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Spatial Distribution and Molecular Diversity of Archaeal Communities in the Extreme Hypersaline Meromictic Brâncoveanu Lake (Transylvanian Basin, Romania)

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ABSTRACT

Dating from the Middle Miocene, the massive halite deposits lying beneath the Transylvanian Basin (Central Romania) have been valuable mineral resources quarried for millennia. Among the numerous hypersaline pit lakes that resulted from this mining, Brâncoveanu Lake is unique by its extreme salinity. Assessment of physicochemical variables, water chemistry and trophic status indicated that Brâncoveanu Lake is a permanently stratified, pH-neutral, NaCl-rich and eutrophied system. We investigated the abundance, molecular diversity and vertical distribution of archaeal community by culture-independent approaches. Additionally, the most relevant environmental parameters shaping the archaeal community composition were evaluated by statistical methods. Archaea appeared to largely outnumber Bacteria; altogether the great prevalence of Halobacteriaceae-related sequences could imply a major contribution of this group to the biogeochemical carbon turnover. The fairly distinct composition of archaeal communities reflects the lake's physicochemical stratification. Among the limnological factors, salinity and oxygen showed a significant impact on determining the composition and structure of archaeal assemblages. Furthermore, Brâncoveanu Lake might harbor novel microorganisms such as members of the recently described phylum Nanohaloarchaea. Overall, this study reported the occurrence of halophilic Archaea in a little explored hydrogeochemical system and provided a better insight into geomicrobiology of meromictic hypersaline pit lakes.

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KEYWORDS
Archaeal community composition; halophilic archaea; hypersaline meromictic lake; physicochemical gradients

Introduction

The permanently stratified (i.e. meromictic) lakes are bodies of water with a strong physicochemical stratification that prevents the intermixing of water layers. As a result of the incomplete water circulation, meromictic lakes commonly contain an upper layer with fluctuating physicochemical conditions (mixolimnion), a boundary stratum (chemocline) and a perennially stagnant deep layer (monimolimnion) (Boehrer and Schultze 2009). Due to the constant vertical zonation of the water column and the steep chemical gradients, the meromictic lakes emerged as important model systems in studying the environmental factors that control the microbial diversity (Barberán and Casamayor 2011; Lauro et al. 2011).

Most of the current understanding of community ecology in hypersaline environments was provided by numerous investigations performed on solar salterns (Oren 2012). As a consequence, information on halophilic communities thriving in the rarer hypersaline meromictic lakes is scarce. In this respect, the extremely hypersaline meromictic Brâncoveanu Lake (Transylvanian Basin, Central Romania) appears as a promising *in situ* model to directly assess the influence of environmental parameters on the resident microbial communities. This lake originates from the salt (i.e. halite) deposits formed by the Paratethys Sea evaporites during the Badenian salinity crisis (12.4–14 Ma) (de Leeuw et al. 2013), being formed on the area of a late medieval bell-shaped salt pit closed at the end of the 17th century (Alexe 2010). Due to the direct contact of the water mass with bare rock salt, this lake is hypersaline in the surface, possibly being one of the most saline lakes of Europe (Alexe 2010). As far as the authors are aware, Brâncoveanu Lake is a new site for microbial research, and the present paper is the first extensive survey of the microbial communities inhabiting the water column.

Taking into consideration the aforementioned rationales and the fact that information regarding the archaeal community assembly in meromictic salt lakes is scarce (Lindström and Langenheder 2012), the present study primarily focused on: (i) the assessment of the abundance, diversity and distribution of archaeal community along the water column; and (ii) the examination of the impact of limnological factors on the structuring and dispersal of archaeal assemblages. To achieve these goals, the vertical distribution and community structure of the Archaea inhabiting Brâncoveanu Lake were investigated by combining community domain-specific quantitative PCR, SSU

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Supplemental data for this article can be accessed on the publisher's website.

rDNA amplicon sequencing and community fingerprinting methods.

Materials and methods

Site description and sample collection

Brâncoveanu Lake is an inland, hypersaline, man-made lake situated at an elevation of 397 m, in Sibiu County at the southern part of the Transylvanian Basin (Central Romania) (45°52′18″N, 24°03′56″E). The lake is a hydrogeologically closed basin, with atmospheric precipitation as the main water input. It has a circular shape, a relatively small surface (approx. 1,600 m^2) and a maximum depth of 14.5 m as on 2010 (Figure 1) (Alexe 2010). During the summer season (late-May to mid-August) the lake is used for recreational purposes. Following a year-round physicochemical survey (data not shown), the lake was found to be a relatively stable environment and the sampling time was chosen in autumn, when human impact on the water stratification was minimal.

The vertical samplings were carried out on October 2012. The measurements of the environmental factors [salinity, temperature, pH, dissolved oxygen (DO) and oxidoreduction potential (ORP)] were performed as described previously (Baricz et al. 2014; Andrei et al. 2015). The water transparency was assessed by a black and white Secchi disk (d = 20 cm). The sampling was completed in agreement with the spatial variation of physicochemical parameters measured *in situ*. Water samples collected for chemical and biological analyses were stored in sterile 2 l polypropylene bottles and transported to the laboratory on ice, within 6 h. Water samples intended for molecular analyses were filtered through 0.22 μm pore size sterile mixed cellulose ester (MCE) membranes (Fioroni, France) and further stored at −20°C until DNA extraction. Molecular analyses were carried out on 11 water samples collected from the following depths: −0.05 m, −0.5 m, −1 m, −2 m, −3 m, −3.5 m, −4 m, −5 m, −7 m, −9 m and −12.5 m. The last depth represented the maximum reached at the sampling site.

