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Contrasting temporal patterns in ammonia-oxidizing archaeal community dynamics in two peri-alpine lakes with different trophic status

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ABSTRACT: We studied the spatiotemporal dynamics of ammonia-oxidizing archaea (AOA) in 2 large, deep peri-alpine lakes (Annecy and Bourget; oligotrophic and mesotrophic, respectively) over 2 yr. Each month, we characterized the structure, richness, and abundance of AOA populations in the epi- and hypolimnion using DGGE and qPCR of the archaea-specific *amoA* gene. Clear vertical patterns were observed in both lakes, with greater values for AOA richness and *amoA* gene abundance in the hypolimnetic layers. AOA community composition and structure was much more stable throughout the year in Lake Bourget than in Lake Annecy. In the upper layers, AOA communities displayed seasonal succession patterns and had greater abundance in winter. The temporal structure showed more pronounced seasonal patterns in Lake Annecy than in Lake Bourget. In the deeper layers of both lakes, the relative abundance of AOA showed no clear temporal pattern. Temporal changes in *amoA* gene composition were correlated with changes in the archaeal 16S rRNA gene in surface waters. Changes in the structure of both genes were not significantly correlated in the hypolimnion, suggesting that the temporal changes in the structure of archaeal communities in the deeper waters might be globally driven by the dynamics of heterotrophic archaea and not archaeal ammonia oxidizers. None of the many environmental variables we measured explained significant amounts of variation in AOA community structure and richness; thus, other factors and processes may exert selective pressures on the structure of these communities. Nevertheless, archaeal *amoA* gene abundance varied with water temperature (negative correlation) and with nitrate concentration (positive correlation). Our study is the first to show that archaeal *amoA* gene abundance is correlated positively with silica and negatively with total nanoflagellate abundance. This suggests that AOA could play a significant role in silica dissolution and regeneration, and points to the possible influence of predation on AOA abundance.

KEY WORDS: Lakes · Ammonia-oxidizing archaea · *amoA* gene · Dynamics · Community structure · Driving factors

INTRODUCTION

At the dawn of the 21st century, anthropogenic activities have considerably altered the nitrogen cycle, increasing the availability of nitrogen compounds in natural ecosystems (Vitousek et al. 1997, Carpenter et al. 1998, Howarth et al. 2000, Galloway & Cowling 2002). Nitrification represents one of the 3 main pathways involved in the production of nitrous oxide (Firestone & Davidson 1989), which is one of the 3 most important greenhouse gases. In the current context of global warming, it is crucial that we better understand the ecology and dynamics of microbial communities involved in the nitrification process.

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The first step of nitrification is the oxidation of ammonia to hydroxylamine, a process that is catalyzed by the protein ammonia monooxygenase, which is synthetized by the expression of the *amoA* gene (Hooper et al. 1997). Historically, ammonia oxidation had only been attributed to bacteria, and gene coding for the amoA enzyme to certain bacterial groups such as *Gamma*- and *Betaproteobacteria*. However, the discovery that this gene is present in the genome of some natural archaeal groups (Venter et al. 2004, Treusch et al. 2005, Könneke et al. 2005, Wuchter et al. 2006, Coolen et al. 2007) has drastically changed the perception of the role of archaea in the nitrification process. The importance of ammonia-oxidizing archaea (AOA) in this biogeochemical process is now established and several studies have even shown that AOA generally outnumber ammonia-oxidizing bacteria (AOB) in a variety of ecosystems, including marine waters (e.g. Bouskill et al. 2012, Zhang et al. 2015), soil (Leininger et al. 2006, He et al. 2007, Taylor et al. 2012) and lakes (Vissers et al. 2013a, Bollmann et al. 2014). A thorough understanding of the role of AOA communities requires knowledge of their spatiotemporal distribution and information on the environmental factors that drive community dynamics. Since the discovery of the critical role of AOA in the nitrification process, a number of studies have been published on the community dynamics of these microbes in marine waters (Francis et al. 2005, Church et al. 2010, Galand et al. 2010, Luo et al. 2014). In lakes, studies dealing with spatiotemporal changes in the distribution of AOA communities are more recent, appearing only since 2009 (Pouliot et al. 2009, Callieri et al. 2009, Llirós et al. 2010, Auguet et al. 2011, 2012, Vissers et al. 2013a,b, Restrepo-Ortiz et al. 2014, Coci et al. 2015). These recent investigations have shown similar trends in terms of spatiotemporal patterns but also great differences between lakes, revealing the complex ecology of AOA in these ecosystems. In general, studies have revealed temporal and vertical patterns in AOA community dynamics, with marked seasonal patterns and increasing abundance and diversity with depth. Mean values for archaea-specific *amoA* gene abundances can vary greatly between lakes (Hayden & Beman 2014), and community dynamics can be influenced by a host of ecological factors, including lake typology, altitude and physical characteristics like temperature, conductivity and nitrogen levels (Auguet et al. 2011, Vissers et al. 2013a,b, Hayden & Beman 2014).

While these studies have provided some interesting data on AOA dynamics in lakes, many examined only intra-annual variation and few compared AOA distribution in several deep lakes with contrasting trophic status. Our previous inter-annual analysis of the whole archaeal community in 2 contrasting deep freshwater lakes, Annecy and Bourget (oligotrophic and mesotrophic, respectively), revealed that members of AOA dominate the archaeal community (Berdjeb et al. 2013). Based on this first investigation and its results, we decided to further explore the spatiotemporal dynamics of AOA in order to better understand AOA distribution patterns and the relationships between environmental factors and AOA communities in lakes. This new study addresses inter-annual AOA community dynamics in the same lakes over 2 consecutive years and is the first to cover such a long time period. We measured many ecological variables (physical, chemical and biological) to better understand the ecology of these microbial communities and to determine what ecological factors influence their dynamics. We used specific ammonia-oxidizing archaeal primer sets in denaturing gradient gel electrophoresis (DGGE) and quantitative real time PCR (qPCR) approaches to analyze AOA community structure in Lakes Annecy and Bourget (see Berdjeb et al. 2011a,b for lake characteristics). We hypothesized that similar temporal patterns would be observed in both lakes, with a clear seasonality in the epilimnion and less clear temporal pattern in the deeper waters. We expected to find a marked vertical structure in both lakes, likely with higher AOA richness and *amoA* gene abundance in the deeper water. We finally hypothesized that temperature and ammonium are the main factors driving the spatiotemporal dynamics of AOA in these lakes.

MATERIALS AND METHODS

Study sites and samples

Water samples were collected from 2 deep perialpine lakes in France: the mesotrophic Lake Bourget and the oligotrophic Lake Annecy, whose characteristics are available in Berdjeb et al. (2011a,b). As previously described (Berdjeb et al. 2013), samples were collected at the reference sampling station of each lake, located above the deepest part of the lake. Samples were put into sterile polycarbonate bottles and kept in the dark at 4°C until being processed immediately upon return to the laboratory. Water samples were collected monthly during 2 yr (2007 and 2008) in the 2 lakes; each time 2 depths were sampled, corresponding to both epi- and hypolimnetic layers (i.e. 2 and 50 m in Lake Bourget, 3 and 45 m in Lake Annecy).

