RESEARCH ARTICLE

Spatio-temporal changes in the structure of archaeal communities in two deep freshwater lakes

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Received 31 January 2013; revised 23 May 2013; accepted 23 May 2013.
Final version published online 26 June 2013.

DOI: 10.1111/1574-6941.12154

Editor: Angela Sessitsch

Keywords
lakes; *Archaea*; dynamics; community structure; driving factors.

Abstract

In this study, we evaluated the driving forces exerted by a large set of environmental and biological parameters on the spatial and temporal dynamics of archaeal community structure in two neighbouring peri-alpine lakes that differ in terms of trophic status. We analysed monthly data from a 2-year sampling period at two depths corresponding to the epi- and hypolimnetic layers. The archaeal communities seemed to be mainly composed of ammonia-oxidizing archaea belonging to the thaumarchaeotal phylum. The spatio-temporal dynamics of these communities were very similar in the two lakes and were characterized by (1) disparities in archaeal community structure in both time and space and (2) no seasonal reproducibility between years. The archaeal communities were regulated by a complex combination of abiotic factors, including temperature, nutrients, chlorophyll *a* and dissolved oxygen, and biotic factors such as heterotrophic nanoflagellates and ciliates. However, in most cases, these factors explained < 52% of the variance in archaeal community structure, while we showed in a previous study that these factors explained 70–90% of the temporal variance for bacteria. This suggests that *Bacteria* and *Archaea* may be influenced by different factors and could occupy different ecological niches despite similar spatio-temporal dynamics.

Introduction

One of the main challenges in aquatic microbial ecology is to understand the role of microbial community structure and its diversity in the functioning of aquatic ecosystems. In these ecosystems, *Archaea* often comprise a significant portion of the microbial life in aquatic systems (Karner *et al.*, 2001; Church *et al.*, 2003; Comte *et al.*, 2006; Auguet & Casamayor, 2008). Initially, *Archaea* were considered to thrive in extreme environments (Fox *et al.*, 1977; Mah *et al.*, 1977; Magrum *et al.*, 1978; De Rosa *et al.*, 1980), but during the past two decades, the perception of *Archaea* as 'extremophiles' has dramatically changed. It is now clear that these prokaryotes are ubiquitously distributed and are found in a variety of temperate environments, including soils (e.g. Bintrim *et al.*, 1997; Buckley *et al.*, 1998; Angel *et al.*, 2009; Bates *et al.*, 2010), marine systems (e.g. DeLong, 1992; Fuhrman *et al.*, 1992; Massana *et al.*, 2000; Wuchter *et al.*, 2006), lakes (e.g. MacGregor *et al.*, 1997; Schleper *et al.*, 1997; Casamayor *et al.*, 2000, 2001; Keough *et al.*, 2003) and rivers (e.g. Crump & Baross, 2000; Galand *et al.*, 2006). Moreover, the recent discovery of their role in ammonium oxidation (Könneke *et al.*, 2005; Wuchter *et al.*, 2006; Coolen *et al.*, 2007; Vissers *et al.*, 2013) has generated new scientific interest and completely altered our understanding of the functioning of the biogeochemical nitrogen cycle (Hatzenpichler, 2012).

Until now, four archaeal phyla have been described: *Euryarchaeota*, *Crenarchaeota* (Woese *et al.*, 1990), *Korarcheota* (Barns *et al.*, 1996) and, more recently, *Thaumarchaeota*, which includes all presently ammonia-oxidizing archaea (AOA) known so far (Brochier-Armanet *et al.*, 2008; Spang *et al.*, 2010). The emergence of culture-independent molecular techniques has led to the recent discovery of a hitherto unsuspected degree of phylogenetic diversity in *Archaea* living in aquatic ecosystems (Schleper *et al.*, 2005; Chaban *et al.*, 2006; Auguet & Casamayor, 2008; Lliros *et al.*, 2008; Casamayor & Borrego, 2009; Hertfort *et al.*, 2009; Auguet *et al.*, 2010, 2012). Freshwater ecosystems have even been identified as one of the largest potential reservoirs of archaeal diversity (Lliros *et al.*,

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2008; Auguet *et al.*, 2010). Among these inland aquatic ecosystems, planktonic archaeal diversity has been primarily described from lakes (Ovreas *et al.*, 1997; Jurgens *et al.*, 2000; Keough *et al.*, 2003). *Archaea* comprise 0.7–20% of the prokaryotic community in lakes (Pernthaler *et al.*, 1998; Jardillier *et al.*, 2005; Callieri *et al.*, 2009; Auguet *et al.*, 2012) and as much as 35% of the prokaryotic plankton in some high latitude and subalpine lakes (Auguet & Casamayor, 2008; Callieri *et al.*, 2009). Their abundance and specific richness have been found to vary between water layers (Ovreas *et al.*, 1997; Callieri *et al.*, 2009; Auguet *et al.*, 2012) and also over time (Lliros *et al.*, 2008; Auguet *et al.*, 2011). These findings suggest that archaeal communities exhibit complex spatio-temporal dynamics in lakes.

While studies investigating the dynamics and diversity of archaeal communities in freshwater lakes have become more numerous in the past decade (e.g. Casamayor *et al.*, 2001; Callieri *et al.*, 2009; Jiang *et al.*, 2009; Pouliot *et al.*, 2009; Auguet *et al.*, 2011, 2012; Vissers *et al.*, 2013), most of these studies have been restricted to a period ranging from several months to just over 1 year. Relatively long-term data (e.g. > 1 year) on the dynamics of archaeal communities in these ecosystems are still scarce (Lliros *et al.*, 2008; Vissers *et al.*, 2013). Moreover, until now, few studies have demonstrated, in a statistically robust way, the relationships between archaeal community dynamics and lacustrine parameters, so there is little information available on what factors drive the dynamics of these communities (Winter *et al.*, 2004; Auguet *et al.*, 2008, 2011; Erguder *et al.*, 2009; Hu *et al.*, 2010; Lliros *et al.*, 2010; Auguet & Casamayor, 2013; Zeng *et al.*, 2012). In particular, we know little about the influence of either physico-chemical or biological variables on the overall archaeal community. Yet such data are crucial to better understand the distribution and dynamics of these communities and, consequently, their role in the functioning of lacustrine ecosystems.

In this study, we conducted a 2-year survey (2007–2008) in two large and deep peri-alpine lakes with contrasting trophic states and environmental conditions (Lakes Annecy and Bourget, France). We carried out monthly sampling of both the epilimnion (depth: 2–3 m) and hypolimnion (depth: 45–50 m). We first examined temporal changes in archaeal community structure at these two depths using PCR-DGGE. Secondly, clone libraries were constructed from some samples collected during the mixing period and some from the stratification period. This yielded an overview of the main operational taxonomic units (OTUs) that mainly composed the archaeal communities in the two lakes. Finally, we performed a robust statistical analysis (CCA) to relate archaeal community distribution to environmental variables.

Materials and methods

Sampling strategy

Water samples were collected from the two largest peri-alpine lakes located in France: the mesotrophic Lake Bourget and the oligotrophic Lake Annecy (whose characteristics are available in Berdjeb *et al.*, 2011a). Samples were collected at the reference sampling station (referred to as point B for Lake Bourget and station GL for Lake Annecy) located above the deepest part of each lake. Samples were put into sterile polycarbonate bottles and kept in the dark at 4 °C until being processed immediately on return to the laboratory (e.g. within the next 5–6 h). For the purpose of this study, water samples were collected monthly during 2 years (2007–2008) in the two lakes. Each time, two depths, corresponding to both epilimnetic and hypolimnetic layers, were sampled (2 and 50 m in Lake Bourget, 3 and 45 m in Lake Annecy).

Physico-chemical variables

The total organic carbon (TOC) and dissolved inorganic nutrient concentrations [e.g. ammonium (NH_4-N), nitrates (NO_3-N) 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.afnor.org). 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 (DO) and chlorophyll *a* concentrations.