The water chemistry was analyzed as described by Andrei et al. (2015). Ammonium and nitrate ions were quantitated 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 methylene blue method following sample fixation with 2% (v/v) Zn-acetate (Trüper and Schlegel 1964) was employed for sulfide detection. Chloride (Cl^-) and bicarbonate (HCO_3^-) anions were measured titrimetrically. The contents of Mg^{2+}, Ca^{2+}, Na^+ and K^+ were measured by inductively coupled plasma atomic emission spectrometry using OPTIMA 5300 DV spectrometer (Perkin Elmer, Norwalk, USA). The total nitrogen (TN) and total organic carbon (TOC) were measured according to EN 12260 and EN 1484, respectively, using the multi N/C 2100 S Analyzer (Analytik Jena, Jena, Germany).

The trophic state indices (TSIs) of the lake were calculated using Secchi depth (m), chlorophyll *a* ($\mu g\ l^{-1}$) and total phosphorous ($\mu g\ l^{-1}$) according to Carlson (1977).

Cell counts, chlorophyll a and total carotenoids analyses

Water samples were fixed in 1% (v/v) glutaraldehyde and filtered through 0.45 μm pore size, black-gridded MCE membrane filters (Fioroni, France). Subsequently, cells retained on 0.45 μm filters were directly stained using 5 μg/ml of 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI) solution and

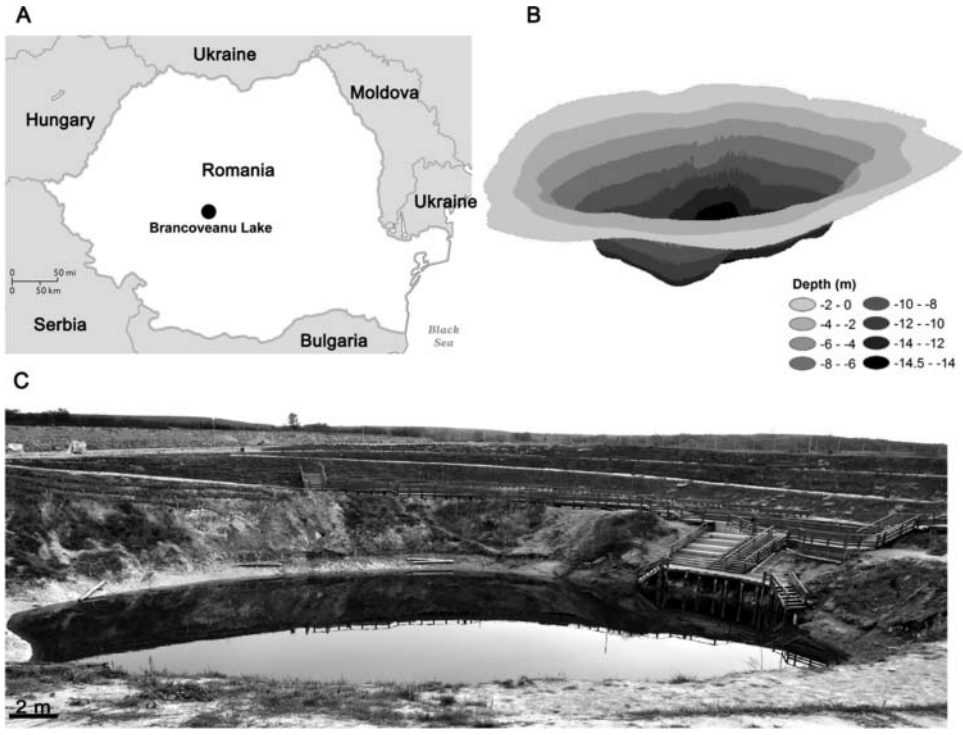

Figure 1. Brâncoveanu Lake sampling site. (A) Map showing the location of the lake within the Transylvanian Basin (Romania). The symbol size is not proportional with the size of the lake. (B) Topo-bathymetric profile of the lake's basin. (C) Photographic image of the study location.

examined by epifluorescence (BX60, Olympus Optical, Tokyo, Japan). Total chlorophyll (TCh) *a* and total carotenoid (TCa) concentrations were determined as follows: water samples of known volume were passed through 0.7 μm pore size glass membrane filters (Fioroni, France) and immediately extracted in hot methanol. The extracts were analyzed spectrophotometrically and pigment concentrations were calculated using equations provided by Wetzel and Likens (2000).

DNA extraction and quantitative PCR (qPCR)

The stored 0.22 μm pore size MCE filters were aseptically cut into small pieces and processed for DNA extraction using the ZR Soil Microbe DNA MiniPrep kit (Zymo Research, Irvine, 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 column by targeting 16S rRNA genes, which was performed as described previously (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 (Table S1).

The efficiency of amplification of the PCR products and data analyses were assessed using the background subtracted data and the LinRegPCR software. Possible correlations between the prokaryotic cell numbers and the environmental factors were evaluated by standard Mantel test with Euclidean distance matrices.

