Bacteria as paleoenvironmental proxies: the study of a cave Pleistocene profile

Cătălina Haidău [1§*], Ionuț Cornel Mirea [1§], Silviu Constantin [1,2], Oana Teodora Moldovan [2,3*]

[1]Emil Racovita Institute of Speleology, București 050711, Romania
[2]Centro Nacional de Investigación sobre la Evolución Humana, Burgos 09002, Spain
[3]Emil Racovita Institute of Speleology, Cluj-Napoca Department, Cluj-Napoca 400006, Romania

§These authors had an equal contribution.

Correspondence to: Cătălina Haidău (haidau.catalina@gmail.com) and Oana T. Moldovan (oanamol35@gmail.com)

Abstract. Caves are well-known archives that preserve valuable information about the past, relevant for reconstructing past climates and environments. We sampled sediments from a 480 cm deep profile. 16S rRNA gene-based metabarcoding analyses were undertaken that complemented lithological logging, sedimentology, and Optically Stimulated Luminescence (OSL) dating. These analyses revealed different sedimentation conditions along the profile with various water inputs. The OSL age of the sediments places the profile between 74.7 ± 12.3 to 56 ± 8 ka (base to top). However, the more recent LGM – Last Glacial Maximum paleofloods might have occurred in the upper and lower passages of the cave. Bacterial compositions changed with depth, from soil bacteria (present in the upper part of the sediment profile) to thermophilic/sulfurous bacteria (abundant in the deeper samples of the profile). Considering the thermophilic bacteria, we could only assume their origin from a surface of hot sulfurous, old thermal springs, or sapropel sediments.

1 Introduction

Caves are known archives that preserve valuable information about climate in deposits and are relevant for the reconstruction of past climate and past environments (e.g., White, 2007; Nejman et al., 2018; Constantin et al., 2021; Bernal et al., 2023; Campaña et al., 2023). Caves are also known as systems with no primary production due to the lack of plants, low nutrient input from the surface, and low in situ production (Howarth and Moldovan, 2018; Kosznik-Kwaśnicka et al., 2022). That also means that the number of biological proxies (fossil bones, fossil invertebrates, pollen) in caves to be used in paleoenvironmental and paleoclimatic studies is relatively low, and they might even be absent (Moldovan et al., 2011, 2016). Therefore, studying bacteria in cave deposits can open an avenue for paleoenvironmental research and fill some gaps related to presumed past processes in the absence of reliable proxies.

Bacteria are crucial in oligotrophic environments such as caves, as they can be primary producers and participate actively in biogeochemical cycles (Talà et al., 2021; Zada et al., 2021; Lange-Enyedi et al., 2022). Microorganisms regulate essential

ecosystem processes such as the biogeochemical cycling of micro- and macronutrients (Uroz et al., 2009; Pester et al., 2012) or are implicated in the formation (Yarwood et al., 2018; Domeignoz-Horta et al., 2021) or decomposition of organic matter (Krishna and Mohan, 2017; Prescott and Vesterdal, 2021). They can adapt and survive depending on abiotic and biotic factors (litter inputs, moisture, temperature; Castro et al., 2010; Wani et al., 2022).

Bacterial community and structure can change over time due to modifications in the physicochemical components of an ecosystem, leading to the development of biogeographical patterns (Malard et al., 2019; Thomas et al., 2019; Bay et al., 2020; Ji et al., 2020). These patterns serve as indicators of past environmental changes. For example, the structure of bacterial communities present at deposition becomes preserved in sediment layers formed under changing environmental conditions (Frindte et al., 2020; Semenov et al., 2020; Barbato et al., 2022). Thus, such environmental conditions could be traced by reconstructing bacterial community structures from different sediment layers (Thomas et al., 2019; Frindte et al., 2020; Semenov et al., 2020; Barbato et al., 2022).

Microbial paleoenvironmental studies on soil and lake/sea sediments are more common than in caves, highlighting the importance of their diversity as an indicator of ecosystem function and environmental conditions for reconstructing the past. For instance, Xu et al. (2022) found microbial communities in lacustrine sediments that provided valuable insights into past environmental and climate changes. Influenced by abrupt environmental shifts, the distinct vertical trends in microbial community structures suggest that these communities responded dynamically to climatic events, such as aridification and cooling, around 8 million years ago. These shifts are also consistent with previous pollen evidence, indicating a transition from forest to steppe vegetation correlated with a significant uplift of the Tibetan Plateau. More et al. (2019) examined the microbial communities in the Black Sea sediments in response to substantial paleoenvironmental changes, mainly focusing on the transition around 5.2 ka. This study highlights bacterial composition changes driven by increased salinity. The research also underlines key microbial metabolic processes, shift from methane metabolism before 5.2 ka to enhanced nitrogen and sulfur metabolisms. These changes correspond with the establishment of modern conditions in the Black Sea.

A study on paleosols (Frindte et al., 2020) that analyzed environmental DNA from different horizons within an arid paleosequence revealed key changes in microbial communities over time. The findings indicate bacterial abundance, diversity, and community composition decline with increasing soil depth and age. However, deviations from this trend were observed in paleosol horizons with higher microbial diversity and abundance, suggesting that advanced soil formation processes may have preserved more diverse microbial communities. The study also identified specific microbial taxa associated with certain soil horizons, proving that some microbial communities from ancient environments remain detectable despite burial.

Regarding the caves, most studies on paleoenvironment focused on proxies such as stable isotopes (Waltgenbach et al., 2021; Weber et al., 2021), fossil bones (Berto et al., 2021; Mirea et al., 2021; Cruz et al., 2023), fossil invertebrates (Moldovan et al., 2011, 2016; Buttler and Wilson, 2018; Romano et al., 2024; Osipova et al., 2022), or pollen (Prieto et al., 2021; Minckley et al., 2023).

Studies on cave microorganisms were performed regarding their diversity (Zhu et al., 2019; Dong et al., 2020; Dominguez-Moñino et al., 2021), associations (Dattagupta et al., 2009; Ma et al., 2021; Zhao et al., 2024), but little attention was given to

their potential as paleoclimate proxies (Epure et al., 2014, 2017; Yun et al., 2016). Furthermore, Epure et al. (2014, 2017) indicated the potential of microorganisms from old cave sediment deposits in paleoenvironment and paleoclimate reconstruction. Zepeda Mendoza et al. (2016) explored the microbial communities within a speleothem, indicating their potential as past biodiversity archives. Metagenomic analysis on a speleothem in a cave near the sea found microorganisms related to soil and marine environments. Michail et al. (2021) revealed a complex and dynamic microbial community from a stalactite core composed of bacteria from the upper-ground environment. As indicated by specific bacteria, the evidence of seasonal climate variations emphasizes environmental factors' role in shaping microbial composition over time. Overall, this research provided valuable insights into the microbial ecology of cave environments and highlights the need for further investigation into the role of microorganisms in cave deposits and paleoclimate reconstruction.

The scope of our study was to investigate the bacterial diversity from a 480 cm deep profile in Muierilor Cave, Romania, where no other biological proxy was found. This cave was studied for its evolution during the last 120 kyr. The combined OSL, AMS[14]C and sedimentology results, together with taphonomical analysis of the Pleistocene mammals' accumulation, indicated that most cave levels were already formed at ~120 ka, with the lower levels functioning periodically as vadose cave passages where sediments from the Galbenul River were deposited (Mirea et al., 2021). The bacteria identified through the 16S rRNA gene-based metabarcoding were also compared to other proxies to help define past environments. Thermophiles and sulfur bacteria were amongst the high-abundance bacteria with depth, which raised questions about their occurrence since the cave is characterized by a temperature much lower than their growth range. The possible sources of our samples are discussed, and the results strongly support the importance of investigating bacteria in old sediments, especially in the absence of other biological proxies. When cross-correlated with other proxies, our findings indicate the deposition conditions and water sources during the Pleistocene/Holocene, bringing new insights into the regional karst evolution.

2 Materials and methods

2.1 Site description and sampling

Muierilor Cave (45°11′31.78′′N and 23°45′14.07′′E)1) is located at ~ 645 m a.s.l. in Baia de Fier, south-western Romania, being one of the most-visited show caves in the country due to its archaeological, paleontological, and mineralogical features (Fig. 1b). It is formed in the Polovragi-Cernădia area (Parâng Mountains complex, Southern Carpathians), where the basement is a combination of metamorphic pre-Alpine formation and granitic bodies (Hann et al., 1986), while the sedimentary deposits are represented by a mix of Upper Paleozoic and Mesozoic limestones, conglomerates, and Cenozoic deposits (gravel, sand, and clay) (Fig 1a). The limestones in this region belong to the Oslea-Polovragi formation, made of white-grey and white limestones that can reach a thickness of 150-250 m, covering a surface of approximately 2 km^2 (Bandrabur and Bandrabur, 2010; Mirea et al., 2021). Muierilor Cave is developed in Upper Jurassic-Lower Cretaceous limestone on the right side of the Galbenul Gorge. The cave system has four distinct levels, with a total length of more than 8000 m, and its cave levels are extended on an elevation range of ~80 m. The significant parts of the cave include the Scientific Reserve (Level 1) and the

Touristic Passage (Level 2) (Mirea et al., 2021). The fossil record in Muierilor Cave is rich, and the long history of excavations (1950-2021) of the upper and lower levels of the cave revealed numerous species. The most significant fossil accumulation is in the Urșilor Passage because of primary and secondary thanatocoenosis (Mirea et al., 2021). The highest density of the fossil remains (~ 200 bones/m2) is reported near the PMP1 excavation site and decreases to no fossils towards the PMP2 test pit (Fig. 1d.).

