www.thonon.inra.chimie.net/page/public/analyses.asp). A conductivity–temperature–depth measuring device (CTD SEABIRD SAB 19 Seacat profiler) and a chlorophyll fluorescence Fluoroprobe (BBE Moaldenke, Germany) were used to obtain vertical profiles of water temperature, conductivity, dissolved oxygen concentration and chlorophyll *a* fluorescence.

Assessment of the In Situ Microbial Community Dynamics

Abundances of virus-like particles (VLP), heterotrophic prokaryotes (mostly bacteria, [8]) and picocyanobacteria were measured by flow cytometry. Briefly, VLP and heterotrophic prokaryotes were fixed with 0.2 µm filtered-glutaraldehyde (0.5% final concentration, grade I, Merck) for 30 min in the dark, until being counted with a FACSCalibur (Becton Dickinson) flow cytometer, using the same protocol as described in Personnic et al. [35] and references therein. To analyze the phytoplankton community dynamics, samples were processed without adding any fixative or dye [35].

P. rubescens abundance was determined following Utermöhl [52] protocol, and by counting 100-µm-length filaments and assuming a mean cell length of 5 µm.

Glutaraldehyde (1% final concentration) was used to fix the flagellates. Samples were filtered (pressure <100 mmHg) on black polycarbonate membranes (diameter, 25 mm; pore size, 0.8 µm), then stained with primuline [2] and stored, for at most a few days, at −20°C until analysis. Slides were examined using epifluorescence microscopy under UV light to count the heterotrophic nanoflagellates, and under blue light to count the autotrophic nanoflagellates at a ×1,250 magnification.

Ciliates were preserved with mercuric bichloride (25%) and identified and counted (within 15 days of sampling) according to the method of Sime-Ngando et al. [47] using an inverted light microscope (Olympus, ×500).

Bacterial Community Structure

Analysis of the bacterial community structure was assessed using DGGE as described by Dorigo et al. [12]. Bacteria were harvested from approximately 250 ml water onto 47 mm diameter, 0.2 µm pore size, polycarbonate white membrane filters (Nuclepore), after a pre-filtration step through 2 µm pore size polycarbonate membrane filters (Nuclepore) to eliminate large eukaryotes and filamentous cyanobacteria. Thus, samples included DNA from free-living heterotrophic bacterioplankton as well as a small fraction of picocyanobacteria. The filters were stored at −80°C until nucleic acid extraction could be carried out, as described in Dorigo et al. [12]. After DNA extraction and quantification according to the absorbance at 260 nm using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific), DNA extracts of the sampled community were then stored at −20°C until PCR amplification. PCR reactions were carried out according to the PCR cycle described in Dorigo et al. [12] and using the Eubacteria-specific primer 358-GC [32] and the universal primer 907rM [40]. PCR products were verified by agarose gel electrophoresis (not shown). DGGE analysis was performed on PCR fragments, essentially as described in Dorigo et al. [12] but by using Ingeny PhorU-2 (Ingeny International) and a linear gradient of the denaturants urea and formamide, which increased from 40% at the top of the gel to 80% at the bottom. Digital images of the gels were obtained using a Kodak DC290 camera, and were then saved for further analysis using the Microsoft Photo Editor Software.

DGGE Pattern Analysis

The DGGE banding patterns were analyzed using the GelCompare II software package (Applied Maths, Kortrijk, Belgium) and after digitalization of the DGGE gels. Briefly, banding patterns were first standardized with a reference pattern included in all gels. Each band was described by its position (Y, in pixel on the image file) and its relative intensity in the profiles (P_i) which could be described as the ratio between the surface of the peak (n_i) and the sum of the surfaces for all the peaks within the profile (N) [14]. This information was used to determine the total number of bands for each profile (S) and to calculate the Simpson evenness index (λ) according to the formula: $\lambda = \sum \left(p_i^2\right)$, where $p_i = n_i/N$ and n_i=number of individuals in species i and N=total number of individuals in all species.

A similarity matrix between densitometric curves of the band patterns was calculated based on the Bray–Curtis index and used to perform moving-window analysis [30] by plotting the correlation between the month x and $x-1$. The $^{\triangle}{}_{t(month)}$ values were calculated as the averages and standard deviations for the respective moving-window analysis curve data points subtracted from the 100% similarity value. The greater the change between the DGGE profiles of month x and $x-1$, the lower the moving-window curve data point and the higher the $^{\triangle}{}_{t(month)}$ values will be.

Statistical Analysis

Comparative analysis of DGGE fingerprints was carried out with the PRIMER 5 software (PRIMER-E, Ltd., UK). Ordination of Bray–Curtis similarities among normalized sample profiles was performed by non-metric multidimensional scaling (MDS). We used this ordination technique to determine the relationships among sample profiles as representative of the bacterial community structure of each sample site. MDS attempts to preserve the ranked order of

the similarity of any two sample communities as an inverse function of the distance between the points representing those communities on the plot [24]. The degree to which the plot matches the similarity matrix can be judged by examining the stress, defined here as Kruskal's stress formula [25], with values lower than 0.1 representing good ordination with little risk of pattern misinterpretation [6]. The prepared MDS plots were used to visualize the relationship between the bacterial communities, as determined by their DGGE profiles, throughout the sampling period.

Additionally, hierarchical agglomerative clustering of Bray–Curtis similarities was performed using the group average method of PRIMER software. To test the null hypothesis, that there was no significant difference between the groups discriminated according to the agglomerative clustering analysis, we conducted an analysis of similarities with the subroutine ANOSIM of PRIMER. ANOSIM is a nonparametric test designed to perform statistical comparisons of multivariate data sets in a manner similar to univariate techniques (ANOVA) [6]. Firstly, ANOSIM calculates the R statistic that displays the degree of separation between groups. Complete separation is indicated by $R=1$, and $R=0$ suggests no separation. Having determined R, ANOSIM, secondly, assigns samples randomly to different groups to generate a null distribution of R (Monte Carlo test) to test whether within-group samples are more closely related to each other than would be expected by chance.