Community fingerprinting

The spatial structure of archaeal assembly was assessed by PCR-denaturing gradient gel electrophoresis (DGGE) using the Archaea domain-specific primer pair A571F/M915R (Table S1). The amplification reactions were performed with a Palm Cycler™ (Corbett Research) and contained the following constituents per 50 μl: 0.2 μM A571F/M915R primers, 0.2 mM of each deoxynucleoside triphosphate (dNTP), 1 × DreamTaq™ Green buffer (Fermentas), 20 mM $MgCl_2$, 0.15 μl template DNA, 1.5 U of DreamTaq™ DNA polymerase (Fermentas, Vilnius, Lithuania), and RNase/DNase-free water to the final volume. The reactions were carried out as following: 120 sec initial denaturation at 92°C, followed by 25 cycles of: 30 sec denaturation at 92°C, 30 sec primer annealing at 54°C and 30 sec extension at 72°C. Amplification was completed by a final extension step at 72°C for 10 min. The reactions were performed in triplicate.

The PCR-amplified DNA products (650 ng) were separated by DGGE on 8% (w/v) polyacrylamide gels (containing 37.5:1 of acrylamide to bisacrylamide) with a linear denaturing gradient ranging from 55% to 67% (the 100% denaturant contains 40% v/v formamide and 7 M urea).

Construction of clone libraries and plasmid sequencing

The choice for construction of clone libraries was based on the spatial separation of archaeal communities inferred from the PCR-DGGE results. Therefore, one clone library (of 96 sequences each) was constructed for each identical cluster obtained by the community fingerprinting analysis. The reaction mixture contained the following components per 50 μl: 0.5 μM forward (Arch21F) and reverse (1397R) primers (Table S1), 0.2 mM of each dNTP, 1 × DreamTaq™ Green Buffer (Fermentas, Vilnius, Lithuania), 20 mM $MgCl_2$, 5 μl template DNA, 1.5 U of DreamTaq™ DNA polymerase (Fermentas), and RNase/DNase-free water to the final volume. The reactions were carried out as follows: 4 min initial denaturation at 95°C, followed by 35 cycles of: 40 sec denaturation at 95°C, 40 sec primer annealing at 56°C and 80 sec extension at 72°C. Amplification was completed by a final 5 min extension step at 72°C. The yields from two independent reactions were pooled together and purified using GeneJet PCR Purification kit (Thermo Scientific, Waltham, MA, USA) according to manufacturer's instructions. Hereinafter, the purified PCR products were quantified and ligated into the pGEM®-T vector (Promega, Madison, WI, USA) at a molar ratio of 1:2 (vector:insert) using T4 DNA Ligase (Promega), and incubated overnight at 4°C, following the manufacturer's instructions. The *Escherichia coli* strain JM109 (Promega) was used as a host for chemical transformation with the ligated vector, after which the bacteria were plated onto Luria-Bertani (LB)/ ampicillin/ isopropyl β-D-1-thiogalactopyranoside (IPTG)/ X-Gal plates and incubated overnight at 37°C. The plasmids from the harvested axenic cultures were purified using the GeneJet Plasmid Miniprep kit (Thermo Scientific) and partially sequenced (192 sequencing reactions) using Sanger method at a commercial company (Macrogen Europe, Amsterdam, The Netherlands).

Bioinformatic and community ecology analyses

The chromatograms produced by the sequencing machine were processed using myRDP Pipeline (Cole et al. 2009). The sequences with minimum length of 600 bp and a Q score > 20 (161 sequences) were downloaded and manually trimmed. Subsequently, they were pooled together and analyzed using Mothur software (Schloss et al. 2009). Furthermore, hypothesis testing and operational taxonomic unit (OTU)-based approaches were used to compare ecological features (e.g., richness, evenness and community similarity) and to evaluate the evidence of structure in the data sets.

To evaluate the key environmental factors that may impact the composition of archaeal communities within the lake, standard Mantel tests were applied. The $OTU_{0.03}$s and environmental factors matrices were calculated using the coefficient of Euclidean distances for quantitative data. The factors tested were: depth, pH, DO concentration, ORP, temperature (T), salinity, TN, nitrate, ammonium, ammoniacal nitrogen, TOC, and phosphate concentrations.

Nucleotide sequence accession numbers

The nucleotide sequences generated in this study, that appertain to the most representative sequence of each $OTU_{0.03}$ (clustering sequences with ≥ 97% similarity), have been deposited in GenBank under the accession numbers KF591546–KF591573.

