



Adaptive response of prokaryotic communities to extreme pollution flooding in a Paleolithic rock art cave (Pindal Cave, northern Spain)

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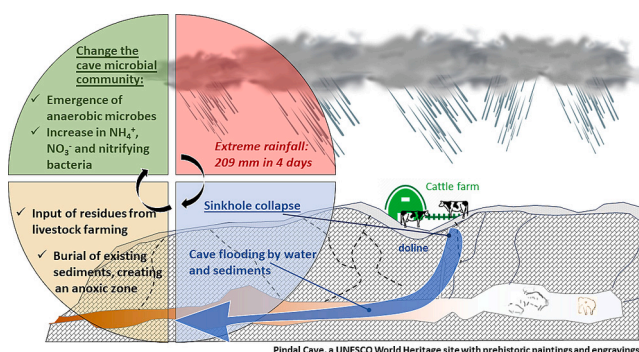
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HIGHLIGHTS

- Extreme rainfall and a sinkhole collapse is responsible for abrupt cave disturbance.
- Residues from livestock farming provokes NH_4^+ and NO_3^- rise in cave sediments.
- Cave flooding by water and sediments changed the subterranean microbial community.
- Anaerobic microbes and nitrifying bacteria emerge in cave sediments.
- Methanotrophy-related functional groups decrease and methanogenic *Archaea* increase.

GRAPHICAL ABSTRACT



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ABSTRACT

A flood event affecting Pindal Cave, a UNESCO World Heritage site, introduced a substantial amount of external sediments and waste into the cave. This event led to the burial of preexisting sediments, altering the biogeochemical characteristics of the cave ecosystem by introducing heightened levels of organic matter, nitrogen compounds, phosphorus, and heavy metals. The sediments included particulate matter and waste from a cattle farm located within the water catchment area of the cavity, along with diverse microorganisms, reshaping the cave microbial community. This study addresses the ongoing influence of a cattle farm on the cave ecosystem and aims to understand the adaptive responses of the underground microbial community to the sudden influx of

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waste allochthonous material. Here, we show that the flood event had an immediate and profound effect on the cave microbial community, marked by a significant increase in methanogenic archaea, denitrifying bacteria, and other microorganisms commonly associated with mammalian intestinal tracts. Furthermore, our findings reveal that one year after the flood, microorganisms related to the flood decreased, while the increase in inorganic forms of ammonium and nitrate suggests potential nitrification, aligning with increased abundances of corresponding functional genes involved in nitrogen cycling. The results reveal that the impact of pollution was neither recent nor isolated, and it was decisive in stopping livestock activity near the cave. The influence of the cattle farm has persisted since its establishment over the impluvium area, and this influence endures even a year after the flood. Our study emphasizes the dynamic interplay between natural events, anthropogenic activities, and microbial communities, offering insights into the resilience of cave ecosystems. Understanding microbial adaptation in response to environmental disturbances, as demonstrated in this cave ecosystem, has implications for broader ecological studies and underscores the importance of considering temporal dynamics in conservation efforts.

1. Introduction

Caves represent unique ecosystems where species-area relationships take shape within niches defined by environmental conditions and resource availability. Some researchers consider caves as “island-like” environments with lower microbial diversity compared to the outside world (Culver and Pipan, 2009; Wu et al., 2015; Lavoie et al., 2017; Ai et al., 2022). Oligotrophic caves offer an important setting for studying microbial community adaptation, where diversity is influenced by available energy and nutrients. This adaptation process encompasses a range of activities, from breaking down scant allochthonous organic carbon delivered by vadose-zone groundwater to autotrophic growth using in situ redox-active compounds (Hershey and Barton, 2018). Seepage water acts as a primary pathway for introducing microbial communities into subterranean environments from the outside. However, the ways microorganisms adapt to this new environment, characterized by stability and limited resources, remain largely unknown.

A fundamental question that remains unanswered is how bacteria existing within complex communities react to environmental changes, and how the surrounding biotic community influences evolutionary pathways as species adapt to new environmental conditions (Scheuerl et al., 2020).

Karst terrains are exceptionally susceptible to flood from groundwater sources due to their low storage and high diffusivity characteristics (Parise et al., 2015). Episodes of intense or prolonged rains can lead to significant increases in water levels within karstic systems (Naughton et al., 2018) leading to abrupt introduction of new sediments, nutrients and microorganisms from outside. Previous works have studied the influence of seepage water entry on the transformations of microbial assemblages in karstic aquifers and epiphreatic subsurface karst water pools (Simon et al., 2001; Shabarova et al., 2013, 2014 among others). Recently, Davis et al. (2020) showed how surface runoff exerts a transformative influence on the structure and functioning of the water cave microbiota. Their findings underlined those anthropogenic contaminants, along with microorganisms introduced from external sources, have the potential to initiate a change in the composition of indigenous cave communities, which could ultimately lead to the replacement of the autochthonous populations. Nevertheless, there are very few instances where its effect on the prokaryotic community inhabiting subterranean sediments has been comprehensively investigated.

This study focuses on examining the impact of an extreme flooding event on the prokaryotic community residing within the sediments of a shallow cave, specifically Pindal Cave in Spain, listed as a UNESCO World Heritage site. Since 2017, this cave has been a subject of research to characterize the microbial populations across different substrates and the interactions between microbes and minerals at the air-rock/speleothem/sediment interface (Martín-Pozas et al., 2022, 2023a). In 1995, a cattle farm was situated atop one of the primary sinkholes within the water catchment area of the cavity, which is directly located above the vertical shaft of the cavity. Between October 19 and 23, 2019, an

extreme episode of rainfall occurred in the area, resulting in a cumulative total of 209 l/m² and producing a strong accumulation of water in the sinkhole. The substantial water accumulation eventually triggered the collapse of the sinkhole, resulting in a rapid influx of surface runoff combined with water and sediments from the cattle farm that temporarily flooded the cave. A week after the flood and following the natural drainage, sediment sampling was conducted and compared with pre-flood samples to assess the immediate impact of the flood on the sub-surface microbial community (including *Bacteria* and *Archaea*). A year later, a second sampling aimed to investigate the medium-term evolution and adaptation of this microbial community to the underground environment.

This flood event presents a distinctive and invaluable opportunity to observe firsthand how established microbial communities react to the abrupt influx of external sediment, nutrients, and microorganisms. Here, we hypothesize that indigenous microbial populations adapted, evolved or potentially reacted to significant environmental disturbances caused by sudden pollution and changes in energy sources due to cave flooding. Furthermore, we propose that these adaptive ways can be elucidated from changes in the composition of the prokaryotic community and their metabolic strategies, which consequently control some biogeochemical processes that occur in cave sediments and ultimately affect the gaseous composition of the subterranean environment. The primary objective is to explore short- and medium-term changes in the composition of the prokaryotic community and how microorganisms adapt their metabolic strategies in response to shifting energy sources over time.

2. Material and methods

2.1. Site

The Pindal Cave (NW Spain, 4°30' W, 43°23' N) is a shallow limestone cave with a length of 590 m and an average width of 25 m. The depth of the galleries in relation to the exterior surface varies between 10 and 35 m. The cave has previously been comprehensively described in detail by Jiménez-Sánchez et al. (2006) and the most recent description of the microclimate of the cave was provided by Martín-Pozas et al. (2023a).

Pindal cave is part of a karst system located within a calcareous massif (Barcaliente Formation, Carboniferous) that has been extensively shaped by coastal morphogenetic processes, as evidenced by the presence of cliffs and multiple levels of wave cuts (Fig. 1), as documented by Goy et al. (2023). The cave is situated beneath one of the marine terrace levels, with an average elevation of 30–68 m above sea level (a.s.l.), serving as the primary catchment area for runoff water from this level and another wave-cut platform situated at +140–170 m a.s.l. This second platform, developed on quartzitic materials with very low permeability (Barrios Formation, Ordovician), contributes to the cave's hydrology.

The Pindal karst system exhibits significant development of karstic-related processes, including poljes, sinkholes, and karstic infill, which

dominate the area occupied by the cave. It is highly likely that most of these features are interconnected with the cave itself. Notably, one of the primary channels draining the Pimiango wave-cut platform, flows directly into a sinkhole located to the southwest of the cave and connected to it by a ponor (Fig. 1). This ponor area has been the site of an active cattle farm since 1995. The main discharge area of this drainage basin is situated within the doline containing the cattle farm and falls within the hydrological limits of the cave's impluvium. An extreme episode of rainfall occurring between October 19 and 23, 2019, led to the collapse of the ponor, resulting in the flood of the cave.

On the administrative side, El Pindal cave is open to the public through limited guided visits, and it is gated and guarded. It was designated a National Monument in 1924 and was granted the status of Property of Cultural Interest in 1985. Since 2008, it has been a part of the prestigious UNESCO List of World Heritage sites, specifically recognized as the Cave of Altamira and Paleolithic Cave Art of Northern Spain. The parietal art of Pindal Cave includes various painted and engraved figures. The main panel, located about 250 m from the entrance, features a prominent doe, intricate strokes, complex symbols, and a Magdalenian group of red paintings surrounded by engraved bison and horses. Just two of the animal figures keep apart from the rest of the panels, both belonging to pre-Magdalenian periods: a horsehead, painted about 120 m from the entrance and a mammoth, 200 m further away (González-Pumariega, 2011).

2.2. Sampling strategies

Location and sample descriptions are detailed in Fig. 2 and Tables S1-S2. To determine the sediment sample locations, cave illumination beacons were utilized, from the entrance (1) to the end of the accessible area (18). The study involved the analysis of sediment samples at

different times, both before and after the flood. The water rose to over 60 cm between lighting beacons 6 and 14, remaining trapped for >24 h (Fig. S1A). Subsequent to natural drainage, this led to the deposition of a centimetric layer of mud sediments covering the area comprised between lighting beacons 6 and 14 (Fig. S2).

The microbiological study focused on one of the areas most affected by the flood (beacon 9). Before the October 2019 flood (in November 2017), we analyzed the *Archaea* and *Bacteria* composition in sediment samples from the area 9, located about 125 m from the entrance. This area featured cave sediments heavily colonized by moonmilk deposits (a common microbial-induced mineralization in caves) and cave sediments without visible moonmilk deposits. At each sampling site, two sediment samples were collected: i) a superficial layer at 0–1.5 cm depth and a deeper layer of sediment at 1.5–3 cm depth (Table S1). Sampling of this area took place one week later (October 2019) and one year later (September 2020). The sampling strategy remained similar to that used before the flood: at each sampling site, two sediment samples were collected: i) a superficial layer of exogenous mud at 0–1.5 cm depth and a deeper layer of cave sediment at 1.5–3 cm depth (Table S2).

Additionally, sediment samples were collected to monitor physico-chemical changes in the areas 9 y 17 after the flood. For this purpose, we compared samples analyzed before the flood (March 2019) with samples obtained one week (October 2019) and one year after the flood (September 2020). The post-flood samples included a one fluvial sediment from an area far from the collapsed doline (beacon 17) and sediments with exogenous mud from the collapsed doline, from the area of the microbial study (beacon 9) (Fig. 2 and Table S2).

All samples were stored in sterile containers and transported at 4 °C to the laboratory. The samples aimed for sequencing analyses were preserved with DNA/RNA Shield™ and stored at –80 °C until further processing.

