Vol. 79: 115–125, 2017
https://doi.org/10.3354/ame01825

AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol

Published online May 9

Unique and highly variable bacterial communities inhabiting the surface microlayer of an oligotrophic lake

Mylène Hugoni[1,2,3], Agnès Vellet[1,2], Didier Debroas[1,2,*]

[1]Laboratoire 'Microorganismes: Génome et Environnement', Clermont Université, Université Blaise Pascal, BP 10448, 63000 Clermont-Ferrand, France

[2]CNRS, UMR 6023, LMGE, 63171 Aubière, France

[3]*Present address:* Université Lyon 1; CNRS, UMR 5557; Ecologie Microbienne; INRA, UMR 1418; 69220 Villeurbanne Cedex, France

ABSTRACT: Surface microlayers (SMLs) play a key role in regulating air–water gas exchange. Few studies have investigated bacterial diversity in this particular zone in freshwater ecosystems compared to that of the epilimnetic water layer, using high-throughput sequencing technologies. The present study examined bacterial community structures in the SML and the epilimnion layers of an oligotrophic high mountain lake, Lake Pavin, France. These 2 habitats harboured distinct bacterial communities, with higher temporal variation observed in the structure of the bacterial community in the SML than in the epilimnion. This arrangement might result from aerosol deposition and/or different levels of solar radiation impacting the lake surface. The presence of typical freshwater bacterial assemblages (i.e. *Alphaproteobacteria* LD12 lineage, and *Actinobacteria* AcI clade) in the epilimnion was also examined. In contrast, the SML was clearly enriched in *Alphaproteobacteria* (i.e. *Rhodospirillales* and *Sphingomonadales*), *Gammaproteobacteria* (i.e. *Pseudomonadales*), and *Firmicutes* (i.e. *Clostridiales*). Notably, analyses conducted on indicator species revealed that *Propionibacterium* and *Acinetobacter* were characteristic in the SML, whereas operational taxonomic units in the epilimnion showed greater diversity. SMLs present a specific bacterial community composition that might originate from the surrounding environment, raising questions about the metabolic adaptations of these organisms in this particular environment.

KEY WORDS: Lake Pavin · Community structure · Surface microlayer · Epilimnion

INTRODUCTION

In aquatic ecosystems, the air–water interface, or surface microlayer (SML), constitutes a specific environment that differs from the underlying water masses. The SML is physico-chemically different from the subsurface (Cunliffe et al. 2011) and is a dynamic system that experiences atmospheric inputs and aerosol transfers, which contain mineral nutrients (nitrogen, phosphorus, and iron) and organic carbon that support the growth of freshwater bacteria (Morales-Baquero et al. 2006). The SML constitutes the primary place for gas exchange between water masses and the atmosphere (Conrad & Seiler 1988, Asher 2005). The enrichment in organic and inorganic compounds (Maki 1993, Liss & Duce 2005) in the SML favours the establishment of microbial communities involved in biogeochemical processes in freshwater systems, such as nutrient and energy cycling. In addition, SMLs are exposed to UV radiation, which can cause direct DNA damage or indirect damage through reactive oxygen species (Cunliffe et al. 2011), therefore representing a specific ecological niche for adapted bacterial communities.

*Corresponding author: didier.debroas@univ-bpclermont.fr

Although studies have been conducted on marine ecosystem SMLs (Agogué et al. 2005, Franklin et al. 2005, Cunliffe et al. 2011), fewer have investigated freshwater ecosystem SMLs (Kalwasińska et al. 2011, Vila-Costa et al. 2013, Sarmento et al. 2015). In lacustrine ecosystems, several studies have focused on microbial diversity in the water column (Auguet et al. 2012, Hugoni et al. 2015), whereas many biological and chemical processes occur at environment interfaces. Comparisons of the freshwater bacterial community composition between the SML and underlying water layers are still scarce (Cunliffe et al. 2009).

In lacustrine ecosystems, some studies have shown that microorganisms inhabit this nutrient-rich air–water interface, including *Bacteria* (Hervas & Casamayor 2009, Hörtnagl et al. 2010) and *Archaea* (Auguet & Casamayor 2008). Hervas & Casamayor (2009) reported the dominance of *Betaproteobacteria* and *Actinobacteria*, which accounted for >75% of bacterial clone libraries from high mountain lakes. They detected bacterial segregation at the clade level, with *Alphaproteobacteria* affiliated with the *Caulobacter-Brevundimonas*-like cluster restricted to the SML, and *Alphaproteobacteria* from the *Rhodopseudomonas*-like cluster restricted to the underlying water. Hörtnagl et al. (2010) reported similar findings in another high mountain lake, with *Betaproteobacteria* and *Actinobacteria* dominating, and showed enrichment of the betaproteobacterial R-BT subgroup in the SML (Hörtnagl et al. 2010). Moreover, *Archaea* account for a non-negligible fraction of the prokaryotic assemblage; Auguet & Casamayor (2008) reported abundances ranging from 3 to 37% of the total DAPI-stained cells in the SML of high mountain lakes in the Pyrenees. Among *Archaea*, *Thaumarchaeota* are involved in a major biogeochemical process; they are ammonia-oxidizers and thus key players of the nitrogen cycle (Pester et al. 2011). This highlights the importance of microbial communities inhabiting the SML, as they play important roles in nutrient and/or energy cycling.

Oligotrophic high mountain lakes provide ideal conditions to study SML community structures because atmospheric loading tends to determine water characteristics (Catalán et al. 2006, Pulido-Villena et al. 2008), nutrients (nitrogen or phosphate) are limited, and the lakes experience weak anthropogenic inputs. A few studies have investigated SML community composition in comparison with epilimnetic community composition, using high-throughput sequencing technologies, and have shown that microbial communities in both SML and epilimnetic waters differed (Vila-Costa et al. 2013, Sarmento et al. 2015). Vila-Costa et al. (2013) reported that *Actinobacteria* and *Betaproteobacteria* dominated under 2 atmospheric aerosol-loading scenarios and suggested that some *Bacteroidetes* and *Thaumarchaeota* were preferentially retrieved in the SML. Similarly, Sarmento et al. (2015) showed consistent differences in picoplankton from the SML and epilimnion abundance, activity, and single-cell physiology, but did not consider temporal variations in bacterial community composition.

In our study, bacterial community composition was analysed in an oligotrophic lacustrine ecosystem (Lake Pavin, France) during spring and summer, in the air–water interface (SML) and underlying waters (epilimnion), by 16S rRNA gene sequencing. We sampled Lake Pavin over time, testing whether bacterial communities in the SML temporally differed from those of corresponding epilimnetic samples.

