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ORIGINAL ARTICLE

Contrasting taxonomic stratification of microbial communities in two hypersaline meromictic lakes

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Hypersaline meromictic lakes are extreme environments in which water stratification is associated with powerful physicochemical gradients and high salt concentrations. Furthermore, their physical stability coupled with vertical water column partitioning makes them important research model systems in microbial niche differentiation and biogeochemical cycling. Here, we compare the prokaryotic assemblages from Ursu and Fara Fund hypersaline meromictic lakes (Transylvanian Basin, Romania) in relation to their limnological factors and infer their role in elemental cycling by matching taxa to known taxon-specific biogeochemical functions. To assess the composition and structure of prokaryotic communities and the environmental factors that structure them, deep-coverage small subunit (SSU) ribosomal RNA (rDNA) amplicon sequencing, community domain-specific quantitative PCR and physicochemical analyses were performed on samples collected along depth profiles. The analyses showed that the lakes harbored multiple and diverse prokaryotic communities whose distribution mirrored the water stratification patterns. Ursu Lake was found to be dominated by Bacteria and to have a greater prokaryotic diversity than Fara Fund Lake that harbored an increased cell density and was populated mostly by Archaea within oxic strata. In spite of their contrasting diversity, the microbial populations indigenous to each lake pointed to similar physiological functions within carbon degradation and sulfate reduction. Furthermore, the taxonomy results coupled with methane detection and its stable C isotope composition indicated the presence of a yet-undescribed methanogenic group in the lakes' hypersaline monimolimnion. In addition, ultrasmall uncultivated archaeal lineages were detected in the chemocline of Fara Fund Lake, where the recently proposed *Nanohaloarchaeota* phylum was found to thrive.
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Introduction

Meromictic (permanently stratified) lakes are distinct hydrological environments characterized by a persistent physicochemical stratification that circumvents deep water recirculation. As a consequence, they commonly comprise an upper stratum where mixing occurs (mixolimnion), a stagnant lower one (monimolimnion) and an interposing boundary layer (chemocline) (Boehrer and Schultze, 2009). These lakes are important model systems for aquatic biology research, because their constant vertical stratification, steep chemical gradients and the presence of oxygen-deprived zones favor niche partitioning among prokaryotic populations, enabling the study of vertical zonations in microbially-mediated biogeochemical cycling (Lauro *et al.*, 2011; Lopes *et al.*, 2011; La Cono *et al.*, 2013).

Ursu and Fara Fund Lakes are 2 of the 41 permanent saline water bodies occurring in the Transylvanian Basin (Romania) that were formed between the eighteenth and nineteenth centuries (Alexe, 2010) on the salt deposits produced by the Paratethys Sea evaporation (during the Badenian

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salinity crisis, ca. 14 Myr ago). These two temperate lakes are unique among the other Romanian salt lakes because of their heliothermy (that is, trapping of solar radiation by a 'saline lens'), size (that is, Ursu Lake is one of the largest heliothermal salt lakes in Europe), conservation status (that is, Fara Fund Lake is a protected area) and lake processes (that is, Ursu Lake is a natural lake whereas Fara Fund Lake is an artificial one) (Alexe, 2010; Máthé *et al.*, 2014). In spite of their different genesis, they belong to the same type of meromictic lakes, where the water column overturn is hampered by strong salinity gradients. Although studies investigating the microbiota and substance turnover in meromictic lakes are available (Biderre-Petit *et al.*, 2011; Habicht *et al.*, 2011; Hamilton *et al.*, 2014), most of the ones concerning the hypersaline water bodies were performed on Arctic (Pouliot *et al.*, 2009; Comeau *et al.*, 2012) or Antarctic (Bowman *et al.*, 2000; Lauro *et al.*, 2011) lakes. Although information on temperate neutral hypersaline meromictic lakes microbiota are scarce, previous investigations on Ursu Lake prokaryotes (using community-level physiological profiling, culture-dependent and PCR–denaturing gradient gel electrophoresis methods) have shown the presence of active and diverse communities (Cristea *et al.*, 2014; Máthé *et al.*, 2014); yet, a detailed molecular diversity study is not available to date. The microbial diversity of Fara Fund Lake has not been previously investigated, and hence the lake could constitute a valuable site for monitoring long-term dynamics of microbial communities that are not under the impact of human activities.

The purpose of this study was to compare the prokaryotic diversity and link it with limnological data in two distinct meromictic hypersaline lakes that differ in their hydric regimen, physicochemical characteristics, genesis and conservation status. This would enable novel inferences regarding the microbial ecology and geochemical cycles in bioenergetically constrained stratified environments. To achieve this aim, the vertical distribution and community structure of the prokaryotes thriving in the two lakes was investigated by combining deep-coverage small subunit (SSU) ribosomal RNA (rDNA) amplicon sequencing, community domain-specific quantitative PCR, chemical analyses and bioinformatics. Concentration and stable C isotope composition of dissolved methane and heavier alkanes were also analyzed for a preliminary assessment of microbial or other sources of hydrocarbons.

Materials and methods

Site description and sampling

Ursu Lake (46°36'15 N; 25°05'09 E) is a natural meromictic lake located at 505 m elevation in the eastern part of the Transylvanian Basin. Having a surface of 41 270 m² and a maximum depth of 18.2 m (Figure 1b), it was formed in a depression caused by an intense process of salt dissolution between 1875 and 1880 (Alexe, 2010; Máthé *et al.*, 2014). Currently, it is exploited for recreational purposes during late-May to mid-August. Fara Fund (45°52'34 N; 24°04'03 E) is an artificial meromictic

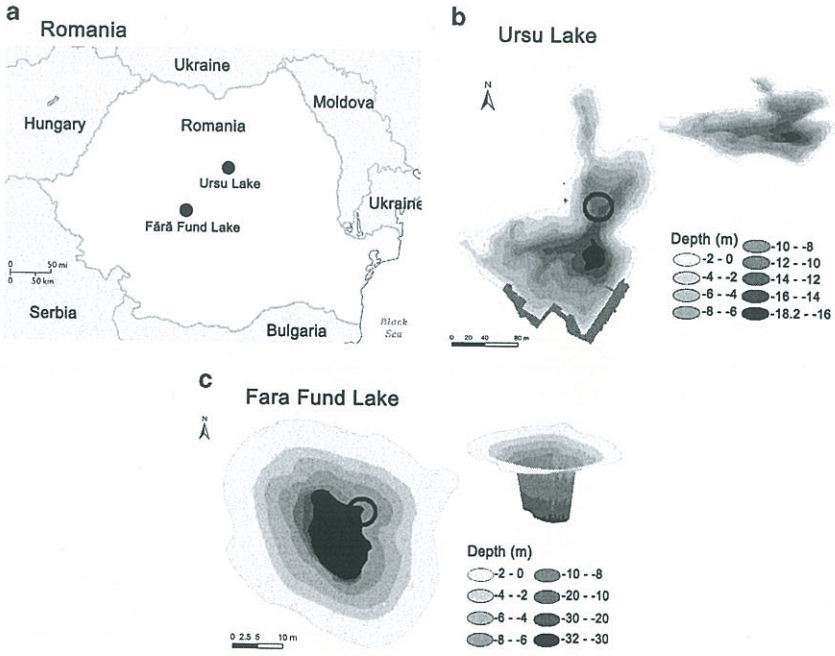


Figure 1 Ursu Lake and Fara Fund Lake sampling sites. (**a**) Map showing the location of the lakes within Transylvanian Basin (Romania). The symbol size is not proportional with the sizes of the lakes. (**b**) Topo-bathymetric profile of Ursu and (**c**) Fara Fund lakes' basins. The circles indicate the sampling points.

pit lake situated at 383 m elevation in the southern area of the Transylvanian Basin (Figure 1a). Having a relatively small surface (~1700 m^2) and a maximum depth of 32 m (Figure 1b), this athalassic (*sensu*, Hammer, 1986) lake was formed in 1775 on the area of a late medieval bell-shaped salt mine that was shut down at the end of the seventeenth century. Presently, it is declared as a protected area, as a consequence of its strong heliothermy (Alexe, 2010).