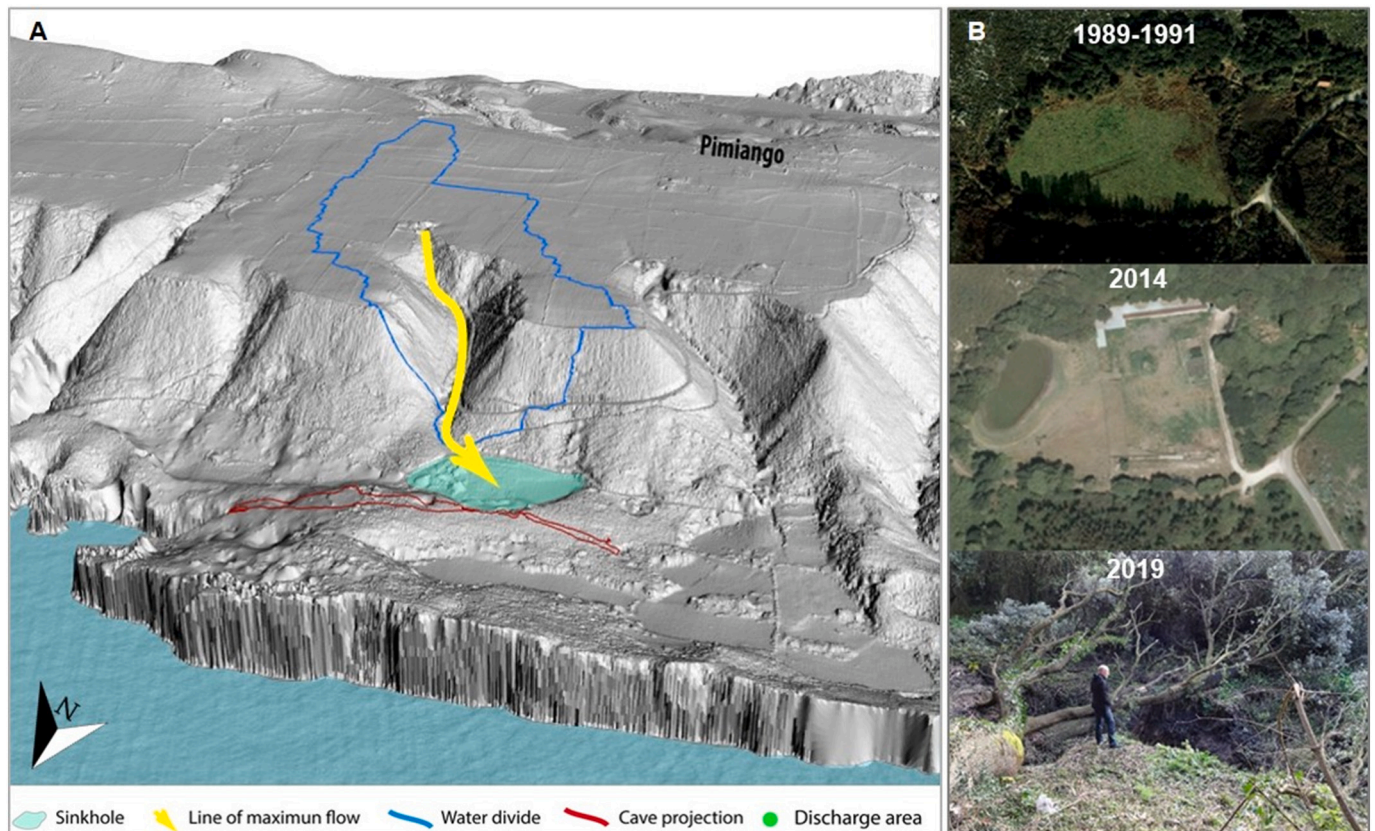


Fig. 1. Location of the main hydrological features in the Pindal karst system. A) 3D view from the NW. The water divide defines the catchment area draining to the sinkhole occupied by the cattle farm. The line of maximum water flow indicates the direct drainage from the catchment area to the sinkhole. B) Evolution of the cattle farm from 1995 to 2014 and the collapsed ponor in 2019.

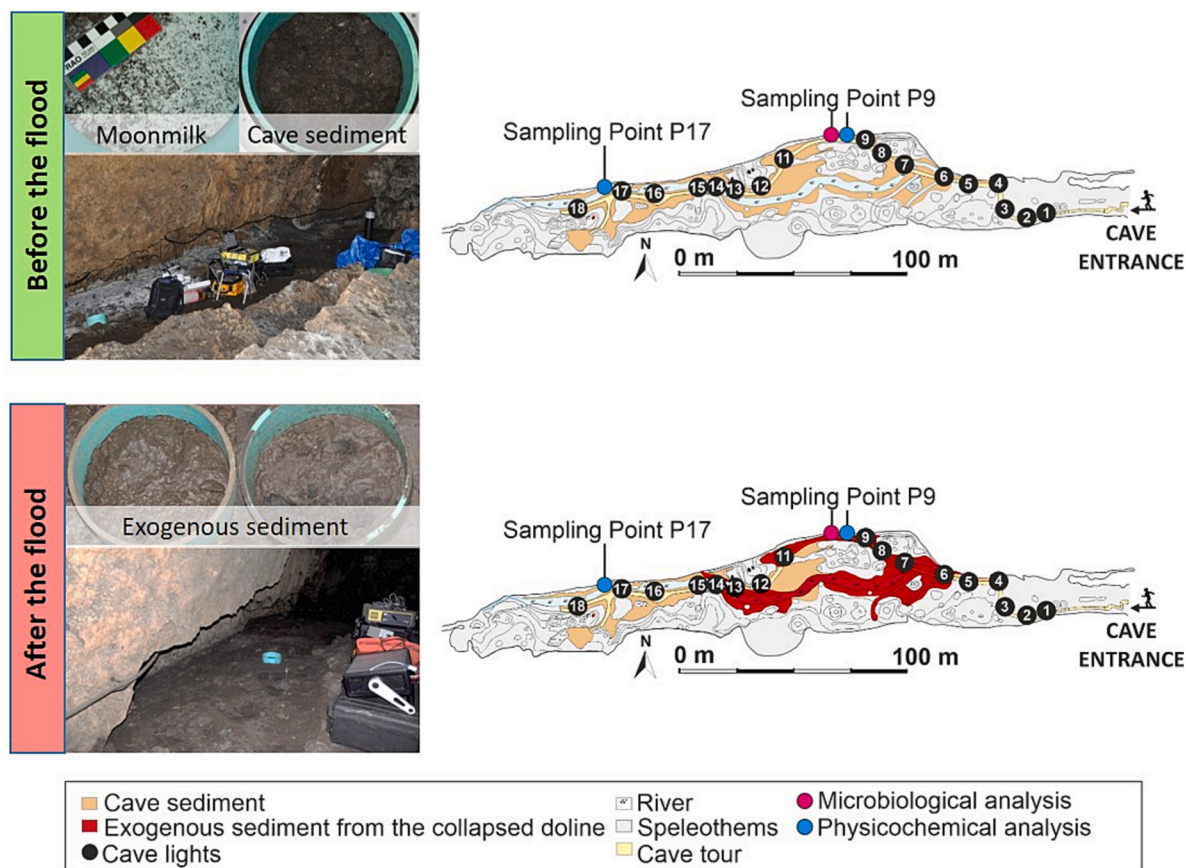


Fig. 2. Cave map indicating the regions impacted by the flood event in 2019, highlighted in red. Pink and blue circle marks the research area.

2.3. DNA extraction, sequencing, and sequence data processing

Total DNA of sediment and moonmilk samples was extracted with a Power Soil DNA Isolation Kit (MOBIO, USA) and FastDNA SPIN Kit (MP Biomedicals, France). The universal primers 341F/805R were used to amplify 16S rRNA (Herlemann et al., 2011). The library preparation was conducted following the protocol detailed by Martín-Pozas et al. (2020). Raw sequences were obtained from an Illumina MiSeq platform in two separate runs at AllGenetics (A Coruña, Spain) and deposited in the Sequence Read Archive (SRA) of NCBI under the project number PRJNA885499.

The analysis of the readings provided by the service of sequencing was performed using bioinformatics software QIIME 2 (Bokulich et al., 2018) and DADA2 (Callahan et al., 2016). Taxonomic assignments were performed by querying the sequence reads against the SILVA SSU 138 reference database (Quast et al., 2013). Raw sequences were processed using QIIME2 software (version 2019.10) following the procedures for quality filtration, demultiplexing, denoising and assigned with the feature-classifier plugin (Bokulich et al., 2018) the SILVA database (release 138) for *Archaea* and *Bacteria*. Alpha diversity was calculated using rarefaction curves and Shannon diversity index. The ASV table was analyzed using the phyloseq library and represented as relative abundances. To assess differences between sampling periods we employed the Kruskal-Wallis test. We tested differences in microbiome beta diversity between samples using permutational multivariate analysis of variance (PERMANOVA) and analysis of similarities (ANOSIM), with 999 permutations and Bray Curtis distance. Indicators taxa of different periods were predicted using the “indicpecies” library, with 999 permutations (Cáceres and Legendre, 2009). The functional MetaCyc pathways that differ between sampling periods were inferred by applying the PICRUSt2 tool to the 16S rDNA sequencing dataset (Douglas et al., 2019). PICRUSt2 downstream analysis to show

statistically significant differences on the predicted functional profile between the sampling periods were performed using the “ggpicrust2” library (Yang et al., 2023).

2.4. Physicochemical properties of sediments

Sediments were chemically characterized following the methods described by Martín-Pozas et al. (2022). The samples were crushed, sieved through a 2 mm sieve, and then ground to <60 mm, before pH, total organic carbon (TOC), organic matter (OM) and nitrogen (N) analyses. Metal and trace element concentrations were determined by digesting the samples with aqua regia and quantified using a VARIAN ICP 720-ES (simultaneous ICP-OES with axially viewed plasma). To assess the physicochemical properties throughout the study period, a 2-dimensional principal component analysis (2D-PCA) scores plot was generated, highlighting the similarities in geochemistry across the sampling periods March 2019, October 2019, and September 2020. Correlograms were created using the ggcorrplot package in R. Additionally, a one-way parametric ANOVA and Welch *t*-test analysis was performed to identify physicochemical properties exhibiting statistically significant differences (*p*-value <0.05) among the three sampling periods and two selected locations.

3. Results

3.1. Physicochemical parameters of sediments

Physicochemical data sets were examined to assess the possible arrival of wastes from the livestock farming located in the sinkhole above the cave. Two locations were selected for the study: Area 17, designated as a control zone with no direct connection to the dairy, and Area 9, suspected of being affected by the cattle farm.

We first explored the relationships between different physicochemical parameters before and after the flood (Fig. 3A). The correlations suggest that the physicochemical relationships showed distinct patterns between before and after the flood. Before the flood, significant positive correlations were observed between calcium carbonate (CaCO_3) and parameters such as organic matter (OM), total organic carbon (TOC), and ammonium (NH_4^+). One year after the flood, changes in pH and other parameters disrupted some of the previously observed correlations, highlighting significant decreases in the relationship between pH and various parameters, including organic matter, total organic carbon, total nitrogen, phosphorus, cobalt, and copper. These changes indicate a potential long-term impact on the system. Correlations provide information on the dynamic nature of the system, reflecting responses to different environmental conditions and disturbances.

Physicochemical data and an initial analysis showed notable variations between two locations (Table 1 and Fig. 3C). Sediments from location 9 showed higher concentrations of OM, total nitrogen Total N (Total N), phosphorus (P), nitrate (NO_3^-), NH_4^+ and metals compared to those from location 17. However, concentrations of NO_3^- and NH_4^+ showed *p*-values close to 0.05 in the Welch's *t*-test, while for the variables Total N, OM, TOC, Ba, Cu, Fe, Ni, Pb, and Zn, the observed differences were statistically significant between the locations.