MATERIALS AND METHODS

Study site, sampling, and environmental parameters

This study was performed at Lake Pavin in the French Massif Central (45° 55′ N, 2° 54′ E). This lake is a meromictic, oligomesotrophic freshwater lake, situated at an altitude of 1197 m, with a maximal depth of 92 m. It is fed by atmospheric precipitation and numerous superficial and sub-lacustrian springs (Viollier et al. 1995). Samples were collected during May, June, July, and August 2011 in both the epilimnion and SML at 3 distinct points (replicates). Samples from the air–water interface were collected using 0.2 µm pore-size polycarbonate filters (Millipore) laid on the water surface for 2 min and stored at −80°C until nucleic acid extraction. Underlying water was collected at 2 m depth using a Van Dorn bottle, and a subsample of water (300 ml) was pre-filtered through 5 µm pore-size polycarbonate filters (Millipore) and collected on 0.2 µm pore-size (pressure <10 kPa) polycarbonate filters (Millipore) before storage at −80°C until nucleic acid extraction. Phosphorus ($P\text{-}PO_4$), nitrate ($N\text{-}NO_3$), nitrite ($N\text{-}NO_2$), and ammonia ($N\text{-}NH_4$) contents were analysed using standard American Public Health Association (2005) methods.

DNA extraction and pyrosequencing

The extraction procedure used was previously described by Lefranc et al. (2005). Briefly, for each zone and each sampling date, 3 filters (replicates) were used and DNA was extracted by digesting the cells

with lysozyme (final concentration: 2 µg $µl^{-1}$) and Proteinase K (100 µg ml^{-1}). Nucleic acids were then separated with phenol/chloroform and precipitated with ethanol. DNA from the 3 filters (replicates) was pooled and DNA yield was quantified using a Nanodrop spectrophotometer (ND 1000). Amplification of the V4-V5 region of the 16S rRNA gene was performed using the universal bacterial primers 563F (5′-AYT GGG YDT AAA GNG-3′; Claesson et al. 2010) and 907R (5′-CCG TCA ATT CMT TTG AGT TT-3′; Lane 1991). Pyrosequencing was achieved by the GINA Platform (Clermont-Ferrand, France) using a Roche 454 GS-FLX system with titanium chemistry.

Bioinformatic analyses

All the pyrosequencing data were checked against the following quality criteria: (1) no ambiguous base: Ns, (2) quality score ≥23 according to the PANGEA process (Giongo et al. 2010), (3) a minimum sequence length of 200 bp, and (4) no sequencing error in the forward primer. The putative chimeras and homopolymers were detected using Uchime (Edgar et al. 2011) and the perl script homopolymer_count.pl (http://alrlab.research.pdx.edu/aquificales/pyrosequencing.html).

The remaining sequences were clustered at a 97% similarity threshold (Kim et al. 2011) with Usearch (Edgar 2010). The seed operational taxonomic units (OTUs) were affiliated by similarity and phylogeny from reference sequences (https://github.com/panammeb/). These bacterial references were extracted from the SSURef SILVA 119 database (Pruesse et al. 2007) according to the following criteria: length >1200 bp, quality score >75%, and a pintail value >50. In addition, the taxonomy of this reference database was modified to take into account typical freshwater lineages defined previously (Newton et al. 2011). After a comparison of the OTUs with the reference sequences by a similarity approach (Usearch tool), trees including OTUs with their closest references were built with FastTree (Price et al. 2010). This process was implemented in the pipeline PANAM (Phylogenetic Analysis of Next-generation AMplicons, https://github.com/panammeb/; Taib et al. 2013). To compare samples among each other, the number of sequences was resampled down to 2700 sequences for each sample (normalization process corresponding to the less abundant sample). The pyrosequencing data reported in this paper have been deposited in the MG-RAST database (http://metagenomics.anl.gov/mgmain.html?mgpage=project&project=mgp18808).

Statistical analyses of sequencing data

Community structure was analysed using a square-root transformed matrix of OTU relative abundance to minimize the impact of highly dominant OTUs and then subject to statistical analyses to compare the structure of the bacterial communities between both layers of Lake Pavin. The dynamics of the bacterial communities were primarily analysed by non-metric multidimensional scaling (NMDS). A stress value was calculated to measure the difference between the ranks on the ordination configuration and the ranks in the original similarity matrix for each repetition. An acceptable stress value should be below 0.1. An ANOSIM and a PERMANOVA were conducted to test the differences in overall bacterial community composition between the SML and epilimnion and to further confirm the results observed in the NMDS plot. All analyses were based on similarity matrices calculated with the Bray-Curtis similarity index.

To explain the temporal variation of bacterial community structure in both layers, a canonical correspondence analysis (CCA) was performed linking community composition (inferred bacterial groups presenting >10 OTUs) and environmental variables (phosphate, nitrite, nitrate, and ammonia concentrations). This analysis was performed with the VEGAN package (http://cran.r-project.org/web/packages/vegan/index.html) in R software. Finally, indicator species (Dufrene & Legendre 1997) were identified using the INDICSPECIES package in R.

RESULTS AND DISCUSSION

Changes in bacterial community structures were evaluated in both the SML and epilimnion of Lake Pavin during spring and summer (from May to August 2011) by 16S rRNA gene 454 pyrosequencing. SML community sampling remains challenging, as many sampling procedures exist for SML collection, and these methods may impact the estimates of microbial diversity (Agogué et al. 2004). In the present study, SML sampling was conducted with polycarbonate membranes that allowed collection of a very thin layer (ranging from 10 to 50 µm) that is highly dependent on the adsorption of microbes to membranes. To avoid sampling biases, 3 polycarbonate filters from each sampling date and each location were pooled (SML and epilimnion) prior to DNA extraction to obtain a 'composite' sample representative for the layer considered. Some other techniques use mesh screens (Auguet & Casamayor 2008, Hörtnagl et al. 2010) or

Table 1. Number of OTUs and sequences retrieved in the epilimnion and surface microlayer. Diversity and richness indices were also calculated. Dates are yyyy/mm/dd

	Epilimnion				Surface microlayer			
	2011/05/10 E1	2011/06/06 E2	2011/07/05 E3	2011/08/23 E4	2011/05/10 SML1	2011/06/06 SML2	2011/07/05 SML3	2011/08/23 SML4
No. of OTUs	447	517	453	429	246	500	218	296
No. of normalized sequences	2700	2700	2700	2700	2700	2700	2700	2700
Chao index	742.5	875.5	691.8	638	338.6	863.2	301.8	376.7
Shannon index	4.929	5.32	5.067	4.677	4.367	5.057	4.326	4.656
Evenness index	0.3092	0.3955	0.3505	0.2505	0.3203	0.3144	0.347	0.3556

glass plates (Reinthaler et al. 2008, Wurl et al. 2009), which present larger sampling zones (20 to 400 µm), implying that the samples collected may be a mixture of both the SML and the underlying waters (epilimnion), constituting potential cross-contaminations.

Singletons were removed from the dataset, and the number of sequences was randomly resampled down to 2700 sequences to compare samples (Table 1). Resulting rarefaction curves indicated that most of the diversity was captured in both layers (Fig. S1 in the Supplement at www.int-res.com/articles/suppl/a079p115_supp.pdf). No significant difference in microbial diversity (Shannon index) between the SML (4.6 ± 0.34) and underlying water (5.0 ± 0.27) was observed (Table 1). Similarly, the number of bacterial OTUs was not statistically different between the 2 layers (Table 1), in agreement with previous studies conducted in high mountain lakes (Vila-Costa et al. 2013, Sarmento et al. 2015).

