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Published in: Environmental Microbiology

Document Version:
Peer reviewed version

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Spatio-temporal study of microbiology in the stratified oxic-hypoxic-euxinic, freshwater-to-hypersaline Ursu Lake

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**Originality-Significance Statement**

Here, we report the functional ecology of a unique lake that formed on an ancient (Mid-Miocene) halite deposit and has oxic, hypoxic and euxinic layers and a freshwater-to-hypersaline water column. Via a combination of phylogenetic characterisation and biophysical and geochemical analyses, we reveal its complexity and dynamism during the annual cycle. Paradoxically, the data also indicates an inherent stability, in relation to the three primary water masses: the upper, low salinity mixolimnion (0-3 m), an intermediate stratum exhibiting a steep halocline and an oxic-anoxic regime shift (3-4 m), and the underlying anoxic and sulphidic hypersaline stratum (4-11 m). We show how salinity/ water activity, irradiance, nutrient availability/ geochemistry interact to determine the microbiology, including seasonally fluctuating, light-dependent communities in the mixolimnion, a stable but diverse population of heterotrophs in the hypersaline stratum, and persistent plate of green-sulphur bacteria that connects these two; evidence of carbon- and sulphur cycling between and within the lake’s three water masses; uncultured lineages in the hypersaline stratum; and that species richness and habitat stability are associated with high redox-potentials. Ursu Lake can be used as a comparator system for other stratified hypersaline systems, and a modern analogue for ancient euxinic water bodies.

**Abstract**

Ursu Lake is located in the Middle Miocene salt deposit of Central Romania. It is stratified, and the water column has three distinct water masses: an upper, freshwater-to-moderately saline stratum (0-3 m), an intermediate stratum exhibiting a steep halocline (3-3.5 m), and a lower, hypersaline stratum (4 m and below) that is euxinic (i.e. anoxic and sulphidic). Recent studies have characterised the lake’s microbial taxonomy, and given rise to intriguing ecological questions. Here, we explore whether the communities are dynamic or stable in relation to
taxonomic composition, geochemistry and biophysics, and ecophysiological functions during the annual cycle. We found: (i) seasonally fluctuating, light-dependent communities in the upper layer (≥0.987-0.990 water-activity), a stable but phylogenetically diverse population of heterotrophs in the hypersaline stratum (water activities down to 0.762), and a persistent plate of green sulphur bacteria that connects these two (0.958-0.956 water activity) at 3-3.5 m; (ii) communities which might be involved in carbon- and sulphur cycling between and within the lake’s three main water masses; (iii) uncultured lineages including Acetothermia (OP1), Candidate Phyla Radiation, Cloacimonetes (WWE1), Marinimicrobia (SAR406), Omnitrophicaeota (OP3), Parcubacteria (OD1), and SR1, in the hypersalina stratum (likely involved in the anaerobic steps of carbon- and sulphur cycling); and (iv) that species richness and habitat stability are associated with high redox-potentials. Ursu Lake has a unique and complex ecology, exhibiting both dynamic fluctuations and stability, and can be used as a comparator system for other stratified hypersaline systems and a modern analogue for ancient euxinic water bodies.

**Keywords:** dynamics and stability, ecosystem function, halophiles, microbial extremophiles, rare taxa, stratified hypersaline lake.

**Introduction**

Meromictic lakes, permanently stratified inland water bodies, occur in a range of geological settings, and can host stratified microbial ecosystems within their chemically distinctive water layers (Boehrer and Schultze, 2008; Lee et al., 2018). Those developed on ancient salt deposits in Central Romania, such as Ursu Lake, have a unique hydrology (Alexe et al., 2018). Ursu Lake has a permanently stratified halocline, from low salt (near-fresh) water at the surface, to saturated bottom brine (Máthé et al., 2014; Andrei et al., 2015; Alexe et al., 2018). Whereas a number of deep-sea haloclines have been well-studied, their microbial ecosystem lacks primary production (e.g. Yakimov et al., 2019), and whereas hypersaline systems such as crystallizer ponds and deliquescing halite have been well-characterised (La Cono et al., 2019), these lack a freshwater upper stratum. Ursu Lake is considered the largest, deepest, geologically...
youngest, continental lake yet documented (Alexe et al., 2018) which is at the same time heliothermal (entrapping the sun’s heat), meromictic, and euxinic (anaerobic and sulphidic). Furthermore, it concomitantly represents a low salt/ high water-activity habitat, intermediate salt-concentration habitat, and hypersaline habitat that are collectively able to host microbes that are primary producers, halophilic heterotrophs, and from other ecophysiological groups.

Ursu Lake has a surface area of 4.12 ha, which is slightly larger than that of the CaCl₂-saturated Don Juan Pond in Antarctica (approx. 3 ha). However, the considerable depth of Ursu Lake means that its volume is almost 500 000 m³ (Alexe et al., 2018) compared with only 3 000 m³ for the Don Juan Pond. The three primary water masses within Ursu Lake are the upper, freshwater-to-moderately saline layer known as the mixolimnion (from 0-3 m), the intermediate stratum exhibiting a steep halocline (from 3-3.5-4 m), and the lower, hypersaline stratum known as the monimolimnion (from about 4 m down to 11-18 m) that is euxinic. These water masses are maintained by a combination of freshwater inputs from rivulets, precipitation, and the underlying evaporite deposit of halite dating from Middle Miocene (Badenian) period, ca. 14 Ma ago (Peryt, 2008; Alexe et al., 2018). There is also evidence that physical chemistry of brines prevents mixing with overlying waters of lower salinity (Thorpe, 1969; Raup, 1970).

Among the 41 salt lakes of the Transylvanian Basin (Central Romania), Ursu Lake is the largest and deepest saline lake of natural origin, and is one of the largest hypersaline, meromictic heliothermal lakes in the world (Andrei et al., 2015; Alexe et al., 2018). Cultivation-based (Máthé et al., 2014; Baricz et al. 2015) and metabarcoding analyses (Andrei et al., 2015; Andrei et al., 2017) indicated higher prokaryotic diversity than in comparable lake systems, mainly owing to the high diversity of Bacteria. There is, however, a paucity of information about seasonal dynamics, biophysics, and ecophysiology of the various microbial communities present in the lake. We carried out the current study to elucidate the complexity of the Ursu Lake ecosystem. The specific aims were to: (i) assess the spatio-temporal dynamics of the prokaryotic communities along the salinity/ water activity and redox gradients, and during the annual cycle, and ascertain environmental variables that can act as determinants of microbial diversity; (ii) seek evidence of nutrient cycling with the lake system; (iii) draw inferences on the
keystone species and functional roles of microbes in this unique freshwater-to-hypersaline lake; and (iv) assess stability versus fluctuation within the overall ecology of the system.

Results and discussion

Ursu Lake is a unique freshwater-to-hypersaline ecosystem

The density-separated, stratified water column of Ursu Lake was maintained throughout the seasons of the annual cycle in relation to the physical, chemical and biological parameters (Fig. 1, Tables S1-S6). Overall, the ionic composition of Ursu Lake resembles that of sea water, with NaCl as the main salt component (Andrei et al., 2015). Determinations of water activity down through the water column according to samples taken in summer 2015 and spring 2016 validated the salinity gradient from surface (0.987-0.990 water activity, relatively close to freshwater) down to 11 m depth (0.763-0.764 water activity, equivalent to saturated brine) (Table S1). By its continuous density-stratification, Ursu Lake can be classified as meromictic (Hammer, 1986; Boehrer and Schultze, 2008).

