Weak influence of paleoenvironmental conditions on the subsurface biosphere of Lake Ohrid in the last 515 ka

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Abstract

Understanding the response of geo- and biosystems to past climatic disturbance is primordial to assess the short to long terms effects of current global change. Lacustrine sediments are commonly used to investigate the impact of climatic change on biogeochemical cycling. In these sediments, subsurface microbial communities play a primordial role in nutrient, organic matter and elemental cycling, but they also can affect the sedimentary record and overprint the original paleoenvironmental signal. Subsurface microbial communities have therefore been investigated to assess the potential connection between microbial diversity and environmental change. Lake Ohrid (North Macedonia, Albania) is the oldest lake in Europe and has been the target of a scientific deep drilling in 2013. The upper 447 m of the 584-m-long sedimentary drill core record obtained from the central part of the lake (DEEP site) is composed of clayey to silty-clayey lithologies differing substantially in terms of carbonate and organic matter content between glacials and interglacials. We investigated the microbial diversity in the retrieved sediment using 16S rRNA gene sequences along the upper ca. 200 m of the DEEP site record spanning ca. 515 ka to assess whether subsurface microbial communities were following a similar trend.

Results show that *Atribacteria, Betaproteobacteria, Bathyarchaeota* and to a lower extent *Dehalococcoidia* phyla structured the community but their occurrence appears to be independent from each other. *Atribacteria* and *Bathyarchaeota* together with *Dehalococcoidia* are commonly encountered in deep lacustrine and marine sediments. Their metabolic versatility is adapted to low energy environments where they can realize the

fermentation of various substrates (sugars, propionate and amino acids). The generation of H_2 from *Atribacteria* and other fermenters can be used by *Dehalococcoidia* and *Bathyarchaeota* for acetogenesis, and even for chemolithoautrophic processes suggested at greater depths. *Betaproteobacteria*-associated sequences were often co-occurring with cyanobacterial sequences that suggest preservation of ancient DNA from the water column or catchment, down to at least 340 ka. In particular, fossil DNA from *Cyanobacteria* in dry glacial intervals may be the relict of ancient blooms of N-fixing clades in periods of nitrogen depletion.

We compared the richness and diversity of all phylotypes with environmental parameters measured in corresponding intervals to test for the relationship between paleoenvironmental conditions, climatic modes and the subsurface biosphere. We found no significant relationship between any phylotype and measured environmental parameters, nor with sediment age or climate patterns. Our preliminary results support a weak recording of early diagenetic processes and their actors by bulk prokaryotic sedimentary DNA in Lake Ohrid, which might suggest dominant turnover and replacement by specialized low-energy clades of the deep biosphere.

Keywords

Bacteria, Archaea, Glacial stages, lake sediment, deep biosphere

1. Introduction

With an age of at least 1.36 million years (Myr) [1], Lake Ohrid is considered to be the oldest lake in Europe [1]. It is located at the border between North Macedonia and the Republic of Albania. Owing to its age, location in the climate sensitive Mediterranean region and its high degree of endemic biodiversity, Lake Ohrid has been targeted for a scientific deep drilling campaign co-sponsored by the International Continental Scientific Drilling Program (ICDP) in 2013. Global and regional scale changes in Pleistocene glacial-interglacial climatic boundary conditions exerted pronounced impacts on the terrestrial and aquatic environments in the lake and its catchment [e.g. 1,2,11–13,3–10]. The main findings suggest that although significant environmental changes are recorded in the catchment and the sediments [2,9], no significant difference can be observed in terms of lake organisms diversification rates [14,15],

94 therefore concluding in a high resilience of the ecosystem in Lake Ohrid. In particular, diatom
95 communities were shown to quickly return to pre-disturbance state after significant tephra
96 fallout from volcanic eruptions (Campi Flegrei caldera) and did not experience evident changes
97 related to short-term climatic events (e.g. Heinrich H4 event) [15]. Similarly, diversification
98 rates of endemic microgastropods were quite constant and led Föller et al. (2015) to suggest
99 that the specific bathymetry, tectonic activity and karst hydrology of Lake Ohrid could buffer
100 environmental changes and contribute to the strong resilience of this ecosystem.
101 Among the organisms susceptible to respond to environmental change in lake systems,
102 prokaryotes have been the subject of increased attention in the past decade. Because Bacteria
103 and Archaea are present everywhere and are relatively sensitive to changes in organic matter
104 inputs, lake stratification, temperature, pH and salinity of lake systems [e.g. 17–19], the study
105 of their diversity in lake sediments has become a means to understand their long-term
106 response to environmental variations. In various lake systems, it has been shown that the
107 living deep biosphere was able to retain information on past climatic conditions [20,21]. In
108 particular, deep scientific drillings into lake sediments have advanced our understanding of
109 low energy systems and highly resilient subsurface microbial communities [22,23]. In Laguna
110 Potrok Aike (Argentina) for example, microbial communities and their imprint differed from
111 glacial to interglacial stages [24,25]. In Lake Van (Turkey), changes in sulfate reduction rates
112 were very sensitive to organic matter quality, varying as a function of changes in
113 environmental conditions [26]. In the hypersaline conditions of the Dead Sea, strong
114 similarities were observed between communities in sediments deposited in very arid
115 conditions, while sediments deposited during more humid periods displayed apparent
116 variability and diversified metabolic potential [27,28]. Such results, all originating from deep
117 scientific drilling projects, have shown that the deep biosphere is a precious tool to evaluate
118 and understand changes in paleoclimatic conditions, along with the variability it may cause in
119 diagenetic processes [29,30].
120 However, these results are still scarce, and more analyses from other lakes must be carried
121 out to validate and potentially generalize the hypothesis of retained sensitivity of the lake
122 subsurface biosphere to paleoclimatic conditions. Indeed, models and studies from other
123 lakes, generally in shallower sediments, have emphasized the strong dominance of low energy
124 taxa, similar to those found in ocean sediments [31]. A second hypothesis is therefore that
125 eventually conditions become too exclusive (i.e. poor in nutrients and in labile organic matter)

and result in the takeover of low energy organisms such as *Bathyarchaeota, Atribacteria, Dehalococcoidia* or other microorganisms that are better adapted to the specificity of deep sedimentary environments [31,32].

In order to test these hypotheses, we have explored the composition of 16S RNA gene sequences from prokaryotic DNA in several sediment intervals along the DEEP site drill core from the central part of Lake Ohrid. By comparing sedimentary microbial diversity and alleged functions with environmental parameters associated to this sediment, we attempt to find links and potential causality between the deep biosphere current structure, and chemical and lithological characteristics of the sediment. We also compare this microbial composition with the magnetic properties of the sediment, as previous work has emphasized a strong shift in diagenetic paramagnetic minerals, likely caused by a change in microbial cycling in the subsurface sediments of the lake [8]. Finally, we tested a link with climate simulation and proxy observation data available for the past 1.36 Myr [1]. Studying the composition and current functions of the deep biosphere of Lake Ohrid should allow deciphering if microbes are more sensitive than eukaryotes to Quaternary changes in paleoenvironmental conditions, or if the low energy environments of the deep subsurface along with buffer capacity of the lake system has had a stronger impact and selected for adapted taxa, regardless of the original conditions in the sediment.