Community composition was analysed based on a normalized dataset, comprising a total of 1673 OTUs (21 000 sequences; Table 1). Even with a limited number of samples, consistent temporal differences in community composition were observed in NMDS analysis (Fig. 1). The NMDS bi-dimensional ordination diagram presented a stress value of 0.0871, which indicates a reliable representation of the original similarity matrix (Bray-Curtis distance). The ordination diagram showed that the bacterial community composition in the SML was considerably more variable (i.e. dynamic) than the community structure in the epilimnion (confirmed with an ANOSIM test, $p = 0.029$, $R = 0.5$; and a PERMANOVA test, $p = 0.028$, $R = 0.38$). Temporal differences between the SML and epilimnetic communities might be related to the *in situ* stressful environmental conditions experienced in the SML (i.e. UV exposure, aerosol concentrations; Agogué et al. 2005, Alonso-Sáez et al. 2006). However, we could not exclude that differences in community composition, with higher variability in the SML, were due to the different sampling strategies used for the SML and underlying water compartment. Indeed, selective adsorption properties of a filter could potentially influence the assessment of bacterial communities in the different zones. Temporal variation of OTU abundance was more important in the SML than in the epilimnetic water (Fig. 2). For example, *Alpha-* and *Betaproteobacteria* and *Bacteroidetes* were slightly enriched in the SML in June compared to the other months, whereas *Gammaproteobacteria* were enriched in May, suggesting that contrasting environmental conditions impact community structure. Further work investigating seasonal

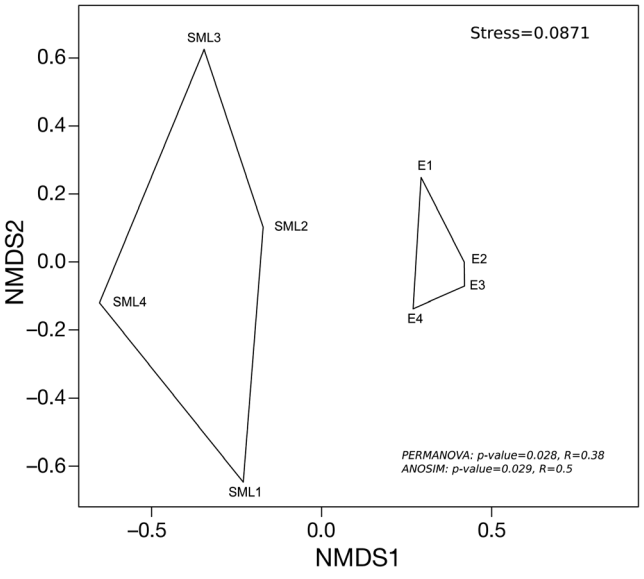

Fig. 1. Non-metric multidimensional scaling (NMDS) ordination of bacterial community structure inferred from 16S rRNA gene counts from main bacterial phyla retrieved in the epilimnion and surface microlayer (*Acidobacteria*, *Actinobacteria*, *Alpha- Beta- Gamma- Delta- Epsilonproteobacteria*, *Armatimonadetes*, BD1-5, *Caldiserica*, Candidate divisions OD1, OP11, TM7, *Bacteroidetes*, *Chlamydiae*, *Chlorobi*, *Chloroflexi*, *Cyanobacteria*, *Deinococcus*, *Fibrobacteres*, *Firmicutes*, *Gemmatimonadetes*, *Lentisphaerae*, *Planctomycetes*, *Spirochaetae*, *Verrucomicrobia*) on the different sampling dates. E1 to 4: epilimnion, SML1 to 4: surface microlayer. See Table 1 for sampling dates

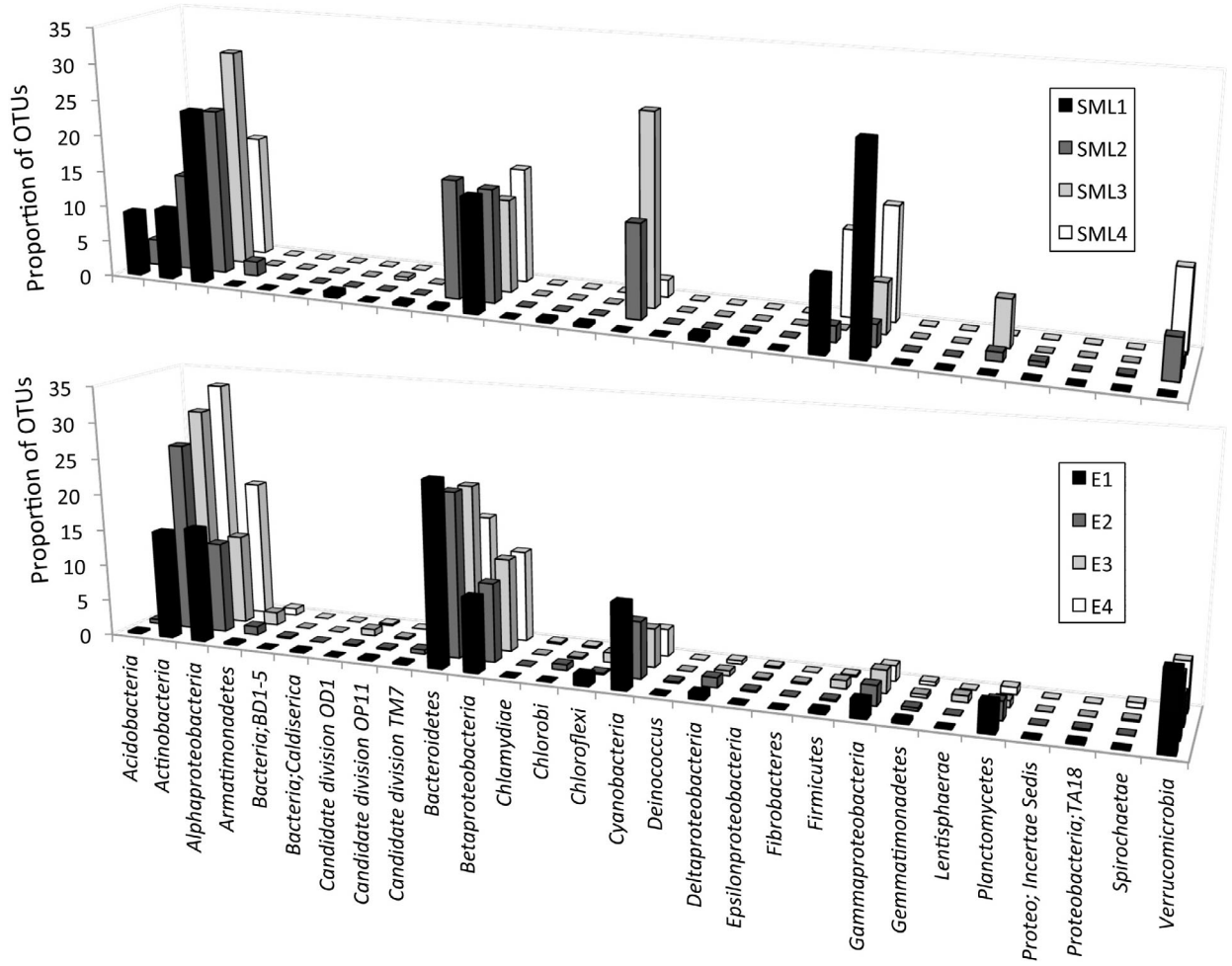

Fig. 2. Phylogenetic affiliation and abundance of OTUs retrieved in the (A) surface microlayer (SML) and (B) epilimnion (E) in Lake Pavin, France. For each layer, the same samples were collected on 10 May, 6 June, 5 July, and 23 August 2011. In each sample, OTUs affiliated with bacterial phyla and/or class were pooled and plotted to visualize temporal variations in bacterial community structure. E1 to 4: epilimnion, SML1 to 4: surface microlayer. See Table 1 for sampling dates

changes over 1 yr and/or inter-annual changes is now needed to increase our understanding of changes in bacterial community composition.

