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RESEARCH ARTICLE

Bacterial and archaeal community structure in the surface microlayer of high mountain lakes examined under two atmospheric aerosol loading scenarios

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Abstract

Bacteria and Archaea of the air–water surface microlayer (neuston) and plankton from three high mountain lakes (Limnological Observatory of the Pyrenees, Spain) were analysed by 16S rRNA gene 454 pyrosequencing (V6 region) in two dates with different atmospheric aerosol loading conditions: (1) under a Saharan dust plume driven by southern winds; and (2) under northern winds with oceanic influence. In general, bacterial communities were richer than archaea, with estimated total richness of *c.* 2500 OTUs for Bacteria and *c.* 900 OTUs for Archaea equivalent to a sequencing effort of *c.* 250 000 and *c.* 20 000 sequences, respectively. The dominant bacterial OTU was affiliated to Actinobacteria. Archaea were one to two orders of magnitude less abundant than bacteria but were more evenly distributed. Apparently, *Bacteroidetes* and *Thaumarchaeota* sequences were preferentially found at the neuston, but no consistent pattern in either total microbial abundance or richness was found in any sample. However, we observed more marked changes in microbial relative abundances between neuston and plankton in the dust-influenced scenario. Higher community dissimilarities between neuston and plankton were also found during the Saharan dust episode, and such differences were higher for Bacteria than for Archaea. Nonetheless, relatively few (< 0.05%) of the neuston sequences matched previously identified airborne microorganisms, and none became important in the dates analysed.

Introduction

Bacteria and Archaea are common components of the microbial plankton but how their community structures and responds to environmental factors are questions poorly explored (Pernthaler *et al.*, 1998; Aller & Kemp, 2008). Bacteria have been considered key components in nutrient cycling in freshwater food webs and with large diversity of species, whereas freshwater Archaea have been traditionally restricted to anaerobic decomposition and methanogenesis and with a limited number of species (Chaban *et al.*, 2006; Newton *et al.*, 2011). This view has been challenged in the last years after the analysis of the large diversity of archaeal ribosomal gene sequences detected in freshwater environments and compiled in databases (see two recent meta-analyses in Auguet *et al.*, 2010; and Barberán *et al.*, 2011) and the potential role assigned to ammonia-oxidizing Thaumarchaeota in the freshwater N cycling (Herfort *et al.*, 2009; Merbt *et al.*, 2011; Auguet *et al.*, 2012).

Lakes in high mountain areas have been recently shown as environments rich in archaeal diversity, and the air–water interface of these lakes as apparently suitable for the development of Thaumarchaeota closely related to ammonia oxidizers (Auguet & Casamayor, 2008; Auguet *et al.*, 2012). Bacteria inhabiting the air–water interface

(bacterioneuston) may also be closely related to airborne bacteria (Hervàs & Casamayor, 2009), and positive effects on bacterial growth and abundance by dust inputs have been shown in lakes (Reche *et al.*, 2009). Catchments in high mountain areas are smaller than in low-land regions and, therefore, atmospheric loadings tend to significantly determine water characteristics (Psenner, 1999; Catalan *et al.*, 2006; Pulido-Villena *et al.*, 2008). These are oligotrophic systems strongly limited by elemental nutrients such as phosphorus and nitrogen, and the absence of direct anthropogenic influences makes them particularly sensitive to changes in the atmosphere and surrounding landscape (Camarero & Catalan, 2012). Because of their oligotrophic nature, microbial communities of high mountain lakes have the potential to be substantially affected by atmospheric inputs of microorganisms and nutrients. In recent years, a significant increase in long-range atmospheric dust transport due to human activities (desertification and changes in land use in geographically distant areas; Moulin & Chiapello, 2006) has been detected, and nutrient inputs from dust (as nitrogen, phosphorus and organic carbon; Morales-Baquero *et al.*, 2006; Mladenov *et al.*, 2011) increase bacterial abundance and production in alpine nutrient-limited waters (Pulido-Villena *et al.*, 2008; Reche *et al.*, 2009). Overall, these conditions make high mountain lakes very sensitive and particularly interesting to explore potential changes in the bacterial and archaeal communities inhabiting surface waters.

In the present study, we have compared the bacterial and archaeal community structure in surface waters of three Pyrenean lakes in two dates in autumn with different atmospheric aerosol loading conditions, that is, under a Saharan dust plume driven by southern winds and under northern winds with oceanic influence. Autumn is a period of the year in which the importance of atmospheric depositions to the lakes' biogeochemistry is exacerbated (Psenner, 1999). The neuston and surface plankton were analysed by high-throughput pyrosequencing of a portion of the 16S ribosomal RNA gene (V6 region) trying to unveil whether or not (1) bacterial and archaeal populations showed different distribution under the two atmospheric scenarios; (2) larger community differences happened in the dust-influenced date; and (3) previously identified airborne microorganisms became important members of the community in the dates analysed.