The upper, slightly saline layer of the mixolimnion (down to 2.5 m and roughly 4.6-7.7% w/v total salinity; Fig 1, Table S1) was characterised by water-activity values (0.975-0.990) that are optimal for growth of many microbes. The most-severe halocline ranged between 3 and 4 m in depth (i.e., the oxic/anoxic transition zone, 0.786-0.958 water activity); and a hypersaline monimolimnion extended from 4 m downwards (0.762-0.776 water activity; 35-37% w/v total salinity) Table S1). The low-to-moderate saline mixolimnion of Ursu Lake is oxygenated and euphotic, and is prone to seasonal fluctuations in chemistry, water activity, and temperature (Table S1). Ursu Lake is heliothermal as the temperature of the underlying water mass (2.5-4 m) reaches ~40°C during summer (Table S1). The upper, slightly saline layer of the mixolimnion (down to 2.5 m and roughly 4.6-7.7% w/v total salinity; Fig 1, Table S1) was characterised by water-activity values (0.975-0.990) that are optimal for growth of many microbes. The most-severe halocline ranged between 3- m depths (i.e., the oxic/anoxic transition zone, 0.786-0.958 water activity); from 4 m downwards, a hypersaline monimolimnion (0.762-0.776 water activity, 35-37% w/v total salinity) was evidenced (Table S1).
The low-to-moderate saline mixolimnion of Ursu Lake is oxygenated and euphotic, and is prone to seasonal fluctuations in chemistry, water activity, and temperature (Table S1). Ursu Lake is heliothermal as the temperature of the underlying water mass (2.5-4 m) reaches ~40°C during summer (Table S1). Plots of salt concentration, dissolved oxygen and redox potential versus depth of the water column indicate that the halocline is more gradual at depths of 3-4 m in the summer season whereas an oxycline and redoxcline occur between 2 and 3 m but are both closer to surface than during other seasons (Table S1). The intermediate stratum (3-4 m deep) is characterised by steep gradients of salinity, pH, light, dissolved oxygen, and redox potential. Beneath this stratum, the lake water becomes more temperature-stable (~20°C), pH-stable (pH~6.0), hypersaline (> 30% w/v salt), and oxygen-depleted (< 0.2 mg O₂ L⁻¹), and is aphotic, highly reduced (> -300 mV), and sulphide-rich (~ 5 mM H₂S) (Fig 1, Table S1).

The stratification of salinity, water activity, and concentrations of total carbon, total nitrogen, sulphate, and ammonium are temporally stable (Fig 1, Tables S2-S5). Total carbon, total inorganic carbon, dissolved carbon and dissolved inorganic carbon concentrations were highly correlated with each other (Pearson's r > 0.9, p = 0.05) and with total nitrogen and total dissolved nitrogen (Fig. S1). Total organic carbon in the mixolimnion was relatively constant during autumn, winter and spring. During the summer, however, total organic carbon increased, probably due to inputs of allochthonous carbon that originate from littoral macrophytes and decayed biomass of invertebrates (Alexe et al., 2018). Throughout the study period, particulate carbon and dissolved organic carbon (also, therefore, total carbon) appeared to accumulate within the 3-5 m stratum (Fig. 1, Table S2). Below 5 m, the concentration of total organic carbon also remained more or less constant. The phosphorus concentration (made up of dissolved and particulate fractions) appeared uniform down to a depth of 2 m, increased below 2 m and was highest between 3 and 5 m and then decreased slightly at lower depths (Fig. 1, Table S4). Accumulation of total carbon, nitrogen, and phosphorous at 3 to 5 m depths is likely to be a consequence of slower settling velocity of organic matter at density interface (Prairie et al., 2015), and low rates of carbon mineralization under the anaerobic, hypersaline conditions (Boehrer et al., 2017). As the highest total cell counts were also recorded in the intermediate stratum (at 3 m depth; these were ~2 x 10⁸ cells mL⁻¹ in summer, autumn, and winter, and ~1.3
Contrasting microbiology is found within the three-strata water column of Ursu Lake

To assess stability and fluctuation within the microbial communities of the mixolimnion, intermediate stratum and monimolimnion, we performed metabarcoding of samples taken at eight discrete depths along the water column during four successive seasons. The sampling points were chosen in correspondence with the stratification of physicochemical parameters, based on in situ measurements, to cover all three main water masses of the meromictic Ursu Lake: mixolimnion (0.5-2 m), intermediary stratum (3 m), hypersaline stratum (3.5-9 m), a sampling strategy in line with those used for other stratified hypersaline systems (Klepac-Ceraj et al., 2012; Yau et al., 2013; Baatar et al., 2016). Our previous analysis (Andrei et al., 2015) revealed that that the permanently stratified Ursu Lake appeared as having a physicochemically...
and biodiversity-stable bottom water, while the upper part of water body appeared more dynamic; this is why sampling was carried out at the two monimolimnetic depth sample points, and more-frequent sample points within the mixolimnetic and transition water strata. By including a second dimension (time), the current study, supported by data from the 32 samplings, provides relevant spatio-temporal insights into the stratified ecosystem of the Ursu Lake.

After quality-filtering and operational taxonomic unit (OTU)-clustering at the 97% sequence identity, we obtained 1,244,800 good-quality reads grouped into 1,649 OTUs. Amongst these, 46 OTUs were affiliated to the Archaea and 1,168 OTUs to the Bacteria. To discriminate between individual OTUs, we grouped the retrieved phylotypes in 'Abundant OTUs' (i.e. ≥ 1%, equivalent to ≥ 389 reads abundance in at least one sample), and the relatively 'Rare OTUs' (i.e. <1% or below 389 reads abundance in all samples). Although not reaching a clear asymptote (Fig. 2A), rarefaction curves indicate that the sequencing effort detected the majority of species possibly present in our samples and increasing sequencing depth uncovers only low abundance taxa. We found 143 Abundant OTUs and 1,506 Rare OTUs accounting for 86.3% and 13.7% of the total number of sequences, respectively. A significant proportion of reads (21.6% of the total, hereinafter referred to as 'unassigned') presented low similarities to characterized prokaryotic lineages. There was considerable variability in the composition of microbial communities down through the water column; 62 Abundant OTUs were unique to the mixolimnion, 21 were unique to the intermediate stratum, and 40 were found only in the monimolimnion. Few Abundant OTUs were common to all three water masses (Fig. 2B), a finding that is consistent with microbial tolerance to different biophysical and nutritional conditions (Yau et al., 2013; Stevenson et al., 2015; Hamilton et al., 2015; Meyerhof et al., 2016).

