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Mineralogy affects prokaryotic community composition in an acidic metal mine

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ABSTRACT

The microbial ecology of acidic mine and sulfide cave ecosystems is well characterised with respect to aquatic communities, typically revealing low taxonomic complexity and dominance by a relatively limited number of cosmopolitan acidophilic bacterial and archaeal taxa. Whilst pH, temperature, and geochemistry are recognised drivers of diversity in these ecosystems, the specific question of a possible influence of substratum mineralogy on microbial community composition remains unanswered. Here we address this void, using 81 subterranean mineral samples from a low temperature abandoned, acidic, sulfide ore mine system at Mynydd Parys (Parys Mountain in English), Wales, UK. Four primary and 15 secondary minerals were identified via x-ray diffraction, each sample containing a maximum of five and an average of two minerals. The mineralogy of primary (e.g. pyrite and quartz) and secondary (e.g. melanterite and pisanite) minerals was significantly correlated with prokaryotic community structure at multiple taxonomic levels, implying that the mineralosphere effect reported in less extreme terrestrial environments is also implicated in driving prokaryotic community composition in extremely acidic, base metal-bearing sulfide mineralisation at Mynydd Parys. Twenty phyla were identified, nine of which were abundant (mean relative abundance >1%). While taxa characteristic of acidic mines were detected, for example *Leptospirillum* (phylum Nitrospirae), *Acidithiobacillus* (phylum Proteobacteria), *Sulfobacillus* (phylum Firmicutes) and *Ferroplasma* (phylum Euryarchaeota), their abundance in individual samples was highly variable. Indeed, in the majority of the 81 samples investigated the abundance of these and other typical acidic mine taxa was low, with 25% of samples devoid of sequences from recognised acidic mine taxa. Most notable amongst the bacterial taxa not previously reported in such environments were the recently cultivated Muribaculaceae family (phylum Bacteroidetes), which often dominated Mynydd Parys samples regardless of their mineralogical content. Our results pose further questions regarding the mechanisms by which taxa not previously reported in such extreme environments appear to survive in Mynydd Parys, opening up research pathways for exploring the biodiversity drivers underlying microbial community composition and function in extremely acidic mine environments.

1. Introduction

The remarkable diversity of minerals, together with their pervasive presence in natural environments, provide an array of niches for microbial colonisation and community development. Not only do minerals provide a physical surface for attachment and potential protection from predators (Cockell and Herrera, 2008), but mineral geochemistry has been demonstrated to exert a profound influence on the composition of the attached microbial community. As a result of variation in mineral dissolution rates and elemental release, different minerals have disparate influences on microbial diversity and activity in the immediate environments (Uroz et al., 2009, 2015) or 'microhabitats' (Carson et al.,

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2009). This 'mineralosphere' effect (Uroz et al., 2015), analogous to the rhizosphere effect in soil, has been confirmed in a diverse range of terrestrial environments and laboratory-based systems. While marine basalt was found to harbour basalt ecotypes not found in the surrounding water (Mason et al., 2007), mineral-inhabiting microbial communities in soils differed amongst minerals (Nishiyama et al., 2012; Uroz et al., 2012; Vieira et al., 2020) and were distinct from those in the bulk soil (Carson et al., 2009; Nishiyama et al., 2012; Uroz et al., 2012). Kotani-Tanoi and colleagues for example, revealed that bacterial community profiles were more similar on soil particles of the same mineralogical composition (quartz, white or yellow feldspar), compared to communities on contrasting soil minerals (Kotani-Tanoi et al., 2007). Likewise, bacterial community profiles in weathered terrestrial Icelandic lava differed between basalt and rhyolite mineralogies, and further differed in a mineralogy between samples with a homogeneous (glassy) or heterogeneous (crystalline) elemental distribution (Kelly et al., 2010, 2011). Elsewhere, individual mineral crystals of feldspar, quartz, muscovite and plagioclase within an Irish granite outcrop hosted significantly distinct bacterial and fungal communities, with many of the ribotypes restricted to one mineralogy (Hutchens, 2009; Hutchens et al., 2010). In a laboratory study using a diverse microbial inoculum, biofilm development and composition on carbonate, silicate and aluminosilicate minerals in flow-through reactors was found to be significantly influenced by mineral composition and less by environmental conditions such as pH and media composition (Jones and Bennett, 2017).

