Distinct Microbial Communities in the Soils, Waters and Speleothems of a Hyperalkaline Cave System

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Key Points:

- Samples from soils, waters, calcites and cave muds were examined to understand the distribution of microbiota in a hyperalkaline cavern.
- The microbial biomass in above-cave soils and cave muds was notably higher than that in speleothem calcites and drip waters.
- Limited in-cave microbial similarities between microhabitats indicated a high variability likely driven by the steep alkalinity gradient.

Abstract

Caves are complex ecosystems with various microbial habitats. Understanding the individual community structures in the key source environments (soils, waters) and those in the sinks (speleothems, cave muds) can help elucidate the extent to which in-cave communities are a function of their source communities, or if they can successfully adapt and diversify locally. Here, we assess the network of microbial communities existing within a unique British cave system. Poole's Cavern is characterised by alkalinity or even hyperalkalinity (pH>9) in its drip waters, creating a series of challenging ecological niches for microbes to survive. Samples of soil, drip water, speleothem and cave sediment were collected for chemical characterization, cell culture assays and DNA sequencing analysis. We show that microbial abundance and diversity were highest in the soils proximal to the cave, and the microbiotas of the hyperalkaline cave chamber were less abundant and less diverse than the cave chamber with lower pH. Proteobacteria and Planctomycetota were the most prevalent bacterial phyla throughout the cave system irrespective of pH, indicating their members are comparatively more metabolically versatile. Predicted essential metabolic pathways were still dominant within all sampling sites, and microorganisms were shown to be capable of utilising various inorganic or simple organic compounds to survive. Interestingly, co-occurrence between Poole's samples was limited, never demonstrating more than 50% similarity. This work highlights the diversity of microbial communities within this extreme environment and the development of microbial niches which reflects the adaptation strategy of microorganisms under alkaline-hyperalkaline conditions.

Plain Language Summary

Caves are complex environments, forming a range of living spaces for microbes. Dissolved inorganic minerals and organic matter are carried by water that flows from the soils above caves to speleothems and into sediments on the ground. Poole's Cavern in the UK is a cave system with very high pH (>9) waters. These waters are not friendly to most microbes living in neutral pH environments and thus possibly foster special microbial communities that can stay alive under high pH conditions. Full of calcite formations and without observable fauna, this cave is not rich in nutrients. We sampled soils above Poole's Cavern, drip waters, speleothems and cave muds to determine the biomass and microbial species in them. We found that the soils outside of this cave had the highest microbial content and diversity. The cave chamber with higher pH waters had fewer microbes than that with lower pH waters. We demonstrate that connectivity of microbial species between in-cave sampling sites was limited, and the community of microbes in each site developed relatively independent finding ways to survive in their respective environment. This study presents the high potential of microbes to evolve and adapt even in conditions almost inhospitable to life.

1 Introduction

Cave and karst systems represent some of the least studied extreme environments on the planet. Caves are in most cases characterised by extreme nutrient deficiency and utter darkness (Dong et al., 2020), characteristics quite inhospitable to life. However, understanding the karst critical zone (Chen et al., 2018) is of fundamental importance to vast numbers of the global population who rely on karst aquifers for fresh drinking water supplies (Stevanovic, 2019). Possibly the least well-

established connections within karst and cave science revolve around the role of microbial communities within karst, their sources, abundance, community structure and their ability to adapt and even thrive in cave environments (Engel, 2010). It is possible that microbial communities play a central role in the karst aquifer system; their utilisation and consumption of key nutrients (C, N, S and P) may help to balance the anthropogenic increase in nutrient loads to many karst environments. With microbes holding such a potentially critical role within the karst geochemical system and wider ecosystem, it is fundamental that we tackle key questions surrounding the "microbial network" (Zhu et al., 2021).

Previous work on cave microbiology is scarce but slowly developing (Fairchild and Baker, 2012; Zhu et al., 2021). The routes to microbial deposition within cave systems are thought to primarily be through the entrainment of microbes along with other organic matter in the soil and the "wash in" of this material through the karst aquifer and into the cave. There is also the potential for aquifer biofilms to develop and contribute to communities later deposited within the cave system. In some systems, cave dwelling animals or human visitors could play a role in adding to the microbial network possibly through aerosol transport and contamination (Dredge et al., 2013; Smith et al., 2013), although this is thought to diminish in importance as you move further from the cave entrance and away from tourist routes (Fairchild and Baker, 2012).

Based on DNA studies, it has been noted that about half of all recognised bacterial phyla have been identified within cave or karst ecosystems (Engel, 2010; White, 2009) and that microbial communities can thrive in these ecosystems with cell counts of up to 10^6 cells/g of rock (Barton and Jurado, 2007). Globally, studies have identified Betaproteobacteria, Deltaproteobacteria, Acidobacteria, Actinobacteria, and Nitrospira throughout cave and karst aquifers, associated in-cave sediments and on cave surfaces, including speleothems (Farnleitner et al., 2005; Ortiz et al., 2013; Pronk et al., 2009; Shabarova and Pernthaler, 2009). The lack of sunlight within cave systems means that these microbes have to be adapted to utilise alternative energy sources. For example, the chemosynthetic fixation of inorganic carbon, degradation of aromatic hydrocarbons and the use of hydrogen sulphide have all been identified as non-photosynthetic energy pathways in use in cave systems (Marques et al., 2019; Sarbu et al., 1996; Wu et al., 2015). These microbial communities may become the primary food base to support other cave adapted species (Sarbu et al., 1996). Not only are these species highly adapted to the cave environment, they also appear specifically adapted to their niche within the cave. A detailed study of caves of the Yunnan-Guizhou Plateau (China) found that cross-cave microbial networks were often similar, however individual "in-cave" habitat niches (air, water, rock and sediment) were found to be quite distinct from one another (Zhu et al., 2019; Zhu et al., 2021), potentially controlled by their different source micronutrients (Cloutier et al., 2017) and mineral chemistry (Wu et al., 2015).

In an attempt to undertake a comprehensive assessment of the microbial network and its key niches within an anthropogenically impacted cave system, we consider possible microbial habitats from the top down, looking at community dynamics within soils, karst aquifer water, sediments and calcite speleothem deposits. This work has focussed upon Poole's Cavern, Buxton, UK. This site was chosen primarily due to the unique hyperalkaline drip waters (pH>9) found within some sections of the cave, the small amount of previous microbial work undertaken locally, and the availability of baseline cave climate data provided as part of the British Cave

Science Centre (BCSC) project. This cave system offers an excellent opportunity to interrogate microbial dynamics in a high pH environment, investigate the unique development of microbial niches and quantify the extent to which there are linkages between these habitats.

Whilst most cave and karst systems have very little known about their inter-niche microbial network, the unique hyperalkaline soil and karst water environments around Buxton have attracted slightly more previous work on extremophile communities. Burke et. al. (2012) profiled a distinct anaerobic alkaliphilic community dominated by a single, unidentified bacterial species within the *Comamonadaceae* family of Betaproteobacteria in the Buxton lime kiln deposits near the Poole's Cavern site. This community appears capable of microbial nitrate reduction with increasing anoxia (Burke et al., 2012). There is also evidence of communities surviving in a non-karst hyperalkaline spring in Buxton. Smith et. al. (2016) sequenced the community in pH 7.3 to pH 13 waters. At pH 13, the sequence library was dominated by the families *Pseudomonadaceae* and *Enterobacteriaceae* of Gammaproteobacteria, with low overall diversity. They concluded that these communities were functioning at a pH of 11-12, but at higher pH (pH 13) these communities remained present, but were unlikely to be active. The only previous work within Poole's Cavern focussed on soil and hyperalkaline drip waters. Blyth et. al. (2014) conducted a study looking at lipid biomarkers: glycerol dialkyl glycerol tetraethers (GDGTs) (Blyth et al., 2014). They found two distinct profiles between the subsurface soils above the cave and the speleothem drip waters in an interior chamber. The drip waters were dominated by branched GDGTs, indicative of a bacterial source, and the soils were dominated by isoprenoid GDGTs, indicative of an aquatic archaeal source. This difference indicates that microbial communities established within the cave (or karst) were distinct from those in the soil zone, suggestive of microbial diversification within the karst system, rather than a simple in-wash from infiltrating soil waters. These previous local studies offer a glimpse into the potential communities that can exist within hyperalkaline waters such as those sampled in Poole's Cavern.

