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Winter planktonic microbial communities in highland aquatic habitats

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Abstract

Winter conditions in aquatic habitats of the temperate zone markedly differ from those present in warmer seasons, nevertheless, relatively scarce information is available on planktonic microbial composition, as sites are not easily accessible and it was supposed traditionally that microbial activity is low during this cold period. Since microorganisms could have great impact on the ecosystem even during winter, we explored various sites in the Eastern Carpathians regarding the abundance and taxonomic composition of planktonic microorganisms. Although many of the studied environments were extreme habitats, planktonic microbial communities were abundant and mostly diverse with the presence of previously unidentified taxa.

Introduction

Information on the composition of winter planktonic microbial communities are relatively scarce compared to the literature data dealing with warmer seasons, since sampling sites are not easily accessible or this period of the year was supposed traditionally to have low microbial activity. However, it has been proven that natural aquatic habitats may harbour abundant planktonic communities in the coldest period of the year (Philips and Fawley 2002; Somogyi et al. 2014), surprisingly even blooms of planktonic phototrophs could emerge (Álvarez et al. 2009; Pálffy et al. 2014; Somogyi et al. 2009; Üveges et al. 2012). Since microorganisms of inland waters have a great impact on biogeochemical cycles, may effect water quality and interact with other aquatic organisms, describing the taxonomy and ecology of microbes present in winter is crucial to understand their role in the ecosystem (Bertilsson et al. 2013).

Eastern Carpathians (Romania) harbour diverse aquatic habitats having different hydrological character and origin. In this region, glacial lakes, meromictic, heliothermal and salt lakes or aquatic environments formed due to volcanic or postvolcanic activity, such as crater lakes, CO₂-rich mineral springs, sulphuric bubbling pools and mudpots, are also found (Magyari et al. 2009; Máthé et al. 2014; Szakács 2010; Szakács and Krézsek 2006). Many of these habitats has been studied previously focusing on the origin and formation of these specific environments (e.g. Begy et al. 2011; Magyari et al. 2009), and some of them has been examined in the last years to reveal the structure of planktonic microbial communities during the productive season (e.g. Borsodi et al. 2013; Máthé et al. 2014). Furthermore, most of them are still unexplored regarding the composition of planktonic microorganisms, and therefore our study serves as the first description of these communities. On the other hand, most microbial studies of seasonally frozen lakes were focused on eukaryotes and obtaining

more data from the prokaryotic communities is essential in the case of these habitats (Bertilsson et al. 2013). Therefore, in this study, the composition of planktonic pro- and eukaryotic microbial communities in these specific aquatic habitats were studied in detail during winter.

Materials and methods

Study sites and sample collection

Samples were collected on 7th February 2013 from a crater lake, Lake St. Ana, from Mohoš peat bog lake and from the sulphuric bubbling pools [Timsós (Apor) Baths] (all located in Ciomad Mountains, Harghita County, Romania), and on 9th February 2013 from the saline lakes, Lake Ursu, Lake Verde and Lake Roşu (all located in Gurghiu Mountains, Mureş County, Romania). Geographic location of sampling sites is given in Supplementary Fig. 1, while detailed maps can be found in Magyari et al. (2009) (map of the crater lake and peat bog), in Borsodi et al. (2013) and Máthé et al. (2014) (map of the saline lakes) and in Supplementary Fig. 2 (map of the sulphuric bubbling pools). A view of sampling sites on the date of sampling is presented in Supplementary Fig. 3. General characteristics of sampling sites are summarized in Table 1.

Physico-chemical analyses

Field measurements for the determination of temperature, pH, specific conductance and dissolved oxygen content (DO) were carried out by an XRX-420 CTD+ type multi-parameter submersible instrument (RBR, Kanata, Ontario, Canada). Additional chemical analyses [concentration of NH_4^+ -N, NO_2^- -N, NO_3^- -N, TN, PO_4^{3-} -P, total organic carbon (TOC), total inorganic carbon (TIC), HCO_3^- -C, Fe, SO_4^{2-} -S, H_2S -S] were performed in the laboratory according to Standard Methods (Eaton et al. 2005) with some special considerations given in Borsodi et al. (2013) and Máthé et al. (2014). Salinity values in the g/L range were determined by the direct gravimetric method from known amount of water samples that were evaporated at 105 °C, while values in the mg/L range were calculated from conductivity data according to the equation given in Keresztes et al. (2012). Chromophoric dissolved organic matter (CDOM, as water colour in Pt units) was determined according to Cuthbert and del Giorgio (1992). Photosynthetically active radiation (PAR) within the water column was measured with a LI-COR quantum sensor (2π). From representative depths, chlorophyll *a* (Chl *a*) concentration was determined spectrophotometrically after hot methanol extraction using the absorption coefficients determined by Wellburn (1994). In Lake Ursu, bacteriochlorophyll *a* (Bchl *a*) and *c* (Bchl *c*) were also determined at different depths according to Biel (1986) and Castenholz (1973), respectively. *In vivo* absorption spectra of the phototrophic communities were also recorded according to Castenholz et al. (1973).

Microscopic analyses

For total bacterial cell counts determination, sample aliquots were fixed with paraformaldehyde solution, stained with DAPI and analysed with epifluorescence microscopy according to the method of Porter and Feig (1980) described in detail by Máthé et al. (2014).

Abundance of picocyanobacteria and picoeukaryotic algae was determined according to MacIsaac and Stockner (1993), while that of larger algae were determined with inverted microscopy (Utermöhl 1958). Abundance values were converted to biomass using average cell dimensions and considering an average cell density of 1 g cm^{-3} .