For this study, we sampled in PMP2 (Figs. 1d and 2), a test pit of 1.5/1.5 m and with a depth of 480 cm, located at the northern end of the Urșilor Passage near the restricted entrance (probably an ancient siphon) towards the Hades Passage (Mirea et al., 2021). There is no evidence of present or former percolation in this cave part, only an important accumulation of finer sediments than in PMP1 before the morphologically restricted entrance towards Hades Passage.

Muierilor Cave is located in the Măgura Hill with forest (broad-leaved forest and mixed forest) and small pasture patches, where few agricultural activities are present (e.g., small herds of grazing cattle). Tourism activities occur mostly in the gorges and in the tourist part of the cave; there are no designed tourist paths above the cave. The few urban settlements are lower in altitude than the cave passages (developing around ~ 400 m.a.s.l.). Therefore, land use and potential anthropogenic influences are absent or very limited.

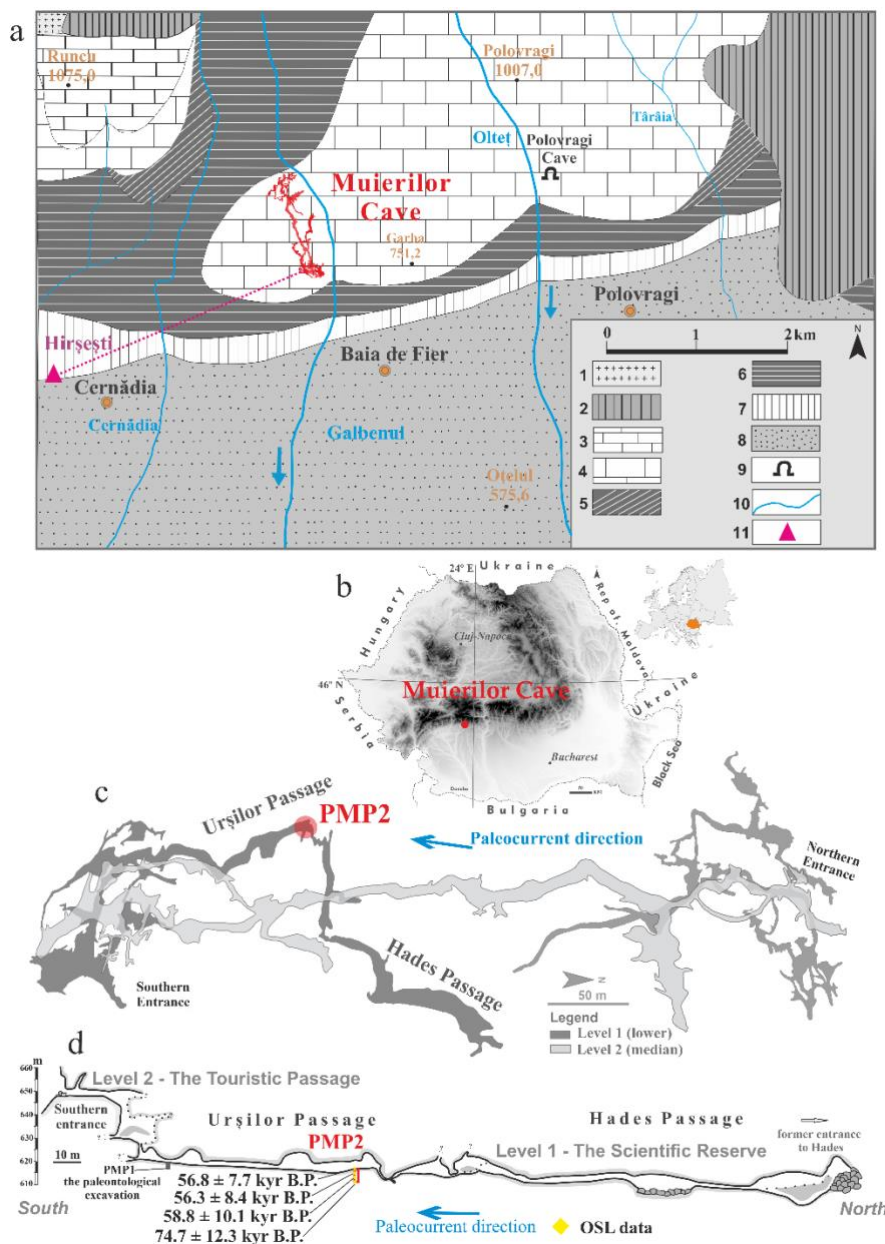
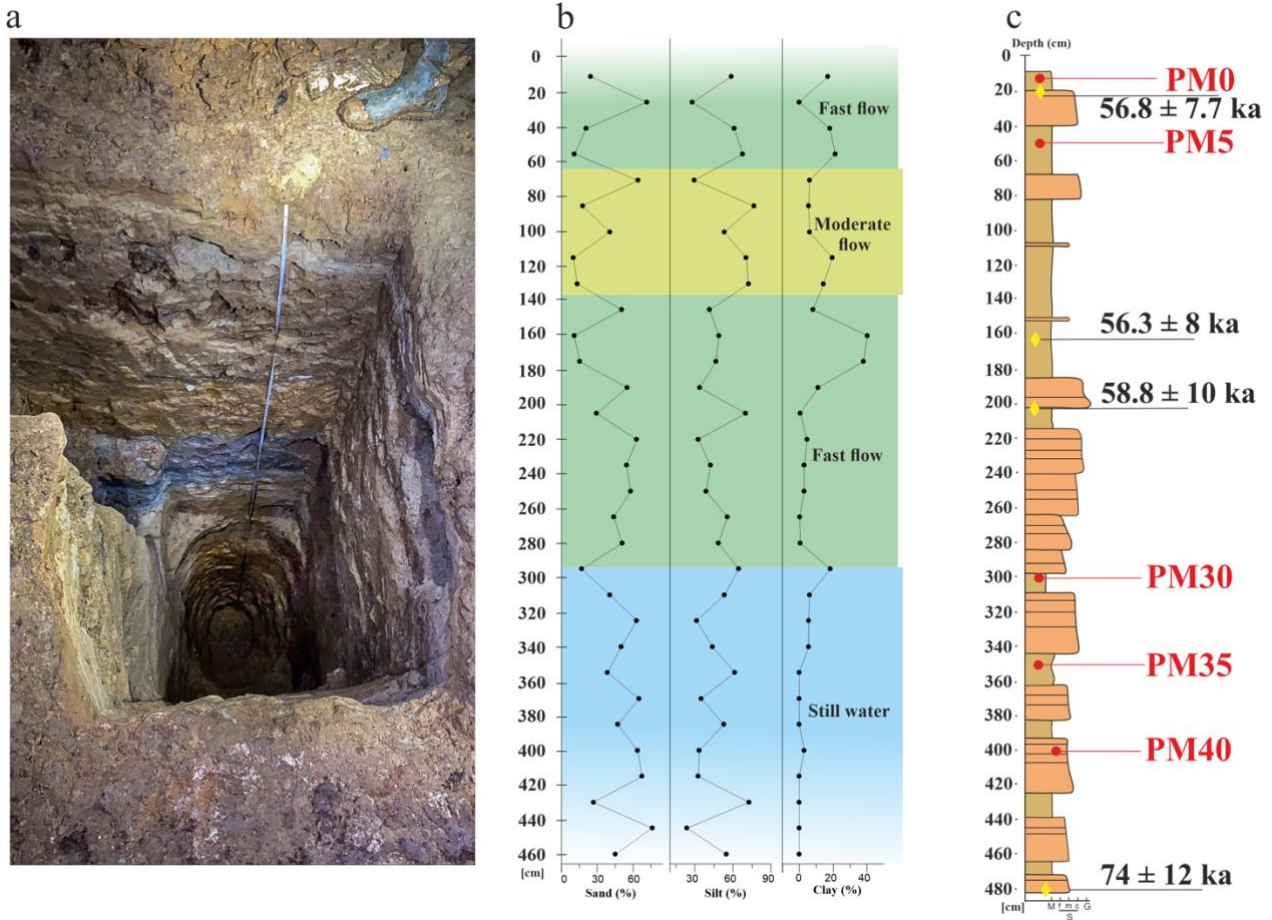



Figure 1: The location of Muierilor Cave and details on the studied profile inside the cave. a. Geological map of the Polovragi - Cernădia area (modified after Diaconu et al., 2008): 1-Parâng Granites; 2-Metamorphic rocks; 3-Early Jurassic (limestone); 4-Late Jurassic (limestone); 5-Late Cretaceous (conglomerates, sandstones, and clays); 6-Early Miocene (marly clays); 7-Middle Miocene (sands and clays); 8-Late Miocene (gravels and sands); 9-Caves; 10-Rivers; 11-Location of the hot springs near Muierilor Cave (according to Ghenea et al., 1981); b. Location of the studied cave in Romania; c. A simplified map of the Muierilor Cave surveyed by the Emil Racovita Institute of Speleology and "Hades" Caving Club (Base map courtesy of Grigore Stelian); d. PMP2 profile with the OSL results (modified after Mirea et al., 2021).

