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RESEARCH

Exploring the Bacteriome Diversity and Use as a Proxy for Climate Change and Human Impacts on Groundwater in Temperate and Tropical Countries

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Abstract

This research investigates bacterial communities in various cave pool water and substrates from Brazil and Romania for their use as indicators of environmental impacts on groundwater. Regional and seasonal differences were observed even if, at the phylum level, common bacteria for both countries were found. Distinct patterns emerged at the genus level due to the different climates (tropical vs. temperate) and ecosystems. Chemoautotrophic conditions define an utterly different groundwater bacteriome than oligotrophic conditions independent of the temperature. Bacteria as a proxy for climate change were explored using seasonal changes in Romanian caves; specific genera become dominant in summer months, such as *Acinetobacter*, *Paeniglutamicibacter*, *Polaromonas*, and *Saccharimonadales*, indicating processes that occur during the low-water season. Climate change, particularly dryness, is expected to exacerbate these variations, threatening the stability of groundwater ecosystems. The research also identified anthropic pollution indicators (*Vogesella*, *Cutibacterium*) and potential decontaminants (*Bacillus*) in Brazilian cave waters. Anthropic pollution indicators, like *Pseudoarthrobacter*. were also found in Romanian caves. Other key bacteria genera, such as *Flavobacterium*, *Pseudomonas*, and *Acinetobacter*, are chemolithotrophs or involved in the nitrogen cycle, which is critical in supplying nutrients for the cave food web. Marked differences between water and substrate microbiomes within the same pools suggested that substrates may play a crucial, underexplored role in groundwater ecosystem processes. Our study found unassigned taxa, 3 phyla, 2 families, and 832 genera (> 40%) in the studied pools. The results underscore the need to further explore groundwater microbiomes as potentially crucial yet fragile ecosystems in the face of climate change and human impacts.

Keywords Metabarcoding · Oligotrophic · Chemoautotrophic · Cave pool · Water · Substrate · Indicators

Highlights.

Similar bacteria phyla but different genera were found in cave pools in Brazil and Romania.

The "summer effect" on the microbiomes was increased abundance of some taxa (e.g., *Acinetobacter*, *Polaromonas*) in different caves.

Brazilian groundwater contains anthropic pollution indicators (*Vogesella*, *Cutibacterium*) and potential decontaminants (*Bacillus*).

Romanian samples contain *Flavobacterium*, *Pseudomonas*, *Polaromonas*, *Rhodoferax* and *Pseudarthrobacter*, which play vital roles in nitrogen cycling, pollutant degradation, and ecosystem stability.

There are significant differences between the bacteriomes in water and substrate from the same pool.

A significant proportion of bacterial taxa remain unassigned, indicating a need for further exploration, especially on the substrate.

Extended author information available on the last page of the article

Introduction

The world's reliance on groundwater and rising concerns about its depletion and contamination have brought us to a critical turning point in recent years. This urgency is heightened by the increasing unpredictability of rainfall, soil moisture, and surface water availability caused by climate extremes [1]. Groundwater is an essential resource for human consumption, agriculture, and industry, and it also represents a unique ecosystem where highly specialised organisms thrive, even in total darkness, with limited energy and scarce nutrients [2]. It sustains diverse microbial and metazoan communities, many of which are strictly endemic and exhibit remarkable adaptations [2–4].

Groundwater biodiversity and its ecological interconnections can serve as valuable indicators of global surface biodiversity patterns [5]. However, despite increasing anthropogenic impacts and climate pressures, groundwater ecosystems have largely been overlooked in global policy agendas [6–9]. This neglect is mainly due to the hidden nature of subterranean water bodies, which has resulted in gaps in our understanding of their biodiversity, distribution, ecological processes and the services they provide [7,10,11]. Large portions of these ecosystems remain inaccessible to humans, with only a limited number of caves, wells and springs serving as "windows" to study groundwater habitats [12].

Despite extensive research on metazoans in groundwater, there is a significant gap in our understanding of microbial diversity and its functions within this ecosystem [13]. This emphasises the pressing need for more comprehensive studies, such as investigating micro-niches in caves, the role of adapted organisms in subterranean food chains, their isolation from surface soil microbiota and their degree of endemism. We have only just begun to uncover knowledge about groundwater microorganisms, and many intriguing organisms have already been discovered, hinting at their diverse applications [14]. One of the challenges in groundwater microbial ecology is the limited information available on boundaries and spatial heterogeneity [15]. Caves provide an opportunity to research groundwater and collect as much information as possible in physically accessible spaces. The water bodies in caves represent only a small fraction of the larger groundwater ecosystem, with many pools being tiny water bodies fed by percolating water from the vadose and epikarstic zones. Nevertheless, these small cave pools are part of aquifers and are accessible for studying their fauna. The physicochemical characteristics of water in these pools can vary [16,17]. Some pools can contain water year-round, fed by relatively constant dripping, while others experience dry periods with seasonal dripping. These variations are influenced by rock permeability, thickness, trajectory, and flow rate [16,18]. However, they represent a reservoir of the incredible diversity of low-range endemic invertebrates and microorganisms that would otherwise remain inaccessible to humans [19–21].

Groundwater invertebrates are typically found in pools with a mixture of fine sediments, silt and clay as substrates. The classic view is that invertebrates in cave pools rely exclusively on nutrients from the surface, mainly through percolation [19–21]. However, numerous studies emphasised the presence of autochthonous bacteria and their possible role in providing nutrients (e.g., [14,22–24]). With new high-throughput techniques, we wanted to investigate the possible roles of bacteria from cave pools as nutrient providers and indicators of changes in temperature and anthropic impacts. In this study, we explore using the most abundant bacteria as key indicators for the state of the environment and their potential as a proxy for climate change and anthropic pollution (agricultural practices, water extraction, deforestation on the surface and tourism inside the caves). Here, we define an indicator taxon as the most abundant bacteria that reflect the abiotic state of the habitat and reveal evidence of an impact, which can be climate change or anthropic pollution. We also compare the microbiomes of water and substrate in cave pools in countries with temperate and tropical climates to assess their biodiversity in the different climates. This approach offers insights into the spatial and temporal variations of water bodies within aquifers. It enhances our understanding of their role in nutrient transfer and the mechanisms that sustain life in low-energy environments. Seasonal variations in the same cave were analysed to evaluate future scenarios involving changes in hydric regimes, water depletion, and increased human impacts.

Material and Methods

The Studied Sites and Sampling Protocol

Samples of pool water (38) and pool substrate (19) were taken from seven Romanian and five Brazilian caves (Figs. 1 and 2, and site description in Supplementary material). The Brazilian samples were taken on a single field trip in which the caves Padre, Brega, Santuário, Lapa d'Água do Zezé and PEA 445 were sampled (Table 1). Single samples were also taken from two Romanian caves, Topolnița and Movile. In Topolnița, only once during our monitoring was water in the pool, and in Movile, a chemoautotrophic cave, the access is limited due to conservation measures. At least four seasonal samplings were done for the rest of the Romanian caves (Muierilor, cu Apă din Valea Leșului, Ferice, Izvorul Tăușoarelor and Cloșani), with a winter overlap in some of the Muierilor Cave stations.

Samples of water and substrate (finely granulated sediments) were taken directly in sterilised Falcon tubes and transported on ice in the laboratory, where they were kept in the refrigerator (− 20 °C) for a few days until extraction. All the samples were taken from pools where invertebrates (primarily crustaceans) were found. Only some samples provided enough DNA for sequencing (Table 1).

DNA Extraction

Before genomic DNA extraction, cells were disrupted using FastPrep-24TM (MP Biomedicals). DNA extraction was performed with the commercial Quick-DNA Fecal/Soil Miniprep kit (Zymo Research) following the producer's protocol. Further, DNA quantification was performed with SpectraMax QuickDrop (Molecular Devices). Extracted DNA was used as a template for exploring the composition of microbial communities and sent for 16S rRNA metagenome sequencing using a commercial company (Macrogen Europe).