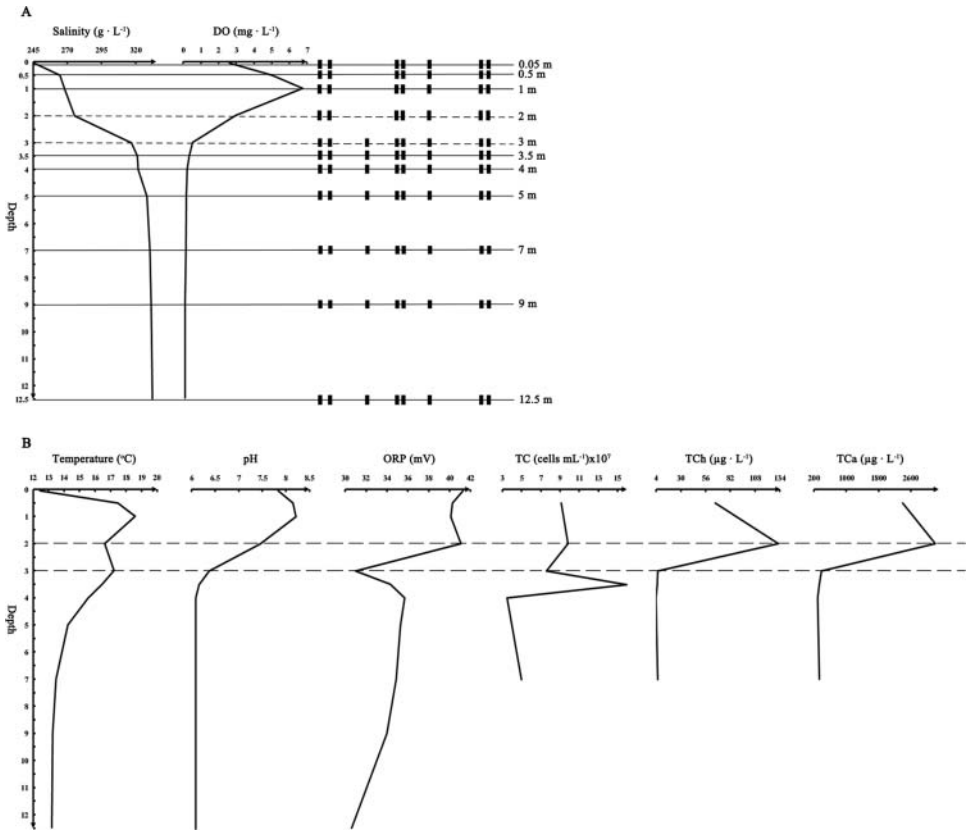

Figure 2. (A) Depth profiles of salinity and oxygen within the water column of the Brâncoveanu Lake (left side) alongside a schematic representation of 16S rRNA genes DGGE community fingerprinting analysis (right side). The numbers in the right side of the figure represent depths of the water column. (B) Vertical distribution of physio-chemical and biological parameters: temperature, pH, ORP, total cell counts (TC), total chlorophyll (TCh) and TCa measured *in situ* along the water column of Brâncoveanu Lake during October 2012. The boundaries of water layer with significant shifts of environmental parameters are marked with dashed lines.

Results

Limnological characterization of the sampling location

The physicochemical parameters measured along the water column of the Brâncoveanu Lake are graphically presented in Figures 2A and B. To study the ionic composition of the water, the sample collected from 7 m depth was considered representative and further investigated. The chemical analysis showed that the main ions were Na^+ (171,000 mg l^{-1}), Mg^{2+} (89 mg l^{-1}), K^+ (240 mg l^{-1}), Ca^{2+} (671 mg l^{-1}), Cl^- (184,000 mg l^{-1}), HCO_3^- (1210 mg l^{-1}) and SO_4^{2-} (3061 mg l^{-1}), indicating that monovalent ions were dominant over the divalent ones.

The estimated salinity varied from 245 g l^{-1} at the surface to 332.5 g l^{-1} at the bottom of the lake (Figure 2A), while the pH changed with depth from slightly alkaline (7.8–8.2) to slightly acidic (ca. 6.1–6.2) (Figure 2B). In this aspect, the lake was categorized as neutral hypersaline environment. The constant extreme salinity of surface waters is additionally supported by our observation that the lake never freezes. The concentration of DO decreased with depth, its level fluctuating from oxic (2.6–6.75 mg l^{-1}) in the upper layers to microoxic (0.1–0.3 mg l^{-1}) at depths under 3 m (Figure 2A). Water temperature peaked at 1 m depth (18.5°C) followed by a sharp decrease below 3 m and steadying at 13–14°C in the monimolimnion (Figure 2B). At the time of sampling, the rapid change of salinity and DO overlapped in the water layer positioned between 2 and 3 m depth.

Other chemical components such as TN and TOC were found in highest concentrations in the mixolimnion and upper part of the chemocline (i.e. 0.5–3 m depth), with a lower but stable levels below the chemocline. Both inorganic phosphate and nitrate peaked at around 3 m depth, while ammonium nitrogen showed a gradual increase in a depth-dependent manner (Supplementary Table S2). Sulfide was below detection limit (<0.04 mg l^{-1}) at all tested depth. At the sampling time, the Secchi depth was 0.65 m resulting in a TSI (SD) of ca. 66. The biological indicators of light-dependent metabolism, the chlorophyll *a* and TCa reached their highest concentration in the photic zone of the lake (down to 1.5–2 m depth) (Figure 2B, Table S2). In addition to TSI (SD), high values of TSI were calculated both for total phosphorous [TSI (TP) = 74.6] and chlorophyll *a* [TSI (CHL) = 72 and 78, at 0.5 and 2 m depth, respectively] indicating that Brâncoveanu Lake is an upper eutrophic/hypereutrophic system.