Assessment of the *in situ* microbial community dynamics

Abundance of virus-like particles (VLP), heterotrophic prokaryotes and small autotrophic eukaryotes (referred to as SMEUK) was measured by flow cytometry using the same protocol as described in the study by Personnic *et al.* (2009). For classical microscopic counts, flagellates and ciliates were prepared according to the protocol detailed in the study by Berdjeb *et al.* (2011a). Flagellates were examined with a Nikon Eclipse TE200 epifluorescence microscope, and ciliates were counted using an Olympus IX50 inverted microscope.

Archaeal community composition

The archaeal community composition was assessed using denaturing gradient gel electrophoresis (DGGE). *Archaea* were harvested from *c.* 250 mL water onto 47-mm-diam-

eter, 0.2-μm-pore-size polycarbonate white membrane filters (Nuclepore) after a prefiltration step through 2-μm-pore-size polycarbonate membrane filters (Nuclepore) to eliminate large eukaryotes and filamentous cyanobacteria. The filters were then stored at −80 °C until nucleic acid extraction. Nucleic acid extraction was performed as described by Dorigo *et al.* (2006) using phenol–chloroform. Molecular weight distribution and purity of the DNA were assessed by 1% agarose gel electrophoresis and quantified by both visual comparison with molecular weight markers in ethidium bromide–stained agarose gels (rough estimate) and optical density measurements using NanoDrop ND-1000 Spectrophotometer (Thermo Scientific). The extracted DNA was then stored at −20 °C until PCR amplification.

PCR and DGGE were performed following the recently developed protocol of Vissers *et al.* (2009) for freshwater samples but with some modifications. We performed nested PCR for DGGE with primer sets 21F-958R (21: 5′-TTC CGG TTG ATC CYG CCG GA-3′; 958: 5′-YCC GGC GTT GAM TCC AAT T-3′) and Parch519-Arch915 (519: 5′-CAG CCG CCG CGG TAA-3′; 915:5′-GTG CTC CCC CGC CAA TTC CT-3′ with a 40-bp GC clamp attached to the 5′ end). PCR conditions were as described in the study by Vissers *et al.* (2009), but PCR amplification was performed in a total volume of 50 μL containing 1× PCR buffer associated with 0.2 mM of each deoxynucleotide, 0.5 μM of each primer, 0.4 mg mL^{-1} of BSA, 1.25 U of Takara LA Taq (Takara Bio Inc.) and 50 ng of extracted DNA for the first PCR. The second PCR was performed using 40 ng of the product of the first PCR purified with the MinElute PCR purification kit (Qiagen). Reactions were performed using the PTC-100 thermocycler (MJ research) with the same temperature cycling than optimized by Vissers *et al.* (2009). Before performing DGGE, the presence of PCR products was determined by analysing 7 μL of products on 1% agarose gels. DGGE was carried out with an Ingeny PhorU-2 system using a 6% (wt/vol) polyacrylamide gel (40–80% gradient). The gel was run at 120 V for 16 h at 60 °C in TAE 1×. The gel was stained during 20 min using SYBR Gold (1/5000 final concentration), and bands were visualized under UV light and photographed using GelDoc (Bio-Rad). Gels were analysed using GelCompar II using a 2% tolerance for band's separation. It is noteworthy here that technical problems during the field campaign impeded the collection of samples for some dates (August and October 2007 at 3 m and 45 m in Lake Annecy; November 2007 and October 2008 at 2 m and April and July 2008 at 50 m in Lake Bourget).

Cloning and sequencing

To have an overview of OTUs that mainly composed the archaeal communities in the two studied lakes, eight samples were chosen and directly sequenced (four in each lake). These samples were chosen from both epilimnetic and hypolimnetic layers and were collected in the two lakes during both mixed and stratified periods (in February and September 2007 at 3 and 45 m in Lake Annecy and in March and July 2007 at 2 and 50 m in Lake Bourget). PCR products were cloned using an Invitrogen cloning kit (TOPO TA cloning) according to the manufacturer's instructions. For each sample, at least 24 positive clones (white colonies) were randomly selected, checked by PCR using the M13 commercial primer and finally sequenced (GATC Biotech). The sequences were then edited, aligned with Genedoc (K. B. Nicholas & H. B. J. Nicholas, http://www.nrbsc.org/gfx/genedoc/) and checked for chimeras using Bellerophon (Huber *et al.*, 2004) and the Ribosomal Database Project (RDP) (Cole *et al.*, 2005). OTUs were defined on the basis of a $\geq$ 98% sequence identity. Rarefaction curves were calculated using PAST software. The Chao1 and abundance-based coverage estimators of species richness were calculated using the software 'ESTIMATES' (http://viceroy.eeb.uconn.edu/estimates). Sequences were subjected to BLAST and the RDP database to determine the level of identity with other archaeal 16S rRNA gene sequences available in GenBank. A phylogenetic tree was constructed from all OTU sequences by neighbour-joining using MEGA5 software. The bootstrap option was used to run 1000 replicates.

Statistical analysis

Comparative analysis of DGGE profiles, based on both presence and intensity of bands, was carried out with the PRIMER 6 software (PRIMER-E, Ltd, UK) after transfer of GelCompare II data. To visualize the relationship between archaeal communities throughout the sampling period, ordination of Bray–Curtis similarities among normalized DGGE profiles was performed by hierarchical agglomerative clustering using unweighted pair group method with arithmetic averages (UPGMA). To test the null hypothesis that there was no significant difference between the groups discriminated according to the Bray–Curtis similarity index, 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; Clarke & Warwick, 2001). 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 archaeal community structure and measured environmental variables, a canonical correlation analysis (CCA) was performed using the software package XLSTAT-ADA. Different variables were submitted to the forward selection procedure, in which the statistical significance of the term was tested by the unrestricted Monte Carlo permutation test (999 permutations). Explanatory variables, with *P*-values larger than 0.05, were excluded from further analyses.

Variation partitioning was used to evaluate whether pure bottom-up variables affected the archaeal community independently of the effect of pure top-down variables. All explanatory variables were divided into three groups. Firstly, we separated the 'pure bottom-up' (1) effect as referring to the control by resources, including nutrients (nitrates, ammonium and orthophosphates). Secondly, we separated 'other physico-chemical variables' (2) (temperature, O_2, TOC, SiO_2 and Chl *a* considered here as organic matter quantity of autotrophic origin rather than a strict biological variable) from the pure bottom-up variables. We called 'environmental parameters' the compilation of pure bottom-up (inorganic nutrients) and other physico-chemical variables. Thirdly, we generated a set of variables related to pure top-down regulation, referring to the control by predators (3) [abundance of viruses, heterotrophic nanoflagellates (HNF) and ciliates]. Each group of explanatory variables was tested independently as well as in combination. Additionally, Spearman's rank pairwise correlations between the environmental variables mentioned above helped to determine their significance for further ecological analysis. Explanatory variables were added until further addition of variables failed to contribute to a significant improvement to the model's explanatory power.