Community composition analysis with DGGE coupled with sequencing

Methods used in the analysis of the bacterial community composition are described in detail in Borsodi et al. (2013) and Felföldi et al. (2009). Briefly, after the extraction of genomic DNA, group-specific PCRs were carried out to amplify partial 16S rRNA gene fragments from Archaea, Bacteria and Cyanobacteria (including also the chloroplast of eukaryotic algae). DGGE runs were performed with 40-70% of denaturants in a 7% polyacrylamide gel, subsequently gel blocks were excised from major bands, which were subjected to DNA extraction and sequence analysis (unfortunately some bands resulted in mixed sequences). Phyllospecies identification was carried out using the EzTaxon-e and GenBank databases (Altschul et al. 1997; Kim et al. 2012). The obtained sequences were submitted to GenBank under the accession numbers KF515277-KF515318.

Results and discussion

Crater lake (Lake St. Ana)

The water column of Lake St. Ana was homogenous, slightly acidic (pH 5.2-5.5) and very clear at the sampling date (Fig. 1A), transparent to the bottom (6 m) with a K_d value of 1.18 m^{-1} and a turbidity value of ~ 0.7 NTU (Table 1 and 2). The lake was covered with a 35 cm thick ice and a 20 cm thick snow, which decreased the ambient PAR by 88% on average. Low ion (specific conductance $\sim 20 \mu S cm^{-1}$), moderate nitrogen (inorganic nitrogen forms $\sim 0.3 mg L^{-1}$, TN $\sim 0.7 mg L^{-1}$), phosphorous ($PO_4^{3-}-P \leq 0.01 mg L^{-1}$) and organic carbon content (TOC $\sim 4.5 mg L^{-1}$) was measured in the lake. Based on the microscopic analysis, the phytoplankton was composed of mainly small cryptophytes (*Cryptomonas* sp., which was also detected by DGGE analysis) (Table 3), however, at the surface layers dinophytes (*Peridinium inconspicuum*) were also detected in a great number (constituting 66% of the total algal biomass). Picocyanobacteria and picoeukaryotic algae were completely absent, however, the latter could be a predominant member of phytoplankton in various water types during winter (Somogyi et al. 2009; Vörös et al. 2009; Weisse 1993). The total absence of the smallest photosynthetic fraction from the plankton of Lake St. Ana is a unique feature of the lake and cannot be easily explained.

The bacterial community based on the DGGE analysis was rather homogenous, however, the archaeal planktonic community at the bottom differed markedly from those of the upper water layers, and in the case of the Cyanobacteria- and chloroplast-specific analysis the pattern of the upper layer was also dissimilar (Fig. 2). The only archaeal phylotype retrieved from this lake belonged to the phylum Thaumarchaeota, while bacterial sequences belonged to the genera *Prostheco bacter* and *Gluconobacter* (Table 3), and both were present in all three studied layers (Fig. 2). Species belonging to *Prostheco bacter* can grow at very low temperature values (down to 1-10 °C), are obligately aerobic and saccharolytic (Krieg et al. 2010), and can be found in freshwater environments (Krieg et al. 2010; Takeda et al. 2008). The recently described species, *Prostheco bacter algae*, was isolated on an agar

medium containing algal metabolites (Lee et al. 2014), therefore, it is possible that the phylotype detected in Lake St. Ana could effectively use dissolved organic matter that was previously released from planktonic algae (*Cryptomonas* and *Peridinium* spp.). The other genus detected by DGGE analysis, *Gluconobacter*, is also an obligately aerobic bacterium, and strains belonging to this genus are similarly able to grow on various carbohydrates (Brenner et al. 2005).

Humic lake (Mohoş peat bog lake)

The peat bog lake was covered by a 4-15 cm thick ice and had high CDOM concentration (599 mg Pt L^{-1}), which caused high light attenuation within the water column with a K_d value of 8.55 m^{-1} . High surface Chl *a* concentration was measured ($81 \mu\text{g L}^{-1}$) that was coupled with the low dissolved oxygen content of the water (0.45, 0.26 and 0.05 mg L^{-1} , which were equal to a saturation of 3.2, 1.8 and 0.3%, at 0.1, 0.4 and 1.0 m water depth, respectively). The low oxygen saturation suggested the absence of the net phytoplankton photosynthesis, possibly because of the low light conditions. Nevertheless, several algae were detected by microscopic analysis, taxa which are well-known inhabitants of cold waters (e.g. Babanazarova et al. 2013; Bertilsson et al. 2013): *Euglena proxima*, *Astasia* sp. (both Euglenophyta), *Chlamydomonas microscopica* (Chlorophyta), *Rhodomonas* sp. (Cryptophyta) and others, representing the biomass of 14000, 2800, 26, 5 and $18 \mu\text{g L}^{-1}$, respectively. Euglenoids are characteristic in small lakes with high organic matter content, while *Chlamydomonas* species were reported to maintain high population densities even in ice-covered humic lakes; both taxa are frequent in small acidic lakes (Reynolds 2006). The detected phytoplankton members are able to grow on different organic carbon sources (Perez-

Garcia et al. 2011), which could contribute to their survival in such light-limited, but organic matter rich habitats.

The dominant phototrophic phylotype was related to *Parachlorella kessleri* (formerly known as *Chlorella kessleri*, Krienitz et al. 2004) based on the sequence analysis of DGGE bands (Fig. 2, Table 3). Cells of this coccoid green algal species are 2.5 to 9 μm in diameter in the laboratory cultures (Yamamoto et al. 2005; Juárez et al. 2011), and therefore usually larger than the pico-size range. The geographical distribution and habitat preference of this species is not well-known, however, recently Juárez et al. (2011) isolated a *Parachlorella kessleri* strain from a volcanic mesothermal sulphurous pond (Laguna Verde, Argentina), which contains free sulphuric acid and has a characteristic pH between 2.5 and 2.8. Therefore, the detection of *Parachlorella* in an acidic peat bog lake (pH 3.95) is not surprising, but adds important data on the occurrence of this chlorophyte species in extreme environments. Furthermore, picocyanobacterial strains belonging to the *Cyanobium gracile* cluster were isolated recently from humic lakes with pH 4.6 and 4.9 in Poland (Jasser et al. 2013). Small-sized coccoid algae have ecological advantage in water bodies with high turbidity or CDOM content, due to the high surface-to-volume ratio of cells and more effective light harvesting machinery compared to larger cells in light-limited environments (Raven 1997; Somogyi et al. 2010). However, pico-sized members of the photoautotrophic plankton were not observed by epifluorescence microscopy.