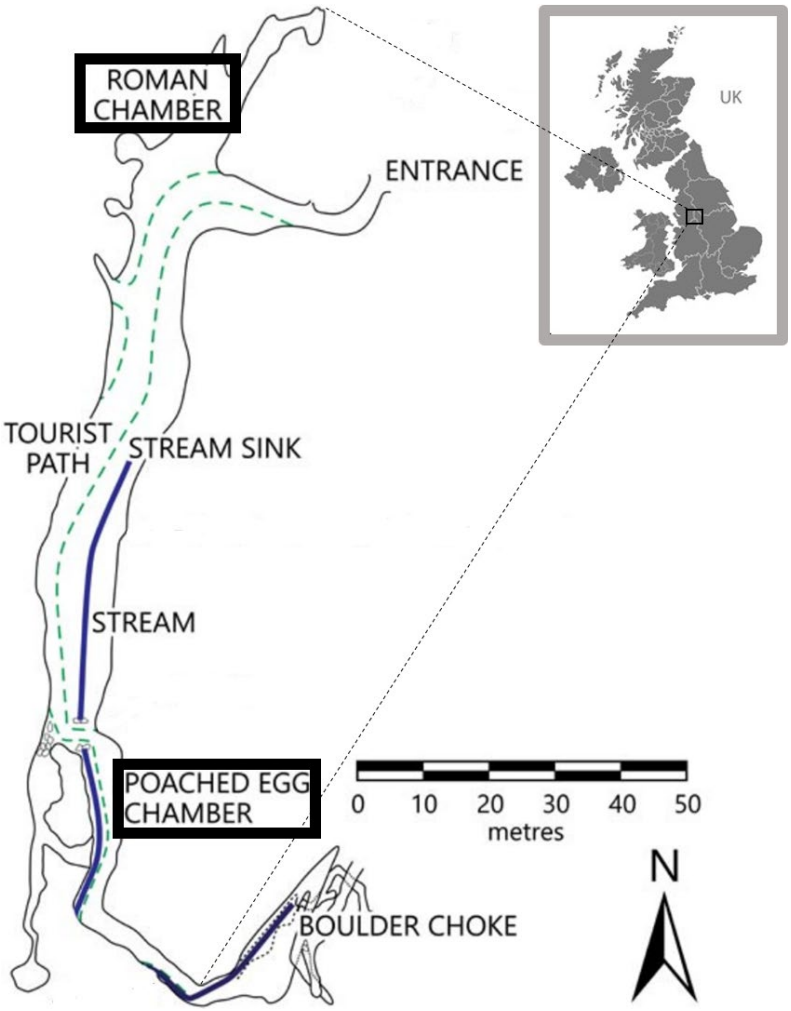
The interior environmental conditions of Poole's Cavern vary remarkably. However, the cavern is a cross-linked integrative ecosystem. Unveiling the individual communities in the key source environments (soils, waters) and those in the sinks (speleothems, muds) will elucidate the development of above-cave and in-cave biodiversity and the adaptive capacity of microorganisms to different but proximal microhabitats (Zhu et al., 2021). This paper will investigate the community network more fully, using culture-dependent experiments and DNA sequencing to answer questions about community viability and structure throughout the soil, karst aquifer, cave and speleothem system.

2 Site Description and Methodology

2.1 Site description

Poole's Cavern is a shallow epigenetic cave evolved from early Carboniferous Bee Low limestones (Hartland et al., 2010b; Newton et al., 2015). The main cave strikes N-S with a total length of 240m, with the main passage terminated by a partially cemented boulder choke (Deakin et al., 1968; Rowberry et al., 2020). The main chambers of interest for sampling (Figure 1a) from this project include Roman Chamber (RC, close to the cavern entrance) and Poached Egg Chamber (PE, deeper into the cavern) (Figure 1b, c). These two chambers are both out of reach from the tourist path

and the public (Figure 1a). Speleothem formations in the PE chamber are characterised by extremely rapid growth rates (10 mm/year) (Hartland et al., 2010b), caused by the super-saturation of carbonate under hyperalkaline conditions. This is due to the leaching of 17th Century lime kiln waste (CaO) above the cave producing $Ca(OH)_2$, which rapidly disassociates to Ca^{2+} and OH^- ions raising the pH (Hartland et al., 2010b). At hyperalkalinity, the hydroxylation of CO_2 dominates, producing a rich carbonate source (Clark et al., 1992) that has facilitated the formation of the large distinctive stalagmites that attract tourists annually (Figure 1c).



(**a**)

(**b**)

(**c**)

Figure 1. (**a**) Locations of Poole's Cavern and its chambers of this study (modified with permissions, from Rowberry et al., 2020). Internal landscapes of (**b**) Roman Chamber (RC) and (**c**) Poached Egg Chamber (PE) [BGS © UKRI].

Above Poole's Cavern exists a well-established, managed deciduous woodland. Soils are organic rich with significant deciduous leaf litter overtopping a ~10-cm layer of organic-rich topsoil and a ~30-cm layer of lime kiln waste (Charles et al., 2015; Hartland et al., 2010a). This lime kiln waste is left over from several lime kilns, which were atop some of the cave chambers. As waters leach the kiln waste they become hyperalkaline. Where leaching waters enter the cave system they form distinctive stalagmite deposits in the chambers below.

No vertebrates including bats were observed in the Poole's Cavern due to the adverse in-cave conditions as well as the mesh on the entrance door. The closure of the cave to tours (more than one year before sampling) during the COVID-19 pandemic provided a unique opportunity to sample the cave in a more natural state, where any microbiological impact from anthropogenic interference is limited.

2.2 Poole's Cavern and sample collection

In total, eight samples were targeted, including soil, water, sediment and calcite. Sampling took place in spring/summer (April) 2021. Two calcite samples (PE High and PE Low) were "grown" in Poached Egg Chamber. These samples were allowed to precipitate onto clean, autoclaved watch glasses, with calcite left collecting for two months before sampling (Figure 1a). One sample (PE Straw) was from a stalactite straw on the ceiling of the PE Chamber. A PE drip water sample (PE Water) was collected over a period of two weeks by attaching a funnel to an actively depositing speleothem ('PE3', as described in (Morgan, 2022)) and routing the captured water into a 20-L jerrican. 6 L of this water sample were filtered through a 0.22 μm Sterivex filter within Poole's Cavern and immediately sealed with Parafilm. RC chamber water was collected only for a single water chemical measurement due to slow drip rates. Additionally, one calcite and one mud sample (RC Calcite and RC Mud) were acquired from Roman Chamber (Figure 1b), with the mud being extracted from the bottom of a seasonally active pool fed by drip waters. This pool contained water at the time of sampling but is known to dry out during the summer months. Moreover, soils above Poole's Cavern and calcareous soils from ~1.7-km southeast of the nearby Brook Bottom valley (53°14'15.2"N, 1°54'35.8"W) were sampled. All samples for microbiological analyses (sequence analysis and cell count analysis) were collected using a sterilised hand auger and containers. For soil and sediment samples, the top 10-cm layers were discarded before sample collection; each soil or sediment sample was a pool of at least three locations.