The analysis through Principal Component Analysis (PCA), highlighted a similar trend in the physicochemical characteristics of the cave sediments before the flood and one week after the flood, while some differences were observed one year after the flood and also between both locations (Fig. 3B). The variables that contribute the most to the observed variability in each principal component are, in the case of Principal Component 1, pH, CaCO_3 , NO_3^- , NH_4^+ , and Pb have higher positive loadings and tend to increase together. Variables with higher positive loadings in Principal Component 2, such as NH_4^+ , P, Ba, Cu, Fe, Mn, and Pb, also tend to increase together.

These findings support the hypothesis that location 9, possibly affected by the waste from the cattle farm, shows a different composition due to different input conditions compared to location 17. Both locations showed changes after the flood, although the magnitude of these changes was more marked in location 9 compared to location 17. Variations in organic matter, total organic carbon, metals, nitrate, and ammonium were more pronounced at location 9. The decrease in Total N, OM, NO_3^- and NH_4^+ one week after flooding is attributed to leaching resulting from the entry of a large amount of water. In contrast, location 9 exhibits an increase in phosphorus levels a week after the flood, while location 17, the variation is not as marked. In both areas, the pH tends to increase a week after flooding and remains elevated one year later. The increases in the concentration of Total N, OM, NO_3^- and NH_4^+ recorded one year after the flood could be explained by the gradual entry of organic matter into the system and its slow decomposition over time, releasing more organic matter and nutrients in the form of NO_3^- and NH_4^+ .

3.2. Microbial diversity

We assessed alpha and beta diversity differences of *Bacteria* and *Archaea* during three different periods: before the flood, one week after the flood, and one year after the flood (Fig. 4). Alpha diversity was significant higher before the flood ($P \leq 0.05$). We used PERMANOVA and ANOSIM on Bray distances to test for dissimilarities in bacterial and archaeal composition among the studied samples (Tables S3-S6). The results revealed that samples clustered by sampling period explained the majority of the observed variation in the communities. This difference between sampling periods was further supported by PCoA, demonstrating that microbial communities in 2019 exhibited greater dissimilarity compared to those in 2017 and 2020.

3.3. Bacterial community

Fig. 5A illustrates the bacterial community composition at the phylum level. Sequencing results showed great changes in microbial composition, after the flood. Before the flood, the most representative phyla were *Pseudomonadota*, *Actinomycetota*, *Chloroflexota*, *Acidobacteriota*, *Planctomycetota*, *Gemmatimonadota*. Immediately after the flood, a substantial increase of *Bacteroidota* and *Bacillota* was observed, accompanied by a general decrease in the abundance of *Acidobacteriota*, *Nitrospirota* and *Methylomirabilota*. However, one year after the flood, *Pseudomonadota*, *Acidobacteriota*, *Methylomirabilota*, *Gemmatimonadota* and *Nitrospirota* were the most abundant phyla while *Actinomycetota*, *Bacteroidota* and *Bacillota* presented very low abundance.

Fig. 6 and Supplementary Table S7 show the composition at the genus level. The most abundant sequences before the flood were affiliated to the lineage wb1-P19 within the phylum *Pseudomonadota* and the genus *Crossiella* within the phylum *Actinomycetota* (Fig. 6A). These sequences were especially abundant in the moonmilk sample (P91-B) and also present in sediment samples. One week after the flood, the percentage of *Crossiella* and wb1-P19 generally decreased as a result of the deposition of exogenous mud layers (P91-F and P93-F) (Fig. 2). However, similar values were maintained in the deeper sample, corresponding to the moonmilk deposit covered by mud (P94-F). One year after the flood, a decrease in *Crossiella* and wb1-P19 was observed in the deeper layers (P92-F and P94-F), while a slight increase was noticed in the surface layers (P91-A and P93-A), compared to the samples from the flood period. A remarkable event was the dramatic increase of the family *Beijerinckiaceae* observed in sample P94-F immediately after flood. However, one year after the flood, these values were reduced to below 1%.

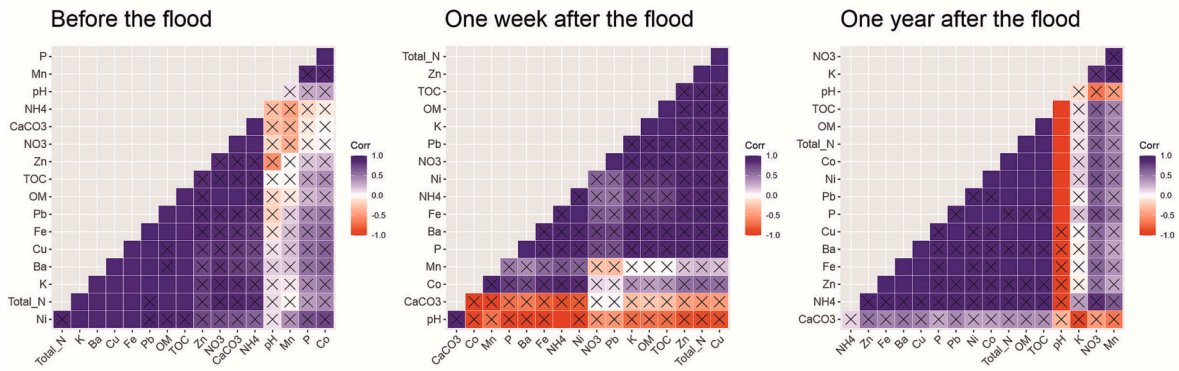
Immediately after the flood, there was a notable decrease in the percentages of other previously dominant sequences affiliated with the phyla *Acidobacteriota* (Subdivision 10), *Chloroflexota* (UTCXF1, RGB-13-53-9, JG30-KF-CM66, uncultured members of the family *Anaerolineaceae*), *Planctomycetota* (*Pirellulaceae*, Pir4 lineage, *Gemmataceae*), *Pseudomonadota* (wb1-P19, PLTA13, IheB2-23), and *Verrucomicrobiota* (*Pedosphaeraceae*). All of these sequences were considered as pristine bacteria affected by the flood (Fig. 6B). One year after the flood the relative abundances of the family *Pedosphaeraceae* and the taxa wb1-P19, PLTA13 were more similar to those found before the flood, indicating a recovering of some taxa along the time.

One week after the flood, there was a significant increase in the relative abundances of specific sequences within the phyla *Actinomycetota* (*Intrasporangiaceae*, *Corynebacteriaceae*), *Bacillota* (*Christensenellaceae* R-7 group, *Bacillus*), *Chloroflexota* (*Longilinea*), and *Pseudomonadota* (*Luteimonas*, *Paracoccus*, *Sphingomonas*, *Thauera*, *Xanthobacteraceae*). These sequences corresponded to bacteria introduced into the cave with the flood (Fig. 6B). Particularly, the genera *Thauera*, *Christensenellaceae* R-7, *Longilinea*, *Fermentimonas*, *Paracoccus*, and the families *Corynebacteriaceae*, *Prolixibacteraceae*, were absent before the flood but exhibited relative abundances over 1% in the superficial exogenous cave sediments (P91-F and P93-F) (Fig. 6A). Furthermore, one year later, these sequences were either absent or nearly absent, suggesting that they were unable to find favorable environmental conditions for long-term survival.

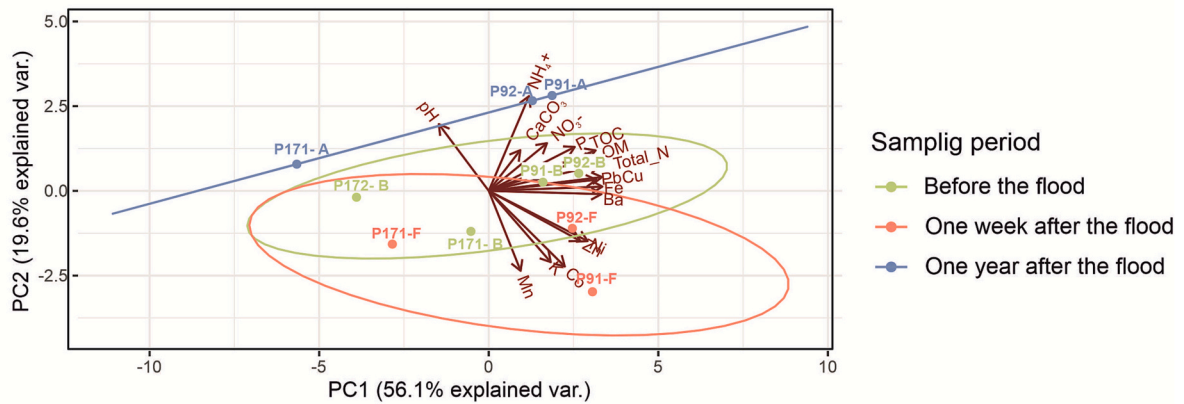
One year after the flood sequences within the phyla *Methylomirabilota* (wb1-A12), *Nitrospirota* (*Nitrospira*), and *Pseudomonadota* (B1-7BS, *Desulfarculaceae*, IS-44, mle1-7, NB1-j, TRA3-20) were found (Fig. 6B). These bacteria presented abundances over 1% in the samples before the flood, which decreased after the flood and increased again one year after the flood (Fig. 6A). The most outstanding increase corresponded to the genus wb1-A12 within the family *Methylomirabilaceae*.

Finally, the prediction tool PICRUST2 was used to examine the functions of prokaryotic communities (Figs. S2-S4). Principal Component Analysis (PCA) and LinDA analysis identified differentially abundant bacteria and archaea between the sampling periods. PCA

A Correlograms of physicochemical features



B PCA of physicochemical features



C Variable physicochemical features

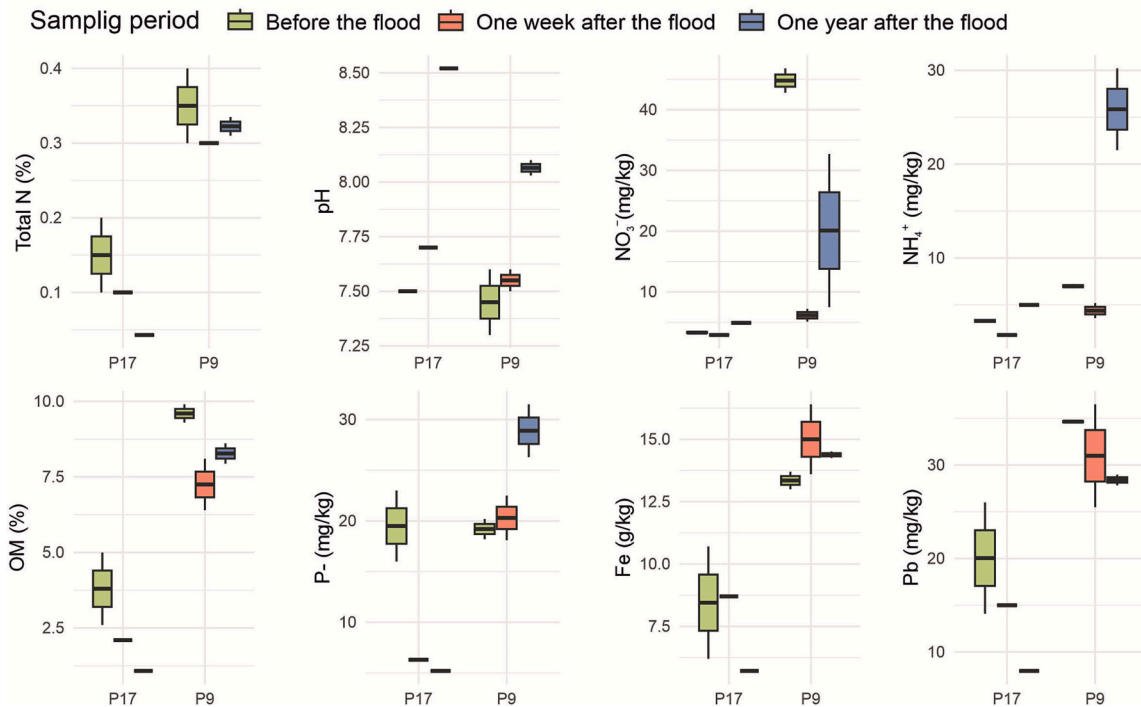


Fig. 3. Physicochemical analyses at each sampling event in two different locations: one directly affected by the doline collapse (P9) and another location far from the area affected by the flood (P17). A) Correlogram of physicochemical features at each sampling event. Blue indicates positive correlations and red negative correlations. The boxes marked with an X indicate p -value of >0.05 for the correlation. B) 2D-PCA scores plot of physicochemical features at each sampling event. Ellipses indicate 95 % confidence intervals. Vectors show how individual physicochemical analyses contribute to overall dataset. C) One-way parametric ANOVA analysis indicating physicochemical features whose levels are significantly different (p -value <0.05).