120 **Figure 2: The analyzed deposits in Muierilor Cave: a. Photo of the PMP2 section in Urșilor Passage seen from above; b. The grain size measurements (modified after Mirea, 2020 and Mirea et al., 2021); c. The position of the samples (red) and the OSL ages (yellow) on the profile (modified after Mirea, 2020 and Mirea et al., 2021).**

2.2 Sediment analysis and chronological framework

125 For the PMP2 profile, Mirea et al. (2021) conducted on-site lithological logging and grain size analyses of sediments. Optically Stimulated Luminescence (OSL) was used to constrain the sediment input and deposition events in the cave passages.

The PMP2 profile was excavated for sedimentology studies. It has a complex structure with sand, silts, and clay levels. It is located at the limit between the Urșilor and Hades passages (Fig. 1). Between 150 and 300 cm in depth; the sediments are alternating between sand and silt (Fig. 2), while higher amounts of clay appear in the upper sector, indicating a change in the

130 source area. In contrast, in the topmost part, alternating clay and sand suggests the persistence of high-energy streams. The anisotropy of magnetic susceptibility (AMS) data (Fig. 2) showed that between 420 and 300 cm in depth, the sediments were deposited from still water (Tauxe et al., 1998), most likely due to a small lateral lake formed on the main cave stream. The

following 150 cm (between 300 and 150 cm) are characterized by deposition under a high energy current flow, a moderate flow current deposited the segment between 150 to 50 cm, and the last 50 cm show a deposition in high, moderate currents (i.e. no particle entrainment). We, therefore, assume that the general flow direction was NE-SW with possible "apparent reversals", such as those due to vortex-type flows generated by cave wall topography.

The base of the sediments in the PMP2 profile has an OSL age of 74.7 ± 12.3 ka (Fig. 1d), while the upper 2 m of the section has a significantly younger OSL age of around 58 ka.

Mirea et al. (2021) thoroughly studied the sediment deposition processes in Muierilor Cave, which highlighted some paleoflood events that had occurred during several time intervals, coinciding with warmer and wetter conditions that led to increased water input into the cave passages.

2.3 Sampling for DNA, extraction, and sequencing

For the microbiome analysis, sediment samples were taken directly into sterile Falcon tubes every 50 cm in the PMP2 profile. To avoid contamination, before sampling, the first few centimetres from the sediment surface were removed with the use of a sterilized utensil, and the sediment was taken directly with a sterile Falcon tube. Sediment samples analyzed in this study were taken from the surface of the pit (PM0) and at -50 cm (PM5), -300 cm (PM30), -350 cm (PM35), and -400 cm (PM40) deep. Not enough genetic material for metagenomics could be extracted for the samples at -100, -150, -200, and -250 cm, most likely due to the high amount of clay or other inhibitors we could not remove. Clay is known for inhibiting microorganisms (McMahon et al., 2016). The samples were transported for further laboratory analysis in an icebox and kept in the freezer at –60°C until extraction. A quantity of 25 mg of sediment was used for DNA extraction.

We used FastPrep-24TM (MP Biomedicals) for cell disruption, and DNeasy PowerSoil (Qiagen) was used for genomic DNA extraction, according to the manufacturer's instructions. DNA was extracted in duplicates and was quantified using SpectraMax QuickDrop (Molecular Devices). Extracted DNA was used as a template and sent for MiSeq 16S V3-V4 Metagenome Sequencing using a commercial company (Macrogen Europe). PCR of the V3-V4 hypervariable regions of the bacterial and archaeal SSU rRNA gene was performed using bacteria-specific primers 341F (5'-CCTACGGGNGGCWGCAG-3') and 805R (5'-GACTACHVGGGTATCTAATCC-3'), according to Illumina's 16S amplicon-based metagenomics sequencing protocol.

2.4 Metabarcoding analysis and tests

Metabarcoding analysis was performed by a commercial company (Macrogen Europe) as follows: samples were analyzed using CD-HIT-OTU (Li et al., 2012) and rDnaTools (Schloss et al., 2009). Merging pairs of short reads was performed with FLASH (1.2.11) (Magoč and Salzberg, 2011). It is designed to merge pairs of reads when the original DNA fragments are shorter than twice the length of reads. CD-HIT-OTU is a multi-step pipeline to generate OTU clusters for ribosomal ribonucleic acid (rRNA) tags from 454 and Illumina platforms. CD-HIT-OTU and rDNATools were used to filter out short reads and extra-long tails; filtered reads were clustered at 100% identity using CD-HIT-DUP. Chimeras were identified and removed.

165   Remaining representative reads from non-chimeric clusters are clustered into OTUs at 97% OTU cutoff. Forward and reverse primers were removed, and for further analysis, reads with a minimum length of 250 nt and a maximum of 301 were retained. The sequencing depth varied between 79,534 and 126,869 sequences per sample, with a median of 112,595. The final dataset consisted of a total of 2,692 OTU from 9 samples.

    Taxonomic assignment and diversity statistics were performed by QIIME-UCLUST using NCBI targeted loci project databases
170 16S RefSeq version 20211127. Representative sequences from each OTU were used to assign taxonomy from phylum to species levels. We acknowledge that this approach has limitations in resolving closely related species due to the short-read lengths and the conserved nature of the targeted regions. Challenges have been documented in the literature (Bailén et al., 2020; Gehrig et al., 2022; Satam et al., 2023), and we recognize that alternative sequencing approaches, such as full-length 16S rRNA gene sequencing, may provide improved taxonomic resolution.

175 We used the updated nomenclature for the Prokaryota taxonomic names according to Oren and Garrity (2021; 2022).

    The raw data were deposited in the NCBI SRA Sequence Read Archive under the BioProject: PRJNA1161469, with BioSample accessions: SAMN43780924, SAMN43780925, SAMN43780926, SAMN43780927, SAMN43780928, SAMN43780929, SAMN43780930, SAMN43780931, SAMN43780932.

    Using Physloseq (McMurdie and Holmes, 2013) in R, alpha diversity indices were calculated, and differences in community
180 composition were evaluated. Tax_glom function (taxonomic agglomeration; McMurdie and Holmes, 2013) generated counts and relative abundances at phylum, family, genus and species levels. Alpha diversity indices such as Shannon, Chao1, and Simpson's were calculated and used to express information about the composition of samples. Shannon considers the weight of each species in an ecosystem and gives a better description of its diversity (Konopiński, 2020). Simpson's diversity index estimates the probability that two randomly selected individuals will be identical in a sample. The less diversity, the greater
185 the likelihood that two randomly chosen individuals will be the same species (Simpson 1949; Zhou et al. 2020). Moreover, an abundance-based estimator of species richness, Chao1 index, was also calculated (Kim et al. 2017). To compare the bacteria diversity in the 5 analyzed sediment samples of the PMP2 profile, we applied the Agglomerative Hierarchical Clustering (AHC) in the XLSTAT (2024.2.2. version) based on the dissimilarity matrix generated with the most 256 abundant species.

3 Results

190 **3.1 Bacteria composition in the sedimentary profile**

We identified *Bacteria* and *Archaea* in the five sediment samples we analysed from the profile at 0, -50, -300, -350, and -400 cm. This diversity was observed only at five out of the nine levels we examined; the rest of the samples did not provide enough DNA.

    Only the domain *Bacteria* was kept for further analysis of microbial composition because the abundance of *Archaea* was very
195 low (under 0.2%), with only one species (the ammonia-oxidizing *Nitrosopumilus ureiphilus*) present in PM0, PM30, PM35,

and PM40. Except for PM40, the other samples provided enough material for duplicates (PM0, PM5, PM30, PM35) for which the mean abundances were used for further analysis.

From 2692 Bacteria OTUs, those with an abundance of over 1% were used for further analysis (see also Table A1). A total of 10 major bacterial phyla were identified in our samples (Fig. 3a), with *Pseudomonadota* (22-62%) being the most abundant in all samples, followed by *Bacillota* (PM0-20%; PM35-23%; PM40-30%), and *Actinomycetota* (PM0-10%; PM5-13%; PM30-20%; PM40-17%). The relative abundance of *Pseudomonadota* decreased with depth, while *Bacillota* and *Actinomycetota* relative abundances increased. *Cyanobacteriota* appeared in surprising relative abundance in PM35 (9%).