All samples for microbiological analysis were transported on ice to BGS Keyworth that day. Samples for DNA analysis were frozen at -20°C on return. Samples for cell culture were shipped cold to the University of St Andrews and stored at 4°C before processing.

Temperature, pH, and electrical conductivity of speleothem drip waters from PE and RC chambers were measured *in situ* at the time of sampling using a WTW Multi 340i handheld multimeter.

2.3 DNA extraction and sequence analysis

Samples for sequence analysis were frozen after sampling and stored at -20°C

for one week before DNA extraction. All implements for molecular analyses were either filter sterilised, autoclaved, flamed, or UV-irradiated to prevent any external contamination. The de-ionised water used was molecular biology grade and nuclease-free. Either ~400 mg of solid material or the Sterivex filter membrane were used as input for DNA extraction. 100 µL DNA were extracted with one negative control using the FastDNA SPIN Kit for Soil (MP Biomedicals, CA, USA) following the manufacturer's instructions. DNA extracts were amplified for barcoded MinION 16S metagenomic sequencing using 16S Barcoding Kit (SQK-RAB204; Oxford Nanopore Technologies, Oxford, UK) and analysed using Flongle R9.4.1 flow cells (Oxford Nanopore Technologies, Oxford, UK). The 16S rRNA primer pair used for amplification was the domain Bacterial-specific forward primer 27F (5′-AGAGTTTGATCCTGGCTCAG-3′) and the universal reverse primer 1492R (5′-GGTTACCTTGTTACGACTT-3′) (Reysenbach et al., 2000). The polymerase chain reaction (PCR) was conducted using C1000 Touch thermocycler (BioRad, CA, USA) with the following conditions – 95°C for 1 min, then 40 cycles of 95°C for 20 s, 55°C for 30 s and 65°C for 2 min, with final extension at 65°C for 5 min. PCR reactions were conducted in triplicate, each consisted of 5 µL LongAmp Taq DNA polymerase (NEB, MA, USA), 0.2 µL barcoded primer, and 0.03-1.6 µL DNA extract, with the remaining volume made up with molecular grade water. The barcoded PCR products were purified using ChargeSwitch PCR Clean-Up Kit (Thermo Fisher Scientific, MA, USA). Amplicon concentration was quantified using an Invitrogen Qubit 3.0 Fluorometer. Only samples with more than 0.05 ng/µL yields (all samples other than PE High and Low) were passed for sequencing on the Oxford Nanopore MinION platform (Oxford Nanopore Technologies, Oxford, UK).

Bacterial sequences were analysed in the framework of the open-source program Quantitative Insights into Microbial Ecology 2 (QIIME 2 version 2021.8) (Bolyen et al., 2019). In the first step, the MetONTIIME meta-barcoding pipeline (version 1.14.0) was applied as an EPI2ME 16S workflow emulator to process MinION sequenced fast5 data in QIIME 2 framework (Maestri, 2021; Maestri et al., 2019). Next, base calling was performed using Guppy 5.0.11, followed by read dereplicating and clustering at 100% identity (Maestri, 2021) against the Silva 138 99% reference database (Quast et al., 2013; Yilmaz et al., 2014). Then, the generated amplicon sequence variants (ASVs) were aligned against the Silva 138 99% reference database to output taxonomy data using q2-feature-classier (Bokulich et al., 2018). To pledge the sequence quality, sequences with confidence > 0.7 were selected for taxonomic identification (Table S1). Based on the taxonomy information, mitochondrial, chloroplast, archaeal, eukaryotic, and unassigned ASVs were excluded prior to the taxonomic classification analysis. Then, the alpha diversity index, Faith's phylogenetic diversity, was computed after rarefaction at sampling depth 92 with alpha-phylogenetic package (Faith, 1992) in QIIME 2.

The improvement of QIIME 2's genome prediction will subsequently optimize accuracy of metabolic inference. Thereafter, potential functions of these 16S sequences were predicted with Phylogenetic Investigation of Communities by Reconstruction of Unobserved States 2 (PICRUSt2) (Douglas et al., 2020) based on individual ASVs. PICRUSt2 has also remarkably improved accury and flexibility for marker gene metagenome inference compared to its predecessor. ASVs at this stage were 3,982 counts. Briefly, reads were assigned into phylogenetic units with HMMER (Eddy and Wheeler, 2007), EPA-ng (Barbera et al., 2019), and GAPPA (Czech and Stamatakis, 2019). The hidden-state prediction was performed with the R package

'caster'. Representative ASVs with nearest-sequenced taxon index (NSTI) more than two were excluded from the output. Finally, microbial metabolic pathways were identified using the Minimal set of Pathways (MinPath) approach (Ye and Doak, 2009) referencing the KEGG Orthology (KO) and MetaCyc pathway database, with the correlation coefficients around 0.8-0.9 between the predicted and observed data (Douglas et al., 2020). Please note the PICRUSt2 inference method is difficult in predicting reduced genomes (e.g., rare species) and cannot perfectly reflect active or even present genes from extracted microbiome, but provides a most likely function based on DNA sequences and related well-understood species (Douglas et al., 2020). After PICRUSt2 filtering, 22 (8 from PE Water, 4 from Soils above Poole's, 9 from Brook Bottom soils, 1 from RC Calcite) out of 3977 sequences were removed from functional group analysis. These 22 sequences belonged to 5 genus *Candidatus Omnitrophus* (Verrucomicrobiota), 1 genus *Brevundimonas* (Alphaproteobacteria), 1 genus *Hydrogenophaga* (Gammaproteobacteria), 1 unidentified Proteobacteria, and 14 unknown bacteria (Table S1).

2.4 Determination of microbial abundance

The estimated cell content of each sample was calculated from the concentration of extracted DNA using the equivalent conversion factor 9×10^{-15} g DNA·cell^{-1} (Kirchman, 2012).

Additionally, microscopic cell counting and cell culture of aseptically collected samples (stored at 4°C) were performed one week after sampling. Each sample was suspended and well-mixed in 1:1 volume (mL):weight (g) autoclaved 0.9% NaCl solution with two drops of Tween 20 to preserve osmotic pressure and detach cells from particles. Several 10× serial dilutions were further prepared. Cell numbers of 100:1 dilutes were counted under an AmScope optical microscope using a Hirschmann hemocytometer in quadruplicate; each replicate picked up the large counting grids at four corners.

For cell culturing experiments, the required volume of each dilute sample was estimated and spread on Plate Count Agar culture plates based on preliminary culturing tests. The recipe for Plate Count Agar was 5 g/L tryptone, 2.5 g/L yeast extract, 1 g/L dextrose and 9 g/L agar. Each sample was plated on these culture plates in quadruplicate. Visible colonies were counted after 10 days of incubation at 21°C in accordance with the growth rate of these microbiotas.

2.5 Statistical techniques

Hierarchical clustering of species from each individual site were performed using the Bray-Curtis dissimilarity index and the unweighted pair group method with arithmetic mean (UPGMA) algorithm in Past 4.08 (Hammer et al., 2001). Next, bivariate correlation coefficients were calculated to understand the relationship between sites. Prior to correlation analysis, normality test was performed to determine whether parametric (Pearson's r) or nonparametric (Kendall's tau) statistical approach should be used. Since the microbial community network closely links with ecosystem function (Finlay et al., 1997), we conducted network analysis on Poole's Cavern microbiota at species level. Samples were combined for the extended Bayesian information criterium graphic lasso (EBICglasso) estimation (Friedman et al., 2008). Using individual sites and intercorrelation as nodes and connecting edges respectively, the co-occurrence pattern of species that represents the microbial network was defined based on their EBICglasso coefficients. The network diagram

and centrality plot of expected influence were drawn using JASP 0.16.2 (https://jasp-stats.org/).