Table 1

Results of the analyses of pH, calcium carbonate, organic matter (OM), macronutrients (N, P, K) and trace elements of the sediment samples. Highlighted data in bold.

Sampling Period	Before the flood (March 2019)				One week after the flood (October 2019)			One year after the flood (September 2020)		
	9		17		9		17	9		17
Location										
Sample	P91-B	P92-B	P171-B	P172-B	P91-F	P92-F	P171-F	P91-A	P92-A	P171-A
pH	7.3	7.6	7.5	7.5	7.5	7.6	7.7	8.0	8.1	8.5
CaCO ₃ (%)	2.3	2.1	0.2	0.2	3.2	3.5	3.5	1.9	9.5	2.3
OM (%)	9.3	9.9	5.0	2.6	6.4	8.1	2.1	8.6	7.9	1.1
TOC (%)	4.7	5.8	2.6	0.8	3.5	4.3	1.1	5.0	4.6	0.6
Total N (%)	0.3	0.4	0.2	0.1	0.3	0.3	0.1	0.3	0.3	0.04
NO ₃ ⁻ (mg/kg)	42.8	46.8	3.1	3.5	5.1	7.2	2.9	32.7	7.5	4.9
NH ₄ ⁺ (mg/kg)	7.2	6.8	3.1	3.5	5.2	3.6	1.8	30.2	21.5	5.0
P- (mg/kg)	18.2	20.2	23.0	16.0	22.5	18.1	6.3	31.5	26.3	5.2
Ba (mg/kg)	62.6	76.5	56.4	35.4	87.8	80.6	50.0	74.4	77.0	30.9
Co (mg/kg)	3.1	4.1	5.0	2.1	7.8	4.4	4.0	3.4	3.1	1.0
Cu (mg/kg)	11.6	13.6	10.4	6.6	14.6	15.1	6.1	14.4	14.4	3.5
Fe (g/kg)	13.0	13.7	10.7	6.2	16.4	13.6	8.7	14.5	14.2	5.7
K (g/kg)	5.2	6.6	4.0	2.1	4.9	5.6	2.7	5.4	5.9	1.9
Mn (mg/kg)	200	201	344	159	680	279	392	259	233	237
Ni (mg/kg)	9.7	11.3	9.6	5.3	17.0	14.3	10.1	10.9	9.8	4.0
Pb (mg/kg)	34.5	34.8	26.0	14.1	25.5	36.5	15.0	29.0	27.8	8.0
Zn (mg/kg)	50.8	38.0	28.8	16.8	67.9	70.5	27.8	32.6	33.7	16.7

conducted using PICRUST2 data, showed the highest variability before the flood, whereas the periods one week after and one year after the flood showed greater similarity (Fig. S2). Interestingly, some ecological functions related to energy metabolism were significantly abundant one week after the flood: denitrification and superpathway of C1 compounds oxidation to CO₂ (Fig. S3).

3.4. Archaeal community

Fig. 5B shows the structure of the archaeal community at the phylum level, while Fig. 7 and Supplementary Table S8 display the genus-level composition. *Thermoplasmata* dominated the community both before and one year after the flood, with high abundances (up to 60 %). However, one week after the flood, there was a significant increase of *Halobacterota* while one year after the flood, *Halobacterota* sequences nearly disappeared.

One week after the flood, exogenous mud samples (P91—F and P93—F) showed increased relative abundances of sequences within the phylum *Halobacterota* (*Methanomicrobiales*, *Methanosaeta*, *Methanolobus*, *Methanosarcina*). Interestingly, two of these sequences, *Methanosaeta* and *Methanosarcina*, were predicted as indicators of the flood and were absent both before and one year after the flood, indicating their association with the entry of material during the flood event in Pindal Cave (Fig. 7B). Related to these methanogenic archaea, PICRUST2 analyses showed that methanogenesis from H₂ and CO₂ was significantly more abundant one week after the flood (Fig. S3).

After the flood, other notable changes in sequence within the phylum *Crenarchaeota*. The family *Nitrosopumilaceae* maintained similar levels during the three periods, while the family *Nitrososphaeraceae* reached proportions over 1 % after the flooding event. The phylum *Thermoplasmata* recovered to similar levels one year later. One of the most abundant sequences within this phylum were related to an uncultured member of the order *Methanomassiliococcales*, with lower abundances in 2017 and 2019 but reaching over 40 % one year after the flood. This order maintained high levels in the three sampling periods, while other uncultured members of the phylum decreased their abundance one week after the flood but exceeded 5 % in all 2020 samples one year after the flood.

4. Discussion

4.1. Flood impact and farm influence on Pindal Cave Ecosystem

Karst terrains are exceptionally susceptible to flood due to a combination of low storage and high diffusivity characteristics of these

aquifers (Parise et al., 2015). Within caves, characterized by a scarcity of exogenous nutrients and energy, microbial communities play a crucial role in the main biogeochemical cycles, essential for maintaining equilibrium and functionality in the subterranean ecosystem. In this context, floods are significant events that can individually remodel cave ecosystems, even more so if they are accompanied by anthropogenic contaminants, as is the case with Pindal Cave. The main objective of the study is to understand the adaptive responses of the underground microbial community to the sudden influx of waste allochthonous material. This research not only contributes to understanding of subterranean microbial ecology, but also has direct implications for the conservation of the cave environment and cultural heritage that make Pindal Cave a unique and valuable site.

In the case of Pindal Cave, the runoff water circulates directly towards the catchment area of Pindal karst system, in channels of little distance and more or less torrential depending on the intensity of the rainfall. Once it reaches that catchment area, it preferentially accumulates in the different karstic depressions (sinkholes) and there, it joins the fall directly over the impluvium zone, infiltrating the carbonate massif (Fig. 2A). This infiltration is favored by the subvertical dip and the high degree of fracturing of the Barcaliente limestone, but in general, percolation occurs progressively due to the filtering effect exerted by the clayey materials that cover the bottom of the sinkholes. The intense rainfall that fell during the previous days and on the October 23rd itself, favored an important accumulation of water in the sinkhole that finally caused the collapse of its natural sink (Fig. 2B). This collapse can still be seen in the external area and caused the sudden entry of a large volume of water affected by its mixture with the slurry pool and the arrival of sludge-sediments largely from the livestock farm.

The analytical data showed the strong influence of the cattle farm in the composition in the area affected by the flood, particularly in terms of organic matter, nitrogen, phosphorous and metals contents. Extensive livestock farming has been shown to have harmful effects on the ground water environment and human health causing pollution by nitrate, heavy metals, antibiotics, and pathogenic microbes (Pasquarell and Boyer, 1995; Boyer and Pasquarell, 1999; Laiz et al., 1999; Kelly et al., 2009; Sahoo et al., 2016). Soil organic matter exhibits a strong affinity for specific metals (such as Co, Cu, Mo, Ni, Pb, and Zn) due to the formation of organometallic complexes that can readily migrate with infiltrating water, resulting in increased metal mobility under acidic conditions, as illustrated in Fig. 3.

The high concentrations of nitrogen compounds, metals, and organic matter content in the site 9 both before and after the flood event (Fig. 3) provides evidence that the impact of the livestock facility on cave

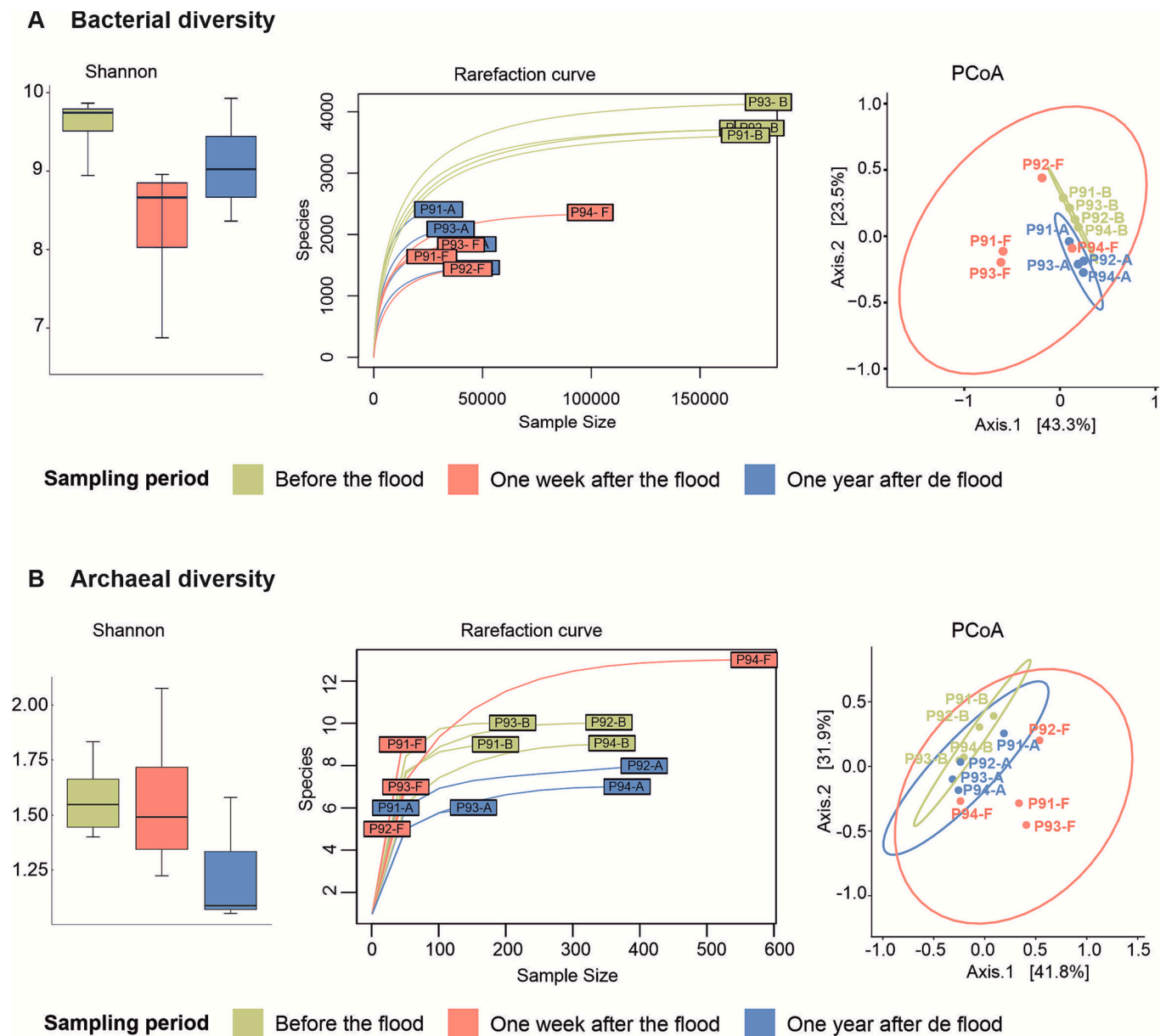


Fig. 4. Diversity of microbial communities before the flood, one week after the flood, and one year after the flood. A) Bacterial diversity for site 9 each sampling period. B) Archaeal diversity for site 9 each sampling period. Microbial alpha diversity was calculated using the Shannon Index and rarefaction curves. Microbial beta diversity was calculated using Principal Coordinates Analysis (PCoA) derived from Bray-Curtis distances among samples of the three sampling periods ($p < 0.05$ by PERMANOVA).

sediments is not of recent origin. Its influence probably exerted a gradual and prolonged influence over time, with a particularly pronounced effect at site 9, where water retention during flood episodes is prolonged. Sediments within this cave zone exhibited distinctively elevated levels of phosphorus both before and subsequent to the 2019 flood event. The observed high levels of phosphorus a week after the flood could be attributed to its relatively immobile nature in soils. Unlike nitrate and ammonium, phosphate tends to form insoluble compounds through precipitation and adsorption to mineral surfaces (Lehmann and Schroth, 2002; Qin et al., 2020). The contrasting behavior of phosphorus, nitrate, and ammonium levels suggests that while the flood event may have caused leaching of some compounds, the mobility and availability of phosphorus were less affected compared to nitrogen compounds. The high levels of phosphorus in site 9 post-flooding support that the flood event led to the entry of external organic material from the cattle farm rich in phosphorus into the cave

system.