Environmental and biological variables

We estimated the most important physico-chemical characteristics of both lakes for the C, N, S and P biogeochemical cycles. Total organic carbon and nutrient concentrations (total nitrogen, NH_4^+, NO_3^-, SiO_2, $PO4_3^-$, and total phosphorus) were measured at each station and date, as previously described and shown in Berdjeb et al. (2011a,b, 2013). A conductivity-temperature-depth measuring device (CTD; Seabird SAB 19 Seacat profiler) and a chlorophyll fluorescence fluoroprobe (BBE Moaldenke) were used to obtain vertical profiles of water temperature, conductivity, dissolved oxygen concentration, and chlorophyll *a* (chl *a*) fluorescence.

Abundances of heterotrophic prokaryotes were measured by flow cytometry (FCM). Briefly, 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 (see Berdjeb et al. 2011a). Glutaraldehyde (1% final concentration) was used to fix flagellates. Samples were filtered (pressure, 100 mm Hg) on black polycarbonate membranes (diameter: 25 mm; pore size: 0.8 µm) and then stained with primulin (Caron 1983) 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 (HNF) and under blue light to count the pigmented nanoflagellates (PNF) at 1250× magnification.

AOA community richness and structure

DNA were harvested from 250 ml water samples onto 47 mm diameter, 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 and quantified using NanoDrop ND-1000 spectrophotometer (Thermo Scientific). The extracted DNA was then stored at −20°C until PCR amplification. AOA richness and structure were assessed using nested PCR and denaturing gradient gel electrophoresis (DGGE). The first PCR mix (50 µl) contained approximately 40 ng of extracted DNA associated with 1× PCR buffer, 0.2 mM of each deoxynucleotide, 4 mM of $MgCl_2$, 1 µM of each primer and 1 U of Platinium *Taq* DNA Polymerase. We performed PCR with the primer sets AOA-amoA-F (5′-CTG AYT GGG CYT GGA CAT C-3′) and AOA-amoA-R (5′-TTC TTC TTT GTT GCC CAG TA-3′) (Wuchter et al. 2006) with conditions as follows: 2 min at 98°C followed by 40 cycles at 94°C for 5 s, at 59°C for 20 s, and 72°C for 15 s, finished by 15 min at 72°C. The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen). The second PCR mix (25 µl) contained approximately 20 ng of the first purified PCR product associated with 1× PCR buffer, 0.2 mM of each deoxynucleotide, 4 mM of $MgCl_2$, 0.5 µM of each primer, 0.4 mg ml^{-1} of bovine serum albumin and 1 U of Platinum *Taq* DNA Polymerase. The second PCR was performed with the primer sets AmoAfiBA (5′-CTG AiT GGG CYT GGA CiT C-3′) and GC AOA-amoA-R (5′-GC clamp-TTC TTC TTT GTT GCC CAG TA-3′) (Cardoso et al. 2013). This second step of the PCR has been performed with a GC-clamp on the reverse primer and with an inosine variant of the Arch amoA-for primer, amoAf-i-BA. This forward primer contained inosine residues instead of degeneracies for molecular detection. As mentioned in Hornek et al. (2006), inosine containing amoA primers significantly helped to reduce the complexity of the DGGE band patterns for pure and environmental samples. The PCR was performed as follows: 5 min at 95°C, followed by 20 cycles at 94°C for 45 s, 51.8°C for 60 s, and 72°C for 60 s, finished by 15 min at 72°C. DGGE were carried out with an Ingeny PhorU-2 system using a 6% (wt/vol) polyacrylamide gel (20 to 50% gradient). The gels were run at 120 V for 10 h at 60°C in TAE 1×, 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 analyzed using GelComparII using a 2% tolerance for band's separation. Briefly, DGGE 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). It is noteworthy here that technical problems during the field campaign impeded the collection of samples for some dates (August and November 2007 at 3 and 45 m, and August 2008 at 45 m in Lake Annecy; November 2007 and July, August and November 2008 at 2 m and January 2007 and April and July 2008 at 50 m in Lake Bourget).

Archaeal *amoA* gene abundances

For each sample, we used qPCR amplification to determine archaeal *amoA* gene abundances. The qPCR conditions were based on Auguet et al. (2011) using the primer sets AOA-amoA-F (5′-CTG AYT GGG CYT GGA CAT C-3′) and AOA-amoA-R (5′-TTC TTC TTT GTT GCC CAG TA-3′) (Wuchter et al. 2006). The qPCR reaction mixture was slightly modified from Auguet et al. (2011) as follows: the reaction mixture contained 10 µl of SYBR Green Master Mix (Qiagen), 1.2 ng of template DNA, 1 µM primers and molecular biology-grade water (Qiagen). The assays were run on the Rotor Gene RG 3000 (Corbett Research). The qPCRs were run for 15 min at 95°C (to activate the *Taq*), followed by 60 cycles (94°C for 15 s, 59°C for 60 s and 72°C for 15 s). The fluorescence signal was read in each cycle after the elongation step at 78°C. All reactions were run in triplicate with standard curves spanning from 10^2 to 10^8 copies of DNA for *amoA* genes. Standard curves were obtained after serial dilutions of previously titrated suspensions of gene amplified by conventional PCR from environmental clones. The efficacy was 0.99 with a r^2 value of 0.998 and a slope of −3.32. The detection limit (DL) was 18 copies of template assay^{-1}. The specificity of reactions was confirmed by both melting-curve analyses and agarose gel electrophoresis to identify unspecific PCR products.

Statistics

For each DGGE gel, ordination of Bray-Curtis similarities was performed by non-metric multidimensional scaling (MDS) to significantly discriminate AOA groups and identify potential seasonality between these groups. The prepared nMDS plots were used to visualize the relationship between the AOA communities, as determined by their DGGE profiles, throughout the sampling period. Comparisons between changes in temporal composition for both 16S rRNA (assessed in our previous study, Berdjeb et al. 2013) and *amoA* genes were performed using a Mantel test in order to assess if the structure of AOA covaries with that of the whole archaeal community, or if these are decoupled. We also determined statistical relationships between the relative abundances of the archaea-specific *amoA* gene (copies ml^{-1}) and environmental variables. Factors driving the seasonal dynamics of the AOA community structure and *amoA* gene abundances were examined using simple or multiple regressions and canonical correspondence analysis (CCA). Statistical analyses were performed using the software package XLSTAT-ada (Addinsoft).