The microbiology of acidic mine (AM) environments has been the focus of intense scrutiny during the past two decades (Bond et al., 2000; Edwards et al., 2000; Brantner et al., 2014; Aliaga Goltsman et al., 2015), resulting in a wealth of information regarding the diversity of planktonic and macroscopic (snotites and streamers) communities and the metabolism and physiology of cosmopolitan acidophilic mine taxa (Hua et al., 2015; Méndez-García et al., 2015; Golyshina et al., 2019). Specific niches such as solid wastes (rock dumps and mineral tailings), drainage streams and snotites have mostly been reported to contain low-complexity microbial communities, characteristically dominated by one or several cosmopolitan chemolithotrophic or heterotrophic acidophilic bacterial and archaeal taxa, a trend observed worldwide both in high- and low-temperature AM systems (Baker and Banfield, 2003; Tyson et al., 2004; Wilmes et al., 2009; Denef et al., 2010; Johnson, 2012). These taxa include species within the phyla Nitrospira (*Leptospirillum*), Proteobacteria (*Acidithiobacillus*, *Ferrovum*, *Acidiphilium*, *Acidocella*), Acidobacteria (*Acidobacterium*), Actinobacteria (*Ferrimicrobium*), and Firmicutes (*Sulfobacillus*), whilst *Ferroplasma* (phylum Euryarchaeota) and uncultivated Archaea often dominate the archaeal communities (Bond et al., 2000; Baker and Banfield, 2003; Coupland and Johnson, 2004; Baker et al., 2010; Kay et al., 2013; Aliaga Goltsman et al., 2015; Pakostova et al., 2020, 2022).

In the abandoned Mynydd Parys mine system (Wales, UK), the majority of previous microbiological studies focused on water bodies within and draining the mines (Walton and Johnson, 1992; Coupland and Johnson, 2004; Kay et al., 2013; Aguinaga et al., 2018). Iron-oxidizing acidophiles were particularly abundant and included both chemolithotrophic (e.g. *Acidithiobacillus ferrivorans, Leptospirillum ferrooxidans* and "*Ferrovum myxofaciens*") and heterotrophic (e.g. *Ferrimicrobium acidiphilum* and *Acidithrix ferrooxidans*) species. Other heterotrophic acidophiles (*Acidiphilium, Acidocella, Acidisphaera* and *Acidobacterium* spp.) were also reported in these studies. Large gelatinous organic growths referred to as "drapes" found within newly-drained subterranean areas within the system were found to be composed primarily of heterotrophic acidophilic bacteria and methanogenic archaea (Johnson, 2012).

While factors such as pH, temperature, redox and osmotic potentials, and concentrations of dissolved oxygen and carbon are recognised constraints on microbial diversity in AM ecosystems (Méndez-García et al., 2015; Huang et al., 2016; Quatrini and Johnson, 2018), a possible role of mineral selectivity in microbial colonisation of solid substrata in these environments has been suggested but not explicitly investigated (Baker and Banfield, 2003; Barton et al., 2015; Uroz et al., 2015; Jones et al., 2017; Blackmore et al., 2018). The aim of the present work was to test the hypothesis that substratum mineralogy is a major determinant in selecting which microbial communities are able to successfully colonize and develop on mineral surfaces in an extreme environment, using the acidic, low-temperature subterranean excavations at the abandoned Mynydd Parys mines as a model environment.

2. Materials and methods

2.1. Study site

Mynydd Parys (Parys Mountain) is an abandoned mine site of historic significance, located in the north-west of Anglesey, Wales, UK (53.22.56 N, 4.21.9 W). Archaeological evidence suggests that copper was mined there in the Bronze Age and again by the Romans. In the 18th century Mynydd Parys became the world's largest copper-producing site with the establishment of two adjacent and interconnected mines (the Parys and Mona mines). These were both initially worked as underground mines, with extensive shaft and adit networks, but later converted to opencast operations. Extractive mining ended in the 1880 s, though copper was still recovered by cementation from waters draining the site until the 1950 s. In 2003, the water table in the flooded underground mines was lowered by pumping out ~274,000 m^3 of metal-rich, acidic (pH ~2.4) groundwater over a 14-week period and opening a low-level drainage channel that had been sealed in the 1950 s (Coupland and Johnson, 2004) allowing access to a vast network of underground mine works.

The geology of the Mynydd Parys area is complex, dominated by a large volcanogenic massive sulfide mineral deposit formed during back-arc submarine volcanism in late Ordovician to early Silurian times and deformed during the Caledonian orogeny. The deposit comprises of layers and lense-like bodies of sulfide minerals dominated by pyrite (FeS_2), chalcopyrite ($CuFeS_2$), sphalerite (ZnS), and galena (PbS) as the major metal-bearing minerals (Jenkins et al., 2000). The microbiologically-accelerated oxidative dissolution of these sulfide minerals, and subsequent hydrolysis of ferric iron, produces sulfuric acid, resulting in a largely unvegetated and, like the waters draining the site, extremely acidic landscape.