Results

Spatio-temporal evolution of environmental and biological parameters

Figure 1 shows spatial and temporal changes in environmental conditions for the 2 years in the two lakes. Thermal stratification in both lakes was apparent from April and maintained until September. Temperature values in the hypolimnion were stable and never exceeded 7 °C, while those in the epilimnetic waters increased up to 25 °C in August 2008 (Fig. 1a). In the epilimnetic layer, DO concentrations peaked in spring at both years in Lake Bourget (exceeding 12 mg L^{-1}), while they remained relatively stable (around 10 mg L^{-1}) in Lake Annecy (Fig. 1b). In the hypolimnetic layer, DO concentrations in Lake Bourget were near those registered at 2 m and displayed the highest values between the end of winter and the early spring. A similar trend was observed in Lake Annecy at 45 m except that DO concentrations were much lower than at 3 m throughout the year 2007. Despite lower concentrations in Lake Annecy, temporal evolution of TOC presented relatively similar trends in the two lakes and was characterized by an increase in the epilimnion from spring (Fig. 1c). TOC concentrations in the epilimnion were often higher than in the hypolimnion except in winter where they displayed similar values ($\approx 2.1 \pm 0.2$ mg L^{-1} in Lake Bourget and 1.7 ± 0.1 mg L^{-1} in Lake Annecy). In the hypolimnion, TOC concentrations were quite stable during the 2 years in the two lakes. NO_3-N concentration was on average much higher in Lake Bourget (0.48 ± 0.2 mg L^{-1}) than in Lake Annecy (0.23 ± 0.1 mg L^{-1}). During the stratification period, a gradual consumption of dissolved NO_3-N was observed in the epilimnetic layer, for both lakes, whereas no seasonal variation was noticed in the hypolimnion (Fig. 1d). For NH_4-N concentrations, peaks (> 12 μg L^{-1}) appeared several times in spring and summer, at 2 m, followed by rapid consumption the month after (Fig. 1e) in Lake Bourget. The highest values of NH_4-N monitored at 50 m were obtained in January 2007 (12 μg L^{-1}) and April 2008 (17 μg L^{-1}), whereas they were very low for the rest of the year. In Lake Annecy, peaks of NH_4-N (> 6 μg L^{-1}) appeared at 3 m (at the end of winter and summer) followed by a rapid consumption the month after (Fig. 1e). The highest values of NH_4-N monitored at 45 m were observed in May 2007 (9 μg L^{-1}) and February 2008 (8 μg L^{-1}). Orthophosphate (PO_4-P) concentrations, in Lake Bourget, never exceeded 5 μg L^{-1} at 2 m, except for two peaks monitored in January 2007 (12 μg L^{-1}) and August 2008 (27 μg L^{-1}). At 50 m, PO_4-P exhibited four peaks higher than 8 μg L^{-1} with three of them recorded only in 2008 (March, June and October). In Lake Annecy, PO_4-P concentrations fluctuated between 0.5 and 5 μg L^{-1} at both depths, excluding the July's peak (11 μg L^{-1}), during 2008 in the hypolimnion (Fig. 1f). Silica (SiO_2) displayed high and temporally stable concentrations in the hypolimnetic layers (2.7 mg L^{-1} in Lake Bourget and 4.1 mg L^{-1} in Lake Annecy, Fig. 1g), whereas a marked seasonality was observed in the epilimnetic layers and seemed to be characterized by an increase during winter. At 2 m in Lake Bourget, significant differences in chlorophyll *a* (Chl *a*) concentrations were recorded between 2007 (0.9 μg L^{-1}) and 2008 (3.7 μg L^{-1}; Mann–Whitney U-test, $n = 12$, $P < 0.001$; Fig. 1h). At 50 m, Chl *a* concentration was

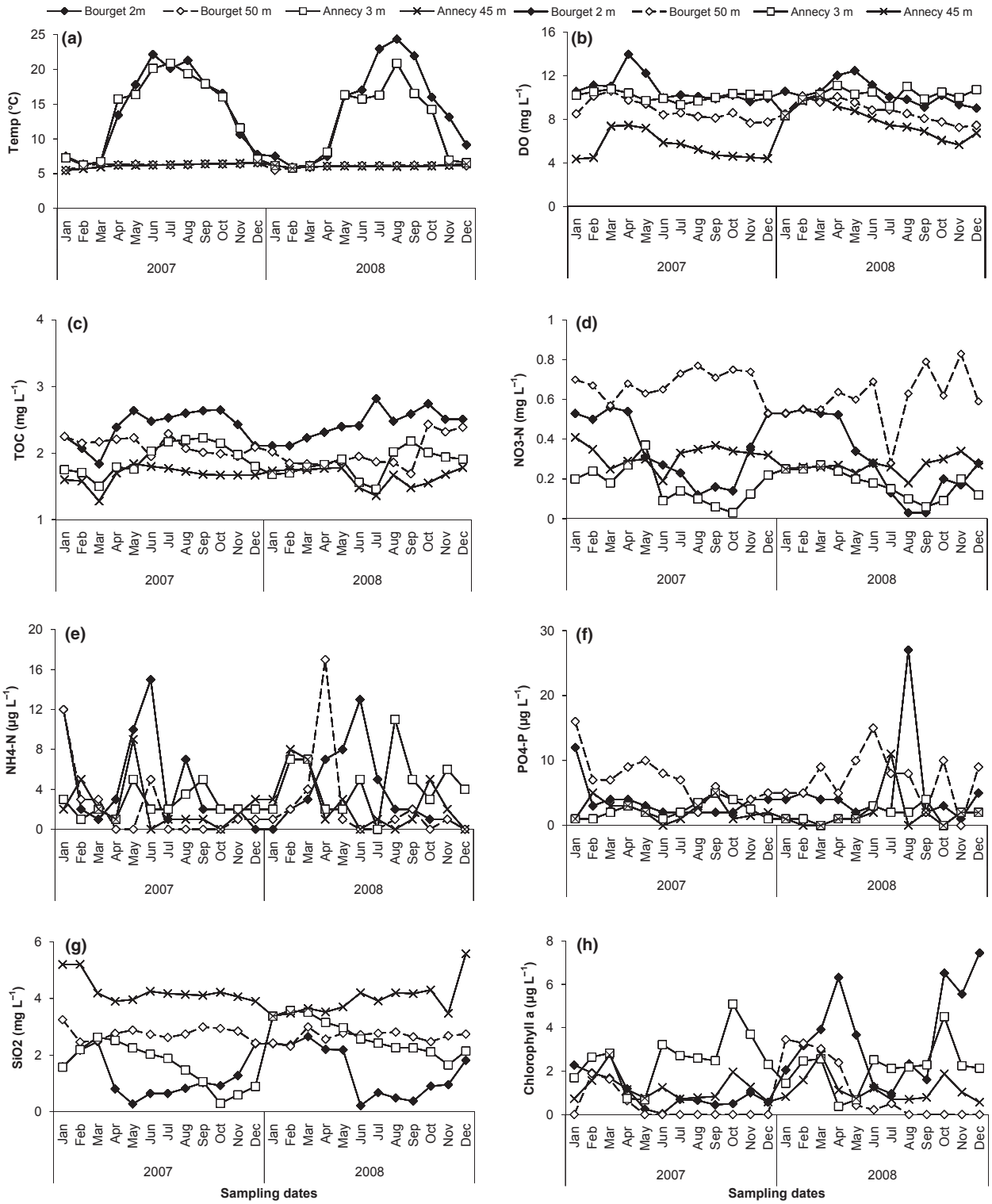

Fig. 1. Temporal evolution of physico-chemical characteristics of lakes Bourget (2 vs. 50 m) and Annecy (3 vs. 45 m). (a) Temperature (Temp, °C); (b) Dissolved Oxygen (DO, mg L^{-1}); (c) Total Organic Carbon (TOC, mg L^{-1}); (d) nitrates (NO_3-N, mg L^{-1}); (e) ammonium (NH_4-N, µg L^{-1}); (f) orthophosphates (PO_4-P, µg L^{-1}); (g) silicates (SiO_2, mg L^{-1}); (h) chlorophyll *a* (Chl *a*, µg L^{-1}).

over the detection limit (> 3.5 μg L^{-1}) only between January and April. Chl *a* concentrations in Lake Annecy displayed a high seasonality at both depths and during the 2 years, marked by three redundant peaks in March, June and October, respectively (Fig. 1h).