To investigate the relationships between bacterioplankton community structure and measured environmental variables, a CCA was performed using the software package CANOCO, version 4.5 for Windows [50]. CCA is an ordination technique that was originally developed to relate community compositions to known (available) variations in the environment [49]. The obtained ordination axes (based on community structure data) are linear combinations of environmental variables that best explain microbial diversity composition data. We first imported OTUs abundance data from spreadsheets using WCanoImp program within the CANOCO package. We then used CANOCO program to perform CCA with species scaling on intersample distances so that samples and environmental variables formed a biplot. To statistically evaluate the significance of the first canonical axis and of all canonical axes together, we used Monte Carlo permutation full model test with 199 unrestricted permutations. Finally, to represent biplots we used the program CANODRAW within CANOCO package for Windows. Additionally, Spearman's rank pairwise correlations between the environmental variables mentioned above helped to determine their significance for further ecological analysis.

Results

Temporal Dynamics of the Physico-chemical and Biological Parameters

A clear seasonal trend was observed at 2 m in the temperature with the lowest values in February (5.9°C) and the highest during summer (24.3°C in August 2008), whereas at 50 m, this parameter remained relatively stable along the study period (around 6.17°C, SD=0.26, $n=24$). At 2 m, dissolved oxygen concentration peaked in spring at both years. At 50 m, dissolved oxygen concentration was near that registered at 2 m and displayed the highest values between the end of winter and the early spring. During the 2 years, TOC concentration remained stable at both depths but displayed significant higher values at 2 m than at 50 m (test t, $p<0.001$, $n=48$). Gradual consumption of dissolved NO_3-N was observed during spring and summer at 2 m, whereas no seasonal variation was remarked at 50 m. Peaks of NH_4-N (>12 μg l^{-1}) appeared several times in summer and spring, at 2 m, followed by a rapid consumption the month after (Fig. 1). The highest values of NH_4-N monitored at 50 m were obtained in January 2007 and April 2008. PO_4-P and TP concentrations fluctuated between 2 and 10 μg l^{-1} and between 5 and 20 μg l^{-1}, respectively (Fig. 1). At 2 m, chlorophyll a concentration was generally below 3 μg l^{-1}. At 50 m, this concentration was significantly lower than at 2 m (t test, $p<0.001$, $n=48$), between 0 and 0.5 μg l^{-1} (Fig. 2). *P. rubescens* biomass was four times higher at 2 m than it was at 50 m, however the same seasonal tendency was observed, with the lowest values during spring–summer and the highest during autumn–winter (Fig. 2). High dynamic in the abundance of picocyanobacteria was observed during the 2 years, at both depths (Fig. 2), with abundance evolving between 6.2×10^2 and 3.8×10^5 cell ml^{-1} at 2 m and between 80 to 8.8×10^3 cell ml^{-1} at 50 m. Heterotrophic prokaryote abundance varied from 4.1×10^5 to 6.6×10^6 cells ml^{-1} at 2 m and from 6.5×10^5 to 2.1×10^6 cell ml^{-1} at 50 m (Fig. 2). In most cases, VLP abundance remained below 3.1×10^8 part ml^{-1} at 2 m and 7.1 10^7 part ml^{-1} at 50 m. Heterotrophic nanoflagellates (HNF) and ciliates abundances averaged 0.7×10^3 cells ml^{-1} and 24.8 cells ml^{-1} respectively at 2 m and 2.2×10^2 cell ml^{-1} and 9 cell ml^{-1}, respectively, at 50 m. Most of the highest abundances of HNF and ciliates coincided with that of the heterotrophic prokaryotes (Fig. 2).

Bacterial Community Structure

At 2 m, a total of 22 individual DGGE bands were detected. The number of DGGE bands varied between 6 (June and September 2007) and 13 per sample (March 2007, February

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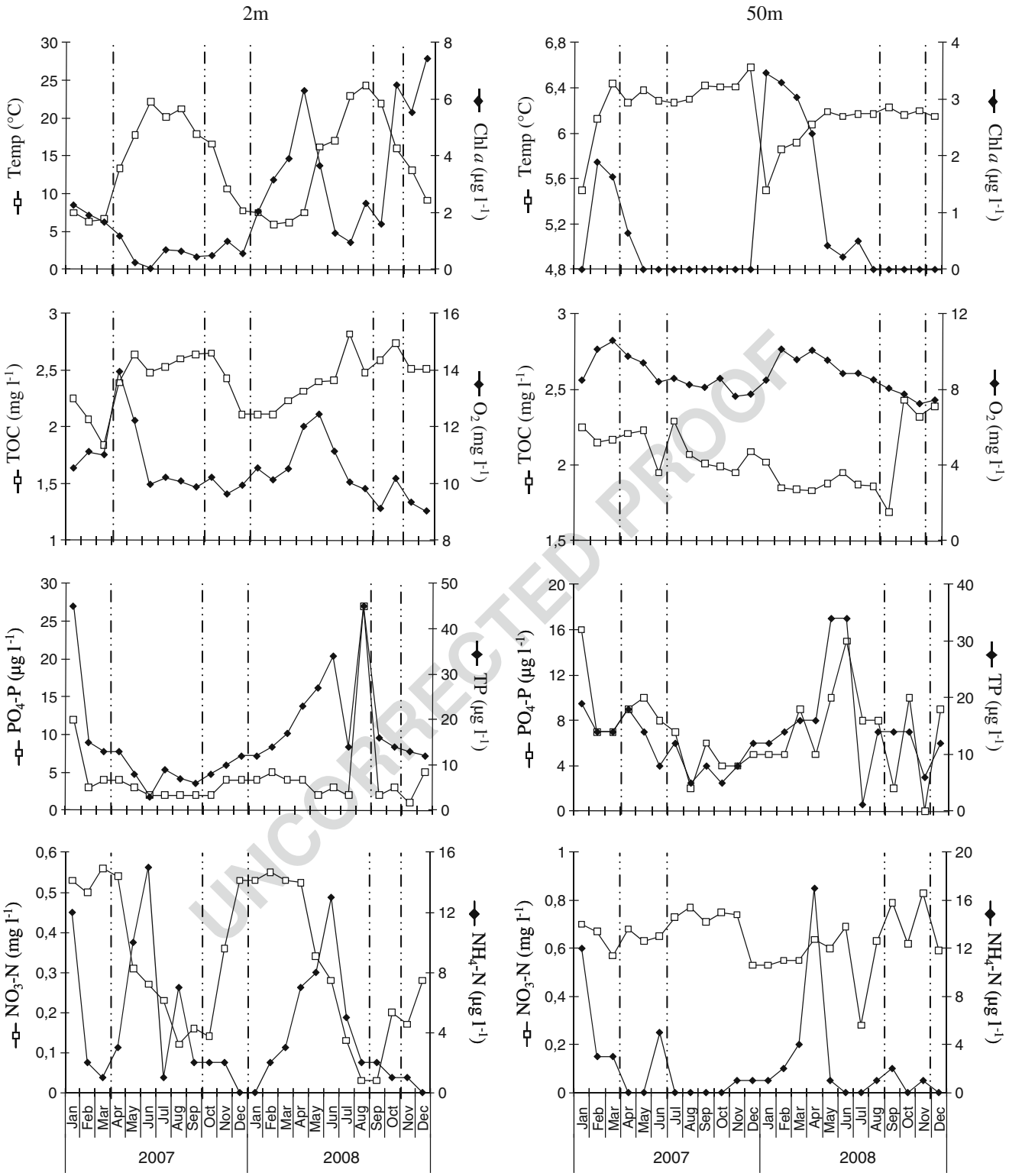