2. Geological and limnological settings

Lake Ohrid covers an area of 358 km^2 at the border between Albania and North Macedonia (Fig. 1A). It is located in a N-S extending pull-apart basin, between the Galicica (East) and Mocra (West) mountain ranges (Fig. 1B), at an altitude of 693 m above sea level (asl). Its mean water depth equals 150 m, with a maximum reached at 293 m. The lake is fed by karstic inflow (55%, [33]), partly originating from neighboring Lake Prespa located 10 km east of Lake Ohrid, small rivers, and direct precipitation on the lake surface. The high amount of nutrient poor karst inflow results in an overall oligotrophic status of the lake.

The DEEP drilling site is located at 243 m water depth, in the central part of the lake (41°02’57’’N, 020°42’54’’E, Fig. 1b). During the SCOPSCO drilling in 2013, several cores were recovered at this site, reaching a terminal depth of 569 m below lake floor (mblf, [34]). The upper 200 m of the DEEP site composite core analyzed herein is composed of a succession of

158 fine grained hemipelagic sediments, with a few (less than 5 cm-thick) intercalated event layers
159 classified as mass wasting deposits and tephra in the presence/absence of microscopic glass
160 shards [2,35]. Three lithotypes were identified in the fine-grained sediments, based on the
161 amount of calcium carbonate: calcareous silty clay, slightly calcareous silty clay and silty clay.
162 These variations are reflected in the calcite and total organic carbon (TOC) content of the
163 deposits. Silty clayey sediments are mostly characterised by low organic matter (OM)
164 concentrations, while OM can be moderate to high in calcareous and slightly calcareous
165 sediments. The sediments appear mottled or massive and lamination is absent, which implies
166 bioturbation and oxygenated bottom water conditions at the time of deposition [2].
167 In silty clay and slightly calcareous silty clay, TOC is predominantly of aquatic origin, as inferred
168 by the C/N ratio [36], while sediments from calcareous silty clay show C/N ratios occasionally
169 above 10, implying somewhat elevated terrestrial OM inputs. However, Francke et al. (2016)
170 suggest that these values may be affected by early diagenetic selective N loss, since the DEEP
171 site is almost completely disconnected to inlet stream supply. Rock-eval analyses on a Late
172 Glacial to Holocene sediment succession retrieved close to the Lini Peninsula (2.5 km to the
173 west of the DEEP site) revealed organic matter mainly of aquatic origin [10]. Lipid biomarker
174 analyses on sediments with similar age retrieved in close proximity of inlet streams however
175 yield dominance of terrestrial organic endmembers [37] which is also supported by C/N ratios
176 >10 in surface sediments close to the major inlets [38].
177 High diatom frustules content, high endogenic calcite concentrations, and overall high OM in
178 the core corresponds to periods of higher primary productivity, likely promoted by higher
179 temperatures and increased supply of nutrients and dissolved ions (Ca, CO_3) from the (karst)
180 catchment, i.e. conditions as they mainly occur during interglacial periods. On the opposite,
181 lower OM, endogenic calcite, and biogenic silica contents were interpreted as periods of lower
182 productivity, coupled with increased OM oxidation and mixing during the winter season
183 [2,5,13]. These conditions are primarily characteristic of glacial periods [2,13].
184
185 ## 3. Material and methods
186
187 ### 3.1. Sampling material
188

Samples for microbial and sediment biogeochemistry analysis were taken from core catchers originating from hole 5041-1B. Immediately after core retrieval, mini cores were taken from the core catchers using pre-cut and autoclaved syringes for microbial analyses. These minicores were then stored at -12 °C until further processing. The ages of the core catcher sediment samples of core 5045-1B were inferred from the published age model [1].

3.2. Sediment chemistry

Biogeochemical data of core catcher samples presented herein were previously published [34]. After freeze-drying, total carbon (TC) and total inorganic carbon (TIC) were analyzed as released CO_2 from powdered material using an DIMATOC 200 (DIMATEC Co.) TOC was calculated as the difference between TC and TIC. Total nitrogen (TN) concentrations were analysed using a Vario MicroCube for this study.

X-ray fluorescence (XRF) analyses were carried on freeze-dried, powdered aliquots (1 g) of the core catcher samples using and ITRAX core scanner (Cox Analytical). The ITRAX core scanner was equipped with a chromium (Cr) X-ray source and was run at 30 kV and 30 mA, with an integration time of 10 s. Data processing was performed with the QSpec 6.5 software (Cox Analytical).

Magnetic property data were taken from Just et al. (2016). Climatic data (including simulated precipitation and temperatures) were taken from Wagner et al. (2019).

3.3. DNA extraction and sequencing

Half a cm^3 of wet sediment was extracted for each sample, using the MOBIO powersoil extraction kit by Qiagen. We realized triplicate DNA amplification of ca. 10 ng of DNA per triplicate using universal primer 515F (5'-GTGYCAGCMGCCGCGGTA-3') and 909R (5'-CCCCGYCAATTCMTTTRAGT-3') for the V4- V5 hypervariable region of the 16S rRNA gene [39], with indexes integrated following the dual-indexing procedure described by Kozich et al. (2013). Pooled triplicate products were then quantified and using Picogreen assay (Life Technologies) and pooled equimolarly (same amount for each sample). The final pool was concentrated with SpeedVac Plus SC110A Savant and purified with CleanNA beads (Moka

221 science) before sequencing was realized by Fasteris (Geneva, Switzerland) on an Illumina
222 Miseq with 2 × 250 cycles, with settings of 7.5 Gb yield (including PhiX), an error rate of 2.5%
223 and Q30 at 75%.
224
225 3.5. DNA sequences processing
226
227 The final analysis error rates were within quality specifications. The workflow included
228 adapters removal using trimmomatic [41], paired-ends reads joining with ea-utils [42], quality-
229 check using FastQC, and samples demultiplexing by Fasteris in-house script. 16S rRNA gene
230 sequences were then processed using Mothur [43]. Samples were dereplicated, aligned, and
231 filtered by length. Chimeras were removed using uchime [44], and taxonomic affiliation was
232 then realized using the method of [45] at a cutoff of 80% against the Silva SSU database 123
233 [46]. Known common contaminants were removed based on the list provided by Sheik et al.
234 (2018). Operational Taxonomic Units (OTU) were then defined at a 97% similarity and used
235 for similarity analysis. Random subsampling was realized based on the smallest number of
236 obtained sequences in one sample after singleton removal.
237 All alpha-diversity indexes were calculated based on OTU matrix using Mothur. The beta-
238 diversity indexes (Local Contribution and Species Contribution to ß-diversity) were calculated
239 from the same matrix with R using formula provided by Legendre and De Cáceres (2013).
240
241 3.6. Data analysis
242
243 All community composition plots and multivariate analyses presented in this article were
244 realized using the decontaminated relative composition based on 16S rRNA gene sequence
245 taxonomy at the phylum level. Diversity profiles were obtained using the decontaminated
246 OTU list using Mothur [43]. Two matrixes (sample vs microbial composition relative
247 percentage at the phylum level and sample vs normalized OTU distribution) were constructed
248 and Principal Coordinate Analyses were run.
249 Three matrices were built for multivariate analyses of sedimentary and community
250 composition (Principal Component Analysis and Canonical Correlation Analysis). To present
251 environmental variables, such data were pooled into 3 different matrixes (lithology, magnetic
252 properties and simulated climatic variables), normalized and a principal component analysis

was obtained using the software PAST [49]. The matrixes were then normalized by subtracted means and compared with a matrix of the relative percentage of each phylum using a CCA with 999 permutations on PAST. The same comparison was conducted with a normalized community matrix at the OTU level. ANOSIM tests were then run to test for the significance of each parameter with the community composition.