The vertical sampling was carried out in October 2013 using an inflatable boat positioned near the center of the lakes (Figures 1b and c). *In situ* measurements (for example, temperature, pH, dissolved oxygen, conductivity and redox potential) were performed using a portable water multiparameter system HI 9828 (Hanna Instruments, Woonsocket, RI, USA). The sampling points were chosen in correspondence with the stratification of physicochemical parameters. From each lake, five water samples (0.5, 2.5, 3.5, 9 and 11 m depth in the Ursu Lake; 0.5, 2, 3, 11 and 13 m depth in the Fara Fund Lake) were collected using a submersible 12 V electric pump, with a flow rate of 166 $cm^3 s^{-1}$ and stored in sterile 2-l polypropylene bottles. The samples were transported to the laboratory, where they were immediately processed. The bottom water samples (9 and 11 m in Ursu Lake; 11 and 13 m in Fara Fund Lake) were used for gas analyses. A fraction of the collected water was vacuum filtered using 0.22 μm pore size sterile polycarbonate filters (47 mm diameter) that were further stored at − 20 °C until DNA extraction.

Analytical techniques

Analyses of water chemical composition were performed at the Research Institute for Analytical Instrumentation, Cluj-Napoca, Romania. Ammonium, nitrate, and nitrite ions were quantified by colorimetry using a Lambda 25 spectrophotometer (Perkin Elmer, Beaconsfield, UK). Sulfate (SO_4^{2-}) ions were measured by ion chromatography on ICS-1500 (Dionex, Sunnyvale, CA, USA). The concentration of sulfides was determined by methylene blue method after fixation of samples with 2% (v/v) Zn-acetate (Trüper and Schlegel, 1964). Chloride (Cl^-), carbonate (as $CaCO_3$) and bicarbonate (HCO_3^-) anions were measured by titrimetric methods. The contents of Mg^{2+}, Ca^{2+}, Na^+ and K^+ were determined by inductively coupled plasma atomic emission spectrometry using OPTIMA 5300 DV spectrometer (Perkin Elmer, Norwalk, CT, USA). The contents of total nitrogen as bound nitrogen (including free ammonia, ammonium, nitrite, nitrate and organic nitrogen, but not dissolved nitrogen gas) was assessed by combustion followed by oxidation to nitric dioxide and subsequent chemoluminescence detection. Organic nitrogen in the form of dissolved organic nitrogen was calculated by subtracting ammonium nitrogen from the total nitrogen quantified by Kjeldahl digestion method (Clesceri *et al.*, 1999). Total organic carbon was determined by sample acidification followed by combustion and infrared detection of CO_2 released. Both parameters (total nitrogen and total organic carbon) were measured according to EN 12260 and EN 1484, respectively, using the multi N/C 2100S Analyzer (Analytik Jena, Jena, Germany). Relative expanded measurement uncertainty was calculated according to International Organization for Standardization—Guide to the expression of Uncertainty Measurement using a coverage factor (k) of 2 ($k=2$), equivalent to a confidence of ~95%. Uc ranged from 4.5% to 13% depending on the compound as follows: 4.5% for Mg^{2+}; 8% for HCO_3^-, particulate Fe and Ca^{2+}; 9.5% for PO_4^{3-}; 9.8% for Na^+; 10% for total nitrogen, total organic carbon, CO_3^{2-}, SO_4^{2-}, HS^- and K^+; 11% for NO_3^- and ON; 13% for NO_2^-, NH_4^+ and Cl^-.

Gas analyses were performed on water samples collected within the monimolimnion of the two lakes (9 and 11 m samples from Ursu Lake; 11 and 13 m samples from Fara Fund Lake). After static headspace extraction at room temperature, a wide range of gaseous hydrocarbons were analyzed at the Instituto Nazionale di Geofisica e Vulcanologia in Rome (Italy). CH_4 was determined by tunable diode laser absorption spectroscopy (West Systems, Pontedera, Italy). Heavier hydrocarbons (C_2H_6, C_2H_4, C_3H_8, n-C_4H_{10}, i-C_4H_{10}, n-C_5H_{12}, i-C_5H_{12} and C_6H_6) were analyzed by a Fourier transform infrared spectrometer (Gasmet DX-4030, Gasmet, Finland; lower detection limit of all hydrocarbons: 3 p.p.m.v.) with a standard spectra library. The stable carbon isotope composition of CH_4 ($\delta^{13}C_{CH4}$) was analyzed by cavity ring-down spectroscopy with a Picarro analyzer G2112-I (Picarro Inc., Santa Clara, CA, USA).

Cell counts, chlorophyll a and total carotenoids analyses

Water samples were fixed in 1% glutaraldehyde and filtered through black, gridded cellulose ester membrane filters (0.45 μm, d = 47 mm). Subsequently, they were stained directly using 5 μg ml^{-1} of DAPI (4',6-diamidino-2-phenylindole, dihydrochloride) solution and examined by epifluorescence (BX60, Olympus Optical, Tokyo, Japan). Chlorophyll *a* and total carotenoids concentrations were determined as described by Wetzel and Likens (1991).

DNA extraction and quantitative real-time PCR (qPCR)

The filters were cut in small pieces (~3–5 mm) using sterile scissors, and processed for DNA extraction using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, CA, USA) and the PowerSoil DNA isolation kit (MoBio Laboratories, Carlsbad, CA, USA), following the manufacturer's instructions. The DNA was extracted in duplicate from each sample and stored at − 20 °C until further use. The qPCR was used to evaluate the relative abundances of Archaea and Bacteria along the water columns of the two lakes, by targeting the SSU rDNA, and was performed as described elsewhere (Baricz *et al.*, 2014). Both archaeal and bacterial reactions were carried out in triplicate using the Archaea 931F/

M1100R (Einen *et al.*, 2008) and Bacteria 338F/518R (Lane, 1991; Muyzer *et al.*, 1993) primer sets.

Amplicon sequencing and analysis
Sequencing of bacterial and archaeal SSU rRNA gene (V4 region) amplicons was performed on the Illumina MiSeq platform (San Diego, CA, USA) using the protocol of Lundberg *et al.* (2013). Sequence data were processed and quality controlled through a combination of the UPARSE and QIIME pipelines (Caporaso *et al.*, 2010a; Edgar, 2013). Cutadapt (Martin, 2011) was used in 'paired-end mode' to trim sequencing primers from the forward and reverse reads while simultaneously discarding those read pairs in which both the forward and reverse primers were not detected (allowed for 10% mismatches for primer search). Paired-ends were merged using the -fastq_mergepairs option of usearch (v.7.0.1001; Edgar, 2013). An in-house python script was used to remove unused barcodes of paired-end sequences that did not survive merging. The QIIME (v1.7) script, split_libraries_fastq.py, was used to demultiplex the sequence data with the quality filter set to zero. Quality control processing and singleton removal was carried out via the UPARSE pipeline (for example, usearch -fastq_maxee 0.5, usearch -sortbysize -minsize 2) including *de novo* and reference-based chimera detection (Edgar, 2013). The resulting operational taxonomic unit (OTU) table was converted to Biological Observation Matrix (BIOM) format (McDonald *et al.*, 2012a). Taxonomy was assigned using the Ribosomal Database Project (RDP) classifier (Wang *et al.*, 2007) against the updated May 2013 '13_5/13_8' Greengenes database (Werner *et al.*, 2011; McDonald *et al.*, 2012b) via the parallel_assign_taxonomy_rdp.py script in QIIME. A phylogeny was constructed using FastTree (Price *et al.*, 2010) from a masked PyNAST (Caporaso *et al.*, 2010b) alignment. The resulting phylogeny was manually rooted to Archaea via Dendroscope (v3; Huson and Scornavacca, 2012). Finally, various diversity metrics were calculated via QIIME. Bacterial and archaeal data were separated into their own OTU tables for independent analysis of each group. Representative sequences of OTUs belonging to *Nanohaloarchaeota* and *Parvarchaeota* candidate phyla (from the sample collected at 3 m depth in Fara Fund Lake) were further investigated by phylogenetic placement against known relatives within the SILVA SSU Ref database (v115; Quast *et al.*, 2013). The representative OTU sequences were aligned with SINA (Pruesse *et al.*, 2012) and then imported into the ARB software package (Ludwig *et al.*, 2004). The sequences were inserted into the SILVA SSU Ref database with domain-specific position variable filters via parsimony insertion.