Figure 1 Temporal evolution of chemical characteristics of Lake Bourget at 2 and 50 m. *Dashed lines* indicate the periods when the shifts in the bacterial community structure was observed. *Chl a* Chlorophyll *a*, *Temp* temperature, O_2 dissolved oxygen, PO_4-*P* orthophosphate (detection limit <0.5 µg of P in PO_4 l^{-1}), NH_4-*N* dissolved ammonium, *TP* total phosphorus, NO_3-*N* dissolved nitrate, *TOC* total organic carbon

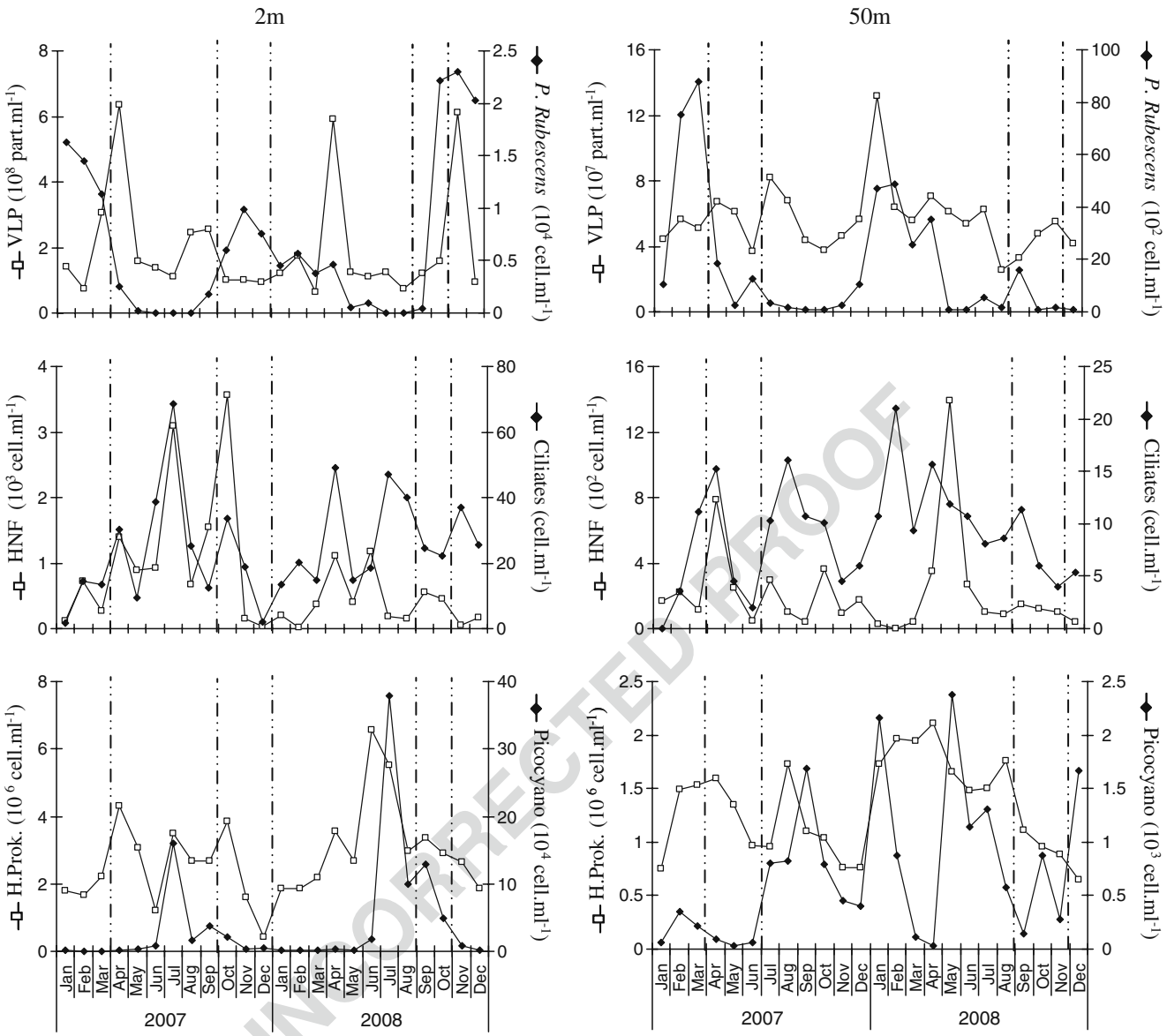

Figure 2 Temporal evolution of microbial communities in Lake Bourget at 2 and 50 m. *Dashed lines* indicate the periods when the shifts in the bacterial community structure was observed. *VLP* Virus-like particles, *P. rubescens* *Planktothrix rubescens*, *HNF* heterotrophic nanoflagellates, *H. Prok.* heterotrophic prokaryotes, *Picocyano* picocyanobacteria