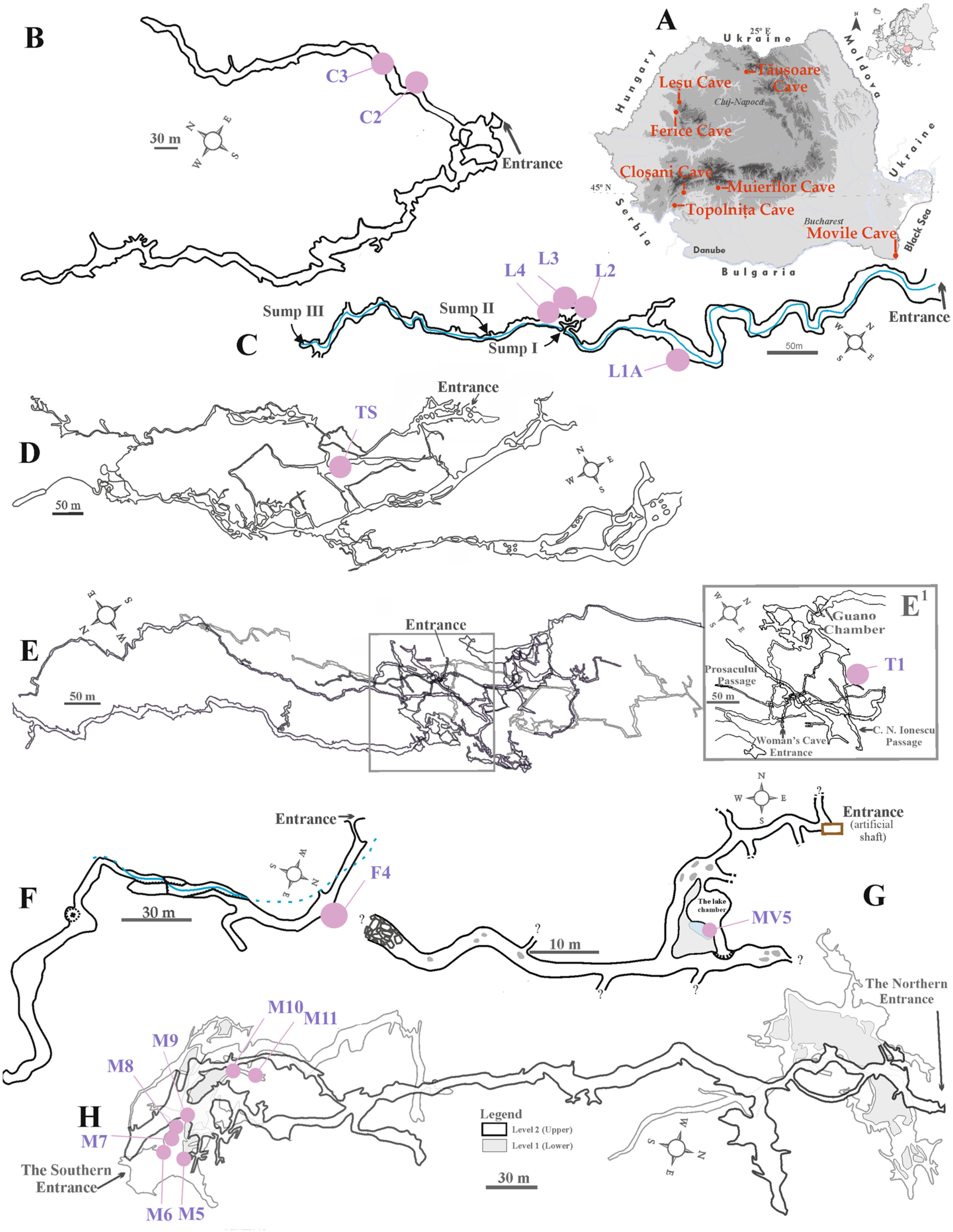


Fig. 1 Location of the studied caves in Romania (**A**) and the position of the sampling points (purple dot) inside the caves: **B** Cloșani Cave, **C** cu Apă din Valea Leșului Cave, **D** Izvorul Tăușoarelor, **E** Topolnița Cave, **F** Ferice Cave, **G** Movile Cave, **H** Muierilor Cave. A description of the sites and caves and details about maps are in Table 1 and the Supplemental material

PCR of the V3-V4 hypervariable regions of the bacterial and archaeal SSU rRNA gene [25] was performed using the bacteria-specific primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′), according to Illumina's 16S amplicon-based metagenomics sequencing protocol. The PCR progressed under the following conditions: Initial denaturation at 95 °C for 3 min, followed by 25 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s, followed by a final extension at 72 °C for 5 min.

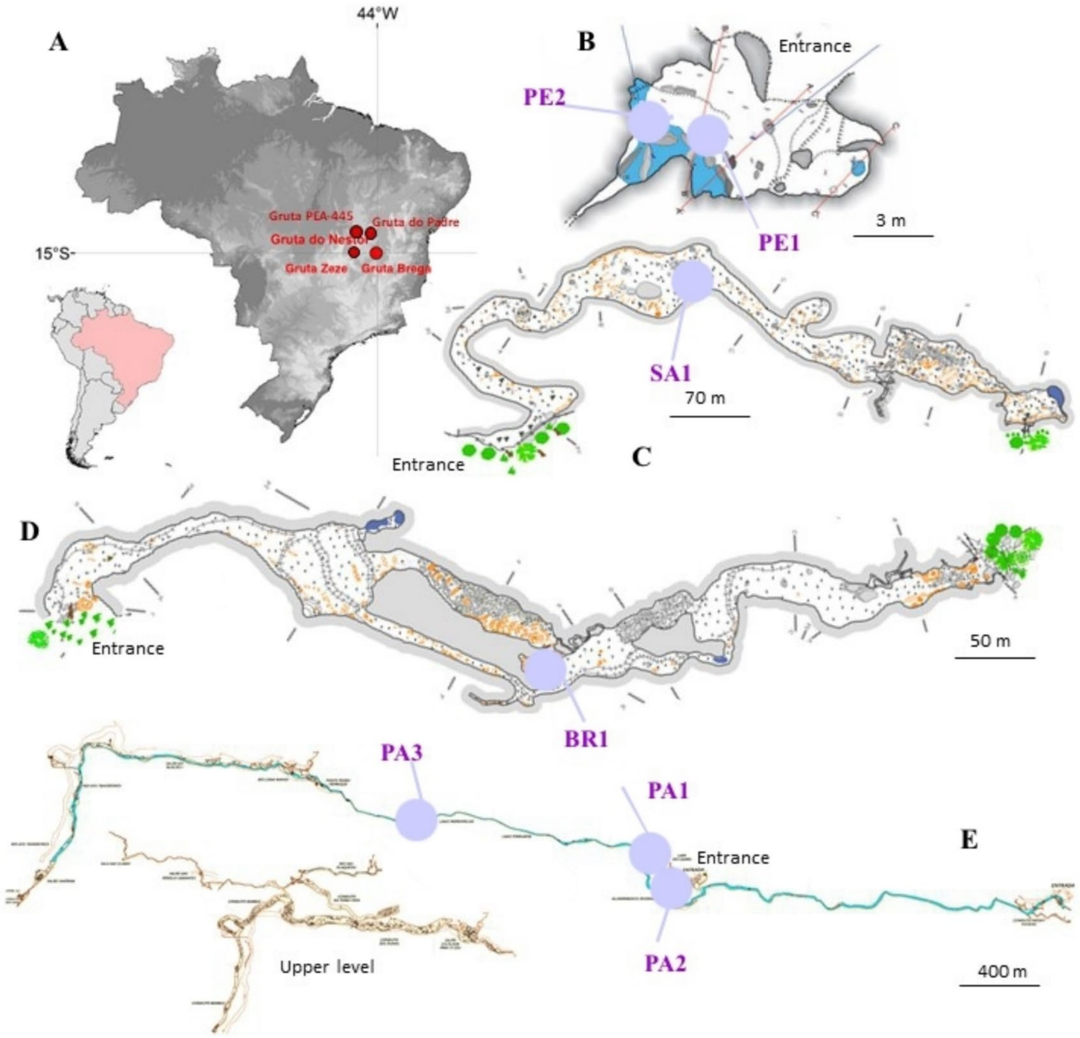


Fig. 2 Location of the studied caves in south-eastern Brazil (**A**) with the caves' map and the studied pools' position (purple dots): **B** PEA 445, **C** Santuário, **D** Brega, **E** Padre. The map of Lapa d'Água do Zezé was not available. A description of the sites and caves is in Table 1 and the Supplemental material

Metabarcoding Analysis

Reads with a minimum length of 250–300 nt were analysed; Cutadapt v2.9 [26] was used to remove sequencing primers and reads with N characters. Further, the DADA2 package [27] implemented in *R* by adapting existing pipelines [28] was used to process paired-end reads from all samples to apply an exact differentiation between actual biological variation and sequencing errors. After primer removal, the resulting paired ends were loaded into the DADA2 pipeline, trimmed, filtered, and merged with a minimum required overlap of 50 nt. Chimeras were removed from merged pairs. Curated ASVs (amplicon sequence variants) were taxonomically classified using the SILVA 138.1 database [29].

Statistical and Other Analysis

For processing differences in community composition and statistics, the Phyloseq package in R ver. 4.0.2 was used [30]. The tax-glom function provided by phyloseq [31] was used for the taxonomic agglomeration to generate counts and relative abundances at the genus, family, and phylum levels. Only 10 counts in at least one averaged sample were considered for abundance estimation for taxa merged at each taxonomic rank. Triplicates have been analysed for each sample, but not all samples provided enough DNA for sequencing. When duplicates or triplicates were available, a mean value was used in the analysis. For the analysis of the microbial composition, only domain *Bacteria* were considered.