Small autotrophic eukaryotic (referred to as SMEUK) abundance was always higher in the epilimnetic layers (Fig. 2a). In Lake Bourget, we observed that mean values of SMEUK abundance were at least 10 times lower in 2007 than that in 2008 in both epilimnetic and hypolimnetic layers (5.36×10^3 and 4.93×10^4 cells mL^{-1} at 2 m in 2007 and 2008, respectively, 2.57×10^2 and 4.84×10^3 cells mL^{-1} at 50 m in 2007 and 2008, respectively). By contrast, in Lake Annecy, mean values of SMEUK abundance were similar from 1 year to another ($3.2 \pm 1.8 \times 10^3$ at 3 m and $6.8 \pm 7.7 \times 10^2$ cells mL^{-1} at 45 m, $n = 24$). Prokaryotic abundance generally displayed a strong temporal dynamics in the epilimnetic layer of Lake Bourget (between 0.4 and 6.5×10^6 cells mL^{-1}) unlike what was observed in Lake Annecy (between 1.5 and 3×10^6 cells mL^{-1}, except for the peak registered in February 2008; Fig. 2b). In the hypolimnion, heterotrophic

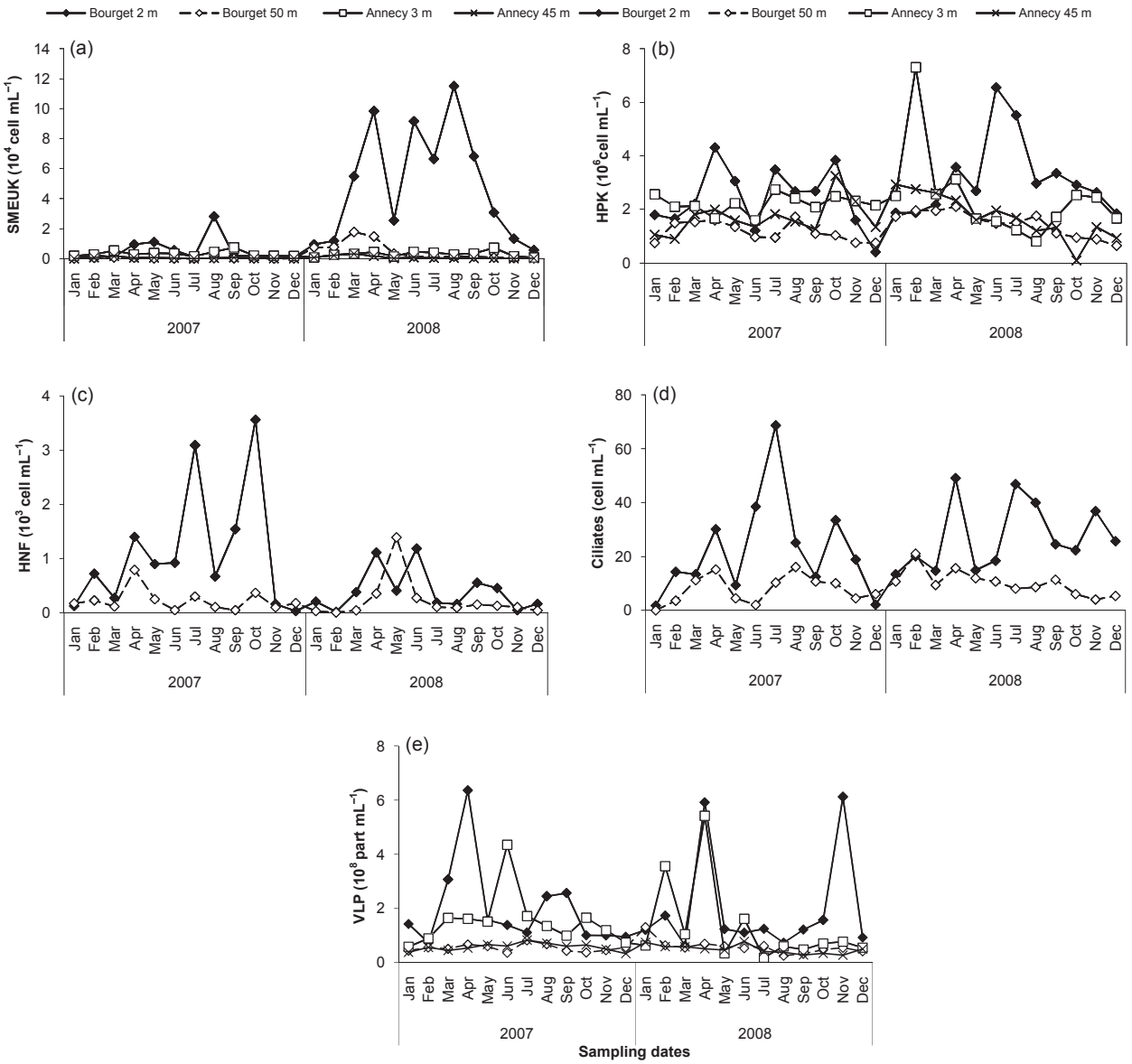

Fig. 2. Temporal dynamics of microbial community abundance in Lake Bourget (at 2 and 50 m) and in Lake Annecy (at 3 and 45 m). (a) small autotrophic eukaryotes (SMEUK, cell mL^{-1}); (b) heterotrophic prokaryotes (HPK, cell mL^{-1}); (c) heterotrophic nanoflagellates (HNF, cell mL^{-1}); (d) ciliates (cell mL^{-1}); (e) virus-like particles (VLP, part mL^{-1}). No data of flagellate and ciliate abundance data were available for Lake Annecy.

prokaryotes did not exceed 2×10^6 cell mL^{-1} in Lake Bourget and 3×10^6 cell mL^{-1} in Lake Annecy.

HNF and ciliates abundance (data only available from Lake Bourget) averaged $0.7 \pm 0.9 \times 10^3$ and 24.8 ± 16 cells mL^{-1}, respectively, at 2 m and $2.2 \pm 3 \times 10^2$ and 9 ± 5 cells mL^{-1}, respectively, at 50 m (Fig. 2c and d). At 2 m, most peaks in HNF and ciliates abundance coincided with those of heterotrophic prokaryotes. In both lakes and during the 2 years, VLP abundance displayed a strong seasonality in the epilimnetic layers characterized by several peaks (mainly in spring and autumn periods), contrary to the hypolimnetic layer (Fig. 2e) where concentrations remained relatively stable.

Archaeal community structure in Lake Bourget

Between 11 and 27 DGGE bands were detected per sample at 2 m in Lake Bourget (Supporting Information, Fig. S1). The DGGE profiles, obtained from samples taken at 50 m, displayed between 10 and 18 bands. From the UPGMA dendrogram, four different clusters were discriminated during the 2-year survey (ANOSIM, $R = 0.75$, $P = 0.01$), at 60% of similarity (Fig. 3a). The two first groups included almost all samples collected in 2007 (2 and 50 m, except February and March at 50 m). The first one incorporated the winter–spring samples at 2 m and all samples at 50 m, whereas the second included only summer–autumn samples at 2 m (except August 2007, which belong to the first group). The third group included almost all samples collected in 2008 at both depths (except August and September at 2 m). The sample collected in August 2008 at 2 m showed the most divergent archaeal structure in the Lake Bourget and was discriminated as the fourth group (Fig. 3a).

Globally, in 2007, significant differences in the archaeal community structure were observed between the upper and deeper waters from June to October (Fig. 3a). Conversely, in 2008, this structure was, in most cases, similar between the two depths except in August and September 2008. At the temporal scale, changes in the archaeal community structure in the epilimnetic layer varied from 1 to 5 months and from 1 to 7 months in 2007 and 2008, respectively (Fig. 3a). In the hypolimnion, this temporal evolution varied from 1 to 9 months in 2007 (between April and December 2007) and until 12 months in 2008 (between January and December 2008).