and December 2008; mean=8, SD=2, n=24; Fig. 3a). The Simpson's evenness index (λ), estimated by both presence and intensity of DGGE bands, varied between 0.13 (March 2007) and 0.3 (January 2007). Seventy-nine percent of the samples presented an evenness index >0.2 (Fig. 3b). We used MDS and hierarchical agglomerative clustering for comparative analysis of DGGE fingerprints. Both analyses showed a recurrent seasonal pattern during the sampling period with four distinct clusters of bacterial communities: January to March 2007 (winter), April to September 2007 (spring–summer), October to December 2007 (autumn–winter) and January to August 2008 (winter to summer; Fig. 4). September and October 2008 samples were found in the same cluster than October to December 2007 samples, as well as November and December 2008 samples with April to September 2007 samples (Fig. 4). The MDS ordination plot stress value was low (0.08) which corresponds to a good ordination with no real prospect of a misleading interpretation [6]. The nonparametric ANOSIM subroutine of PRIMER confirmed the robustness of these four clusters (R=0.991, p=0.001). Bray–Curtis similarity values varied from 30 to 87.5% (mean=67%±17%, n=24; data not shown). According to the moving-window analysis (Fig. 3c), the most significant rate of changes in bacterial community structure was registered between February and May in 2007 (53%) and between August and September in 2008 (60%). The level of bacterial community dynamics between the other months did not exceed 40%, with a

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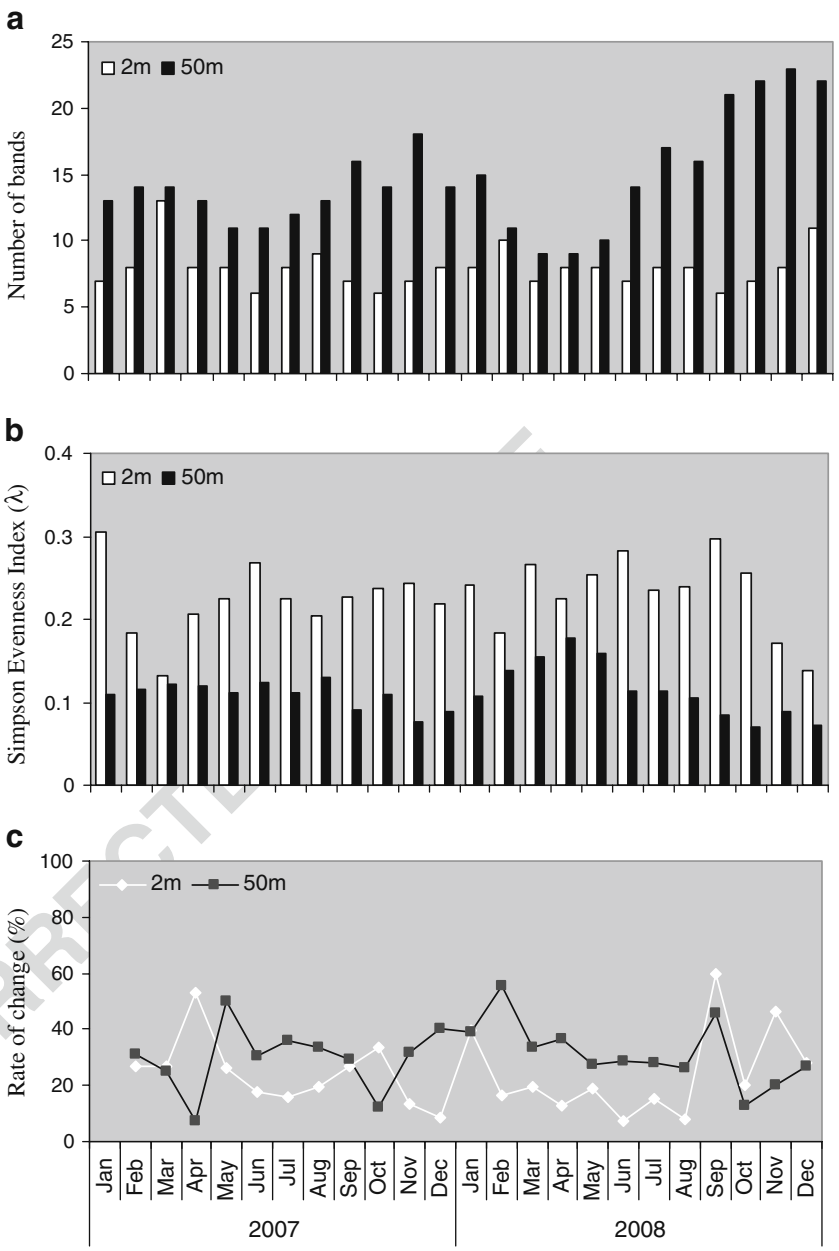

Figure 3 Temporal changes in the number of bands (**a**), in the Simpson (λ) evenness (**b**) and the level of dynamics (**c**, moving-window analysis (%)) of the bacterial community between 2007 and 2008 in Lake Bourget at 2 vs. 50 m according to PCR-DGGE analysis

monthly rate of change about 24% (±14%), which represented a medium level of community dynamics (according to Marzorati et al. [30]).

The number of individual DGGE bands observed at 50 m was higher than that at 2 m with 32 and 22 bands, respectively (Fig. 3a). At 50 m, the number of bands per samples varied from 9 (March and April 2008) to more than 20 (September to December 2008). The Simpson's evenness index values were in contrast much lower at 50 m, varying between 0.07 (October 2008) and 0.17 (April 2007; Fig. 3b). MDS ordination plots and cluster analysis showed a clear separation between bacterial community structure originated from 2 to 50 m (Fig. 4). At 50 m, only three clusters could be distinguished: January to April 2007 (winter to spring), May to July 2007 (summer) and August 2007 to August 2008 (1 year). The last cluster could be separated into two sub-clusters including samples from August 2007 to January 2008 (summer to winter) and samples from February 2008 to August 2008 (winter to summer; Fig. 4). Samples from September to November 2008 were found to be similar to the May to July 2007 cluster, but formed a separate sub-cluster (Fig. 4). Bray–Curtis similarity values among samples ranged from 36 to 85%, with a mean value of 64.5%±18% (data not shown). Moving-window analysis revealed that the bacterial community structure at 50 m shifted over time with a monthly rate of change up to 28%±10%. The most significant change in the bacterial community structure (more than