We constructed principal coordinate analyses (PCoA) and correlation matrices to compare microbiome diversities

Table 1 Water (W) and substrate (L) samples for microbiome analyses from the Romanian and Brazilian cave pools. Temperature measurements were done with a Hanna Combo Instrument

Country	Cave Used abbr	Code	Station no	Month(s) year	Water temperature (°C)	Potential human impacts
Brazil	Padre	WPA2jul	2	July 2019	24	Agriculture, Tourism
		LPA1jul	1	July 2019		
		LPA3jul	3	July 2019		
	Brega	LBR1jul	1	July 2019	22	Agriculture, Tourism
	Santuário	LSA1jul	1	July 2019	22	Agriculture, Tourism
	PEA445	WPE2jul	2	July 2019	27	Agriculture
		LPE1jul	1	July 2019		
	Lapa d'Água do Zezé -Zeze-	LZE1jul	1	July 2019	25	Water removal
		LZE2jul	2	July 2019		
		LZE3jul	3	July 2019		
Romania	Muierilor -Muieri-	WM5feb WM5may	5	February 2019 May 2019	9.7	Bat colonies, fossil bones, guano
					9.6	
		WM6nov18	6	November 2018	9.6	
		WM7nov18	7	November 2018	9.5	
		WM8nov18	8	November 2018	9.5	
		WM9nov18 WM9aug WM9nov	9	November 2018 August 2019 November 2019	9.2	
					10.2	
					9.1	
		WM10may WM10aug WM10nov	10	May 2019 August 2019 November 2019	9.2	
					9.9	
					9.1	
		WM11feb	11	February 2019	9.3	
	Cu Apă de la Leșu -Lesu-	WL1Amay WL1Aaug WL1Anov	1A	May 2019 August 2019 November 2019	8.2	Bat colonies
					8.9	
					8.0	
		WL2may WL2aug	2	May 2019 August 2019	8.5	
					9.3	
		LL2may LL2aug	2	May 2019 August 2019	8.5	
					9.3	
		WL3mar WL3may	3	March 2019 May 2019	8.1	

Table 1 (continued)

Country	Cave Used abbr	Code	Station no	Month(s) year	Water temperature (°C)	Potential human impacts
					8.8	
		LL3mar	3	March 2019	8.1	
		LL3may		May 2019		
		LL3aug		August 2019		
					8.8	
					9.1	
		WL4may	4	May 2019	8.7	
		LL4aug	4	August 2019	9.1	
	Ferice	WF4mar	4	March 2019	10.1	Bat colonies, tourism
		WF4may		May 2019	11.4	
		WF4aug		August 2019	12.1	
		WF4nov		November 2019	10.6	
		LF4mar	4	March 2019	10.1	
		LF4may		May 2019	11.4	
		LF4aug		August 2019	12.1	
	Izvorul Tăușoarelor -Tausoare-	WTSmar	-	March 2019	2.7	-
		WTSsep		September 2019	7.1	
	Topolnița -Topolnita-	WT1mar	1	March 2019	12.2	-
	Cloșani -Closani-	WC2feb	2	February 2019	11.0	Bat colonies, tourism
		WC2may		May 2019	11.2	
		WC2aug		August 2019	14.1	
		WC2nov		November 2019	10.9	
		LC2aug	2	August 2019	14.1	
		LC2nov		November 2019	10.9	
		WC3feb	3	February 2019	12.0	
		WC3may		May 2019	11.2	
		WC3aug		August 2019	12.7	
		WC3nov		November 2019	11.2	
	Movile	WMV5dec	5	December 2019	21.5	-

between regions, microhabitats or seasons. PCoA used a dissimilarity matrix, and the analyses were performed using XLSTAT 2024.2.2. Bubble plots were done in R ver. 4.4.2.

The Piper diagram for chemical characteristics was constructed using the application by ref [32].

Results

Pool Water Physicochemical Variation

The Piper diagram shows the separation of the Movile sample (Fig. 3; explanations in the Supplementary material). The other samples are more similar in terms of physicochemical characteristics. However, clustering can be observed for the other caves except for the Muieri samples, which had a larger variation of their chemical characteristics.

Pool Water Microbiome Diversity and Abundance

In all the 38 sequenced water samples, 1031 Bacteria ASVs and 12 Archaea ASVs were identified. More than half of the bacteria belonged to *Pseudomonadota*, 23% to *Bacteroidota* and 10% to *Actinobacteriota* (Figs. 4A,B and S1) in the Romanian samples. The two Brazilian samples mainly differed by replacing *Bacteroidota* with *Firmicutes* among the three dominant phyla. *Firmicutes* were dominant in a single March Lesu sample (WL3). Another exception was Movile Cave (WMV5), which was almost equally dominated by *Campilobacterota* (41%) and *Pseudomonadota* (54%). One phylum, more abundant in Tausoare, was unassigned.

From the 317 bacteria families, the most abundant were *Flaviobacteriaceae* (18%), *Pseudomonadaceae,* and *Comamonadaceae* (each with 14%), followed by unassigned families that represented 8% of the total identified ASVs. *Flavobacterium* and *Pseudomonas* dominated with variation according to the sample and season in the Romanian samples, while the two Brazilian samples had more abundant *Bacillus*, *Aquicella*, *Vogesella* and *Cutibacterium*. *Flavobacterium* was especially abundant in Muieri (May), Ferice (August and November) and Closani (February) (Table S1). *Flavobacterium* was present but in very small abundance in Topolnita and Movile caves. In Topolnita, *Aquabacterium* was the most abundant, while in Movile *Thiovirga*, *Aquabacterium* is known from drinking water biofilms [33]. *Thiovirga* was far dominating the microbiome in Movile, together with an unassigned genus. *Thiovirga* is a chemolithoautotrophic sulphur-oxidizing bacterium found in other caves and springs with sulfidic waters [34–36]. *Brevundimonas* and *Duganella* were abundant only in two stations in Lesu in May samples. Given the small number of samples we analysed, no particular bacterium was abundant in Brazilian waters. *Bacillus* and *Vogesella* dominated in PEA445 and *Aquicella* and *Cutibacterium* in Padre.

Pool Substrate Microbiome Diversity and Abundance

The 19 substrate samples had 1029 identified Bacteria ASVs and 21 identified Archaea ASVs. From the 52 bacteria phyla, *Pseudomonadota* (30%) was dominant in the samples of both countries (Fig. 4C,D and S1). It was followed by *Actinobacteriota* and *Firmicutes* (12% and 10%, respectively) in the 8 Brazilian samples and by *Patescibacteria* (16%) and *Acidobacteriota* (11%) in the 11 Romanian samples. The 320 bacteria families were by far dominated by unassigned families (36%), followed by *Pseudonocardiaceae* (5%), *Nitrosomonadaceae* and cvE6 (each with 4%). The most abundant genera were *Pseudomonas*, followed by four unassigned genera for the Romanian samples and *Bacillus*, *Acinetobacter* and an unassigned genus for the Brazilian samples (Table S2). *Pseudomonas* was abundant in Lesu, while an unassigned genus dominated Ferice and Closani.

Inter-continental and Intra-continental Variations of Diversity

Inter-continental differences are illustrated in the Venn diagram (Fig. 5), which shows the few common elements between bacteriomes in Romanian and Brazilian cave water and the original elements in water and substrate in each country. Romanian water samples had the most unique genera, followed by Brazilian and Romanian substrates and Brazilian waters. However, the bias in the results must be mentioned since the more numerous the samples, the more bacteria were identified. The Romanian water samples were the most numerous in this study.

The most abundant common taxa in the Brazilian and Romanian water samples include *Bacillus*, *Pseudomonas*, *Nitrospira*, MND1 and an unassigned genus in *Vicinamibacteraceae* (Table S1). While the first two genera are generalists, *Nitrospira* and MND1 were found in caves involved in carbon fixation, nitrification and ammonia oxidation.

The substrate showed more common genera for Romania and Brazil, even if most of the taxa remained unidentified at the genus level (Table S2).

Water vs Substrate Bacteriomes

We compared nine Romanian water and substrate samples from the same station and month (L3mar, F4mar, L2may, L3may, F4may, L2aug, F4aug, C2aug, C2nov). The number of identified phyla was slightly higher in substrates, and the dominant phyla were more diverse (Fig. 6A,B). Only *Pseudomonadota*, *Firmicutes* and *Actinobacteriota* were the common dominant phyla in substrate and water (Fig. 6C).

The differences between the two micro-habitats were even sharper at the genus level (Fig. 6D). All of the dominant genera in the substrate were unassigned, while in water, the dominant was *Flavobacterium*, *Pseudomonas*, *Polaromonas*, *Rhodoferax*, *Pseudoarthrobacter*, *Paeniglutamicibacter*, *Acinetobacter* and *Brevundimonas*. The unassigned genera belong to *Verrucomicrobiota* (cv6E family) and *Patescibacteria* (orders Ca. *Magasanikbacteria* and *Saccharimonadales*). Members of the *Patescibacteria* superphylum are highly adapted to low food and lack of light in the groundwater [37].

Seasonal Variations in the Pool Bacteriomes

To compare seasons, we analysed the pools' bacteria community variations in four Romanian caves (Muieri, Lesu, Ferice and Closani), where data were available for at least two seasons. Correlations between seasonal samples in the same station were calculated (Table 2) for each cave. In Muieri, significant differences were in station 5 (February vs. May) and slightly significant in station 10 (August vs. November). In Lesu, only stations 2 (May vs. August) and 3 (March vs. May) were significantly different. Ferice samples are seasonally similar if only the most abundant bacteria are considered. Closani station 3 had seasonal significant differences, and station 2 for February vs. May, May vs. August and May vs. November.