Based on field observations and a hydrological model, water from livestock farming enters the cave through an upwelling point at site 13 (Fig. 2). From there, its influence gradually extends towards the north wall and the cave exit affecting, to a greater or lesser extent a considerable portion of the accumulated sediments from site 6 to the sites 14–15–16. The composition of sediments in the site 17 suggests that their origin is different and that external recharge must occur to the NW of the cattle farm.

4.2. Comparative study of microbial communities before and after the flood

Our microbiological analysis reveals a substantial shift in microbial community composition between samples collected before and one week after the flood event (Fig. 5). The most abundant taxa before the

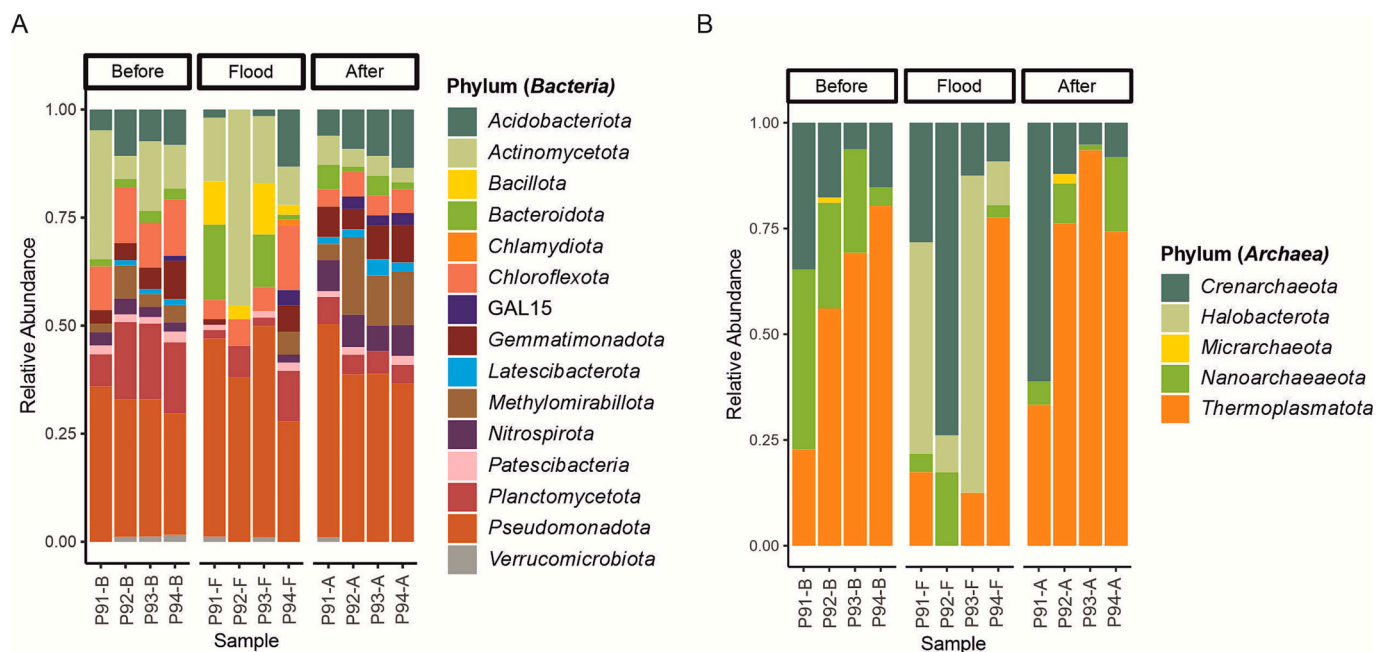


Fig. 5. Relative abundance of dominant phyla (over 1 %) in Pindal Cave during three sampling periods. Before: Before the flood (2017). Flood: one week after the flood (2019). After: one year after the flood (2020). A) Bacteria. B) Archaea.

flood were the genera *Crossiella* and wb1-P19 within the bacterial population, and the family *Nitrosopumilaceae*, uncultured *Thermoplasmata*, and uncultured *Nanoarchaeota* within the archaeal population (Figs. 6 and 7). These results were consistent with previous microbiological studies in Pindal Cave and other limestone and volcanic caves, where the high abundance of *Crossiella* and wb1-P19 sequences was associated with the presence of biofilms and mineral deposits induced by bacteria (Martín-Pozas et al., 2022, 2023a, 2023b). Both genera are commonly found in caves and have been identified as keystone members (Zhu et al., 2019; Jurado et al., 2020). Although there is limited information available on its metabolism, the gammaproteobacterial genus wb1-P19 (*Nitrosococcaceae*) has been phylogenetically clustered with sulfur or nitrite-oxidizing autotrophic bacteria (Holmes et al., 2001; González-Riancho, 2021). Other abundant groups represented by the phyla *Planctomycetota*, *Methyloirabillota* and *Gemmatimonadota* were retrieved in caves together with other phyla with low abundances (GAL15, *Latescibacterota*, *Patescibacteria*, *Verrucomicrobia*) (Jurado et al., 2020; Cheng et al., 2023a).

Archaeal communities, although less studied in caves compared to bacteria, can provide valuable information about the ecosystem. *Crenarchaeota* were found to be dominant in caves (Ai et al., 2022; Martín-Pozas et al., 2022). Although this phylum was not the most abundant in Pindal Cave, it presented abundances over 5 % within the archaeal community. Recently, *Nitrosopumilaceae* and *Nitrososphaeraceae* have been identified as a dominant ammonia-oxidizing archaeon in karst ecosystems and *Thermoplasmata*, were identified as important predictor group inside the caves, which was consistent with our results (Wu et al., 2021; Cheng et al., 2023a). These results indicate that the microbial communities that existed before the flood were characteristic of a natural cave environment. This information establishes the baseline for comprehending the alterations induced by the flood.

The comparative study of cave sediment microbial communities at site 9 confirms that the flood introduced sediments mixed with slurry, probably from the nearby livestock farm. The microbiological study shows a great change in the composition of the microbial communities between the samples before and one week after the flood event (Fig. 5). The most notable change in microbial communities consisted of the presence of the bacterial phyla *Bacillota* and *Bacteroidota*, as well as methanogenic *Archaea*, which were absent in the samples prior the flood

(Fig. 5). The phylum *Bacillota* was barely represented by the genus *Bacillus* in the 2017 samples. In contrast, in the 2019 samples it became one of the most abundant phyla in flood sediments coinciding with the presence of *Christensenellaceae* (gut group R-7), *Proteinclasticum* (*Clostridiaceae* family), *Lachnospiraceae*, and *Ruminococcaceae*. The *Clostridiaceae* family, known for its pathogenic *Clostridium* species, and the *Ruminococcaceae* family have been previously found in the intestines of many mammals and in waste from bovine farms (Pandey et al., 2018; Mutungwazi et al., 2022). The *Christensenellaceae* (gut group R-7) family and the aforementioned groups are commonly detected as core members of the rumen microbiota in the Global Rumen Census project (Henderson et al., 2015). The *Bacteroidota* phylum in flood sediments is represented by *Fermentimonas* (*Bacteroidales* order) and *Chryseobacterium* (*Flavobacteriales* order), which have also been associated with waste from bovine farms (Pandey et al., 2018).

The flood also introduced methanogenic *Archaea* that are typically associated with ruminants (Henderson et al., 2015; Huang et al., 2016; Li et al., 2019; Shaw et al., 2019). Some archaea, such as *Methanosaeta*, *Methanolobus*, and *Methanomassiliicoccales*, have also been found in freshwater sediments (Cozannet et al., 2021; Chakkiath et al., 2012; Conrad et al., 2006). All mentioned *Archaea*, except one unidentified group within the *Methanomassiliicoccales* order, were not present in the sediments before the flood or in the sediments one year later (Fig. 7). Therefore, its presence was clearly associated with the flood. Specifically, the presence of *Methanomicrobiales* and *Methanosarcina* in the flooded sediments suggests an entry of material with slurry from the cattle farm.

Additionally, certain bacterial families and genera, including *Corynebacteriaceae* family and the genera *Thauera* and *Longilinea* from the *Actinomycetota*, *Pseudomonadota*, and *Chloroflexota* phyla, respectively, showed a significant presence in the flood sediment samples, indicating a connection with the farm waste. *Corynebacteriaceae* family members are found in various environments, including soil, mammal skin, bat guano in caves and the human gut (Tauch and Sandbote, 2014; Bernard, 2012; De Leon et al., 2018). The genus *Thauera*, a facultative anaerobic group of bacteria, is abundantly present in the mud sample and can degrade aromatic compounds in diverse environments. They are crucial denitrifiers and play an important role in nitrogen removal in wastewater ecosystems, including dairy farm wastewater (Liu et al., 2013;