Material and methods

Sample collection, DNA extraction and microscopic counts

Samples were obtained from three geographically close oligotrophic lakes located at different altitudes within the same catchment [Lake Llebreta (LLB), 1620 m; Lake Llong (LLG), 2010 m; and Lake Redó d'Aigüestortes (RAT), 2117 m] in September and November 2007. The lakes belong to the long-term ecological research (LTER) site 'Limnological Observatory of the Pyrenees' (Spanish Pyrenees, 42º 33′3″N, 0º 53′25″E) in the protected area of Aigüestortes National Park. These lakes are highly oligotrophic (Table 1), small (< 10 ha), shallow (< 13 m deep) and ice-covered for 4–7 months every year (typically from December to April). Neuston samples were collected from the surface microlayer (SML) using a nylon screen sampler (see Auguet & Casamayor, 2008 for details). Plankton samples were collected from the underlying water (UW, top 1 m integrated sample) over the deepest part of the lakes. Chemical analyses [N species and dissolved organic carbon (DOC)] were carried out as recently described (Auguet *et al.*, 2011). Standard deviations were < 1% for nitrite and DOC and < 10% for ammonium and nitrate. The three lakes have a marked oligotrophic nature with dissolved organic carbon concentrations always below 1.5 mg L^{-1} (Table 1).

Table 1. Environmental data, CARD-FISH counts and number of sequences and OTUs obtained after V6 tag sequencing for bacteria (BAC) and archaea (ARC), in the lakes sampled

						BAC FISH		ARC FISH		BAC #V6				ARC #V6			
						Cells ($\times 10^3$) mL^{-1}		Cells ($\times 10^3$) mL^{-1}		#seqs		#OTUs		#seqs		#OTUs	
Lake	Month	DOC mg L^{-1}	NO_3^- µM	NH_4^+ µM	NO_2^- µM	N	UW	N	UW	N	UW	N	UW	N	UW	N	UW
LLB	September	1.5	14.0	0.6	0.1	158.0	138.2	15.0	14.7	33930	30195	462	459	1402	1405	194	196
	November	0.6	15.0	0.5	0.1	106.0	86.9	2.3	3.2	14268	31139	683	989	757	1199	143	156
LLG	September	1.4	4.2	0.8	0.1	227.0	285.1	13.5	14.4	31191	27618	837	790	6024	2004	566	166
	November	ND	6.1	0.5	0.1	74.3	59.6	0.3	1.8	25439	24307	1100	786	517	3444	144	245
RAT	September	0.8	8.8	0.2	0.1	164.0	187.4	5.2	11.2	28125	26662	780	795	4184	1480	233	165
	November	ND	9.2	0.4	0.1	86.9	51.1	0.4	2.8	30174	35978	802	775	1136	2002	198	308

N, neuston; UW, underlying water; ND: not determined.

Sampling dates matched two different atmospheric scenarios. One was at the end of summer (September 18) immediately after a 5-day Saharan dust outbreak episode. The other was in fall (November 12) after a 2-week period free of Saharan dust (Fig. 1). Dust plumes were tracked using images from the BSC-DREAM8b (Dust REgional Atmospheric Model) operated by the Barcelona Supercomputing Center (http://www.bsc.es/earth-sciences/mineral-dust-forecast-system). In the previous 7 days before sampling, wet deposition (estimated by measuring the volume of water accumulated in a permanent collector located 200 m away from LLB) averaged 0.85 L m^{-2} per day in September and 0.28 L m^{-2} per day in November.

For microscopic counts, a volume of 7 mL was fixed in triplicate overnight at 4 °C with formaldehyde (2% final concentration). The microbial cells were collected on polycarbonate filters (0.2 μm pore size, GTTP Millipore) and stored at −20 °C until further processing in the laboratory. Filters were embedded in 0.1% (w/v in MilliQ water) low gelling point agarose to avoid high cell losses during the DAPI and CARD-FISH procedures (Pernthaler *et al.*, 2002). Once dried (40 min at 37 °C) and dehydrated in 95% ethanol, filters were cut in sections. One section of each filter was stained with DAPI (1 μg mL^{-1} for 10 min in the dark), rinsed twice with MilliQ water and dehydrated with 80% ethanol before mounting on slides and counting by epifluorescence microscopy. Filter sections for CARD-FISH counts were processed as previously described in Pernthaler *et al.* (2002) and Sekar *et al.* (2003) using the oligonucleotide probes EUB338 (5′-GCTGCCTCCCGTAGGAGT-3′; targeting most of Bacteria, 55% formamide concentration) and ARCH915 (5′-GTGCTCCCCCGCCAATTCCT-3′; mostly targeting Archaea, 50% formamide concentration). For cell wall permeabilization, filters were incubated with lysozyme (0.05 M EDTA, pH 8.0; 0.1 M Tris-HCl; 10 mg mL^{-1} lysozyme, Fluka) for 1 h at 37 °C, washed three times in MilliQ water and dehydrated in 95% ethanol. Additional permeabilization was carried out with achromopeptidase [0.01 M NaCl; 0.01 M Tris-HCl, pH 8.0; achromopeptidase (60 U mL^{-1})] for 30 min at 37 °C. Standard deviations in cell counts were < 10%.