Furthermore, based on the sequence analysis of excised DGGE bands, one archaeal species *Methanosaeta harundinacea* and one bacterium species, *Polynucleobacter necessarius* were identified (Table 3). The latter species is a chemo-organotrophic bacterium with growth temperature down to 5 °C and was reported from a broad variety of freshwater habitats (including humic-rich environments) with 20-60% relative abundance of total bacteria (Hahn et al. 2009; Pernthaler 2013), and according to the intensity of the DGGE

band (Fig. 2), this species was an important component of the bacterioplankton in the studied Mohoš peat bog lake. The presence of a strictly anaerobic methanogenic archaeon, *Methanosaeta* (Ma et al. 2006), corresponded well with the low dissolved oxygen content of the water, however, bulk methanogenic activity within the lake was most probably located in the deeper anoxic zones or in the sediment (Bertilsson et al. 2013; Pernthaler, 2013). *Methanosaeta* could be the dominant methanogenic archaeon in permanently cold environments (e.g. Antarctic sediments), and since psychrophilic methanogens were reported to have their maximal methane production rates at 6 °C (Fuchs et al. 2013), the contribution of winter methanogenesis to the release of methane to the atmosphere (which as a greenhouse gas implicates possible climate influence) could be presumed in the study site. However, the produced methane is not necessarily released, since it could be oxidized to carbon-dioxide within the lake, even in the anaerobic regions (Bertilsson et al. 2013; Pernthaler 2013).

Sulphuric bubbling pools [Timsós (Apor) Baths]

Only one of three investigated sulphuric bubbling pools had ice cover at the time of sampling (Apor 2) (Table 1, Supplementary Fig. 3), the ones uncovered had slightly higher temperature value (0.3, 2.6 and 2.6 °C in Apor 2, 4 and 6, respectively), probably due to the intensive bubbling and the warming effect of the upwelling gas. We hypothesize that this gas had high CO or CO₂ content that was indicated by the high inorganic carbon content (~400 mg L⁻¹) of the water (Table 2). On the other hand, moderate amount of oxygen was also detected (3.33, 8.14, 4.71 mg L⁻¹ which were equal to 23.3, 60.5 and 35.1% saturation, respectively in pools Apor 2, 4 and 6). All sites had high ion (>2000 µS cm⁻¹ specific conductance) and sulphate concentration (109-273 mg L⁻¹) and acidic pH (2.4-4.8), this latter resulted in the low hydrogen carbonate content of the water (Wetzel 2001). These pools also contained moderate

or high amount (depending on the pool) of whitish sediment at the bottom (Supplementary Fig. 3F), which could be easily stirred up, most probably sulphur compounds or precipitated carbonates.

Probably the combination of these extremities led to the almost complete lack of phototrophic microorganisms in these habitats, phototrophic cells and chlorophyll *a* were not detectable by traditional methods (Table 2). However, by DNA based analysis *Navicula* (Bacillariophyta) and *Arthrospira* (formerly known as *Spirulina*, Cyanobacteria) phylotypes were retrieved from these environments (Table 3). Many diatoms, such as the members of the genus *Navicula* are well-known for tolerating harsh environmental conditions and could be found in many cold and extreme habitats (Seckbach 2007). Although the common view is that cyanobacteria are seldom or even absent from acidic environments such as our sampling sites (Seckbach 2007), there are some reports on that *Spirulina*-like filamentous cyanobacteria could be present in lakes with pH ~3 (Steinberg et al. 1998). However, the most possible explanation for the presence of algal DNA in the sulphuric bubbling pools is an allochthonous origin: surrounding habitats, where filamentous blue-green algae or diatoms could be found, e.g. tree leaves, barks, lichens; or the stone rim of these pools (Round 1981) (Supplementary Fig. 3EF).

The single, but dominant archaeal phylotype based on the DGGE analysis (Fig. 2) belonged to *Ferroplasma acidiphilum* (Table 3), which is an aerobic, autotrophic (cf. the high inorganic carbon content of these pools, ~400 mg L⁻¹) (Table 2), Fe²⁺-oxidizing archaeon with a pH optimum of 1.7 (Golyshina et al. 2000). The predominant bacterial phylotype in the sites having the lowest pH (Apor 2 and 6) (Table 2, Fig. 2) was *Acidithiobacillus ferrooxidans* (Table 3), an acidophilic and obligately autotrophic species, which could oxidize iron and various sulphur compounds with a pH optimum around 2.5 (Brenner et al. 2005; Hedrich and Johnson 2013). However, *Metallibacterium scheffleri* was detected in only

one site (Apor 2) (Table 2, Fig. 2), which is an acid-tolerant heterotrophic bacterium, capable for iron reduction (Ziegler et al. 2013). Therefore, a cycle with iron-oxidizing and iron-reducing bacteria were detected in this environment, although their activity could be very low during winter, since both were reported to be a mesophilic species and does not show growth at 4 °C (Brenner et al. 2005; Ziegler et al. 2013).

Saline lakes (Lake Ursu, Lake Verde and Lake Roşu)

In Lake Ursu, a deep chlorophyll maximum was detected at approximately 3.0 m depth. An intensive green coloured layer was visible to the naked eye from 2.97 to 3.17 m when a water column was taken out with a special transparent tube sampler apparatus (Márialigeti et al. 2014). The K_d value of the upper 2.5 m water column was 1.26 m^{-1} . At the deeper layers, the attenuation increased rapidly: between 2.5 and 2.75 m, the K_d was 3.16 m^{-1} ; between 2.75 and 3.0 m, it increased to 25.5 m^{-1} . As a result, extremely light-limited conditions were present in the green coloured layer [at 2.75 m depth, only 2% of the surface PAR was detected (Fig. 3)].