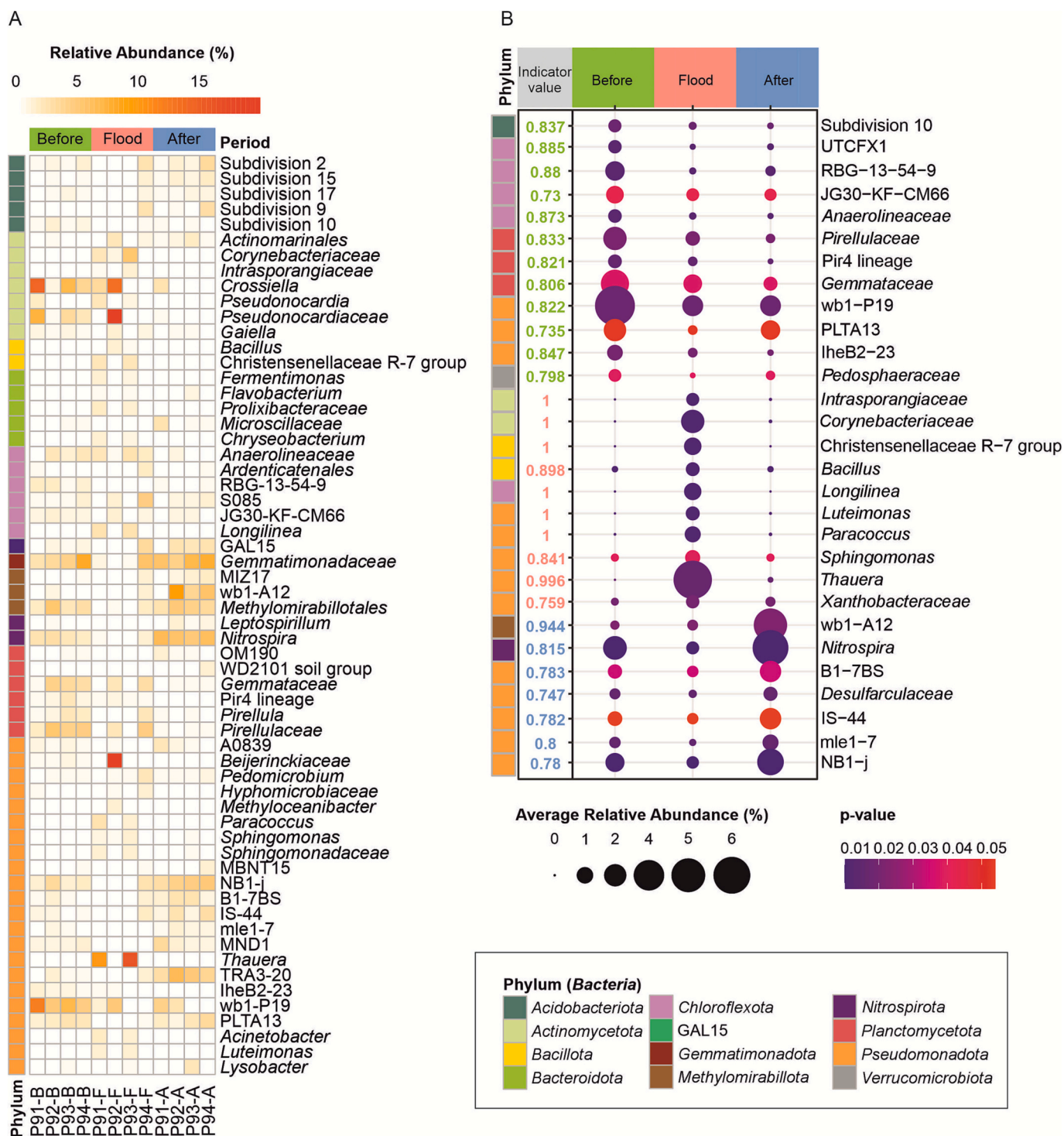


Fig. 6. Relative abundance of the dominant bacterial taxa in cave during three different sampling periods: Before: Before the flood (2017). Flood: one week after the flood (2019) After: one year after the flood (2020). A) Heatmap with relative abundances of the most abundant taxa with the least abundant represented in white and the most abundant in red. B) Bubble plot showing the average of relative abundance of the indicator taxa of different sampling periods based on an IndVal analysis. The legend located at the bottom right indicates the classification at the phylum level.

Yokoyama et al., 2016). The order *Anaerolineales*, specifically *Longilinea*, encompasses anaerobic bacteria isolated from methanogenic consortia (Yamada et al., 2007). Other bacteria (*Intrasporangiaceae*, *Luteimonas*, *Paracoccus*, *Xanthobacteraceae*, *Sphingomonas*) found in significant proportions in the flood samples also appear to be associated with the flood event. These bacteria are commonly found in anaerobic environments, as well as in caves and soils (Yokoyama et al., 2016; De Mandal et al.,

2017; Rangseekaew and Pathom-Aree, 2019; Martin-Pozas et al., 2020), so their presence could be associated with the flood, although not necessarily with the cattle farm.

Our findings underscore the impact of the flood event on shaping the composition and potential metabolism of the microbial communities in the cave. The decline in pathways related to the oxidation of C1 compounds aligns with the decrease in methanotrophic bacteria (IheB2-23).

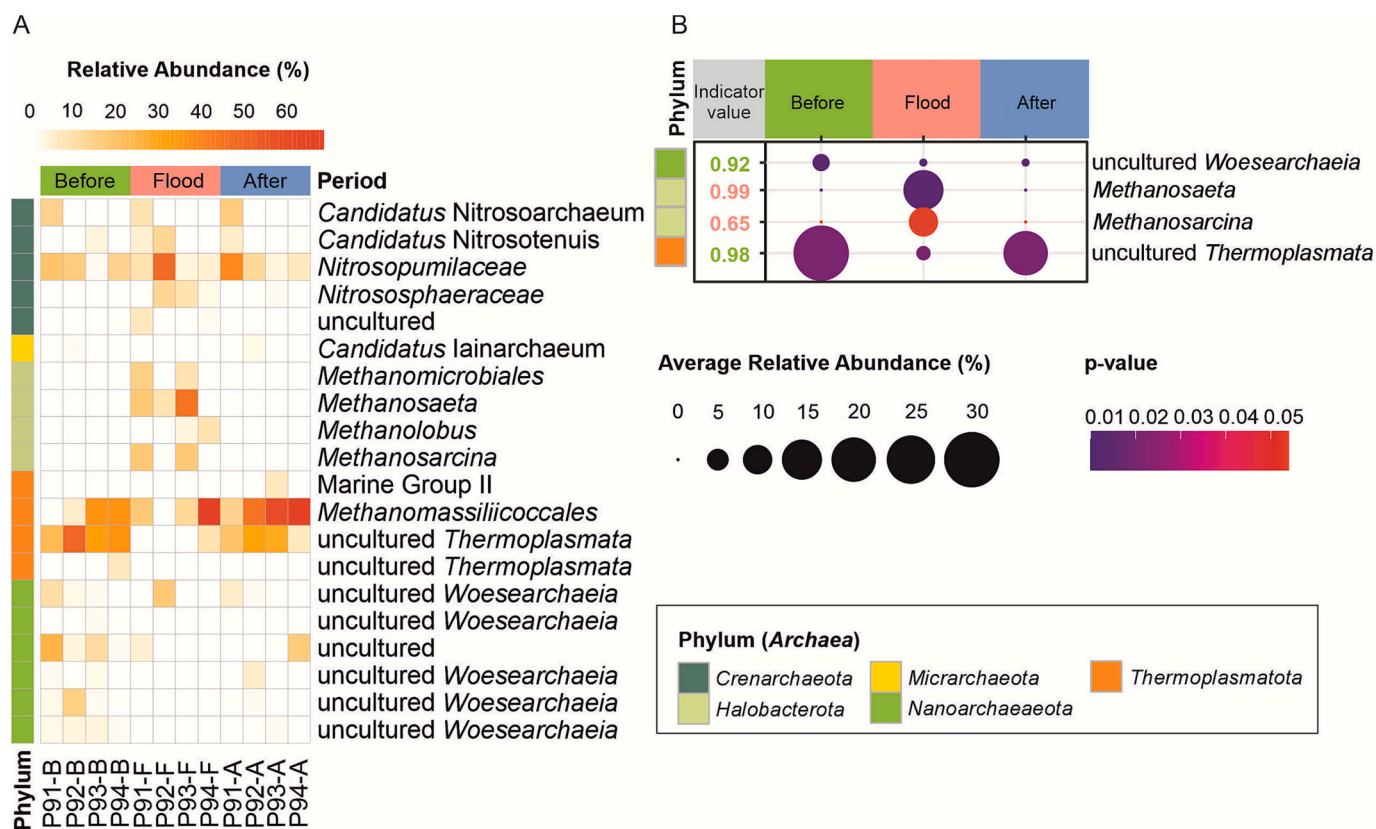


Fig. 7. Relative abundance of the archaeal taxa in cave during three different sampling periods: Before: Before the flood (2017). Flood: one week after the flood (2019) After: one year after the flood (2020). A) Heatmap with relative abundances with the least abundant represented in white and the most abundant in red. B) Bubble plot showing the average of relative abundance of the indicator taxa of different sampling periods based on an IndVal analysis. The legend located at the bottom right indicates the classification at the phylum level.

On the contrary, an increase in denitrification and methanogenesis processes corresponds to increased abundances of denitrifying bacteria such as *Thauera* and methanogenic archaea such as *Methanomicrobiales* and *Methanosarcina*. Understanding the functions of microbial communities is crucial to know how microbial communities influence ecosystems. (Toole et al., 2021). Although limitations in bioinformatic prediction based on 16S-rRNA gene profiles, in comparison to shotgun sequencing, are clear, PICRUSt2 has large representation among studies with different sample types and provides valuable insights into potential ecological functions within soil microbial communities. In Pindal Cave, PICRUSt2 predictions were consistent with information available in the literature about some groups of bacteria and archaea, offering valuable insights into the ecological consequences of such disturbances. Overall, these findings suggest that the flood transported microbial contaminants from the cattle farm, modifying the endemic cave communities.

4.3. Adaptive response of prokaryotic communities in cave sediments over the medium term

Almost all *Bacteria* and *Archaea* that appeared with relatively high abundances during the period of flood in the exogenous sediments have completely disappeared or are barely detectable one year later (Figs. 6–7). Most of these microorganisms were associated with anaerobic conditions. Therefore, their decrease is indicative of a significant shift in the redox state within the cave sediments explained by the transition from flooded reducing sediment conditions to dry oxic sediments.

Methanogens, crucial for anaerobic degradation in the global carbon cycle, displayed limited activity in caves. Aerobic gammaproteobacterial methanotrophs typically represented the methane cycle, as observed

in Pindal Cave before the flood (Cheng et al., 2023b). Interestingly, the flood led to the appearance of methanogens and a decrease in aerobic oxidizing bacteria (AOB). However, this shift was not sustained, as traditional methanogenic archaea mostly disappeared a year later. Conversely, there has been a proliferation of uncultured members of the *Thermoplasmatata* class and *Methanomassiliococcales* order. Although these archaea share genetic similarities with traditional methanogens, they comprise a metabolically diverse group capable of obtaining energy through other pathways such as heterotrophy, sulfur and hydrogen metabolism, denitrification, and fermentation, which could explain their long-term survival in the cave.

In the case of methanotrophic bacteria, there is an increase in potential anaerobic methane-oxidizing bacteria (DAMO), which are nitrite-dependent. This was the case of wb1-A12, in the family *Methylomirabilaceae*, which accommodate bacteria that couple anaerobic methane oxidation with nitrite reduction (Ettwig et al., 2010). These DAMO are not usually abundant as AOB in caves, but their ability to withstand low levels of oxygen has recently been demonstrated (Li et al., 2020). Their ability to grow under aerobic conditions, coupled with the observed increase in inorganic nitrogen, would explain their persistence one year after the flood.

One year after the flood, the levels of organic matter in the flooded areas have remained stable, with similar levels of carbon and nitrogen (Table 1). However, inorganic nitrogen forms, specifically ammonium and nitrate, increased. The source of bioavailable nitrogen in the flooded area of Pindal Cave appears to be related to the surface input from the doline where the cattle farm was established. The ammonium from urea in manure quickly volatilizes, while feces contain organic N that is more stable and slowly released. During the flood, a potential increase in the degradation pathways of organic compounds was observed (Table S3).

Some of these compounds, such as creatinine or amino acids, can be degraded by soil bacteria, such as *Corynebacterium* and *Flavobacterium*, releasing ammonia as a byproduct (Dubos and Miller, 1937; Koyama et al., 1990). Alternatively, bioavailable nitrogen could be produced by diazotrophs; however, a significant increase in nitrogenase activity has not been detected in PICRUST2 analyses. Therefore, the increase in nitrates and ammonia observed a year later was the result of mineralization activity of sediment microorganisms capable of transforming organic N into ammonia. Subsequently, this increase in ammonia levels elevated the abundance of nitrifying (Wang et al., 2018). The main groups of nitrifiers were ammonia oxidizing archaea (*Nitrosopumilaceae*, *Candidatus Nitrosotenuis*, *Candidatus Nitrosarchaeum*, *Candidatus Nitrososphaera*, *Nitrososphaeraceae*), ammonia oxidizing bacteria affiliated to the family *Nitrosomonadaceae* (IS-44, MND1, mle1–7), nitrite-oxidizing bacteria (*Nitrospira*) and NB1-j (De Voogd et al., 2015).