RESULTS AND DISCUSSION

Environmental context

A complete description of the spatial and temporal changes of physico-chemical and biological variables in Lakes Annecy and Bourget for the studied period are available in Berdjeb et al. (2013). We have summarized the most important physico-chemical characteristics of both lakes for the C, N, S and P biogeochemical cycles. Briefly, thermal stratification in both lakes was observed from April to September. Hypolimnetic temperatures were stable and never exceeded 7°C, while those recorded in the epilimnetic waters increased up to 25°C. NO_3^- concentrations were on average much higher in Lake Bourget than in Lake Annecy. During the stratification period, a gradual consumption of dissolved NO_3^- was observed in the epilimnetic layer of both lakes, whereas no seasonal variation was observed in the hypolimnion. For NH_4^+ concentrations, peaks (never exceeding 12 and 6 µg l^{-1} in Lake Bourget and Lake Annecy, respectively) appeared several times in spring and summer, followed by rapid consumption the following month in the epilimnetic layer of both lakes. In the hypolimnetic layers, the highest concentrations of NH_4^+ were obtained in January 2007 (12 µg l^{-1}) and April 2008 (17 µg l^{-1}) in Lake Bourget, and in May 2007 (9 µg l^{-1}) and February 2008 (8 µg l^{-1}) in Lake Annecy, whereas concentrations were very low for the rest of the year. Silica (SiO_2) displayed high and temporally stable concentrations in the hypolimnetic layers, whereas a marked seasonality was observed in the epilimnetic layers and seemed to be characterized by an increase during winter. In Lake Bourget (no data available for Lake Annecy), at 2 m, HNF and PNF averaged 0.7 ± 0.9 × 10^3 and 2.2 ± 3.4 × 10^3 cells ml^{-1}, respectively, while at 50 m they averaged 0.2 ± 0.1 × 10^3 and 0.06 ± 0.04 × 10^3 cells ml^{-1}, respectively. The dynamics of heterotrophic bacterial communities are described elsewhere (Berdjeb et al. 2011a).

Vertical and temporal patterns in AOA community richness and structure

With the advent of next-generation sequencing (NGS), fingerprinting approaches are now used less

and less for microbial community analysis. However, while they have lower resolution than NGS, these inexpensive approaches detect and preserve general patterns of microbial community structure (Gobet et al. 2014, van Dorst et al. 2014). They have similar capacities to capture significant biological patterns and correlate environmental variables with biological distributions. We thus used a fingerprinting approach (DGGE) to describe overall distribution patterns in AOA communities.

Lake Annecy

In Lake Annecy, we observed a total of 16 and 11 different bands among all samples at 3 m in 2007 and 2008, respectively, and 24 and 23 bands at 45 m in 2007 and 2008, respectively. The number of bands varied between 7 and 12 per sample at 3 m. At 45 m, the number of bands per sample varied between 9 and 16 (Fig. 1).

The corresponding MDS ordinations obtained for the different DGGE gels are presented in Fig. 2. As indicated by the goodness of fit test (≤0.1) on Kruskal stress values, distances in MDS ordination reflect relatively well the magnitude of similarity in DGGE patterns between samples. In Lake Annecy, the first temporal changes in AOA community structure in 2007 and 2008 were observed from a 50% Bray-Curtis similarity index (Fig. 2A–D). We observed marked shifts in AOA community structure from 1 to 3 mo (5 shifts) and from 2 to 4 mo (4 shifts) in 2007 and 2008, respectively at 3 m in Lake Annecy (Fig. 2A,B). The temporal structure followed clear seasonal patterns with 3 distinct groups. The first included winter samples while the second incorporated spring and autumn samples. The third group only contained AOA communities sampled in July. In the deeper layers, we observed shifts in AOA community structure every 1 to 3 mo and every 5 to 7 mo in 2007 and 2008, respectively, at 45 m in Lake Annecy (Fig. 2C,D).

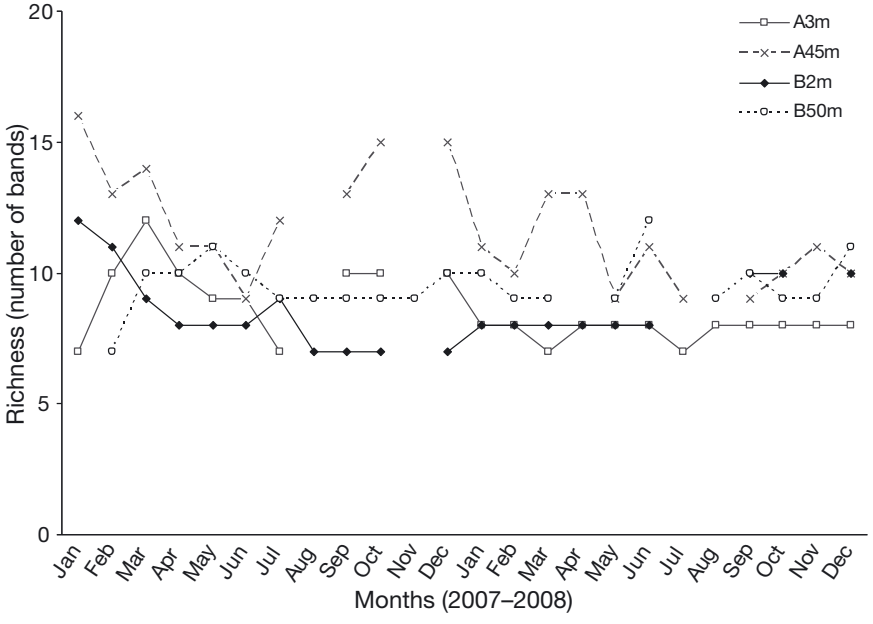

Fig. 1. Temporal evolution of the number of DGGE bands in the epi- and hypolimnion of lakes Annecy and Bourget. White squares: Annecy (A) 3 m; crosses: Annecy 45 m; black diamonds: Bourget (B) 2 m; white circles: Bourget 50 m. Note that technical problems during the field campaign impeded the collection of samples for some dates (August and November 2007 at 3 and 45 m and August 2008 at 45 m in Lake Annecy; November 2007 and July, August and November 2008 at 2 m and January 2007 and April and July 2008 at 50 m in Lake Bourget)

Lake Bourget

We detected between 7 and 12 bands per sample at 2 m in Lake Bourget (Fig. 1). The DGGE profiles, obtained from samples taken at 50 m, displayed between 7 and 12 bands. In lake Bourget, a total of 16 and 12 different bands were counted among all samples at 2 m in 2007 and 2008, respectively, and 13 and 12 bands at 50 m in 2007 and 2008, respectively.

Considering the Bray-Curtis similarity values, the first temporal changes in Lake Bourget appeared from 60 and 70% at 50 m in 2007 and 2008, respectively, while they appeared from 75 and 85% at 2 m in 2007 and 2008, respectively (Fig. 2E–H). These contrasted results suggest an AOA community structure annually much more stable in Lake Bourget than in Lake Annecy. In Lake Bourget at 2 m, changes appeared every 3 to 5 mo (3 shifts) and 1 to 6 mo (3 shifts) in 2007 and 2008, respectively (Fig. 2E,F). Temporal patterns were less clearly defined than in Lake Annecy, with 2 distinct groups in 2007 and 2008. In the deeper layers of Lake Bourget, changes appeared every 4 to 7 mo and every 1 to 3 mo in 2007 and 2008, respectively (Fig. 2G,H).