Archaeal community structure in Lake Annecy

At 3 m in Lake Annecy, the number of DGGE bands varied between 9 and 22 per sample (Fig. S2). At 45 m, the number of bands per sample varied from 7 to 16. According to the UPGMA dendrogram, seven different clusters (ANOSIM one factor, $R = 0.84$, $P = 0.01$) characterized the temporal dynamics of the archaeal community structure in Lake Annecy, at 50% of similarity (Fig. 3b). Note that groups including April 2007 and May–June–September 2007 at 3 m presented the most divergent archaeal structures (< 30% of similarity). Globally, in 2007, significant differences in the archaeal community structure were observed between the upper and deeper waters from March to September (Fig. 3b). Conversely, in 2008, this structure was, in most cases, similar between the two studied depths except in September (3 m) and December (45 m). At the temporal scale, changes in the archaeal community structure in the epilimnetic layer of the Lake Annecy varied from 1 to 2 months and from 1 to 8 months in 2007 and 2008, respectively (Fig. 3b). In the hypolimnion, this temporal evolution varied from 1 to 7 months and from 1 to 9 months in 2007 and 2008, respectively.

Archaeal community composition assessed by cloning–sequencing

The eight clone libraries (from both lakes), performed on samples in 2007, yielded 197 archaeal sequences corresponding to 11 different OTUs (GenBank accession numbers: from KC797158 to KC797168) with ≥ 98% sequence identity. The rarefaction curves and chao1 values highlighted that we did not obtain a sufficient number of sequences to detect the whole archaeal OTU richness in our samples. It was the case for samples from Lake Annecy in February and September at 3 m (Fig. S3). For the other samples, the sampling effort seemed to be high enough to recover all the archaeal diversity. As only one or two OTUs were found among all the sequences, this last result did not match with the results obtained by the DGGE approach that always revealed several bands per sample. These contradictory results between the two approaches could suggest that the number of clones analysed was not enough to recover all the archaeal diversity. As shown by the phylogenetic tree (Fig. 4), all archaeal OTUs found in our lakes belonged to the thaumarchaeotal phylum. Among the 11 archaeal OTUs, only two (OTU1 and OTU2), belonging to the thaumarchaeotal group 1.1a, represented more than 5% of the archaeal sequences and appeared in at least two samples (Fig. 5). The nine others often represented < 5% of the sequences and/or were always found in only one sample. The OTU1 was found in all sequenced samples and represented at least 40% of the total sequences obtained (Fig. 5). It displayed a high degree of similarity (99%) with archaeal sequences found in many different aquatic environments including lakes, reservoirs, deep sea hydrothermal plumes or again the oxygen minimum zone of the subarctic

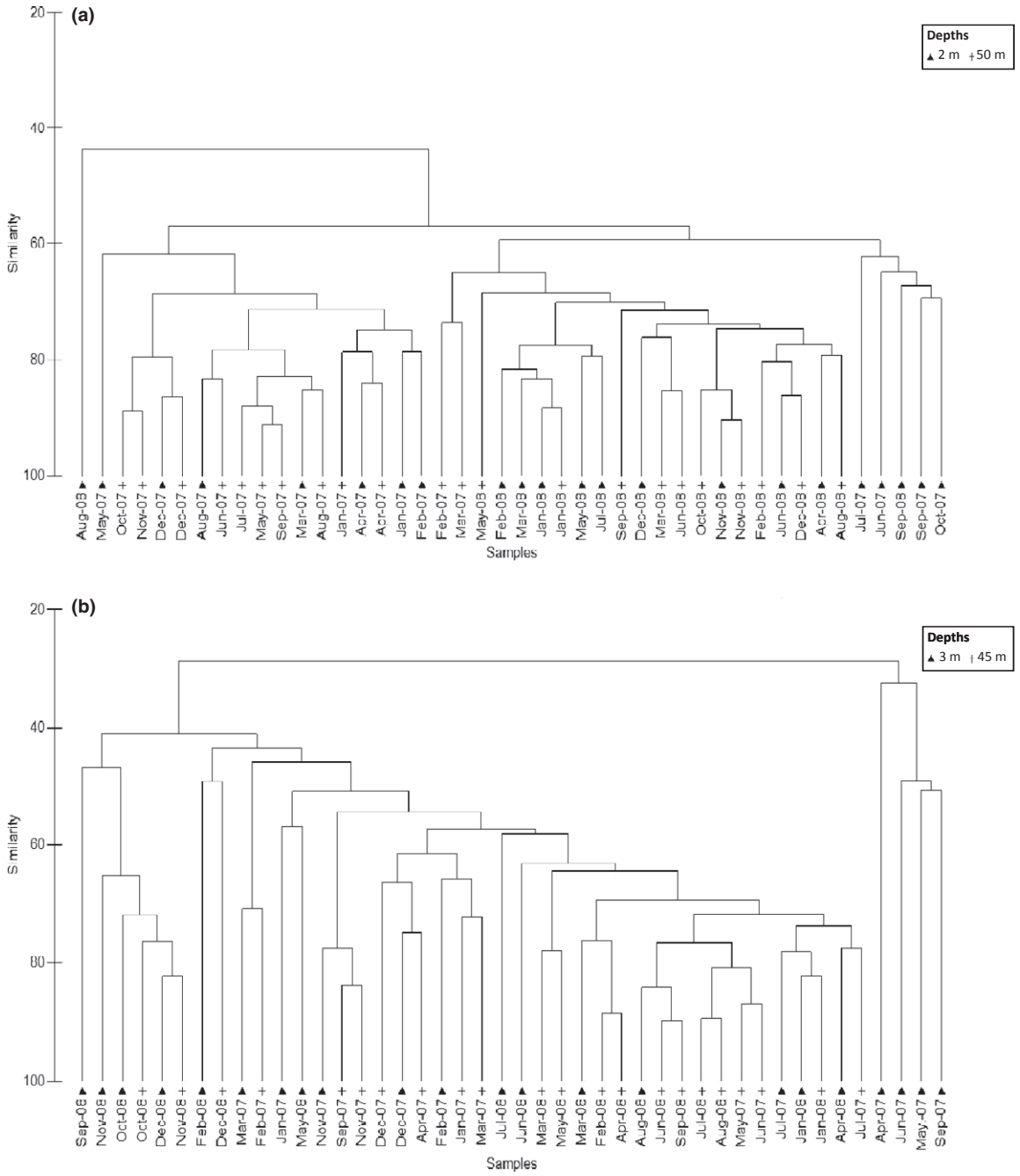

Fig. 3. Dendrograms obtained by UPGMA clustering of DGGE banding patterns from lakes Bourget (a) and Annecy (b). Similarity is expressed as a percentage of the Bray–Curtis index.

Pacific Ocean (Fig. 4). This OTU was assigned (99% of similarity) to the 16S rRNA gene sequence of *Nitrosopumilus maritimus*. The OTU2 showed, as OTU1, a high degree of similarity (99%) with ammonia-oxidizing archaeal sequences found in different environments such as lakes and soils (Fig. 4).

Archaeal community structure in relation to environmental and biological variables

The complex influence of environmental and biological parameters on changes in archaeal community structure was statistically demonstrated using direct multivariate