2.2. Sample collection

Ninety-four mineral samples were collected aseptically from walls (n = 87) and ceilings (n = 7; at the side, in close proximity to the wall) of subterranean chambers within the Myndd Parys mine system, at depths of up to forty-five fathoms (~82 m) below the land surface. The majority of samples were retrieved in the course of five visits to the site from 2015 to 2016: 20th May, 2nd September, 13th and 27th January, and 24th February (18, 21, 8, 18 and 14 samples, respectively), representing summer (March-May), autumn (September-November) and winter (December-February) seasons The remaining samples were retrieved on 13th May, 19th June, 15th and 29th July, 23rd November and 23rd December (1, 2, 3, 1, 4 and 4 samples, respectively). Using visual clues such as colour, texture and location (the latter referring to recorded geological information of the location), samples were retrieved in order to represent a range of mineralogies from multiple locations (shown in Fig. S1). Areas covered by ochreous (ferric iron-rich) amorphous precipitate were avoided so that only exposed mineral surfaces were retrieved. Samples were obtained by directly coring into or scraping the surface of the deposit using a sterile 50 ml tube, or by dislodging samples into sterile Whirl-Pak® bags using a flame-sterilized rock hammer. The retrieved samples, ranging in size from < 1 g to circa 5 g, were frozen at − 20 °C within 2 h of collection.

2.3. Mineral identification

Semi-quantitative mineral identifications were determined by Powder X-Ray Diffraction (PXRD) (Ali et al., 2022) at National Museum Wales Cardiff using a PANalytical X'PertPRO diffractometer fitted with a sample spinner, equipped with an X'Celerator solid state detector. Samples were ground to a fine powder using an agate mortar and pestle, then spread evenly across the surface of a zero-background silicon wafer. Acetone was used to aid the spreading of the powder and to adhere it to the surface of the wafer prior to insertion into a steel holder. The operating conditions were set at 40 kV and 30 mA using Cu Kα radiation. Each sample was analysed at room temperature across an area of 20 mm^2 for a total of 2 min 54 s through the range 6.5°2θ to 75.066°2θ. A nickel filter was placed in the diffracted beam path in order to reduce fluorescence caused by iron-bearing samples. Data was processed using HighScore (version 4.1) software (PANalytical). Where ambiguity existed regarding mineral identity, additional elemental analyses were performed as described in the supplemental material.

While the size and/or nature of the mineral samples (water solubility) precluded analysis of pore water chemistry and pH analyses, selected samples were subjected to ion chromatography (IC; Dionex) and/or atomic absorption spectroscopy (AAS; Varian SpectrAA 220FS) analysis to assist with or confirm PXRD mineral identification results. Those samples containing exclusively hydrated iron sulfates were dissolved in acidic deionised water, while samples containing water-insoluble minerals were subjected to acid digestion using a modified aqua-regia protocol (Hu and Qi, 2014). Sample elemental concentrations were calculated with reference to elemental standards which were run prior to, during and post-sample analysis.

2.4. DNA extraction, PCR and sequencing

DNA was extracted from a ~0.2 g of each freshly thawed sample, using a sterile spatula to transfer to extraction tubes. Extraction was performed using a MoBio PowerSoil® DNA extraction kit (Qiagen) according to the manufacturer's instructions, except that horizontal vortexing was modified to provide 2 × 7.5 min steps, cooling on ice between. As contamination controls for kit components, two extractions using sterile 13 mm diameter filters were included. All extracts were subjected to NanoDrop™ analysis to determine DNA concentration and purity, and standard PCR amplification using primers 27f and 1492r (Lane, 1991) to check for amplifiable DNA. Controls described above proved negative. Library preparation and core amplicon (16 S rRNA genes; V4 region) sequencing using Illumina MiSeq (150 bp paired-end) were performed by the Earth Microbiome Project (Thompson et al., 2017) according to standard EMP protocols (Caporaso et al., 2012). Samples described in this study were run on a lane with additional samples not described here.

2.5. Sequence denoising and OTU clustering

All initial sequence analysis steps and calculation of alpha diversity indices were executed in QIIME (v1.9.0) (Caporaso et al., 2010). Following paired end joining (join_paired_ends.py), sequences were demultiplexed and minimal quality filtered using the split_libaries_fastq.py command (Phred default=3). Chimeras were identified with usearch61 (identify_chimeric_seqs.py) and removed (filter_fasta.py). Operational taxonomic units (OTUs) were identified at 97% sequence similarity using a usearch-based open-reference OTU picking strategy (Rideout et al., 2014). OTUs comprising less than 0.005% of sequences were removed from the dataset using the filter_otus_from_otu_table.py command (Bokulich et al., 2013). OTU taxonomy was assigned against the SILVA database (release 128) (Quast et al., 2013). Illumina sequence data used in this study have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB35982.