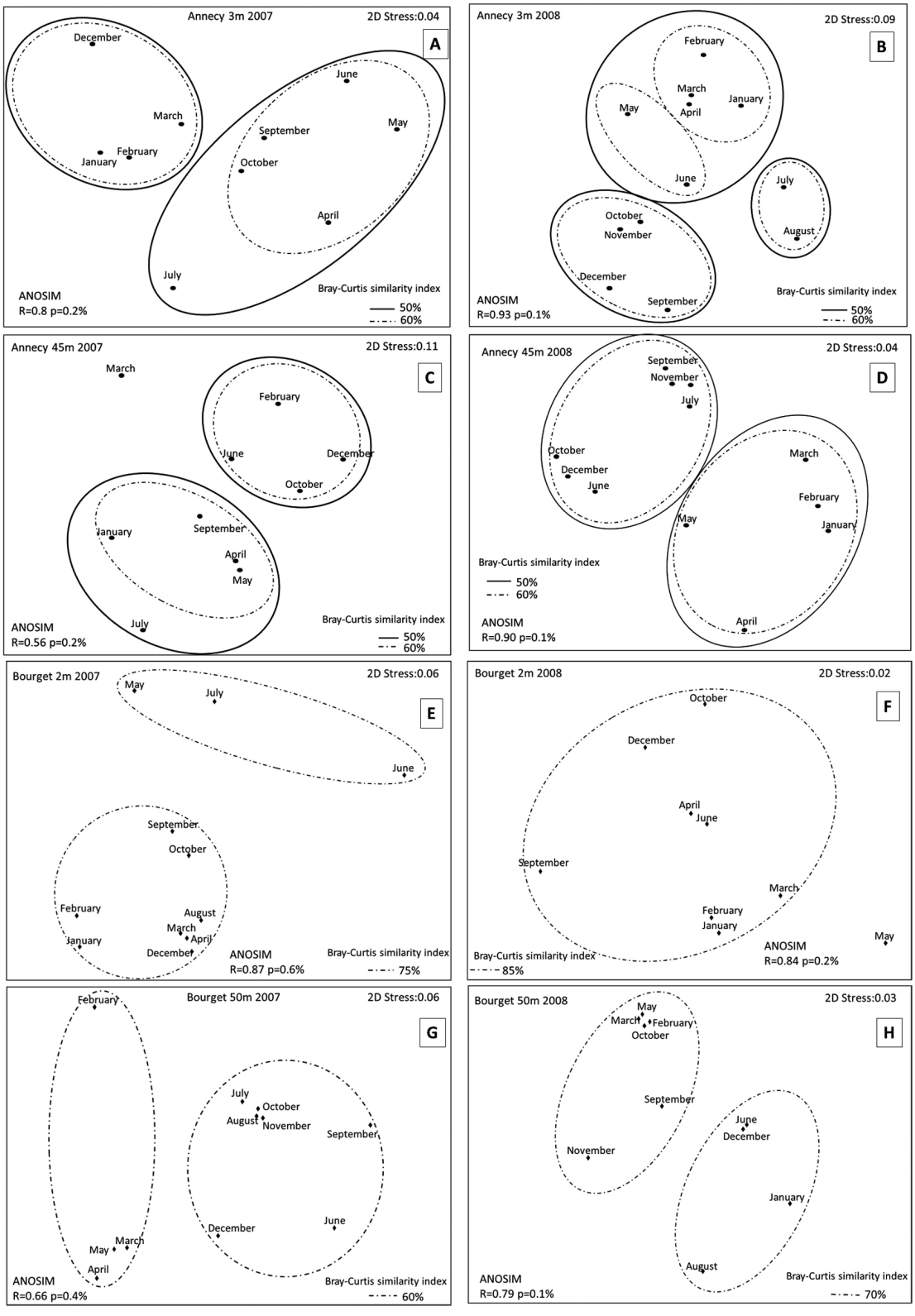

Fig. 2. Non-metric multidimensional scaling (nMDS) ordination obtained for DGGE gels for (A–D) Lake Annecy and (E–H) Lake Bourget. Ordination of Bray-Curtis similarities among normalized sample profiles was performed by MDS. We used this ordination technique to determine the relationships among sample profiles as representative of the ammonia-oxidizing archaeal (AOA) community structure of each sample site

Lake Annecy versus Lake Bourget

Based on examples from the gel pictures focused on the dominant DGGE bands (see Fig. A1 in Appendix 1), several bands appear to be common to many samples. The main differences among these dominant bands are linked to the intensity that varies between samples (months, depth, years and lakes). This could suggest that the composition of the dominant AOA phylotypes are similar from one month to another, from one depth to another or from one lake to another, but with different abundances. However, our 2 yr analysis comprised 85 different samples. The analysis of this large number of samples required the use of several DGGE gels. Gel-to-gel comparisons do not guarantee that corresponding bands in different gels can be correctly identified, especially when the communities are complex. Gel-to-gel comparisons could be risky, and to avoid too much speculative conclusions, we decided to describe only the patterns of diversity and compare the temporal patterns observed between each depth in each lake from one year to another.

The AOA richness spectra were similar in both lakes, with the highest values mainly found in the hypolimnion (Fig. 1). These findings are consistent with previous results for other stratified lakes (Auguet et al. 2011, 2012, Vissers et al. 2013b). This could be attributable to the vertical heterogeneity of the habitat and reflect differences between these layers in the factors and processes that drive AOA communities (Shade et al. 2008 and references therein). Microbial growth rates, abundances and biomasses, rates of biological processes, pH levels and seasonal nutrient depletions are generally higher in the epilimnion than in the hypolimnion of lakes, due in part to higher temperatures in upper waters. This, combined with lower competition for resources in the hypolimnia, might help clarify the fact that the estimate of AOA richness was lower in the epilimnia than the hypolimnia. In addition, upper layers are characterized by higher irradiances and thus a higher UV intensity. This physical factor, known to considerably damage DNA, and the reported photoinhibition of AOA (Merbt et al. 2012) would consistently explain the lower richness in the epilimnetic layers of the 2 lakes.