Estimation of the prokaryotic cells numbers

Two methods were employed for the estimation of prokaryotic cell densities: qPCR and DAPI staining. The medium amplification efficiencies estimated for the archaeal and bacterial standard curves were 1.825 and 1.867, respectively. The mean PCR efficiency achieved by the amplification of environmental DNA with archaeal and bacterial primers was also comparable (1.851 and 1.809, respectively). A post-PCR melting curve analysis

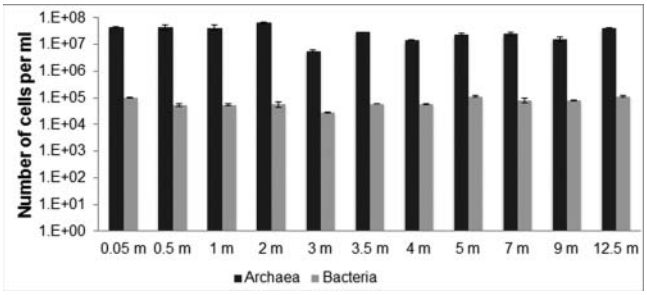

Figure 3. Archaeal and bacterial cell numbers per ml as estimated by quantitative PCR performed on DNA extracted from water samples collected from 0.05 to 12.5 m depth. The reactions were performed in triplicate as described in the text. The data points represent means, and the error bars their standard deviations. Note the different logarithmic scales for Archaea and Bacteria.

established that all the reactions produced specific amplification. Overall, in the water samples collected from various depths, the estimated bacterial numbers ml^{-1} ranged from 2.8×10^4 to 1.1×10^5, while values of archaeal numbers ml^{-1} ranged from 5.7×10^6 to 6.5×10^7 (Figure 3). It is worth noting that the estimation of DAPI-stained cells showed higher values in mixolimnion followed by a maximum just below chemocline and a decreased density in deeper layer (Figure 2B; Table S2). The total cell numbers estimated by DAPI staining gave roughly similar values ($3.4–16 \times 10^7$ cells ml^{-1}) as those that resulted from the summing of qPCR results.

Standard Mantel tests showed correlations ($p < 0.05$) between archaeal cell numbers and ORP, pH and salinity, while the vertical distribution of bacterial cell density correlated with temperature (Supplementary Table S3).

Community fingerprinting

The presence of Archaea as a significant proportion of the total prokaryotic population, as implied by enumeration methods, supported our choice for deciphering the spatial distribution, structure and composition of archaeal communities in the water column of Brâncoveanu Lake. The profiles generated by the PCR-DGGE revealed that minor differences in archaeal community composition might occur in relation to depth. The obtained banding patterns were relatively similar, with one additional band appearing in samples originating from −3 m to −12.5 m (see Figure 2A). This observation faintly suggested that the taxonomic composition of archaeal assemblages might change below 2 m. In an effort to elucidate the spatial grouping of archaeal assemblages inferred from the fingerprinting method, one sample from two separate depths (−0.5 m and −7 m, respectively) was selected for the construction of 16S rRNA gene clone libraries.

Analysis of archaeal communities via the parsimony and UniFrac tests

The similarity between the two archaeal clone libraries constructed from −0.5 m and −7 m samples was examined using a phylogenetic lineage-sorting test (P-test). Although the fingerprinting methods indicated that the two archaeal communities inhabiting the water column of the lake were only slightly dissimilar, the P-test (P-score = 37, $p < 0.0001$) applied to clone libraries indicated the existence of distinct archaeal communities.

The result obtained from the unweighted and weighted UniFrac tests (score = 0.8197, $p < 0.001$; and score = 0.4053, $p < 0.001$, respectively) established that the two clone libraries contained archaeal communities with different compositions and phylogenetic structures. As the discrimination between the two archaeal communities appeared stronger using the unweighted UniFrac metric, it was assumed that the communities are more dissimilar in composition. Together, the results of the parsimony and UniFrac tests validated the observation that the clone libraries constructed from surface (−0.5 m) and deep (−7 m) water layer harbored distinct archaeal assemblages, and that they were not subcommunities of each other.

Analysis of archaeal diversity present in clone libraries

The calculated Good's coverage estimator indicated that at the OTU level of 3% dissimilarity we sampled approximately 88% of the community is represented by the −0.5 m sample and 85.7% of the community is represented by the −7 m sample. The calculated values of evenness for the $OTU_{0.03}$s belonging to the two clone libraries were found to be relatively low (Simpson's indices of 0.208 and 0.213 for the −0.5 m and −7 m samples, respectively) indicating that the majority of sequences were assigned to a few $OTU_{0.03}$s, and the rest of them had low abundances. The evenness results corroborated with UniFrac analyses and indicate that the differences between the archaeal assemblages were caused by the occurrence of rare $OTU_{0.03}$s. Furthermore, the Mantel test results showed that salinity ($R = 0.936$, $p < 0.001$), ORP ($R = 0.809$, $p < 0.001$), and DO ($R = 0.768$, $p < 0.001$) were strongly correlated with community matrix.

The analyzed clone libraries comprising 161 sequences revealed 28 $OTU_{0.03}$s, with a richness of 14 for the −0.5 m sample and 18 for the −7 m sample. Four $OTU_{0.03}$s (DE09024D08, DE09025D10, DE09025C10 and DE09025G03) representing 130 cloned sequences were shared between the two sampled layers. All the archaeal $OTU_{0.03}$s with one exception belonged to Halobacteriaceae family (Figure 4). The exception, designated as OTU DE09025B06, was classified as belonging to the phylum Nanohaloarchaea as revealed by SILVA taxonomy browser and the phylogenetic tree (Figure 4). Out of the total number of $OTU_{0.03}$s, 19 received classification to the genus level being grouped into 11 genera (Supplementary Table S4). The classified $OTU_{0.03}$s presented an irregular distribution between the two communities with 4 and 10 genera being retrieved from −0.5 m and −7 m sample, respectively. *Halorubrum, Halobellus* and *Natronomonas* were the only three genera shared between the clone libraries. Results also indicated that *Halorubrum*-related sequences decreased with depth, while those affiliated to *Natronomonas* increased.