For DNA analyses, 500 mL of water were prefiltered *in situ* through a 40-μm mesh to remove large organisms and then sequentially filtered on 5- and 0.2-μm-pore-size polycarbonate filters (47 mm diameter, Nuclepore). Filters were stored in lysis buffer (40 mM EDTA, 50 mM Tris, pH 8.3, 0.75 M sucrose) at −20 °C until further processing in the laboratory. Filters from the < 5 μm fraction were incubated with lysozyme, proteinase K and sodium dodecyl sulphate (SDS), and DNA was extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1 vol : vol : vol) and with chloroform/isoamyl alcohol (24 : 1, vol : vol) as previously described (Demergasso *et al.*, 2008).

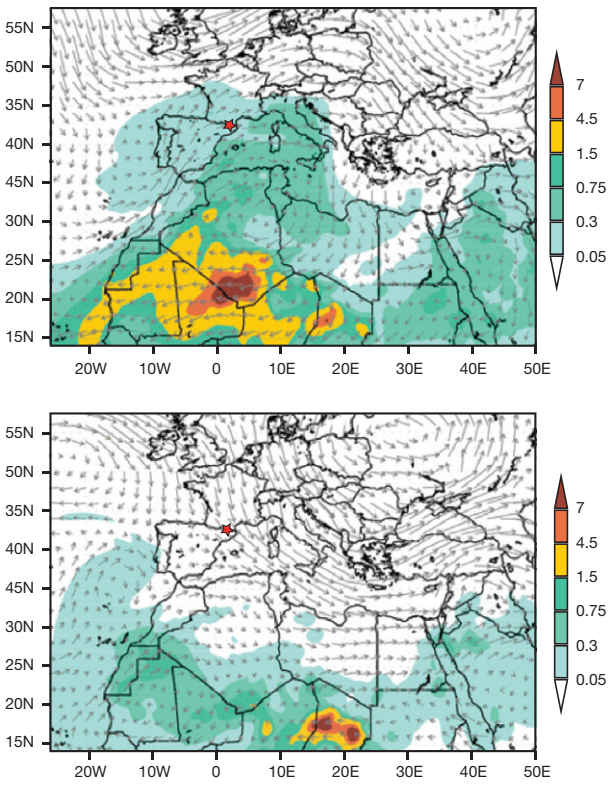

Fig. 1. Dust loads (in g m^{-2}) and wind vector trajectories at 3000 m height on 16 September (top) and 12 November (bottom) 2007. Images from BSC-DREAM8b model (http://www.bsc.es/projects/earthscience/BSC-DREAM/). Location of the lakes labelled with a red star.

DNA amplification and sequencing

Community composition was determined by 454 pyrosequencing of a PCR-amplified region of the 16S rRNA gene. We used the Roche GS FLX sequencing which on average provided fragments < 100 bp, and we selected fragments containing the hypervariable region V6 using a mix of five forward primers (967F) and four reverse primers (1046R) for Bacteria, and the forward primer 958F and a mix of two reverse primers (1048R) for Archaea (Sogin *et al.*, 2006). Current technology can provide longer fragments (Loman *et al.*, 2012), although previous studies have shown minimal differences of taxonomy obtained at the class level when comparing different hypervariable regions of 16S rRNA gene (V3, V6) and the full length sequence (Huse *et al.*, 2008). Tagged primers were used to discriminate samples. For Bacteria, the reaction was carried out with Ready-To-Go PCR beads (Amersham Pharmacia, Piscataway, NJ) with

0.1 μM final concentration of forward and reverse primer mixtures. PCR conditions were as follows: 5 min at 94 °C, followed by 30 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min and primer extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. For Archaea, the reaction mix consisted of 0.5 U of Invitrogen TM (Carlsbad, CA) High Fidelity Platinum® *Taq* polymerase and the buffer L from MasterAmp™ PCR optimization kit, 2× PCR PreMixes (Epicentre Biotechnologies, Madison, WI) with 0.1 μM final concentration of forward and reverse primer mixtures. PCR conditions were as follows: 3 min at 94 °C, followed by 35 cycles of denaturation at 94 °C for 50 s, annealing at 55 °C for 50 s and primer extension at 72 °C for 1.3 min, and a final extension at 72 °C for 10 min. PCR products were cleaned using the QIAquick PCR Purification kit (Qiagen) followed by the Agencourt AMPure XP kit (Agencourt Bioscience, Beckman Coulter). The Roche GS FLX sequencing run yielded a total of 25.6 Mbp split in 407 112 reads (63-bp average length). All 454 sequences can be accessed from the CAMERA database (http://camera.calit2.net/) under project id CAM_P_0000910.

Data analysis

Raw sequences were quality-based, trimmed and clustered at a 95% identity threshold using CD-HIT (Li & Godzik, 2006). Singletons were removed to prevent inflation in diversity estimates (Kunin *et al.*, 2009). As observed in other studies (e.g. Quince *et al.*, 2011), we checked for differences between denoiser vs. our own data purging protocol and found them to be very minor (details not shown). OTU taxonomic affiliation was determined by pairwise Smith–Waterman alignments to the Ribosomal Database Project (RDP; http://rdp.cme.msu.edu/). We identified sequences at the phylum level for *Actinobacteria*, *Firmicutes* and *Bacteroidetes*, and at the class level for *Proteobacteria* (i.e. *Alpha, Beta and Gamma*). A minimum of 90% sequence identity with an overlap of 80% of the input sequence length was required for taxonomic assignment. For Archaea, we specifically checked whether V6 fragments would be correctly assigned in a BLAST analysis against RDP. Thus, 200 randomly selected full 16S rRNA gene sequences (1400 bp in length) previously identified at the phylum level were trimmed to select the V6 region. These control fragments were blasted against RDP following the protocol described above. Up to 95% of the trimmed V6 reads yielded the same phylum assignment by both pipelines. To identify Thaumarchaeota sequences (a group recently renamed as differentiated from Crenarchaeota), all the OTUs classified as either unidentified Archaea or Crenarchaeota by RDP were further checked in the Greengenes database (http://greengenes.lbl.gov/).