Finally, potential functions were obtained using the online tool METAGENassist [50] based on taxonomic affiliation of obtained OTUs. A heatmap was built using Pearson distance and Ward clustering algorithm after unmapped and unassigned reads were excluded, along with OTUs appearing in only 10% of the samples. Data filtering was done using interquantile range. Row-wise (sample by sample) normalization was performed using the median, while column-wise normalization was done by auto-scaling (mean-centered and divided by the standard deviation for each variable).

The matrices are available in supplementary material, and the complete list of OTUs and sequences can be downloaded from NCBI Genbank (MT066494 - MT067558) and on the Open Science Framework data repository (https://osf.io/s9e2q/).

4. Results

4.1. Lake and sediment characteristics

Due to the low sampling resolution, sedimentary characteristics display a relatively scattered pattern along depth (Fig. 2), but conserve a strong relationship with climatic patterns (warm vs cold periods) (Fig. 3). A plot of the principal components explaining 62 + 13 % of the variance shows that TIC and Ca vary together (Fig. 3). TOC and the C/N ratio also have a similar behavior. Detrital elements Ti, K, Al and Si are anticorrelated to TOC. Fe, As and Mn have quite similar behavior with each other, but seem not correlated to sediment depth. Overall, there is a marked distinction between samples that have high TOC, C/N ratio, Ca and TIC, and others that have higher Mn, As, Fe, Ti, K, Al and Si values. The former mainly belong to interglacial stages, while the second are generally from glacial periods. Three remarkable samples can be identified based on their environmental parameters' characteristics: the samples at 191.9 and 29.1 m, which have high Fe/Mn ratio values, and the sample at 4.7 m, which has low Fe/Mn and high As and Mn.

Magnetic properties have been described in detail in Just et al. (2016). The displayed PCA here explains 28+38 % of the variance (Fig. 3). Magnetic susceptibility (kappa) and hard Isothermal remanent magnetization behave similarly. They show a slight anticorrelation with depth. The other properties seem independent from each other. No clear cluster can be observed for the samples. Samples between 4.7 and 29.1 m are characterized by high kappa and HIRM. The shallowest sample, at 1.8 m, is rather characterized by a high S ratio and high saturation isothermal remanent magnetization (SIRM). Samples below 95.8 m bear a higher imprint of greigite, marked by high ΔGRM/ΔNRM. No clear distinction is observed in terms of glacial vs interglacial stages.

4.2. Microbial community composition and variation

The number of reads obtained from the profile varies largely and has to be taken into account when analyzing the structure of the community. Reads drop significantly with depth, in particular below 60 m (Fig. 2). This distribution is correlated with the decrease in the number of taxa (OTUs), although it is not exactly similar. However, diversity indexes are not related to read numbers. Evenness steadily increases with depth, but the Shannon index remains quite high all along the 200 m of profile, and only drops below 4 at 95.8 m and 201.9 m. Otherwise, it remains close to 4.5 and even 5 throughout the core. Local contribution to beta diversity peaks at 9.6 m in association with an increase of evenness. It then sharply decreases and follows a general increasing trend with depth, with a second maximum at 95.8 m correlated to high dominance and minimum evenness.

Based on PCoA results, three main phyla seem to significantly drive the structure of the deep biosphere community (Fig. 4): *Bathyarchaeota*, *Atribacteria* and *Betaproteobacteria*. They are all uncorrelated to each other and vary independently. Other obtained phyla that show significant relative percentages are *Alphaproteobacteria*, *Dehalococcoidia*, members of *Actinobacteria* group OPB41, and to a lesser extent, *Physisphaerae*, *Gammaproteobacteria*, *Cyanobacteria*, *Bacteroidetes* and *Acidobacteria* (Fig. 5). Two samples are marked by a high relative abundance of *Betaproteobacteria* members: 9.6 m and 147.9 m (Figs. 5 and 6). *Cyanobacteria* are also abundant in these layers. *Atribacteria* abundance increases with depth, while *Bathyarchaeota* and *Dehalococcoidia* vary a lot with depth (Fig. 6). No clear cluster is observable regarding community composition along the profile. Samples from glacial

intervals at 7.2, 12.4, 19.1, 54.3 and 68.8 m have similar compositions to samples from interglacial or transitional intervals at 1.8, 4.7, 39.9 and 95.8 m (Fig. 4). Species contribution to beta-diversity is mostly carried by OTUs associated to *Bathyarchaeota* (39% of the first 40 OTUs), with *Atribacteria* (9%), *Gammaproteobacteria* (9%) and Clostridia (8%) having an important contribution too (Fig. 7).

Results from METAGENassist analyses only allowed assigning functions to a rather small percentage of OTUs (25% for metabolisms). They show that samples at 7.2, 9.6 and 12.4 m have a higher proportion of organisms associated to aquatic habitats. Higher sporulation is observed for deep samples at 109.5, 179.4, 191.9 and 201.9 m, along with enhanced motility (147.9, 191.9 and 201.9 m). Just like diversity, large variations are observed for metabolisms (Fig. 8). Sulfate reducer and sulfide oxidizers dominate between 12.4 and 29.1 m, and in samples at 54.3 m. Dehalogenation follows a similar occurrence. Sulfate reducers are also largely present at 95.8 m with nitrite reducers. Sulfide reducers are dominant at 134.7 m, along with N fixators and nitrite reducers. CO_2 fixation seems to dominate in the deep layers at 147.9, 164.8 and 201.8 m. Hydrogen production is always associated to this CO_2 fixation. Outlier sample at 9.6 m is dominated by aromatic hydrocarbon degradation, sulfur oxidation and metabolizing organisms. Sulfur metabolizing functions are also dominant at 1.8 m and 39.9 m. Finally, methanogenic functions are observed between 12.4 and 39.9 m, and dominate particularly at 19.1 m.

We plot a canonical correlation analysis of a selection of these parameters (excluding magnetic properties) against microbial community composition at the phylum level (Axis 1: 38.56 % of variance, axis 2: 29.4 % of variance; Fig. 9). We observe a rough anti-correlation between phyla *Alphaproteobacteria*, *Betaproteobacteria* and *Cyanobacteria* with TOC content and simulated precipitation. *Actinobacteria* OPB41*, Gammaproteobacteria* and *Atribacteria* seem to increase relatively with depth and age of the sediment, unlike *Phycisphaerae*, *Anaerolinae* or *Deltaproteobacteria*. However, multivariate analysis comparing environmental parameters and phyla or OTU matrix did not yield significant results based on ANOSIM tests ($p>0.05$), and therefore all tested hypotheses of a significant influence of environmental parameters (magnetic properties, sedimentary composition or simulated climatic variations) on microbial diversity were rejected.