Bacteria genera in water showed a different pattern for each cave, as seen in the PCoA analysis (Fig. 7B). Water samples in Ferice are seasonally separated in space. However, the differences are significant only for the increase in *Polaromonas* in August in both caves (Fig. 7A). Closani

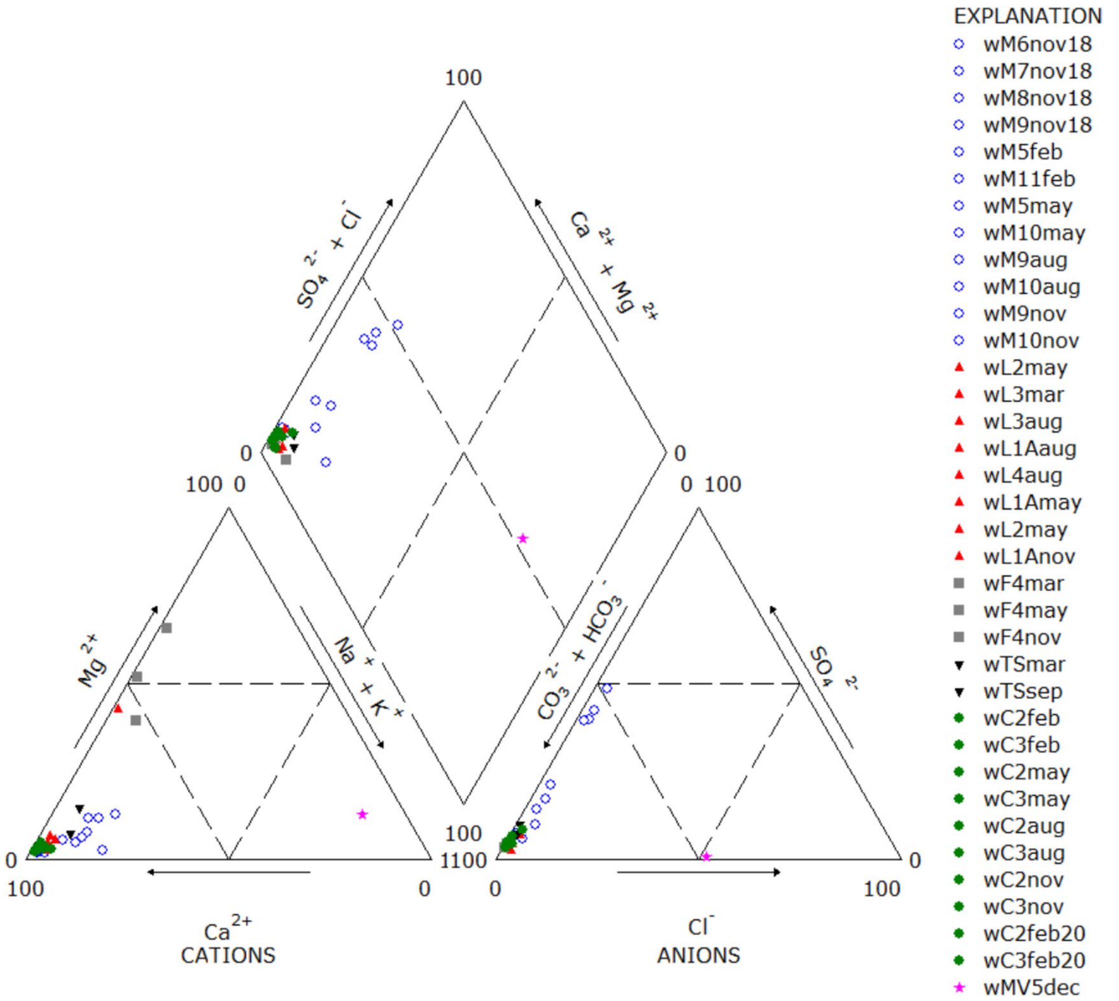


Fig. 3 Piper trilinear diagram showing chemical characteristics for water samples in the Romanian analysed pools. See Table 1 for abbreviations

samples are more or less clustered, with some of the winter samples better separated in space. These winter samples had a high abundance of *Flavobacterium*. Lesu seasons are clustered, much like Muieri, which had an exceptional May sample in station 5. This sample was characterised by a decrease in *Pseudomonas* and *Rhodoferax* with an increase in *Polaromonas* (Fig. 7A).

Like water, seasonal substrate samples are better separated in Ferice along both axes in the PCoA space (Fig. 7D). Closani samples were separated along F2 due to different abundances of *Nitrospira* and a non-assigned genus. Lesu had clustered seasons except for August and May samples when an increase in *Pseudomonas* and cvE6 was seen. *Pseudomonas* is ubiquitous, and cvE6 was found in aquatic environments [38] and moonmilk in caves [39]. August samples in Ferice had increased *Patescibacteria* (an unassigned genus belonging to the unclassified ABY1) and of an unassigned phylum. *Patescibacteria* are ultra-small bacteria with limited metabolic capacities, found in diverse environments, including aquifers [40,41]. In the Closani August sample, there was an increase in an unassigned genus belonging to *Saccharimonadales*, an order identified in cave sediments [42].

Discussion

Our research has uncovered significant patterns in the bacterial communities of pool water despite the unbalanced sample number collected from Romania's temperate and Brazil's tropical climates. The typical abundant phyla in both regions are similar to those in other karst caves or substrates (i.e. [43–45]). However, at the genus level, they were different, characterising the water and substrates in both countries, located on different continents and climates. These findings have important implications for studying microbial diversity in cave ecosystems, especially in the context of less-known cave microbial biodiversity in Brazil [46]. While continental

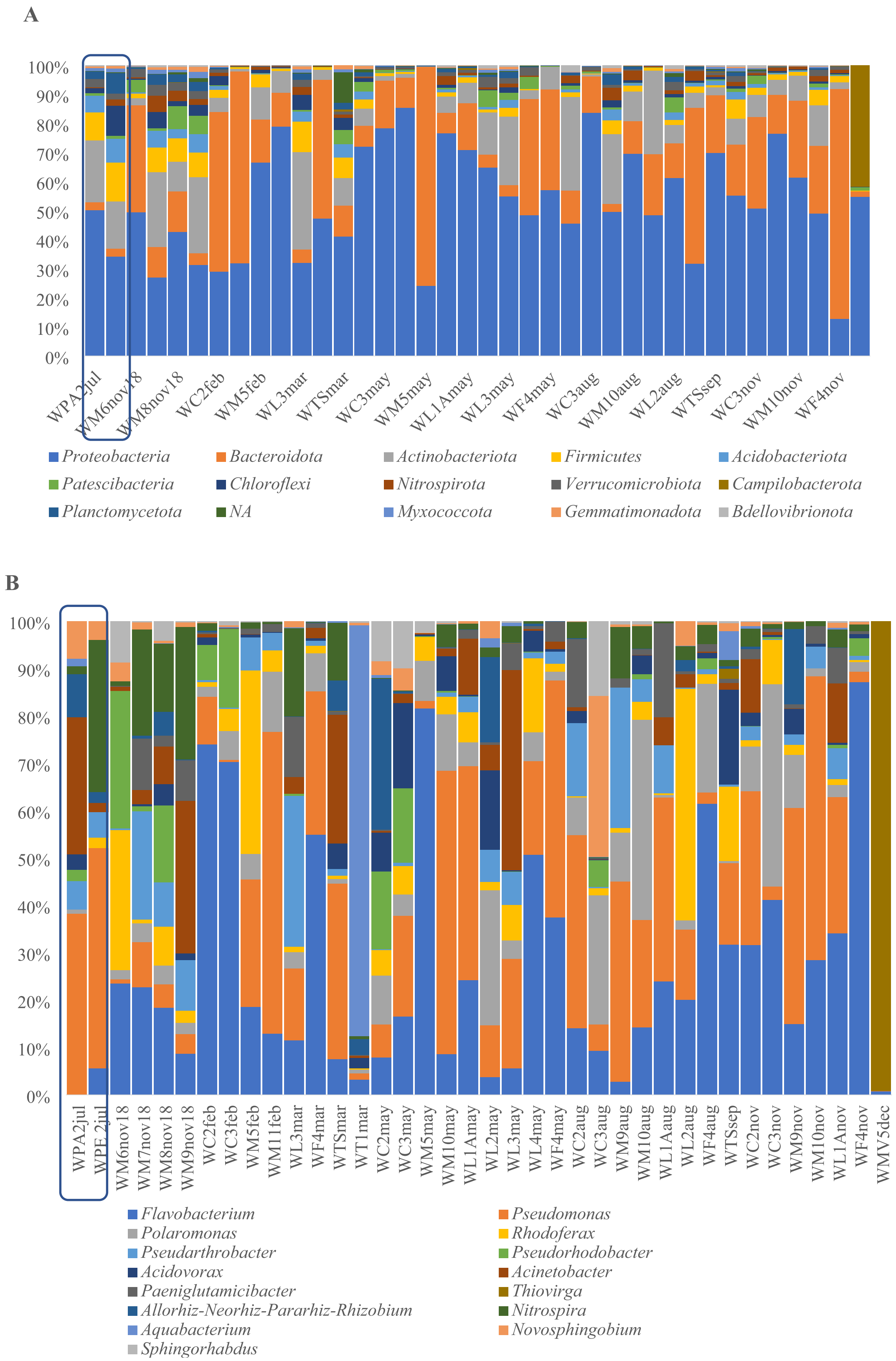


Fig. 4 The most abundant taxa in the studied cave microhabitats: phyla (**A**) and genera (**B**) in pool water and phyla (**C**) and genera (**D**) in the pool substrate. Brazilian samples are in rectangles. See Table 1 for abbreviations