The flood episode in the cave offered a unique opportunity to elucidate how microorganisms adapt their metabolic strategies in response to sudden pollution and shifting energy sources over time. In conclusion, the adaptive responses observed in prokaryotic communities in the medium term revealed an increase in nitrifying activity while other original cave groups previously associated with moonmilk deposits (*Crossiella* and wb1-P19) and related to the presence of ammonium in caves showed a decrease in abundance (Martín-Pozas et al., 2022; Cheng et al., 2023a). Our results suggest that the microbial community present before the flood has not recovered one year after the flood and that its long-term evolution requires further investigation.

5. Conclusions

The comparative study of Pindal Cave sediments before and after a flood event revealed significant impacts on biogeochemical properties and the prokaryotic community. The flood, triggered by extreme rainfall and resulting from the collapse of a sinkhole located directly above the cave, caused the deposition of exogenous sediments within the cave. These sediments included waste from livestock situated over the sinkhole, resulting in the burial of pre-existing cave sediments. Analytical data highlighted the persistent influence of livestock farming on sediment composition within the cave, facilitating the input of substantial amounts of available organic matter and organic nitrogen. Elevated levels of available phosphorus as well as a few heavy metals were also observed.

Despite the significant leaching of most organic compounds, the affected area exhibited elevated levels of phosphorus one week after the flood. This event led to a notable alteration in both the structure and functionality of the sediment microbial community, resulting in a significant reduction in the diversity of prokaryotes. In the short term, one week after the flood, a significant increase in denitrifying bacteria and bacterial groups typically found in mammalian intestinal tracts, dairy farm manure, and previously unseen methanogenic archaea were observed within the cave.

In the medium term, a notable increase in nitrifying bacterial populations affiliated with the *Nitrosomonadaceae* family and the *Nitrospira* genus was observed, probably related to the higher levels of nitrate and ammonium observed one year after the flood. This analysis also revealed that certain original bacterial groups, such as *Crossiella*, *wb1-P19*, and *Methylomonaceae*, among others, did not show signs of recovery.

The results of this study played a decisive role in stopping cow breeding activities in 2021 to prevent future adverse effects on the cave. In conclusion, this study highlights the need for exhaustive environmental management to preserve the integrity of karstic caves such as Pindal Cave. The protection of this cave extends beyond its cave paintings; it implies the preservation of a unique and vulnerable underground ecosystem. The decision to stop livestock activity near the cave constituted an essential step in short-term protection. However, it is crucial to recognize the importance of future research to understand changes induced by human activity and to develop sustainable strategies that

ensure the long-term integrity of this valuable cave.

CRediT authorship contribution statement

Tamara Martín-Pozas: Writing – review & editing, Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Soledad Cuezva:** Writing – original draft, Validation, Supervision, Investigation, Formal analysis. **Angel Fernandez-Cortes:** Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Investigation, Funding acquisition. **Maria Gonzalez-Pumariega:** Supervision, Investigation. **Javier Elez:** Visualization, Investigation, Formal analysis. **Elsa Duarte:** Visualization, Investigation. **Marco de la Rasilla:** Supervision, Resources, Investigation. **Juan Carlos Canaveras:** Investigation, Formal analysis. **Cesareo Saiz-Jimenez:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Formal analysis. **Sergio Sanchez-Moral:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.scitotenv.2024.171137>.

References

- Ai, J., Guo, J., Li, Y., Zhong, X., Lv, Y., Li, J., Yang, A., 2022. The diversity of microbes and prediction of their functions in karst caves under the influence of human tourism activities—a case study of Zhijin Cave in Southwest China. *Environ. Sci. Pollut. Res.* 29, 25858–25868. <https://doi.org/10.1007/s11356-021-17783-x>.
- Bernard, K.A., 2012. The genus *Corynebacterium* and other medically relevant coryneforms-like bacteria. *J. Clin. Microbiol.* 50, 3152–3158. <https://doi.org/10.1128/jcm.00796-12>.
- Bokulich, N.A., Kaehler, B.D., Rideout, J.R., Dillon, M., Bolyen, E., Knight, R., Huttley, G. A., Caporaso, G., 2018. Optimizing taxonomic classification of marker-gene amplicon sequences with QIIME 2's q2-feature-classifier plugin. *Microbiome* 6, 90. <https://doi.org/10.1186/s40168-018-0470-z>.
- Boyer, D.G., Pasquarell, G.C., 1999. Agricultural land use impacts on bacterial water quality in a karst groundwater aquifer. *J. Am. Water Resour. Assoc.* 35, 291–300. <https://doi.org/10.1111/j.1752-1688.1999.tb03590.x>.
- Cáceres, M.D., Legendre, P., 2009. Associations between species and groups of sites: indices and statistical inference. *Ecology* 90, 3566–3574. <https://doi.org/10.1890/08-1823.1>.