5. Discussion

5.1 Dominant taxa and associated metabolisms in the deep Ohrid sediment

Lake Ohrid sediments bear an original and diverse subsurface microbial community, based on the analysis of 16S rRNA gene sequences (Figs. 2 and 5). Three main phyla have been identified, two from the bacterial domain and one from the archaeal domain (Fig. 5). *Betaproteobacteria* seem to play a significant role in the structuration of the subsurface community and are mainly occurring in two specific samples that largely differ from the others (i.e. 9.6 m, and 147 m; Fig. 5). These two samples have different taxonomic compositions resulting in different results in terms of metabolic prediction (Fig. 8). While the 9.6 m sample seems to be dominated by naphtalene, chitin and aromatic hydrocarbon degradation, along with sulfur related metabolisms (potentially sulfur oxidizers), the 147 m sample mainly exhibits hydrogen production and carbon dioxide fixation. Such metabolisms are common in low energy deep biosphere samples, where phyla like *Atribacteria* produce H_2 as a fermentative product [51]. The 9.6 m sample seems to be dominated by an oxic habitat community (as suggested by the varied organic matter degradation metabolic capacities outlined by METAGENassist, Fig. 8). As a consequence, we suggest that most of the DNA extracted from this sample associates with high amounts of terrestrial OM thereby likely also containing soil microbes masking the subsurface biosphere contribution in this level. Conversely, this sample exhibits minimum TOC that could coincide with oxidative conditions at the time of deposition [2]. Hence, we suggest preservation of ex-situ microbial DNA rather than this sample being representative for an in situ sedimentary microbial community.

The two other most significant phyla observed in Ohrid sediments belong to the archaeal candidate division *Bathyarchaeota* and the bacterial division *Atribacteria*. These are both common phyla in sedimentary environments at depth [52], and particularly in the marine realm [e.g. 32], where their occurrence has been associated with strong adaptations to low energy environments and varied fermentative abilities. *Atribacteria* have been suggested to perform primary fermentation of carbohydrates and secondary fermentation of organic acids (propionate among others), leading to the production of H_2 [32,51]. *Bathyarchaeota* are more enigmatic as they have been hypothesized as organoheterotrophic and autotrophic acetogens [53], potentially able to perform dissimilatory nitrite reduction to ammonium. Lloyd et al. (2013) also suggested they could degrade detrital proteins. Finally, CH_4 production was also

hypothesized for this clade [55]. These two phyla appear as the most important contributors to beta diversity among the 40 first OTUs contributions to SCBD (Fig. 7). They likely bear a strong role in the deep subsurface of Lake Ohrid and are often associated with *Dehalococcoidia* phylum sequences, which form a common deep biosphere clade, in particular in marine sediments. Kawai et al. (2014) hypothesized anaerobic respiration of organohalides for the *Chloroflexi* clade, but their catabolic reductive dehalogenation ability has been questioned by the study of several assembled genomes, which suggested they had a strictly anaerobic organotrophic or lithotrophic lifestyle. Sewell et al. (2017) suggested their involvement in reductive dehalogenation with H_2 as an electron donor and linked them to homoacetogenic *Chloroflexi*, which could connect their activity to other deep biosphere taxa like H_2 producers *Atribacteria*, often presented as syntrophs [51] and potentially to acetoclastic methanogens. Samples that have high *Atribacteria* and *Bathyarchaeota* relative abundance often bear reads associated to *Deltaproteobacteria*, *Aminicenantes* and *Bacteroidetes* (Figs. 4 and 5). Their metabolic abilities cannot be easily constrained using our method, but their occurrence has often been acknowledged in the deep subsurface [32]. Potential association with sugar fermentation coupled with Mn and Fe reduction was hypothesized for *Bacteroidetes* members [see in 32], but this does not come out in our METAGENassist simulation (Fig. 8). However, they likely have energy conservative metabolisms allowing them to remain present in extreme deep lacustrine sediments [25]. Based on sedimentary intracellular DNA analysis, *Deltaproteobacteria*, *Bathyarchaeota* and *Clostridia* were shown to be part of the growing communities with depth in ferruginous Lake Towuti, suggesting they are well adapted to the deep subsurface environment [58].

Based on our METAGENassist simulation (Fig 8), samples between 12.4 to 29.1 m and at 54.3 m carry a strong similarity in metabolic potential, encompassing ammonia oxidation, dehalogenation (likely supported by *Dehalocccoidetes*), sulfate reduction, sulfide oxidation, xylan degradation and methanogenesis. All metabolisms seem hard to conjugate in one single sample, as some are strictly anaerobic while others require oxygen. Apart from the fact that a major fraction of observed OTUs could not be linked to any functional potential, it is likely that our METAGENassist simulation is biased by the contribution of archived sedimented DNA from the catchment and water column. It could be the case of soil derived *Acidobacteria*, or water derived *Alphaproteobacteria* or *Physisphaera* for example. The contribution of *Betaproteobacteria* and *Cyanobacteria* suggests likewise.

5.2 Diversity changes along depth

Observations of diversity changes from the most significant taxa fails to exhibit a clear pattern along depth. Except for *Atribacteria* (Fig. 6)*, Gammaproteobacteria* and OPB41 (Fig. 8) that tend to increase in relative abundance with depth (below 10 m), the relative abundance of common deep subsurface taxa such as *Bathyarchaeota* or *Dehalococcoidia* does not exhibit a clear trend. This is reflected in the varied alpha and beta-diversity indexes used (Fig. 2). Regardless of the number of OTUs, Shannon index remains relatively high although a gentle decrease is observed with depth and corresponds likely with an increase of evenness that should be associated to the increasing contribution of energy-conservative taxa. Decrease of read number also suggests biomass and DNA quality decrease with depth. This is similar to the diversity profiles observed down to 80 m in freshwater lake Laguna Potrok Aike [25]. However it is worth noticing that this diversity is lower compared to what has been observed in shallow lake sediments (first m) [e.g. 31,59]. Local contribution to beta diversity is very high for the sample at 9.6 cm, as expected given its peculiarity in microbial community. Below 40 m, a general increase can be observed towards the deepest layers, that could be associated to a general depletion of less adapted taxa and a relative increase in the low-energy taxa such as *Bathyarchaeota* members, which carry much of the SCBD. Going deeper, we conclude that we tend to lose the diversity that has been provided by the sedimenting DNA in paleolake Ohrid. Low energy, well adapted slow growers common in deep subsurface environments necessarily take over in terms of relative abundance, as described by Kirkpatrick et al. (2019) in the marine realm, or Wurzbacher et al. (2017) in higher depth of lake sediments. In the deep sediments of Lake Ohrid, this pattern is roughly carried by *Atribacteria* and OPB41 (Fig. 9). These two phyla are known to catabolize sugars, suggesting availability of this substrate and their catabolic products with depth [61]. They were also shown to express several subsistence mechanisms in deep environments. In particular, *Atribacteria* has the ability to produce *de novo* amino acids and export them in very low energy environments, likely halting cell growth and suggesting metabolic interdependencies [61]. *Gammaproteobacteria* relative abundance also seems to increase with age or depth of the sediment, but the poor taxonomic affiliation of members of this genus prevents any further interpretation on this basis.