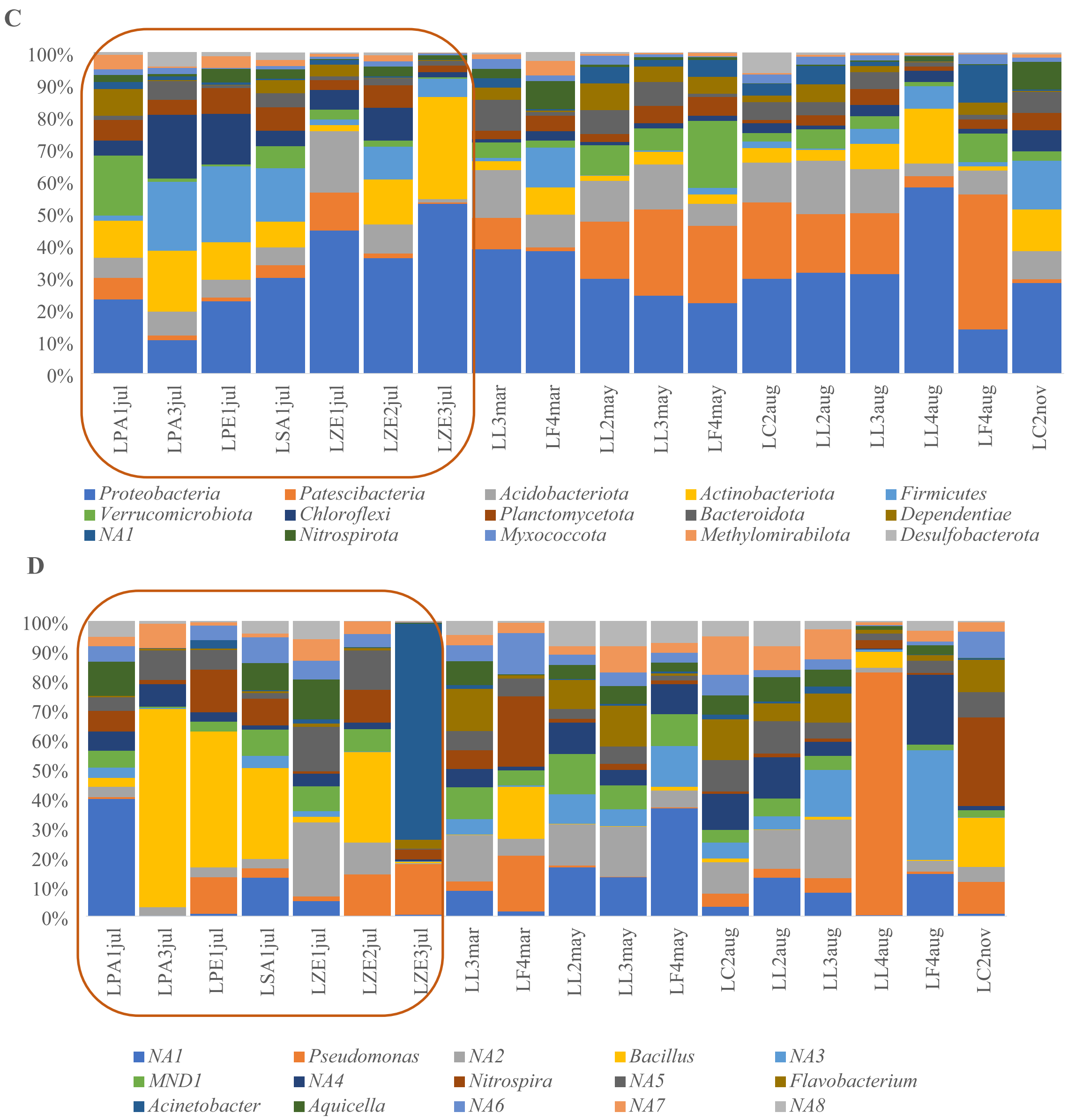


Fig. 4 (continued)

endemics at the genus and higher systematic levels are recognised among cave invertebrates [47], our current understanding of microbial endemicity still needs to be improved for a continental analysis.

Movile Cave stands out from the other sites we studied due to its sulphurous water, which supports a chemoautotrophic ecosystem [48]. This cave's microbiome differs significantly from tropical caves at similar temperatures. Groundwater bacteriomes are more similar in oligotrophic environments that characterise most caves worldwide, regardless of temperature. Temperature alone does not notably separate the bacteriomes of tropical and temperate groundwater, nor do physicochemical characteristics serve to differentiate the samples from oligotrophic caves. Although the Brazilian samples were relatively isolated in the analysis, they were more or less like Romanian samples, even in the colder Tausoare waters. All were different from the Movile sample.

Using seasonal variations in the same cave as a proxy for climate change, we noticed significant shifts in bacterial communities throughout the year, particularly in dominant taxa found in water and substrate. For example, the genus *Paeniglutamicibacter* was predominant only in the Lesu and Closani caves during summer, while *Novosphingobium* and *Sphingorhabdus* were dominant at station 3 of Closani in August. These last genera are aerobic and have been isolated

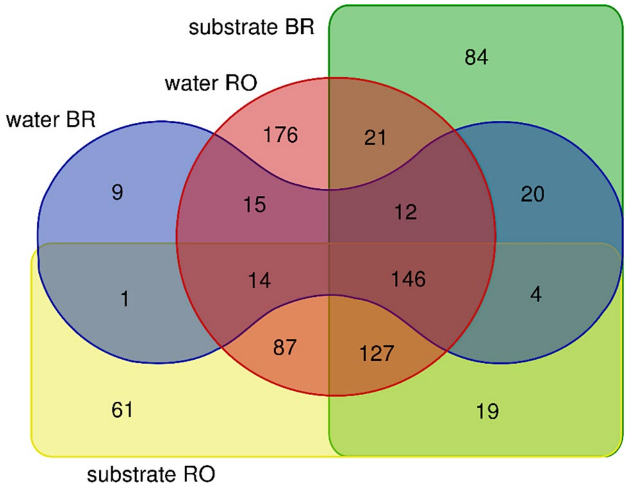


Fig. 5 Venn diagram of the bacteria shared genera in water and substrate from Brazilian and Romanian cave pools

from various environments; *Paeniglutamicibacter* found in other caves is known to precipitate calcite [49,50], a phenomenon that could intensify during low water season as in Romanian caves. In Closani station 2, there was also increased abundance in *Saccharimonadales* in August. This taxon is involved in the phosphorous cycle [51] in a station nearest a bat colony inside Closani Cave.

Interestingly, we observed changes in some key bacterial populations across all seasons, and no apparent summer effect was noted in any caves. Groundwater bodies are stable microhabitats that maintain their characteristics, particularly without human impact [52]. Variations in percolation rates, rock thickness, permeability, and other natural factors contribute to seasonal changes in some caves and sampling stations. For instance, increased levels of *Acinetobacter*, a possible pathogenic but also involved in phosphorous removal [53] from the water in August and November at Lesu Station 1A, particularly in May at station 3, may correlate with heightened bat activity. Nonetheless, short-term changes can be difficult to detect; therefore, monitoring bacteriome diversity should occur for longer intervals. Climate change acts as a region-dependent variable, producing effects like drying of the cave pools. Dryness is likely to emerge as a significant consequence of climate change, with unpredictable impacts on groundwater microbiomes, especially those already affected by human activities.