- Callahan, B.J., McMurdie, P.J., Rosen, M.J., Han, A.W., Johnson, A.J.A., Holmes, S.P., 2016. DADA2: high-resolution sample inference from Illumina amplicon data. *Nat. Methods* 13, 581–583. <https://doi.org/10.1101/024034>.
- Chakkiath, P.A., Murrell, J.C., Shouche, Y.S., 2012. Molecular diversity of methanogens and identification of *Methanobolus* sp. as active methylophilic *Archaea* in Lonar crater lake sediments. *FEMS Microbiol. Ecol.* 81, 43–51. <https://doi.org/10.1111/j.1574-6941.2011.01274.x>.
- Cheng, X., Xiang, X., Yun, Y., Wang, W., Wang, H., Bodelier, P.L., 2023a. *Archaea* and their interactions with bacteria in a karst ecosystem. *Front. Microbiol.* 14, 1068595. <https://doi.org/10.3389/fmicb.2023.1068595>.
- Cheng, X., Zeng, Z., Liu, X., Li, L., Wang, H., Zhao, R., Tuovinen, O.H., et al., 2023b. Methanotrophs dominate methanogens and act as a methane sink in a subterranean karst cave. *Sci. Total Environ.* 164562 <https://doi.org/10.1016/j.scitotenv.2023.164562>.
- Conrad, R., Erkel, C., Liesack, W., 2006. Rice cluster I methanogens, an important group of *Archaea* producing greenhouse gas in soil. *Curr. Opin. Biotechnol.* 17, 262–267. <https://doi.org/10.1016/j.copbio.2006.04.002>.
- Cozannet, M., Borrel, G., Roussel, E., Moalic, Y., Allieux, M., Sanvoisin, A., et al., 2021. New insights into the ecology and physiology of *Methanomassiliicoccales* from terrestrial and aquatic environments. *Microorganisms* 9, 1–31. <https://doi.org/10.3390/MICROORGANISMS9010030>.
- Culver, D.C., Pipan, T., 2009. Caves, as islands. In: Gillespie, R.G., Clauge, D.A. (Eds.), *Encyclopedia of Islands*. University of California Press, Berkeley, pp. 150–153.
- Davis, M.C., Messina, M.A., Nicolosi, G., Petralia, S., Baker IV, M.D., Mayne, C.K., et al., 2020. Surface runoff alters cave microbial community structure and function. *PLoS One* 15, e0232742. <https://doi.org/10.1371/journal.pone.0232742>.
- De Leon, M.P., Montecillo, A.D., Pinili, D.S., Siringan, M.A.T., Park, D.-S., 2018. Bacterial diversity of bat guano from Cabalorisa cave, Mabini, Pangasinan, Philippines: a first report on the metagenome of Philippine bat guano. *PLoS One* 13, e0200095. <https://doi.org/10.1371/journal.pone.0200095>.
- De Mandal, S., Chatterjee, R., Kumar, N.S., 2017. Dominant bacterial phyla in caves and their predicted functional roles in C and N cycle. *BMC Microbiol.* 17, 1–9. <https://doi.org/10.1186/s12866-017-1002-x>.
- De Voogd, N.J., Cleary, D.F., Polonia, A.R., Gomes, N.C., 2015. Bacterial community composition and predicted functional ecology of sponges, sediment and seawater from the thousand islands reef complex, West Java, Indonesia. *FEMS Microbiol. Ecol.* 91, fiv019. <https://doi.org/10.1093/femsec/fiv019>.
- Douglas, G.M., Maffei, V.J., Zaneveld, J., Yurgel, S.N., Brown, J.R., Taylor, C.M., et al., 2019. PICRUSt2: an improved and extensible approach for metagenome inference. *BioRxiv preprint*. <https://doi.org/10.1101/672295>.
- Dubos, R., Miller, B.F., 1937. The production of bacterial enzymes capable of decomposing creatinine. *J. Biol. Chem.* 121 (2), 429–445. [https://doi.org/10.1016/S0021-9258\(18\)74276-2](https://doi.org/10.1016/S0021-9258(18)74276-2).
- Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., et al., 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* 464, 543–548. <https://doi.org/10.1038/nature08883>.
- González-Pumariega, M., 2011. La cueva de El Pindal, 1911–2011. Estudio de su arte rupestre cien años después de Les Cavernes de la Région Cantabrique. Ménsula, Pola de Siero, 212 pp.
- González-Riancho, C., 2021. Análisis descriptivo y funcional de las colonias microbianas visibles que crecen en la cueva de Altamira, enfocado al diseño de medidas de control. Ph.D. thesis. University of Cantabria.
- Goy, J.L., Zazo, C., Elez, J., Martínez-Graña, A.M., Cañaveras, J.C., Dábrio, C.J., Duarte, E., De Bustamante, I., Sánchez Moral, S., 2023. Geomorphological evolution and mapping of the littoral of Asturias and Cantabria (northern Spain) in the area of El Pindal Cave: relations between coastline and karstic morphologies. *J. Maps* 19, 2196447. <https://doi.org/10.1080/17445647.2023.2196447>.
- Henderson, G., Cox, F., Ganesh, S., Jonker, A., Young, W., Janssen, P.H., 2015. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. *Sci. Rep.* 5, 14567. <https://doi.org/10.1038/srep14567>.
- Herlemann, D.P.R., Labrenz, M., Jürgens, K., Bertilsson, S., Waniek, J.J., Andersson, A.F., 2011. Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea. *ISME J.* 5, 1571–1579. <https://doi.org/10.1038/ismej.2011.41>.
- Hershey, O.S., Barton, H.A., 2018. The microbial diversity of caves. In: Moldovan, O.T., Kováč, L., Halse, S. (Eds.), *Cave Ecology*. Springer Nature Switzerland, Cham, pp. 69–90. https://doi.org/10.1007/978-3-319-98852-8_5.
- Holmes, A.J., Tujula, N.A., Holley, M., Contos, A., James, J.M., Rogers, P., et al., 2001. Phylogenetic structure of unusual aquatic microbial formations in Nullarbor caves, Australia. *Environ. Microbiol.* 3, 256–264. <https://doi.org/10.1046/j.1462-2920.2001.00187.x>.
- Huang, X.D., Martínez-Fernández, G., Padmanabha, J., Long, R., Denman, S.E., McSweeney, C.S., 2016. Methanogen diversity in indigenous and introduced ruminant species on the Tibetan plateau. *Archaea* 2016, 5916067. <https://doi.org/10.1155/2016/5916067>.
- Jiménez-Sánchez, M., Bischoff, J.L., Stoll, H., Aranburu, A., 2006. A geochronological approach for cave evolution in the Cantabrian coast (Pindal Cave, NW Spain). *Z. Geomorphol.* 147, 129–141.
- Jurado, V., Gonzalez-Pimentel, J.L., Miller, A.Z., Hermosin, B., D'Angeli, I.M., Tognini, P., et al., 2020. Microbial communities in vermiculations of an alpine cave. *Front. Earth Sci.* 8, 586248.
- Kelly, W.R., Panno, S.V., Hackley, K.C., Martinsek, A.T., Krapac, I.G., Weibel, C.P., et al., 2009. Bacteria contamination of groundwater in a mixed land-use karst region. *Water Qual Expo Health* 1, 69–78. <https://doi.org/10.3389/feart.2020.586248>.
- Koyama, Y., Kitao, S., Yamamoto-Otake, H., Suzuki, M., Nakano, E., 1990. Cloning and expression of the creatinase gene from *Flavobacterium* sp. U-188 in *Escherichia coli*. *Agri. Biol. Chem.* 54 (6), 1453–1457.
- Laiz, L., Groth, I., Gonzalez, I., Saiz-Jimenez, C., 1999. Microbiological study of the dripping waters in Altamira cave (Santillana del Mar, Spain). *J. Microbiol. Methods* 36 (1999), 129–138. [https://doi.org/10.1016/S0167-7012\(99\)00018-4](https://doi.org/10.1016/S0167-7012(99)00018-4).
- Lavoie, K.H., Winter, A.S., Read, K.J.H., Hughes, E.M., Spilde, M.N., Northup, D.E., 2017. Comparison of bacterial communities from lava cave microbial mats to overlying surface soils from Lava Beds National Monument, USA. *PLoS One* 12, e0169339. <https://doi.org/10.1371/journal.pone.0169339>.
- Lehmann, J., Schroth, G., 2002. Nutrient leaching. In: *Trees, Crops and Soil Fertility: Concepts and Research Methods*. CABI publishing, Wallingford UK, pp. 151–166. <https://doi.org/10.1079/9780851995939.0151>.
- Li, Z., Wang, X., Zhang, T., Si, H., Xu, C., Wright, A.-D.G., et al., 2019. Heterogeneous development of methanogens and the correlation with bacteria in the rumen and cecum of sika deer (*Cervus nippon*) during early life suggest different ecology relevance. *BMC Microbiol.* 19, 129. <https://doi.org/10.1186/s12866-019-1504-9>.
- Li, Y., Wang, J., Hua, M., Yao, X., Zhao, Y., Hu, J., et al., 2020. Strategy for denitrifying anaerobic methane-oxidizing bacteria growing under the oxygen-present condition. *Sci. Total Environ.* 742, 140476 <https://doi.org/10.1016/j.scitotenv.2020.140476>.
- Liu, B., Mao, Y., Bergaust, L., Bakken, L.R., Frostegård, A., 2013. Strains in the genus *Thauera* exhibit remarkably different denitrification regulatory phenotypes. *Environ. Microbiol.* 15, 2816–2828. <https://doi.org/10.1111/1462-2920.12142>.
- Martin-Pozas, T., Sanchez-Moral, S., Cuezva, S., Jurado, V., Saiz-Jimenez, C., Perez-Lopez, R., et al., 2020. Biologically mediated release of endogenous N₂O and NO₂ gases in a hydrothermal, hypoxic subterranean environment. *Sci. Total Environ.* 747, 141218 <https://doi.org/10.1016/j.scitotenv.2020.141218>.
- Martin-Pozas, T., Cuezva, S., Fernandez-Cortes, A., Cañaveras, J.C., Benavente, D., Jurado, V., et al., 2022. Role of subterranean microbiota in the carbon cycle and greenhouse gas dynamics. *Sci. Total Environ.* 831, 154921 <https://doi.org/10.1016/j.scitotenv.2022.154921>.
- Martin-Pozas, T., Fernandez-Cortes, A., Cuezva, S., Cañaveras, J.C., Benavente, D., Duarte, E., et al., 2023a. New insights into the structure, microbial diversity and ecology of yellow biofilms in a Paleolithic rock art cave (Pindal Cave, Asturias, Spain). *Sci. Total Environ.* 897, 165218 <https://doi.org/10.1016/j.scitotenv.2023.165218>.
- Martin-Pozas, T., Gonzalez-Pimentel, J.L., Jurado, V., Laiz, L., Cañaveras, J.C., Fernandez-Cortes, A., et al., 2023b. *Crossiella*, a rare *Actinomycetota* genus, abundant in the environment. *Appl. Biosci.* 2, 194–210. <https://doi.org/10.3390/applbiosci2020014>.
- Mutungwazi, A., Ijoma, G.N., Ogola, H.J.O., Matambo, T.S., 2022. Physico-chemical and metagenomic profile analyses of animal manures routinely used as inocula in anaerobic digestion for biogas production. *Microorganisms* 10, 671. <https://doi.org/10.3390/microorganisms10040671>.
- Naughton, O., McCormack, T., Gill, L., Johnston, P., 2018. Groundwater flood hazards and mechanisms in lowland karst terrains. *Geol. Soc. Lond. Spec. Publ.* 466, 397–410. <https://doi.org/10.1144/SP466.9>.
- Pandey, P., Chiu, C., Miao, M., Wang, Y., Settles, M., del Rio, N.S., et al., 2018. 16S rRNA analysis of diversity of manure microbial community in dairy farm environment. *PLoS One* 13, e0190126. <https://doi.org/10.1371/journal.pone.0190126>.
- Parise, M., Ravbar, N., Živanović, V., Mikszewski, A., Kresic, N., Mádl-Szőnyi, J., et al., 2015. Hazards in karst and managing water resources quality. In: Stevanović, Z. (Ed.), *Karst Aquifers-Characterization and Engineering*. Springer, Cham, pp. 601–687. https://doi.org/10.1007/978-3-319-12850-4_17.
- Pasquarell, G.C., Boyer, D.G., 1995. Agricultural impacts on bacterial water quality in karst groundwater. *J. Environ. Qual.* 24, 959–969. <https://doi.org/10.2134/jeq1995.00472425002400050026x>.
- Qin, X., Guo, S., Zhai, L., Pan, J., Khoshnevisan, B., Wu, S., Liu, H., et al., 2020. How long-term excessive manure application affects soil phosphorous species and risk of phosphorous loss in fluvo-aquic soil. *Environ. Pollut.* 266, 115304 <https://doi.org/10.1016/j.envpol.2020.115304>.
- Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., et al., 2013. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic Acids Res.* 41, D590–D596. <https://doi.org/10.1093/nar/gks1219>.
- Rangseekaew, P., Pathom-Aree, W., 2019. Cave Actinobacteria as producers of bioactive metabolites. *Front. Microbiol.* 10, 387. <https://doi.org/10.3389/fmicb.2019.00387>.
- Sahoo, P.K., Kim, K., Powell, M.A., 2016. Managing groundwater nitrate contamination from livestock farms: implication for nitrate management guidelines. *Curr. Pollut. Rep.* 2, 178–187.
- Scheuerl, T., Hopkins, M., Nowell, R.W., Rivett, D.W., Barraclough, T.G., Bell, T., 2020. Bacterial adaptation is constrained in complex communities. *Nat. Comm.* 11, 754. <https://doi.org/10.1007/s40726-016-0033-5>.
- Shabarova, T., Widmer, F., Pernthaler, J., 2013. Mass effects meet species sorting: transformations of microbial assemblages in epiphreatic subsurface karst water pools. *Environ. Microbiol.* 15, 2476–2488. <https://doi.org/10.1111/1462-2920.12124>.
- Shabarova, T., Villiger, J., Morenkov, O., Niggemann, J., Dittmar, T., Pernthaler, J., 2014. Bacterial community structure and dissolved organic matter in repeatedly flooded subsurface karst water pools. *FEMS Microbiol. Ecol.* 89, 111–126. <https://doi.org/10.1111/1574-6941.12339>.
- Shaw, G.T.-W., Weng, C.-Y., Chen, C.-Y., Weng, F.C.-H., Wang, D., 2019. A systematic approach re-analyzing the effects of temperature disturbance on the microbial community of mesophilic anaerobic digestion. *Sci. Rep.* 9, 6560. <https://doi.org/10.1038/s41598-019-42987-0>.

- Simon, K.S., Gibert, J., Petitot, P., Laurent, R., 2001. Spatial and temporal patterns of bacterial density and metabolic activity in a karst aquifer. *Arch. Hydrobiol.* 67–82 <https://doi.org/10.1127/archiv-hydrobiol/151/2001/67>.
- Tauch, A., Sandbote, J., 2014. The family *Corynebacteriaceae*. In: Rosenberg, E., DeLong, E.F., Lory, S., Stackebrand, E., Thompson, F. (Eds.), *The Prokaryotes*. Springer, Berlin, pp. 239–277. https://doi.org/10.1007/978-3-642-30138-4_187.
- Toole, D.R., Zhao, J., Martens-Habbena, W., Strauss, S.L., 2021. Bacterial functional prediction tools detect but underestimate metabolic diversity compared to shotgun metagenomics in Southwest Florida soils. *Appl. Soil Ecol.* 168, 104129 <https://doi.org/10.1016/j.apsoil.2021.104129>.
- Wang, F., Chen, S., Wang, Y., Zhang, Y., Hu, C., Liu, B., 2018. Long-term nitrogen fertilization elevates the activity and abundance of nitrifying and denitrifying microbial communities in an upland soil: implications for nitrogen loss from intensive agricultural systems. *Front. Microbiol.* 9, 2424. <https://doi.org/10.3389/fmicb.2018.02424>.
- Wu, Y., Tan, L., Liu, W., Wang, B., Wang, J., Cai, Y., et al., 2015. Profiling bacterial diversity in a limestone cave of the western Loess Plateau of China. *Front. Microbiol.* 6, 244. <https://doi.org/10.3389/fmicb.2015.00244>.
- Wu, F., Zhang, Y., He, D., Gu, J.-D., Guo, Q., Liu, X., et al., 2021. Community structures of bacteria and archaea associated with the biodeterioration of sandstone sculptures at the Beishiku Temple. *Int. Biodeter. Biodegr.* 164, 105290 <https://doi.org/10.1016/j.ibiod.2021.105290>.
- Yamada, T., Imachi, H., Ohashi, A., Harada, H., Hanada, S., Kamagata, Y., et al., 2007. *Bellilinea caldijistulae* gen. nov., sp. nov. and *Longilinea arvoryzae* gen. nov., sp. nov., strictly anaerobic, filamentous bacteria of the phylum *Chloroflexi* isolated from methanogenic propionate-degrading consortia. *Int. J. Syst. Evol. Microbiol.* 57, 2299–2306. <https://doi.org/10.1099/ijs.0.65098-0>.
- Yang, C., Mai, J., Cao, X., Burberry, A., Cominelli, F., Zhang, L., 2023. ggpicrust2: an R package for PICRUST2 predicted functional profile analysis and visualization. *Bioinformatics* 1 (39(8)), btad470. <https://doi.org/10.1093/bioinformatics/btad470>.
- Yokoyama, K., Yumura, M., Honda, T., Ajitomi, E., 2016. Characterization of denitrification and net N₂O-reduction properties of novel aerobically N₂O-reducing bacteria. *Soil Sci. Plant Nutr.* 62, 230–239. <https://doi.org/10.1080/00380768.2016.1178076>.
- Zhu, H.-Z., Zhang, Z.-F., Zhou, N., Jiang, C.-Y., Wang, B.-J., Cai, L., et al., 2019. Diversity, distribution and co-occurrence patterns of bacterial communities in a karst cave system. *Front. Microbiol.* 10, 1726. <https://doi.org/10.3389/fmicb.2019.01726>.