Previous investigations of meromictic lakes in Central Romania revealed rich phototrophic plankton assemblages formed of mostly marine-derived, halotolerant Chlorophyta, Cryptophyta, Haptophyta, and picocyanobacteria (Keresztes et al., 2012; Máthé et al., 2014; Şuteu et al., unpublished data). We detected significant densities of picoeukaryotes and picocyanobacteria in the euphotic, oxic and slightly saline upper layer of Ursu Lake (Table S6).
Taxonomic assignment of eukaryotic reads in the samples collected from depths of 0.5, 1 and 2 m indicated the prevalence of Chlorophyta, Cryptophyta, and Stramenopiles, primary producers that apparently reached their maximum abundance during spring and summer (Fig. S2). The oxic and slightly saline (roughly 4.6-7.7% w/v) mixolimnion (0.5–2 m) host oxygenic phototrophs (Synechococcus sp.) and aerobic heterotrophs belonging to the Bacteroidetes (Cytophagia, Flavobacteriaia - Cryomorphaceae and Flavobacteriaceae families, [Rhodotermii], [Saprospirae], and Sphingobacteria), Alphaproteobacteria (mainly Pelagibacteraceae, Rhodobacteraceae, and Rhodospirillaceae families), and the highly diverse Gammaproteobacteria (Aeromonadales, Chromatiales, Legionellales, Thiohalorhabdallaes, Vibrionales, and sequences affiliated to HTCC1288). Taxonomically, the mixolimnetic community is consistent with other meromictic lakes, of freshwater or saline nature (Lehours et al., 2005; Gies et al., 2014; Baatar et al., 2016). Betaproteobacteria (mostly Alcaligenaceae family) reached maximum abundance at 0.5 m (Fig. 3). Recent metagenomic surveys revealed that phytoplankton blooms in the open ocean mostly influence the proliferation of heterotrophic taxa pertaining to Flavobacteriia (i.e. Polaribacter), or Alpha- and Gammaproteobacteria (Delmont et al., 2015; Teeling et al., 2016). Similarly, data on the microbiome specifically associated to Synechococcus sp. shows that prokaryotes affiliated to Alphaproteobacteria, Bacteroidetes (i.e. Flavobacteriales, Sphingobacteriales) and Gammaproteobacteria may have a role in shaping the cyanobacterial niche-space (Callieri et al., 2017).

The mixolimnetic community (constructed from rRNA genes) included some Abundant OTUs related to Mollicutes, Opitutae (Puniceicoccaceae family), Planctomycetes, SR1 candidate phyla, and Verrucomicrobia (Verrucomicrobiaceae family) (Fig. 3 and 4, Table S8). Taxa in the Planctomycetes (Planctomyces sp.) and Verrucomicrobia (Verrucomicrobiaceae, Coraliomargarita sp.) are generally less abundant but nevertheless increased in abundance following the summer peak in phytoplankton. Genomes of Planctomyces have been found to be enriched in carbohydrate-active enzymes (i.e. sulfatases) and are presumed to be involved in the initial breakdown of the sulphated heteropolysaccharides produced by various algae (Woebken et al., 2007), subsequently using their carbon skeletons as an energy source (Glöckner et al., 2003). Similarly, Puniceicoccaceae representatives have been shown to
assimilate exudates from *Synechococcus* sp. in euphotic waters (Nelson and Carlson, 2012). Given their widespread distribution and heterotrophic lifestyle, members of the *Verrucomicrobiaceae* might be involved in the utilization of phytoplankton-derived organic matter, either by protein assimilation or by polysaccharide hydrolysis (Orsi *et al*., 2016).

Down through the intermediate stratum, there is not only a transition from low salinity to hypersaline conditions, but also from euphotic to aphotic, and oxic to anoxic conditions. This stratum is, therefore, highly heterogeneous and accommodates ecophysically diverse taxa. Twenty Abundant OTUs detected the intermediate stratum were affiliated to classes found in the mixolimnion (*Actinomycetales*, *Alphaproteobacteria*, *Gammaproteobacteria*, *Nitriliruptoria*, *Opitutae*, [Rhodotermi], *Verrucomicrobia*, candidate phylum SR1) or monimolimnion (*Bacteroidia*, *Chlorobia*, *Clostridia*, *Deltaproteobacteria*, *Synergistetes*, candidate phylum *Patescibacteria* - OD1) (Fig. 3). *Halobacteria* (Euryarchaeota phylum), *Defferibacteres*, *Spirochaeta* and one candidate lineage *Cloacimonetes* - WWE1 were only abundant in the intermediate layer, at 3 m. The relative abundance of green sulphur bacteria-related reads (those assigned to genus *Prosthecochloris*, family *Chlorobiaceae*) peaked at 3 m depth (up to ~30%) and decreased in the 3.5- and 4-m-depth samples (about 8 and 6%, respectively; Fig. 3 and Table S9). This finding is consistent with patterns observed in other meromictic lakes where anoxygenic phototrophs dominate the intermediate stratum (Lehours *et al*. 2005; Degermendzhy *et al*., 2010; Lauro *et al*., 2011). The predominance of green sulphur bacteria reads at 3-3.5 m was corroborated by the high chlorophyll concentrations, high total cell counts, and high concentration of bacteriochlorophyll pigments, according to thin-layer chromatography (data not shown). These photoautotrophs contribute considerably to oxidative sulphur cycling and carbon photo-assimilation in stratified lakes (Guyouneaud *et al*., 2001). Furthermore, the diagenetic products of bacteriochlorophylls and other pigments (e.g. isorenieratene) derived from green sulphur bacteria and found in sediments, have been used as biomarkers for ancient (e.g. Quaternary) photic-zone euxinia events (Damsté *et al*., 2001; Meyer and Kump, 2008). Micro-oxic conditions were recorded in the intermediate layer (3-3.5 m); nevertheless, we believe that the aerobic phylotypes identified exist within the necromass that sinks downwards from the mixolimnion. Similarly, the aerobic taxa detected in the euxinic
(lower part) of the monimolimnion (i.e. *Cyanobacteria*, represented by *Synechococcus* sp.-related reads, were abundant in each of the three distinct water masses) may represent necromass and/or inactive biomass that has drifted down from the mixolimnion. Within the intermediate stratum (3-3.5 m), we detected taxa involved in methanotrophy and Fe/Mn metabolism (Tables S8 and S10). *Methylococcales* (represented by OTU244) oxidise and assimilate C1 compounds (Paul *et al.*, 2016) while the rarely microaerophilic *Defferribacterales* (represented by OTU50) can use various terminal electron acceptors including Fe(III), Mn(IV), S(0), Co(III), and nitrate, and some could use fermentative metabolism. Although there have been earlier reports of these taxa in saline habitats (Schneider *et al.*, 2013; Paul *et al.*, 2016), whether they are metabolically active within the hypersaline stratum of Ursu Lake remains unclear. The presence of Abundant OTUs assigned to sulphate-reducing bacteria (*Desulfovibrionales* and *Desulfobacterales* related OTUs) in the samples from the intermediate stratum (3 m depth) is consistent with studies on differential oxygen tolerance of sulphate-reducing bacteria, some taxa being able to survive in the oxic zones of microbial mats (Jonkers *et al.*, 2003). The high-salt (>30% w/v), oxygen-depleted stratum (3.5-9 m) favours abundant phylotypes related to *Methanobacteria*, *Bacilli* (*Lactobacillales* order), *Lentisphaerae*, and candidate phylum *Acetothermia* (OP1) that may be involved in anaerobic carbon cycling.