Discussions

Geochemical and limnological characteristics of Brâncoveanu Lake

The chemistry of the lake's water column was found to be a consequence of halite diapirism and the sedimentary input of

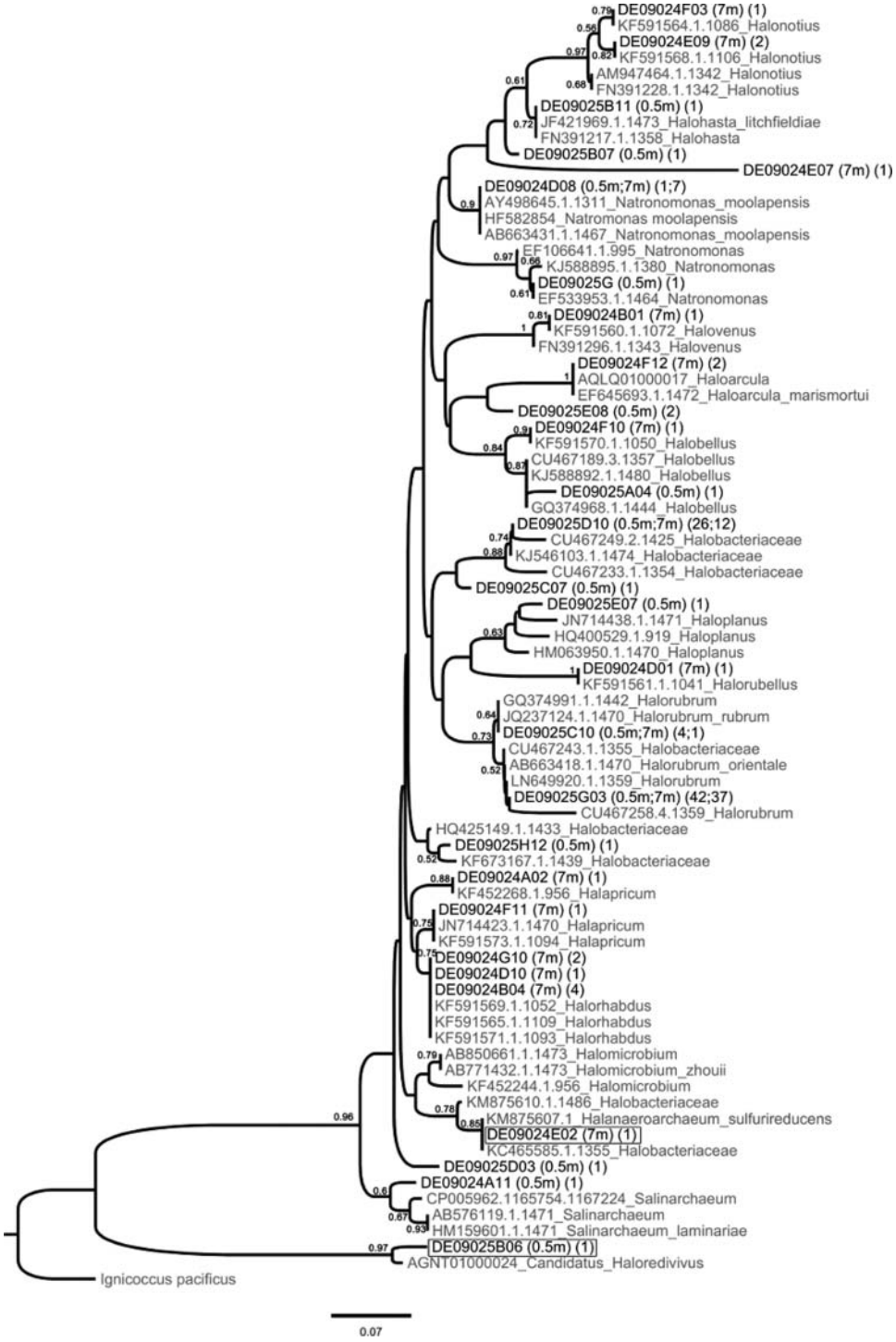

Figure 4. Neighbor-joining phylogenetic tree based on 16S rRNA partial gene sequences showing the relationships between the OTUs (defined at a 97% sequence identity cutoff; black color) recovered from Brâncoveanu Lake and their closest SILVA database matches (gray color). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the tree. Bootstrap values over 50% are shown next to the tree branches. The first brackets indicate the depth where the OTU was observed and the second ones indicate the OTU's abundance. Scale bar indicates 7% sequence dissimilarity.

clastic materials that molded the stratigraphy of the Transylvanian Basin (Har et al. 2010). As a result, elevated levels of alkali and alkaline earth cations (e.g. sodium, potassium, magnesium and calcium) might be credited to halide dissolution and to the silicate minerals weathering (data not shown).

From the perspective of the vertical distribution of salinity, the majority of Transylvanian Basin hypersaline water bodies (i.e. 41 permanent salt lakes) have a discrete three-layered structure, with the upper strata presenting a significantly lower salt concentration (i.e. <100 g l^{-1}) in comparison with the subsequent two (Alexe 2010; Baricz et al. 2014; Baricz et al. 2015; Andrei et al. 2015). The measurements carried out at Brâncoveanu Lake showed that its surface water is remarkably hypersaline (ca. 245 g l^{-1}) with the salinity gradient sharply increasing between 2 and 3 m depth (halocline) followed by a monotonous increase to the bottom

of the lake. As a consequence, the lake appeared rather double-layered with highly salt-rich surface waters, making it unique among other hypersaline meromictic lakes from its geographic region. Explanations might reside both in the direct contact of water with the rock salt and the absence of any continuous freshwater supply. Additionally, the human activity during summer bathing may well contribute to the homogenization of the hypersaline surface with the meteoric water.