Among the total OTUs, 732 (3923 sequences) were exclusively retrieved in the epilimnion, and 622 (7581 sequences) were exclusively in the SML, whereas 319 OTUs (10 096 sequences) were found in both layers (i.e. shared; Fig. 3A). The present study supports a classical view of bacterial community composition (phyla or class level; Newton et al. 2011) retrieved from the epilimnion and shared fraction dataset (Fig. 3B), with *Bacteroidetes*, *Actinobacteria*, *Betaproteobacteria*, and *Alphaproteobacteria* OTUs dominating (24%, 21%, 19%, and 12% of the OTUs, respectively, in the shared fraction, and 21%, 22%, 7%, and 17% of the OTUs, respectively, in the epilimnion). Interestingly, indicator species from the epilimnion (Table 2) were mainly affiliated with the previous groups but also with cyanobacterial-chloroplast-related sequences, and *Gemmatimonadetes*, *Gammaproteobacteria*, or *Verrucomicrobia* phyla. These last phyla that contained indicator species were less represented among the OTUs, with *Cyanobacteria* and *Verrucomicrobia* representing 10% and 8% of the overall OTUs, respectively, in the shared fraction, and 7% and 10%, respectively, in the epilimnion. A previous study on bacterial community composition over 2 consecutive years conducted in the Lake Pavin epilimnetic layer showed that *Actinobacteria* and *Betaproteobacteria* were dominant (Boucher et al. 2006), as seen in the SML of freshwater lakes (Hörtnagl et al. 2010). The results reported here are congruent with the idea of freshwater groups that are ubiquitous in epilimnetic waters. Biogeographic patterns have been shown to exist in microbial communities, and the spatial distribution of bacterial assemblages in ecosystems is still

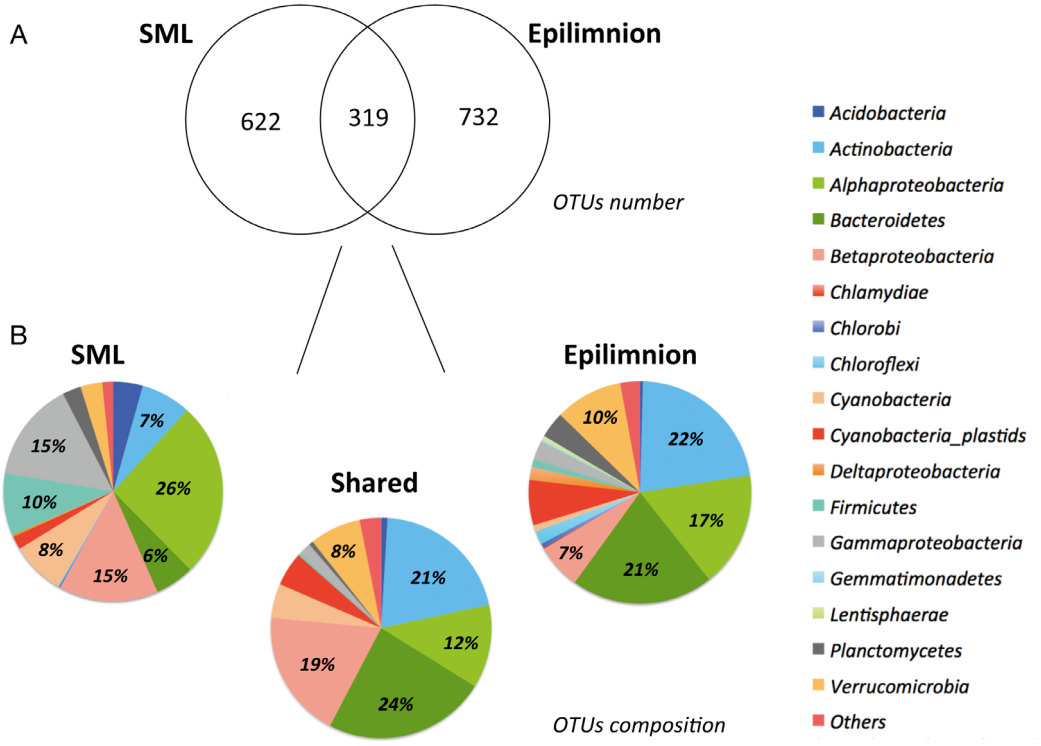

Fig. 3. (A) Number of specific and shared OTUs from surface microlayer (SML) and epilimnion. (B) Comparison of OTU abundance associated with SML or epilimnion and shared OTUs retrieved in both layers. OTUs were screened for their specificity to each layer and the OTUs affiliated with the same phyla and/or class were pooled to evaluate which ones are specific for each layer and/or shared

Table 2. Indicator OTUs (with their respective abundance) identified in both layers. In the epilimnion, 32 OTUs were statistically enriched, while in the surface microlayer, only 4 OTUs were characteristic (p = 0.028)

Affiliation	No. of affiliated OTUs	Indicator value
Surface microlayer		
Actinobacteria_Actinobacteria_Propionibacteriales_Propionibacteriaceae_Propionibacterium	3	1.00
Gammaproteobacteria_Pseudomonadales_Moraxellaceae_Acinetobacter	1	1.00
Epilimnion		
*Actinobacteria_Acidimicrobia_Acidimicrobiales_Acidimicrobiaceae*_CL500.29.marine.group	1	1.00
*Actinobacteria*_AcIV_Iluma.A2	1	0.993 to 1.00
Actinobacteria_Actinobacteria_Frankiales_Sporichthyaceae	4	1.00
*Actinobacteria_Actinobacteria_Frankiales_Sporichthyaceae*_hgcI.clade	7	0.981 to 1.00
Bacteroidetes_Flavobacteria_Flavobacteriales_Cryomorphaceae_Fluviicola	1	0.996
*Bacteroidetes_Flavobacteria_Flavobacteriales*_NS9.marine.group	1	0.996
*Bacteroidetes_Sphingobacteria_Sphingobacteriales*_env.OPS.17	1	0.988
*Bacteroidetes_Sphingobacteria_Sphingobacteriales*_LiUU.11.161	1	1.00
*Cyanobacteria*_Chloroplast	1	1.00
Gemmatimonadetes_Gemmatimonadetes_Gemmatimonadales_Gemmatimonadaceae_Gemmatimonas	2	1.00
Alphaproteobacteria_Caulobacterales_Caulobacteraceae_Caulobacter	3	0.968 to 1.00
*Betaproteobacteria_Burkholderiales*_BetaI_Lhab	1	1
Betaproteobacteria_Burkholderiales_Comamonadaceae_Acidovorax	1	0.957
Betaproteobacteria_Burkholderiales_Comamonadaceae_Chlorochromatium	3	0.985 to 1.00
Gammaproteobacteria_Xanthomonadales_Xanthomonadaceae_Stenotrophomonas	3	1
*Verrucomicrobia*_Sta2.35	1	0.985

under active debate. Indeed, some authors have suggested that many of the OTUs are commonly retrieved in freshwater ecosystems (Lindström et al. 2005), whereas others have suggested that some species are ecosystem-specific, with a limited geographic distribution, because they are dependent on the trophic status or input of allochthonous communities through rivers or catchment areas (Urbach et al. 2001).