Alphadiversity patterns revealed by our analyses indicate that prokaryotic richness is enhanced in the stable conditions of euxinic monimolimnion (Fig. S4). Community stability over time is supported by analysis of similarity (ANOSIM, R=-0.007, p=0.445). The four-way set difference analysis (Fig. 2C) indicated that 38 Abundant OTUs (accounting for 63% of reads associated to all Abundant OTUs) were detected all year-round (Table S10). However, temporal variation was apparent from the distribution of OTUs unique to each season (11 in July and 13 in November 2015; 19 in February and 18 in April 2016). Albeit that these OTUs were phylogenetically related to phylotypes encountered throughout the year (Fig 2C, Table S10), the mixolimnion appears as a more seasonally fluctuating environment compared to the deeper strata, as evidenced by the succession of aerobic heterotrophic *Bacteroidetes*, *Proteobacteria* and *Verrucomicrobia* bacterial clades (Fig. S3, Table S10). *Aeromonadales* affiliated OTUs were abundant in spring, *Chromatiales* in spring and autumn, while *Gammaproteobacteria*
OTUs affiliated to the *Legionellales*, *Methylococcales*, and *Thiohalorhabdinales* were abundant in autumn. Most of *Bacteroidetes*-affiliated OTUs exhibited high abundances in all seasons, except *Sphingobacteria* which was abundant in spring, and *Cytophagia-Roseivirga* which was abundant in spring and summer. *Defferibacteres* and *Planctomycetia* OTUs were detected at higher numbers in spring, SR1 was abundant in autumn and winter, while *Cloacimonetes* (WWE1) was abundant in winter (Table S10).

We have detected increases in abundance for OTUs affiliated to *Flavobacteriaceae* (*Psychroflexus* sp. and *Polaribacter* sp.) spring and winter, and a significant proportion of reads pertaining to *Cryomorphaceae* throughout the year (Tables S8). Members of *Flavobacteria*, such as *Polaribacter* sp. can degrade complex algal polysaccharides via the production of diverse glycoside-hydrolases (Teeling et al., 2016). On the other hand, Delmont et al. (2015) suggest involvement of *Cryomorphaceae* in algal decomposition, as a particular functional gene pool related to virulence and host invasion was found in *Cryomorphaceae* bacteria associated to a *Phaeocystis* bloom. We observed seasonal fluctuations of abundant OTUs affiliated to *Cytophaga-Roseivirga*, *Rhodobacteraceae* (*Alphaproteobacteria*), and *Vibrio* sp. (*Gammaproteobacteria*) (Table S8). It is possible that the succession of distinct bacterial population to be related to the diversity of algal (phytoplankton) exudates, as no individual bacterial species is known to possess all of the genes required for complete degradation of all naturally occurring polysaccharides (Teeling et al., 2016).

Salt concentration and water activity, pH, and redox determine the fine-scale ecology of Ursu Lake

For samples taken down through the 1-37% (w/v) salt water column, downstream alpha- and beta-diversity analyses were performed. All 32 samples (at eight discrete depths and during four seasons) for which a total of 2,469,892 reads (with a mean length of 441 bp per read) were recovered. Due to the variations in the number of reads per sample, the rarefaction and diversity analyses were performed at the smallest sequencing depth (i.e. 38,900, representing a total of 1,244,800 high-quality reads). In hypersaline systems, biodiversity is determined, and can be constrained, by salt-induced cellular stress which, in turn, relates to adaptation to ionic...
conditions, osmotic stress and water activity (Laron and Belovsky, 2013; Alves et al., 2015; Stevenson et al., 2015; Lee et al., 2018, Merlino et al., 2018). Prokaryotic richness was highest in the extreme conditions, including low water activity values of the hypersaline stratum (0.763-0.764 at 11 m; Table S1). This finding is consistent with recent evidence showing that the water-activity of NaCl-saturated brines is thermodynamically mid-range rather than extreme (Stevenson et al., 2015; 2017; Lee et al., 2018). In Ursu Lake, the impact of environmental parameters on prokaryotic diversity is illustrated by high spatial variations with increases of up to two-fold in calculated richness as depth decreases, e.g. the chao1 indices ranged between 350-587 for the mixolimnion (0.5-2 m) and 785-1055 for the hypersaline stratum (4 -9 m), and the observed species (OS) indices increased from ~300 in the mixolimnion to >700 in the hypersaline stratum (Fig. S4, Table S11). Increasing richness with depth (higher salinity, lower water activity) was reported in other meromictic saline lakes, including high-latitude coastal lakes (Lauro et al., 2011) and inland alkaline saline (Dimitriu et al., 2008), pH-neutral saline (Baatar et al., 2016) and hypersaline lakes (Baricz et al., 2014; Andrei et al., 2015). The richness indices correlated strongly with salinity, total carbon, total nitrogen, sulphate, sulphides, ammonium, and phosphate concentrations (Fig. 5A, 5B, Table S12). Limited temporal variations in richness and evenness raise the prospect that no significant changes occurred during the sampling period, the seasonal homogeneity endorsing the independence of microbial community from seasonal blooms of phytoplankton.

Evenness indices (Shannon-H and Simpson-S) showed similar values down through the water column and there were no apparent correlations with any of the measured physicochemical parameters (Fig. 5A) However, the reduced evenness (Table S11) at the oxic/anoxic interface during autumn (November 2015) and winter (February 2016) could be a manifestation of a large prokaryotic population development. Amplicon sequencing data (see below) revealed the high abundance of a single OTU affiliated to green sulphur bacteria at a depth of 3 m. In stratified aquatic ecosystems, at depths where oxygen concentration reaches very low levels, the anoxygenic phototrophs affiliated to green (Lauro et al., 2011; Laybourn-Parry et al., 2014) or purple sulphur bacteria (Rogozin et al., 2010; Klepac-Ceraj et al., 2012) become dominant.
The Mantel test performed for the physicochemical data and beta-diversity (based on Bray-Curtis distance matrix) indicated the significant impact (Mantel $r = 0.8219$, $p = 0.001$) of selected environmental factors on the prokaryotic community composition. PCoA ordination plot, based on Bray-Curtis dissimilarity matrices, was used to test for patterns and to further identify the contribution of environmental factors in shaping the spatial and temporal variability of the microbial community. PCoA ordination (carried out to order/arrange samples characterized by values on multiple variables) separated samples into three distinctive depth-dependent zones (Fig. 5B). The mixolimnetic microbial community is impacted by the effects of pH (that can be used as a proxy for photosynthetic activity of phytoplankton) and redox potential (a proxy for dissolved oxygen), whereas the monimolimnetic microbiome is affected by salinity and availability of reduced nutrients. The depth-related decrease of pH and dissolved oxygen are presumably driven by mineralization of organic matter. Accordingly, depth-related decreases in pH, oxygen and nutrients, and increases in salinity apparently select for anaerobic halophilic community (Meyerhof et al., 2016).