2.6. Statistical analysis

Core diversity analysis was undertaken following normalisation to 1071 sequences, which represented the maximum number of sequences permitting the inclusion of all samples containing at least 1000 sequences (n = 81 samples) after the above processing steps. Processing steps thus required the removal of thirteen of the original samples. Differences in community richness, evenness, and composition between the 81 remaining samples using the Bray-Curtis dissimilarity, were calculated in the R package "vegan" (Dixon, 2003). Non-parametric Spearman's correlations were used to determine relationships between taxa and mineral abundance. Mineral effects were assessed by PERMANOVA (999 permutations) using the adonis function, including Season as a categorical variable and mineral abundance as co-correlates. Community shift based on these minerals were calculated using the envfit function with Season as strata. All analyses were conducted using both mineral abundance (included in the main text) and minerals as binary (presence/absence). The results were significantly positively correlated for the envfit correlations (Rho = 0.74, $p < 0.001$) and Adonis (Rho = 0.76, $p < 0.001$) analyses. We therefore included only the analysis with mineral abundance in the main text.

2.7. Enrichment of acidophiles

A small number of mineral samples were subjected to enrichment in a 9 K broth of pH 1.9–2.2, as described elsewhere (Golyshina et al., 2016). Medium was inoculated with circa 0.1–0.2 g of sample and incubated at room temperature, without shaking. Water samples retrieved from a subterranean pool (not shown) in the mine system were also subjected to enrichment. At intervals ranging from 2 to 12 months, enrichment aliquots were inoculated into a fresh 9 K medium. To determine microbial community composition, 2 ml aliquots were filtered through 0.2 μm filters, the latter subjected to DNA extraction, sequencing and data processing performed as described above, with the omission of the final filtering step to remove rare OTUs (<0.005% abundance).

3. Results and discussion

3.1. Mineral identification

The primary aim of this study was to investigate the effect of mineralogy on prokaryotic community composition underground within the low-temperature acidic environment of an abandoned base metal mine, focusing on primary and secondary minerals. Our sampling strategy (Table S1; Fig. S1) was successful in retrieving a wide diversity of minerals - 19 in total amongst the 81 samples that yielded usable DNA data. Quartz (SiO_2) and pyrite (FeS_2) were the most frequent primary minerals encountered along with, to a lesser extent, chalcopyrite ($CuFeS_2$) and galena (PbS), in keeping with the geology of the mine (Table 1 and Table S2). Melanterite ($Fe^{2+}(H_2O)_6SO_4{\cdot}H_2O$), pisanite (Fe, Cu)$SO_4{\cdot}7H_2O$; a Cu-bearing variety of melanterite), hydronium jarosite ($(H_3O)Fe^{3+}3(SO_4)2(OH)_6$), gypsum ($CaSO_4{\cdot}2H_2O$) and aluminite ($Al_2(SO_4)(OH)_4{\cdot}7H_2O$) were the most abundant secondary minerals, formed post-mining, principally by the oxidative weathering and alteration of primary sulfides through a process known as pyrite decay (Baars, 2019). The concurrent generation of sulfuric acid from sulfide oxidation produces low pH liquors and can result in the generation of acid mine drainage (AMD). Hydrated sulfates dominated the secondary mineral species, but with iron oxy-hydroxide (FeO(OH); goethite) also present. Alteration of host rock explains the presence of the silicate minerals muscovite ($KAl_2(Si_3AlO_{10})(OH)_2$), chlorite ($ClO_2$) and kaolinite ($Al_2Si_2O_5(OH)_4$). The PXRD data (Table S2) were supported (where tested) by elemental analyses using IC and/or AAS and the spectra from a subset of the samples is provided in Fig. S2.

Individual samples comprised of between one (n = 36) and five (n =

Table 1
Significant mineral-phylum and mineral-OTU associations of Mynydd Parys minerals.