Members of the *Bacteroidetes* phylum (previously known as the *Cytophaga-Flavobacterium-Bacteroides* group) represent important heterotrophs in aquatic ecosystems, and they are involved in organic carbon cycling. These bacteria are commonly retrieved in freshwater lakes and rivers (McCammon et al. 1998, Böckelmanna et al. 2000) and in marine systems (Cottrell & Kirchman 2000, Humphry et al. 2001). *Actinobacteria* represent up to 20% of bacterial OTUs retrieved in both the epilimnion and shared fractions (Fig. 3). Many previous studies have shown that *Actinobacteria* are abundant and ubiquitous in freshwater ecosystems, accounting for up to 50% of the bacteria in the epilimnion (Glöckner et al. 2000) and containing several cosmopolitan freshwater clades (Newton et al. 2007), with some, like AcI, accounting for >50% of DAPI-stained cells from a lacustrine epilimnion (Newton et al. 2007). This clade has been previously retrieved in lacustrine ecosystems such as Lake Baikal or Lake Fuchskuhle (Glöckner et al. 2000), and it is distinct from marine *Actinobacteria* clusters (Rappé et al. 1999). Our study showed that, among *Actinobacteria*, OTUs affiliated to both the AcI lineage (including the hcgI clade) and the CL500-29 marine group were always the most abundant ones (Table S1 in the Supplement) in both epilimnion and shared fractions. The presence of the CL500-29 marine group was surprising, as it has been retrieved primarily in estuarine and delta ecosystems which were ecosystems at the interface between marine and freshwater (Stepanauskas et al. 2003), while Lake Pavin was exclusively freshwater. Regarding the AcI lineage, a study has suggested that this lineage is very active and successful in planktonic assemblages in mountain lakes (Warnecke et al. 2005). No AcII OTU was detected in our work, in accordance with a previous study that has not found this taxon in freshwater ecosystems (e.g. Lake Redon) (Hervas & Casamayor 2009). In the same study, the authors did not detect AcIV OTUs, whereas our study identified 13 OTUs specific to the epilimnion fraction (Table S1 in the Supplement). Moreover, many actinobacterial strains are pigmented, and it has been proposed that these pigments, such as proteorhodopsins, are important for acquiring energy from solar radiation (Warnecke et al. 2005, Sharma et al. 2008). They also may have a competitive advantage in relieving grazing pressure because of their relatively small size (Pernthaler et al. 2001, Tarao et al. 2009, Newton et al. 2011). The great abundance of *Betaproteobacteria* in the epilimnion and shared fractions is not surprising, as they are usually reported as the dominant group in freshwater lakes (Newton et al. 2011), based on *in situ* hybridization and PCR-dependent methods (Glöckner et al. 2000, Barberán & Casamayor 2010). However, a recent study uncovered potential biases associated with the use of the 907R 'universal' primer (probably related to an underestimate of *Epsilonproteobacteria* involved in the sulphur-oxidizing process), showing the need for complementary approaches to evaluate the abundance of specific taxa in bacterial communities (Llorens-Marès et al. 2016). Many betaproteobacterial members are cultivated in association with other microorganisms, allowing a thorough characterization of their metabolic abilities. *Betaproteobacteria* are known to respond rapidly to organic and inorganic nutrient availability (Šimek et al. 2006, Pérez et al. 2015). It has been suggested that *Betaproteobacteria* and *Actinobacteria* have different temporal niches, with *Betaproteobacteria* abundant when mixing events occur in the water column (during June and July in the present study) and more nutrients are available, whereas *Actinobacteria* show less fluctuation in their abundance, suggesting that these microorganisms are more efficient in consuming less organic carbon (Glöckner et al. 2000), as reported during August in our study. This was confirmed by a CCA, which showed that *Alpha-*, *Beta-*, and *Gammaproteobacteria* are linked with the phosphate content in the ecosystem, whereas *Actinobacteria* and *Bacteroidetes* are mainly associated with ammonia and nitrite concentrations (Fig. 4), suggesting that shifts in environmental parameters may favour the presence of different bacterial groups.

Notably, the bacterial community specific to the SML consisted not only of lineages classically inhabiting freshwater ecosystems but also of taxa rarely detected in these ecosystems (Fig. 3B). *Alphaproteobacteria* were more abundant (26% of the OTUs) than *Betaproteobacteria* (15% of the OTUs; Figs. 2 & 3), in contrast with findings from previous studies showing that *Betaproteobacteria* and *Actinobacteria* dominated in the SML of high mountain lakes (Hervas & Casamayor 2009, Vila-Costa et al. 2013). *Alphaproteobacteria* are usually less represented in bacterioplankton in alpine freshwater lakes than *Betaproteobacteria* (Glöckner et al. 2000, Zwart et al. 2002). *Alphaproteobacteria* have been shown to

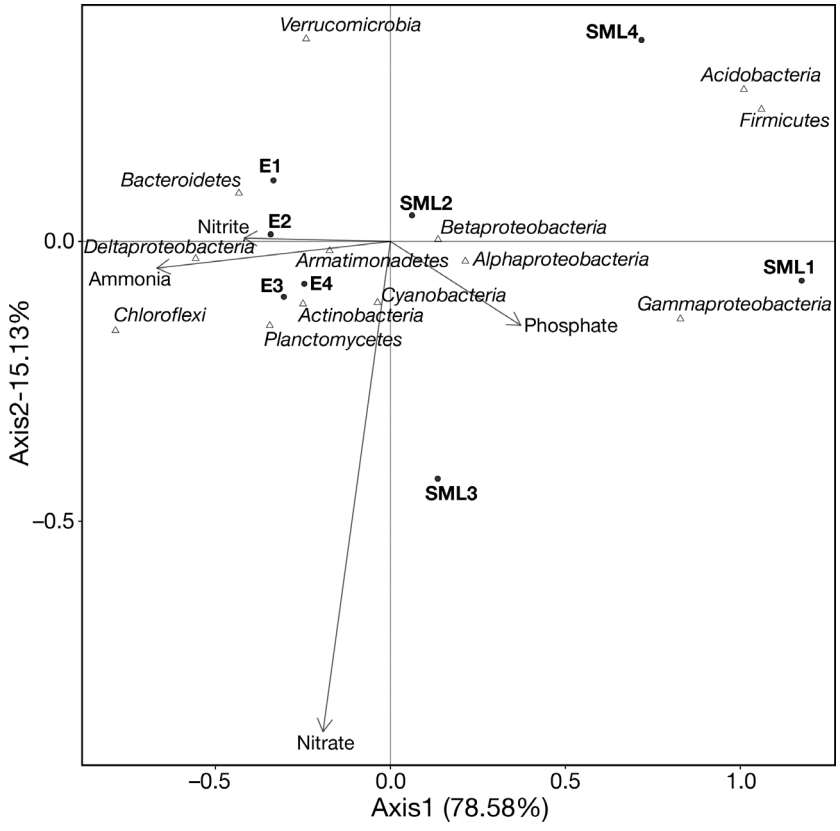

Fig. 4. Canonical correspondence analysis (CCA) plot of main bacterial groups (represented by >10 OTUs) linked to environmental parameters. E1 to 4: epilimnion, SML1 to 4: surface microlayer. See Table 1 for sampling dates