Nucleotide sequence accession number
All sequence data are available through the National Center for Biotechnology Information (NCBI) via Sequence Read Archive (SRA) database, under accession numbers: SRR1569016, SRR1560515, SRR1560527, SRR1569024, SRR1560549, SRR1569028, SRR1560574 and SRR1560593.

Data not shown in the manuscript were deposited at the Dryad Digital Repository (www.datadryad.org) and are referenced in the text using the following doi: 10.5061/dryad.2gm06.

Results

Limnological characterization of the meromictic lakes
Physicochemical measurements were performed along water columns, and the samples were collected in agreement with the lakes' stratification patterns (Figure 2). The chemical and biological characteristics of the water collected from Ursu and Fara Fund lakes are shown in Table 1. Among the several hydrocarbons analyzed, only methane was detected in the suboxic monimolimnia, with concentrations and stable C isotope composition of $\sim 0.2\,mg\,l^{-1}$ and $\delta^{13}C_{CH4}$: −59.7 to −63.1‰ in the Ursu Lake, and 0.9 $mg\,l^{-1}$ and $\delta^{13}C_{CH4}$: −62.1 to −63.0‰ in the Fara Fund Lake, respectively (Table 2). All alkanes from C_2 to C_6 were below the detection limit (3 p.p.m.v. in the headspace in equilibrium with water).

The measured transparency values (Secchi depths) for the two lakes were 1.9 m for Ursu and 1.5 m for Fara Fund. These measurements were further used to estimate the euphotic depth (Z_{eu}) as described by Wetzel and Likens (1995), and the results indicated that the euphotic zone extended to the superior part of the monimolimnion in Ursu Lake (Z_{eu}: 4.6 m) and to the lower limit of the chemocline in Fara Fund Lake (Z_{eu}: 3.6 m). Chlorophyll *a* concentrations (expected to be a substitute for phytoplankton-derived autochthonous organic carbon input) varied between lakes and depths and ranged from 1.43 to 394.8 $\mu g\,l^{-1}$. Noteworthy, the chlorophyll *a* values peaked at the chemocline in both Ursu and Fara Fund lakes.

The vertical profile of temperature (Figure 2) revealed that the water strata were in a transitional state between direct and inverse thermal stratification, with the highest temperatures registered in the chemocline region for both lakes. The monimolimnia were suboxic, had a lower pH, and a higher redox potential and salinity than the overlaying water masses, corroborating with our previous measurements (doi: 10.5061/dryad.2gm06). Notably, the redox potential of the Ursu Lake was more reduced and the vertical salinity profiles fluctuated over a wider range than the water in Fara Fund Lake (Figure 2).

The levels of total organic carbon were similar within and between the lakes and varied from 38.2 to 66.1 $mg\,l^{-1}$. As the level of the total nitrogen increased with depth, mostly because of rise in ammonium concentration, the organic nitrogen showed an inverse pattern, decreasing in the deeper strata of both lakes after the chemocline. The water

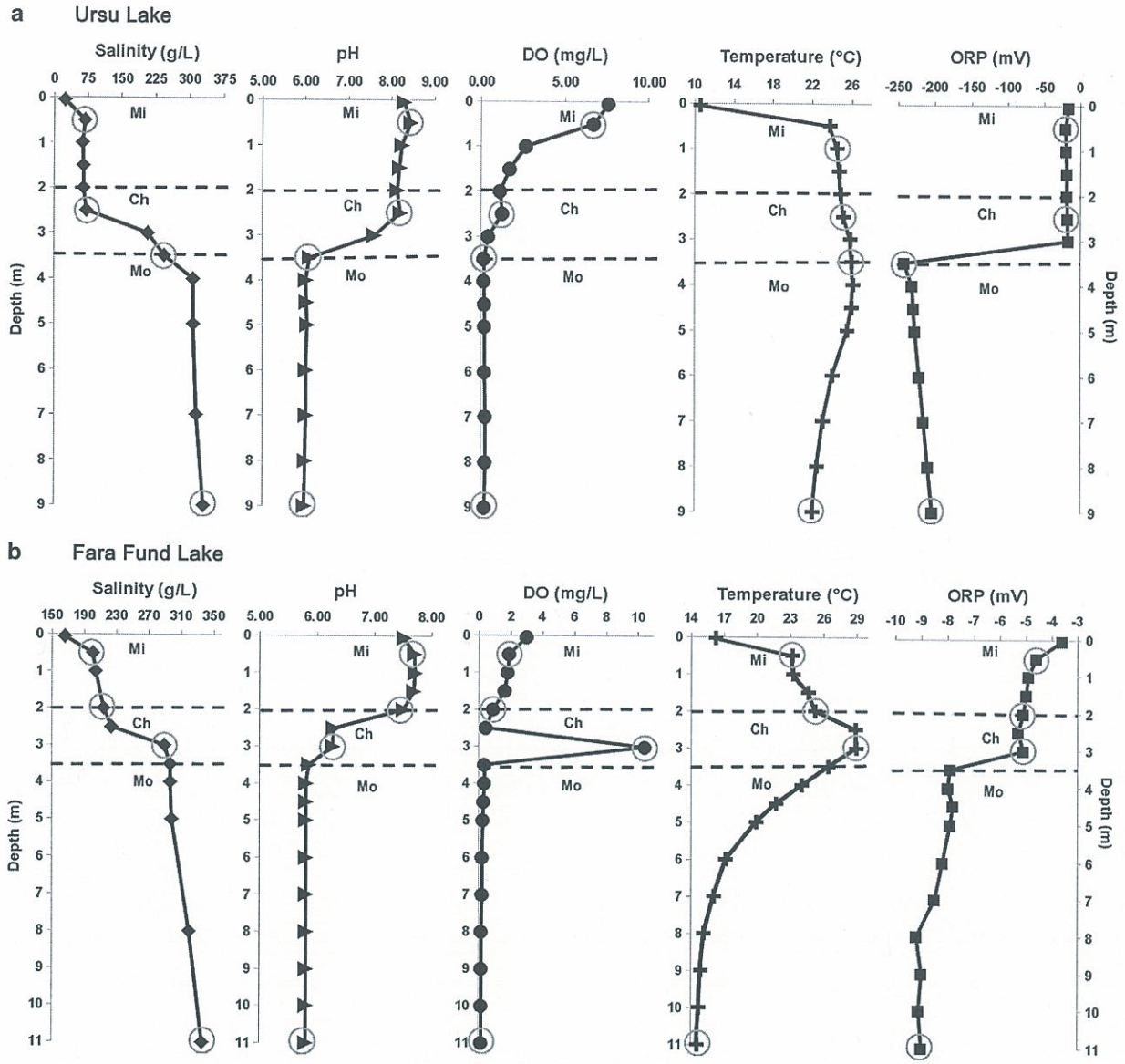


Figure 2 Vertical distribution of physicochemical parameters (salinity, dissolved oxygen, pH, temperature and reduction potential) measured *in situ* along the water columns of Ursu (**a**) and Fara Fund (**b**) lakes during October 2013. The three water layers resulted from physicochemical partitioning were separated by dashed lines. Sampling points for DNA and chemical analyses are indicated by circles. Ch, chemocline; DO, dissolved oxygen; Mi, mixolimnion; Mo, monimolimnion; ORP, oxidoreduction potential.

ionic composition analyses showed that the monovalent cations (Na^+ and K^+) were dominant over the divalent ones (Mg^{2+} and Ca^{2+}). The main anions were Cl^-, SO_4^{2-}, HCO_3^- and PO_4^{3-}, revealing a composition similar to that of sea water.