Depth-specific conversion between the two dominant pigments, Chl *a* and Bchl *c*, was observed in the Lake Ursu water column with *in vivo* spectrum analysis (Fig. 4) at the absorption maximum of the two pigments, at 675-680 and 740 nm, respectively (Castenholz 1973). Chl *a* dominated exclusively from the surface to around 2.0 m depth with values between 13 and $26 \mu\text{g L}^{-1}$ (Table 2). The characteristic peak of Bchl *c* was observable from 2.5 m, and this latter pigment became the major type at 3.0 m depth, where the aforementioned green coloured layer could also be recognized visually. Assuming that at 3.0 m, the 'Chl *a* + Bchl *c*' content of the methanolic extract (in this case, curves of the two pigment overlap with a maximum at the red region at 663 and 667 nm, respectively; Castenholz 1973) was composed of only Bchl *c*, the Bchl *c* content was ninety times higher

than the Chl *a* concentration at 2.5 m (47.3 compared to 4233 $\mu\text{g L}^{-1}$). Besides this, moderate amount of Bchl *a* was also detected at 2.75 m and 3.0 m depth (10 and 264 $\mu\text{g L}^{-1}$, respectively), which pigment had a slightly visible peak maximum at ~ 805 nm in the *in vivo* absorption curves (Fig. 4). Based on these results, aerobic photoautotrophic microorganisms dominated the upper 2.7 m layer of Lake Ursu, but below that anaerobic phototrophs were present in a huge amount.

Two distinct *Cryptomonas* sp. dominated the phytoplankton in the upper zone of Lake Ursu, with decreasing total biomass in the function of depth (804, 435, 54 and 35 $\mu\text{g L}^{-1}$ in depth 0.5, 1.5, 2.0 and 2.5 m, and were absent at depth 2.75 m and below). Cryptophytes were also detected by the sequence analysis of the excised DGGE bands from this zone (Fig. 2, Table 2), and were reported to be characteristic in lakes during winter, even at low-light conditions (Bertilsson et al. 2013). In the deeper aerobic zone, having a peak at 2.75 m, another group of photosynthetic microorganisms, the photoautotrophic picophytoplankton (picocyanobacteria and picoeukaryotic algae) dominated the planktonic community in Lake Ursu (Fig. 3). The abundance of picoeukaryotic algae continuously increased with the depth from 0.3×10^5 cells mL^{-1} (at 0.5 m) to 5.2×10^5 cells mL^{-1} (at 2.75 m). The same trend was observable for phycocyanin-rich picocyanobacteria, but with considerably lower abundance values (0.005 - 1.1×10^5 cells mL^{-1}). Phycoerithrin-rich picocyanobacteria were completely absent, as it was reported in the case of other saline eutrophic lakes in the same region (Somogyi et al. 2014). In Lake Ursu, the deepest, picoplankton-rich aerobic layer (around 2.5-2.7 m depth) had higher salinity (11.9-18.9%) compared to the uppermost layer of the lake (6.8% at 0.5 m) (Fig. 1B, Table 2). Former studies (Schapira et al. 2010; Somogyi et al. 2014) reported that eukaryotic algae are the dominant in the picophytoplankton of saline environments between salinities 5 and 13%, which corresponded well with our observations.

Surprisingly, in the upper anaerobic part of the water column, picophytoplankton were also found in significant number (Fig. 3): the abundance of picoeukaryotes was 4.8×10^5 cells mL^{-1} and 17.3×10^5 cells mL^{-1} at 3.0 and 3.5 m, while that of picocyanobacteria was 0.92×10^5 cells mL^{-1} and 0.66×10^5 cells mL^{-1} at 3.0 and 3.5 m depths, respectively. The anaerobic milieu, high sulphide content and no available light suggest that sinking of cells generated these high abundance values. Sinking of particulate organic matter was observed in this lake, since partially degraded leaves and other organic debris were visible several times below 3 m depth (István Máthé and Károly Márialigeti, unpublished data), most probably due to the dramatic increase in salinity-caused density values. Presence of small-sized phototrophic cells in the anoxic monimolimnion of other saline lakes in the same region was reported recently (Somogyi et al. 2014), and the same sinking mechanism was hypothesized for occurrence of a significant picocyanobacterial population in the anoxic, sulphidic water layers of the hypersaline Mono Lake, California (Budinoff and Hollibaugh 2007).

Unfortunately, in this study we failed to identify members of picophytoplankton taxonomically with DGGE, e.g. these phylotypes represented bands from which sequence analysis was not successful or due to the low copy number of the ribosomal operon in the genomic DNA compared with larger eukaryotic algae (Shi et al. 2011). Samples taken on different sampling dates (spring 2009 and 2010) from Lake Ursu contained picocyanobacteria belonging to the marine *Synechococcus* clade (Anikó Mentés and Tamás Felföldi, unpublished results), while pico-sized eukaryotic algae were represented by the genus *Picochlorum* (Máthé et al. 2014). The green layer was dominated by the strictly anaerobic phototrophic green sulphur bacterium, *Prosthecochloris* (Fig. 2, Table 3), which could use reduced sulphur compounds as electron donor, and has the characteristic pigment Bchl *c* (Imhoff 2003), as it was detected in the *in vivo* absorption spectrum of water taken from 3.0 m depth (Fig. 4).

Our study has highlighted that a complex interaction of various factors (temperature, light intensity, oxygen and sulphide concentration, salinity, sinking of cells, etc.) determined the actual recorded distribution and abundance of phototrophic microorganisms in the stratified, heliothermal, saline and deep Lake Ursu.