3 Results

3.1 Water chemistry

The temperatures of PE Water and RC Water were 7.3±0.3 °C (n=4) and 9.3 °C (n=1), respectively. Electrical conductivities of each water type were 1216.5±57.5 µS/cm (n=4) and 600.0 µS/cm (n=1), respectively. pH values were 12.2±0.1 (n=5) and 9.3 (n=1), for PE Water and RC Water respectively.

3.2 Microbial abundance and diversity

Sites where samples were collected for microscopic cell counting and cell culture experiments were: PE High, PE Low, PE Water, PE Straw, Soils above Poole's, and Brook Bottom soils. PE Low had the lowest detectable microbial content as reflected in <0.05 ng/µL and in all cell count methods; the extracted DNA concentration from PE High was also below the detection limit of Qubit fluorometer (0.05 ng/µL), although its countable microbes were more abundant than PE Water and PE Straw. Conversely, the microbial abundance of Soils above Poole's Cavern and at Brook Bottom, were consistently the highest (Table 2).

Table 1

Environmental Parameters of PE and RC Chambers within Poole's Cavern.

Features	PE	RC
pH	12.2±0.1 (n=5)	9.3 (n=1)
Temperature (°C)	7.3±0.3 (n=4)	9.3 (n=1)
Conductivity (µS/cm)	1216.5±57.5 (n=4)	600.0 (n=1)
Hydroxide (mg/L)	144.4 (n=1)	-
Carbonate (mg/L)	11.6 (n=1)	-
Total alkalinity (mg/L)	156.0 (n=1)	-
Orthophosphate (mg/L)	0.018±0.001 n=3)	-

Note. RC chamber did not have enough liquid sample for environmental characterization.

Table 2

The Concentrations (Mean ± Standard Error) of Microbial Cells Determined by Plate Count Agar Cell Culture, Microscopic Cell Counting, and Soil DNA Conversion; Faith's Phylogenetic Diversity Indices of Poole's Cavern Samples.

Samples	Viable Cultivable Cell Counts (CFUs/g)	Microscopic Cell Counts (cells/g)	Soil DNA Equivalence (cells/g)	Faith's Phylogenetic Diversity
PE High	$(2.36\pm1.67)\times10^{4}$	$(1.28\pm0.50)\times10^{8}$	$<1.39\times10^{6}$	-
PE Low	$(6.00\pm1.47)\times10^{2}$	$(3.44\pm1.64)\times10^{7}$	$<1.39\times10^{6}$	-
PE Water	$(5.13\pm2.81)\times10^{3}$	$(2.81\pm1.43)\times10^{7}$	5.89×10^{7}	16.21
PE Straw	$(2.41\pm1.53)\times10^{4}$	$(2.50\pm1.29)\times10^{7}$	2.40×10^{7}	17.68
Soils above Poole's	$(1.29\pm0.24)\times10^{7}$	$(4.14\pm1.11)\times10^{8}$	1.61×10^{9}	24.35
Brook Bottom soils	$(7.83\pm2.72)\times10^{5}$	$(1.83\pm0.78)\times10^{8}$	1.61×10^{9}	26.22
RC Calcite	-	-	4.36×10^{7}	22.49
RC Mud	-	-	2.73×10^{8}	22.12

Note. RC Calcite and Mud did not have enough sample for cell culture. PE High and Low did not have enough extractable DNA for sequencing.

Based on sequence analysis, PE Water had the lowest phylogenetic diversity index (16.21), followed by PE Straw (17.68). Brook Bottom soils (26.22) and Soils above Poole's (24.35) had the highest diversity indices. The two RC samples, calcite (22.49) and mud (22.12) were intermediate to these (Table 2).

3.3 Taxonomic phylogeny

Proteobacteria were the most abundant phyla and displayed similar relative abundance among six samples (Figure 2). Proportions of unclassified bacteria were high in all samples. PE Water had abundant Actinobacteriota and Bacteroidota, while PE Straw had higher abundances of Firmicutes and Zixibacteria (Figure 2). Consistent with phylogenetic diversity indices, PE Water and PE Straw had the least diverse communities while soils from Brook Bottom and above Poole's had the most diverse communities (Table 2). Planctomycetota, Verrucomicrobiota, Acidobacteriota, Myxococcota and Nitrospirota were abundant in the solid samples (mud, soil, calcite), while PE Water clearly had lower abundances of these groups (Figure 2).

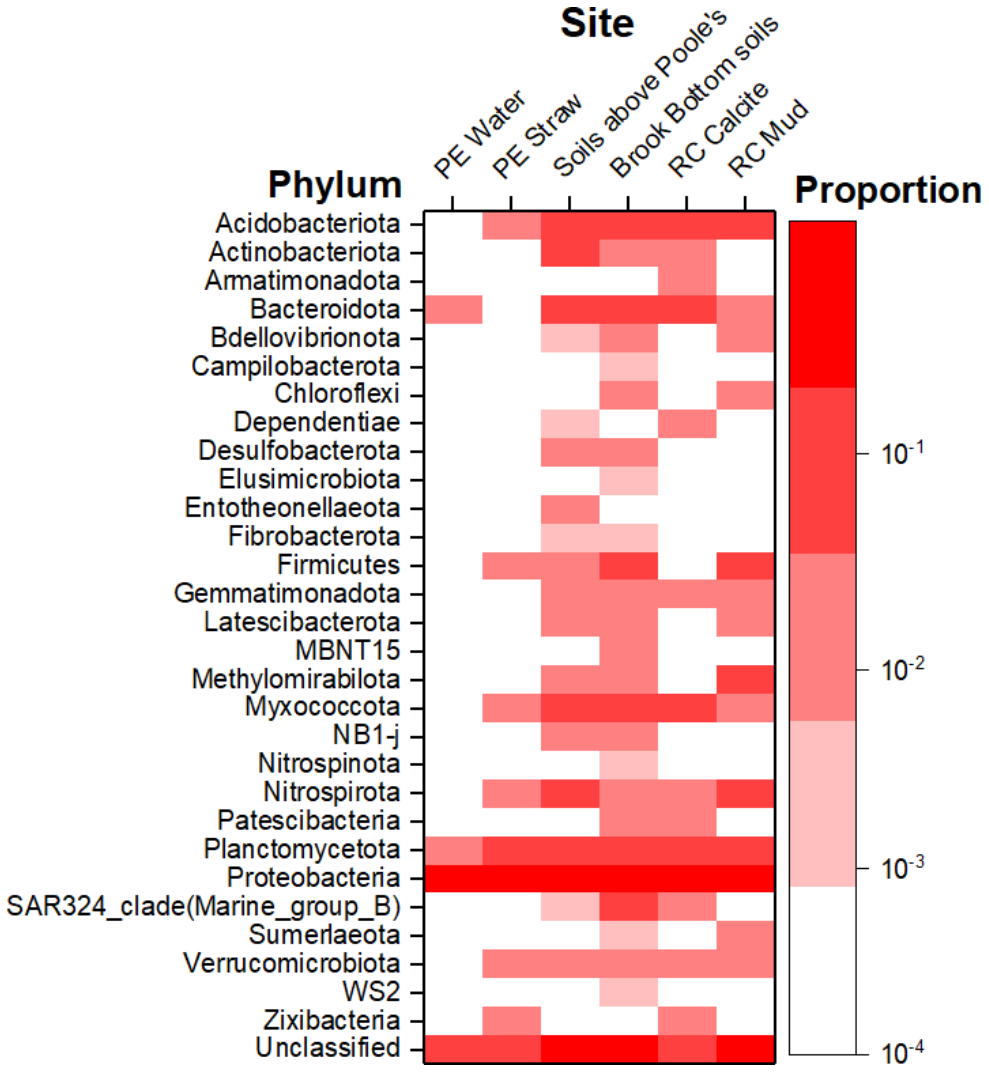


Figure 2. Proportions of taxonomic groups of bacteria from Poole's Cavern samples.