Sequencing statistics

Paired-end sequencing of 16S rDNA hypervariable region V4 amplicons generated 697 529 high-quality reads (347 773 for Bacteria and 349 756 for Archaea) with an average length of 254 nt. The number of sequences per sample ranged from 64 650 (3.5 m depth sample from Ursu Lake) to 141 896 (2 m depth sample from Fara Fund Lake) (Supplementary Table S1). In order to test the reproducibility of the sequencing method, one sample (9 m depth from Ursu Lake) was analyzed in duplicate, and the results showed that the obtained phylogenetic profile was almost identical between replicates (weighted normalized UniFrac distance = 0.029). In addition, Pearson's correlation coefficients between the qPCR results and the number of archaeal and bacterial sequences obtained showed positive significant correlations for both Ursu ($r = 0.735$, $P < 0.05$) and Fara Fund ($r = 0.728$, $P < 0.05$) lakes.

Table 1 Biological and chemical characteristics of the water samples from Ursu and Fara Fund lakes

Parameter	*Ursu Lake Depth (m)*				*Fara Fund Lake Depth (m)*			
	0.5	*2.5*	*3.5*	*9*	*0.5*	*2*	*3*	*11*
TCh ($\mu g\,l^{-1}$)	14.91	394.80	67.16	18.78	1.83	1.43	4.01	2.82
TCa ($\mu g\,l^{-1}$)	668.13	1624.35	975.62	587.08	290.82	299.19	165.67	150.50
NH_4^+-N ($mg\,l^{-1}$)	0.3	11.2	33.2	30.2	6.3	7.4	22.7	33.3
NO_3^--N ($mg\,l^{-1}$)	0.30	0.45	0.55	0.24	0.56	1.14	0.83	0.95
NO_2^--N ($mg\,l^{-1}$)	0.36	0.55	0.27	0.14	<0.05	<0.05	<0.05	<0.05
ON ($mg\,l^{-1}$)	2.0	2.2	1.4	0.6	3.1	2.5	1.3	1.0
TN ($mg\,l^{-1}$)	3.34	16.88	34.80	33.63	10.28	11.18	25.23	29.40
TOC ($mg\,l^{-1}$)	41.4	48.4	66.1	45.9	58.6	54.5	38.2	49.1
[a]CO_3^- ($mg\,l^{-1}$)	200	245	1170	968	100	163	652	682
HCO_3^- ($mg\,l^{-1}$)	366	427	1830	1647	153	183	915	1220
Cl^- ($mg\,l^{-1}$)	41 000	44 000	165 000	205 000	128 000	131 000	180 000	217 000
SO_4^{2-} ($mg\,l^{-1}$)	231	299	724	1363	1269	1247	1792	1534
HS^- ($mg\,l^{-1}$)	<0.04	<0.04	151	114	<0.04	<0.04	<0.04	36
PO_4^{3-} ($mg\,l^{-1}$)	0.62	6.14	8.81	3.75	0.80	<0.34	0.47	0.34
K^+ ($mg\,l^{-1}$)	31.8	53.6	57.0	68.4	119	128	110	105
Fe ($mg\,l^{-1}$)	0.177	0.413	0.632	0.642	0.132	0.069	0.225	11.1
Ca^{2+} ($mg\,l^{-1}$)	112	246	738	967	422	568	1057	1183
Mg^{2+} ($mg\,l^{-1}$)	20.1	27.6	35.4	101	96	122	143	145
Na^+ ($mg\,l^{-1}$)	26 300	26 500	109 000	136 000	78 000	85 800	117 500	137 300

Abbreviations: NNH_4^+, ammonium nitrogen; ON, organic nitrogen; TCa, total carotenoids; TCh, total chlorophyll *a*; TN, total nitrogen; TOC, total organic carbon.
[a]Carbonate anions were determined as $CaCO_3$.

Table 2 Dissolved methane concentration and its stable C isotope composition

Site	*Depth (m)*	*Dissolved CH_4 ($mg\,l^{-1}$)*	*$\delta^{13}C_{CH4}$‰ (VPDB)*
Ursu Lake	9	0.26	−59.7
	11	0.24	−63.1
Fara Fund Lake	11	0.92	−62.1
	13	0.91	−63.0

Abbreviation: VPDB, Vienna Pee Dee Belemnite standard.
C_2 to C_6 hydrocarbons, not shown in the table, were below the detection limit (3 p.p.m.v. in the headspace in equilibrium with water).

Abundance and vertical distribution of prokaryotic communities

DAPI epifluorescence microscopy showed that in both lakes the mixolimnion was largely populated by small-sized (0.5–1 μm) coccus-shaped organisms, and that the monimolimnion was characterized by an increased abundance of medium-sized (2–4 μm) morphotypes (for example, vibrios and spirilla) (doi: 10.5061/dryad.2gm06).

For both lakes, the maximum prokaryotic abundance, estimated by qPCR, was found within the euphotic zone and on average Fara Fund Lake (mean cell numbers per ml = $4.56 \times 10^7 \pm 28.2 \times 10^4$) harbored higher cell density than Ursu Lake (mean cell numbers per ml = $1.54 \times 10^7 \pm 7.4 \times 10^4$). In Ursu Lake the bacterial cell abundance ranged from 3.6 to 66.5×10^6 cells per ml, and the archaeal ones from 8.1 to 24.1×10^5 cells per ml (Figure 3). In this lake the bacterial density increased with depth between 0.5 and 2.5 m, but subsequently decreased steadily to the lowermost sampling point, whereas the archaeal numbers increased with depth throughout the water column. In Fara Fund Lake, the bacterial and archaeal densities ranged from 1.6 to 24.6×10^6 cells per ml, and from 4.4×10^5 to 1.4×10^8 cells per ml respectively (Figure 3). In Fara Fund Lake the bacterial numbers per ml gradually decreased from the surface to 11 m deep whereas the archaeal abundance slightly increased from 0.5 (archaeal cell numbers per ml = $1.46 \times 10^8 \pm 7 \times 10^5$) to 2 m depth (archaeal cell numbers per ml = $1.49 \times 10^8 \pm 6.2 \times 10^5$) to continuously decrease throughout the water column.

The results of standard Mantel tests performed on domain-specific qPCR data (Supplementary Table S2) revealed that archaeal abundances within Ursu Lake were significantly positively correlated with both salinity ($R = 0.949$, $P < 0.05$) and sulfate concentrations ($R = 0.672$, $P < 0.05$), whereas bacterial abundances were positively correlated with nitrite concentrations ($R = 0.84$, $P < 0.05$). The same tests indicated that the archaeal abundances in Fara Fund Lake were positively correlated with total carotenoid concentrations ($R = 0.993$, $P < 0.05$), whereas bacterial abundances were positively correlated with organic nitrogen concentrations ($R = 0.952$, $P < 0.05$) (Supplementary Table S2).