Contrary to Lake Ursu, in the shallow, small Lake Verde and Lake Roşu, phytoplankton consisted of two *Dunaliella* sp. with total biomass values of 1144 and 663 $\mu\text{g L}^{-1}$, respectively. This genus is a well-known planktonic alga of various saline aquatic environments (Seckbach 2007) and is characteristic in other salt lakes in this region (Keresztes et al. 2012; Somogyi et al. 2014), but surprisingly was not present in Lake Ursu, although all three lakes are directly connected with small channels (Máthé et al. 2014). Characteristic differences were also found between the bacterial communities of the upper zone of Lake Ursu and that of Lake Verde and Lake Roşu (Fig. 2), since the latter lakes were dominated by *Marinobacter psychrophilus* (Gammaproteobacteria), while Lake Ursu by *Albidiferax ferrireducens* and *Polaromonas glacialis* (Betaproteobacteria). Nevertheless, *Marinobacter psychrophilus* and *Polaromonas glacialis* have similar properties, both are psychrophilic (could grow even at 1 °C), aerobic, heterotrophic bacteria, which are able to grow in the presence of NaCl (Margesin et al. 2012; Zhang et al. 2008). However, *Albidiferax ferrireducens* is also heterotrophic, but only psychrotolerant (shows significant growth even at 4 °C with optimum growth temperature at ~25 °C) and a facultatively anaerobic species, which could use nitrate or ferric ion as electron acceptor for anaerobic growth (Finneran et al. 2003; Ramana and Sasikala 2009). Therefore, all three species could be active members of the bacterioplankton even in winter. Former studies of Lake Ursu (Máthé et al. 2014) and Lake Roşu (Borsodi et al. 2013) confirmed the importance of other bacteria in these saline lakes, members of the genera *Halomonas*, *Psychroflexus* and *Pseudoalteromonas* were dominant during spring 2009 in the planktonic microbial communities. None of them were

detected in the winter samples analysed in this study, however it is worth to mention that some of the excised DGGE bands resulted in mixed sequences, therefore some potentially important planktonic bacteria were not identified.

Most of the retrieved archaeal genotypes were affiliated with the order Halobacteriales, which are inhabitants of various saline environments and are aerobic heterotrophs, while some of them are capable for anaerobic growth, e.g. with nitrate or fumarate as electron acceptor (Oren 2006), as possible in the deeper zones of Lake Ursu (Fig. 2, Table 3). The detected phylotypes were distantly related to validly described species with a common occurrence of the same Halopelagius-related genotype in all three studied saline lakes [(Fig. 2, Table 3) and based on former studies (Borsodi et al. 2013; Máthé et al. 2014), contributed also significantly to the archaeal communities in Lake Ursu and Lake Roşu in spring]. Besides this, the studied environments could be the potential source of other new prokaryotic species, since many of the detected genotypes were distantly related to validly described species and their closely related sequences were exclusively uncultured (e.g. the dominant phylotypes in the deep anaerobic zone of Lake Ursu, B13 and B14) (Fig. 2, Table 3).

Planktonic microbial communities of the studied aquatic habitats in the winter environment

Compared to the warmer seasons, in general, aquatic habitats of the temperate zone have two main distinguishing features: they receive less solar radiation (number of sunshine hours and sunlight intensity are lower, ice and snow cover further hinders light transmission) and has lower water temperature values. However, concentration of nutrients could be present at relatively high level (Table 2), which supports the growth of planktonic organisms.

Ice cover has fundamental impact on the physico-chemical properties of the under-ice environment, since it prevents atmospheric input of particulate matter and gas exchange, hinders heat loss and wind shear thus lowers circulation and the exchange with the surrounding terrestrial habitats; furthermore, the low solar radiation is further decreased, especially in the case of the presence of a snow cover (Bertilsson et al. 2013). These circumstances create markedly different environment for the phototrophic members of the plankton compared to those present in warmer seasons of the year. PAR was lowered by almost 90% in the case of Lake St. Ana in our study, where both ice and snow were present at the date of sampling. Additionally, in the case of the Mohoş peat bog lake, the high CDOM content further lowered the available light for photosynthesis. Therefore, algae may decrease their photosynthetic activity or survive this low-light and low-temperature environment in a dormant form even in the water column (like dormant cells of *Peridinium inconspicuum* in Lake St. Ana) or supply their requirements in a heterotrophic lifestyle (like possibly some of the genera detected in Mohoş peat bog lake). Humic substances of peat bog lakes could be decomposed by prokaryotes to smaller molecules, e.g. to acetate, which could be a good substrate for heterotrophic algal growth (Perez-Garcia et al. 2011) in the aerobic zone, and for the methanogenic archaea in the deep anaerobic zones (Ma et al. 2006). However, it should be mentioned that, prokaryotic species both could be the competitors of eukaryotic primary producers for nutrients and depend on the organic carbon produced by them (Pernthaler 2013); for the latter, *Prostheco bacter* detected in Lake St. Ana could be an example. Prosthecae on the cells may serve not only for the reduction of sinking but for facilitating nutrient uptake in oligotrophic environments through increasing the surface-to-volume ratio of cells (Madigan et al. 2012). Furthermore, mixotrophic phytoplankton can account for a significant removal of bacterial cells in lakes (Bertilsson et al. 2013). Additionally, motile cells (such as *Cryptomonas* in Lake St. Ana and Lake Ursu) may have selective advantage

during winter, since they can compensate sinking and find patches of nutrients (Bertilsson et al. 2013).

Under the ice, special stratification may evolve during winter, and due to the lack of the mixing effect of wind, density is the key factor that determines this process. However, in the case of Lake Ursu, salinity determines stratification, including the measured differences of temperature values. Such stratification of many physicochemical parameters determines the vertical distribution of planktonic microorganisms, as it was observed for the phototropic community within a 1-m-wide zone (Fig. 3), or in general, comparing the whole bacterial and archaeal communities of different depths (Fig. 2).