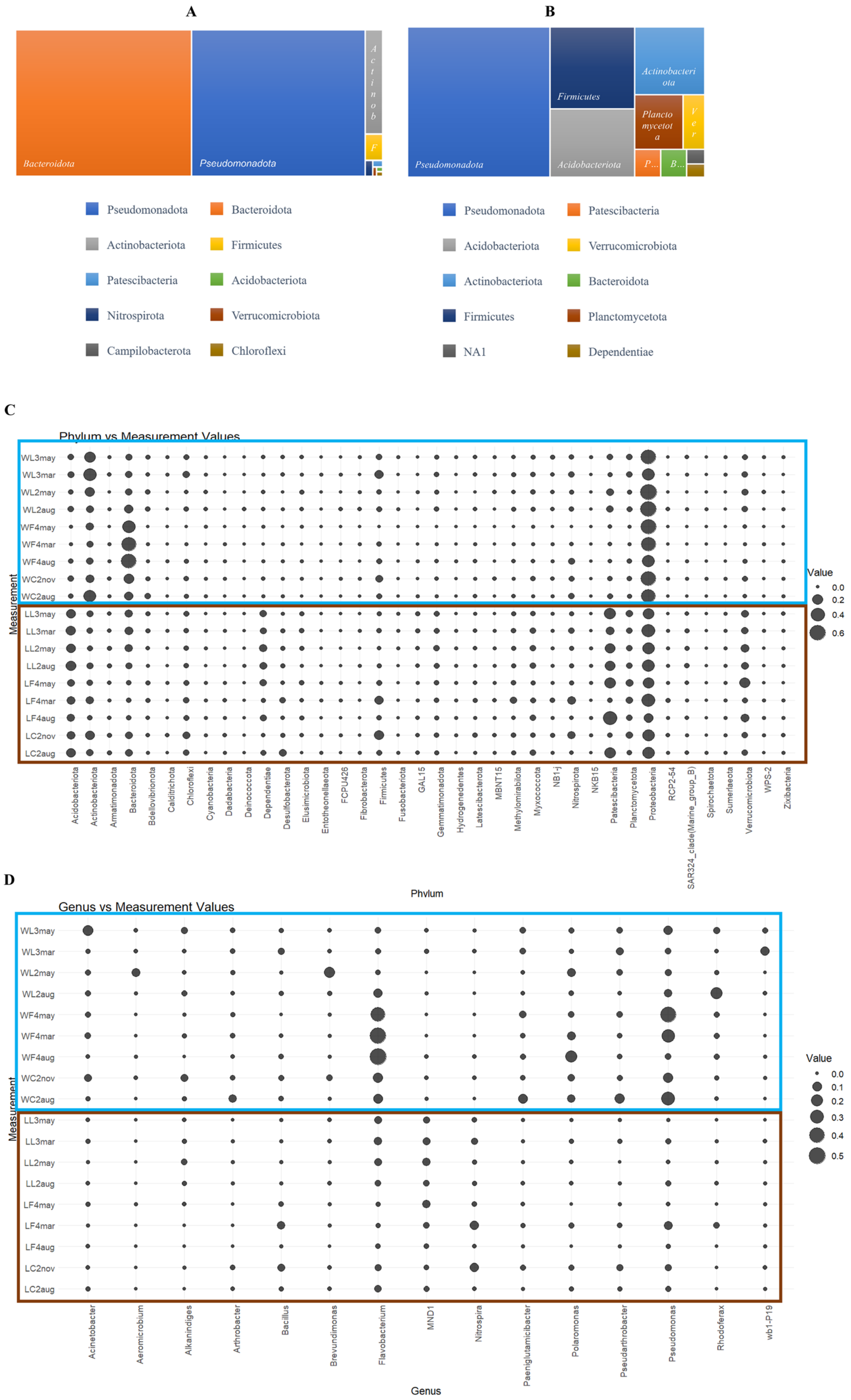
We observed the drying of pool water in summer in many of the studied caves. Groundwater temperature and availability influence biochemical processes [54]. The small water bodies within caves, supplied by surface percolation and hosting highly adapted organisms, are the first to experience drought and a decline in water quality. Changes in the environment can promote the growth of pathogens [55] or other bacteria (*Acinetobacter*), as seen in our data.

Another indicator of ecosystem change was the presence of *Polaromonas* in many August samples, which coincided with low water levels and increased hypoxia. Taubert et al. [56] demonstrated that *Polaromonas* and other genera utilised sulphur oxidisers to support autotrophic growth, enabling these microbes to use electron acceptors during periods of low oxygen, such as during dry months in Muieri, Ferice and Cloşani caves. The observed decrease in summer precipitation directly corresponds to climate changes in the area, as discussed in another Romanian cave study where some percolation points were utterly suppressed first during summer and later for the whole year [57]. Suppose such shifts in water inflow and temperature persist. In that case, we must consider how resilient the ecosystem will be, particularly in the small water bodies representing microhabitats within the karstic aquifers. Assessing the impact of climate change on these difficult-to-access habitats remains a complex challenge.

The two Brazilian cave water samples (Padre and PEA445) show a mixture of possible pollution indicators (*Vogesella* found dominant in groundwater of contaminated landfills [58] and waters with high nitrate concentration [59]), human indicators (*Cutibacterium*) and potential decontaminants (*Bacillus*). *Bacillus* modulates pH and oxygen levels in water through mineralisation and nitrification, mineralisation that also results in nutrient production. It can also remove phosphorus from water (see review in [60]).

The Brazilian pool substrate microbiomes displayed similarities to the water content found in Padre and PEA 445, mainly due to the abundance of *Bacillus*. In the Lapa d'Água do Zezé cave, one of the sampling stations featured a high concentration of *Acinetobacter*, with no explanation so far. Another prevalent bacterium, *Crossiella*, is commonly found in caves worldwide (e.g. [39,61]) and plays a crucial role in carbon sequestration and biotechnology as a source of active compounds [63].

In Romanian water samples, certain key bacteria are vital for the ecosystem of groundwater bodies. *Flavobacterium* was identified as the most abundant bacterium. It is a known denitrifier commonly found in contaminated groundwater from landfill sites [58]. *Pseudomonas*, a ubiquitous genus that includes species found in caves, is engaged in the nitrogen cycle and biomineralisation and inhibits the growth of bacteria and pathogens [14]. The third most abundant genus was the mixotrophic *Polaromonas*, which has metabolic capabilities for CO_2 fixation, reflecting the complexity of subsurface food webs [62]. This psychrotolerant genus is primarily known from polar regions [63] and is associated with organic acids. Some strains are nitrogen-fixing; others can utilise hydrogen and carbon dioxide as their primary energy sources and carbon during autotrophic growth (e.g. [64]). *Rhodoferax*, an iron (Fe)-reducing bacterium, has been noted in cave pools [65] and isolated from other freshwater environments [66].


A
Bacteroidota
Pseudomonadota
Actinob
F
Pseudomonadota
Bacteroidota
Actinobacteriota
Firmicutes
Patescibacteria
Acidobacteriota
Nitrospirota
Verrucomicrobiota
Campilobacterota
Chloroflexi
B
Pseudomonadota
Firmicutes
Acidobacteriota
Actinobacteriota
Planctomycetota
Ver
P...
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Pseudomonadota
Patescibacteria
Acidobacteriota
Verrucomicrobiota
Actinobacteriota
Bacteroidota
Firmicutes
Planctomycetota
NA1
Dependentiae
C
Phylum vs Measurement Values
Measurement
WL3may
WL3mar
WL2may
WL2aug
WF4may
WF4mar
WF4aug
WC2nov
WC2aug
LL3may
LL3mar
LL2may
LL2aug
LF4may
LF4mar
LF4aug
LC2nov
LC2aug
Value
0.0
0.2
0.4
0.6
Acidobacteriota
Actinobacteriota
Armatimonadota
Bacteroidota
Bdellovibrionota
Calditrichota
Chloroflexi
Cyanobacteria
Dadabacteria
Deinococcota
Dependentiae
Desulfobacterota
Elusimicrobiota
Entotheonellaeota
FCPU426
Fibrobacterota
Firmicutes
Fusobacteriota
GAL15
Gemmatimonadota
Hydrogenedentes
Latescibacterota
MBNT15
Methylomirabilota
Myxococcota
NB1-j
Nitrospirota
NKB15
Patescibacteria
Planctomycetota
Proteobacteria
RCP2-54
SAR324_clade(Marine_group_B)
Spirochaetota
Sumerlaeota
Verrucomicrobiota
WPS-2
Zixibacteria
Phylum
D
Genus vs Measurement Values
Measurement
WL3may
WL3mar
WL2may
WL2aug
WF4may
WF4mar
WF4aug
WC2nov
WC2aug
LL3may
LL3mar
LL2may
LL2aug
LF4may
LF4mar
LF4aug
LC2nov
LC2aug
Value
0.0
0.1
0.2
0.3
0.4
0.5
Acinetobacter
Aeromicrobium
Alkanindiges
Arthrobacter
Bacillus
Brevundimonas
Flavobacterium
MND1
Nitrospira
Paenglutamicibacter
Polaromonas
Pseudarthrobacter
Pseudomonas
Rhodoferax
wb1-P19
Genus

◂**Fig. 6** Comparison between the water and substrate microbiomes in Lesu, Ferice and Closani samples. The first 10 dominant phyla in the substrate (**A**) and water (**B**) show that two phyla were dominant in water, while the substrate shared dominance of more phyla. Bubble plots for the common most abundant phyla (**C**) and genera (**D**) in water and substrate. Water (W) = blue rectangles, substrate (L) = brown rectangles. Taxa that were not assigned were excluded from the analyses in **C** and **D**. See Table 1 for abbreviations

Pseudarthrobacter, abundant in the highly visited Muierilor and Cloşani caves, is capable of degrading organic pollutants under aerobic conditions. Its abundance decreases as anthropic pollution intensifies [58], making it a significant indicator of water quality. The genera mentioned above were found to be abundant in multiple caves.

In contrast, some genera were key indicator taxa in only one or two caves. For example, *Acinetobacter* was found in Lesu and is known for its role in pollutants' decomposition [67,68] and as a candidate for nitrogen removal from water [69,70]. *Paeniglutamicibacter*, found in Lesu and Cloşani, *Brevundimonas*, a bacterium related to phosphate [71] and human impact in a show cave [72], was also found in Lesu. *Brevundimonas* are unique in their ability to survive under simulated Martian conditions [73].