Our study builds on our previous work, in which the same DNA samples were used to characterize the whole archaeal community structure in both Lakes Annecy and Bourget (Berdjeb et al. 2013). Overall, when we compared the different data sets (surface 2007–2008 and hypolimnia 2007–2008 in both lakes), changes in structure for both 16S rRNA and *amoA* genes were significantly correlated only in the upper layers (Mantel tests; A3m2007 $r = 0.32$, $p < 0.05$; A3m2008 $r = 0.49$, $p < 0.0001$; B2m2007 $r = 0.32$, $p < 0.05$; and B2m2008 $r = 0.3$, $p < 0.05$). In the deeper layers, the structure of AOA did not covary with that of the whole archaeal community as no correlations between these genes were observed in the hypolimnia (A45m2007-2008 and B50m2008, $r \leq 0.17$, $p > 0.05$) except in Lake Bourget at 50 m in 2007 ($r = 0.44$, $p < 0.001$). Temporal co-variations between the structure of AOA and the whole archaeal community in the upper layers are consistent with Auguet et al. (2011). This is also consistent with our previous findings, which indicated that the archaeal communities seemed to be mainly composed of AOA (Berdjeb et al. 2013) and suggest that the temporal changes in the structure of archaeal communities in the surface waters might be mainly driven by the dynamics of archaeal autotrophic ammonia oxidizers. By contrast, the lack of correlations in the hypolimnetic layers indicates that the temporal changes in the deeper waters would be globally driven by the dynamics of heterotrophic archaea.

In contrast to what we expected, different intra-annual patterns in AOA community structure were observed in the upper layers of the 2 lakes, with succession seasonal patterns in Lake Annecy and much more stable AOA communities in Lake Bourget. This is despite the 2 lakes both being peri-alpine, morphologically similar (monomictic, deep, approximately the same area) and geographically close (approximately 50 km apart). Surface AOA communities in both lakes are therefore exposed to comparable levels of environmental stressors such as temperature, UV intensity and day duration. While such variables linked to season could easily explain the temporal dynamics of surface AOA communities observed in Lake Annecy, the lack of clear seasonal patterns in the upper layers of Lake Bourget suggests that these factors alone cannot account for variability in AOA community temporal dynamics. The contrasting results observed for the 2 lakes suggest that trophic status might also have a major influence and play a crucial role in shaping AOA community structure. This hypothesis has already been mentioned by Yang et al. (2016), who compared the dynamics of archaeaplankton abundance with sampling site and time in freshwater lakes of different trophic status. They found correlations between variables associated with the trophic status of lakes and archaeaoplankton richness, and concluded that these archaeal communities in freshwater lakes might be determined by

trophic status. We should also note that the water retention time in Lake Bourget (10 yr on average) is 2 times higher than in Lake Annecy (5 yr), which could also explain the differences in the temporal patterns observed in the upper layers of both lakes. In the hypolimnetic layers, no clear temporal pattern was observed in either of the studied lakes during the 2 yr study period: AOA temporal structure was much more variable, random and non-recurrent than in the epilimnion. Such higher temporal variability in the hypolimnion and the lack of clear temporal patterns in these deeper layers has already been reported in Lakes Annecy and Bourget for the entire archaeal community (Berdjeb et al. 2013), for the entire bacterial community (Berdjeb et al. 2011a) and for *Planctomycetes*, a non-dominant bacterial phyla (Pollet et al. 2011), suggesting that this may be a 'rule' for prokaryotes at different taxonomical levels in deep perialpine lakes. According to both CCA and regression analysis, estimated abiotic variables do not explain a significant portion of the temporal variability in AOA structure in Lakes Annecy and Bourget ($p > 0.05$), suggesting that these variables do not operate as direct forces in shaping the temporal dynamics of AOA in these systems. Studies on the temporal dynamics of microbial community composition have yielded key insights into the drivers of microbial dynamics in different aquatic systems (e.g. Berdjeb et al. 2011a, Chow et al. 2013, Tinta et al. 2015). For example, in microbial communities in aquatic ecosystems, despite the importance of environmental forcing functions such as temperature, nutrient supply and physical mixing, which galvanize these communities and determine bulk properties and general features of community composition, there exists a complex network of interrelationships (e.g. cooperation, competition, mutual dependency, predator–prey relationships) that have self-regulatory effects (Fuhrman et al. 2015). Given our results, it would seem important to now explore 'co-occurrence networks' in AOA communities in order to identify possible symbiotic associations and connections between different community members, to more fully understand the factors controlling AOA ecological processes in lacustrine systems.

Spatiotemporal changes in archaeal *amoA* gene abundances

As revealed by the qPCR approach, the copy numbers of the archaea-specific *amoA* gene are on the same order of magnitude as those commonly reported for other lakes (Llirós et al. 2010, Auguet et al. 2011, 2012, Vissers et al. 2013a). The relative abundances of the archaea-specific ammonia monooxygenase (*amoA*) gene are presented in Fig. 3. In Lake Bourget, gene copy numbers ranged from below qPCR detection limits (hereafter, 0) to 1.95×10^3 copies ml^{-1} at 2 m, and from 0 to 47.35×10^3 copies ml^{-1} at 50 m. In Lake Annecy, copy numbers ranged from 0 to 9.09×10^3 copies ml^{-1} at 3 m, and from 0 to 27.14×10^3 copies ml^{-1} at 45 m. Note that for most surface samples collected between May and September in both lakes, the relative abundance of the *amoA* gene was below the detection limit (Fig. 3).

Throughout the 2 yr study period, the archaea-specific *amoA* gene number was lower in surface waters and tended to increase with depth in both lakes (Fig. 3). AOA vertical distribution appeared similar to that reported from previous studies performed in deep lakes (Callieri et al. 2009, Pouliot et al. 2009, Llirós et al. 2010, Maitreyee 2013, Vissers et al. 2013a, Restrepo-Ortiz et al. 2014). A similar vertical distribution pattern has also been reported in marine waters (Church et al. 2010). For many samples, the archaea-specific *amoA* gene number was below the detection limit particularly in the upper layers of both lakes. This result raises a question about the interpretation of the diversity analysis in the context of such low abundance. This could get close to the questions about the 'rare biosphere', which is composed of highly diverse members at low abundance. The most obvious influence of the rare biosphere members is their ability to act like a seed bank. Organisms that may be ideally adapted to conditions in another time could eventually thrive by just waiting for better environmental conditions. Based on this ecological theory explaining the dynamics of the rare biosphere, we could hypothesize that despite their low abundances observed during some months, probably due to unfavorable environmental conditions, AOA communities could act like a seed bank, maintaining their diversity waiting for good conditions. In both lakes, the relative abundance of the archaea-specific *amoA* gene globally displayed seasonal patterns in the upper layers, with greater abundances found in winter (December to April in 2007 and 2008 in Lake Bourget, and in 2008 in Lake Annecy), generally followed by a strong decrease from spring to autumn (Fig. 3A,C). This spring and summer decrease corresponds to an increase in diatom abundance (data not shown), which could be strong competitors for the available resources, and the abundance of potential predators such as nanoflagellates and ciliates (see in Berdjeb et al. 2013).