Analysis of similarity (ANOSIM) supported a depth-related grouping of samples ($R = 0.666$, $p = 0.001$) indicating a significant spatial difference between the prokaryotic communities at different depths, as well as the clustering of samples into three strata ($R = 0.989$, $p = 0.001$), a situation depicted by prior studies of meromictic lakes, although internal environmental gradients, specific to each lake, may determine spatial separation of communities (Lauro et al., 2011; Klepac-Ceraj et al., 2012; Baatar et al., 2016). In addition, ANOSIM indicated no significant difference in community composition relative to season of sampling ($R = -0.007$, $p = 0.445$).

**Low-abundance microbes flourish in Ursu Lake**

The species distribution pattern indicates the presence of a considerable number of low-abundance phylotypes in the water column of Ursu Lake (Fig. 2D). Rarefaction analysis (Fig. 2A), as well as richness indicators (Fig S4, Table S11), suggest an increase in the prokaryotic diversity down through the water column, that may be seen as the outcome of increasing proportion of Rare phylotypes. Out of the 1649 OTUs, 1506 (91%) are Rare (i.e. present in
abundances below 1%). We found that 401 Rare OTUs (24.3% of total number of OTUs) are shared between all three water strata (Fig. 2E). Interestingly, the proportion of Rare OTUs in Ursu Lake’s microbiome, that are confined to their respective strata (mixolimnion, intermediate or hypersaline), is nearly double in the monimolimnion (339 OTUs, 20.5%) compared to the mixolimnion (0.5 to 2 m samples) (196 OTUs, 11.8%), and only 39 (2.4%) rare phylotypes were unique to the intermediate stratum (3 m samples) (Fig. 2E).

Within the detection limits of the analysis, approximately 48% (790 OTUs) of all Rare OTUs identified in Ursu Lake were confirmed as present during each season (Fig. 2F), their phylogenetic composition being similar to the composition of the abundant community members. Nonetheless, we observed an increase in taxonomic diversity of Rare OTUs compared to Abundant phylotypes (Fig. S5, Table S13) particularly in the euxinic water samples (4-9 m) where sequences affiliated to bacterial (Acidobacteria, Armatimonadetes, Elusimicrobia, Fibrobacteres, Gemmatimonadetes), and archaeal (Crenarchaeota, DSEG, Parvarchaeota, and Thermoplasmata) lineages, were detected. Several candidate Rare phyla were detected in both the intermediate stratum of the water column and the monimolimnion: BRC1, NKB19, OP3, SAR406, TM6, WPS2, WWE1 (Fig. S5; Table S13). Rare taxa exhibited patterns of seasonality, as OTUs pertaining to WWE1, [Thermi], BRC1, SAR406, NKB19, and Gemmatimonadetes were detected in summer, Parvarchaeota and [Thermi]-related OTUs in autumn, while Elusimicrobia, Fibrobacteres, OP3, WWE1, and WPS2 predominated in winter (Fig. S5). It is nevertheless likely that some community changes may occur below the threshold for detection. Any populations that diminish to levels that are undetectable (≤1-10 reads) may be dormant or quiescent, and / or stressed by biotic or abiotic factors, and either in decline or lag phase until they recover during subsequent seasons. It is also possible that there are occasional extinctions (and, indeed, occasional introductions or evolution of new phylotypes) for some of the Rare taxa within the microbiome. (Fig. S5). One intriguing finding was that several lineages that are thought uncultivable (i.e. Parvarchaeota, OP3, OP8, OP9, OP11, TM6, TM7) were found only as Rare microbes. This is corroborated by the phylogenetic diversity of uncultivated microbes found in the bottom organic-rich (sapropelic) sediment of Ursu Lake (Andrei et al., 2017).
Our analyses indicated a 1:10 ratio between Abundant- and Rare taxa within the microbial communities of Ursu Lake; a ratio that is not surprising given that low abundance phylotypes, by definition, represent the majority of the microbial diversity usually present in individual ecosystems (Pedrós-Alió, 2012; Lynch and Neufeld, 2015). Down through the three strata of the Ursu Lake water column, the majority of Rare phylotypes were affiliated to Actinobacteria, Bacteroidetes, Firmicutes, Proteobacteria, and Verrucomicrobia (Table S13). The phylogenetic similarities at high-taxonomic level (i.e. class) of Abundant- and Rare microbes in Ursu Lake samples (Table S8, Table S13) provides evidence that rare phylotypes are active in situ, even when the methodology does not distinguish directly between cells that are metabolically inactive and active (Galand et al., 2009). The increased diversity of the Rare phylotypes in the euxinic monimolimnion (4-9 m), compared to the upper strata, could be related to higher metabolic diversity and various substrates (i.e. highly diverse carbon sources, electron donors and terminal electron acceptors) availability in the anoxic strata of stratified freshwater (Lehours et al., 2005; Gies et al. 2014), alkaline (Dimitriu et al., 2008) or hypersaline lakes (Klepac-Ceraj et al., 2012; Andrei et al. 2015).

Whereas low water activity and hypersalinity select for halophilic taxa (Lee et al., 2018), the salt-saturated waters of Ursu Lake have a remarkably high bacterial diversity (Fig. 3-4). Among the 1649 OTUs, only two archaeal OTUs were detected as Abundant, one affiliated to Halobacteria (in the intermediate stratum), and the other related to Methanobacteriales in the monimolimnion). The majority of the small number of halophilic microbes that are capable of growth in the water-activity range 0.635-0.755 are members of the Class Halobacteria (Stevenson et al., 2015). The rest of the Ursu Lake archaeal phylotypes, affiliated to Bathyarchaeota (Crenarchaeota of TACK superphylum), Aenigmarchaeota and Parvarchaeota (DPANN superphylum) were identified solely among the Rare OTUs (Table S13). It has been suggested that the Rare taxa may serve as ‘seed bank’ that harbours and provides ecophysiological diverse microbes that maintain or enhance the metabolic diversity and functional roles of the in situ communities (Lynch and Neufeld, 2015). Yet, compared to the surface waters, the increased number of Rare OTUs in the stable conditions of Ursu Lake’s monimolimnion suggests that they are less efficient, yet active as resource competitors (Hugoni
et al., 2013). It appears that the monimolimnion of Ursu Lake are enriched in endemic Rare phylotypes (Elusimicrobia, Fibrobacteres, Lentisphaeria) and candidate phyla (BRC1, Cloacimonetes, Dependentaiæ, Hydrogenedentæ, Omnitrophicæota, Marinimicrobia, WPS2, (Table S13). It is clear that these microbes are ubiquitous within anoxic, sulphur and organic carbon-rich environments, but their ecophysiological roles have yet to be determined (Hamilton et al., 2016; Llorens-Marès et al., 2016).

Putative spatio-temporal function of the Ursu Lake ecosystem

The geochemically stratified waters of meromictic lakes host disparate microbial communities, and microbe-driven processes, so can perform the complete cycling of nutrients (Lauro et al., 2011; Barberán and Casamayor, 2011; Meyerhof et al., 2016). The high taxonomic diversity of Ursu Lake’s prokaryotic community argues for complex interactions between chemistry, biophysics and ecophysiology of the lake’s microbiome (Hallsworth, 2018), some of which can be elucidated using SSU rRNA gene-based diversity (Aßhauer et al., 2015; Knight et al., 2018).