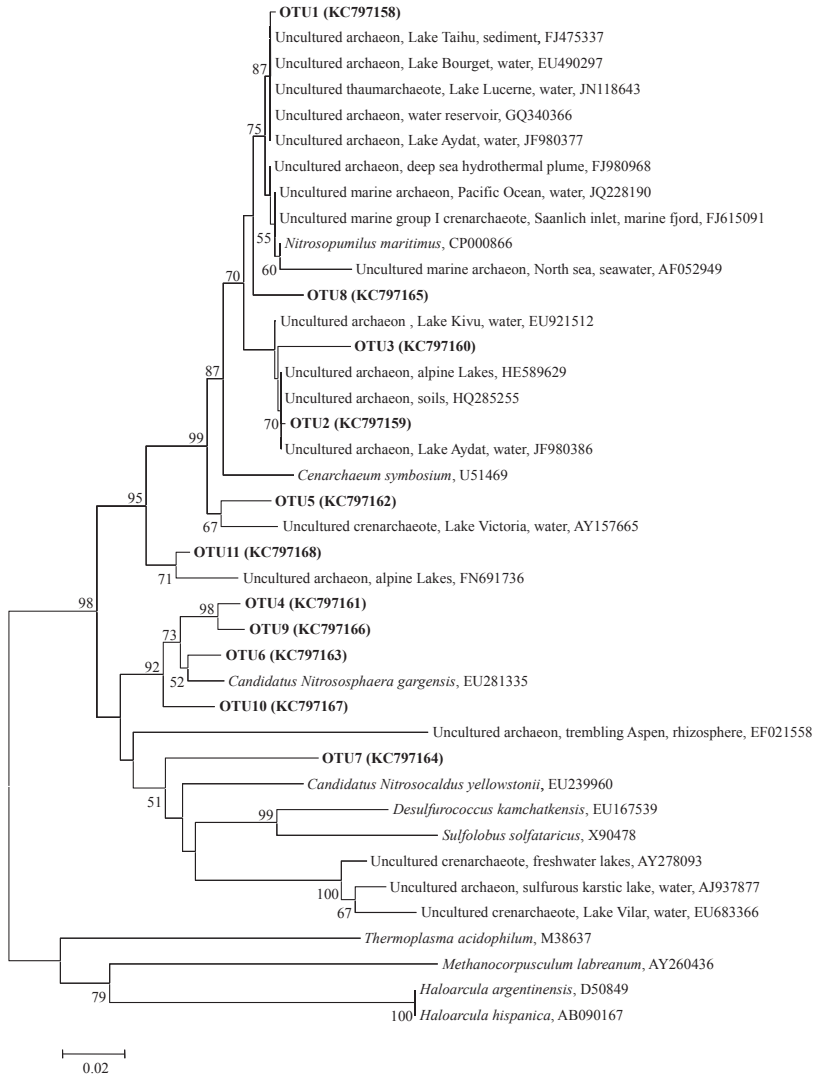

Fig. 4. Phylogenetic tree of archaeal 16S rRNA gene as retrieved by nested PCR amplification and gene cloning–sequencing of samples of both lakes Annecy and Bourget.

gradient analyses. We first performed CCA using both environmental parameters and biological counts as constrained variables of the temporal changes in archaeal community structure in Lake Bourget (Fig. 6a and b). Note here that, at 2 m, TOC was excluded of the CCA due to its strong correlation with temperature and NO_3-N concentrations ($R^2 = 0.76$, $n = 24$, $P < 0.01$ and $R^2 = 0.83$, $n = 24$, $P < 0.01$, respectively). According to CCA analysis, 51.9% (ratio between the sum of all canonical eigenvalues and the total inertia) of the variance of the archaeal community structure was explained by a combination of environmental (temperature, Chl *a*, NH_4-N, NO_3-N and PO_4-P) and biological (SMEUK, HNF, ciliates and VLP) factors at 2 m (Fig. 6a), as indicated by the ratio between the sum of all canonical eigenvalues and the total inertia (Table S1). At 50 m, temperature, DO, TOC, NH_4-N, NO_3-N and PO_4-P coupled with SMEUK, ciliates and VLP counts variables explained 63.9% of the variance of the archaeal community structure (Fig. 6b, Table S1). Monte Carlo test for first and all canonical axes was highly significant ($P < 0.01$), indicating that the parameters selected were good explanatory variables of the archaeal community structure. The cumulative percentage of variance of the species–environment relationship indicates that the first and second canonical axes accounted for 29.4% and 23.4% of this variance, respectively, at 2 m and for 35.1% and 19.3% of this variance, respectively, at 50 m (Fig. 6a and b). Subsequent axes accounted for < 17% of the variance each and are not considered further here. At 2 m, the first canonical axis was positively correlated with temperature, ciliates, HNF and VLP and negatively correlated with NO_3-N concentrations (Fig. 6a). The second canonical axis, which seemed to define a clear separation in archaeal community structure between 2007

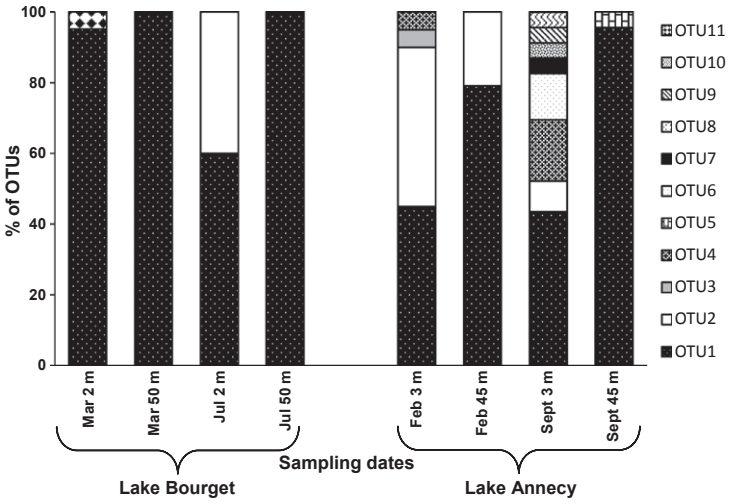

Fig. 5. Changes in the relative proportions (%) of archaea operational taxonomic units (OTUs) detected in March and July 2007 at 2 and 50 m in Lake Bourget and in February and September 2007 at 3 and 45 m in Lake Annecy.