Prokaryotic α-diversity patterns

To compare the α-diversity indices across lakes and depths, we normalized the sequence number of each sample to 5411 for Archaea and 5953 for Bacteria (the fewest among the data set) through random

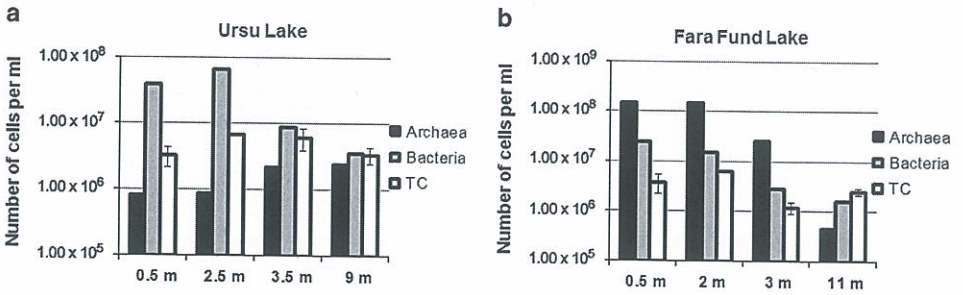


Figure 3 Prokaryotic cell numbers per ml as estimated by quantitative PCR (black and gray columns) and DAPI counts (white columns) for the Ursu (**a**) and Fara Fund (**b**) lakes. The sampling depths from each lake are represented on the abscissa. The columns represent means, and the error bars represent their s.d. values. TC, total cell counts.

resampling. Two Ursu Lake samples (0.5 and 2.5 m) were excluded from the Archaea data set, as they contained 267 and 340 reads respectively (Supplementary Table S1). Sequences with ≥97% similarity were grouped into OTUs and used for the diversity estimations. In addition, the normalized data set was shown to cover ~98.3—99.9% of the species richness as indicated by Good's estimator (Supplementary Table S3).

The α-diversity levels in Ursu Lake (for both Archaea and Bacteria) typically increased with depth as proven by each of the measurement indexes used: divergence-based phylogenetic diversity (Faith's phylogenetic diversity), OTU-based observed species or the quantitative Shannon's index (Table 3). Furthermore, these indices showed that α-diversity was higher for Bacteria than for Archaea. In Fara Fund the archaeal α-diversity increased from surface to the bottom of the lake for all the three indices used (phylogenetic diversity PD, observed species and Shannon), whereas the bacterial one decreased monotonously along the water column till 3 m, after which it rose at 11 m deep. The α-diversity metrics indicated that, in general, the Bacteria were more diverse than Archaea (Table 3).

Archaeal α-diversity of the two hypersaline lakes, measured as phylogenetic diversity (Table 3), was positively correlated with ammonium nitrogen ($r=0.921$, $P<0.05$), Cl^- ($r=0.844$, $P<0.05$), Na^+ ($r=0.848$, $P<0.05$), depth ($r=0.884$, $P<0.05$), total nitrogen ($r=0.862$, $P<0.05$) and HCO_3^- ($r=0.848$, $P<0.05$) and negatively with pH ($r=-0.874$, $P<0.05$) and organic nitrogen ($r=-0.828$, $P<0.05$) (Supplementary Table S4). In contrast, the bacterial phylogenetic diversity values were positively correlated with HCO_3^- ($r=0.74$, $P<0.05$) and total nitrogen ($r=0.712$, $P<0.04$) and negatively with dissolved oxygen ($r=-0.792$, $P<0.05$) and the redox potential ($r=-0.773$, $P<0.05$) (Supplementary Table S4).

Table 3 Archaeal and bacterial estimates of α-diversity metrics for the samples collected from Ursu and Fara Fund lakes

Lake	*Depth (m)*	*α-Diversity indices*		
		PD	*OS*	*S*
Archaea				
Ursu	3.5	8.512	73	2.715
Ursu	9	8.821	93.14	3.346
Fara Fund	0.5	2.388	27.13	0.21
Fara Fund	2	3.637	46.23	0.302
Fara Fund	3	4.209	56.63	2.992
Fara Fund	11	10.418	124.71	4.062
Bacteria				
Ursu	0.5	12.9	112.42	4.644
Ursu	2.5	20.903	188.12	4.994
Ursu	3.5	27.31	250.02	4.996
Ursu	9	29.426	272.32	5.456
Fara Fund	0.5	16.468	124.26	3.741
Fara Fund	2	15.018	101.93	3.284
Fara Fund	3	9.782	55.23	2.299
Fara Fund	11	23.77	193.76	3.536

Abbreviations: OS, observed species; PD, phylogenetic diversity; S, Shannon.

Prokaryotic diversity and community structure in Ursu and Fara Fund lakes

We assigned taxonomy for the tags using the RDP classifier with the GreenGenes database, via QIIME software package. OTU (≥97% similarity clusters) rarefaction curves reached a clear asymptote (Supplementary Figure S1), and the Good's coverage estimator indicated that we sampled between 99.7% and 99.9% of the species richness in each sample (Supplementary Table S3). From the total number of sequences (Supplementary Table S1), ~3% could not be classified at domain rank and ~5.7% could not be attributed to any particular phylum.

Ursu Lake. In Ursu Lake, the OTUs were found to belong to both prokaryotic domains, and their abundance increased continuously with depth, ranging from 180 at 0.5 m deep to 575 at the bottom of the lake (Supplementary Table S1). The taxonomy results showed that Ursu Lake harbored a wider spectrum of prokaryotic lineages than Fara Fund Lake, and that they were affiliated with 2 Archaea (that is, *Euryarchaeota* and *Parvarchaeota*), and 20 Bacteria phyla (that is, *Actinobacteria*, *Bacteroidetes*, *Chlamydiae*, *Chlorobi*, *Cyanobacteria*, *Deferribacteres*,

Firmicutes, GN02, *Gemmatimonadetes*, *Lentisphaerae*, OD1, OP1, *Planctomycetes*, *Proteobacteria*, SR1, *Spirochaetes*, *Tenericutes*, *Verrucomicrobia*, WWE1 and *Thermi*) (Supplementary Table S5).

The fraction of archaeal reads present in the 0.5 and 2.5 m samples was low (between ~0.4% and 0.5% of total reads). Therefore, the prokaryotic assemblages at these depths will be considered as comprising exclusively of Bacteria. The majority of archaeal OTUs were classified within *Euryarchaeota* that covered ~8% of the sequences from 3.5 m and 26% from the ones in the 9 m sample. The same tendency was observed in *Parvarchaeota* that increased in abundance from the chemocline (~0.6%) to the monimolimnion (~2%) (Supplementary Table S5). The bacterial taxonomy at phylum level showed that the abundance of *Proteobacteria* remained relatively constant throughout the water column, ranging between ~16% and 19%. Even though their vertical distribution was relatively uniform, the samples from mixolimnion and the upper part of the chemocline preponderantly comprised *Gammaproteobacteria* and *Alphaproteobacteria*, in contrast to the lower part of the chemocline and monimolimnion where *Deltaproteobacteria* prevailed (Figure 4). *Actinobacteria* abundance decreased from 0.5 m (~50%) to 3.5 m (~4%) and slightly increased in the 9 m sample (~5%). A similar pattern was also observed for *Bacteroidetes*, whose abundance decreased from the mixolimnion (~26%) through chemocline (~19% and 5%, respectively) and increased afterwards in the monimolimnion (~11%). Furthermore, a significant part of the sequences from 3.5 m (~22%) and 9 m (~14%) were affiliated with *Firmicutes* (Supplementary Table S5).

Fara Fund Lake. Overall, the numbers of OTUs in Fara Fund Lake fluctuated between samples from 127 to 474 (Supplementary Table S1) and were found to appertain to both Archaea and Bacteria domains. The majority of the sequences in the data set were found to be affiliated to the phyla *Euryarchaeota*, *Parvarchaeota*, *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, OD1 and OP1 (Supplementary Table S5). In addition to the prevailing phyla, OTUs belonging to *Lentisphaerae*, *Deferribacteres*, OP3 and *Spirochaetes* were present within the lake's water column.