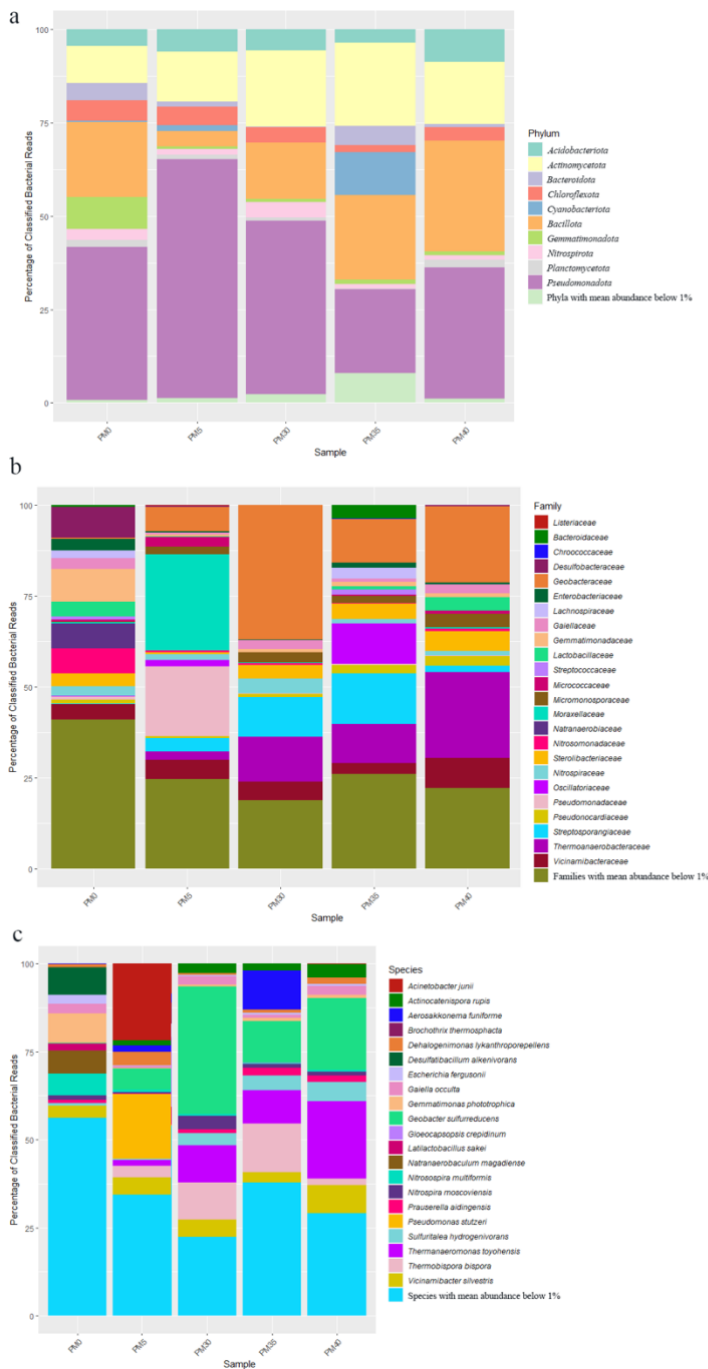



Figure 3 The relative abundance of phyla (a), families (b), and species (c) in the analyzed sediment samples of Muierilor Cave (abundance >1%).

The family composition (Fig. 3b) differed when comparing the upper with the deeper samples. In PM0, *Gemmatimonadaceae* and *Desulfobacteraceae* (8%), and *Natranaerobiaceae* (6%) were the most abundant, while in PM5 *Moraxellaceae* (26%),

Pseudomonadaceae (19%), and *Geobacteraceae* (6%). With deeper samples, the family relative abundances were quite similar, with *Geobacteraceae* (PM30-36%; PM35-12%; PM40-20%), *Thermoanaerobacteraceae* (PM30-12%; PM35-11%; PM40-23%) and *Sterolibacteriaceae* (PM30-3%; PM35-5%; PM40-5%) amongst the highest. *Streptosporangiaceae* (PM30-11%; PM35-14%; PM40-2%) and *Vicinamibacteraceae* (PM30-5%; PM35-3%; PM40-8%) were present in the highest abundance in the two samples. *Oscillatoriaceae* was the third highest in PM35 (11%; very low in the other samples), while *Sterolibacteriaceae* in PM40 (5%; PM30- 4%; PM35-5%).

The most abundant species (Fig. 3c) from surface sample PM0 were *Gemmatimonas phototrophica* (7%), *Desulfatibacillum alkenivorans* (7%), and *Natranaerobaculum magadiense* (6%), while in PM5 (-50 cm) *Acinetobacter junii* (21%), *Pseudomonas stutzeri* (18%), and *Geobacter sulfurreducens* (6%) where the most abundant. As in the case of family abundances, species relative abundances are similar in deeper samples, with *Geobacter sulfurreducens* (PM30-28%; PM35-10%; PM40-18%) and *Thermanaeromonas toyohensis* (PM30, PM35-8%; PM40-19%) present in all three samples with high abundances. *Thermobispora bispora* (PM30-8%; PM35-12%) was in high abundance in the first two bottom samples (-300 cm; -350 cm) while *Sulfuritalea hydrogenivorans* in the last two bottom samples (PM35-4%; PM40- 5%; -350 cm; -400 cm). Also, *Nitrospira moscoviensis* was found in high abundance in PM30 (3%; PM35, PM40-0.1%), and *Actinocatenispora rupis* in PM40 (3%; PM30, PM35-2%). *Vicinamibacter silvestris* was one of the most abundant bacteria throughout our profile (3-7%). A cyanobacterium *Aerosakkonema funiforme* in high abundance in PM35 (9%; PM0-0.1%; PM5-1%; PM30-0.04%) was absent in PM40.

The Chao1 (Table 1) indicated that species richness was relatively higher in the surface samples (PM0 and PM5) than in deeper samples (PM30, PM35, PM40). The Shannon and Simpson diversity indices showed that surface samples (PM0) had a more diverse bacterial community than deeper samples (PM5; PM30, PM35, PM40).

Table 1 Diversity indices of sediment samples from Muierilor Cave.

Sample	Diversity indices		
	Chao1	Shannon	Simpson
PM0	342.49	4.57	0.97
PM5	395.15	3.26	0.82
PM30	266.04	3.60	0.92
PM35	231.71	3.66	0.93
PM40	211.5	3.73	0.92

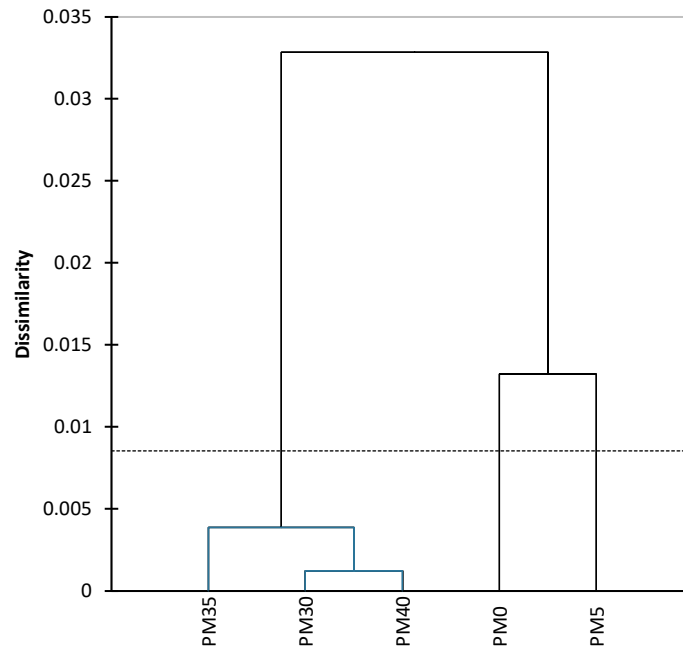



Figure 4 Dendrogram of the Agglomerative Hierarchical Clustering (AHC) on bacteria species diversity in the PMP2 sediment samples of Muierilor Cave.

235 **3.2 The distribution of bacteria in the sedimentary profile with depth/age**

During our investigation of bacterial distribution with depth in the sedimentary profile (Figs. 3-6), we found a diverse range of species associated with biogeochemical cycles, particularly sulfur (S) and iron (Fe). Additionally, we identified species specific to various environments, including soil, water, and even human-associated habitats, as well as those adapted to extreme

conditions,   such   as   thermophilic   and   halophilic   environments.