Alpha-diversity was measured by the abundance-based estimators Chao1, ACE and Good's coverage, and the Shannon and evenness indexes. Beta-diversity analyses were based on Bray–Curtis distance measures for community similarity and represented in nonmetric multidimensional scaling (NMDS) plots. Correlations between bacterial and archaeal community Bray–Curtis matrices were assayed with a Mantel test, while PERmutational Multivariate ANOVA (McArdle & Anderson, 2001) was used to elucidate the factors (i.e. lake, layer or date) significantly structuring the communities. All statistical analyses were carried out in R (http://www.r-project.org/) with the vegan package version 1.15 (Oksanen *et al.*, 2009).

Identification of sequences from airborne bacteria and archaea

We assembled a data set of 16S rRNA genes from known airborne bacteria and archaea obtained from a previous study in the same area (Hervàs *et al.*, 2009, and unpublished data for Archaea from the same published experiments available in GenBank). The data set contained 46 bacterial sequences matching *Gammaproteobacteria* (12 sequences), *Actinobacteria* (10), *Alphaproteobacteria* (7) and *Betaproteobacteria* (17), and 9 archaeal sequences (accession numbers from HE964958 to HE964965).

Results

For the whole data set, we obtained 339 026 bacterial and 25 554 archaeal 16S rRNA gene V6 fragments, which averaged 772 bacterial OTUs and 226 archaeal OTUs per sample (see details in Supporting Information, Tables S1 and S2). This sequencing effort captured most of the richness for each domain (> 95% coverage estimates) both in neuston and plankton. The cumulative number of species recorded as a function of sampling effort reached asymptote at *c.* 2500 OTUs for Bacteria and *c.* 900 OTUs for Archaea equivalent to a sequencing effort of *c.* 250 000 bacterial and *c.* 20 000 archaeal V6 fragments (Fig. 1). For all the samples, we consistently observed higher richness in the bacterial than in the archaeal assemblages (Table 1), and not significant differences were found between water layers in any of the dates (*t*-test, $P > 0.05$). After microscopic counts, the combination of ARC and EUB probes accounted on average for close to 90% of the total number of cells detected by DAPI. Archaea showed one to two orders of magnitude lower abundances than bacterial cells, and any consistent pattern in the abundance distribution both between

neuston and plankton and between dates was observed either for Bacteria or for Archaea (Table 1).

Rank-abundance curves for the complete set of data indicated dominance of a single bacterial OTU (Fig. 1) further identified as a member of the Actinobacteria (see below). Conversely, archaeal assemblages were dominated equally by several OTUs (for details see Supporting Information, Fig. S1b). Thus, despite the higher richness present in the bacterial assemblage, archaeal communities were more evenly distributed (evenness = 0.8 ± 0.0 for Archaea, 0.5 ± 0.1 for Bacteria) and overall were more diverse (Shannon diversity index = 4.5 ± 0.4 for Archaea, 3.6 ± 0.5 for Bacteria) (Tables S1 and S2). However, we did not find any significant differences in diversity, and similar profiles were observed for rank-abundance curves between neuston and plankton for both Bacteria and Archaea (Fig. S1a and b).

In-depth analysis of taxonomic composition (all samples pooled) indicated that *Actinobacteria* dominated the bacterial amplicons (40.7 ± 12.8% of total bacterial sequences), followed by *Betaproteobacteria* (28.6 ± 10.1%) and *Bacteroidetes* (13.8 ± 9.2%). Very few representatives of *Firmicutes*, *Alpha-* and *Gammaproteobacteria* were found (see details in Fig. 2). The archaeal amplicons were dominated by *Euryarchaeota* (84.2 ± 8.7%), whereas Thaumarchaeota and Crenarchaeota accounted for 13.8 ± 8.6 and 1.9 ± 1.0%, respectively. Interestingly, we observed different distribution ratios (i.e. changes in relative abundance between neuston and plankton) for the different taxa in the two dates examined. In November (Saharan dust-free scenario), differences for the bacterial taxa were always < 1×, whereas up to 4× difference was observed for Thaumarchaeota sequences (Fig. 3). Conversely, in September, differences among lakes were more marked for the different taxa, being *Bacteroidetes* preferentially distributed in the neuston and *Firmicutes* in the plankton for the three lakes. LLB showed the most conspicuous differences (i.e. > 2×) with *Betaproteobacteria* and *Bacteroidetes* with higher ratios in the neuston, and *Actinobacteria* and *Firmicutes* in the plankton (Fig. 3).