The dominant bacterial families displayed very different patterns among Poole's Cavern sampling sites (Table 3). The most dominant family was always from Proteobacteria however differed among the samples: *Xanthomonadaceae* was the most dominant family in PE Water (28.6%), which also included high abundances of *Comamonadaceae* (26.1%). *Comamonadaceae* was the most dominant in PE Straw (16.3%), Soils above Poole's (5.8%), and RC Calcite (8.3%); *Nitrosomonadaceae* was the most dominant in Brook Bottom soils (10.8%) and RC Mud (14.1%). Other dominant families came from Planctomycetota, Nitrospirota, Bacteroidota, Methylomirabilota, Firmicutes, Sumerlaeota, Acidobacteriota and Zixibacteria (Table 3).

Table 3

Proportions of Dominant Bacterial Families (>2% in At Least One Site, Bolded) from Poole's Cavern Samples.

Family	PE Water	PE Straw	Soils above Poole's	Brook Bottom soils	RC Calcite	RC Mud
Nitrosomonadaceae (Proteobacteria)	0.8%	**11.4%**	**2.9%**	**10.8%**	**6.7%**	**14.1%**
Sphingomonadaceae (Proteobacteria)	**12.6%**	0.0%	1.1%	0.0%	**3.3%**	0.0%
Caulobacteraceae (Proteobacteria)	**15.7%**	**3.6%**	0.0%	0.1%	0.0%	0.0%
Xanthomonadaceae (Proteobacteria)	**28.6%**	0.0%	0.4%	0.4%	**4.2%**	0.0%
Xanthobacteraceae (Proteobacteria)	1.0%	**3.0%**	**4.0%**	1.3%	0.8%	**2.2%**
Comamonadaceae (Proteobacteria)	**26.1%**	**16.3%**	**5.8%**	**3.6%**	**8.3%**	**2.2%**
Hyphomicrobiaceae (Proteobacteria)	0.0%	**6.6%**	0.7%	0.2%	0.8%	0.0%
Beijerinckiaceae (Proteobacteria)	**2.9%**	0.6%	0.0%	0.1%	0.0%	0.0%
Methylophilaceae (Proteobacteria)	0.1%	0.0%	0.7%	0.0%	**2.5%**	0.0%
Rhodobacteraceae (Proteobacteria)	0.0%	0.0%	1.4%	0.0%	**4.2%**	0.0%
Burkholderiaceae (Proteobacteria)	0.4%	**2.4%**	0.4%	0.0%	0.0%	1.1%
Sutterellaceae (Proteobacteria)	0.0%	0.0%	0.0%	0.1%	**3.3%**	1.1%
Solimonadaceae (Proteobacteria)	0.1%	0.0%	0.0%	0.0%	0.0%	**3.3%**
Rhizobiales Incertae Sedis (Proteobacteria)	0.0%	0.0%	**2.2%**	0.4%	0.8%	**3.3%**
Gemmataceae (Planctomycetota)	0.0%	1.2%	**5.0%**	1.5%	0.0%	**2.2%**
Phycisphaeraceae (Planctomycetota)	0.0%	**7.8%**	0.0%	0.2%	0.0%	0.0%
Nitrospiraceae (Nitrospirota)	0.0%	**2.4%**	**3.2%**	1.0%	1.7%	**4.3%**
Chitinophagaceae (Bacteroidota)	0.0%	0.0%	**4.3%**	1.4%	1.7%	1.1%
Methylomirabilaceae (Methylomirabilota)	0.0%	0.0%	0.0%	0.1%	0.0%	**3.3%**
Planococcaceae (Firmicutes)	0.0%	0.0%	0.4%	0.8%	0.0%	**2.2%**
Bacillaceae (Firmicutes)	0.0%	0.0%	0.7%	1.4%	0.0%	**2.2%**
Sumerlaeaceae (Sumerlaeota)	0.0%	0.0%	0.0%	0.2%	0.0%	**2.2%**
Vicinamibacteraceae (Acidobacteriota)	0.0%	0.0%	**2.2%**	0.7%	0.8%	0.0%
Zixibacteria (Zixibacteria)	0.0%	**2.4%**	0.0%	0.0%	0.8%	0.0%

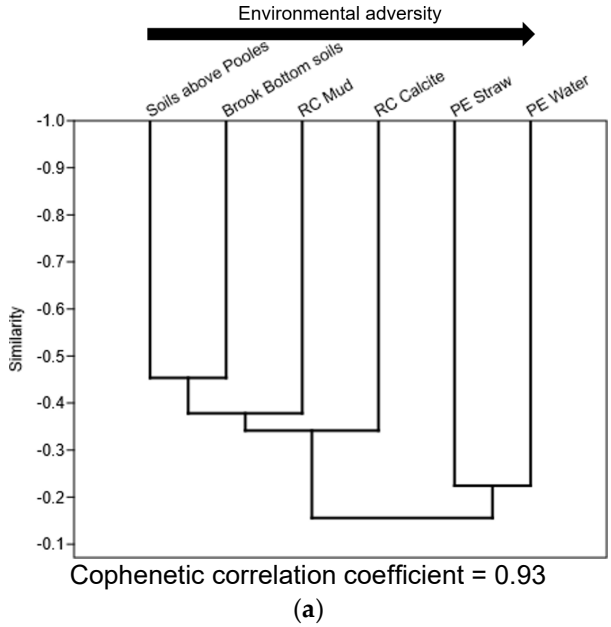
Note. Nomenclatures follow updated Silva 138 database.

3.4 Ordination analyses of study sites

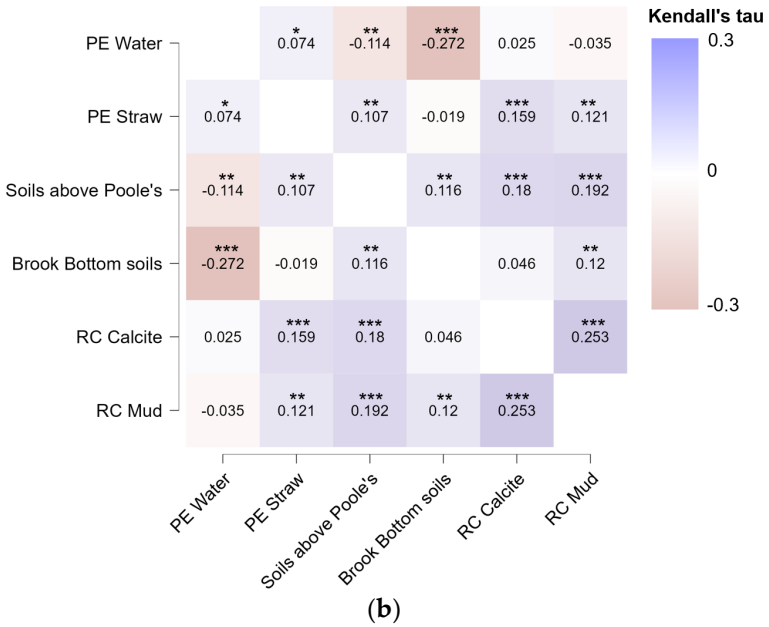
Based on the hierarchical clustering analysis of the sequence data, soils above Poole's Cavern were the most similar to Brook Bottom soils (~45% similarity) (Figure 3a). Their cluster was the most similar to RC Mud (~38% similarity), and then to RC Calcite (~32% similarity). PE Straw and PE Water were clustered together but with the lowest level of similarity (~22% similarity).