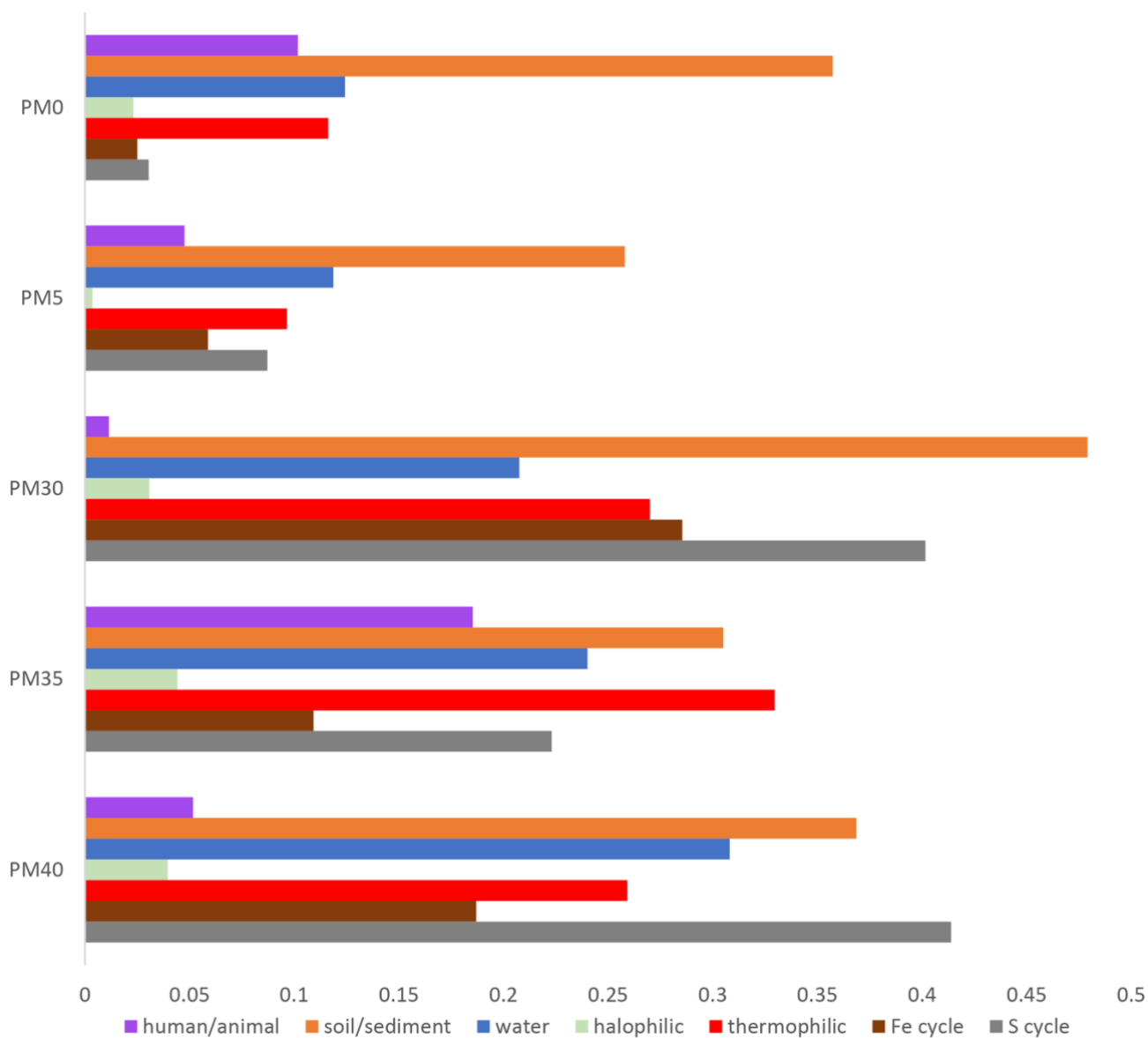



Figure 5 Distribution of bacteria categories with depth in the PMP2 sediment profile of Muierilor Cave.

The AHC analysis shows a strong separation of samples PM0 and PM5 from the other samples in the profile (Fig. 4) and a significant separation of the first two samples. The three lower samples are separated from each other at lesser significance.

245   The family and species abundance in the profile's first two depths (PM0; PM5) comprised bacteria commonly found in surface environments. Since some of them were identified throughout the profile, the plausible explanation is the input from the surface. For example, *Roseisolibacter agri*, an agricultural soil bacterium (Pascual et al., 2018), was found only in PM5 and PM35. Moreover, the presence of halophilic and halotolerant bacteria (*Aidingibacillus halophilus* - PM30, PM40; *Algiphilus aromaticivorans* – PM5, PM30; *Halomonas lactosivorans* and *Saccharopolyspora deserti* – in all samples) could be linked to

250   the rhizosphere, because it may have plant growth promoting characteristics (Reang et al., 2022). Furthermore, another link to the surface environments might be the presence of animal or human-related bacteria. Such bacteria were found in higher abundance in PM0 and very low or absent in the other samples. For instance, *Escherichia fergusonii*, which causes diseases in humans and animals (Gaastra et al., 2014), was found in all samples, with the highest abundance in PM0 (2%; and under 0.4% in the other samples); human gut bacteria (Pianta et al., 2017; Hosomi et al., 2022) such as *Blautia wexlerae* and *Prevotella*

255   *copri* were found in low abundance in PM0 and PM35, while *Megamonas funiformis* previously found in human faeces (Sakon et al., 2008), was found in PM0, PM5, and PM35.

The levels with bacteria related to slow flow or still water while from the fast flow levels no DNA could be extracted.

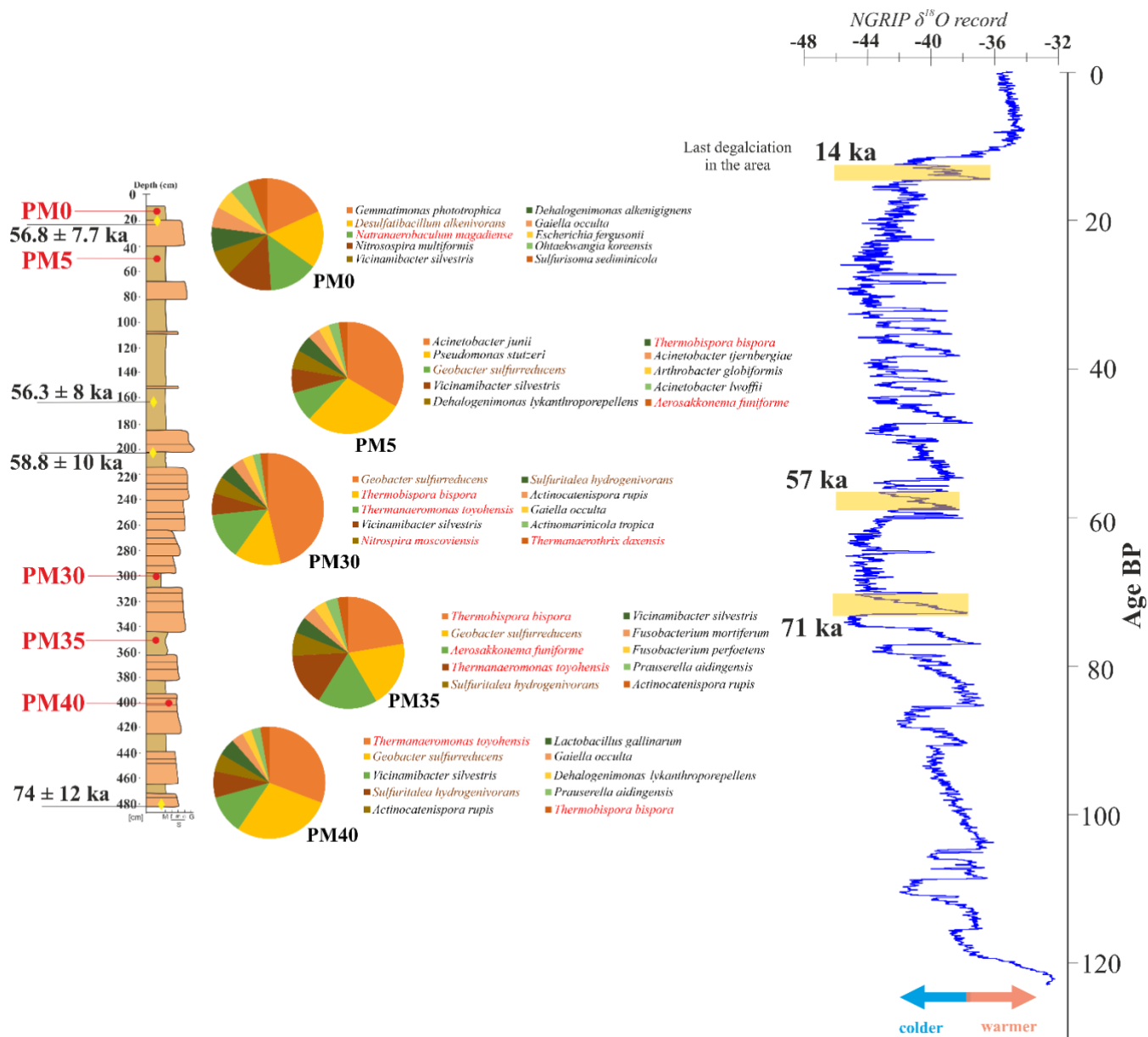



Figure 5 The distribution of bacteria at different depths in the analyzed Muierilor Cave deposits (left) compared to the isotopic oxygen and temperature variations (right; modified after North Greenland Ice Core Project, 2004); only the first 10 most abundant species in each sample (red-thermophiles, brown-involved in the S cycle) are represented.