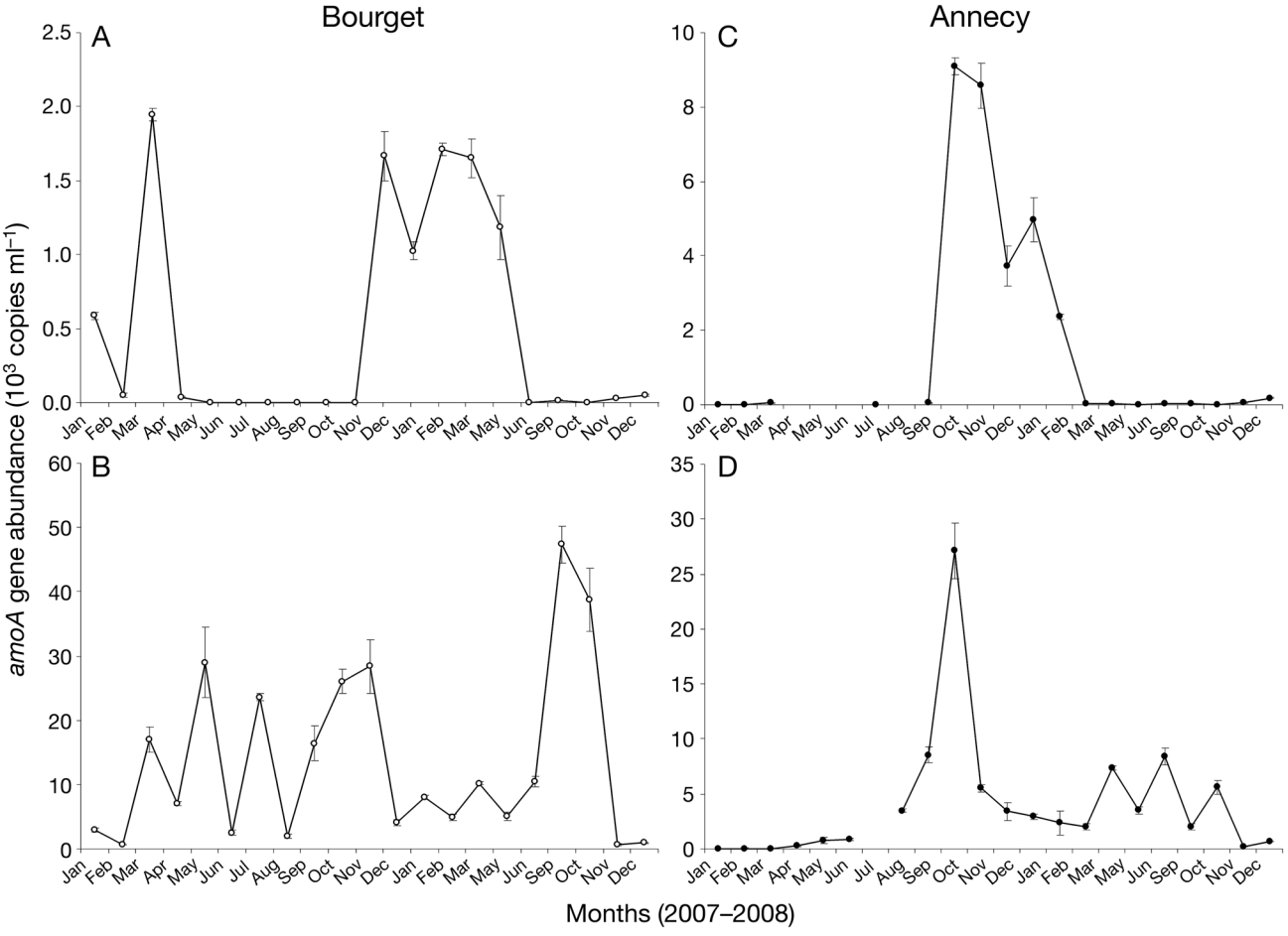

Fig. 3. Average abundances (±SE) of archaea-specific *amoA* gene (copies ml^{-1} normalized to extraction yields) from January 2007 to December 2008 in Lake Bourget at (A) 2 m and (B) 50 m and in Lake Annecy at (C) 3 m and (D) 45 m. '0' represent the value when the *amoA* gene abundances were below the detection limit. ●: Lake Annecy; ○: Lake Bourget

No clear temporal pattern was observed for the hypolimnetic layers, with relative abundances much more variable and unpredictable than in the epilimnion (Fig. 3B,D). Very similar patterns have been described in other aquatic ecosystems (Vissers et al. 2013a for Lake Lucerne; Galand et al. 2010 for coastal marine waters). Interestingly, Auguet et al. (2011) highlighted an opposite pattern in high mountain lakes, i.e. higher *amoA* gene abundances in summer. Despite the specific environmental conditions in the Auguet et al. (2011) study, these contrasting results make it difficult to draw a clear picture of temporal patterns in AOA abundance in surface waters of aquatic ecosystems, particularly in lakes. Clearly, more investigations are needed to better understand the dynamics of these micro-organisms in these ecosystems.

We statistically tested for correlations between *amoA* gene abundance and a variety of environmental variables, including temperature, dissolved oxygen, pH, total organic carbon, chl *a*, nitrate, ammonium, phosphate and silicate. We also looked for correlations with various biological variables, including concentrations of picocyanobacteria, viruses, nanoflagellates and ciliates. The significant correlations ($p < 0.05$) are presented in Fig. 4. Our statistical analyses suggest that copy numbers of archaea-specific *amoA* gene, and thus AOA dynamics, could be driven by several environmental factors (temperature, nutrients) as well as biological factors (nanoflagellates). As previously reported by Vissers et al. (2013a) for another large and deep alpine lake (Lake Lucerne), the environmental factors usually known to influence AOA dynamics (pH, ammonium, nitrite and oxygen concentrations) showed no significant impact in lakes Bourget and Annecy. We obtained a strong negative correlation between water temperature and relative abundances of the archaea *amoA* gene for values ranging between 5.4 and 22.9°C ($p < 0.001$ in both lakes; Fig. 4A). Such a negative relationship has also been previously reported (Vissers et al. 2013a, Restrepo-Ortiz et al. 2014). Interestingly, in their micro-