Mineral	Chemical composition	No. of Samples	P-value < 0.05		P-value < Bonferroni cutoff[a]	
			OTUs[b]	Phyla[b]	OTUs[b]	Phyla[b] (*Genus*[c])
Primary minerals						
Galena	PbS	2	63	10	6	Actinobacteria, Euryarchaeota, Proteobacteria (*Acidithiobacillus.*), WPS-2
Chalcopyrite	$CuFeS_2$	4	66	9	1	Proteobacteria (*Stentrophomonas)*
Pyrite	FeS_2	18	34	3	0	
Quartz	SiO_2	35	74	11	0	
Secondary minerals						
Aluminite	$Al_2(SO_4)(OH)_4{\cdot}7\ H_2O$	9	64	9	3	Actinobacteria, Proteobacteria
Amorphous		7	90	14	23	Crenarchaeota, Acidobacteria, Actinobacteria, AD3, Bacteroidetes, Chlamydiae, Firmicutes, Planctomycetes, Proteobacteria, TM6
Antlerite	$Cu_3(SO_4)(OH)_4$	1	69	7	41	Actinobacteria, AD3, Chlamydiae, Chloroflexi, Planctomycetes, Proteobacteria WPS-2
Brochantite	$Cu_4SO_4(OH)_6$	3	74	8	16	Actinobacteria, AD3, Bacteroidetes, Chloroflexi, Firmicutes, Planctomycetes, Proteobacteria,
Chalcanthite	$CuSO_4{\cdot}5\ H_2O$	4	63	4	6	Bacteroidetes, Firmicutes, Proteobacteria
Goethite	FeO(OH)	3	56	11	10	Actinobacteria, Chlamydiae, Euryarchaeota, Firmicutes, Nitrospirae (*Nitrospira*), Proteobacteria
Chlorite	ClO_2	6	74	12	3	Chloroflexi, Proteobacteria
Gypsum	$CaSO_4{\cdot}2\ H_2O$	10	43	4	0	
Hydronium Jarosite	$(H_3O)Fe^{3+}_3(SO_4)_2(OH)_6$	13	82	13	3	Actinobacteria, Nitrospirae (*Leptospirillum*)
Jarosite	$KFe^{3+}_3(SO_4)_2(OH)_6$	5	73	13	1	Bacteroidetes (*Candidatus Amoebophilus*)
Kaolinite	$Al_2Si_2O_5(OH)_4$	2	44	6	2	Firmicutes
Melanterite	$Fe^{2+}(H_2O)_6SO_4{\cdot}H_2O$	17	65	8	2	Firmicutes (*Sulfobacillus*)
Muscovite	$KAl_2(Si_3AlO_{10})(OH)_2$	7	45	7	1	Proteobacteria (*Devosia*)
Native sulphur	S_8	1	6	1	1	Firmicutes
Pisanite	$(Fe, Cu)SO_4{\cdot}7\ H_2O$	9	31	3	1	Gammaproteobacteria
Siderotil	$Fe(SO_4){\cdot}5H_2O$	2	38	4	6	Proteobacteria
Unidentified*		1	79	14	13	Acidobacteria, Firmicutes, Proteobacteria

[a] Bonferroni cut-off 2.02E-06.
[b] Number of OTUs (and the phyla they represent) significantly correlated with sample mineralogy at the relevant P-value (0.05 or Bonferroni).
[c] Genus affiliations provided where exclusive for that phylum for a given mineral.

1) identifiable minerals (median 2). These included one sample (DNA.65) composed of a complex mixture of crystallized Al, Cu and Zn-bearing phases whose mineralogy was not possible to characterise with the techniques used. However, an elemental analysis confirmed the presence of Al (284 mg/kg), Cu (49 mg/kg) and Zn (11 mg/kg), in addition to Fe (11 mg/kg), Mg (10 mg/kg) and Ca (4 mg/kg), with trace amounts of K and Mn (~1 mg/kg). Seven samples, six of which were sourced from Fe-rich stalactites, provided no crystalline signature and were thus labelled as amorphous for the purpose of subsequent statistical analyses. One or more of these samples however may have contained poorly crystalline mineral phases such as schwertmannite (Jiménez et al., 2019).

3.2. Prokaryotic community composition

Bacterial and archaeal members of microbial communities were identified via high-throughput sequence analysis of 16 S rRNA gene amplicons, derived from community DNA extracts. Table S3 provides the initial number of sequences, % of chimeric sequences, together with the pre- and post-filtering number of sequences and Chao1 richness estimator for each sample. Following chimera-checking and quality-filtering ~1.1–44 × 10^3 sequences remained per sample. A total of 771 OTUs were identified among the 81 samples (listed in Table 2). Of these 18.9% (146) were present in a single sample only, their abundance averaging 1.5% (median 1.1%) in those samples. Abundant OTUs comprising at least 1% of total sequences in one or more of the samples, accounted for 57.6% (444) of the detected OTUs. Individual samples contained 19–171 OTUs (median=56; Tables 2) and 3–17 phyla (median=7) in non-rarefied data, and rarefaction analysis indicated an adequate sampling of most communities at a cut-off of 1071 sequences (Fig. S3). Differences in the evenness of prokaryotic communities was observed, the most abundant OTU in a sample accounting for between 5.7% and 78.7% of sequences (median 19.6%). Unassigned sequences accounted for < 1% of amplicons on average.