5.3 Impact of environmental parameters on current communities

Multivariate analyses coupled with ANOSIM tests failed to identify specific external parameters that were significantly linked to given OTU or phyla relative abundance. We can however identify some covariance based on Fig. 9. In particular, *Atribacteria* and OPB41 members were identified as being increasingly dominant with depth. Metatranscriptomics, metabolomics and single cell genomics studies from deep sediments of the Baltic Sea have highlighted the adaptations and metabolic activity allowing *Actinobacteria* group OP41 and *Atribacteria* to remain active in low energy environments like the deep sediments of Lake Ohrid [61].

Samples between 12.4 to 29.1 m and at 54.3 m are all from glacial intervals. They exhibit a mix of metabolic potential involving anaerobic and aerobic processes (Fig. 8). While anaerobic degradation processes coincide with sedimentary conditions, the presence of sequences associated to aquatic habitats, xylan degraders, N-fixers, *Betaproteobacteria* and *Cyanobacteria* fits quite well with the Ohrid depositional model in which glacials are characterized by lower productivity and enhanced input of soil sediments from the catchment. This also coincides with low TOC, TIC and C/N levels, that have been associated to glacial stages with lower productivity and enhanced detrital inputs in Lake Ohrid [2,5]. Consequently, the obtained DNA in these layers could result in a mix of archived sedimentary DNA, and active OM anaerobic degraders.

Of special interest is the occurrence of *Cyanobacteria* in samples at 9.6 m dated at 24 ka and at 147.9 m at 340 ka. As *Cyanobacteria* are not expected to be active in the deep sediment, relative cyanobacterial increase in samples from glacial periods is likely associated to an increase in archived fossil DNA. In temperate lakes, limited nutrient and in particular N-deficiency has consensually been shown to support blooms of N-fixing *Cyanobacteria* [62,63]. This could explain the increased presence of *Cyanobacteria* in the 9.6 m and 147.9 m samples of Lake Ohrid, along with low C/N ratio [64], since dry and cold conditions during glacial periods likely caused nutrient depletion in Lake Ohrid [4]. However, most cyanobacterial sequences obtained from these intervals could not be affiliated to a given genus, and those that were affiliated mainly belong to *Cyanobium*, which seems to lack N-fixing genes [65]. Some work on fossil sedimentary DNA possibly dovetailed with characteristic pigment analysis

could therefore reveal information on the evolution of Lake Ohrid's productivity and planktonic communities in relation with Quaternary changes of nutrient availability.

5.4 Lake Ohrid specificity

Lake Ohrid is characterized by marked changes in sedimentary composition between glacial and interglacial periods [2,13][2], which contribute to the use of the Lake Ohrid sedimentary record for powerful paleoclimatic reconstructions [e.g. 1]. However, the study of the lake biosphere also highlights the strong resilience of the planktonic to benthic communities to major climatic events [14,15]. Based on our DNA data, the behavior of the deep biosphere and the parameters controlling their diversity are quite complex to disentangle. First of all, the limits of the environmental data available are significant. While bulk sedimentary XRF and magnetic data can provide key information regarding sedimentary processes at a macroscale, they lack the second order precision that could help unravel early diagenetic processes, which could be better addressed using for example pore water chemistry and stable isotope composition.

Links with changes in diagenetic conditions, identified by Just et al. (2016), could not be confirmed. Based on a difference in early diagenetic precipitates (shifts from ferrimagnetic iron sulfides to siderites at 320 ka, ca. 140 m), the authors suggested higher sulfate concentration in the lake before 320 ka. This would have permitted a deeper penetration of sulfate in the sediment and favored formation of iron sulfide via sulfate reduction. After 320 ka, rapid depletion of sulfate in the shallow sediments of the lake may have permitted the formation of siderite through methanogenesis dominance in the shallow sediments. We observe a general peak in the presence of potential sulfate reducers between 30.83 ka and 316.43 ka (although samples at 39.9, 68.8 83.5 and 109.5 m do not bear this signal). Before 320 ka, no peak in potential sulfate reducers nor methanogens could be identified. Moreover, no obvious dichotomy between methane-driven vs sulfur-driven cycling in the 16S rRNA gene composition of the sediments were observed. This can be due either to a suppression of the potential methanogenic or sulfate reducer genetic signatures with time. The sulfate-methane transition zone is indeed generally constrained to the first centimeter of the sediment [31,32] and while some signatures could be retained with burial [24], the continued microbial activity in the deep sediment may lead to turnover of the dominant communities and overall

suppression of the initial signal. We may also miss their presence through the use of non-specific 16S rRNA gene sequencing. Targeting and quantifying functional genes associated to sulfate reduction (*dsrA*) or methanogeneis (*mcrA*) in the archived DNA pool of the deep Ohrid sediment could provide valuable insights on this question.

Interestingly, samples older than 320 ka indeed support different metabolic potential than younger ones. In particular, hydrogen production and carbon dioxide fixation are the main metabolisms highlighted by our simulation (Fig. 8). The extent to which this might be related to a change in cycling from sulfate- to methane-driven microbial cycling in the first place remains unresolved. Potential microbial OM consumption by sulfate reduction and subsequent fermenting processes may have depleted OM to a more important extent than in methane-driven microbial communities. The lack of labile OM available as a carbon source for deep sedimentary communities below 135 m may lead to a shift towards more dominant chemolithoautotrophic metabolisms. The conjunction of H_2 production along with CO_2 fixation directs towards a potential niche for hydrogenotrophic methanogenesis or acetogenesis. Such processes have been suggested in the past for deep lacustrine sediments [66] and deep marine sediments and hydrothermal systems [67,68].

Intracellular vs extracellular DNA extraction methods have shown their value in the study of deep life in lacustrine settings [58]. Such methods could confirm that *Cyanobacteria* and *Betaproteobacteria*, significantly influencing the compositions of samples from 9.6 m and 147.9 m, are inherited from dead cell biomass. It would also allow discriminating between transported-archived vs active-dormant living microbes in the deep sediment of Lake Ohrid, since spore-forming or motility abilities seem to increase with depth.

Finally, a significant part of the community diversity is held by phylotypes adapted to low energy environments, which suggests that Lake Ohrid deep biosphere is likely alive until ca. 515 ka ages (ca. 200 mblf), and that these phylotypes have partly erased a potential microbial signature that could have been inherited through paleoclimatic conditions.

6. Conclusion

Based on 16S rRNA gene sequences, the subsurface biosphere composition of Lake Ohrid is dominated by low energy microbial communities common to deep sedimentary settings, regardless of their marine or lacustrine origin. *Bathyarchaeota, Atribacteria*, and

Dehalococcoidia play a strong role in structuring this subsurface community beta diversity. The ability of these communities to adapt to low energy environments has likely erased the potential original paleoenvironmental, paleolimnological and early diagenetic signals that Lake Ohrid sediments have recorded, except for water column or soil DNA archiving during dry glacial periods. Unlike other lacustrine systems, it seems that the strong resilience of Lake Ohrid's ecosystem and/or the peculiar limnological characteristics of this lake basin do not allow for the conservation or transfer of a specific microbial community in these sedimentary archives.