be more resistant to predation, and particularly to microeukaryote grazing, and it has been suggested that in low-nutrient environments, such as oligotrophic lakes, they might be favoured because of their ability to compete for resources (Newton et al. 2011). This could explain the dominance of this phylum in the SML of Lake Pavin, which is an oligotrophic ecosystem barely impacted by anthropogenic inputs and surrounding leaching. Among the *Alphaproteobacteria*, the most represented OTUs were affiliated with *Rhizobiales*, *Rhodospirillales*, and *Sphingomonadales* (Table S1 in the Supplement; Fig. 5). Among the *Rhodospirillales*, 51 OTUs were affiliated with the genus *Acidocella*, which was first isolated from a naturally acidic shallow lake with high heavy-metal content in Mexico and has been retrieved in acid mine drainage environments with high heavy-metal levels (Johnson et al. 2001, Hallberg et al. 2006). Among the *Sphingomonadales* OTUs, the GOBB3-C201 freshwater cluster consisted of 21 OTUs in the SML of Lake Pavin. This group was first retrieved in Crater Lake, Oregon, USA (Page et al.

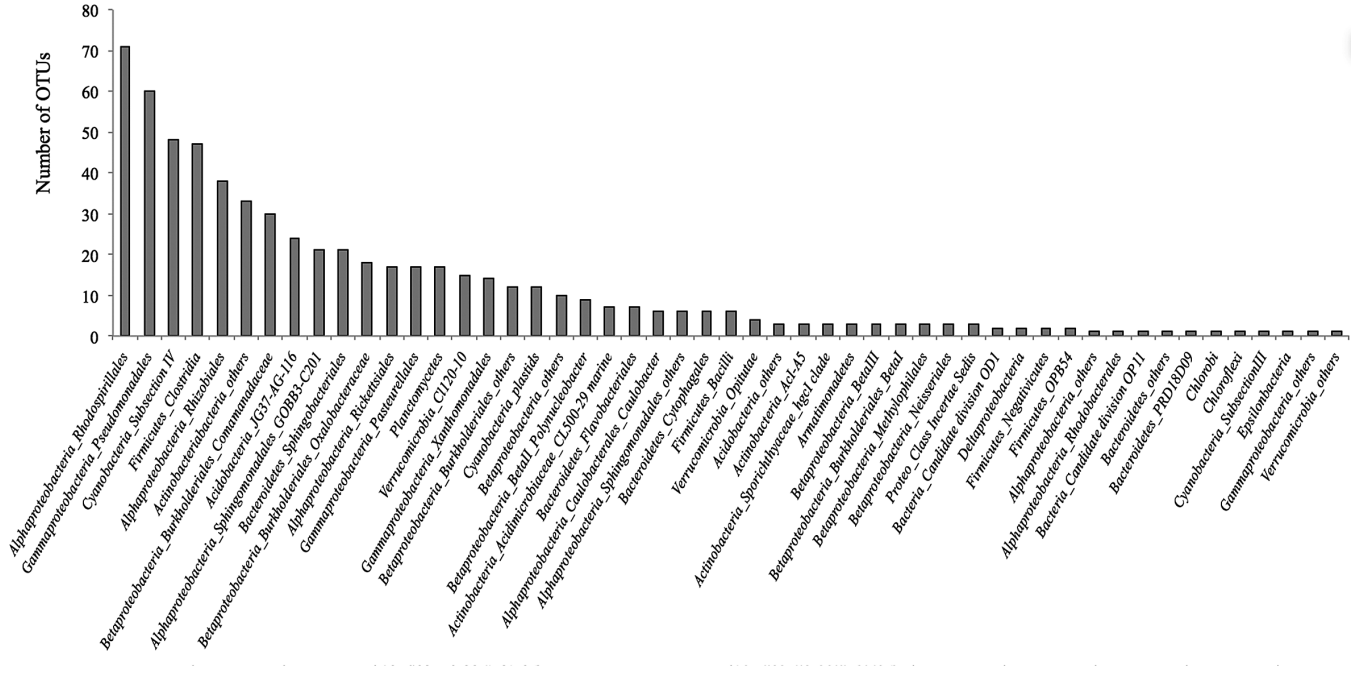

Fig. 5. Rank-abundance curve of specific OTUs retrieved in the surface microlayer

2004) and is typically associated with lacustrine or riverine ecosystems (Likens 2010), suggesting the presence of freshwater-specific OTUs clusters. The present study showed a clear enrichment in *Gammaproteobacteria* and *Firmicutes* OTUs in the SML dataset (15% and 10% of the OTUs, respectively; Fig. 3). *Gammaproteobacteria* are rarely detected in lakes and are usually found in marine ecosystems and saline lakes (Wu et al. 2006). *Gammaproteobacteria* might come from the surrounding environment through aerosol deposition. The great abundance of *Gammaproteobacteria* in the SML fraction can be explained by their resistance to sunlight and particularly to UV radiation exposure (Alonso-Sáez et al. 2006, Santos et al. 2012). These results indicated the presence of specific bacteria that might be adapted to multi-stress conditions related to the SML. A previous study showed that among the *Gammaproteobacteria*, strains isolated from UV exposure experiments were related to *Pseudomonas* in the SML, whereas in underlying water masses, strains belonged to *Acinetobacter* or *Psychrobacter* (Santos et al. 2012). This is congruent with our study, which showed that the most abundant gammaproteobacterial OTUs were affiliated with *Pseudomonas*, *Acinetobacter*, and *Haemophilus* (Table S1 in the Supplement). In our work, *Acinetobacter* constituted an indicator species in the SML (Table 2), suggesting its importance in this zone. Recently, Hervas & Casamayor (2009) proposed that *Acinetobacter* members could be indicators of airborne transport in dust from the Sahara; however, in the present study, we have no information on aerosol deposition in Lake Pavin. *Haemophilus* are mostly commensals of the upper respiratory tract of birds and mammals, and their presence in the SML can be explained by the presence of animal excreta. Interestingly, among *Firmicutes*, the most abundant OTUs in our study were affiliated with *Clostridiales*, which are among the most abundant bacterial families in the mammalian gut and are strictly anaerobic (Lopetuso et al. 2013). Moreover, those *Bacteria* have a resistant stage, spores, that protect them in unfavourable environmental conditions. Their presence might reflect the existence of dead cells, and further studies based on an analysis of 16S rRNA transcript abundance would be useful to understand their role in the SML of Lake Pavin. The present study also showed that 3 OTUs related to *Propionibacterium* (*Actinobacteria*) were enriched in the SML (Table 2). However, *Propionibacterium* is a commensal group from human skin, and we could not exclude cross-contamination.