A recent study of Baricz et al. (2014) focusing on the prokaryotic communities of the saline Lake Ocnei (which is, similarly to Lake Ursu, also a heliothermal lake) showed that members of the domains Archaea and Bacteria were present there usually in similar cell densities, with abundance values $\sim 10^7$ cells mL⁻¹. In March and April 2009, total cell counts were $\sim 10^6$ – 10^7 cells in Lake Ursu (Máthé et al. 2014), as the values detected for the winter plankton in this study (Table 2), which indicated that, at least regarding their abundance, the winter planktonic community could be very similar to those of the warmer periods of the year. However, in general, pelagic microbial biomass is usually lower in ice-covered than in the warmer periods of the year (Bertilsson et al. 2013). It should be noted that Lake Ursu had a special thermal regime during winter with maximal temperature ~ 21 – 23 °C in the hypolimnion, and the layer above this zone may serve as a temperature refugium for the spring planktonic community (Table 2).

Metabolic activities could be also significantly different among the seasons. As temperature decreases during autumn and winter, cells adapted to the warmer period could enter to a dormant state (which is not always coupled with obvious morphological changes), which allows to become resistant to cold environments and to survive with a minimal

metabolic rate (Fuchs et al. 2013). During this study, both potentially actively growing and inactive (or dead) microorganisms were detected; *Prosthecobacter* in Lake St. Ana, *Marinobacter* and *Polaromonas* in Lake Ursu are examples for the first type, while *Parachlorella* in Mohoš peat bog lake and *Peridinium* in Lake St. Ana for the second one. In general, the turnover of bacterial biomass is reported to be surprisingly high, 4-10 days, in seasonally frozen lakes during winter (Bertilsson et al. 2013), which contradicts with traditional view that in this time microorganisms could be characterized with a rather low metabolic activity.

Furthermore, many of the studied sites are extreme environments (e.g. low pH, high salinity), which could be characterized with special trophic interactions. Recently Somogyi et al. (2014) studied eight lakes of the same Salt Region in Transylvania, where our studied salt lakes are also located, and found that heterotrophic nanoflagellates, the main grazers of picocyanobacteria and picoeukaryotic algae, were completely absent there contrary to some other hypersaline habitats (e.g. Park et al. 2003). On the other hand, brine shrimp (*Artemia* sp.) could be found in high abundance during the warmer periods of the year in these lakes (Borsodi et al. 2013; Ionescu et al. 1998; Máthé et al. 2014), however, according to our best knowledge, members of winter food web has not been identified previously in these habitats. The top down control of planktonic primary production could be slight or even absent in some of the studied environments, since multicellular eukaryotes are known to tolerate less the extremities compared to microorganisms (Rothschild and Mancinelli 2001). Therefore, the special environmental conditions associated with the winter season coupled with the unknown effect of grazers provides special circumstances for the planktonic microorganisms in these aquatic habitats, which could be an interesting topic for future investigations. Further studies may also help to reveal the causes of unusual phenomena observed during this winter

survey: complete lack of picophytoplankton in Lake St. Ana and Mohoš peat bog lake, and lack of *Dunaliella* sp. in Lake Ursu.

Conflict of interest

The authors declare that they have no conflict of interest.

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TABLE AND FIGURE LEGENDS

Table 1 General and on-site measured characteristics of sampling sites

Table 2 Chemical and biological characteristics of samples

Table 3 Identified sequences retrieved from excised bands of various group-specific DGGEs (shown in Fig. 2) from winter water samples from the Eastern Carpathians

Fig. 1 Depth profiles of major physicochemical variables in **(a)** Lake St. Ana on 7th February 2013 and **(b)** in Lake Ursu on 9th February 2013. The reduction fold of original values are shown in brackets

Fig. 2 DGGE patterns of winter planktonic microbial communities in the aquatic habitats of the Eastern Carpathians examined with different group-specific analyses. Arrowheads mark excised bands (results of sequence analysis of the reamplified DNA is shown in Table 3)

Fig. 3 Depth distribution of total microbial cell count, picophytoplankton, photosynthetically active radiation (PAR) and chromophoric dissolved organic matter (Pt colour) in the upper 3.5 m layer of Lake Ursu on 9th February 2013. P-Euk – picoeukaryotic algae; P-Cya – picocyanobacteria

Fig. 4 *In vivo* absorption spectra of Lake Ursu water samples taken from different depths on 9th February 2013

TABLES

Table 1 General and on-site measured characteristics of sampling sites.

Name	L. St. Ana	Mohoş	Apor 2	Apor 4	Apor 6	L. Ursu	L. Verde	L. Roşu
Type	crater lake	peat bog lake	sulphuric bubbling pool	sulphuric bubbling pool	sulphuric bubbling pool	deep, heliothermal, meromictic saline lake	shallow saline lake	shallow saline lake
Altitude (m, a.s.l.) ^a	950	1050	930	932	934	502	505	505
GPS coordinates (sampling point)	N 46.12552 E 25.88744	N 46.13603 E 25.90145	N 46.11465 E 25.94965	N 46.11474 E 025.94968	N 46.11501 E 025.94965	N 46.6035 E 25.08539	N 46.3621 E 25.0558	N 46.3623 E 25.0520
Max. water depth (m)	6.0 ^b	1.2 ^c	0.25 ^b	0.15 ^b	0.67 ^b	17.18 ^b	1.20 ^a	2.10 ^a
Surface area (m ²)	193,000 ^a	212.7 ^a	10.37 ^b	0.18 ^b	4.33 ^b	41,270 ^a	291 ^a	1406 ^a
Volume (m ³)	250,00 ^a	1298 ^a	2.59 ^b	0.015 ^b	2.90 ^b	262470 ^a	159 ^a	1163 ^a
Ice thickness (cm) ^b	35	4-15	0-2	0	0	2	0	0
Snow cover (cm)	20	2-8	0-5	0	0	0-1	0	0
Secchi depth (cm) ^b	tr	18	tr	tr	tr	196	n.m.	n.m.