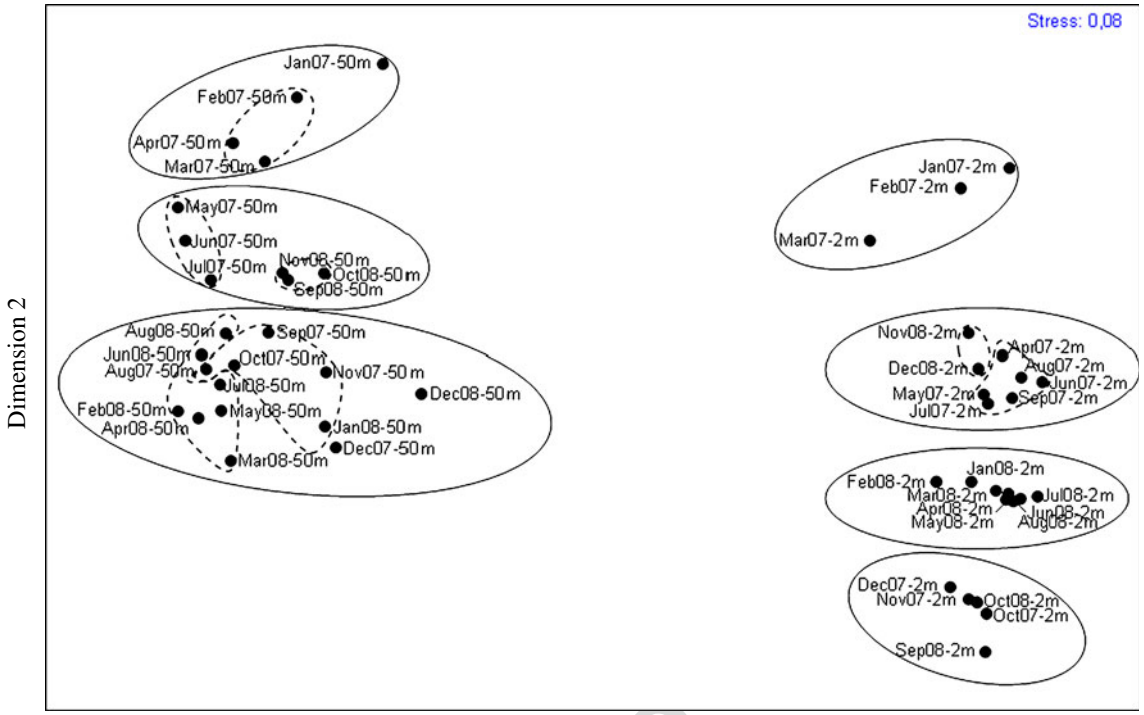

Figure 4 Multidimensional scaling (MDS) plot of the bacterial community structure composition as determined from PCR-DGGE profiles based on Bray–Curtis similarity index for Lake Bourget at 2 and 50 m, between 2007 and 2008. Stress value: 0.08

40%) were observed between April and May in 2007, January and February in 2008 and August and September 2008 (Fig. 3c).

Bacterial Community Structure in Relation to Environmental Variables at 2 m

The transition between the four bacterial groups observed at 2 m depth was concomitant with changes in several physico-chemical and biological variables. Transition from winter to spring–summer 2007 clusters (March–April) took place alongside a marked increase in both TOC and NH_4-N (by factor 1.3 and 3 respectively), a peak in oxygen (14 mg l^{-1}) and a decrease in NO_3-N (Fig. 1). At this period, the heterotrophic prokaryote abundance doubled in parallel with an important decrease in *P. rubescens* and a clear peak in VLP (Fig. 2). Transition from spring–summer to autumn 2007 clusters (September and October) occurred when surface waters became nutrient limited (Fig. 1). This period was also marked by an increase in both *P. rubescens* and bacterial grazer abundances, especially for HNF cells that reached the highest value of the 2 years (3.5×10^3 cell ml^{-1}; Fig. 2). Transition from autumn 2007 to winter–spring–summer 2007–2008 clusters (December 2007 and January 2008) took place during NH_4-N depleted period and low abundance of heterotrophic prokaryotes (Fig. 2) but an increase in chlorophyll *a* concentration (by factor 3; Fig. 1). During this period, marked decrease in the abundance of *P. rubescens* was observed after a peak in November. Changes in bacterial community from August to September 2008 occurred in a period of limited nutrients (both NO_3-N and NH_4-N) and during a rapid decrease of TP concentrations (by factor 2.8; Fig. 1). Changes in bacterial community structure from October to November 2008 took place under limit detection of NH_4-N and PO_4-P but during elevated chlorophyll *a* (around 6 µg l^{-1}) and *P. rubescens*, as well as important VLP and ciliates abundances (Fig. 2). During this period, the abundance of cyanobacteria decreased after a peak in September.

The complex influence of physico-chemical and biological parameters on changes in bacterial community structure was statistically demonstrated by using direct multivariate gradient analyses. We first performed CCA using both physico-chemical parameters and predator counts as constrained variables of the temporal changes of bacterial community structure at 2 m. A strong Spearman's rank pairwise correlation between NO_3-N and TN (R^2=0.92, $p<$ 0.01), between PO_4-P and TP (R^2=0.72, $p<0.001$), allowed us to use NO_3-N as a proxy of TN and TP as a proxy of PO_4-P. Temperature, NO_3-N, NH_4-N and TP coupled with HNF, ciliates and VLP counts variables explain 61.2% of the temporal bacterial community structure variance, as indicated by the sum of all canonical eigenvalues (Table 1). The cumulative percentage of variance of the species–

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t1.1 **Table 1** Summary of results from canonical correspondence analyses of the bacterioplankton community structure data when constrained by physico-chemical, physico-chemical and predators variables at 2 m and 50 m

t1.2 Environmental variables	2 m						50 m			
t1.3	Physico-chemical		Nutrients		Physico-chemical and predators		Physico-chemical		Physico-chemical and predators	
t1.4	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2	Axis 1	Axis 2
t1.5 Total inertia	0.518		1.530		1.530		1.296		1.296	
t1.6 Sum of all canonical eigenvalues	0.469		0.393		0.612		0.462		0.596	
t1.7 Eigenvalues	0.260	0.084	0.256	0.079	0.271	0.114	0.221	0.109	0.274	0.125
t1.8 Species–environment correlations	0.805	0.835	0.805	0.801	0.834	0.695	0.837	0.881	0.896	0.904
t1.9 Cumulative percentage variance of										
t1.10 Species data	17.0	22.5	16.7	21.9	17.7	25.2	17.0	25.4	21.1	30.7
t1.11 Species–environment relation	55.5	73.3	65.2	85.2	44.3	62.9	47.8	71.3	45.9	66.8