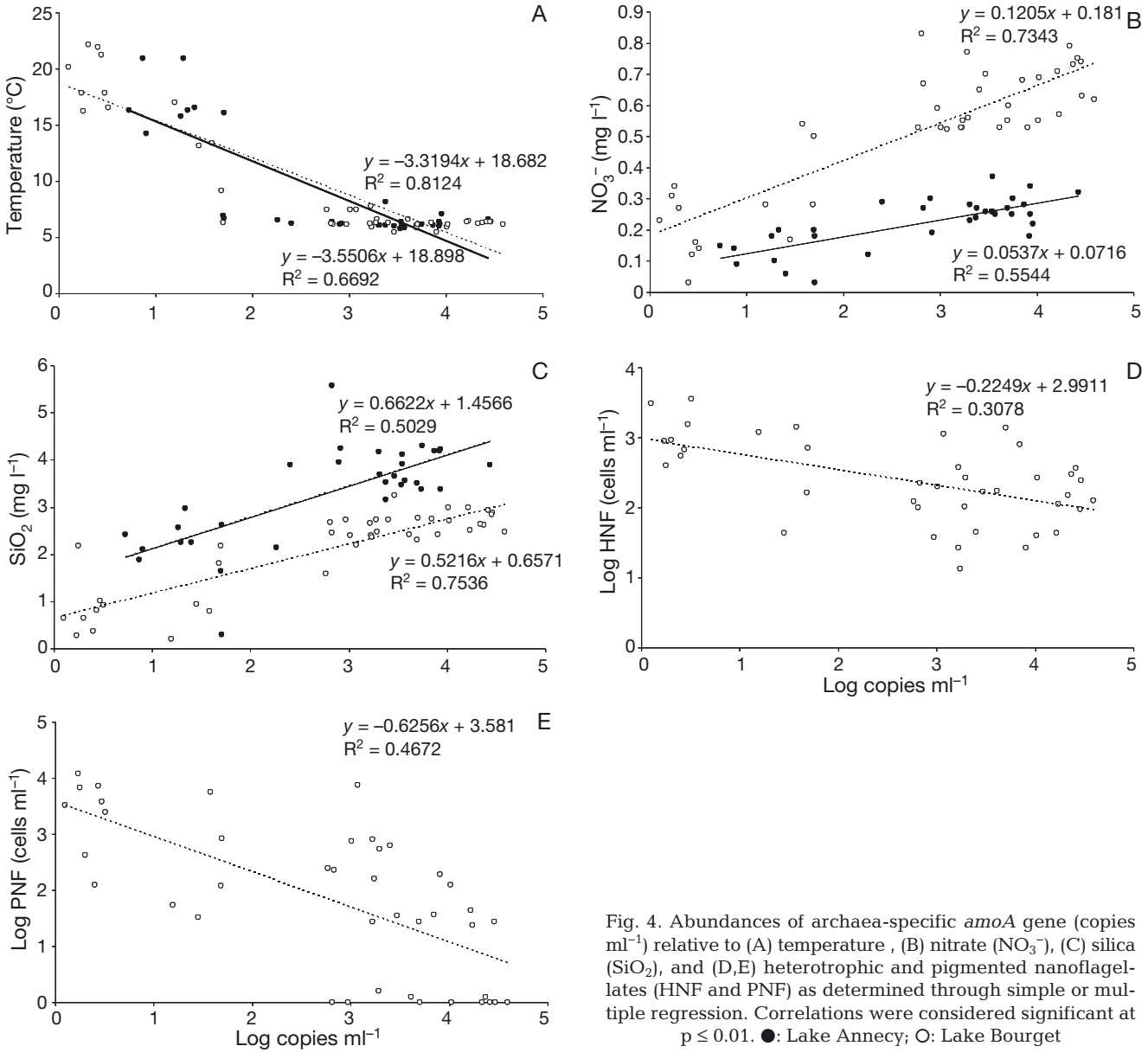

Fig. 4. Abundances of archaea-specific *amoA* gene (copies ml^{-1}) relative to (A) temperature , (B) nitrate (NO_3^-), (C) silica (SiO_2), and (D,E) heterotrophic and pigmented nanoflagellates (HNF and PNF) as determined through simple or multiple regression. Correlations were considered significant at $p \leq 0.01$. ●: Lake Annecy; ○: Lake Bourget

cosm experiment, Zeng et al. (2014) described an increase in the abundance of the archaeal *amoA* gene for temperatures ranging from 15 to 35°C. Combined with our results, this may suggest a parabolic relationship between temperature and archaeal *amoA* gene abundance, with lowest abundances found at water temperatures ranging between 20 and 25°C. Results from Vissers et al. (2013a) and Restrepo-Ortiz et al. (2014) are consistent with this hypothesis. Temperatures of around 20 ± 5°C are known to be optimal for AOB growth (Pintar & Slawson 2003), which would explain the fact that AOB outnumbered AOA during summer stratification in the surface waters of Lake Lucerne (Vissers et al. 2013a).

While ammonium is known to be the main substrate oxidized by AOA, and its concentration may explain a significant part of the changes in AOA abundance (Auguet et al. 2011), we found no correlation between the relative abundance of archaeal *amoA* gene and ammonium concentrations. This lack of a relationship has already been observed for lakes (Vissers et al. 2013a) and marine waters (Galand et al. 2010). However, ammonium concentrations were extremely low in both Lake Annecy and Lake Bourget, and it is known that AOA can grow at very low ammonium concentrations, perhaps below the detection limit. This would make it very difficult to accurately establish the relationship between ammonium

concentration and AOA abundance. Furthermore, as previously observed by Llirós et al. (2010), we found that *amoA* gene abundance was strongly correlated with nitrate concentration ($p < 0.001$, $n = 41$ in Lake Bourget and $p = 0.001$, $n = 33$ in Lake Annecy; Fig. 4B). Nitrate concentration is a variable associated with the trophic status of lakes. This high correlation between nitrate and AOA abundance strengthens our hypothesis that trophic status can influence AOA community dynamics.

Rarely estimated in previous studies, silica concentrations showed an unexpected strong positive correlation with archaeal *amoA* gene numbers in both lakes ($p < 0.001$; Fig. 4C). Correlations between total bacteria abundances and silica concentrations were tested and no significant correlation was observed ($p > 0.05$). Pedneault et al. (2014) reported a strong relationship between silica and archaeal *amoA* gene number in the Arctic Ocean. Another study detected archaeal *amoA* gene phylotypes in iron-silica-rich microbial mats in deep sea hydrothermal fields (Kato et al. 2009), and silicate concentration emerged as a highly significant explanatory variable for the spatial structure of free-living AOA communities in a river estuary (Zhang et al. 2014). In contrast, Bidle & Azam (2001) did not find any relationship between archaea and silica concentration in marine waters, and concluded that the archaea do not play a significant role in silica regeneration. However, these authors used the Arch21F/Arch958R primers to identify microbial communities colonizing diatoms detritus, while in silico analyses using the Silva database show that this primer pair yields a low recovery of sequences for *Archaea* (only 11%) and *Thaumarchaeota* (19%). This could obscure any real link between AOA and silica. To the best of our knowledge, our study is the first to show a correlation between silica concentration and archaeal *amoA* gene abundance in lacustrine systems. This relationship is intriguing, but caution is needed before a direct link can be made between *amoA* gene abundance and silica concentration. We are aware that this correlation could simply reflect co-variation with other unmeasured variables, especially since there appears to be no known physiologic or metabolic pathway that could account for this interaction with silica. However, bacteria are known to utilize the organic matter derived from oceanic primary production by varied strategies, including attack on dead and living diatoms by using hydrolytic enzymes (Smith et al. 1992). In the same way, Bidle & Azam (1999) reported that Silica dissolution accompanied, and was caused by, bacterial colonization and hydrolytic attack. Based on these findings, we could venture the hypothesize that AOA might similarly be involved in silica dissolution using hydrolytic enzymes. Even though this correlation would have to be confirmed by experimentally testing the influence of various silicate concentrations on AOA communities, our findings suggest a potential role of AOA in silica dynamics and regeneration, and constitute a very promising research avenue in our bid to better understand the functioning of microbial networks.