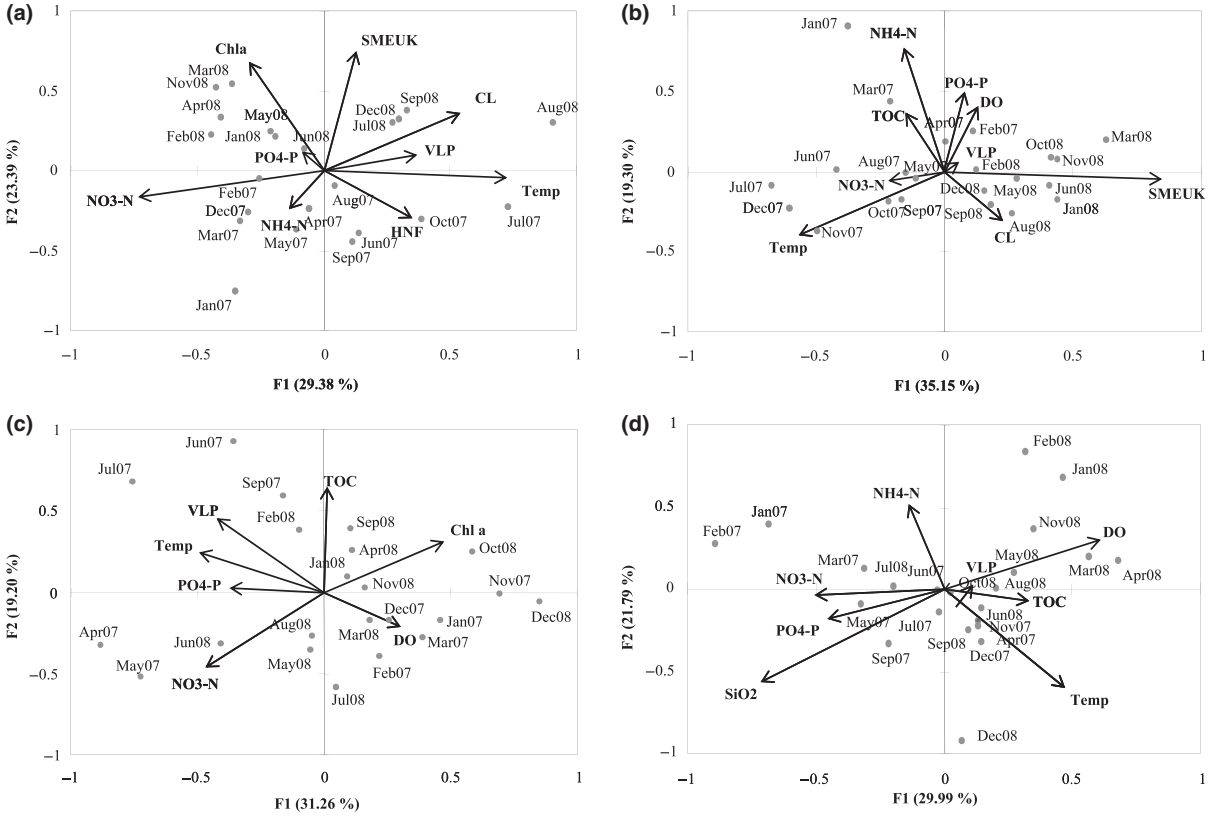

Fig. 6. Canonical correspondence analysis of archaeal community structure from samples in Lake Bourget at 2 m (a) and 50 m (b) and Lake Annecy at 3 m (c) and 45 m (d), using physicochemical and biological parameters. Arrows point in the direction of increasing values of each variable. The length of the 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 structure is influenced by the environmental parameter represented by that arrow. Chl *a*, chlorophyll *a*; Temp, temperature; DO, dissolved oxygen; TOC, total organic carbon; PO_4-P, orthophosphates; NH_4-N, ammonium; NO_3-N, nitrates; SiO_2, silicates; SMEUK, small autotrophic eukaryotes; HNF, heterotrophic nanoflagellates; CL, ciliates; VLP, virus-like particles.

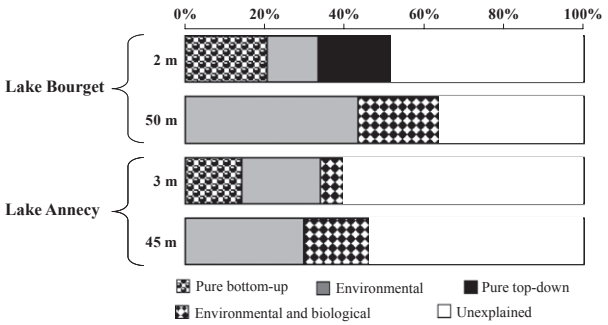

Fig. 7. Variation partitioning analysis of data sets from lakes Bourget (2 and 50 m) and Annecy (3 and 45 m). Pure bottom factors were nutrients (ammonium, nitrates, orthophosphate). Environmental factors included both physico-chemical and pure bottom-up variables. In Lake Bourget, physico-chemical variables were temperature, chlorophyll *a* for 2-m samples and temperature, DO and TOC for 50-m samples. Biological factors: small autotrophic eukaryotes, ciliates, heterotrophic flagellates and viruses for 2-m samples and small autotrophic eukaryotes, ciliates and viruses for 50-m samples. Pure top-down: ciliates, heterotrophic flagellates and viruses for 2-m samples. In Lake Annecy, physico-chemical variables were temperature, DO, TOC, chlorophyll *a* for 3 m samples and temperature, DO, TOC, for 45 m samples. Biological factors included only viruses at both depths.

and 2008 (Fig. 6a), was positively correlated with Chl *a* and SMEUK. At 50 m, the first canonical axis was positively correlated with SMEUK and ciliates and negatively correlated with temperature and to a lower extent with NO_3-N concentrations (Fig. 6b). The second canonical axis was rather positively correlated with NH_4-N, DO and PO_4-P.

In Lake Annecy, the CCA analysis was performed by considering the environmental parameters and VLP as constrained variables of the temporal changes in archaeal community structure. According to CCA analysis, 40.0% of the variance of the archaeal community structure was explained by a combination of environmental (temperature, DO, TOC, Chl *a*, NO_3-N and PO_4-P) and biological (VLP) factors at 3 m (Fig. 6c), as indicated by the ratio between the sum of all canonical eigenvalues and the total inertia (Table S1). At 45 m, environmental factors (temperature, DO, TOC, SiO_2, NO_3-N, NH_4-N and PO_4-P) coupled with VLP explained 46.3% of the variance of the archaeal community structure (Fig. 6d, Table S1). Monte Carlo test for first and all canonical axes was highly significant ($P < 0.01$), indicating that the parameters selected were good explanatory variables of the archaeal community structure. Note here that SMEUK abundance was excluded of the CCA due to the fact that their addition made the CCA not significant for the both depths. The cumulative percentage of variance of the species–environment relationship indicates that the first and second canonical axes accounted for 31.2% and 19.2% of this variance, respectively, at 3 m and for 29.9% and 21.8% of this variance, respectively, at 45 m (Fig. 6c and d). Subsequent axes accounted for < 13% of the variance each and are not considered further here.

According to the partitioning analysis (Fig. 6 and Table S1), environmental factors (pure bottom-up and physico-chemical parameters) explained between 29.8% and 43.3% of the total variance in the four samples fitted for diversity (two depths × two lakes; Fig. 7 and Table S1). Between five and seven variables were included in the set of environmental parameters selected by the forward selection (Fig. 6). None of the models including pure bottom-up parameters only (nutrients) explained significantly the variation in the hypolimnion of both Lakes, while these parameters explained 20.6% of the total variance at 2 m in Lake Bourget and 14.1% at 3 m in Lake Annecy. In the Lake Bourget, pure top-down control explained significantly 18.5% of the total variation at 2 m, while it was no significant at 50 m. Finally, variation partitioning indicated that, in average, 49.5% (SD = 10, $n = 4$) of the observed variance in the temporal dynamics of archaeal community structure in both lakes remained unexplained (Fig. 7).

Discussion

We used a nested PCR-DGGE approach to characterize the spatio-temporal dynamics of archaeal communities in Lakes Annecy and Bourget. We are aware that such a two-step PCR may introduce more bias than a one-step PCR and limit the representativeness of our results for archaeal specific richness. It is also true that cloning and sequencing (or pyrosequencing) would have provided more in-depth data on archaeal community composition in the two lakes. However, due to the high number of samples obtained, we decided that these last methods were not the best options. Furthermore, nested PCR has already been used in many studies dealing with the dynamics and structure of microbial communities such as *Archaea* (Vissers *et al.*, 2009) and *Bacteria* (Boon *et al.*, 2002; Dar *et al.*, 2005; Giloteaux *et al.*, 2010). Most of the authors of these studies asserted that the added PCR step did not change either the number or the intensity of DGGE bands compared with the one-step PCR (e.g. Boon *et al.*, 2002; Dar *et al.*, 2005). Likewise, Vissers *et al.* (2009), using the same fingerprinting approach to study archaeal community composition in lakes, demonstrated that the archaeal sequences obtained in direct PCR were also recovered from nested reactions. This indicates that the nested approach does not miss any archaeal sequence found with one-step PCR. Furthermore, the above-cited

researchers showed that the specificity of the nested PCR was much higher than that of the direct method. Consequently, we concluded that using the nested PCR prior to DGGE was a reliable and convenient method for analysing archaeal structure in Lakes Annecy and Bourget.

Archaeal community composition

Even though the number of clones we analysed was low, the sequencing of a restricted number of samples allowed us to obtain a first overview of the OTUs that mainly composed the archaeal communities in the two lakes. The phylogenetic tree of the OTUs as identified from cloning–sequencing (Fig. 4) shows that the archaeal sequences belong to the thaumarchaeotal group. This is similar to the findings of Vissers *et al.* (2013) for another perialpine lake (Lake Lucerne, Switzerland). The most represented OTUs (OTU1 and 2) were affiliated with other thaumarchaeotal sequences within the group 1.1a. Their presence in a range of environments, including lakes, marine ecosystems and soils, underlines their ubiquity and suggests they possess physiological capabilities that enable them to develop and grow in a wide range of conditions. Moreover, their high incidence in the archaeal communities of lakes Annecy and Bourget suggests, indirectly, that AOA may play a significant role in the nitrification process in these two lakes. However, this hypothesis will need to be confirmed by quantifying the specific abundance of AOA or the expression of the *amoA* gene.