^a based on the data given in: Magyari et al. (2009); Alexe (2010); Diaconu and Mailat (2010); Romanescu et al. (2010); Begy et al. (2011); Borsodi et al. (2013); Máthé et al. (2014)

^b measured in this study during sample collection

^c above the underwater decaying *Sphagnum* layer

n.m. – not measured; tr – transparent water to the bottom

Table 2 Chemical and biological characteristics of samples

sampling site	L. St. Ana			Mohoş	Apor 2	Apor 4	Apor 6	L. Ursu								L. Verde	L. Roşu
sampling depth (m)	0.4	2.5	5.5	0.4	0.1	0.1	0.1	0.1	0.5	1.5	2.7	3.0	3.5	5.0	8.0	0.1	0.1
temperature (°C)	1.7	4.5	4.1	0.6	0.3	2.6	2.6	1.4	10.7	12.2	17.1	19.4	21.4	23.0	22.3	1.6	1.3
spec. el. cond. ($\mu\text{S cm}^{-1}$)	19	23	19	109	2392	2091	2495	46,200	95,000	98,400	154,700	194,300	233,700	239,100	242,600	110,000	156,900
pH	5.23	5.20	5.53	3.95	2.93	4.77	2.41	8.97	8.58	8.33	7.37	7.10	6.26	6.25	6.22	7.55	7.85
salinity (mg L ⁻¹)	8.1	9.8	8.0	45.5	1,000	870	1,040	4,800	69,700	73,200	147,800	217,800	301,700	314,300	322,600	86,200	151,300
NH ₄ ⁺ -N (mg L ⁻¹)	0.08	<0.01	<0.01	<0.01 ^a	1.50	1.69	1.14	<0.01	0.05	0.02	17.0	n.m.	28.5	32.9	28.4	2.80	2.49
NO ₂ ⁻ -N (mg L ⁻¹)	<0.01	<0.01	<0.01	0.02 ^a	0.01	0.02	0.01	<0.01	0.03	0.15	<0.01	n.m.	<0.01	0.01	<0.01	0.05	0.05
NO ₃ ⁻ -N (mg L ⁻¹)	0.25	0.23	0.23	n.m.	1.8	1.7	1.9	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.	n.m.
TN (mg L ⁻¹)	0.7	0.6	0.8	3.2	5.5	5.6	3.1	1.9	2.1	2.4	20.9	n.m.	32.8	46.2	28.4	6.9	5.3
PO ₄ ³⁻ -P (mg L ⁻¹)	0.01	0.01	<0.01	0.04 ^a	1.9	0.19	0.36	0.13	0.09	0.15	4.5	n.m.	4.3	3.9	3.2	0.04	0.04
TOC (mg L ⁻¹)	4.5	4.5	4.7	73	14.6	14.3	16.5	1.2	7.3	<0.1	45.7	n.m.	20.9	54.4	14.9	21.2	19.3
DOC (Pt colour, mg L ⁻¹)	3.15	n.m.	n.m.	599	n.m.	n.m.	n.m.	n.m.	15.7	19.1	61.8	284	72.8	n.m.	n.m.	n.m.	n.m.
TIC (mg L ⁻¹)	0.3	0.6	0.5	<0.1	422	414	384	10	72	58	173	n.m.	240	202	202	29	24
HCO ₃ ⁻ -C (mg L ⁻¹)	n.m.	n.m.	n.m.	<7	<7	<7	<7	11	84	67	201	n.m.	280	235	235	33	28
Fe (mg L ⁻¹)	0.10	0.12	0.09	0.62 ^a	1.16	0.46	0.50	0.07	0.05	0.05	<0.01	n.m.	0.62	0.45	0.27	0.15	0.16
SO ₄ ²⁻ -S (mg L ⁻¹)	1.0	1.3	<0.01	<0.1 ^a	273	172	109	6.7	70	92	270	n.m.	394	406	457	46	47
H ₂ S-S (mg L ⁻¹)	n.m.	n.m.	n.m.	n.m.	27	52	0	11	23	n.m.	81	n.m.	247	222	207	132	43
Chl <i>a</i> ($\mu\text{g L}^{-1}$)	1.8	1.9	1.1	81.3	<0.5	<0.5	<0.5	n.m.	13.2	24.7	405 ^b	5440 ^b	142.3	n.m.	n.m.	n.m.	n.m.
total bacterial cell count (10 ⁵ cells mL ⁻¹)	13.9	9.11	8.06	21.9	2.23	3.24	31.8	49.4	278	235	n.m.	538	289	259	163	85.3	77.4

^a intensive water color (i.e. high concentration of dissolved organic material) may alter the results of photometric detection

^b the majority is Bchl *c* according to the *in vivo* absorbance spectrum

n.m. – not measured

Table 3 Identified sequences retrieved from excised bands of various group-specific DGGEs (shown in Fig. 2) from winter water samples from the Eastern Carpathians