4 Discussion

The identified bacteria species from the first two sediment samples (PM0 and PM5 at 0 cm and -50 cm, respectively) are common in soils, indicating a direct input from the surface during the last fast flow episode(s). *Gemmatimonadaceae,*

265 *Desulfobacteraceae,* and *Natranaerobiaceae* were the most abundant families in PM0 and were commonly found in water, marine sediments, and soil (Chee-Sanford et al., 2019). Representatives of *Gemmatimonadaceae*, involved in the N cycle, are soil species (*Gemmatimonas aurantiaca*; Chee-Sanford et al., 2019) commonly found in agricultural soils (*G. kalamazoonesis*; Jia et al., 2022) or are phototrophic (*G. phototrophica*; Koblížek et al., 2020). *Desulfobacteraceae* representatives were isolated from oil-polluted sediments, being involved in the S cycle (*Desulfatibacillum alkenivorans, Desulfatiferula berrensis*; Hakil

270 et al., 2014; Ding et al., 2024), and *Natranaerobaculum magadiense* from *Natranaerobiaceae* was isolated from soda lake sediments, and it is obligately alkaliphilic, anaerobic, thermo-, and halotolerant (Zavarzina et al., 2013).

In PM5, representatives of one of the most abundant families (*Moraxellaceae*) such as *Acinetobacter tjernbergiae* was commonly isolated from activated sludges (Yang, 2014), being involved in the P cycle and may have potential applications in the biological removal of phosphates (Täuber et al., 2022). *Psychrobacter aquimaris* is a halophilic bacterium isolated from

275 seawater (Zhang et al., 2021). Denitrifying bacteria of *Pseudomonadaceae* were found in our samples (*Pseudomonas stutzeri*; Feng et al., 2020), and others previously isolated from marine sediments presenting an antagonistic activity (nitrification; *P. glareae*; Romanenko et al., 2015). *Geobacteraceae* representatives were mostly isolated from sediments except for *Geoalkalibacter subterraneus*, which was isolated from petroleum reservoir water (Greene et al., 2009). Representatives of this family are metal-reducers such as *G. subterraneus* - an anaerobic Fe(III)- and Mn(IV)-reducing bacterium (Greene et al.,

280 2009); *G. ferrihydriticus* - an alkaliphilic, iron-reducing bacterium, isolated from lake sediments (Zavarzina et al. 2020); *Geobacter argillaceus* - a Fe(III)-reducing bacterium, isolated from subsurface kaolin strata (Shelobolina et al., 2007) and *G. sulfurreducens* is capable of reducing different forms of Fe(III), Mn(IV), U(VI), elemental sulfur, fumarate and malate (Engel et al., 2020), and was isolated from surface sediment of a hydrocarbon-contaminated soil (Caccavo et al., 1994). *Vicinamibacter silvestris* (*Vicinamibacteraceae*), a soil bacterium isolated from subtropical savanna soil (Huber et al., 2016)

285 and from agriculture and residential (park) soil (Kim et al., 2022), was found in high relative abundances in all samples. With depth, there was also an increase in the relative abundance of *Bacillota* and *Actinomycetota*. This might be due to their resistant spores (Hashmi et al., 2020; Hazarika and Thakur, 2020), enabling the persistence unaffected by the environment for more extended periods (Hanson et al., 2012; de Rezende et al., 2013). For the deeper samples (PM30, PM35, PM40) that mark the transition from fast flow to still water, the relative abundances of families and species are quite similar amongst all three

290 depths. *Thermoanaerobacteraceae* representatives are thermophilic, involved in different biogeochemical cycles, and were isolated from various substrates. Found in all three depth samples are *Brockia lithotrophica*, isolated from a terrestrial hot spring and is involved in the S cycle (Perevalova et al., 2013), *Thermanaeromonas toyohensis* isolated from a geothermal aquifer (Mori et al., 2002), and *Moorella stamsii* previously isolated from a digester sludge (Alves et al., 2013). *Desulfovirgula thermocuniculi* (PM30) was previously isolated from a geothermal underground mine (Kaksonen et al., 2007), and

295 *Carboxydothermus hydrogenoformans* (PM30; PM35) grows with CO as their sole carbon source, was previously identified from a hot swamp (Henstra et al., 2004; Wu et al., 2005). The abundance of this family increases with depth.

Along the profile (see also Fig. 6), there is a transition of abundant species from soil Fe-reducing species (0 and -50 cm) to thermophilic involved in S cycle bacteria (-300 cm, -350 cm, and -400 cm), with the most abundant *Geobacter sulfurreducens,*

Thermanaeromonas toyohensis, and *Sulfuritalea hydrogenivorans*. The identified thermophilic bacteria involved in biogeochemical cycle of S cycle are *Thiobacter subterraneus* (PM0, PM5; found in a hot aquifer by Hirayama et al., 2005), *Thermodesulfovibrio hydrogeniphilus* (all samples; found in a hot spring by Haouari et al., 2008), *Thermosulfurimonas dismutans* (PM5, PM30; deep-sea hydrothermal vent; Slobodkin et al., 2012), *Thioprofundum lithotrophicum* (PM0, PM5, PM30, PM40; hydrothermal field; Mori et al., 2011). Bacteria involved in both the Fe and S cycles are *Acidiferrobacter thiooxydans* (PM0; an acidophilic, thermo-tolerant, copper mine drainage; Ma et al., 2022), and in Fe cycle *Aciditerrimonas ferrireducens* (PM0, PM5, PM30, PM40; acidophilic, geothermally heated field related with fumaroles emitting sulfurous gasses; Itoh et al., 2011). Sulphur can naturally occur in caves, also due to the presence of fossil bones and organic decay (Onac et al., 2011; Audra et al., 2019; Misra et al., 2019; Haidău et al., 2022); its biogeochemical cycle being driven by various microbial metabolic activities, including sulphate reduction and oxidation (Holmer and Storkholm, 2001; Takahashi et al., 2011; Fike et al., 2015; Zhu et al., 2021). With specific bacteria in our samples, we would conclude that their source must be a hot sulfurous environment around the cave.

Streptosporangiaceae with the thermophilic representative, *Thermobispora bispora*, was present in all samples but with higher abundance in PM30 and PM35. This bacterium was isolated from soil (Slobodkina et al., 2017). *Sterolibacteriaceae* representative *Sulfuritalea hydrogenivorans*, previously isolated from freshwater lakes, increases in abundance with depth. It can oxidize thiosulfate, sulfur, or hydrogen and degrade aromatic compounds (Sperfeld et al., 2019). *Nitrospira moscoviensis* was previously isolated from a heating system and was reported to be moderately thermophilic (Edwards et al., 2013).

The cyanobacterium *Aerosakkonema funiforme* (*Oscillatoriaceae*), found in all samples except for PM40, was previously isolated from a mesotrophic water reservoir (Thu et al., 2012), and from a hot spring microbial mat (Moreno et al., 2023), indicating its survival at high temperatures. The increase of this cyanobacterium might be correlated with abrupt warming ~ 70,000 years ago (Lang et al., 1999) following a cold episode that might have promoted the blooming of this taxon before being transported underground.

Additionally, lower abundance thermophiles were identified in the lower samples, like *Thermanaerothrix daxensis, Caldilinea tarbellica* found in deep hot aquifers (Grégoire et al., 2011), or in hot springs *Thermoanaerobaculum aquaticum* (Losey et al., 2013), *Thermincola carboxydiphila* (Sokolova et al., 2005), and *Carboxydothermus islandicus* (Novikov et al., 2011). Bacteria tolerating high temperatures, such as *Gaiella occulta* (deep mineral water aquifer; Albuquerque et al., 2011), were also found. A hydrogeological map of the area (Ghenea et al., 1981) included several mineral springs near Muierilor Cave, including thermal springs (Săcelu and Ciocadia) at less than 20 km distance. Some of them are well-known in the region for having therapeutic properties (Săcelu). In contrast, others were hard to identify in the field because of their low flow rate (Hîrșești see Fig. 1a). Direct proof of the existence of a thermal spring near Muierilor Cave (upstream of Galbenul River) is difficult to demonstrate due to the complex morpho-dynamic evolution of the river slopes. By the abundance of thermophilic bacteria, we presume that the hot spring was present in the area and was the source of old input(s) of water in the cave. Although high concentrations of S and Fe can originate from fossil bones and organic decay in caves (Audra et al., 2019; Misra et al., 2019; Haidău et al., 2022), the thermophiles point to a different possible source, a hot spring.