Relatively abundant in the substrates of some Ferice and Cloşani samples was *Nitrospira*, a chemolithoautotrophic [74] or mixotrophic [75] and nitrite-oxidizer [76]. *Nitrospora* was abundant in cave sediments [77] and moonmilk [78] and, together with MND1, is involved in carbon fixation, two-step nitrification [76] and ammonia oxidation [79,80] in caves. *Nitrospira* can also utilise urea (as bat colonies are present in both caves) as a nutrient source, allowing it to assimilate CO_2. At the same time, the ammonia and organic by-products enable coexistence with other microbes [76]. Identified in ferromanganese nodules, soils and caves, MND1 from the *Nitrosomonadaceae* family may serve different functions depending on environmental conditions [80,81].

An intriguing finding is the significant difference between the bacterial communities in the water and substrate of the

Table 2 Pearson's correlation matrices for the seasonal Bacteria abundances in the water of each of the studied Romanian pools. Only the most abundant 24 genera were included in the analyses. See Table 1 for abbreviations

	WM5feb	WM5may	WM9aug	WM9nov	WM10may	WM10aug	WM10nov
WM5feb	-	0.341					
WM5may	0.341	-					
WM9aug	0.410		-	0.700			
WM9nov	0.516		0.700	-			
WM10may					-	0.577	0.921
WM10aug					0.577	-	0.476
WM10nov					0.921	0.476	-

	WL1Amay	WL1Aaug	WL1Anov	WL2may	WL2aug	WL3may	WL3mar
WL1Amay	-	0.796	0.783				
WL1Aaug	0.796	-	0.880				
WL1Anov	0.783	0.880	-				
WL2may				-	-0.143		
WL2aug				-0.143	-		
WL3may						-	0.061
WL3mar						0.061	-

	WF4may	WF4aug	WF4nov	WF4mar
WF4may	-	0.546	0.583	0.892
WF4aug	0.546	-	0.942	0.857
WF4nov	0.583	0.942	-	0.876
WF4mar	0.892	0.857	0.876	-

	WC2feb	WC2may	WC2aug	WC2nov	WC3feb	WC3may	WC3aug	WC3nov
WC2feb	-	0.128	0.306	0.693				
WC2may	0.128	-	-0.022	0.028				
WC2aug	0.306	-0.022	-	0.723				
WC2nov	0.693	0.028	0.723	-				
WC3feb					-	0.462	0.151	0.698
WC3may					0.462	-	0.226	0.272
WC3aug					0.151	0.226	-	0.452
WC3nov					0.698	0.272	0.452	-

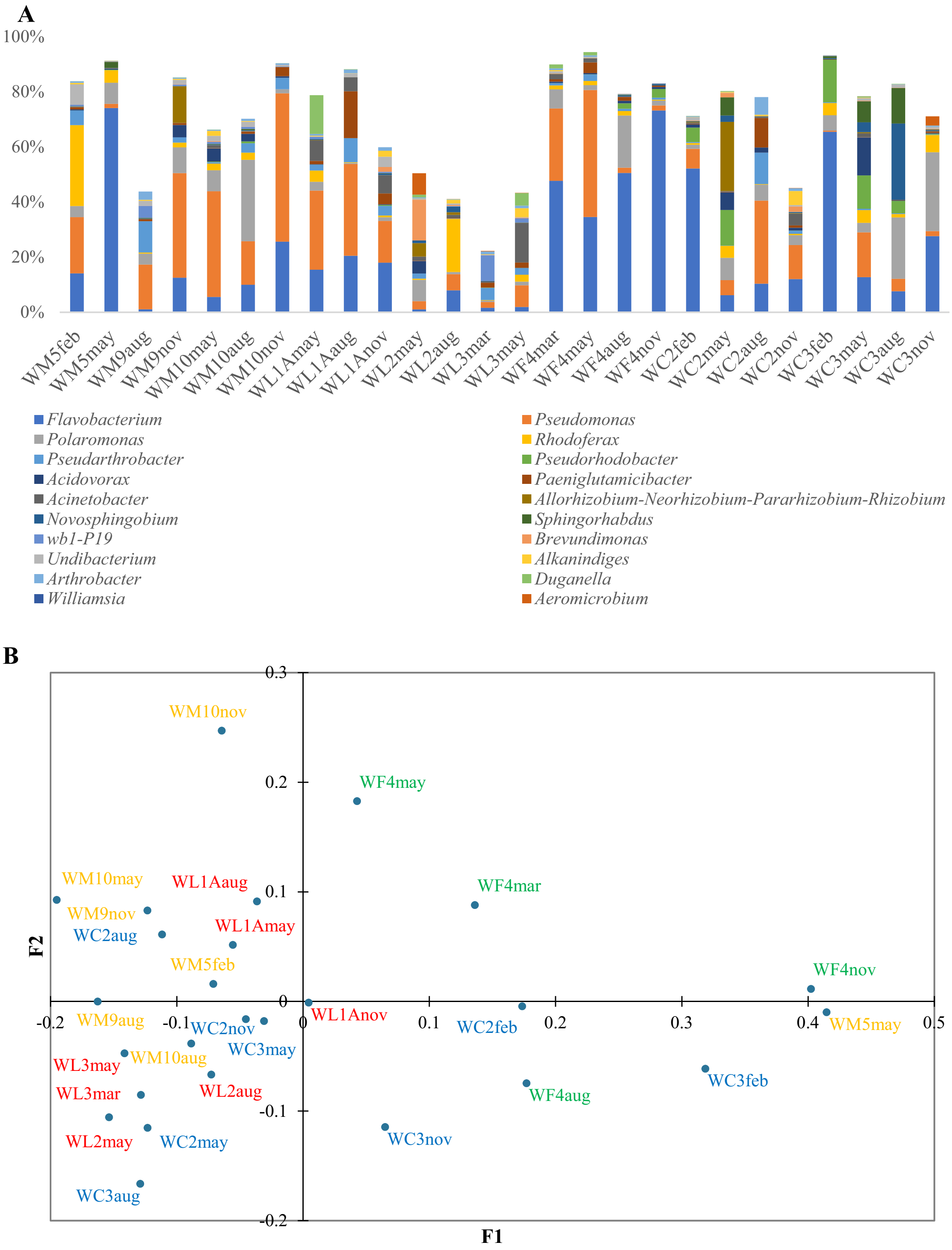

Fig. 7 The seasonal analysis of the most abundant genera of the microbiome in four of the Romanian sites: **A** Bar representation of the seasonal variation in water; **B** PCoA for the seasonal microbiomes in water; **C** bar representation of the seasonal variation in the substrate; **D** PCoA for the seasonal microbiomes in the substrate. See Table 1 for the samples' abbreviations and Supplementary Table S3

same pool. We anticipated that the bacteriomes in these overlapping micro-niches would be more similar. While bacteria from the substrate are also present in the water, they exist at much lower concentrations, possibly due to a small amount of water in the substrate samples. Nevertheless, the dominant bacteria in the substrate remain unclassified;

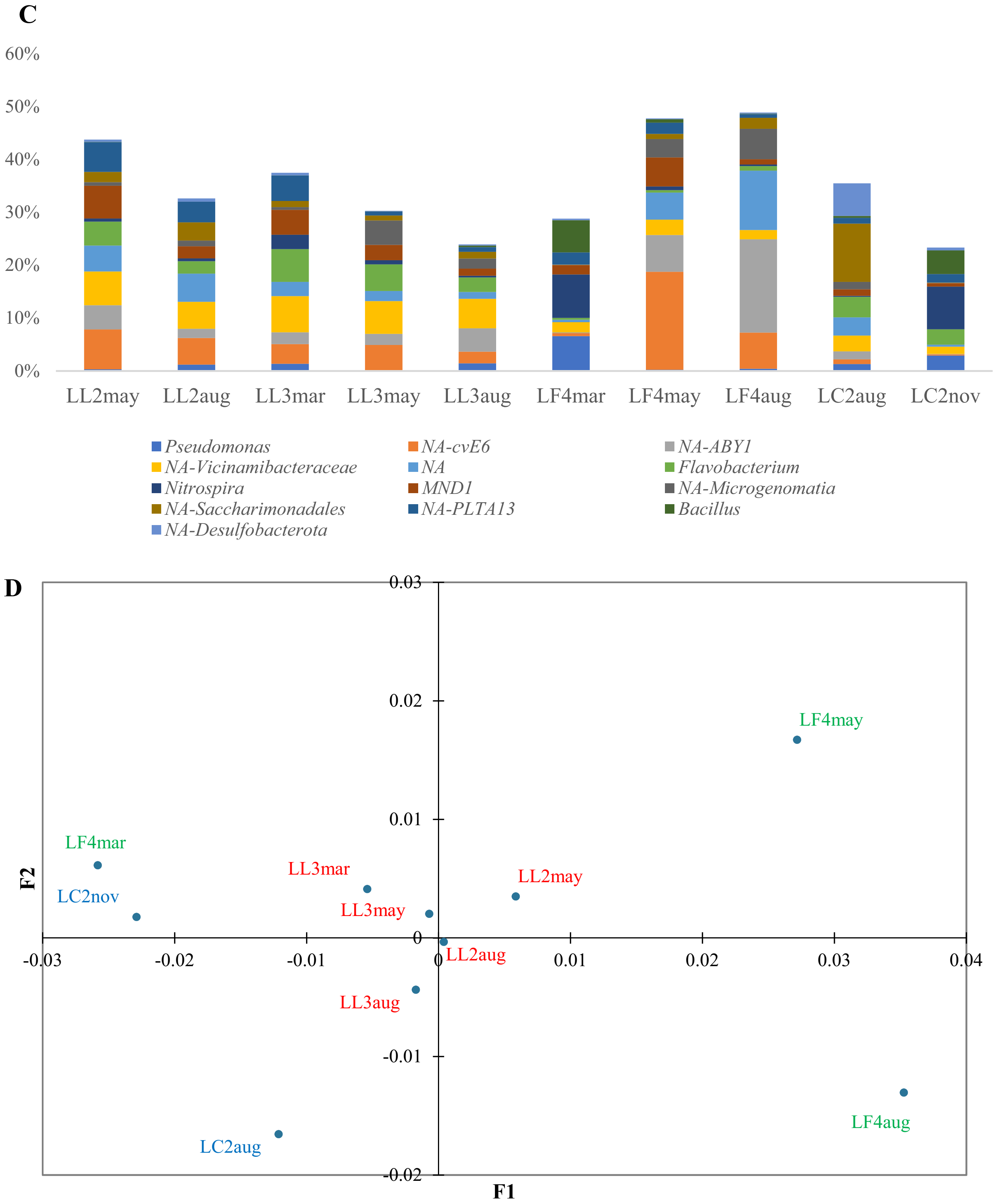

Fig. 7 (continued)