Within the euphotic zone of Ursu Lake, we identified a wide range of heterotrophic microorganisms (Actinobacteria, Alphaproteobacteria, Flavobacteria, and Gammaproteobacteria; Fig. 3), capable of working as consortia to degrade complex organic substrates derived from halotolerant phytoplankton (Teeling et al., 2016). Sulphur oxidisers are known to be active under moderately high salinities (up to 4 M NaCl) and microaerophilic conditions (Oren, 2011), so we believe that the sulphur oxidisers identified in Ursu Lake in the 0.5-3 m (Fig. 6) are active in situ. The large population of Prosthecocloris t a depth of 3 m that was observed regardless of season suggests the oxic/anoxic interface might be the main site for sulphur compounds turnover in Ursu Lake.

In the euxinic bottom water (below 4 m), the sinking particulate organic carbon (Table S1), most of which originates from above (Boehrer et al., 2017), is probably utilised as a substrate by Halanaerobiaceae and other obligately halophilic fermenters, Delsulfohalobiaceae and other sulphate-reducers, methanogens (e.g. Methanobacteriales) (Fig. 3, 6), and Acetothermia (OP1), Omnitrophicæota (OP3) and other candidate taxa (Fig. 6, Table S13). The Halanaerobiaceae is known to contain some of the most extreme halophiles, such as
*Halanaerobium lacusrosei* that is capable of metabolism and growth at water activity values below that of saturated NaCl (Stevenson *et al*., 2015). Sulphate-reducing bacteria, mainly *Deltaproteobacteria*, and methanogenic functional types affiliated to *Methanobacteriales*, occur together in the hypersaline stratum although are known competitors for acetate; the available substrate.

Compared to the upper 3 m of the water column, in the 3.5-9 m portion, uncultivated microbes such as *Parcubacteria* (OD1) and *Acetothermia* (OP1) are abundant (Fig. 3, 6, Table S8). Moreover, 26 out of the 143 Abundant OTUs (21% of total reads) exhibited low similarities to characterized prokaryotic lineages (referred to as ‘unassigned’). The phylogenetic tree constructed by selecting the closest 20 sequence neighbours from the SSU/LSU 132 SILVA database (Fig. 6) indicated that a fraction of unassigned OTUs (Fig. 6, Table S14), Abundant in the monimolimnion, may be related to the Candidate Phyla Radiation (CPR). CPR bacteria have small genome size (often < 1 Mb) and exhibit multiple metabolic limitations, such as partial tricarboxylic acid cycle, lack of electron transport chain complexes, incomplete biosynthetic pathways for nucleotides and amino acids (Brown *et al*., 2015). All these suggest a co-dependent lifestyle in which these putative obligate fermenters obtain the much needed metabolites from the symbiotic bacterial or archaeal partner and reciprocate by providing fermentation end products (Wrighton *et al*., 2014; Nelson *et al*., 2015; Brown *et al*., 2015; Castelle *et al*., 2018). However, CPR representatives with respiratory and fermentative capacities as well as nitrogen and fatty acid metabolism have been detected by genome analysis (Castelle *et al*., 2017). The *Acetothermia* (OP1) affiliated OTUs in the monimolimnion of Ursu Lake are related to the candidate KB1 bacterial group, that has been consistently found in deep-sea hypersaline anoxic lakes (Nigro *et al*., 2016). Brine enrichments and radiotracer experiments that used 14C-labeled glycine betaine (Yakimov *et al*., 2013), support the idea that these uncultured microorganisms assimilate glycine betaine as carbon and energy source. According to Yakimov *et al*. (2013), in anoxic environments, the KB1 bacteria perform the reductive cleavage of glycine betaine, simultaneously producing acetate and trimethylamine, the latter supporting extremely halophilic methylotrophic methanogens. In addition to fermentative metabolism and co-dependent lifestyle (Hu *et al*., 2016; Hao *et al*., 2018),
metagenomic studies of *Acetothermia* and *Parcubacteria* microbes have also predicted their involvement in sulphide production (Wrighton *et al*., 2014; Gies *et al*., 2014).

In the phylogenetic tree, the 15 of the unassigned OTUs cluster together (Fig. 6, Table S14). The closest 20 sequence neighbours from the SSU/LSU 132 SILVA database for these 15 OTUs are affiliated to the *Patescibacteria* superphylum, which is known to contain *Parcubacteria* (OD1), *Saccharibacteria* (TM7), *Gracilibacteria* (GN02), and *Microgenomates* (OP11) (Peura *et al*., 2012). These fermentative microbes are known to thrive in anoxic, organic matter-rich environments, and are likely involved in cycling of C, H, and S (Wrighton *et al*., 2014). This is consistent with studies of nutrient cycling by *Parcubacteria* and *Microgenomates* that are associated to sulphur-rich ecosystems (Harris *et al*., 2004; Peura *et al*., 2012). Throughout the year (Table S8, Table S14), Abundant Marinimicrobia (SAR406)- and Cloacimonetes (WWE1)-related OTUs were found in the 3-9 m portion of Ursu Lake’s water column. The *Marinimicrobia* are thought to anaerobically digest recalcitrant polysaccharides such as cellulose (Limam *et al*., 2014) and *Cloacimonetes* proposed to perform S cycling within marine systems (Wrighton *et al*., 2014). Both candidate phyla were predicted to take part in syntrophic interactions in methanogenic environments; i.e. fermentative degradation of amino acids (*Marinimicrobia*) or propionate (*Cloacimonetes*) (Nobu *et al*., 2016). Genome-level studies indicated an eukaryote (amoeba)-dependent (parasitic) lifestyle of *Dependentiae* (TM6) (Yeoh *et al*., 2016), which could explain their maximum abundance in the intermediate stratum during the phytoplankton bloom in November 2015, while being part of the monimolimnetic’s Rare taxa. Omnitrophicaeota (OP3) apparently thrive in anoxic environments, so share the habitat of methanogens. Metagenomic analyses indicated that OP3 is a diverse group comprising syntrophic bacteria with an anaerobic-respiring metabolism fuelled by formate or H₂ (Glöckner *et al*., 2010).

Overall, our analyses revealed that the three water masses (characterised by differences in the thermodynamic parameter water activity) influence community stability and shape of Ursu Lake’s microbiome, which is dominated by a taxonomically diverse microbial community involved in carbon and sulphur cycling.
Conclusions
Ursu Lake has revealed the complexity of its microbial assemblages and community structure, and that salinity, pH, redox, and reduced nutrients are correlated with spatio-temporal variability of microbial populations in its unique ecosystem. In the low- to moderate salinity (1 to 10% w/v salt), euphotic upper two meters we found seasonal fluctuations in dissolved chemical species, water activity as well as temperature, which favoured a diverse community of mesophilic and halotolerant microbes. Moreover, species richness increases in the stable conditions of the hypersaline stratum (> 30% w/v salt), inhabited by a phylogenetically diverse community of extremely halophilic microbes. A biomass-dense community of green sulphur bacteria in the intermediate stratum connects the two contrasting water masses, regardless of season. During the annual cycle, fluctuations are limited, and the highly reduced conditions of the hypersaline water mass seem to enhance habitat diversity.