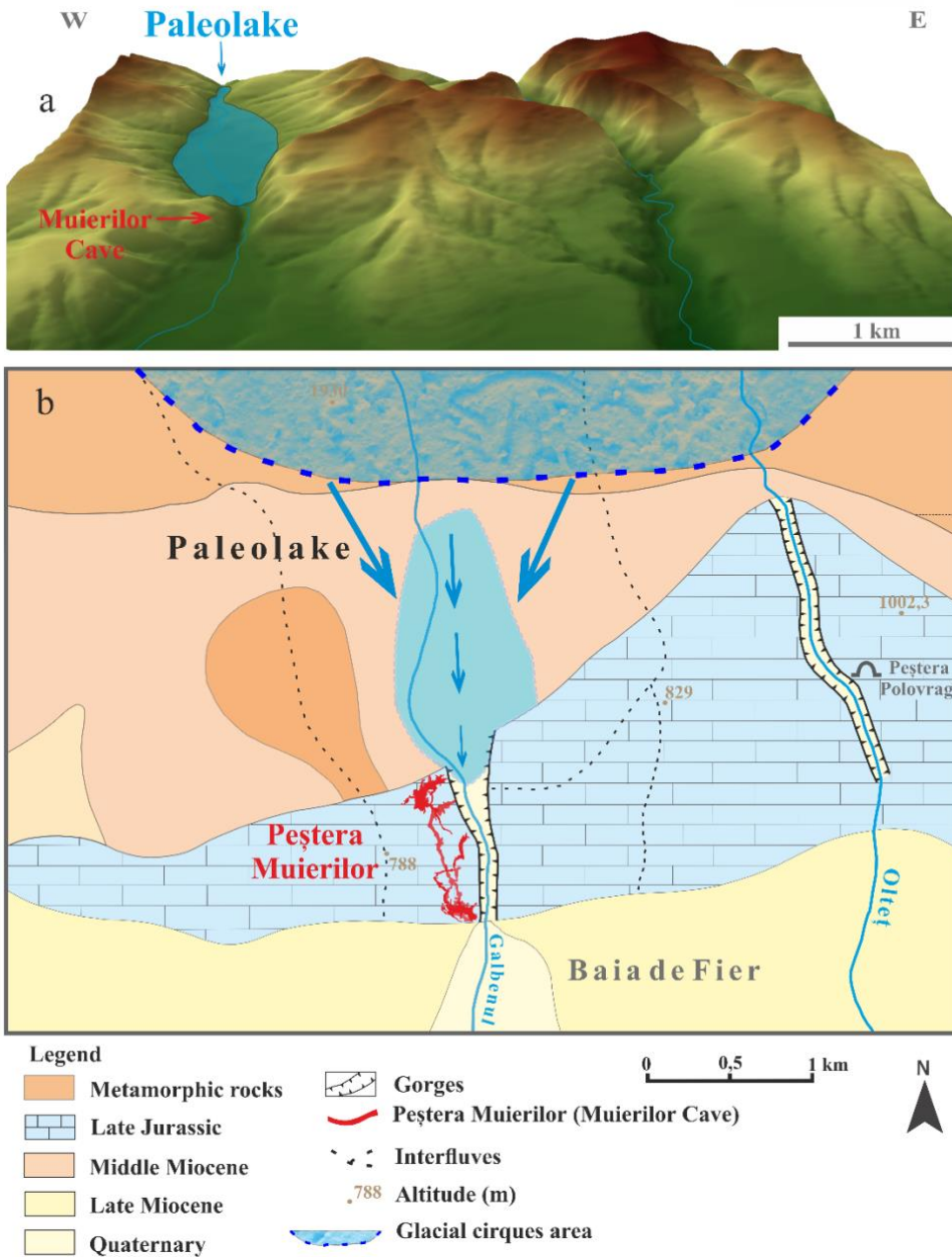



Figure 7 Digital elevation model of the relief near Muierilor Cave (a) with the hypothetical position of the paleolake relative to Muierilor Cave and the flow direction during periods of high-water input from the upstream mountains (b; modified after Lupu & Ion, 1962).

Nevertheless, we do not rule out other possible sources and inputs, such as lacustrine organic sediments near the cave system. In certain conditions, sapropel sediments may form in small freshwater lakes (Leonova et al., 2019). Lupu & Ion (1962) reported the presence of a former lake upstream of the cave system, with intermittent inflows in the cave passages related to the water availability from the snow and ice melting in the high mountains (Parâng Mountains; Fig. 7). Sapropels are characterized mainly as biogenic lake sediments, sludge sediment composed of organic matter and traces of clay, sand, or calcium carbonate (Leonova et al., 2019) with high concentrations of S amongst others (Mg, Fe, Ca) (Taran et al., 2018; Bogush et al., 2022). Moreover, Bogush et al. (2022) found that sulphate-reducing bacteria in a sapropel core from a lake near Baikal increased with depth, probably because such bacteria are important decomposers of organic matter. Thermophilic bacteria are crucial in decomposition, especially when temperatures reach 70°C for several weeks (Finore et al., 2023). Furthermore, S in our samples could result from the intensive decomposition, thermophiles being active in organic matter mineralization, and releasing inorganic nutrients (González et al., 2023).

The extensive clay deposits in the cave passages can also be related to the inputs from the former lake upstream of the cave system, a possible low-flow episode(s). Even though the OSL uncertainties span thousands of years, other proxies (e.g., fossil remains, speleothems) dated from the cave passages constrained sediment deposition with the flooding events from the MIS 5 through the Holocene (Mirea et al., 2021). These sedimentation stages (episodes) can be associated with different climate events from the MIS 5 to the Holocene (Pleistocene), with warmer periods characterized by water and sediment input in the cave.

Given the limestone's isolation within surrounding magmatic and metamorphic rocks, it is unlikely that bacteria were transported over long distances (no more than 2 km) via the epikarst network. Moreover, PMP2 is in an area of the cave with no proof of present or former percolation and no speleothems. It is near a former siphon between two different cave passages. Microbial communities in caves are shaped by the constant input from the surface environments (Wu et al., 2015). Therefore, the possibility of such bacteria being sourced from the surface is high. Thermophiles are thought to survive only in high-temperature habitats like compost heaps (Finore et al., 2023), hot springs (Benammar et al., 2020; Kochetkova et al., 2022), or deep-sea hydrothermal vents (Miroshnichenko and Bonch-Osmolovskaya, 2006; Zeng et al., 2021). However, they were also found in cool and temperate soils (Portillo et al., 2012; González et al., 2015; Santana and González, 2015; Santana et al., 2020; Milojevic et al., 2022), strengthening the idea of microbial dispersal and the possibility of tracking their movement (Müller et al., 2014; Rime et al., 2016; Comte et al., 2017; Bell et al., 2018; Walters et al., 2022). Thermophiles can disperse on short or long distances from hot sources by water or wind (Portillo and González, 2008; Hubert et al., 2009; Perfumo and Marchant, 2010; Portillo et al., 2012; Bell et al., 2018). Soil is also a possible source. Thermophiles in temperate soils were considered vegetative viable organisms (Portillo et al., 2012; González et al., 2023), with the potential involvement in biogeochemical reactions (González et al., 2015, 2023). Recent studies on soil microbiota have included thermophiles as a permanent component despite their strict ecological requirements (Portillo et al., 2012; Santana and González, 2015; González et al., 2023). Thermophiles that inhabit the upper soil layers are believed to grow and show significant enzymatic activity during hot days (>30°C) to produce and stock extracellular enzymes that can help their activity under stress conditions (such

as lower-temperature, dryness) (Milojevic et al., 2022; Gomez et al., 2021). For example, thermophiles showed enzymatic activity for more than 100 days per year at around 37°N in Seville, Spain, and even only 1-2 hot days per year at 52°N in Cambridge, UK (Santana and González, 2015). There are ~ 40 hot days in Romania per year (Micu et al., 2015), and thermophilic bacteria can survive in the soil. During extreme events, the thermophilic enzymes could decompose soil organic

375 matter into smaller compounds (Santana et al., 2020), releasing N as ammonium (Portillo, et al., 2012) and S as sulfate (Portillo et al., 2012; Santana et al., 2021), at a higher rate than soil mesophiles (Portillo et al., 2012), indicating that S cycle in soils is performed mainly by them (Santana et al., 2021). A possibly high abundance of thermophiles in the soil could explain their high abundance in the cave.

The presence of bacteria involved in Fe and S cycles in all our samples that date from the last interstadial could have different

380 explanations. The deposit of fossil bones, or guano (Misra et al., 2019; Haidău et al., 2022) in the cave can be an essential source of these bacteria. Detrital clay (Audra et al., 2019) can be another source of these bacteria. The depositional condition of the fossil remains from the Urşilor Passage contributed to a rapid burial (e.g., fast flow phases), resulting in a slow diagenetic process with few mineral exchanges (mostly apatite-related minerals) on long-term sedimentation. Different minerals in the upper levels were related to phosphate-rich deposits (bone and guano degradation; Haidău et al., 2022) at the same level as the

385 studied profile. The interconnected passages of the cave on different levels (upper and lower) by shafts can influence the concentration of various minerals by the mixed sediment inputs.

Tracing the effects of these paleofloods on bacterial communities within the cave passages was challenging, as reconstructing the precise environmental conditions during those periods is difficult. Moreover, this study serves as supplementary information aimed at enhancing our understanding of past environments in the region. The key events contributing to sediment

390 are the paleofloods that occurred during warmer and wetter conditions in the region. Most of our bacterial findings correlate with these events, indicating a significant influx of bacteria from outside the cave. No other major processes, such as bioturbation or collapse, have been identified in relation to the analyzed sediments.

Moreover, the bacterial abundance growth with depth could be correlated with the age of the sediments and be linked to more stable phases of the cave passages evolution when the sedimentation processes developed under a slow energy environment

395 (Mirea, 2020; Mirea et al., 2021). Mirea et al. (2021) showed that the top sediments within the Urşilor Passage are linked with the warm condition of the Bølling–Allerød interstadial, the last inflow around ~14.7 ka. Thus, it explains the different bacterial compositions correlated with sediment type and age.