Code (Acc. No.)	Sampling site	Closest species ^a [Major taxonomic group]	Similarity (%)	Sequence length (nt)
Archaea				
A1 (KF515277)	L. St. Ana	<i>(Nitrososphaera gargensis)</i> [Thaumarchaeota/Nitrososphaerales]	88.2	408
A2 (KF515278)	Mohoş	<i>Methanosaeta harundinacea</i> [Euryarchaeota/Methanosarcinales]	98.9	348
A3 (KF515279)	Mohoş	<i>Methanosaeta harundinacea</i> [Euryarchaeota/Methanosarcinales]	99.0	410
A4 (KF515280)	Apor 4	<i>Ferroplasma acidiphilum</i> [Euryarchaeota/Thermoplasmatales]	100	325
A5 (KF515281)	L. Ursu	<i>(Nitrososphaera gargensis)</i> [Thaumarchaeota]	83.3	216
A6 (KF515282)	L. Ursu	<i>(Halopelagius longus)</i> [Euryarchaeota/Halobacteriales]	93.7	458
A7 (KF515283)	L. Ursu	<i>(Halopelagius longus)</i> [Euryarchaeota/Halobacteriales]	93.8	483
A8 (KF515284)	L. Ursu	<i>(Salarchaeum japonicum)</i> [Euryarchaeota/Halobacteriales]	93.5	384
A9 (KF515285)	L. Ursu	<i>(Salarchaeum japonicum)</i> [Euryarchaeota/Halobacteriales]	94.1	410
A10 (KF515286)	L. Roşu	<i>(Halopelagius longus)</i> [Euryarchaeota/Halobacteriales]	93.8	483
A11 (KF515287)	L. Roşu	<i>(Halopelagius longus)</i> [Euryarchaeota/Halobacteriales]	93.2	381
Bacteria				
B1 (KF515288)	L. St. Ana	<i>Prostheco bacter (vanneervanii)</i> [Verrucomicrobia/Verrucomicrobiales]	96.8	156
B2 (KF515289)	L. St. Ana	<i>Gluconobacter (roseus/oxydans)</i> [Alphaproteobacteria/Rhodospirillales]	95.3	403
B3 (KF515290)	Mohoş	<i>Polynucleobacter necessarius</i> [Betaproteobacteria/Burkholderiales]	100	395
B4 (KF515291)	Mohoş	<i>(Acidomonas methanolica)</i> [Alphaproteobacteria/Rhodospirillales]	93.2	385
B5 (KF515292)	Apor 2	<i>Metallibacterium scheffleri</i> [Gammaproteobacteria/Xanthomonadales]	100	155
B6 (KF515293)	Apor 6	<i>Acidithiobacillus ferrooxidans</i> [Gammaproteobacteria/Acidithiobacillales]	100	444

B7 (KF515294)	L. Ursu	<i>Albidiferax ferrireducens</i> [Betaproteobacteria/Burkholderiales]	98.4	450
B8 (KF515295)	L. Ursu	<i>Polaromonas glacialis</i> [Betaproteobacteria/Burkholderiales]	99.3	153
B9 (KF515296)	L. Ursu	<i>Prosthecochloris vibrioformis</i> [Chlorobi/Chlorobiales]	98.3	408
B10 (KF515297)	L. Ursu	<i>Prosthecochloris vibrioformis</i> [Chlorobi/Chlorobiales]	97.3	441
B11 (KF515298)	L. Ursu	<i>Prosthecochloris vibrioformis</i> [Chlorobi/Chlorobiales]	97.0	437
B12 (KF515299)	L. Ursu	<i>Prosthecochloris vibrioformis/aestuarii</i> [Chlorobi/Chlorobiales]	98.0	298
B13 (KF515300)	L. Ursu	<i>(Alkaliflexus imshenetskii)</i> [Bacterioidetes/Bacteroidales]	84.3	408
B14 (KF515301)	L. Ursu	<i>(Aestuariicola saemankumensis)</i> [Bacteroidetes/Flavobacteriales]	86.5	304
B15 (KF515302)	L. Verde	<i>Marinobacter psychrophilus</i> [Gammaproteobacteria/Alteromonadales]	98.7	453
Cyanobacteria and plastids				
C1 (KF515303)	L. St. Ana	<i>Cryptomonas ovata</i> NIES274, plastid [Cryptophyta/Cryptomonadales]	97.5	323
C2 (KF515304)	L. St. Ana	<i>Cryptomonas ovata</i> NIES274, plastid [Cryptophyta/Cryptomonadales]	97.5	323
C3 (KF515305)	L. St. Ana	<i>Cryptomonas ovata</i> NIES274, plastid [Cryptophyta/Cryptomonadales]	97.5	322
C4 (KF515306)	L. St. Ana	<i>Cryptomonas ovata</i> NIES274, plastid [Cryptophyta/Cryptomonadales]	97.5	323
C5 (KF515307)	L. St. Ana	<i>(Opitutus terrae)</i> [Verrucomicrobia/Opitutales]	92.4	328
C6 (KF515308)	L. St. Ana	<i>(Opitutus terrae)</i> [Verrucomicrobia/Opitutales]	92.7	344
C7 (KF515309)	Mohoş	<i>Parachlorella kessleri</i> SAG 211-11g, plastid [Chlorophyta/Chlorellales]	99.0	306
C8 (KF515310)	Mohoş	<i>Parachlorella kessleri</i> SAG 211-11g, plastid [Chlorophyta/Chlorellales]	98.7	311
C9 (KF515311)	Apor 2	<i>Navicula</i> sp. C21, plastid [Bacillariophyta/Naviculales]	99.4	323
C10 (KF515312)	Apor 2	<i>Navicula</i> sp. C21, plastid [Bacillariophyta/Naviculales]	99.4	323
C11 (KF515313)	Apor 6	<i>Arthrospira</i> (formerly known as <i>Spirulina</i>) <i>fusiformis/platensis/maxima/indica</i> [Cyanobacteria/Oscillatoriales]	99.7	322
C12 (KF515314)	Apor 6	<i>(Carboxydocella thermautotrophica)</i> [Firmicutes/Thermolithobacterales]	83.8	319

C13 (KF515315)	L. Ursu	<i>Guillardia theta</i> , plastid [Cryptophyta/Pyrenomonadales]	99.1	322
C14 (KF515316)	L. Ursu	<i>Guillardia theta</i> , plastid [Cryptophyta/Pyrenomonadales]	99.0	298
C15 (KF515317)	L. Ursu	<i>Cryptomonas ovata</i> NIES274, plastid [Cryptophyta/Cryptomonadales]	97.5	323
C16 (KF515318)	L. Roşu	<i>Synedra/Dickieia/Detonula/Haslea</i> [Bacillariophyta/various]	97.8	323

^a in the case of Archaea- and Bacteria-specific DGGE, type strains based on EzTaxon search while for cyanobacteria and eukaryotes, closest species based on Blast search (excluding uncultured sequences) are shown (with strain codes, if available); distant relationships are indicated with parentheses: in the case of prokaryotes >95% pairwise nucleotide sequence similarity for genus and >97% similarity for species level were assumed as suggested by Tindall et al. (2010), in the case of eukaryotes, no such general threshold values were applied