Abundance and molecular diversity of archaea in the water column

Although among the organisms inhabiting hypersaline waters, halophilic Archaea seem to prevail (Andrei et al. 2012), the Bacteria representatives play crucial roles in many highly saline ecosystems (Oren 2012). The qPCR-based estimation of archaeal and bacterial cell numbers indicated the prevalence of Archaea in the total prokaryotic population inhabiting hypersaline Brâncoveanu Lake, in a similar fashion as reported for other meromictic salt lakes situated in the same geographic region (Baricz et al. 2014; Andrei et al. 2015). Although the bacterial cell numbers were comparable with those typically described for high-salt environments (Demergasso et al. 2008), it appeared that they constituted less than 0.5% of the total prokaryotes inhabiting Brâncoveanu Lake. High Archaea to Bacteria ratio was also reported in hypersaline sediments of Salton Sea (California), where Archaea accounted for up to 99% of the total prokaryotes (Swan et al. 2010), but our present finding of high archaeal proportion cannot be matched in the known literature due to the paucity of studies focused on the microbial communities of hypersaline meromictic lakes.

Our qPCR estimates for Archaea were found to be positively correlated with ORP, pH and salinity, and the results are corroborated by previous observations of salinity-driven change of archaeal cell numbers in the meromictic hypersaline Ocnei Lake (Baricz et al. 2014). The reduction of archaeal cell numbers throughout the water column could be attributed to their light-dependent heterotrophic metabolism, and their decrease alongside ORP could be attributed to the Halobacteriaceae preference for an aerobic life style (Andrei et al. 2012).

As suggested by the constructed clone libraries, the vast majority of the abundant archaeal population inhabiting Brâncoveanu Lake is related to Family Halobacteriaceae (Class Halobacteria). Our findings indicated that 2 $OTU_{0.03}$s, accounting for more than 50% of the sequences recovered from the clone libraries (Table S4), belonged to *Halorubrum sp*., a genus that prevailed at both sampled depths (−0.5 and −7 m). These results are corroborated by previous work reporting isolation of *Halorubrum* strains in the epilimnion (−0.5 m) and upper monimolimnion (−3.5 m) of Brâncoveanu Lake (Baricz et al. 2015). However, due to its relative ease in cultivation and cosmopolite distribution in hypersaline habitats, this genus is frequently described as being part of haloarchaeal communities (Benlloch et al. 2001; Oh et al. 2010). Moreover, the abundance of the *Halorubrum*-related sequences in hypersaline water column of Brâncoveanu Lake is substantiated by the observations that members of this genus are widespread at salinities close to saturation (Oren 2011) and outcompeted by other haloarchaeal genera at lower salinities (Youssef et al. 2012). *Halorubrum*-dominated communities were reported for solar salterns (Burns et al. 2004; Pasić et al. 2005), hypersaline sediments (Bowman et al. 2000) and monimolimnetic brine of meromictic lake Ocnei (Baricz et al. 2014).

Members of Family Halobacteriaceae (haloarchaea) are obligately halophilic, predominantly aerobic heterotrophs that consistently contribute to the biogeochemical cycling of carbon and nitrogen in saline systems (Andrei et al. 2012; Ventosa et al. 2014). Haloarchaeal involvement in the sulfur turnover in hypersaline anoxic habitats was only recently inferred (Sorokin et al. 2015). Interestingly, one sequence (GenBank Acc. No KF591563.1) retrieved from microoxic layer (−7 m) of Brâncoveanu Lake has close relatedness (ca. 98%) with *Halanaeroarchaeum sulfurireducens*, a recently described haloarchaeon with novel sulfur-dependent metabolic capabilities (Figure 4).

One archaeal $OTU_{0.03}$ (OTU DE09025B06) was classified by SINA (SILVA Incremental Aligner) as belonging to uncultured Nanohaloarchaea phylum (Table S4). This sequence, that did not cluster within Halobacteriaceae, was further analyzed and found to be 88% identical with "Candidatus Haloredivivus G17" (Figure 4), a virtual archaeon reconstructed using a combination of metagenomic and single-cell genome sequencing approaches (Ghai et al. 2011). Therefore, Brâncoveanu Lake seems to accommodate phylotypes related to the recently designated halophilic putative class of 'Nanohaloarchaea'. These newly identified microorganisms have eluded detection until recently due to their ultrasmall size (Narasingarao et al. 2012), appearing to be abundant in hypersaline environments worldwide (Zhaxybayeva et al. 2013). The finding of Nanohaloarchaea-related sequences in Brâncoveanu Lake could provide insights into their geographic distribution and phylogenetic diversity, and possibly offer indications concerning their contribution to the microbial assemblages and biogeochemical cycling.

From a total of 28 $OTU_{0.03}$s, 8 were designated as uncultured or unclassified at genus level, pointing Brâncoveanu Lake as a potential reservoir of novel microorganisms. However, among these potential novel phylotypes, OTU DE09025D10 was frequently present in both clone libraries, comprising over 23% from the total sequences, in contrast with the rest, which were largely found in proportion of less than 1%, suggesting the presence of novel Halobacteriaceae within the rare ecosystem of this meromictic lake.