The results presented here depicted the SML of a high mountain lake as a distinct microhabitat that seems to favour colonization by specific bacterial groups. The abundance of *Gammaproteobacteria* and *Firmicutes* raises many questions about their origins, as some taxa are believed to be specific to marine waters. Our results lead to several questions about the colonization success of bacteria inhabiting the SML and which factors control their abundance and activity in lacustrine ecosystems.

Acknowledgements. We thank Stéphanie Grizard (University of Lyon, France) and Danis Abrouk (iBio platform, UMR CNRS 5557 Ecologie Microbienne) for helpful discussions on statistical analyses.

LITERATURE CITED

Agogué H, Casamayor EO, Joux F, Obernosterer I and others (2004) Comparison of samplers for the biological characterization of the sea surface microlayer. Limnol Oceanogr Methods 2:213–225

Agogué H, Casamayor EO, Bourrain M, Obernosterer I, Joux F, Herndl GJ, Lebaron P (2005) A survey on bacteria inhabiting the sea surface microlayer of coastal ecosystems. FEMS Microbiol Ecol 54:269–280

Alonso-Sáez L, Gasol JM, Lefort T, Hofer J, Sommaruga R (2006) Effect of natural sunlight on bacterial activity and differential sensitivity of natural bacterioplankton groups in northwestern Mediterranean coastal waters. Appl Environ Microbiol 72:5806–5813

American Public Health Association (2005) Standard methods for the examination of water and wastewaters, 21st edn. Centennial Edition. APHA, American Water Works Association, Water Environmental Federation, Washington, DC

Asher W (2005) The sea-surface microlayer and its effect on global air–sea gas transfer. In: Liss PS, Duce RA (eds) The sea surface and global change. Cambridge University Press, Cambridge, p 121–172

Auguet JC, Casamayor EO (2008) A hotspot for cold crenarchaeota in the neuston of high mountain lakes. Environ Microbiol 10:1080–1086

Auguet JC, Triadó-Margarit X, Nomokonova N, Camarero L, Casamayor EO (2012) Vertical segregation and phylogenetic characterization of ammonia-oxidizing Archaea in a deep oligotrophic lake. ISME J 6:1786–1797

Barberán A, Casamayor EO (2010) Global phylogenetic community structure and β-diversity patterns in surface bacterioplankton metacommunities. Aquat Microb Ecol 59:1–10

Böckelmanna U, Manza W, Neub T, Szewzyka U (2000) Characterization of the microbial community of lotic organic aggregates ('river snow') in the Elbe River of Germany by cultivation and molecular methods. FEMS Microbiol Ecol 33:157–170

Boucher D, Jardillier L, Debroas D (2006) Succession of bacterial community composition over two consecutive years in two aquatic systems: a natural lake and a lake-reservoir. FEMS Microbiol Ecol 55:79–97

Catalán J, Camarero L, Felip M, Pla S and others (2006)

High mountain lakes: extreme habitats and witnesses of environmental changes. Limnetica 25:551–584

Claesson MJ, Wang Q, O'Sullivan O, Greene-Diniz R, Cole JR, Ross RP, O'Toole PW (2010) Comparison of two next-generation sequencing technologies for resolving highly complex microbiota composition using tandem variable 16S rRNA gene regions. Nucleic Acids Res 38:e200

Conrad R, Seiler W (1988) Influence of the surface microlayer on the flux of nonconservative trace gases (CO, H_2, CH_4, N_2O) across the ocean-atmosphere interface. J Atmos Chem 6:83–94

Cottrell MT, Kirchman DL (2000) Natural assemblages of marine proteobacteria and members of the *Cytophaga-Flavobacter* cluster consuming low- and high-molecular-weight dissolved organic matter. Appl Environ Microbiol 66:1692–1697

Cunliffe M, Harrison E, Salter M, Schäfer H, Upstill-Goddard RC, Murrell JC (2009) Comparison and validation of sampling strategies for the molecular microbial analysis of surface microlayers. Aquat Microb Ecol 57:69–77

Cunliffe M, Upstill-Goddard RC, Murrell JC (2011) Microbiology of aquatic surface microlayers. FEMS Microbiol Rev 35:233–246

Dufrene M, Legendre P (1997) Species assemblages and indicator species: the need for a flexible asymmetrical approach. Ecol Monogr 67:345–366

Edgar RC (2010) Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26:2460–2461

Edgar RC, Haas BJ, Clemente JC, Quince C, Knight R (2011) UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27:2194–2200

Franklin MP, McDonald IR, Bourne DG, Owens NJP, Upstill-Goddard RC, Murrell JC (2005) Bacterial diversity in the bacterioneuston (sea surface microlayer): the bacterioneuston through the looking glass. Environ Microbiol 7: 723–736

Giongo A, Crabb DB, Davis-Richardson AG, Chauliac D and others (2010) PANGEA: pipeline for analysis of next generation amplicons. ISME J 4:852–861

Glöckner FO, Zaichikov E, Belkova N, Denissova L, Pernthaler J, Pernthaler A, Amann R (2000) Comparative 16S rRNA analysis of lake bacterioplankton reveals globally distributed phylogenetic clusters including an abundant group of actinobacteria. Appl Environ Microbiol 66: 5053–5065

Hallberg KB, Coupland K, Kimura S, Johnson DB (2006) Macroscopic streamer growths in acidic, metal-rich mine waters in north Wales consist of novel and remarkably simple bacterial communities. Appl Environ Microbiol 72:2022–2030

Hervas A, Casamayor EO (2009) High similarity between bacterioneuston and airborne bacterial community compositions in a high mountain lake area. FEMS Microbiol Ecol 67:219–228

Hörtnagl P, Pérez MT, Zeder M, Sommaruga R (2010) The bacterial community composition of the surface microlayer in a high mountain lake. FEMS Microbiol Ecol 73:458–467

Hugoni M, Domaizon I, Taib N, Biderre-Petit C and others (2015) Temporal dynamics of active Archaea in oxygen-depleted zones of two deep lakes. Environ Microbiol Rep 7:321–329

Humphry DR, George A, Black GW, Cummings SP (2001) *Flavobacterium frigidarium* sp. nov., an aerobic, psychrophilic, xylanolytic and laminarinolytic bacterium from Antarctica. Int J Syst Evol Microbiol 51:1235–1243

Johnson DB, Rolfe S, Hallberg KB, Iversen E (2001) Isolation and phylogenetic characterization of acidophilic microorganisms indigenous to acidic drainage waters at an abandoned Norwegian copper mine. Environ Microbiol 3:630–637

Kalwasińska A, Kęsy J, Wilk I, Donderski W (2011) Neustonic versus epiphytic bacteria of eutrophic lake and their biodegradation ability on deltamethrin. Biodegradation 22:699–707

Kim M, Morrison M, Yu Z (2011) Evaluation of different partial 16S rRNA gene sequence regions for phylogenetic analysis of microbiomes. J Microbiol Methods 84: 81–87

Lane D (1991) 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. John Wiley & Sons, Chichester, p 115–175

Lefranc M, Thénot A, Lepère C, Debroas D (2005) Genetic diversity of small eukaryotes in lakes differing by their trophic status. Appl Environ Microbiol 71:5935–5942