The Archaea domain was found to dominate the mixolimnion and chemocline samples (that is, 0.5, 2 and 3 m) where it accounted for >90% of the

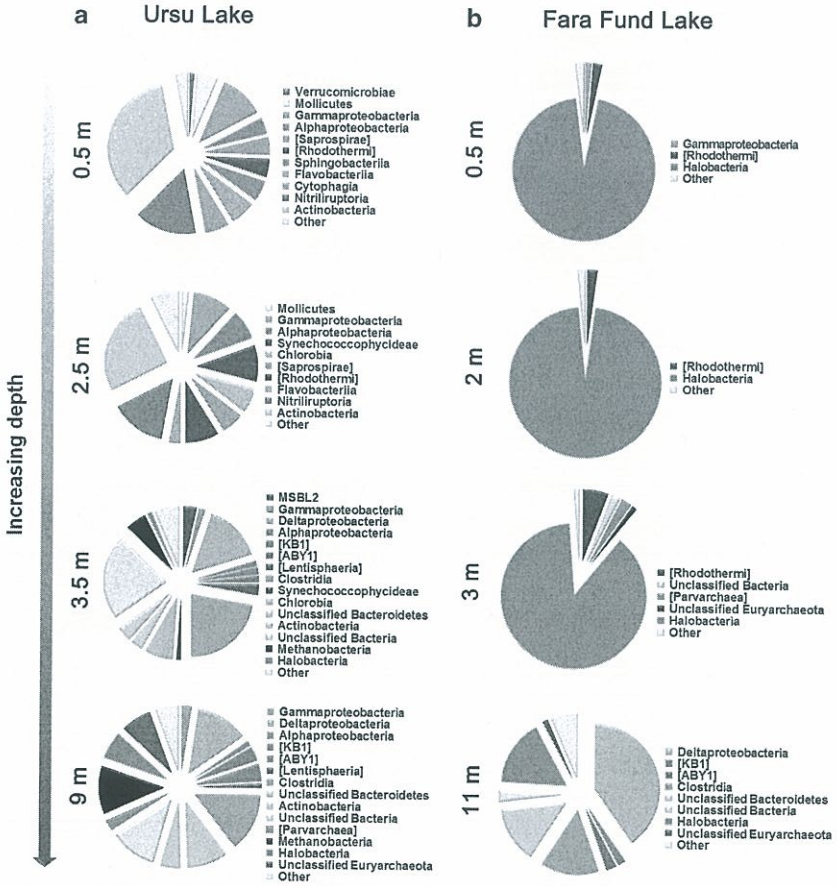


Figure 4 Class-level taxonomic profiles of the prokaryotic communities using 16S rRNA gene sequences.

sequences, in contrast with the monimolimnion, where it represented ~20% of the prokaryotic community reads (Supplementary Table S5). The *Euryarchaeota* phyla covered the vast majority of reads in the 0.5 m (~95%), 2 m (~96%) and 3 m (~89%) samples, and accounted for ~18% of the sequences in the monimolimnion. In contrast, the number of *Parvarchaeota* reads peaked at 3 m depth (~2%), while being poorly represented in the rest of the lake (0.02–0.7%). The percentage of *Proteobacteria* sequences decreased with depth from 1.9% in the 0.5 m sample to 0.57% in the 3 m sample, and increased afterwards to ~42% in the 11 m sample. Within the *Proteobacteria*, the most abundant class in the mixolimnion and chemocline was *Gammaproteobacteria*, substituted in monimolimnion by *Deltaproteobacteria* that covered ~40% of the total prokaryotic reads (Figure 4). A similar pattern was observed in the abundance of *Bacteroidetes* that increased from ~3% in the mixolimnion to ~15% at 11 m deep. Furthermore, a significant part of the sequences from the monimolimnion were affiliated with *Firmicutes* (~14.4%), OD1 (~2.7%) and OP1 (2%) (Supplementary Table S5).

Discussion

Water chemistry

The water chemistry of the lakes was found to be a reflection of halite diapirism and of the sedimentary input of clastic materials that shaped the Transylvanian Basin stratigraphy (Har *et al.*, 2010). As a consequence, the raised levels of alkali and alkaline cations (for example, sodium, potassium, magnesium and calcium) found within the monimolimnia of both lakes could be attributed to halide dissolution (that is, halite) and to the weathering of tectosilicate (that is, anorthite and albite), inosilicate (that is, ferro-actinolite) and phyllosilicate (that is, vanadian and barian muscovite, clinochlore) under acidic pH conditions (doi: 10.5061/dryad.2gm06).

Both Ursu and Fara Fund lakes presented a perennially strong stratification of the water column with contrasting chemical compositions between the mixolimnion and monimolimnion. The higher particulate iron concentrations found in the lower parts of the water column were most probably caused by the sedimentation of ferric oxyhydroxides within the oxic strata. Even though the suboxic conditions may favor their reductive dissolution, with the generation of dissolved Fe^{2+}, the presence of HS^- in the monimolimnion might induce FeS formation, thereby restricting its role as terminal electron acceptor for organic matter oxidation (Cappellen *et al.*, 1998; Hurtgen *et al.*, 1999). Ammonium reached the highest concentrations in the deep anoxic layers of the lakes, indicating a higher rate of mineralization than assimilation, coupled with a higher stability in the acidic pH range. The depth profiles of ammonium concentration were comparable for both Ursu and Fara Fund lakes, and similar to the ones described in other stratified lakes (Auguet *et al.*, 2012; La Cono *et al.*, 2013; Yau *et al.*, 2013). The vertical profiles of nutrients (sulfate, sulfide, nitrite, nitrate and phosphate) were found to be a result of sedimentation, biogeochemical cycling or conservative mixing (Pasche *et al.*, 2009), and were also comparable with those found in other studied lakes (Lepère *et al.*, 2010; La Cono *et al.*, 2013; Marteinsson *et al.*, 2013).

Dissolved CH_4 concentrations in Ursu and Fara Fund lakes (Table 2) were found to be within the typical range observed in the monimolimnion of several types of lakes (for example, Schubert *et al.*, 2010), including the hypersaline Big Soda Lake (Iversen *et al.*, 1987). The monimolimnion of a lake typically shows CH_4 with a stable C isotope composition ($\delta^{13}C_{CH4}$) that is higher (^{13}C-enriched) compared with that in the deeper anoxic CH_4-producing layers (generally $< -65‰$), and lower (^{13}C-depleted) compared with that in the shallower water ($> -50‰$; see, for example, Iversen *et al.*, 1987), because of moderate CH_4 oxidation. The monimolimnion $\delta^{13}C_{CH4}$ values of Ursu and Fara Fund (from −59.7 to −63.1‰; Table 2) are consistent with this general trend. The slight increase of $\delta^{13}C_{CH4}$ values at lower depths (a difference of +3.4‰ from 11 to 9 m in the Ursu Lake, and +0.9‰ from 13 to 11 m in the Fara Fund Lake) combined with a practically constant CH_4 concentration could suggest that the eventual CH_4 oxidation by methanotrophs is minimal within the monimolimnion. The values confirm a microbial origin, likely related to a fermentation pathway, as typically found in lakes (see, for example, Whiticar *et al.*, 1986). The absence of higher n-alkane hydrocarbon gases (C_2–C_6) suggests the lack of significant geological seepage of thermogenic gas from the lake sediments (typically ^{13}C-enriched, with $\delta^{13}C_{CH4} > -50‰$). But seepage of microbial geological (fossil) methane, which is C_2–C_6 free and isotopically indistinguishable from modern biologic CH_4, cannot be excluded. Such a microbial seepage, releasing CH_4 with $\delta^{13}C < -60‰$ is in fact quite common in the Transylvanian Basin (Etiope *et al.*, 2009; Spulber *et al.*, 2010; Ionescu, 2015). In this respect, radiocarbon analyses of CH_4 would be necessary to reveal a possible geological component of CH_4 in the lakes. Given the high salinity of the water, it is likely that methane in Ursu and Fara Fund lakes is dominantly formed via a specific fermentation pathway such as methylotrophic methanogenesis (Kelley *et al.*, 2012) the only metabolic pathway shown to occur at salinities comparable with those found in the lakes' monimolimnion (Andrei *et al.*, 2012).

Vertical patterns in prokaryotic abundances

Although among the prokaryotes living in extreme hypersaline waters the most abundant are usually considered to be Archaea (Andrei *et al.*, 2012),

recently it has become indisputable that representatives of the domain Bacteria play key metabolic roles in high-salt ecosystems (Oren, 2012).