The impact of limnological factors on archaeal community distribution

Foregoing studies found salinity to be the most important environmental factor controlling the worldwide distribution of both Bacteria (Lozupone et al. 2007) and Archaea (Auguet et al. 2010). Furthermore, it was found to be a key factor affecting prokaryotic diversity in salt-rich environments such as the hypersaline lake Chaka (Jiang et al. 2007), athalassohaline sediments (Oueriaghli et al. 2013) and the hypersaline Great Salt Lake (Meuser et al. 2013). Likewise, ORP and DO were also found to control the composition of aquatic microbial communities (Edlund et al. 2008; Hollister et al. 2010; Amaral-Zettler et al. 2011; Meuser et al. 2013).

Salinity was a major limnological factor influencing the archaeal assemblages in Brâncoveanu Lake, in spite of its extremely high salt concentration. Our results are in agreement with recent work of Baricz et al. (2014) on Transylvanian hypersaline stratified lake Ocnei, but tend to be in opposition with previous findings which reported that the influence of salinity on microbial communities would be less important at local scales, particularly in systems that are already salt-rich (Hollister et al. 2010). However, it is worth stressing that our study was performed on water column and not on sediments (as in the Hollister et al. experiment), highlighting the fundamental differences between their microbial assemblages, as well as in the forces shaping the structure of their resident prokaryotic communities. Furthermore, our results suggested that the environmental filters acted more robustly on the rarer taxa than on the abundant ones, indicating that the lake's scarce $OTU_{0.03}$s were more sensitive to environmental variations.

Influence of salinity, ORP and DO on the abundance, distribution and composition of archaeal assemblages in Brâncoveanu Lake could be attributed to the environmental heterogeneity and to their metabolic diversity. Zonation of the water column into oxic mixolimnion and microoxic and much saltier monimolimnion might create different potential niches for the archaeal populations. In addition to tested physicochemical parameters, input of organic carbon could significantly modulate the abundance and composition of heterotrophic microbial population (Schippers et al. 2012). In-field observation of the absence of continuous water inflow and scarce vegetation in the catchment area seconded by calculated Carlson's TSI values showing the upper eutrophic/hypereutrophic state suggested that lake's autotrophic communities might consistently contribute to the organic carbon production. These findings were also supported by our observation of picophytoplanktonic population comprising eukaryotic algae (such as *Dunaliella*-like cells, Supplementary Figure S1) and cyanobacteria, the latter counted at densities of $0.4–8 \times 10^5$ cells ml^{-1} in mixolimnion and chemocline (our unpublished data).

Although the lake monimolimnion had higher salt and ammonium concentrations and was microoxic (Table S2), the highest archaeal diversity was found to be present here (Figure 4). We consider that this zonation of archaeal communities could be attributed to the downward metabolite fluxes that favored niche diversity through the maintenance of various nutrient sources. Furthermore, we consider that low oxygen environments have the capacity to maintain a higher variety of energetic pathways, thus leading to the retention of higher ecological diversity coupled with lower interspecific competition (Humayoun et al. 2003). These hypotheses are sustained by the exclusive positioning of the *Haloarcula, Halorhabdus* and *Halanaeroarchaeum*-related sequences in the O_2-depleted monimolimnetic brine, which is in accordance with the microaerophilic or anaerobic metabolism documented in these haloarchaea (Andrei et al. 2012; Sorokin et al. 2015). Thus, the variation in Archaea community membership and structure could be attributed to environmental variables, indicating that environmental filtering was an important mechanism involved in the generation of the observed spatial distribution pattern.

Nonetheless, 16S rRNA gene-based analyses do not provide direct evidence regarding the metabolism of the detected OTUs, but useful information may be inferred by matching taxa to known taxon-specific biochemical functions. Therefore, by considering the stratification of the environmental parameters, the high abundance of Archaea in the prokaryotic communities, and the taxonomy results, we could assume that the abundant haloarchaeal populations of Brâncoveanu Lake are responsible for significant part of the biogeochemical cycling of carbon and perhaps nitrogen and sulfur despite the extreme salinity of the entire water column.

Conclusions

Several limnological (e.g. extreme salinity, double-layered stratification, upper eutrophic state) and hydrogeological (e.g. direct contact of water mass with bare salt rock) characteristics provide the distinctiveness of Brâncoveanu Lake among the saline Transylvanian pit lakes. The prokaryotic population of the lake appeared to be dominated by halophilic Archaea affiliated to the Family Halobacteriaceae. *Halorubrum*-related sequences were most frequently recovered whereas ca. 30% of the cloned sequences remained unclassified or pertained to uncultured taxa. These findings might allow anticipating relevant roles of haloarchaea in the biogeochemical turnover of C and, to some extent, of N and S in Brâncoveanu Lake. In spite of the hypersaline nature of the entire water column, the haloarchaeal-dominated population is apparently parted into two communities dwelling the upper, oxic and deeper, anoxic layers, respectively. The spatial distribution and the phylogenetic configuration of the archaeal communities were shaped primarily by the salinity. Overall, the hydrogeochemical and biological peculiarities of Brâncoveanu Lake raise the perspectives for novelty in the understanding of geomicrobiology of hypersaline aquatic ecosystems.

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