Since none of the relative abundances of microbiomes from sampling sites demonstrate a normal distribution pattern, Kendall's tau correlation coefficients were calculated and depicted in Figure 3b. Intriguingly, the PE Water sample significantly anticorrelated with the two soil samples – Soils above Poole's and Brook Bottom soils. This trend indicated that most of the dominant microbial members in the least adverse soil samples outside Poole's Cavern diminished in the harsh hyperalkaline water, while most of the rare species in external soils became dominant in the in-cave hyperalkaline water. Besides, the microbial community of PE Water was moderately associated with PE Straw, while PE Straw was highly associated with RC Calcite, and RC Calcite was highly associated with RC Mud. Additionally, the two external soil samples were correlated with each other as well as the samples from the RC chamber.

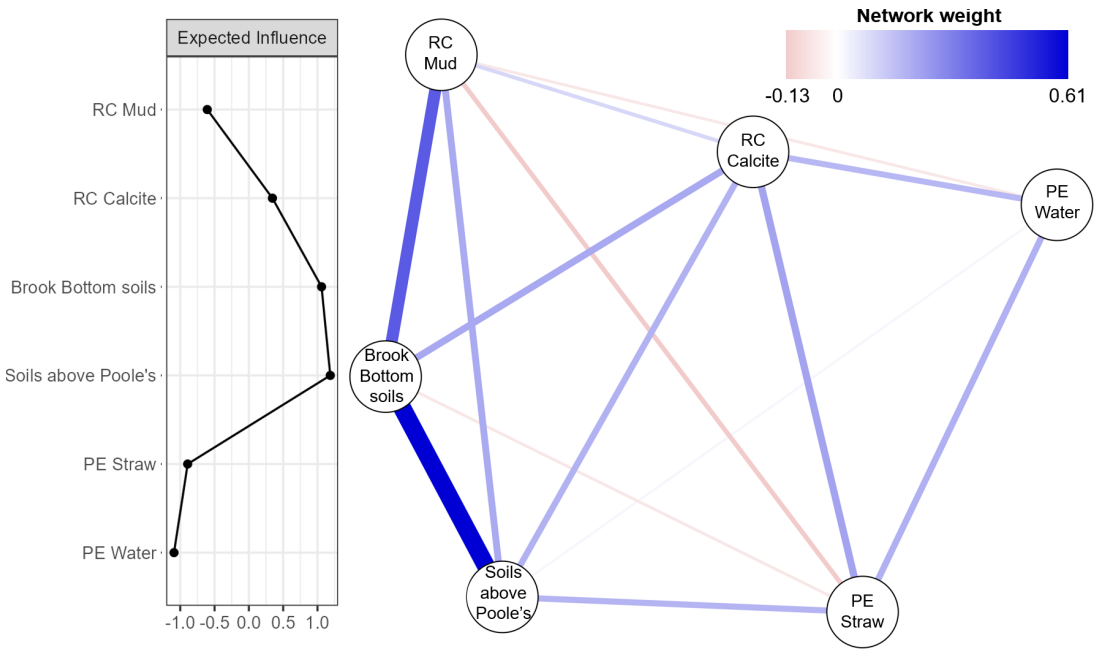
Moreover, network analysis that visualises the co-occurrence between species demonstrated that PE Water were the most marginal point that had the weakest connection with other sites; PE Straw was slightly less marginalized; two RC samples Mud and Calcite were more centralized. Centrality plot (represented by the expected influence index) delineated that Soils above Poole's was the most influential site that was positively linked with other sites, followed by Brook Bottom soils (Figure 3c).



(**a**)



(**b**)



(**c**)

Figure 3. (**a**) Hierarchical clustering of Poole's Cavern samples using Bray-Curtis dissimilarity index based on bacterial species; (**b**) Kendall's tau correlation heatmap (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$); (**c**) centrality and network plots.

By closely inspecting microbial classes, in-cave niches were distinct from Brook Bottom soils primarily due to abundance differences in Acidobacteriae, Holophagae, Vicinamibacteria, Acidimicrobiia, Thermoleophilia, Bacteroidia, Anaerolineae, Desulfuromonadia, Syntrophia, Bacilli, Clostridia, Latescibacteria, MBNT15, Methylomirabilia, Myxococcia, Polyangia, Planctomycetes, Alphaproteobacteria and Gammaproteobacteria. On the other hand, in-cave niches were distinct from Soils above Poole's primarily due to Vicinamibacteria, Acidimicrobiia, Bacteroidia, Polyangia, Nitrospiria, Phycisphaerae, Alphaproteobacteria and Gammaproteobacteria.

3.5 Metabolic pathway analyses

Of the different metabolic pathways identified in the Poole's Cavern samples, half were common across all samples and most involved the biosynthesis of essential biomolecules (Figure 4). The most common of these metabolic pathways were: amine and polyamine biosynthesis, amino acid biosynthesis, aromatic compound biosynthesis, carboxylate degradation, cell structure biosynthesis, cofactor and vitamin biosynthesis, fatty acid and lipid biosynthesis, fatty acid and lipid degradation, nucleoside and nucleotide biosynthesis, nucleoside and nucleotide degradation, secondary metabolite biosynthesis, secondary metabolite degradation, aminoacyl-tRNA charging, fermentation, nucleic acid processing, pentose phosphate pathways, photosynthesis, respiration and tricarboxylic acid (TCA) cycle.

PE Water, as the sole liquid sample, showed higher proportions of aldehyde degradation, amino acid degradation and aromatic compound degradation, but lower relative proportions of alcohol degradation, carbohydrate biosynthesis, polymeric compound degradation, C1 compound utilisation and assimilation, electron transfer and glycolysis (Figure 4). PE Straw had relatively

lower proportions of alcohol degradation, aldehyde degradation and detoxification/antibiotic resistance than other sites. Soils above Poole's had a higher proportion of cofactor, prosthetic group and electron carrier degradation. Brook Bottom soils had higher proportions of alcohol degradation and Entner-Duodoroff pathways. RC Calcite had a higher proportion of amine and polyamine degradation, while RC Mud had a lower proportion of glycan degradation.

Figure 4. Proportions of predicted metabolic pathway superclasses by PICRUSt2 in reference to the MetaCyc metabolism database.

The percentages of salinity-related pathways ranged from 1.03% in PE Water to 1.21% in RC Mud. The percentages of inorganic nutrient metabolism ranged from 1.45% in Brook Bottom soils to 1.76% in PE Straw (Table 4). Some pathways relevant to nitrate, sulphate and phosphate metabolisms contain assimilatory nitrate reduction, denitrification, nitrifier denitrification, assimilatory sulphate reduction, sulphur oxidation and methylphosphonate degradation.

Table 4

Percentages of Predicted Pathways Related to Salinity and Inorganic Nutrient Metabolisms.