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781 Figure and figure captions.
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783 Fig. 1: Map of the location of Lake Ohrid (a), and of the DEEP drilling site (b) at the border
784 between N Macedonia and Albania.
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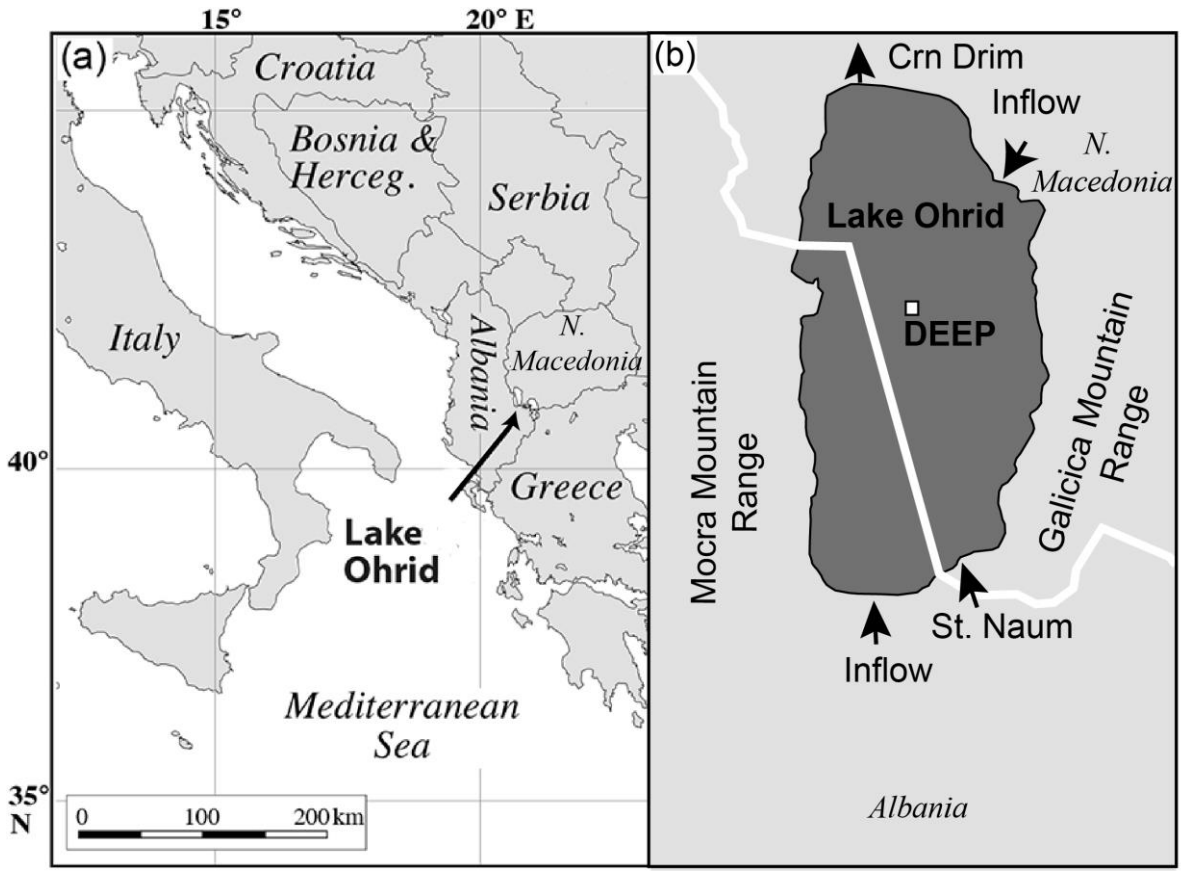
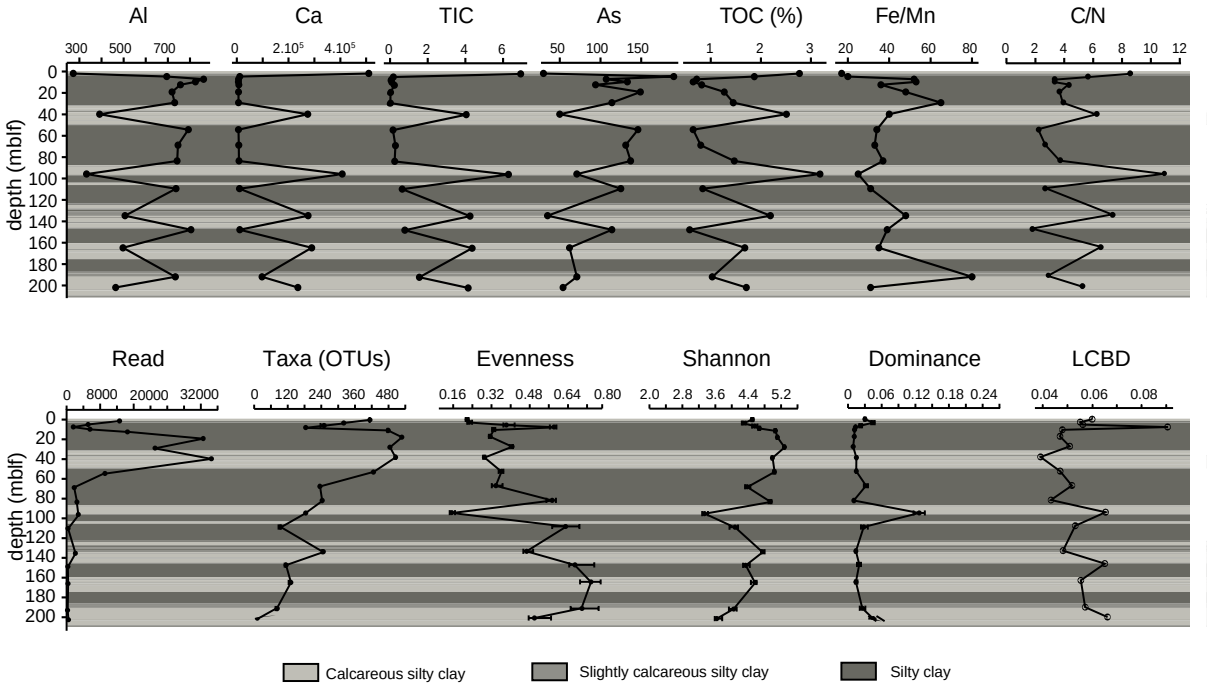


Fig. 2: Profiles of elemental composition and ratio along the core, with corresponding sedimentary facies as described by Francke et al. (2016), and diversity profiles including sequencing read number, OTU number, OTU richness, Shannon diversity index, evenness and local contribution to beta diversity (LCBD) along the core.