Linking Ursu Lake ecosystem functioning with SSU marker gene-based diversity provided clues on putative trophic relationships among components of microbial communities that are capable of complete C and S biogeochemical cycling. Abundant and rare uncultured lineages including Acetothermia (OP1), Candidate Phyla Radiation, Parcubacteria (OD1), SR1, Cloacimonetes (WWE1), Marinimicrobia (SAR406), and Omnitrophicaeota (OP3), are likely to be key players in the anaerobic degradation of carbon- and sulphur compounds in the euxinic, hypersaline stratum. The high diversity of low-abundance microbes in euxinic conditions suggests that, despite the thermodynamic constraints imposed by low water activity (including the energetic burden of osmotic adjustment), the availability of electron donors and organic nutrients are associated with microbial diversity.

Experimental procedures
Sample collection
Water samples collected from 8 discrete depths (0.5, 1, 2, 3, 3.5, 4, 5, 9 m) on four occasions (July 2015 – corresponding to summer season; November 2015 – autumn; February 2016 - winter; April 2016 – spring) were sub-sampled to determine molecular diversity, biological, physical, and chemical characteristics. In total, 32 (8 x 4) samples were analysed during 2015-
2016 survey. The sampling points were chosen in correspondence with the stratification of physicochemical parameters, based on \textit{in situ} measurements, to cover all three water strata of the meromictic Ursu Lake (mixolimnion, intermediate stratum, hypersaline stratum). For environmental monitoring, the physical and chemical parameters were measured from 0.1 to 11 m of depth. Water samples were collected using a submersible 12-V electric pump, with a flow rate of 9.9 L\textperiodcentered min\textsuperscript{-1}, and stored in sterile 2 L polypropylene bottles. To avoid cross-contamination, prior to collecting the samples, the inside of the pump-tubing system was purged with water from each depth for at least 15 minutes (~150 L total purged volume) until physicochemical parameters stabilized following a procedure adapted from Harter \textit{et al.} (2014). Constant flow rate (9.9 L min\textsuperscript{-1}) was applied with no change between purging and water sampling. The samples were kept cool and light-protected and transported to laboratory in less than 6 hours.

**Biological parameters**

For total prokaryotic cell counts, water samples were fixed in 1% glutaraldehyde and filtered on 0.45-\(\mu\text{m} \) pore-sized, black-gridded MCE filters (Fioroni, France). The filters were stained directly using DAPI (4',6-diamidino-2-phenylindole, dihydrochloride, 2.5 \(\mu\text{g mL}^{-1}\) solution) and examined by epifluorescence microscopy (BX60, Olympus Optical, Japan). Picophytoplankton (picoeukaryotes and picocyanobacteria) cell counts were estimated based on autofluorescence of fresh samples by using a BX60 Olympus microscope. Total photosynthetic pigments (chlorophyll \(a\) and total carotenoids) were estimated by spectrophotometric measurements of methanol extracts, as described by Wetzel and Likens (2000).

**In situ measurements and chemical profiling**

\textit{In situ} measurements (e.g. temperature, pH, dissolved oxygen, and oxidation/reduction potential) were performed using a portable water multi-parameter system HI 9828 (Hanna Instruments, USA). Salinity (g L\textsuperscript{-1}) was estimated based on electrical conductivity values measured with a Multi 340i multi-parameter (WTW, Germany), with a built-in temperature correction. Vertical profile of photosynthetic active radiation (PAR, 400-700 nm) was recorded.
with a spherical irradiance probe connected to a calibrated quantum irradiance meter (ULM-500, Walz GmbH, Germany). Main ionic content of water column in Ursu Lake was detailed elsewhere (Andrei et al., 2015). More than 90% of total ions were represented by Na\(^+\) and Cl\(^-\) followed by K\(^+\), Mg\(^{2+}\), Ca\(^{2+}\), and carbonates and sulphate as minor anions and cations, respectively (Andrei et al., 2015). Total alkalinity was measured by titration with HCl to the methyl orange indicator endpoint. Total nitrogen (TN) and total dissolved nitrogen (TDN) including free ammonia, ammonium, nitrite, nitrate, and organic nitrogen were analysed by catalytic combustion followed by oxidation of nitrogen monoxide to nitrogen dioxide with ozone and subsequent chemiluminescence detection. Total particulate nitrogen (TPN) was calculated by subtracting TDN from TN. Dissolved organic nitrogen (DON) was calculated by subtracting ammonium nitrogen (N-NH\(_4^+\)), nitrite and nitrate from TN. Total carbon (TC) and total inorganic carbon (TIC) were determined in unfiltered samples, while dissolved carbon (DC) and dissolved inorganic carbon (DIC) in samples filtered through 0.45-µm pore size PTFE filters, by catalytic combustion and infrared detection of CO\(_2\) using a Multi N/C 2100S Analyser (Analytik Jena, Germany). Total organic carbon (TOC) and dissolved organic carbon (DOC) were obtained by subtracting TIC from TC and DIC from DC, respectively. Ammonium nitrogen by salicylate method and sulphides by methylene blue method after fixation of samples with 2% (v/v) Zn-acetate (Trüper and Schlegel, 1964) were determined spectrophotometrically (Lambda 25, Perkin-Elmer, USA). Sulphate (SO\(_4^{2-}\)), nitrite (N-NO\(_2^-\)) and nitrate (N-NO\(_3^-\)) were measured by ion chromatography on 761 Compact IC (Metrohm, Switzerland). Methane was measured as indicated in Ionescu et al. (2017). Total phosphorous (TP) and total dissolved phosphorous (TDP) were determined in unfiltered and respectively, filtered (0.45-µm pore size; PTFE) samples after digestion with 65% HNO\(_3\), by OPTIMA 5300 DV multichannel inductively-coupled plasma optical emission spectrometer (Perkin-Elmer, USA). Total particulate phosphorous (TPP) was calculated by subtracting TDP from TP. Five-day biochemical oxygen demand (BOD\(_5\)) was measured in the freshly collected samples (i.e. within 6 hours of sampling) from oxygenated surface water (i.e. down to 4 m depth). Total BOD\(_5\) was calculated as the difference between the DO measured initially and after incubation of samples in 300-ml BOD bottles, at 20\(^\circ\)C for 5 days, using an Oxi 740 meter (WTW, Germany). Water-activity values of water
samples (10 discrete depths) were measured as described in Stevenson et al. (2015) using a Novasina Humidat IC-II water-activity machine (Axair Ltd, Pfäffikon, Switzerland) at the same temperature at which the samples were collected.