Next, we explored differences at the community level through beta-diversity patterns analysed by NMDS plots (Fig. 4). For Bacteria, PERMANOVA analyses indicated that the lake from which the samples were collected was the main driver clustering the communities ($R^2 = 0.32$, $P < 0.005$). In turn, for Archaea, diversity patterns were not influenced by lake and only feebly structured by sampling date ($R^2 = 0.13$, $P < 0.05$). A consistent general trend, however, was observed for both Bacteria and Archaea communities of the neuston compared to underlying waters for the three lakes. Community dissimilarity between the layers (measured as Bray–Curtis distance between each pair of points) was significantly higher in

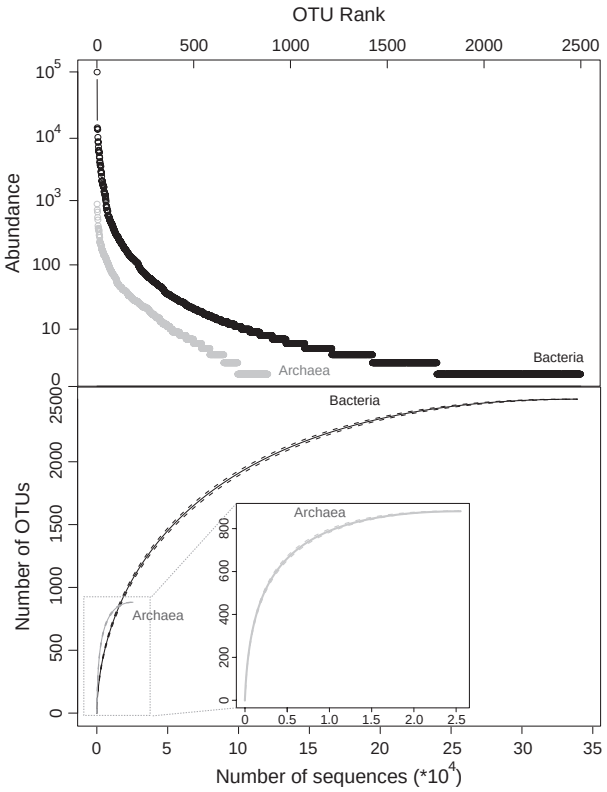

Fig. 2. Rank-abundance (top) and rarefaction curves (bottom) for bacterial and archaeal high mountain lake communities, all samples pooled. The dotted lines represent the deviation across 1000 permutations. Note the different scales.

September (dust-influenced period) than in November (dust-free period) ($P < 0.01$, paired *t*-test) and was more pronounced for Bacteria than for Archaea (Fig. 5, upper). Conversely, the richness between layers showed no significant differences ($P = 0.635$, paired *t*-test) (Fig. 5, lower). Overall, no correlation was found between similarities of bacterial and archaeal community compositions when the Bray–Curtis distance matrices were compared ($r_M = -0.09$, $P = 0.625$, Mantel test).

Finally, we explored the *in situ* presence of airborne immigrants using previously known airborne bacteria and archaea collected in the Pyrenees region (sequences from Hervàs *et al.*, 2009 and from GenBank accession numbers HE964958 to HE964965). The V6 deep sequencing data set was blasted against the airborne immigrants list. Overall, < 0.05% of the sequences matched airborne microorganisms.

Discussion

A number of studies suggest that inland water bodies are among the environments harbouring the highest number of microbial taxa, both Bacteria (Barberán & Casamayor,

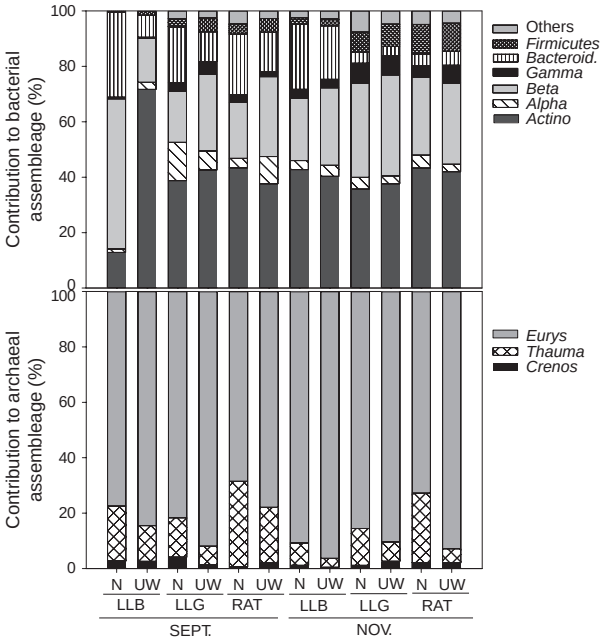

Fig. 3. Taxonomic composition of bacterial and archaeal communities measured as relative abundances of each phylum in the total V6 sequences. Proteobacteria are identified at class level. ACTINO: Actinobacteria. ALPHA: Alphaproteobacteria. BETA: Betaproteobacteria. GAMMA: Gammaproteobacteria. BACTEROID:: Bacteroidetes. CRENOS: Crenarchaeota. THAUMA: Thaumarchaeota. EURYS: Euryarchaeota.