Physico-chemical variables were temperature, nitrate, ammonium and total phosphorus for 2 m samples and nitrate, ammonium, dissolved oxygen and chlorophyll *a* for 50 m samples. Predators were ciliates, HNF and viral abundance

environment relationship indicates that the first and second canonical axes accounted for 44.3% and 18.6% of this variance respectively (Fig. 5). Consequent axes accounted for less than 13% of the variance each, and are not considered further here. The first canonical axis is highly negatively correlated with HNF, ciliates, temperature (ca., less than −0.5) and to a lesser extent with VLP (ca., −0.2) positively correlated with NO_3-N, TP (ca., >0.8) and to a lesser extent with NH_4-N (ca., 0.2). The first axis clearly organizes the temporal evolution of the samples from 2007 whereas the temporal evolution of 2008 samples were more spread out along the second canonical axis (Fig. 5).

Other CCA were performed to investigate more precisely the relative contribution of nutrients or predators to the

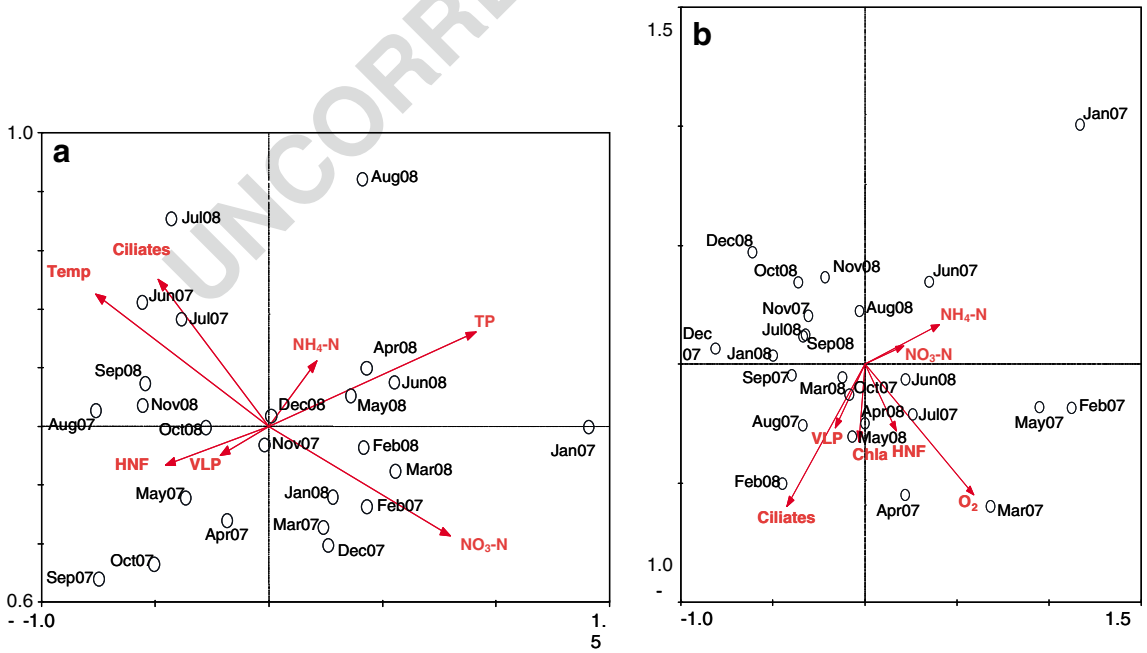

Figure 5 Canonical correspondence analysis of bacterioplankton community structure from samples from 2 (**a**) and 50 m (**b**) using physico-chemical and biological parameters. *Arrows* point in the direction of increasing values of each variable. The *length of the arrows* indicates the degree of correlation with the represented axes. The position of samples relative to arrows is interpreted by projecting the points on the arrow and indicates the extent to which a sample bacterial community composition is influenced by the environmental parameter represented by that arrow. *Chl a* Chlorophyll *a*, *Temp* temperature, O_2 dissolved oxygen, *HNF* heterotrophic nanoflagellates, *VLP* virus-like particles

observed changes in bacterial community structure. A model utilizing only NO_3-N, TP, and NH_4-N concentrations could statistically explain 39.3% of the variance (sum of all canonical eigenvalues; Table 1). Another model using NO_3-N, TP, and NH_4-N concentrations together with temperature could statistically explain 46.9% of the variance (Table 1). However, the model considering ciliate, HNF and VLP abundances could not significantly explain the variance of the bacterial community structure ($p>0.1$). Variation partitioning indicated that 24% of the observed variance in the 16S rRNA data set remained unexplained at 2 m depth.

Bacterial Community Structure in Relation to Environmental Variables at 50 m

Changes in community structure from January–April 2007 cluster to May–July 2007 cluster coincided with NH_4-N depletion (undetected), a significant drop both in chlorophyll *a* concentration and *P. rubescens* abundance (Fig. 1) but also in both ciliates and HNF abundance (by factors 4 and 3, respectively; Fig. 2). The transition occurring in summer 2007 (July–August) was concomitant with NH_4-N depletion and to a decrease in both TP and PO_4-P concentrations (by factor 2.4 and 1.4, respectively; Fig. 1). Over this period, the abundance of heterotrophic prokaryotes doubled, ciliates peaked at 16 cell ml^{-1} whereas both HNF and VLP displayed a strong decrease (by factor 3 and 1.2, respectively). Changes in the bacterial community structure from August to September 2008, coincided with high concentration of both TP and NO_3-N (14 $\mu g\ l^{-1}$ and 0.8 $\mu g\ l^{-1}$ respectively) but limited NH_4-N concentration (2 $\mu g\ l^{-1}$) and an important decrease in PO_4-P (by factor 4; Fig. 1). During this period, the abundance of cyanobacteria decreased by factor 5. No significant growth in the abundance of both predators was observed. The last transition in the bacterial community structure was observed at the end of autumn (November–December) and coincided with depleted NH_4-N conditions, an increase in the concentration of PO_4-P (by factor 9) and a decrease in the concentration of NO_3-N (by factor 1.4). The abundances of both heterotrophic prokaryotes and VLP dropped during this period by factors 1.2 and 1.4, respectively.