DNA extraction, sequencing and bioinformatics analyses
Water samples were vacuum-filtered through 0.45-μm pore size MCE membranes (Fioroni, France), until clogging of the membrane, therefore the volume of the filtered water varied depending on the biomass content (100-500 mL). Three membrane filters were obtained for each sample, and from every individual membrane filter, DNA was extracted using the ZR Soil Microbe DNA kit (Zymo Research, Irvine, CA, USA), according to manufacturer's instructions. The extracted DNA was pooled together. MiSeq 16S V3-V4 Metagenome Sequencing was performed by a commercial company (Macrogen Inc., South Korea), using MiSeq Reagent Kit v3 Illumina and Nextera XT Index Kit for library preparation. The V3-V4 hypervariable regions of bacterial and archaeal SSU rRNA gene were PCR amplified by using primer 341F (5’-CCTACGGGNGGCWGCAG-3) and 805R (5’-GACTACHVGGGTATCTAATCC-3’), using the following PCR program: 95°C for 3 minutes, 25 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 30 seconds, and 72°C for 5 minutes, based on Illumina's 16S amplicon-based metagenomic sequencing protocol. The amplicon size achieved was 464 bp. Raw sequences (SRA accession number PRJNA395513) were processed using the QIIME and Usearch v8 pipelines (Caporaso et al., 2010; Edgar, 2013). The pair-end reads were joined in QIIME, followed by quality filtration, dereplication and singleton removal in Usearch v8. Both de novo and reference chimera checking were performed in Usearch v8, using the last version of the Greengenes database ('13_8') as a reference (DeSantis et al., 2006). The taxonomy was assigned for the representative OTUs (441 bp) in QIIME using the default classifier (Greengenes) against the last version of the Greengenes database ('13_8'), using 'species-level' OTU cut-off (97% sequence identity). The taxonomy was added to the OTU-table with the biom-format package, and the mitochondrial and plastidial sequences were filtered out of the final OTU table. To minimise the uncertainties in OTU affiliation, sequences from each OTU were also queried against SILVA-LTP (Yilmaz et al., 2014), RDP (Cole et al., 2014) and EMBL-
ENA databases (Leinonen et al., 2011). For 16S rRNA genes classified as chloroplasts taxonomy was assigned using Greengenes database. The relative phylogenetic placement of the Abundant unassigned OTUs was determined using the online tool SINA 1.2.11 (Pruesse et al., 2012) and the SSU/LSU 132 SILVA database (Quast et al., 2013). The closest 20 neighbours for each query sequence were retrieved from the database, and were further used for a phylogenetic tree construction with the Neighbor-Joining method (Saitou and Nei, 1987) in Mega7 (Kumar et al., 2016). The Kimura 2-parameter method (Kimura, 1981) was used in computing the evolutionary distances. The analysis involved 309 nucleotide sequences with a total of 263 positions in the final dataset. The tree was graphically processed in FigTree v1.4.3 (k).

Data analysis

Rarefaction was carried out at a sequencing depth of 38,900 sequences per sample, followed by α- and β-diversity estimation in QIIME. The Pearson's correlations between the physicochemical parameters of the water and the alpha-diversity indices were calculated in R with the cor function of the 'stats' package. These correlations were plotted in R using the corplot function of the corrplot package, significant correlation ($p < 0.05$) being selected with an in-home function. Euclidean distances for physicochemical parameters were computed with the vegdist function of the 'vegan' package in R, and the Mantel test (999 permutations) was run to evaluate the environmental impact on the microbial diversity. Data was log transformed prior to analysis. The variance inflation factor was computed for all the physicochemical parameters, and in order to decrease the collinearity of the explanatory variables, those with an inflation factor greater than 10 were not considered for further analyses. For observing the biodiversity variation in time and space according to different environmental physicochemical parameters, a PCoA was generated for the Bray-Curtis distance matrix, using the cmdscale and envfit functions of the 'stats' and 'vegan' packages in R. The environmental factors were fitted onto an ordination with the 'envfit' function of the 'vegan' package in R, being scaled by their correlation to the distance matrix. The significance of the samples grouping according to the season, the depth of the water column and the layer of the lake was evaluated by the analysis
of similarity (ANOSIM) using 999 permutations (Clarke, 1993) in QIIME (Caporaso et al., 2010). Phyla and classes with abundances higher than 1% of the sequencing library for each depth and season were plotted using the inkspot function of the 'rioja' package in R.

**Acknowledgements**

This work was supported by grants of the Romanian National Authority for Scientific Research, CNCS–UEFISCDI (project numbers PN-II-ID-PCE-2011-3-0546 and PN-III-P4-ID-PCE-2016-0303), the Natural Environment Research Council (NERC, UK) (grant no. NE/E016804/1), and the Department of Agriculture, Environment and Rural Affairs (DAERA, Northern Ireland). HLB was additionally supported by STAR-UBB Advanced Fellowship-Intern (Babeş-Bolyai University). We are grateful to Fülöp-Nagy János (Sovata) for the permission to enter the study area. The authors declare that there are no conflicts of interest.

**References**


**Figures Legends**

**Figure 1.** Depth-related profiles and seasonal dynamics of environmental variables in Ursu Lake. The water masses were delineated by a rough estimation based on the observed sharp changes in physical (temperature, PAR) and chemical (pH, salinity, DO) parameters.

**Figure 2.** Distribution of Abundant and Rare OTUs within the three water masses. (A) Rarefaction curves constructed on the observed number of species. Rarefaction curves are presented by depth, as seasonal average of observed species (B) Spatial distribution of shared and unique Abundant OTUs. (C) Seasonal distribution of shared and unique Abundant OTUs. (D) Species distribution patterns of OTU0.03. (E) Spatial distribution of shared and unique Rare OTUs. (F) Seasonal distribution of shared and unique Rare OTUs.
**Figure 3.** Depth-related distribution of prokaryotic community at phylum and classes level based on OTU abundances. All taxa exhibiting abundances >1 % are presented.

**Figure 4.** Seasonal variations in the distribution of abundant phyla and classes based on relative OTU abundances. All taxa exhibiting abundances >1 % are presented.

**Figure 5.** Patterns of alpha and beta diversity for the Ursu Lake prokaryotic community. (A) Correlations of alpha diversity indices with selected environmental variables. (B) Principal coordinates analysis (PCoA) of Bray-Curtis distances for the samples collected during a one year survey of Ursu Lake. Environmental drivers of beta diversity patterns are pictured on the PCoA graphic.

**Figure 6** Putative functional groups inhabiting Ursu Lake. Neighbour-Joining phylogenetic tree indicates the phylogenetic placement of abundant OTUs with low similarity to reference sequences in selected databases.
Figure 1
Figure 2

118x70mm (762 x 762 DPI)
Figure 3

40x29mm (762 x 762 DPI)
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<td>[Saprospirae]</td>
</tr>
<tr>
<td>Actinobacteria</td>
<td>[Rhodotherm]</td>
</tr>
<tr>
<td>Nitriliruptoria</td>
<td>Sphingobacteria</td>
</tr>
<tr>
<td>Unassigned</td>
<td>Flavobacteria</td>
</tr>
<tr>
<td>Bacteroides</td>
<td>Cytophaga</td>
</tr>
<tr>
<td>Euryarchaeota</td>
<td>Bacteroides</td>
</tr>
</tbody>
</table>

**Abundance (%)**

10 25 50

July November February April

Figure 4

43x41mm (762 x 762 DPI)
Figure 5

44x51mm (762 x 762 DPI)
Figure 6