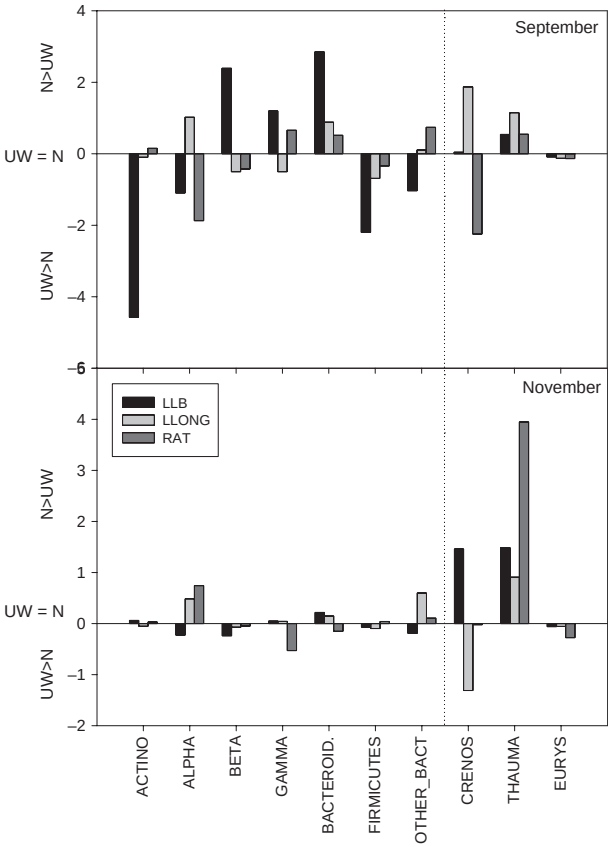

Fig. 4. Ratio of relative abundances of each phylum to the total V6 sequences in the neuston (N) and plankton from underlying waters (UW). To scale the numbers similarly, the figure shows the ratio as N/UW for N>UW and as UW/N for UW>N. ACTINO: Actinobacteria. ALPHA: Alphaproteobacteria. BETA: Betaproteobacteria. GAMMA: Gammaproteobacteria. BACTEROID: Bacteroidetes. CRENOS: Crenarchaeota. THAUMA: Thaumarchaeota. EURYS: Euryarchaeota.

2010; Tamames *et al.*, 2010) and Archaea (Casamayor *et al.*, 2001; Auguet *et al.*, 2010). The high diversity is usually attributed, on the one hand, to the wide physicochemical gradients that biota experience in these environments and, on the other hand, to the larger habitat isolation as compared, for instance, with oceans (Reche *et al.*, 2005; Barberán & Casamayor, 2010). Curiously, high-altitude lakes located in remote areas have as predominant community members cosmopolitan phylotypes often recovered from other worldwide distributed alpine and glacier regions, showing a quite similar common community structure despite the large geographical distances among them (Catalan *et al.*, 2006; Sommaruga & Casamayor, 2009). In spring and summer, nutrient inputs from melting snow and high photosynthesis rates, respectively, are important nutrient sources for the microbial plankton (Camarero *et al.*, 1999). In the late summer and fall period, shallow, poorly vegetated Pyrenean lakes are, however, more influenced by daily atmospheric loadings. Both bacteria and archaea are common components of the microbial plankton of these lakes but how much do dynamics and community structures resemble each other remains poorly understood.

After deep sequencing, we found the composition of the bacterial community in general agreement with what has been typically found in freshwaters when assessed by near-full-length 16S rRNA gene cloning and sequencing (Newton *et al.*, 2011; and see a recent meta-analysis by Barberán & Casamayor, 2010). The dominant bacterial OTU was affiliated with Actinobacteria, an abundant and ubiquitous phylum in freshwater systems, including high-altitude lakes (Glockner *et al.*, 2000; Warnecke *et al.*, 2005; Hervàs & Casamayor, 2009; Hörtnagl *et al.*, 2010; Newton *et al.*, 2011). *Betaproteobacteria* are also abundant, and different members of this group can use both autochthonous nutrient sources such as algal exudates but also terrigenous DOM inputs (Pernthaler *et al.*, 1998; Pérez & Sommaruga, 2006; Šimek *et al.*, 2008, 2011; Nelson *et al.*, 2009). In the Pyrenees lakes studied here, Actinobacteria dominated the community from July to November as assessed by fluorescence *in situ* hybridization (FISH) (A. Hervàs and E.O. Casamayor, unpublished data). Factors that have been proposed to explain the