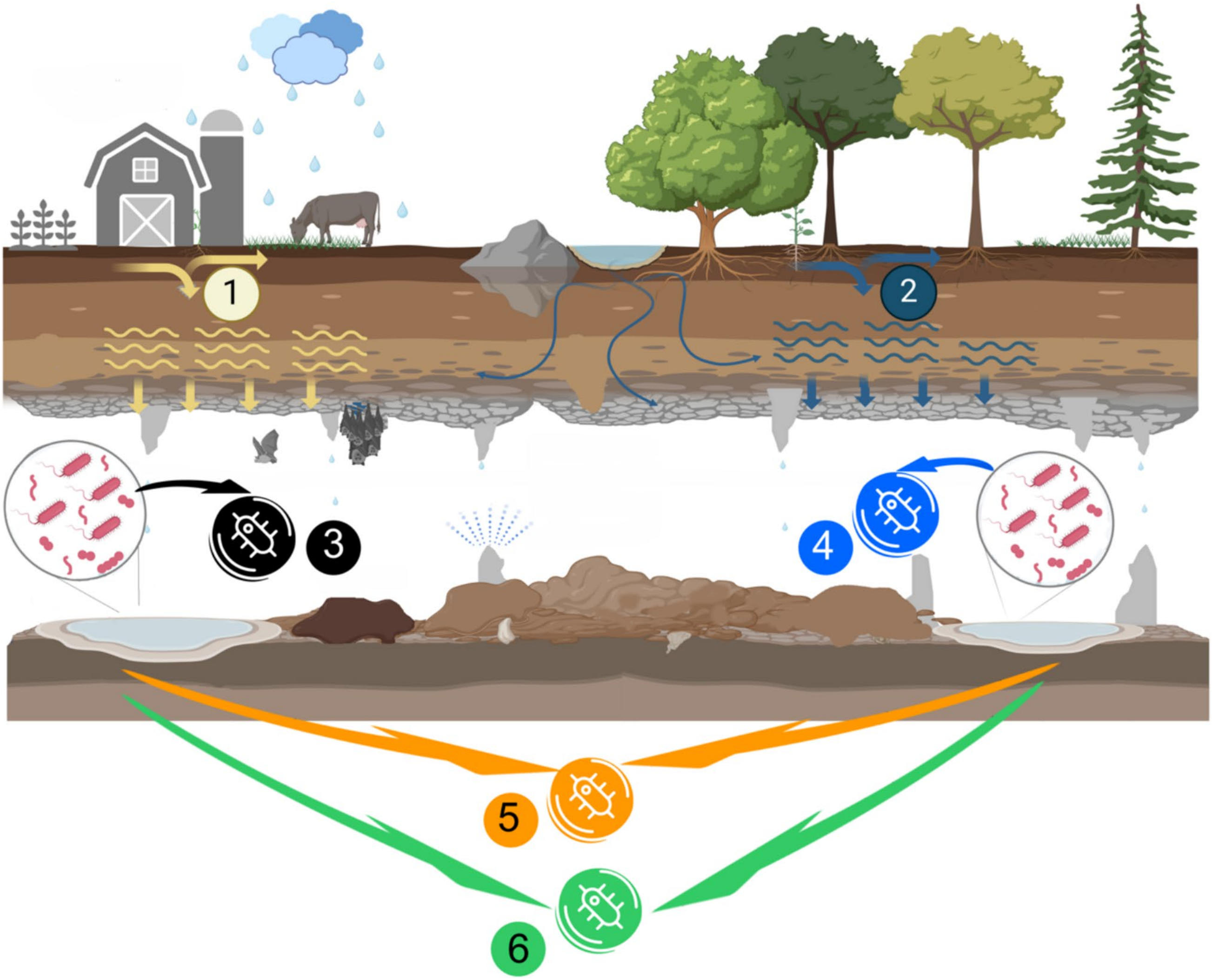

Fig. 8 Section through a cave showing the distribution of polluted (**1**) and unpolluted (**2**) percolation-fed pools with the different bacteriomes in pools and their proposed roles; (**3**) climate change proxy: *Acinetobacter*, *Polaromonas*; (**4**) anthropic pollution indicator: *Brevundimonas*, *Vogesella*; (**5**) nutrient source: *Flavobacterium*, *Nitrospira*, *Polaromonas*, *Pseudomonas*; ecosystem services (depollution, organic pollutants degradation, nitrogen removal, CO_2 assimilation, biomineralization, proxy for astromicrobiology): *Bacillus*, *Brevundimonas*, *Crossiella*, MND1, *Nitrospira*, *Novosphingobium*, *Polaromonas*, *Pseudoarthrobacter*, *Pseudomonas*, *Sphingorhabdus*. Created in https://BioRender.com

identifying these organisms and exploring their physiological roles could be crucial for understanding food chains and other ecological processes in caves. Further investigation into the substrate's microbial community is necessary to explore potential applications and deepen our understanding of this often-overlooked micro-environment.

Various authors have emphasised the stability over time and the space monotony of the subterranean environments [82]. However, groundwater fauna is highly fragmented [83,84], and the remarkable heterogeneity of the subterranean environment is reflected in inter-site and inter-cave differences, even within the same region or season. The microbial diversity and dynamics serve as an effective measure of a highly dynamic and variable environment. Subterranean microorganisms are often overlooked when assessing the diversity of underground ecosystems [85]. Groundwater should receive increased research attention, as it represents a significant reservoir and one of the last relatively undisturbed ecosystems, at least in certain areas. For instance, our study revealed that 1 phylum, 1 family and 398 genera (38.6%) were unassigned in the water samples. We identified 2 phyla, 1 family and 434 genera (42.18%) unassigned in the substrate. The unique composition and an elevated number of unassigned taxa thriving in cave pool substrates underscore the subterranean environment's remarkable diversity, even at a centimetre scale.

Chemolithotrophic bacteria and those in the nitrogen cycle are critical in supplying nutrients for the cave food web (Fig. 8). This function is frequently underappreciated in groundwater processes. Some of the identified taxa also contribute to depollution and carbon sequestration, serving as indicators of groundwater quality and as producers of active compounds. Other bacteria are candidates as indicators of climate change as their abundance is enhanced during dry periods and depleted oxygen. Despite their importance, the diversity of roles and interactions within this ecosystem remains poorly understood, yet they are essential for effective groundwater management. Their ability to adapt to environmental changes is significant in climate change research. Our short-term study observed no drastic fluctuations in the abundance of key bacteria during the summer; however, seasonal monitoring was a preliminary attempt to assess their behaviour during rising temperatures and diminished percolation, which could lead to increased pathogens that threaten aquifer endemic fauna.

Conclusion

This study explored the potential of bacteria as a proxy for climate change and human impacts from small groundwater bodies. Monitoring seasonal dynamics in cave pools might be an avenue to explore the use of key bacteria as indicators of groundwater micro-habitat resilience under climate change or human impacts. Monthly monitoring and long-term studies might be a better approach in such studies in capturing processes on this fine scale level.

Nevertheless, our study has implications for conserving small groundwater bodies and their substrates, commonly found in caves and often left unprotected unless they exhibit exceptional beauty. Conservation strategies and water resource policies are crucial for preserving all elements of groundwater ecosystems as integral parts of aquifers. The current climate change scenario accentuates this urgent need for groundwater management plans, especially as many regions globally confront water crises and declining water quality. Prolonged periods of dryness, coupled with increased human impacts, can disrupt the functioning of groundwater micro-ecosystems, potentially endangering the survival of endemic invertebrate species.

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Author Contribution O.T.M. designed the research, interpreted the results, and wrote the first draft of the manuscript. E.L. made the chemical analysis. R.L.F. and M.S.S wrote the description of Brazilian sites, I.C.M. wrote the description of Romanian sites. All the authors contributed to and approved the manuscript.

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Data Availability The raw data were deposited in the NCBI SRA Sequence Read Archive.

Declarations

Competing Interests The authors declare no competing interests.

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