As expected, Archaea dominated the mixolimnion and chemocline samples from Fara Fund, harboring similar numbers as that reported within other meromictic salt lakes (Baricz *et al.*, 2014). Even though the bacterial abundance was comparable to that typically described for high-salt environments (Demergasso *et al.*, 2008; Baricz *et al.*, 2014), the fact that they constituted ~78% of the monimolimnion community was unanticipated, as previous studies reported the dominance of Archaea at salinities $>300\,g\,l^{-1}$ (Ghai *et al.*, 2011; Baricz *et al.*, 2014). We assume that the increased abundance of Bacteria in the deeper water strata could be achieved by outcompeting Archaea in suboxic hypersaline conditions, as the majority of halophilic archaeal species are aerobic (Andrei *et al.*, 2012). In Ursu Lake, a different vertical distribution pattern of prokaryotic abundance was observed, as Bacteria clearly dominated the water column communities. The relative abundance of Archaea in the 0.5 (~2%) and 2 m (1.2%) depth assemblages was minor, and could be attributed to the lower salinity levels found at these depths (that is, $\sim 70\,g\,l^{-1}$). Similar findings were reported in other saline lakes (Yau *et al.*, 2013; Baricz *et al.*, 2014) or shallow ponds (Ghai *et al.*, 2011), and are supported by the fact that most haloarchaeal species lyse at salt concentrations of $<100\,g\,l^{-1}$ (Andrei *et al.*, 2012). As the salinity increased over $240\,g\,l^{-1}$ (at 3.5 m depth) the proportion of the Archaea in the communities was higher, ranging between ~20% (at 3.5 m depth) and ~40% (at 9 m depth), but nonetheless lower than the bacterial one. We argue that although the salinity premise was supported, the suboxic environment favored thriving of the Bacteria. Overall, the dissimilar prokaryotic abundance patterns found in the two lakes could be attributed to their different physicochemical milieus, as well as the different metabolic requirements of Archaea and Bacteria domains (as shown by correlation analyses; Supplementary Table S4).

Prokaryotic diversity

A general ecological principle states that as the factors in the environment become more extreme, the species that might be harbored will be less diverse (Frontier, 1985). Nevertheless, even though the reproducibility of this pattern is uncertain for microbial communities, several studies found evidence for a declining tendency in prokaryotic diversity along increasing salinity gradients (Benlloch *et al.*, 2002; Hollister *et al.*, 2010). Strikingly, both archaeal and bacterial diversity increased along with the salinity values from surface to the bottom of the Ursu Lake. In Fara Fund Lake, the increase of archaeal diversity paralleled the salinity trend, whereas the bacterial one decreased from surface through chemocline and subsequently rose in the deeper water strata. The decrease of Bacteria diversity from surface to 3 m deep recorded in Fara Fund Lake overlapped with an increased abundance of *Salinibacter* sequences (~5%), indicating that it outcompeted the other taxa. Thus, the observed shift could be attributed to changes in the bacterial ecological niches through the euphotic zone.

The lake's monimolimnion may be considered a more 'extreme' environment by comparison with the mixolimnion, for the reason that it has higher concentrations of salts ($>300\,g\,l^{-1}$), sulfide (26–114 $mg\,l^{-1}$) and ammonium ($>30\,mg\,l^{-1}$) that have accumulated as a result of extended meromixis. In spite of the metabolic constraints possibly associated with these factors, the highest prokaryotic diversity was found to be present here. The elevated phylogenetic diversity may be attributed to the downward metabolite fluxes that favor niche diversity through the maintenance of various nutrient sources. Furthermore, visual and microscopy observations showed increased levels of suspended particulate and floc matter in the lakes' monimolimnia that may contribute to increased phylogenetic divergence through habitat diversification (Tang *et al.*, 2009). Another hypothesis may be that low oxygen environments have the capacity to maintain a higher variety of energetic pathways than oxygen-rich environments, leading to retention of higher ecological diversity coupled with lowered interspecific competition (Humayoun *et al.*, 2003). This postulation is corroborated by our results that indicate strong negative correlations between Bacteria diversity and dissolved oxygen concentrations (Supplementary Table S4). Furthermore, the prokaryotic taxonomic composition showed that oxygen gradients have an obvious effect of separating aerobic from anaerobic taxa, allowing oxygen-sensitive nitrogen and sulfur (or methane) cycles to occur in the monimolimnion.

Community physiology inferences in Ursu and Fara Fund lakes

The taxonomy results showed a clear vertical separation throughout the water column of both Archaea and Bacteria taxa (Figure 4). Furthermore, these findings are corroborated with other studies that detected disparate communities in the mixolimnion versus monimolimnion of other meromictic lakes (Barberán and Casamayor, 2011; Lauro *et al.*, 2011; Comeau *et al.*, 2012). In addition, the overall organization of the prokaryotic communities was found to be typical for stratified lakes with regard to depth distribution of photosynthetic and redox conditions (Lauro *et al.*, 2011; Comeau *et al.*, 2012; Edberg *et al.*, 2012; Lentini *et al.*, 2012; Yau *et al.*, 2013).

Although 16S metabarcoding analyses do not provide direct evidence on the functional status of

detected phylotypes, useful information might be deduced from the abundance and distribution of 16S gene reads belonging to major metabolic groups (Supplementary Figure S2 and Supplementary Table S6). Furthermore, suppositions on the putative metabolic activity could also be inferred from the stratification of environmental parameters and nutrient profiles.

Oxygenic photoautotrophic cyanobacteria (mainly *Synechococcus* spp.) together with significant populations of unicellular algae (Máthé *et al.*, 2014) and anoxygenic photosynthesizers (Supplementary Table S6) might be responsible for the primary productivity in the upper layer (down to 2.5 m deep) of Ursu Lake. We consider that the accumulated organic carbon might be subsequently metabolized by both heterotrophic aerobic (*Actinobacteria*, *Bacteroidetes*, *Gammaproteobacteria* and *Planctomycetes*) and anaerobic Bacteria (*Halanaerobiales* and *Clostridiales*) as well as Archaea (*Halobacteriales*). In comparison with Ursu Lake, the photic zone of Fara Fund Lake hosts smaller communities of phototrophic microorganisms as shown by the low chlorophyll *a* content reported in this study (see Table 1) and by previous works (Keresztes *et al.*, 2012). Thanks to our microscopy and molecular analyses (doi: 10.5061/dryad.2gm06), we consider that oxygen levels in the euphotic zone of Fara Fund Lake were mostly generated by the photosynthetic activity of *Dunaliella* algae (see also Somogyi *et al.*, 2014). Microbial communities capable of organic carbon degradation were found to be distributed along the water column of the lake, spreading from the oxic (*Halobacteriales* and *Nanohaloarchaeota*) to the suboxic layers (*Halanaerobacter, Halanaerobium* and *Halorhabdus*) (Supplementary Table S6).

Taxonomic analyses revealed that the coexistence of phototrophy with anaerobic sulfur oxidation might be facilitated by the presence of purple sulfur bacteria and green sulfur bacteria in Ursu Lake and by purple sulfur bacteria of *Ectothiorhodospiraceae* family (that is, mainly *Halorhodospira* spp.) in Fara Fund Lake (Supplementary Table S6). In Ursu Lake, 16S reads belonging to purple sulfur bacteria of the Class *Chromatiales* (*Chromatiaceae* and *Ectothiorhodospiraceae*) were found in the epilimnion and upper chemocline, whereas those affiliated to green sulfur bacteria of the Class *Chlorobia* (*Prosthecochloris*) were found to be prevalent within the O_2/HS^- (redox) transition zone (as also shown by Máthé *et al.*, 2014). The occurrence of aerobic sulfur-oxidizing bacteria in the moderately saline mixolimnion of Ursu Lake might be an indicator of an active aerobic or microaerophilic sulfur-oxidation metabolism performed by members of *Thiotrichales* (for example, *Thiomicrospira*) and *Chromatiales* (for example, *Halothiobacillus*). In both lakes, the abundance of 16S sequences indicated that two *Deltaproteobacteria* families (*Desulfohalobiaceae* and *Desulfobacteraceae*) dominated by far the monimolimnetic sulfate-reducing communities (Supplementary Table S6).