At 50 m, a strong Spearman's rank pairwise correlation between NO_3-N and TN ($R^2=0.77$, $p<0.01$), between PO_4-P and TP ($R^2=0.62$, $p<0.01$), allowed us to use NO_3-N as a proxy of TN and TP as a proxy of PO_4-P to perform CCA together with the rest of physico-chemical parameters. Dissolved oxygen, chlorophyll *a*, NO_3-N, NH_4-N, and TP coupled with HNF, ciliates and VLP explained 59.6% of the temporal bacterial community structure variance at 50 m, as indicated by the sum of all canonical eigenvalues (Table 1). The cumulative percentage variance of the species–environment relationship indicates that the first and second canonical axes account for 47.8% and 23.5% of this variance respectively (Fig. 5). Subsequent axes accounted for less than 13% of the variance each, and are not considered further here. The first canonical axis is highly negatively correlated with ciliates (ca. less than −0.5) and to a lesser extent with VLP (ca. −0.2) and positively correlated with dissolved oxygen (ca. >0.8) and to a lesser extent with NH_4-N, NO_3-N, and HNF (ca. 0.2). An additional model using NO_3-N, TP, and NH_4-N together with temperature, dissolved oxygen and chlorophyll *a* could statistically explain 46.9% of the variance (sum of all canonical eigenvalues; Table 1). Adding ciliates, HNF and VLP abundances could not significantly explain the variance of the bacterial community structure ($p>0.1$). Nevertheless, in contrast to 2 m, the model using only NO_3-N, TP, and NH_4-N could not significantly explain the variance of the bacterial community structure. Variation partitioning indicated that 40.4% of the observed variance in the 16S rRNA data set remained unexplained at 50 m depth, i.e. almost twice more than what we found at 2 m.

Discussion

Due to the large set of samples used to characterize major shifts inside the bacterioplankton community structure in Lake Bourget in 2007 and 2008, genetic fingerprints (DGGE in this study) were considered as more adequate tools than time-consuming cloning (inventory) approaches. Nevertheless, we are aware that such fingerprinting techniques are PCR-based and so may be subjected to potential PCR bias/drawbacks such as heteroduplex or chimera formation [23], the choice of annealing temperature, the DNA quantity used [41]. However, as stressed by Sekigushi et al. [43], the effects of bias can be minimized when, as here, relative changes are studied within a single environment. In addition, it should be kept in mind that DGGE fingerprints reflect the microorganism populations that are present at high concentrations. Casamayor et al. [3] reported that the number of bands is indeed related to the number of populations that account for more than 0.3–0.4% of the total cell counts. Thus, results given by DGGE fingerprints certainly do not represent the total species richness in the samples but, rather, a standardized measure of richness [15].

Temporal Scales of Changes in the Epi- and Upper Hypolimnion Bacterial Community Structure

One of the major findings of this study was to observe that bacterial community structure of Lake Bourget showed pronounced temporal shifts in both epi- (2 m) and upper hypolimnion (50 m), but also very long steady-state periods. Moreover, we did not find, in both epi- and

hypolimnitic layers, repeatedly seasonal patterns, during our 2-year monthly sampling but rather dramatic changes occurring at given periods (between 49% of Bray–Curtis similarity (SD=8, n=5) at 2 m and 45% of Bray–Curtis similarity (SD=8, n=8) at 50 m. Conflicting dramatic vs. gradual changes on bacterial community structure have been observed in several lakes. Some authors observed a dramatic change in the bacterial community structure over time, especially in the stratified summer period [51, 53, 54], while other studies reported a more gradual change [1, 26, 27]. Such apparent discrepancy may be due to the sampling time scale of each study, distinct lake community, sensitivity of the molecular fingerprinting method or due to distinct environmental constraints.

Few attempts have been made to assess the inter-annual bacterial structure dynamics in lacustrine ecosystems. To the best of our knowledge, Boucher et al. [1], Nelson [33], Shade et al. [44], Yannarell et al. [54] are among the few who have examined the bacterial community dynamics over several years (between two and six) in freshwater systems. No repeatable pattern from 1 year to another was observed, in only some cases, for bacterial community composition [1, 26]. Our results extend this view since we also observed a clear variability between 2007 and 2008 in Lake Bourget, in both time and space. However, this result contrasted with the clear seasonal reproducibility patterns of the bacterial abundance, reported in this study (Fig. 2) and previously by Personnic et al. [35], suggesting thus that the annual repetition or variation scheme for this parameter is probably more complex than hitherto assumed.

Interestingly, we observed very few changes in bacterial community structure for relatively long period of time, ranging from 8 months in the epilimnion to 13 months in the hypolimnion (Fig. 4). A steady state of the bacterial community structure (more than 2 months) was already observed in other lacustrine ecosystems [51, 53] while the expecting bacterial doubling time is generally lower than 3 days in lacustrine ecosystems [9]. Boucher et al. [1] concluded to a relative steady state of lacustrian bacterial community structure for relatively long periods of time, during which growth and losses of each population compensate each other to reach a stable community structure. This does not mean that the bacterial community may not react rapidly to environmental changes since we found a minimum time scale of less than a month in both epi- and hypolimnion with pronounced changes in community assemblages.

Environmental Factors Driving the Bacterial Community Structure

In order to determine the relative importance of top-down vs. bottom-up control, Gasol et al. [17] reviewed different empirical and experimental ways in which factors that control the abundance, production and growth rate of bacterial community in aquatic systems have been considered. They concluded that bacterial growth appears to be top-down regulated in most nutrient-poor environments and bottom-up regulated in the richer ones. However, these authors suggested that bacterial community composition could be more affected by top-down factors in the richest environments. Although various studies have described the structure of bacterial communities in various ecosystems, few have attempted to determine factors controlling the temporal changes. The important finding of this study was to statistically demonstrate that a complex array of physico-chemical and biological parameters was the driving force behind the temporal shifts of the bacterial community structure in the mesotrophic Lake Bourget, and these variables could explain 61.2% and 59.6% of the temporal bacterial community changes at 2 and 50 m, respectively.