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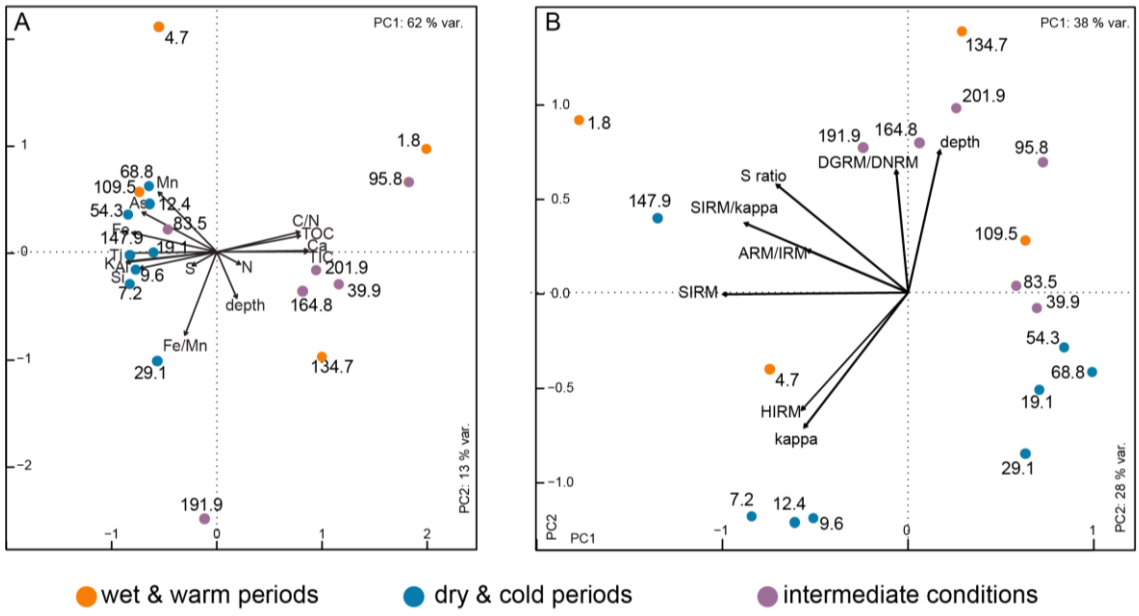
795 Fig. 3: Principal component analysis of elemental composition of the core (A) and magnetic
796 properties (B) along the core. Numbers correspond to sample depth (in m), and colors code
797 for wet and warm periods, mainly corresponding to interglacials (orange), dry and cold periods
798 generally corresponding to glacial stages (blue), and intermediate conditions for transitional
799 climatic stages (purple), based on data by [1,2,5,8]



800

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Fig. 4: Relative abundance of 16S rRNA gene sequences per sample at the phylum level, and corresponding estimated ages for each sample.

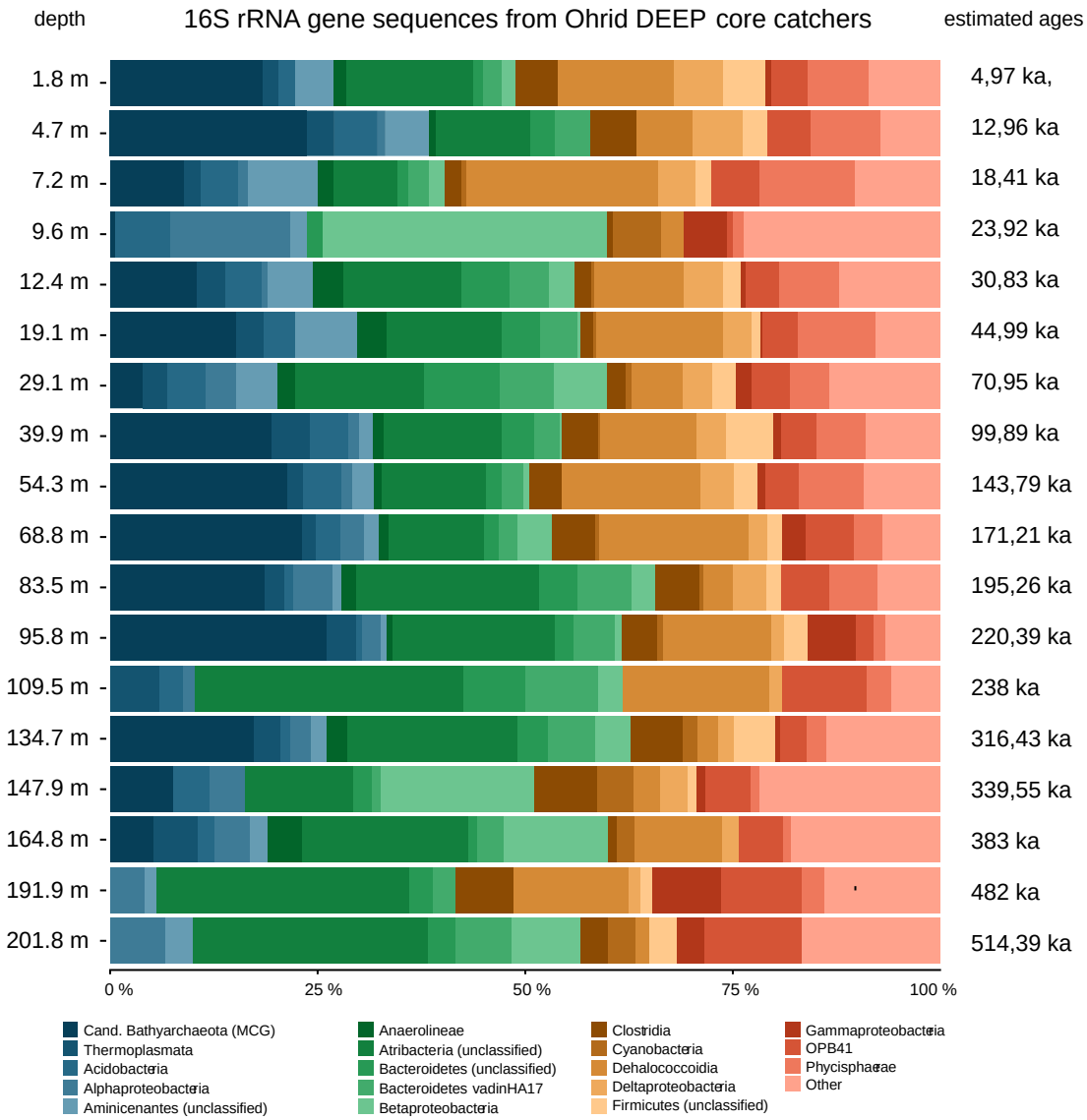

Fig. 5: Principal coordinate analysis of microbial community composition at the phylum level. Colors code for wet and warm periods, mainly corresponding to interglacials (orange), dry and cold periods generally corresponding to glacial stages (blue), and intermediate conditions for transitional climatic stages (purple), based on data by [1,2,5]