We discovered that several bacterial taxa, if active, could be involved in various steps of nitrogen cycling (Supplementary Figure S2) such as nitrogen assimilation (carried out by members of the *Rhodospirillaceae* family) and heterotrophic denitrification (performed by representatives of *Halomonadaceae* and *Rhodospirillaceae*). In addition, in Fara Fund Lake we found sequences related to extremely halophilic denitrifying lithoautotrophic sulfur-oxidizing bacteria *Thiohalorhabdus* sp. (Sorokin *et al.*, 2008). Potential for ammonification could be inferred in Ursu Lake where we found 16S reads assigned to *Desulfurispirillum alkaliphilum* (family *Chrysiogenaceae*), an anaerobic dissimilatory nitrate-reducing and halotolerant bacterium that utilizes elemental sulfur as electron acceptor (Sorokin *et al.*, 2007).

As for methanotrophy, the only taxa capable of methane metabolization (*Methylococcales*) were detected at moderate salinity solely in Ursu Lake (Supplementary Table S6).

Furthermore, the Fe/Mn metabolism seemed to be faintly supported by 16S reads belonging to *Deferribacteraceae* (genus *Deferribacter*) and *Desulfuromonadaceae* (primarily *Desulfuromonas*) that were detected below the chemocline of Ursu Lake (Supplementary Table S6). However, most of these anaerobic bacteria were identified in marine and estuarine sediments and are capable of using Fe^{3+}, Mn^{4+}, nitrate or sulfur as electron acceptors, playing a role in the organic matter diagenesis (Roden and Lovley, 1993). In spite of the fact that 16S rRNA gene sequences belonging to *Deferribacteres* were identified in other hypersaline habitats (Ferrer *et al.*, 2012), no evidence that these iron reducers could function under high-salt concentration was shown to date.

Although both lakes were found to harbor prokaryotes capable of biogeochemical cycling (carbon, sulfur and nitrogen), the inferred physiology of Fara Fund Lake's microbiota was shown to be less diverse than the one found in Ursu Lake. This finding may be attributed to the site-specific conditions and larger environmental gradients (for example, salinity and oxidoreduction potential) found in Ursu Lake that supposedly favored higher niche variety and metabolic diversity. By matching taxa to known taxon-specific biogeochemical functions, we were able to identify a close correspondence between the functional specialists and environmental gradients. Although a coherent picture could be sketched for several steps of C, S and N cycling in Ursu and Fara Fund lakes (for example, carbon fixation and organic carbon degradation, sulfur oxidation and sulfate reduction and nitrogen assimilation and denitrification), other aspects of ecosystem element cycling were beyond the purpose of this study and remained to be addressed by more specific approaches (for example, *in situ* measurements of the production/consumption rate for nutrients, strain isolation, targeting of functional marker genes and shotgun metagenomics).

Methanogenesis

Biogenic methane production in hypersaline conditions is thermodynamically challenging because of bioenergetic constraints (Oren, 2011). We found that sequences matching taxa attributed to methanogenesis were present in the suboxic strata of the lakes, and represented between ~0.6 (in Fara Fund Lake) and ~12% (in Ursu Lake) of the prokaryotic communities. Most of the reads were classified to the *Methanobacteriales* or the euryarchaeal candidate division MSBL1. As the majority of halophilic methanogenic Archaea are classified within *Methanosarcinales* (Andrei *et al.*, 2012), with *Methanobacteriales* usually being hydrogenotrophic (Bonin and Boone, 2006), we searched for related DNA sequences from other environments (Supplementary Table S7). The most abundant OTUs belonging to *Methanobacteriales* or MSBL1 were highly similar (95–99%) to unclassified sequences recovered from other hypersaline meromictic lakes, solar salterns sediments or deep sea-hypersaline anoxic basins, indicating the existence of a yet-undescribed methanogenic group present in extreme hypersaline habitats. Although this assumption is supported by a recent study on Medee brines (Yakimov *et al.*, 2013), in which MSBL1 candidate division was shown to be responsible for the methanogenic fermentation of trimethylamine, further experiments using cultivation and ^{14}C-assimilation are needed to unambiguously describe the methanogenesis in these high-salt ecosystems.

Ultrasmall, uncultivated archaeal lineages thriving in the chemocline of Fara Fund Lake

The Archaeal communities from the oxic zone of Fara Fund were found to be typical for extreme hypersaline aquatic environments, in terms of community composition, as they were dominated by *Halobacteriales* phylotypes (Ghai *et al.*, 2011). Aside from a clear separation from the monimolimnion assemblages, the communities also showed a vertical variation. As the samples from 0.5 and 2 m depths were mainly dominated by *Halobacteriaceae* reads, related to an environmentally derived DNA sequence from a solar saltern (Baati *et al.*, 2010), the 3 m depth sample contained a high abundance of reads belonging to the MSP41 group (~47.7%). Moreover, a V4 SSU rDNA phylogenetic tree suggested that the 3 m community consists of OTUs related to candidate genera *Nanosalinarum*, *Nanosalina* and *Haloredivivus* (Supplementary Figure S3) (Ghai *et al.*, 2011; Narasingarao *et al.*, 2012). In addition to the MSP41 group, which was recently proposed as phylum *Nanohaloarchaeota*, the community also contained OTUs related to the *Parvarchaeota* (2.3%) phylum (Rinke *et al.*, 2013). Even though we know that the prokaryotes from the mentioned putative phyla have an ultrasmall cellular size and aerobic heterotrophic lifestyles (Baker *et al.*, 2010; Narasingarao *et al.*, 2012), their role and metabolic contribution to the microbial assemblages is uncertain. Furthermore, even though the nanohaloarchaea appear to be abundant in hypersaline environments and to have a worldwide distribution (Zhaxybayeva *et al.*, 2013), data on the presence of *Parvarchaeota* in hypersaline lakes are missing. Thus, as far as we know, this is the first report of an archaeal community dominated by the MSP41 group, as well as the first one recording the co-occurrence of *Nanohaloarchaeota* and *Parvarchaeota*.

Conclusions

To our knowledge, the current metabarcoding study represents the first attempt to use deep amplicon sequencing to assess the extent of prokaryotic diversity occurring in hypersaline meromictic lakes. The employed polyphasic methodology provided a powerful snapshot of the vertical distribution of archaeal and bacterial communities, and could serve further as a framework for functional studies.

Even though both lakes are meromictic and extremely saline, their distinctive subtle limnological variances were found to be reflected in their prokaryotic communities' structure and composition. Thus, Ursu Lake was found to be dominated by Bacteria and to have a greater prokaryotic diversity than Fara Fund Lake that harbored an increased cell density and was dominated mostly by Archaea (within the oxic strata). Regardless of the energetic constraints imposed by the high ionic strength, both lakes were found to be colonized by diverse microbial communities whose physiological inferences generally pointed toward organic carbon degradation and sulfur reduction pathways.

In spite of the high salinity, the Bacteria were found to be more diverse than Archaea, indicating that our current view on microbial diversity in hypersaline ecosystems needs to be broadened. We also found that the prokaryotic communities harbored a wide variety of taxa, and contained a large proportion of OTUs with no close relatives, showing that these poorly investigated environments are potential sources of novel microorganisms. By matching taxa to known taxon-specific biogeochemical functions, we were able to sketch parts of biogeochemical cycles, but further functional studies are needed to describe nutrient cycles near the thermodynamic limits of life.

Conflict of Interest

The authors declare no conflict of interest.

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