Variation partitioning was used in this study to separate top-down from bottom-up effects on the temporal dynamic of bacterial community structure. The results of these analyses suggest no difference between depths, in Lake Bourget. Pure bottom-up-related variation was important (46.9% at 2 m vs. 46.2% at 50 m) at 2 and 50 m whereas pure top-down-related variation was not observed at both depths, which indicate that top-down control was less important than bottom-up control in driving the temporal changes of the bacterial community structure in the epi- and hypolimnion. Jardillier et al. [22] are among the few studies which evaluated the relative importance of bottom-up and top-down factors in temporal change of bacterial community structure and composition in lacustrine systems. These authors found that bottom-up control of the bacterial community composition are much stronger than top-down control in lakes. Our results are consistent with their results; however, the originality of the present study is to unravel such question in space (two different depths) and over two complete years. We demonstrated the importance of bottom-up factors as the main control of the temporal dynamics of the bacterial community structure as well as in the hypolimnion than in the epilimnion, and to lesser extent, the combined action of both top-down and bottom-up factors.

Bottom-Up Control

Our results showed that, even if bacterial community structure displayed high dissimilarity between the two depths, bottom-up factors explained about the same percentage of total variation at the two depths, suggesting the same sensitivity of the bacterial community to the environmental and biological parameters whatever the depth examined in Lake Bourget. The high proportion of the bacterial community structure variance (between 30%

and 64%) explained by the environmental parameters have been reported in many studies [1, 34, 55]. At 2 m, 39.3% of the temporal variability was explained by nutrient patterns only, whereas at 50 m, the model testing different combinations of nutrient concentrations alone did not give any significant explanation of the temporal changes in the BCS, which may indicate the complexity of the interactions driving the bacterial community dynamic and structure in the hypolimnion [8] and could also explain the difference in BCS between the two depths observed in this study.

The relatively strong relation between bacterial community structure and temperature is not an unexpected finding in the epilimnion. Several field investigations showed that temperature covaries with the structure and composition of bacterial community in lacustrine systems in temperate regions [29, 44]. Since mechanistic relationships cannot be revealed by statistical relationships alone, it could be possible that difference in temperature about 19°C, such as that between the winter cold water and the summer warm water in Lake Bourget could select different taxa by favoring the growth of some specific phylotypes and thus diversity in relation to species tolerance/optimum [29]. Owing to the relative stability of temperature at 50 m (Fig. 1), no such relation was found. We observed, rather at this depth, high statistical relationship between bacterial community structure and the concentration of dissolved oxygen, suggesting that the hypolimnion bacterial community could be more strongly driven by electron acceptor availability, as previously suggested by Shade et al. [45], inducing probably different metabolic pathways which may, to a large extent, explain the evolution of the bacterial structure [10].

The source and composition of organic matter pool in aquatic systems have been shown repeatedly to be related to bacterial community structure and function (e.g., [33]). In Lake Bourget, such relationships seemed to be significant only at 50 m. In fact, the concentration of Chl *a* seemed to contribute significantly to explain the variance only at 50 m, which indicate the importance of such autotrophic organic matter source on the bacterial community in the hypolimnion. As the organic matter is less biodegradable in the hypolimnion than in surface [4], we may suppose that the flow of autotrophic organic matter from surface water to the bottom, as observed in the dynamic evolution of both Chl *a* (Fig. 1), during the autumn–winter mixing could affect the bacterial community structure, at 50 m. In Lake Bourget, *P. rubescens* has been dominating the phytoplanktonic biomass since 1996 [19] and because we found a significant correlation between Chl *a* and *P. rubescens* abundance (R^2=0.53, p<0.001, n=48), we supposed that the autotrophic organic matter mentioned above was mainly represented by these filamentous cyanobacteria.

Nutrient concentrations may directly influence bacterial biomass [5] as well as community structure [22, 39] through effects on growth [31]. The low contribution of picocyanobacteria to the total number of clones (less than 1% of the total sequences obtained by PCR-cloning-sequencing) previously reported in Lake Bourget [12, 18] could probably not explain the statistical relation between nutrient elements and BCS. However, a significant relationship between BCS and nutrients may also arise from co-variation of nutrient concentrations with phytoplankton. Since autotrophic organisms such as *P. rubescens* can take up a large fraction of nutrients [19], it may at the same time influence the bacterial structure dynamics [31, 34].

Top-Down Control

Our study revealed that among the main mortality agents of the bacterial community previously identified in Lake Bourget (i.e. HNF, ciliates and viruses), ciliates seemed to be the principal top-down factors implicated in the dynamic evolution of the BCS. This result confirmed the role of ciliates in bacterial community structure shifts, previously reported by Comte et al. [8] who observed an important drop of filamentous *Cytophaga–Flavobacteria* and an increase in β-proteobacteria in summer in Lake Bourget. The importance of ciliates in shaping the structure of bacterial communities has already been reported in lacustrine systems [27, 28, 31]. For instance, Tadonléké et al. [48] reported that ciliates may have a greater impact on the more active bacterioplanktonic cells by selective grazing, outweighing HNF, and significantly shaping the bacterial community structure *in fine*. The weak correlation between HNF and BCS in lacustrine systems compared to ciliates has also been reported by Muylaert et al. [31]. According to these authors, a relatively small grazing impact of HNF on bacteria in lakes studied may explain the absence of a relationship with changes in the bacterial community composition. Although it has been reported in Lake Bourget that HNF are important grazers of bacteria [8] and could be responsible together with viral lysis of about 70% of the bacterial mortality in this lake [20, 36], we suggest that the weak coupling observed here between BCS and HNF/viruses could be the result of factors that drive strongly the dynamic evolution of these two biological compartments [16].

Conclusion

Temporal scale variations at which free-living bacterial community changes occur is an important query in lacustrine microbial ecology. Our results suggest that the temporal variation of bacterial community structure was

visible in both epi- and hypolimnion layers but with different time scales and they underline the importance of several years of sampling effort to have a consistent picture of the shifts in bacterial community structure in lake environments. This study identifies important environmental and biological drivers, at different stratum, that should be included in the comprehension of the BCS behavior. These drivers consisted in both bottom-up and top-down factors, including temperature, dissolved oxygen and nutrients (bottom-up) as well as ciliates, HNF and viruses (top-down). The importance of these forces varied according to periods and depths. Some were secondary to others, but all played a role in shaping the free-living bacterial community structure in Lake Bourget and explained the same percentage (around 60%) of the variance at the two depths, suggesting the same sensitivity of the bacterial community to the environmental and biological parameters in this ecosystem.

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