Likens GE (2010) Plankton of inland waters. Academic Press

Lindström ES, Kamst-Van Agterveld MP, Zwart G (2005) Distribution of typical freshwater bacterial groups is associated with pH, temperature, and lake water retention time. Appl Environ Microbiol 71:8201–8206

Liss P, Duce R (2005) The sea surface and global change. Cambridge University Press, Cambridge

Llorens-Marès T, Triadó-Margarit X, Borrego CM, Dupont CL, Casamayor EO (2016) High bacterial diversity and phylogenetic novelty in dark euxinic freshwaters analyzed by 16S tag community profiling. Microb Ecol 71: 566–574

Lopetuso LR, Scaldaferri F, Petito V, Gasbarrini A (2013) Commensal Clostridia: leading players in the maintenance of gut homeostasis. Gut Pathog 5:23

Maki J (1993) The air–water interface as an extreme environment. In: Ford TE (ed) Aquatic microbiology: an ecological approach. Blackwell Scientific Publications, Oxford, p 409–439

McCammon SA, Innes BH, Bowman JP, Franzmann PD and others (1998) *Flavobacterium hibernum* sp. nov., a lactose-utilizing bacterium from a freshwater Antarctic lake. Int J Syst Bacteriol 48:1405–1412

Morales-Baquero R, Pulido-Villena E, Reche I (2006) Atmospheric inputs of phosphorus and nitrogen to the southwest Mediterranean region: biogeochemical responses of high mountain lakes. Limnol Oceanogr 51:830–837

Newton RJ, Jones SE, Helmus MR, McMahon KD (2007) Phylogenetic ecology of the freshwater *Actinobacteria* acI lineage. Appl Environ Microbiol 73:7169–7176

Newton RJ, Jones SE, Eiler A, McMahon KD, Bertilsson S (2011) A guide to the natural history of freshwater lake bacteria. Microbiol Mol Biol Rev 75:14–49

Page KA, Connon SA, Giovannoni SJ (2004) Representative freshwater bacterioplankton isolated from Crater Lake, Oregon. Appl Environ Microbiol 70:6542–6550

Pérez MT, Rofner C, Sommaruga R (2015) Dissolved organic monomer partitioning among bacterial groups in two oligotrophic lakes. Environ Microbiol Rep 7:265–272

Pernthaler J, Posch T, Šimek K, Vrba J and others (2001) Predator-specific enrichment of actinobacteria from a cosmopolitan freshwater clade in mixed continuous culture. Appl Environ Microbiol 67:2145–2155

Pester M, Schleper C, Wagner M (2011) The Thaumarchaeota: an emerging view of their phylogeny and ecophysiology. Curr Opin Microbiol 14:300–306

Price MN, Dehal PS, Arkin AP (2010) FastTree 2 — approximately maximum-likelihood trees for large alignments. PLOS ONE 5:e9490

Pruesse E, Quast C, Knittel K, Fuchs BM, Ludwig W, Peplies J, Glöckner FO (2007) SILVA: a comprehensive online resource for quality checked and aligned ribosomal RNA sequence data compatible with ARB. Nucleic Acids Res 35:7188–7196

Pulido-Villena E, Reche I, Morales-Baquero R (2008) Evidence of an atmospheric forcing on bacterioplankton and phytoplankton dynamics in a high mountain lake. Aquat Sci 70:1–9

Rappé MS, Gordon DA, Vergin KL, Giovannoni SJ (1999) Phylogeny of Actinobacteria small subunit (SSU) rRNA gene clones recovered from marine bacterioplankton. Syst Appl Microbiol 22:106–112

Reinthaler T, Sintes E, Herndl GJ (2008) Dissolved organic matter and bacterial production and respiration in the sea-surface microlayer of the open Atlantic and the western Mediterranean Sea. Limnol Oceanogr 53:122–136

Santos AL, Baptista I, Lopes S, Henriques I and others (2012) The UV responses of bacterioneuston and bacterioplankton isolates depend on the physiological condition and involve a metabolic shift. FEMS Microbiol Ecol 80: 646–658

Sarmento H, Casamayor EO, Auguet JC, Vila-Costa M, Felip M, Camarero L, Gasol JM (2015) Microbial food web components, bulk metabolism, and single-cell physiology of piconeuston in surface microlayers of high-altitude lakes. Front Microbiol 6:361

Sharma AK, Zhaxybayeva O, Papke RT, Doolittle WF (2008) Actinorhodopsins: proteorhodopsin-like gene sequences found predominantly in non-marine environments. Environ Microbiol 10:1039–1056

Šimek K, Hornák K, Jezbera J, Nedoma J and others (2006) Maximum growth rates and possible life strategies of different bacterioplankton groups in relation to phosphorus availability in a freshwater reservoir. Environ Microbiol 8:1613–1624

Stepanauskas R, Moran MA, Bergamaschi BA, Hollibaugh JT (2003) Covariance of bacterioplankton composition and environmental variables in a temperate delta system. Aquat Microb Ecol 31:85–98

Taib N, Mangot JF, Domaizon I, Bronner G, Debroas D (2013) Phylogenetic affiliation of SSU rRNA genes generated by massively parallel sequencing: new insights into the freshwater protist diversity. PLOS ONE 8:e58950

Tarao M, Jezbera J, Hahn MW (2009) Involvement of cell surface structures in size-independent grazing resistance of freshwater Actinobacteria. Appl Environ Microbiol 75:4720–4726

Urbach E, Vergin KL, Young L, Morse A, Larson GL, Giovannoni SJ (2001) Unusual bacterioplankton community structure in ultra-oligotrophic Crater Lake. Limnol Oceanogr 46:557–572

Vila-Costa M, Barberan A, Auguet JC, Sharma S, Moran MA, Casamayor EO (2013) Bacterial and archaeal community structure in the surface microlayer of high mountain lakes examined under two atmospheric aerosol loading scenarios. FEMS Microbiol Ecol 84:387–397

Viollier E, Jézéquel D, Michard G, Pèpe M, Sarazin G, Alberic P (1995) Geochemical study of a crater lake (Pavin Lake, France): trace-element behaviour in the monimolimnion. Chem Geol 125:61–72

Warnecke F, Sommaruga R, Sekar R, Hofer JS, Pernthaler J (2005) Abundances, identity, and growth state of actinobacteria in mountain lakes of different UV transparency. Appl Environ Microbiol 71:5551–5559

Wu QL, Zwart G, Schauer M, Kamst-van Agterveld MP, Hahn MW (2006) Bacterioplankton community composition along a salinity gradient of sixteen high-mountain lakes located on the Tibetan Plateau, China. Appl Environ Microbiol 72:5478–5485

Wurl O, Miller L, Röttgers R, Vagle S (2009) The distribution and fate of surface-active substances in the sea-surface microlayer and water column. Mar Chem 115:1–9

Zwart G, Crump BC, Agterveld MPK, Hagen F, Han S (2002) Typical freshwater bacteria: an analysis of available 16S rRNA gene sequences from plankton of lakes and rivers. Aquat Microb Ecol 28:141–155

Editorial responsibility: Karel Šimek, České Budějovice, Czech Republic

Submitted: December 2, 2016; Accepted: March 16, 2017
Proofs received from author(s): May 4, 2017