Metabolism Site	Salinity	Inorganic nutrient metabolism
PE Water	1.03%	1.61%
PE Straw	1.16%	1.76%
Soils above Poole's	1.17%	1.58%
Brook Bottom soils	1.20%	1.45%
RC Calcite	1.14%	1.70%
RC Mud	1.21%	1.51%

4 Discussion

Cave deposits, including speleothems, are prominently influenced by factors external to the cave system. Inorganic minerals and organic molecules are released from the soil, aquifer and bedrock and flushed into the cave system, where they are re-deposited on speleothem surfaces. Therefore the "source" region is often defined as the soils and waters above the cave and the vector of transport the percolating waters that eventually enter the cave. Whilst clear connections can often be seen between physical events (large rainfalls or autumnal flushes) with influxes of inorganic nutrients (Borsato et al., 2007; Wynn et al., 2014) and organic carbon (Baker et al., 1993; Webb et al., 2014), less is known on how this continuous transfer of nutrients and minerals and indeed microbial cells will influence the makeup of the in-cave microbial network (Zhu et al., 2019). Additionally, the delivery of nutrients driven by environmental alterations such as UV fluorescence laminations can vary notably on a secular basis (Webb et al., 2014) or an annual basis (Baker et al., 1993). Understanding the individual community structures in the key source environments (soils, waters) and those in the sinks (speleothems, muds) can help elucidate the extent to which in-cave communities are a function of their source communities, or if they can successfully adapt and diversify forming microhabitats or niches (Zhu et al., 2021).

4.1 Microbial community structures in different microhabitats

Proteobacteria represent the dominant phyla in all the samples collected within and around Poole's Cavern. Most sites and sample types have a high abundance (if not dominant) of previously discovered *Comamonadaceae* (Table 3), which is unsurprising as they are a versatile bacterial family, including anaerobic denitrifiers, hydrogen oxidisers, ferric iron reducers, photoautotrophs, photoheterotrophs and aerobic organotrophs (Burke et al., 2012; Willems, 2014). Apart from the dominance of Proteobacteria in all sites, the microbial community structure from soils, waters, mud and speleothems in this hyperalkaline cave environment (Hartland et al., 2010b) varied remarkably (Figure 2 & Table 2). As a rule, the biodiversity and abundance of bacteria in karst ecosystems were less than soils above the cavern, indicating a stronger selection pressure at the community level within the cave (Bassil et al., 2020).

4.1.1 Soils

The soil systems above Poole's Cavern are by far the most diverse in terms of community structure, due to the organic rich and more neutral nature of the soils and the lack of requirement for highly specialised microbial communities to develop. Only the Soils above Poole's Cavern were characterised by *Gemmataceae*, *Chitinophagaceae* and *Vicinamibacteraceae* (Table 3). The bacterial families found here, but not elsewhere, suggests a community well adapted to the temperate forest ecosystem overlying the cave and include *Gemmataceae* which are aerobic chemoorganotrophic bacteria that inhabit soils, wetlands, and freshwater (Kulichevskaya et al.,

2020); *Chitinophagaceae* which degrade chitin or even hydrolyse cellulose (Rosenberg, 2014), and *Vicinamibacteraceae* which are neutrophilic and psychrotolerant chemoheterotrophs (Huber and Overmann, 2018). The dominance of these specific families within the soil means they can be used as tracers for "downstream" environments, helping us to identify if these communities can be flushed through the soil system and into the cave, and once washed in if they can still function/thrive in this unique environment of high pH waters and complete darkness.

4.1.2 Cave Waters

PE Water was the least diverse of all the samples collected, immediately indicating a disconnect between the highly diverse soils above the cave and the water emanating from the karst aquifer. Microbes in this water sample with pH>12 were even plausibly not metabolically active (Smith et al., 2016), however were still viable as demonstrated by the presence of culturable microorganisms. Only four families, *Sphingomonadaceae*, *Xanthomonadaceae*, *Comamonadaceae* and *Caulobacteraceae* make up over 80% of the bacteria found within this water (Table 3). The sole bacterial family that dominates PE Water only were *Beijerinckiaceae* (Table 3), a group of nitrogen fixers and methanotrophs (Morawe et al., 2017). Whilst we are limited to assessing a single sample, there was also no evidence for the potential tracer families unique to the Soil above Poole's (*Gemmataceae*, *Chitinophagaceae* and *Vicinamibacteraceae*) within the drip water. This disconnect is clear in Figure 3a and 3b where there is no clear linkage between the soils above Poole's Cavern and the drip water emanating in PE. This, and the reduction of culturable microorganisms in the water sample, indicates that leaching of microbial communities from the soil to the aquifer is limited or that during transit through the karst, significant community filtering/ restructuring occurs. In the drip waters two of the most dominant families (*Sphingomonadaceae* and *Xanthomonadaceae*) are known to degrade aromatic compounds (Balkwill et al., 2003) and reactive oxygen species (Saddler and Bradbury, 2005), quite different from the dominant soil bacteria. This reduction in the diversity of microorganisms, and change in metabolic pathway, all indicate a significant reduction in suitable micronutrients and environmental conditions to support a diverse microbial community within the karst aquifer and resultant drip waters. These are significant observations when considering the karst aquifer as a potential filter or zone of consumption for enhanced nutrient leachates from anthropogenic activities.

4.1.3 Speleothem calcite

As mentioned in the sample collection section, the stalagmite samples from Poole's Cavern (PE High and Low) were grown on glass plates (watch glasses) and collected over a relatively short time period (two months). These samples gave an opportunity to assess the rates of biodiversification and the extent to which these "new" calcite samples varied from, for example, the straw speleothem from the same chamber. Unfortunately, the PE High and Low calcite samples showed the lowest extractable DNA abundance (Table 2) and there was not enough extracted DNA to perform taxonomic phylogeny assessments. This lower abundance could be due to a limited time for community development (probably due to rapid drip washing rates) or could be a function (such as partially metabolic inhibition) (Smith et al., 2016) of a high pH feeding water (pH 12.2), far more alkaline than Roman Chamber (pH 9.3) (Table 2).

Where we could perform an assessment of the dominant bacterial families within the cave system, including PE Straw and RC Calcite, we found differences in the bacterial makeup

of these sampling sites. *Rhodobacteraceae*, *Sutterellaceae*, and *Methylophilaceae* were characteristic families in the RC Calcite sample. *Rhodobacteraceae* are a universal but less dominant marine family (Pohlner et al., 2019). *Sutterellaceae* are asaccharolytic anaerobic to microaerophilic bacteria (Morotomi, 2014). *Methylophilaceae* are oligotrophs that consume simple organics such as methanol or methylamine through dehydrogenases. The genomic evolution of *Methylophilaceae* to adapt to environmental changes is primarily driven by horizontal gene transfer (Salcher et al., 2019). These bacteria demonstrate essential traits to fit in the oxygen- and nutrient-deficient in-cave conditions.

In comparison, *Caulobacteraceae* were enriched in PE Straw (3.6%) and PE Water (15.7%); they are motile, with the presence of prosthecae and flagella, and alkaline phosphatase is ubiquitous within this family to adapt to the high pH environment (Abraham et al., 2014). This co-dominance indicates some interconnectivity between the drip waters and the speleothem calcite developing from them and a clear adaption to the hyperalkaline environment in which they survive. *Zixibacteria*, *Hyphomicrobiaceae*, *Phycisphaeraceae* and *Burkholderiaceae* were dominant bacterial families only in the PE Straw sample. *Zixibacteria* have previously been detected in moonmilk cave deposits (Maciejewska et al., 2018). *Hyphomicrobiaceae* can perform denitrification or mixed-acid fermentation under anaerobic conditions (Oren and Xu, 2014). *Burkholderiaceae* are saprophytic microbes (Coenye, 2014). *Phycisphaeraceae* are nitrate reducing species discovered in marine algae that have alkaline phosphatase (Fukunaga et al., 2009). Metabolic pathways of inorganic nutrient metabolism within cave environments hold important status for understanding the nutrient content of speleothem calcite (Wynn et al., 2021; Wynn et al., 2008). The finding of these microbial inhabitants of the straw but not in the PE Water is significant, implying that this community has developed independently within the cave, and not controlled by the water source feeding the precipitating calcite.