A total of twenty phyla were identified, nine of which were abundant (mean relative phylum abundance >1%), although most of the remaining phyla were abundant in at least one sample (Table 3). The three most abundant phyla were Bacteroidetes, Firmicutes and Proteobacteria, at ~27%, 25% and 19% mean relative sample abundance, respectively (Table 3; Fig. 1). With one exception (discussed below), the dominant abundant phyla have similarly been encountered in other acidic mine and AMD-impacted environments, including subterranean pools and an acidic surface stream at Mynydd Parys (Baker and Banfield, 2003; Johnson, 2012; Jiménez et al., 2019). Firmicutes and Proteobacteria phyla, which were recovered from all 81 samples, were dominated by well-documented acidophile genera (e.g. *Sulfobacillus* and *Acidiphilium*; Table 3), as were the Nitrospirae (*Leptospirillum*) and Euryarchaeota (*Ferroplasma*) phyla, while the dominant taxa within the Actinobacteria and Acidobacteria could only be resolved to order level. Interestingly, Bacteroidetes were detected in 80 samples and with the highest mean relative abundance (~27%), with the maximum observed abundance of ~71%. This phylum is rarely encountered in acidic mine environments and has not been previously reported at such high abundance in subterranean acidic mine samples elsewhere, or indeed above ground at Mynydd Parys (Aliaga Goltsman et al., 2015; García-Moyano et al., 2015; Chen et al., 2016; Mesa et al., 2017; Aguinaga et al., 2018; Nural Yaman et al., 2021). It has however been identified as abundant in mine waste in Canada, comprising up to 14.7% of sequences (Pakostova et al., 2020), and in above ground tailings in an Armenian copper mine, where *Lutibacter* (family Flavobacteriaceae) were dominant (43% relative abundance) (Margaryan et al., 2019). OTUs affiliated with the family Muribaculaceae (formerly S24–7) dominated the Bacteroidetes at Mynydd Parys and were present in 95% of samples at abundances of up to ~60% (Fig. 2). Of the 771 OTUs identified in this study that with the greatest sample frequency were members of the Muribaculaceae family (accounting for 17.3%), followed by *Sulfobacillus* (OTU of 16.0% sample frequency).

Muribaculaceae were, until recently, known only from sequence-

Table 2
Total and abundant OTUs (97% sequence identity) and their acidic mine (AM) associations in Mynydd Parys minerals.

Sample[a]	Total OTUs	Abundant OTUs[b]	Phyla represented[c]	Sequences represented (%)[c]	AM sequences (%)[d]	Minerals identified	Sample[a]	Total OTUs	Abundant OTUs[b]	Phyla represented[c]	Sequences represented (%)[c]	AM sequences (%)[d]	Minerals identified
DNA.1	52	9	5	83.7	76.8	M	DNA.63	89	9	3	89.1	89.1	Q+H
DNA.24	82	7	3	75	64.4		DNA.131	88	12	6	83.3	69.8	
DNA.25	47	14	6	88.8	56.9		DNA.134	67	30	6	89.4	20.7	
DNA.27	39	21	4	96.1	3.7		DNA.170	82	9	5	92.5	82	
DNA.29	40	16	4	95.3	69.9		DNA.183	169	18	6	78.9	15.5	
DNA.30	37	19	5	95.8	0		DNA.140	56	16	5	90.7	58.8	Q+Py
DNA.31	47	24	4	95.1	41		DNA.160	44	20	4	92.3	28.7	
DNA.32	44	26	5	96.1	4.7		DNA.163	71	12	5	83.3	56.4	
DNA.33	38	24	4	98.1	0		DNA.171	41	4	3	96.8	96.8	
DNA.34	40	23	6	96.7	35.4		DNA.177	50	7	3	93.2	84.6	
DNA.37	61	7	6	87.1	81.5		DNA.60	120	15	5	80.6	0	Q+An
DNA.38	39	6	2	90.3	83.2		DNA.137	80	37	8	88.3	9.8	Q+J
DNA.83	26	20	5	98.6	2.6		DNA.184	54	33	6	96.7	6.8	Q+K
DNA.84	49	24	6	95.6	9.6	P	DNA.59	19	5	3	89.4	0.0	Gy+Q
DNA.87	46	20	4	91.9	48.4		DNA.51	37	26	5	97	5.8	Gy+H
DNA.126	31	4	3	99.2	97.6		DNA.55	44	23	3	92.4	0	Al+Gy
DNA.128	43	20	3	95.9	7.5		DNA.132	46	28	5	96.7	14.4	P + Py
DNA.129	39	23	4	96.9	0		DNA.133	44	27	5	96.6	0	Si+P
DNA.130	56	28	6	95.1	0		DNA.135	71	27	4	82.1	12.8	Cl+J
DNA.96	55	37	8	97.5	3.6	Al	DNA.54	119	21	9	80.7	10.1	Mu+Cl+H
DNA.150	57	24	7	93.7	1.6		DNA.57	34	20	4	97.8	0	
DNA.178	73	28	8	88.9	11.7		DNA.23	72	37	11	92.6	6.6	Mu+Gy+Q
DNA.179	61	33	8	94.2	12.2		DNA.80	65	33	4	92.7	0	Ca+P + Si
DNA.97	85	29	8	84.7	6.2	A	DNA.90	57	23	3	93.4	56.2	Ca+H+Q
DNA.98	126	16	6	68.3	11.6		DNA.64	171	15	8	76.7	22.4	Q+Py+Ga
DNA.99	93	6	3	93.4	93.4		DNA.26	31	2	1	92.5	92.5	Q+Py+S
DNA.100	153	14	6	82.4	8.8		DNA.35	45	8	4	89.9	81.6	Q+M+Py
DNA.101	64	26	11	87.1	1.1		DNA.165	56	23	6	88.3	41.2	Q+Py+Co
DNA.148	27	18	4	98.6	0		DNA.172	80	5	2	89.3	85	Mu+Q+K
DNA.182	168	16	8	78.7	4.4		DNA.62	56	30	5	93.9	2.3	Gy+Q+H
DNA.52	46	27	5	97.2	0	Go	DNA.49	110	10	4	83.3	1.8	Al+Gy+M
DNA.149	51	28	5	94	0		DNA.53	93	14	5	86.1	64.1	H+Q+J
DNA.159	145	17	7	80.1	4.4		DNA.50	86	13	4	89.7	2.0	Al+Gy+Py
DNA.138	70	26	6	83	4.7	Ca	DNA.180	103	26	11	75.7	35	Al+Q+Cl
DNA.139	59	31	8	94.3	10.5		DNA.127	62	23	5	94.1	0	Mu+Gy+Q+HJ
DNA.61	54	23	7	91.5	54.2	Gy	DNA.166	159	18	7	74.7	0	Q+B+Py+Co
DNA.65	53	27	6	95.3	0	U	DNA.173	40	24	3	97.5	0	
DNA.169	57	25	5	93.6	0	Q	DNA.56	123	14	4	88.7	0	Q+Mu+Cl+H
DNA.28	52	29	4	93.8	1.2	M+ Py	DNA.58	25	3	2	97.9	97.9	Py+Q+Ga+Co
DNA.39	52	16	4	89	63.6		DNA.94	81	19	7	78.5	16.4	Py+H+Al+Q
							DNA.176	46	23	4	94.2	0	Mu+Q+B+Cl+Py