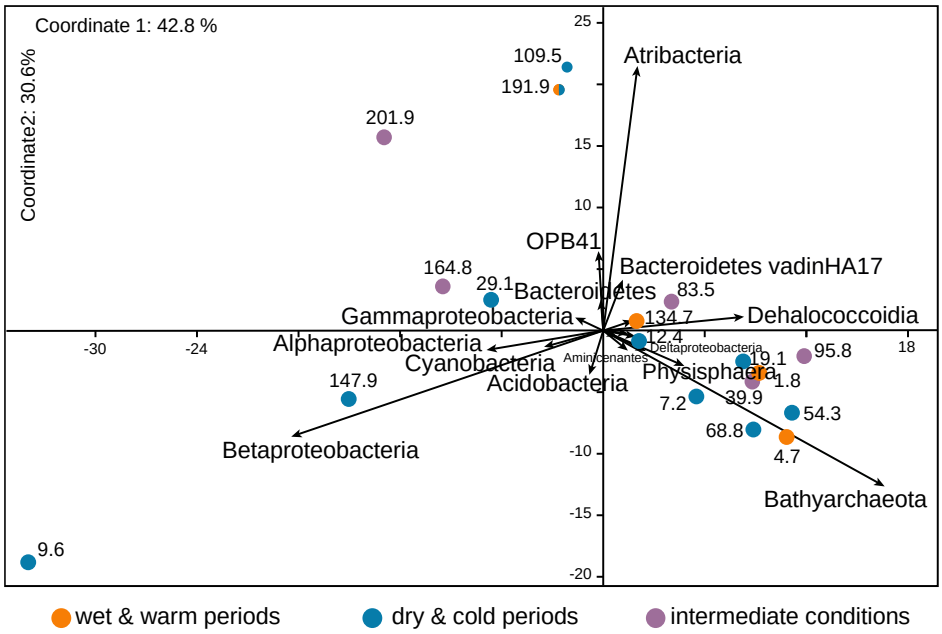

Fig. 6: Relative abundance profiles (read %) of the main microbial phyla along the core as estimated by PCoA. Colors are the same as those used for Fig. 6.

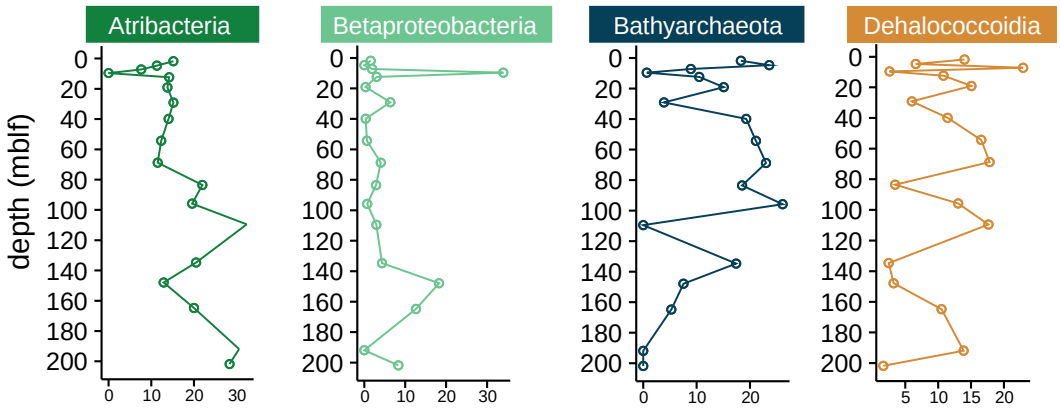

Fig. 7: Species contribution to betadiversity (SCBD) per OTU, and contribution and taxonomic assignment of the 40 first OTUs. Colors are the same as those used in Fig. 4.

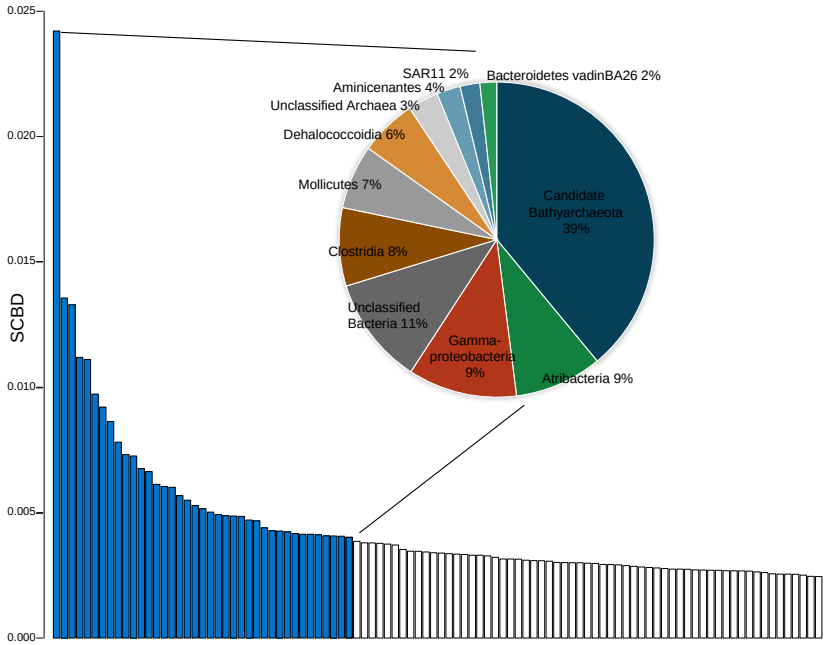

Fig. 8: Heatmap of potential metabolisms obtained from METAGENassist, with corresponding estimated ages.

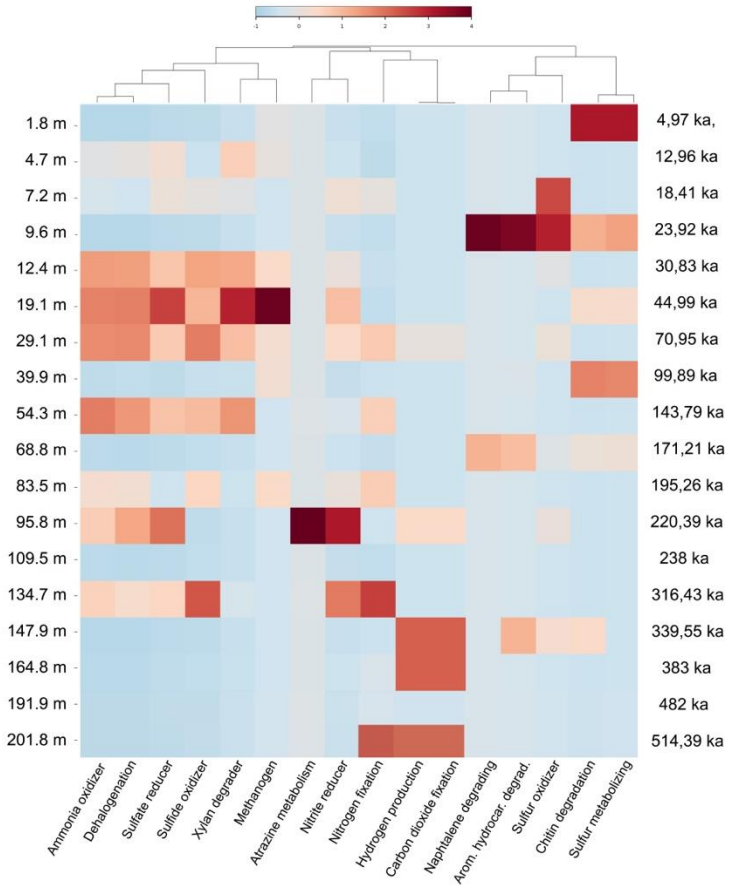

Fig. 9: Canonical correlation analysis involving various paleoclimatically relevant proxy [1,2] and microbial phyla for the DEEP Ohrid sediment. Colors code for wet and warm periods, mainly corresponding to interglacials (orange), dry and cold periods generally corresponding to glacial stages (blue), and intermediate conditions for transitional climatic stages (purple).