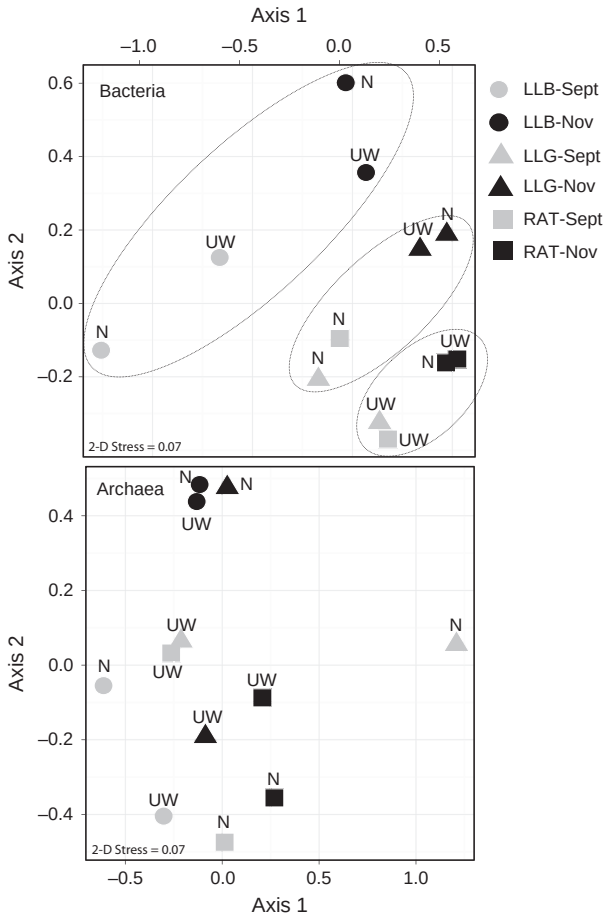

Fig. 5. NMDS plots based on Bray–Curtis distance matrices of bacterial and archaeal communities. Stress measures (i.e. an indication of the mismatch between the Bray–Curtis distance and the location in ordination space) are indicated. Ovals represent significant different community structures (PERMANOVA $R^2 = 0.32$, $P < 0.005$). N: neuston. UW: plankton from underlying water. Sept: September. Nov: November.

dominance of this group are diverse. At the local level, *Actinobacteria* may have a competitive advantage potentially related to a higher resistance to UV radiation (Warnecke *et al.*, 2005) and grazing pressure relief because of their relatively small size (Pernthaler *et al.*, 2001; Tarao *et al.*, 2009; Newton *et al.*, 2011). Furthermore, *Actinobacteria* can have proteorhodopsins that may help them obtain extra energy directly from solar radiation (Sharma *et al.*, 2008). At the regional level, a high percentage of bacteria found in lakes could come from inlet and stream inputs (Lindström *et al.*, 2006; Crump *et al.*, 2007; Logue & Lindström, 2008) and soils (Crump *et al.*, 2012), and *Actinobacteria* are typically a very abundant group in soils and could be introduced to the lakes from terrigenous environments. In fact, the retention time of water in this set of shallow connected lakes is less than a month (Auguet & Casamayor, 2008), which makes the microbial communities sensitive to the rate of immigration of bacteria from the drainage area (Logue & Lindström, 2008). However, it has been shown that the most important clades within *Actinobacteria* (acI and acII) represent autochthonous components of freshwater microbial assemblages (Warnecke *et al.*, 2004).

Contrasting with results from Aller & Kemp (2008), we found that Shannon diversity index was higher for archaea than for bacteria, related to the dominance of a single bacterial phylotype that substantially reduced bacterial evenness. Shannon indices for Archaea in the Pyrenean lakes were in the upper range of those measured in other environments, such as polar waters, using similar methodology (Galand *et al.*, 2009; Alonso-Sáez *et al.*, 2011), and higher than those estimated by clone libraries (Auguet *et al.*, 2010). This result, therefore, agrees with previous studies identifying high-altitude lakes as hot spots of archaeal diversity (Auguet & Casamayor, 2008; Auguet *et al.*, 2010). Unfortunately, high-throughput taxonomic classification of Archaea at the class level is not currently possible because many of the available archaeal reference sequences are not identified at this taxonomic level. In our study, the archaea assemblage was dominated by Euryarchaeota. This result diverged quite significantly from recent studies reporting the dominance of archaea belonging to the Thaumarchaeota phylum, particularly ammonia-oxidizing archaea (AOA) from the Marine 1.1a and SAGMGC-1 lineages, in several oligotrophic alpine lakes of the central Spanish Pyrenees (Auguet & Casamayor, 2013). Discrepancy between the primers used (i.e. primers 21f – 958r for cloning and 958arcF-1048arcR for the V6 region) may certainly affect these results as discussed elsewhere (Llirós *et al.*, 2008). The new sequencing technology yielding longer reads (> 400 bp) and the use of different primers sets, combined with PCR-independent techniques (e.g. metagenomics), will probably resolve potential differences regarding phylogenetic affiliations. Nevertheless, Thaumarchaeota were found to contribute at higher percentage of V6 sequences in the neuston than in plankton of underlying waters, agreeing with Auguet & Casamayor (2008) that higher abundances of Thaumarchaeota are present in the SML of a number of alpine lakes possibly related to their capacity to oxidize ammonia (Auguet & Casamayor, 2013). This is closely related to the fact that atmospheric nitrogen deposition is the main source of nitrogen in remote mountain catchments, and NH_4 concentrations in the rain are *c.* 30-fold higher than mean values measured in the water of these lakes (Auguet *et al.*, 2011).