In Lake Bourget, we found a moderately negative correlation between archaeal *amoA* gene abundance and both HNF and PNF ($p < 0.01$; Fig. 4D,E). The AOA mortality rate attributed to nanoflagellates is still unknown in aquatic ecosystems. Bonilla-Findji et al. (2009) hypothesized a potential effect of flagellate grazing on archaeal community composition. In our previous study of lakes Bourget and Annecy, we estimated that 18.5% of temporal variability in archaeal community structure could be explained by top-down regulation (Berdjeb et al. 2013). Because we showed in this previous investigation that members of AOA dominated the archaeal community in both lakes, our 2 complementary studies and statistical analyses suggest the potential involvement (direct or indirect) of nanoflagellates, and thus the important role of protistan predation in shaping AOA dynamics. We also found that PNF were negatively correlated with AOA abundance (Fig. 4E). PNF are dominated by mixotrophs in both lakes Bourget and Annecy (Domaizon et al. 2003, Comte et al. 2006, Jacquet et al. 2014); in particular, by Chrysophytes such as *Dinobryon* spp. This mixotrophic algal group obtains energy either from resources or by feeding on decaying or living cells. In lakes Bourget and Annecy, blooms of *Dinobryon* are commonly observed during summer and early autumn (e.g. Comte et al. 2006, Jacquet et al. 2014), which coincides with the AOA decline period observed in this study, suggesting that mixotrophic *Dinobryon* may play a role in controlling AOA abundance, either by grazing or through competition for resources. This finding is especially relevant as *Dinobryon* species are commonly among the dominant phytoplankton species during the summer in many freshwater lakes. This hypothesis is supported by the recent study of Ballen-Segura et al. (2017), who investigated the feeding behavior of 3 species of mixotrophic flagellate species (*Rhodomonas* sp., *Cryptomonas ovata* and *Dinobryon cylindricum*) via their food vacuole contents in field populations of a high mountain lake. Their results provide field evidence that contrasting selective feeding exists between coexisting mixotrophic flagellates under the same environmental conditions, that some prokaryotic groups

may be preferentially impacted by phagotrophic pressure in aquatic microbial food webs, and that archaea were the preferred prey.

The spatial and temporal resolution of our study is the most common interval in major aquatic ecosystem time-series studies, and it allowed us to assess the driving environmental forces which vary strongly at these scales. We are aware that fully assessing AOA community dynamics requires studying the community at multiple time scales (daily to weekly, monthly to seasonal, inter-annually) and using experimental approaches to better understand associations with some variables (i.e. silicates). Our study has identified a number of variables associated with the temporal variation of archaeal *amoA* gene abundance and provides valuable insights into the monthly to inter-annual dynamics in AOA community structure as well as the vertical distribution of this assemblage in lakes. Our findings point to the complexity of processes affecting AOA communities in peri-alpine lakes and suggest many promising avenues for further exploration. In particular, there are a number of questions that could be answered using experimental approaches: (1) How does silica influence AOA dynamics and *vice versa*? (2) What is the AOA mortality rate due to grazers? (3) Does the lack of a relationship between viruses and AOA abundance mean AOA communities are resistant to infection?

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Appendix 1

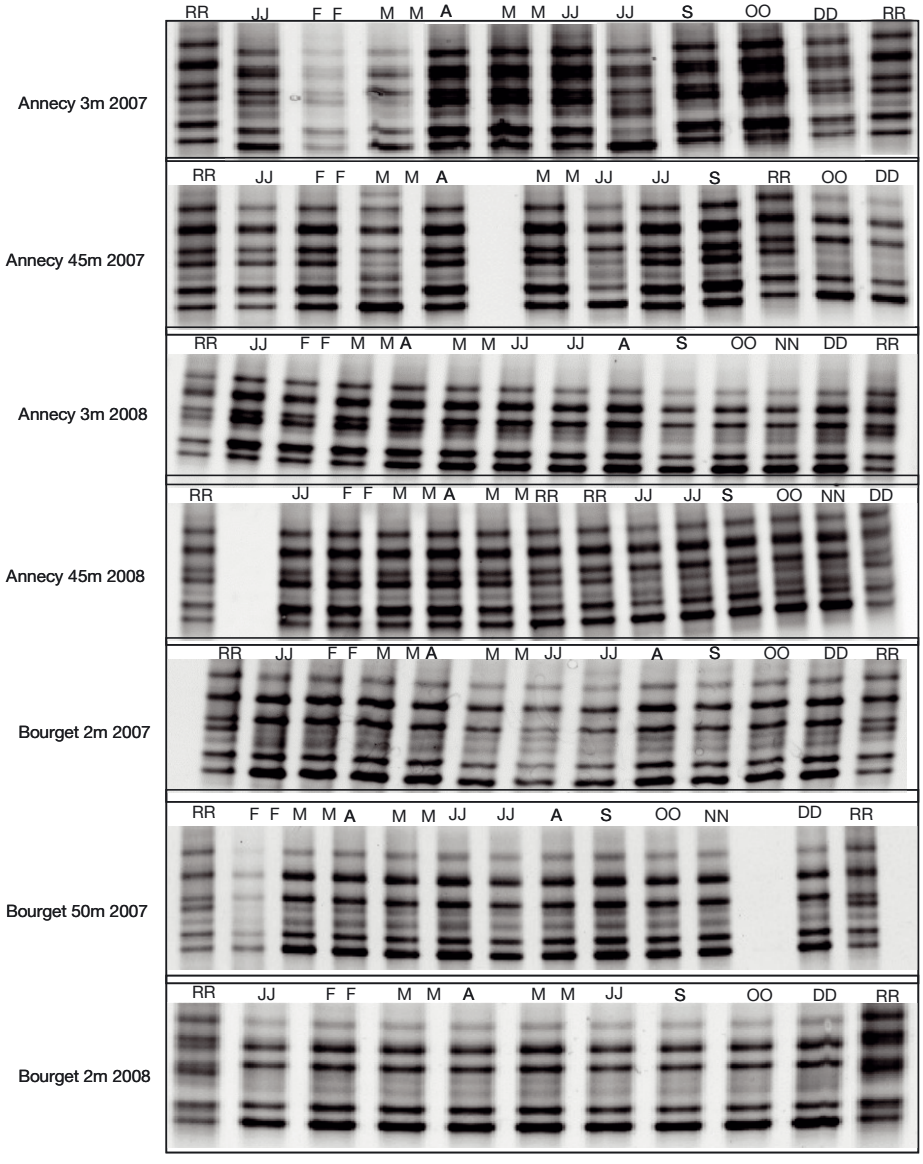

Fig. A1. Gel pictures of the dominant DGGE bands for each lake, depth, year and month

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