5 Conclusions

The bacterial composition of a 480 cm deep profile in Muierilor Cave presented a clear difference between the upper (PM0,

400 PM5) and bottom (PM30, PM35, PM40) samples. The composition changes with depth, from the dominance of soil-specific, Fe-reducing bacterial species to the dominant thermophilic, involved in S cycle bacteria. The presence of bacteria involved in Fe and S cycles can be due to the presence of an abundance of fossil bones in the cave, probably brought inside the cave

together with the sediments during the episodic paleofloods events associated with the end of MIS 5a and MIS 3 (Pleistocene). Thermophiles found in higher abundance in the lower part of the profile could originate from a warm water source in the area or from the soils above the cave during a warmer period. Still, their origin is yet to be determined. The presence of lacustrine organic sediments (sapropelic sediments) near the cave system can also be considered.

The study shows that bacteria in cave deposits can be used in a multi-proxy archive to understand sediment sources and the climate during deposition, as was proposed for other cave sites and organisms (Epure et al. 2014, 2017; Moldovan et al. 2011, 2016). It shows that for old sediments with complex depositional histories, Bacteria can offer new information at the time of deposition that can support or add to the entire understanding of the paleoenvironments.

Appendix A

Table A1. The most abundant bacteria in the analyzed sediments (PM0, PM5, PM35, PM40).

Phylum	Class	Family	Species	PM0	PM5	PM30	PM35	PM40
Pseudomonadota	*Gammaproteobacteria*	*Acidiferrobacteraceae*	*Acidiferrobacter thiooxydans*	0.011	0	0	0	0
Pseudomonadota	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter junii*	0	0.213	0	0	0.001
Pseudomonadota	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter lwoffii*	0.002	0.018	0	0	0
Pseudomonadota	*Gammaproteobacteria*	*Moraxellaceae*	*Acinetobacter tjernbergiae*	0	0.022	0	0	0
Actinomycetota	*Actinomycetia*	*Micromonosporaceae*	*Actinocatenispora rupis*	0	0.013	0.021	0.016	0.032
Actinomycetota	*Acidimicrobiia*	*Iamiaceae*	*Actinomarinicola tropica*	0	0.002	0.013	0.006	0.008
Pseudomonadota	*Gammaproteobacteria*	*Aeromonadaceae*	*Aeromonas veronii*	0.019	0	0.002	0.002	0.005
Cyanobacteriota		*Oscillatoriaceae*	*Aerosakkonema funiforme*	0.001	0.016	0	0.092	0
Pseudomonadota	*Gammaproteobacteria*	*Algiphilaceae*	*Algiphilus aromaticivorans*	0	0	0.011	0	0
Actinomycetota	*Actinomycetia*	*Micrococcaceae*	*Arthrobacter globiformis*	0.004	0.019	0	0	0.004
Pseudomonadota	*Betaproteobacteria*	*Zoogloeaceae*	*Azoarcus olearius*	0.019	0.002	0.003	0.002	0.007
Bacillota	*Clostridia*	*Thermoanaerobacteraceae*	*Brockia lithotrophica*	0	0	0.011	0.004	0.008
Chloroflexota	*Dehalococcoidia*		*Dehalogenimonas alkenigignens*	0.027	0.001	0.003	0	0

Phylum	Class	Family	Species					
Chloroflexota	*Dehalococcoidia*		*Dehalogenimonas lykanthroporepellens*	0.006	0.034	0.004	0.008	0.016
Pseudomonadota	*Deltaproteobacteria*	*Desulfobacteraceae*	*Desulfatibacillum alkenivorans*	0.065	0	0	0	0
Pseudomonadota	*Alphaproteobacteria*	*Rhodospirillaceae*	*Dongia mobilis*	0.014	0.001	0	0	0
Pseudomonadota	*Gammaproteobacteria*	*Enterobacteriaceae*	*Escherichia fergusonii*	0.022	0.001	0.001	0.003	0.004
Bacillota	*Clostridia*	*Oscillospiraceae*	*Faecalibacterium prausnitzii*	0.018	0	0	0	0
Pseudomonadota	*Alphaproteobacteria*	*Hyphomicrobiaceae*	*Filomicrobium fusiforme*	0	0.001	0	0	0.010
Fusobacteriota	*Fusobacteriia*	*Fusobacteriaceae*	*Fusobacterium mortiferum*	0	0	0	0.020	0
Fusobacteriota	*Fusobacteriia*	*Fusobacteriaceae*	*Fusobacterium perfoetens*	0	0	0	0.019	0
Actinomycetota	*Rubrobacteria*	*Gaiellaceae*	*Gaiella occulta*	0.023	0.004	0.018	0.008	0.021
Gemmatimonadota	*Gemmatimonadetes*	*Gemmatimonadaceae*	*Gemmatimonas phototrophica*	0.071	0.002	0.004	0.007	0.008
Pseudomonadota	*Deltaproteobacteria*	*Geobacteraceae*	*Geobacter sulfurreducens*	0.001	0.055	0.278	0.103	0.175
Bacillota	*Bacilli*	*Lactobacillaceae*	*Lactobacillus gallinarum*	0.003	0	0	0	0.030
Bacillota	*Bacilli*	*Lactobacillaceae*	*Latilactobacillus sakei*	0.017	0	0	0	0
Verrucomicrobiota	*Verrucomicrobiae*	*Verrucomicrobia subdivision 3*	*Limisphaera ngatamarikiensis*	0	0.003	0.010	0.001	0
Bacteroidota	*Bacteroidia*	*Bacteroidaceae*	*Mediterranea massiliensis*	0	0	0	0.015	0
Bacillota	*Clostridia*	*Natranaerobiaceae*	*Natranaerobaculum magadiense*	0.055	0	0	0	0
Pseudomonadota	*Betaproteobacteria*	*Nitrosomonadaceae*	*Nitrosospira multiformis*	0.052	0.005	0.003	0	0.005
Nitrospirota	*Nitrospira*	*Nitrospiraceae*	*Nitrospira japonica*	0.011	0.010	0	0	0
Nitrospirota	*Nitrospira*	*Nitrospiraceae*	*Nitrospira moscoviensis*	0.011	0.004	0.029	0.009	0.008
Bacteroidota	*Cytophagia*	*Fulvivirgaceae*	*Ohtaekwangia koreensis*	0.022	0	0	0	0
Bacillota	*Clostridia*	*Peptococcaceae*	*Pelotomaculum thermopropionicum*	0	0	0.011	0	0.003

Bacillota	Clostridia	Peptostreptococcaceae	Peptacetobacter hiranonis	0	0	0	0.011	0
Actinomycetota	Actinomycetia	Pseudonocardiaceae	Prauserella aidingensis	0.007	0.001	0.007	0.019	0.016
Pseudomonadota	Alphaproteobacteria	Xanthobacteraceae	Pseudolabrys taiwanensis	0.002	0.010	0	0	0
Pseudomonadota	Gammaproteobacteria	Pseudomonadaceae	Pseudomonas stutzeri	0	0.181	0	0	0
Bacillota	Bacilli	Sporolactobacillaceae	Scopulibacillus darangshiensis	0.005	0	0.003	0.010	0.006
Pseudomonadota	Betaproteobacteria	Sterolibacteriaceae	Sulfurisoma sediminicola	0.022	0	0.001	0	0
Pseudomonadota	Betaproteobacteria	Sterolibacteriaceae	Sulfuritalea hydrogenivorans	0.005	0.002	0.026	0.036	0.047
Pseudomonadota	Deltaproteobacteria	Syntrophaceae	Syntrophus aciditrophicus	0.018	0	0	0	0
Bacillota	Clostridia	Thermoanaerobacteraceae	Thermanaeromonas toyohensis	0	0.015	0.081	0.082	0.189
Chloroflexota	Anaerolineae	Anaerolineaceae	Thermanaerothrix daxensis	0	0	0.013	0.006	0
Actinomycetota	Actinomycetia	Streptosporangiaceae	Thermobispora bispora	0	0.031	0.081	0.120	0.016
Chloroflexota	Anaerolineae	Anaerolineaceae	Thermomarinilinea lacunifontana	0.003	0.002	0.006	0.001	0.010
Planctomycetota	Planctomycetia	Thermoguttaceae	Thermostilla marina	0.010	0.001	0.001	0	0
Actinomycetota	Vicinamibacteria	Vicinamibacteraceae	Vicinamibacter silvestris	0.029	0.044	0.037	0.025	0.069

Data availability

415 The raw data were deposited in the NCBI SRA Sequence Read Archive under the BioProject: PRJNA1161469, with BioSample accessions: SAMN43780924, SAMN43780925, SAMN43780926, SAMN43780927, SAMN43780928, SAMN43780929, SAMN43780930, SAMN43780931, SAMN43780932.

Author contribution

OTM designed the study; CH made the extractions; CH, OTM, and ICM wrote the manuscript; OTM, ICM and SC made the
420 paleoclimatic interpretation; all authors read and approved the manuscript.

Competing interests

The authors declare that they have no conflict of interest.

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