[a] Sample retrieval locations are shown in Fig. S1.
[b] OTUs present in one or more samples at a relative frequency of at least 1%.
[c] Phyla and % of total sequences represented by abundant OTUs.
[d] The % of total sequences affiliated with cosmopolitan acidic mine taxa. Minerals identified were as follows: A=amorphous, Al=aluminite, An=antlerite, Br=brochantite, Ca=chalcanthite, Cl=chlorite, Co=chalcopyrite, Ga=galena, Go=goethite, Gy=gypsum, H=hydronium jarosite, J=jarosite, K=kaolinite, M=melanterite, Mu=muscovite, P = pisanite, Py=pyrite, Q=quartz, S=native sulfur, Si=siderotil, U=unidentified. The chemical composition of minerals is provided in Table 1.

Table 3
Phyla and proteobacteria classes detected in Mynydd Parys samples in order of decreasing mean relative abundance (as %).

Mean relative abundance	Lower (non-zero) relative abundance	Upper relative abundance	Sample frequency	Domain; Phylum (*Proteobacteria Class)*	Dominant Taxa[a]
26.95	0.06	70.65	96.3	Bacteria; Bacteroidetes	*family* Muribaculaceae (S24–7)[b]
25.26	0.01	94.84	100	Bacteria; Firmicutes	*Sulfobacillus, Desulfosporosinus*
19.30	0.64	74.81	100	Bacteria; Proteobacteria:	See below
8.31	*0.02*	*53.93*	*84.0*	*Alphaproteobacteria*	*Methylobacterium, Acidiphilium, Acidocella*
6.19	*0.07*	*29.18*	*96.3*	*Gammaproteobacteria*	*Acidithiobacillus*
3.40	*0.01*	*74.68*	*93.8*	*Betaproteobacteria*	*order* Burkholderiales
0.89	*0.12*	*8.11*	*33.3*	*Deltaproteobacteria*	*families* Syntrophobacteraceae, Bdellovibrionales
0.32	*0.02*	*9.47*	*19.8*	*Unclassified Proteobacteria*	No further taxonomic resolution
0.19	*0.01*	*9.07*	*42.0*	*Epsilonproteobacteria*	*Helicobacter*
8.63	0.01	72.93	79.0	Bacteria; Actinobacteria	*orders* Acidimicrobiales, Actinomycetales
7.44	0.01	76.71	64.2	Archaea; Euryarchaeota	*Ferroplasma, Thermogymnomonas*
3.17	0.02	69.54	45.7	Bacteria; Nitrospirae	*Leptospirillum*
2.29	0.02	19.30	46.9	Bacteria; Acidobacteria	*order* Acidobacteriaceae
2.00	0.04	40.79	22.2	Bacteria; Chloroflexi	*family* Thermogemmatisporaceae
1.79	0.01	21.14	59.3	Bacteria; Verrucomicrobia	*Akkermansia*
0.60	0.07	9.39	27.2	Bacteria; Tenericutes	*Anaeroplasma, family* Mycoplasmataceae
0.51	0.01	5.45	39.5	Bacteria; Cyanobacteria	*order* Streptophyta
0.43	0.15	12.46	14.8	Bacteria; Planctomycetes	*order Gemmatales*
0.37	0.01	12.11	16.0	Bacteria; WPS-2	No further taxonomic resolution
0.37	0.02	9.46	28.4	Unassigned (Phylum-level)	No further taxonomic resolution
0.28	0.07	10.61	12.3	Archaea; Crenarchaeota	*order* NRP-J
0.20	0.02	2.90	23.5	Bacteria; AD3	*class* JG37-AG-4
0.17	0.02	3.30	12.3	Bacteria; Deferribacteres	*Mucispirillum*
0.13	0.02	1.81	24.7	Bacteria; Chlamydiae	*Candidatus Rhabdochlamydia*
0.07	0.48	5.38	3.7	Bacteria; Fusobacteria	*Fusobacterium*
0.02	0.01	0.55	14.8	Bacteria; TM6	*class* SJA-4
0.01	0.10	0.19	3.7	Bacteria; Elusimicrobia	*order* Elusimicrobiales