Due to the nature of the PE Straw sample this may only reflect part of the potential community in these calcite drip samples. Although some researchers have found minimal variety within calcite samples in a cave system (Dhami et al., 2018), more of them have found reduced similarity (Mendoza et al., 2016; Ortiz et al., 2013; Park et al., 2020; Van de Kamp, 2004). Despite this ambiguity the differences in the communities between PE Straw and PE Water reflect a disconnect between the water and some areas of the cave system.

4.1.4 Sediments/ mud

The RC Mud sample has a similar level of diversity as the RC Calcite collected from the same chamber, with *Xanthobacteraceae* (2.2%) and *Nitrospiraceae* (6.7%) slightly dominant in RC Mud, similar to Soils above Poole's. However, RC Mud had the most bacterial families many of which were not found in other in-cave samples (Table 3), i.e., *Solimonadaceae*, *Methylomirabilaceae*, *Planococcaceae*, *Bacillaceae* and *Sumerlaeaceae*. One of the dominant families, *Nitrospiraceae*, are nitrite oxidisers (Daims, 2014) capable of metabolising under hyperalkaline conditions (Daebeler et al., 2020), reflective of the conditions even in the RC sediments which are fed by hyperalkaline waters. The family *Solimonadaceae* is primarily found in soils and freshwater; many species of *Solimonadaceae* can decompose chemical pollutants such as chlorinated compounds (Zhou et al., 2014). *Methylomirabilaceae* are denitrifying methanotrophs that couple anaerobic methane oxidation with nitrite reduction to nitrogen (Ettwig et al., 2010). *Planococcaceae* are more abundant in terrestrial habitats rather than aquatic or marine environments (Gupta and Patel, 2020). *Bacillaceae* are endospore-forming bacteria (Vos

et al., 2011). *Sumerlaeaceae* are rarely reported but they have been identified in other extreme environments, including cold arid deserts and deep-sea basins (Fang et al., 2021). As above, these families can all adapt to soils or moister environments. More dominant families suggest that the less alkaline mud below speleothems can foster a more diverse community.

The dominant bacterial families of Poole's Cavern microbiomes were not different from most cave and karst systems (Engel, 2007, 2010; Farnleitner et al., 2005; Ikner et al., 2007; Northup et al., 2003; Pronk et al., 2009), although far fewer common families are identified (Bassil et al., 2015). These findings highlight that there are significant adaptations found within the cavern in comparison to the soils or waters feeding the in-cave locations. Disconnections between sites suggest that the microbial communities in the soil-karst-speleothem-sediment system are not part of a continuum, but only a partial continuum, with opportunity for adaptation and unique community development specific to the immediate environmental conditions and substrates on which they are supported.

4.2 Microbial network between sites

As discussed above, alkaliphilic microbial dwellers of Poole's Cavern samples possessed various metabolic functions to consume simple inorganic or organic nutrients and to adapt to changes in ions (Table 4). The main bacteria taxa in Poole's Cavern are similar to neutral to slightly alkaline cave environments (Hershey and Barton, 2018; Tomczyk-Zak and Zielenkiewicz, 2016). Some bacterial taxa were common between sites, and some subgroups such as *Methylophilaceae* develop new metabolic functions by horizontal gene transfer (possibly to adapt to the different environments of RC Mud and RC Calcite, Table 4 & Figure 3b, c). Hence, the network between microbial communities from different microhabitats can be crucial in a complex cave system. The anticorrelation between PE Water and two external soil niches implied that dominant bacterial inhabitants in milder environments lost their advantages in a hyperalkaline aquatic setting (Figure 3b). Additionally, proximity of PE Straw to the Soils above Poole's in the correlation and network graphs suggest a rapid hydrological connection from the surface (Figure 3b, c), implying less time for species specialisation. Despite the PE Straw being more hyperalkaline at the drip site, the dry surface conditions of the straw feed the microbes in a similar manner to RC Calcite regarding microbial compositions (Figure 3b).

However, whilst commonality of bacterial taxa between sites was present, and functional likeness was evidenced in specific circumstances, this connectivity was weak (network weight < 0.21) among in-cave sites and the change in ecological niches can lead to shifts in microbial consortia (Park et al., 2020). Slightly different from previous microbiological studies of less alkaline caves (Dhami et al., 2018; Hershey and Barton, 2018; Tomczyk-Zak and Zielenkiewicz, 2016), both the hierarchical clustering (similarity < 45%) and network analyses displayed relatively lower similarity among different sampling sites (Kruskal-Wallis H of inter-site difference = 443.8, $p < 1.1\times10^{-93}$), indicating that microbial communities of these sites interacted and affected each other less often (Figure 2 and Figure 3). Despite reduced microbiota connections, the positive associations between RC Calcite and RC Mud, as well as PE Water and PE Straw manifested the presence of a spatial continuum within Poole's Cavern chambers (Figure 3b), as Blyth et al. (2014) also proposed that organic molecules such as biolipids in cave speleothems and associated soils were likely derived indigenously (Blyth et al., 2014).

Since the connectivity between microhabitats in Poole's Cavern was not high, each microbial group from its respective ecological niche has to rely on its own metabolisms. Within

this soil-water-speleothem-sediment continuum, microbes evolve and adapt to their respective microhabitats along the environmental adversity gradient (Figure 3a), gradually forming distinct communities, possibly also affected by their unique external sources that others are not exposed to. Due to the changes in pH and nutrients, multiple functions were constructed *in situ*. Complete independent metabolic function structure from each sampling site was detected using the PICRUSt2 metagenome inference method (Figure 4). Almost all sites shared similar proportions of essential biosynthesis pathways for producing amino acids, nucleotides, fatty acids, cell structure materials, and amines. Other pathways varied among sites to adapt to their unique environmental conditions. Microorganisms in each site were capable of utilising various and sometimes even unusual inorganic or organic compounds to survive (Charles et al., 2015). The dominance of pathways is closely related to environmental properties. The higher proportions of salinity-related response pathways in mud samples than in the water sample (Table 4) were probably due to evaporation during spring/summer. PE Straw and RC Calcite samples were less trophic than the soils (Dhami et al., 2018), and thus had more percentages of inorganic molecule consuming pathways. The evolution rates of microbial communities are quick in these susceptible microhabitats, and bacteria are very versatile to develop strategies to survive in the Poole's Cavern system.

5 Conclusions

Poole's Cavern in the UK is famous for its hyperalkaline speleothem deposits and anthropogenically impacted karst formation history. Extremophilic microorganisms were considered to dominate this region. In this study, the abundance, taxonomy and functions of microbial communities from four types of samples (soil, water, speleothem and mud) were examined to understand the distribution of microbial communities and their connections. The aqueous conditions in Roman Chamber, located close to the cave entrance, were warmer, less saline, and less alkaline than Poached Egg Chamber. Consequently, Roman Chamber nurtured more abundant and diverse microbial communities. We discovered limited similarity between different niches with community structure demonstrating a high variability among sampling sites, feasibly driven by the steep alkalinity gradient. Each site possessed a relatively independent complete essential metabolic structure due to the demands of self-sustainability. This is the first study to identify microbial taxa that can adapt to an alkaline-hyperalkaline cave ecosystem and inhabit different niches of a cavern and provide a basis to determine the controls on these niches and potential seasonal variability. Investigations of alkaliphilic microorganisms contributed to the understanding of global biosphere and microbe-environment interactions in karst and cave science.

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Open Research

The 16S rRNA gene sequences used for taxonomic analysis and functional pathway inference in this study are available in the NCBI BioProject database via the accession number PRJNA807843 [BGS © UKRI]. A supplemental table Table S1 is available at the link: https://doi.org/10.6084/m9.figshare.20113907.v1.

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