Patterns in archaeal community structure

Few studies have assessed interannual archaeal structure dynamics in lacustrine ecosystems. To the best of our knowledge, only one study has specifically examined archaeal community dynamics over several years ($\geq$ 2 years) in lakes (Lliros *et al.*, 2008), and this study used only limited sampling (e.g. $<$ 4 samplings per year). Thanks to monthly sampling over two consecutive years, and our study shows that archaeal community dynamics are, in fact, not reproducible from 1 year to the next in either time or space. Our results therefore call for caution when drawing conclusions about patterns in archaeal community structure in lakes, particularly when the ecosystems are sampled over only 1 year (or less). In fact, to fully understand community dynamics and put exceptional events into perspective, it may be necessary to conduct much longer surveys ($\geq$ 3 years).

We observed strong differences in archaeal community structure during the thermal stratification in 2007, with richness (e.g. number of DGGE bands) higher in the epilimnetic layer. These vertical differences are in accordance with the observations of Lliros *et al.* (2008), Pouliot *et al.* (2009) and, more recently, those of Auguet *et al.* (2012), both of whom found pronounced differences in archaeal community composition along depth. However, two of these studies were carried out in meromictic lakes, where the redox gradient seems to be the main factor responsible for the vertical differentiation observed in these communities (Lliros *et al.*, 2008; Pouliot *et al.*, 2009). In our study, the epi- and hypolimnetic layers were continuously oxygenated over the 2 years (Fig. 1), suggesting that some additional environmental factors shape the archaeal community structure along the vertical scale. Interestingly, we did not find, in the epi- and hypolimnetic layers of the two lakes, seasonal patterns that consistently repeated themselves from 1 year to the next. We found that the archaeal community structure exhibited pronounced temporal shifts on a monthly basis in both the epilimnetic and hypolimnetic layers in addition to very long steady-state periods (lasting up to 12 months in the hypolimnetic layer of Lake Bourget). Furthermore, temporal variation in the banding patterns of samples from the hypolimnetic layers of the two lakes were, in most cases, lower than those of samples from the epilimnetic layers. All of these observed differences between the upper and deeper layers may be due to vertical habitat heterogeneity, suggesting that the factors and processes that drive archaeal community structure vary between layers.

Key environmental factors

The effects of physico-chemical variables on archaeal community structure were significant for all analyses performed (Fig. 7). Temperature and DO, already known to have significant effects on archaeal communities (Pouliot *et al.*, 2009; Auguet *et al.*, 2011), had a strong effect on the spatio-temporal dynamics of archaeal communities in lakes Annecy and Bourget. Chlorophyll *a* concentrations were significantly linked to archaeal community dynamics in the epilimnetic layers (Fig. 6a and c). Due to their autotrophic activity, we could suppose that AOA (the most frequently occurring archaeal group in the two lakes) are outcompeted for resources by other autotrophs (Murray *et al.*, 1998). However, due to the ability of some AOA to take up organic carbon compounds (Ingalls *et al.*, 2006; Prosser & Nicol, 2012; Stahl & de la Torre, 2012), it is also possible that this archaeal group could use autotrophic matter as an organic carbon source. According to our CCA analysis, and as previously observed for bacterial communities (e.g. Jardillier *et al.*, 2005; Berdjeb *et al.*, 2011a, b), inorganic nutrients were also significant parameters in the temporal dynamics of archaeal communities in both lakes. For the epilimnetic layers, 20.6% (Lake Bourget) and 14.1% (Lake Annecy)

of the temporal variability were explained by nutrient concentrations alone, whereas in the hypolimnion, the model testing different combinations of nutrient concentrations alone did not give any significant explanation of the temporal changes in the archaeal community structure (Fig. 7). This may be explained by the different metabolic pathways between epilimnetic and hypolimnetic archaeal communities, as it has already been shown for deep marine ecosystems (Ingalls *et al.*, 2006; Agogué *et al.*, 2008; Varela *et al.*, 2011). Our results emphasize, once again, the importance of nutrient concentrations to prokaryote distribution in the surface waters of peri-alpine lakes. However, in contrast to bacterial communities, the mechanisms whereby these nutrients determine archaeal community structure are still unclear, although some studies have demonstrated that *Archaea* may act as chemolithotrophs using ammonia (Francis *et al.*, 2005; Könneke *et al.*, 2005; Beman *et al.*, 2008) or other reduced inorganic compounds (Auguet *et al.*, 2008) as their energy source. Our study also shows that in the epilimnetic layer, potential mortality agents such as viruses, HNF and ciliates (top-down factors) may play a significant role in determining archaeal community structure, explaining up to 18.5% of temporal variability in archaeal community structure (Figs 6a and b and 7). To the best of our knowledge, no information is currently available on the actual grazing impact of HNF and ciliates on archaeal community structure and composition. Only Bonilla-Findji *et al.* (2009) have hypothesized a potential effect of flagellate grazing on archaeal community composition. Our results suggest, however, that these microbial components may be an important factor in shaping archaeal distribution (Fig. 6), suggesting that it would be useful in future research to estimate the grazing rate of these potential predators on archaeal communities. Viruses may also influence archaeal community dynamics through their lytic activity, as previously reported by Winter *et al.* (2004). These authors showed that archaeal OTUs exhibited variable responses to the presence of viruses and concluded that the individual members of pelagic archaeal communities may be affected by virioplankton to varying degrees, leading to changes in community structure.

Interestingly, the spatio-temporal dynamics of archaeal communities appear to display similar trends to those observed for lacustrine bacterial communities (Berdjeb *et al.*, 2011a, b and references therein), suggesting, as previously proposed by Auguet *et al.* (2010), that the same types of factors control the distribution of all 'prokaryotes'. However, using direct gradient multivariate ordination analyses, our previous study showed that 70–90% of temporal variance of bacterial communities in lakes Annecy and Bourget could be explained by a combination of biotic and abiotic parameters (Berdjeb *et al.*, 2011a). However, these same factors explained, in most cases, $< 52\%$ of temporal variance in archaeal communities in the two lakes, suggesting that bacterial and archaeal assemblages may occupy different ecological niches.

Despite the high number of variables considered in this study, our variation partitioning revealed that between 36% and 60% of the temporal change in archaeal community structures remained unexplained. However, we did not take into account pH, sulphide, solar irradiance (day length) and UV, all variables that are known to, or deemed likely to, affect the dynamics and/or metabolism of archaeal communities (Murray *et al.*, 1998; Erguder *et al.*, 2009; Pagaling *et al.*, 2009; Ajon *et al.*, 2011). These variables, known to vary greatly, particularly in the epilimnetic layers of lakes, may account for some of the unexplained variation in archaeal community structure that we found, and so we will take these variables into account in future studies. Nevertheless, our findings do clearly support the assertion that in deep freshwater lakes, a complex combination of environmental and biological factors, including temperature, O_2, inorganic nutrients, TOC and Chl *a*, viruses, HNF and ciliates, drive the distribution of archaeal communities. The observed significant associations among a variety of complex variables, both biotic and abiotic, suggest there may be a large number of possible ecological mechanisms responsible for archaeal distribution patterns. It will be the task of future studies to tease apart these mechanisms.

Acknowledgements

L. Berdjeb and T. Pollet were granted for their PhD by a French-Algerian cooperation fellowship completed by INRA and by a French Ministry research doctoral grant, respectively. This study is a contribution to the French ANR AQUAPHAGE project.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. DGGE band profiles of archaeal 16S rRNA gene for samples obtained from 2007 (a) and 2008 (b) in Lake Bourget.
Fig. S2. DGGE band profiles of archaeal 16S rRNA gene for samples obtained from 2007 (a) and 2008 (b) in Lake Annecy.
Fig. S3. Rarefaction curves and Chao1 values obtained from each sequenced sample in the two studied lakes.
Table S1. Summary of results from canonical correspondence analyses of the archaeal community structure data when constrained by environmental and biological factors at 2 and 50 m in Lake Bourget and at 3 and 45 m in Lake Annecy.