Interestingly, we observed that neuston and plankton communities were less similar in September than in November. This was more pronounced for Bacteria than

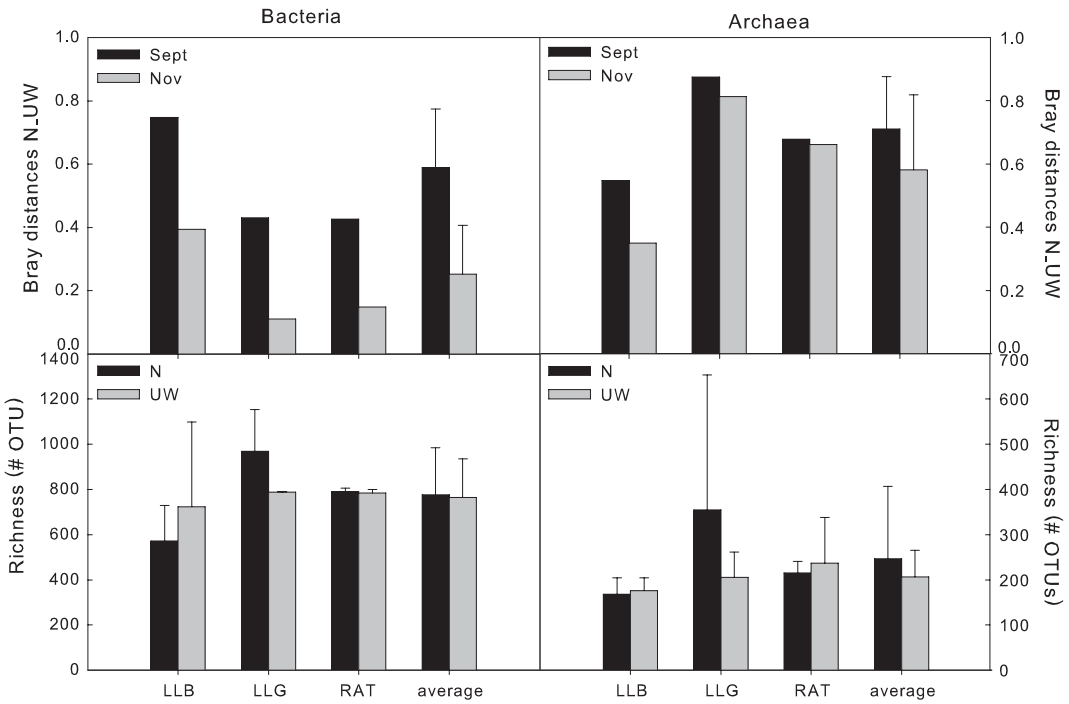

Fig. 6. Bray–Curtis distances (upper) and average taxonomic richness (lower) between neuston (N) and plankton from underling water (UW) by lake and month. Sept: September. Nov: November. Error bars in the lower indicate standard deviation between September and November.

for Archaea. These results are in agreement with previous studies showing higher dissimilarities between bacterioneuston and bacterioplankton composition than between the Archaea inhabiting both layers (Cunliffe *et al.*, 2008, 2009), although some contradictory results are found in the literature (Cunliffe *et al.*, 2011). These studies together suggest a different reactivity of Bacteria and Archaea to environmental factors. We observed that in November, when atmospheric dust deposition was minimal, neuston and plankton had more similar bacterial composition than in September, when a Saharan atmospheric dust plume was on the area. It has been reported that Saharan dust atmospheric depositions can have two main effects on the bacterial communities: (1) a fertilization effect, enriching the upper layers with nutrients and organic carbon (Hervàs *et al.*, 2009; Reche *et al.*, 2009; Mladenov *et al.*, 2011); and (2) an inoculation effect, adding airborne bacteria that can colonize the waters if the conditions turn favourable (Kellogg & Griffin, 2006; Hervàs *et al.*, 2009). Previous studies had shown both a strong influence of the fertilization effect in high-altitude areas (Morales-Baquero *et al.*, 2006; Ortega-Retuerta *et al.*, 2007; Pulido-Villena *et al.*, 2008; Mladenov *et al.*, 2009; Reche *et al.*, 2009) and the potential of airborne immigrants to develop *in situ* after an intercontinental air travel (Hervàs *et al.*, 2009; Reche *et al.*, 2009). We explored the presence of airborne immigrants *in situ* using the deep sequencing data set but $< 0.05\%$ of the total sequences matched airborne microorganisms. This result supports the view that their influence is low (Jones *et al.*, 2008) and that airborne bacteria and archaea reaching high-altitude lakes remain as rare taxa in the ecosystem, part of the 'seed bank' that has the potential to colonize the lake if the conditions turn favourable (Hervàs *et al.*, 2009). Overall, no significant differences in richness were observed between neuston and plankton for the period studied but consistent differences in community composition were observed not directly related to the introduction of allochthonous microorganisms in the SML. The time frame and *in situ* conditions that would potentially stimulate successful colonization of such airborne microorganisms remain to be determined in future studies.

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Supporting Information

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

Fig. S1. Rank-abundance curves for Bacteria (Fig. S1a) and Archaea (Fig. S1b) by lake and layer. Llbr: Lake Llebreta, Llng: Lake Llong, RAT: Lake Redo d'Aigüestortes. N: neuston. UW: plankton from underlying waters. Nv: November. Spt: September.

Table S1. Diversity indices for bacterial communities.

Table S2. Diversity indices for archaeal communities.

Table S3. Enumeration of OTUs from the neuston (N) in September that were present in the plankton community of the underlying water (UW) in September or the N or UW in November.