[a] Taxonomy provided to genus level where possible, otherwise to the nearest phylogenetic resolution. Only the most abundant taxa are listed for each phylum.
[b] Family Muribaculaceae were formerly known as S24–7.

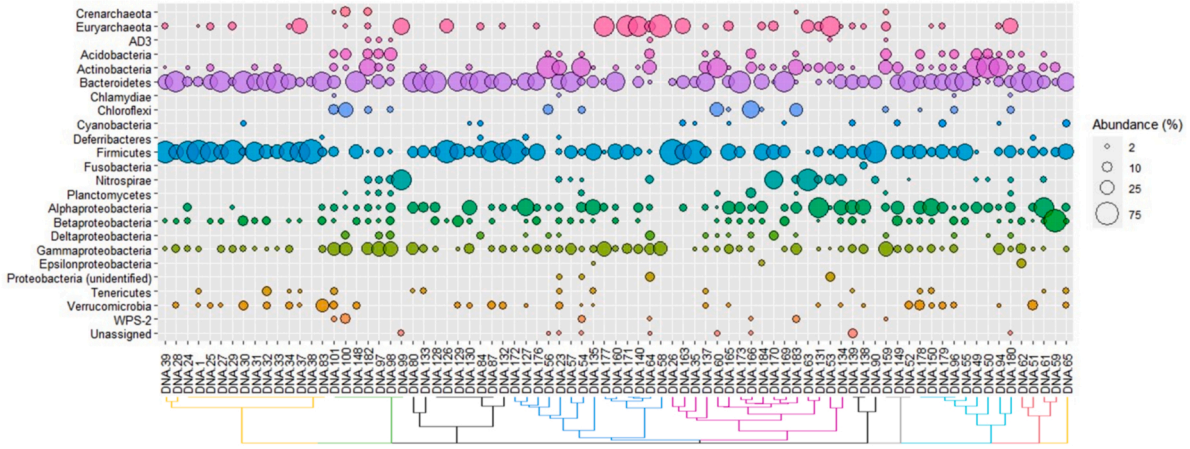

Fig. 1. Phylum-level composition of prokaryotic communities in Mynydd Parys mineral samples. Individual phyla (and proteobacteria classes) are represented by bubbles, the size of which is representative of the abundance (%) of the phylum in the sample, as determined by Illumina sequencing. Samples are organised based on hierarchial clustering (hclust) by sample mineralogy (Table S2), where the 10 coloured clusters shown (left to right) contain samples composed entirely of or are largely dominated (%) by the following minerals: Melanterite (yellow), Amorphous (green), Pyrite (black), Quartz and Muscovite (dark blue), Quartz (pink), Calcite (black), Goethite (green), Aluminite (light blue), Gypsum (red) and unidentified mineralogy (yellow).

based studies and currently (3 November 2021) comprise six known genera and three candidate genera (Schoch et al., 2020). All genera known are primarily reported from mammalian guts thus far, with no cultured environmental representatives (Lagkouvardos et al., 2016, 2019; Park et al., 2021). Likewise, other less abundant phyla in Mynydd Parys mineral samples sometimes contained significant proportions of taxa most commonly found, to date, in gut microbiomes, the most notable being Verrucomicrobia and the genus *Akkermansia* specifically (Table 3; Fig. 1). While it is possible that the sizeable presence of Muribaculaceae in mineral communities underground at Mynydd Parys is due to contamination via water infiltration from the surface, DNA originating from dead cells and/or persistent environmental DNA, this is unlikely given that separate cultivation efforts proved the viability of family Muribaculaceae in sequential enrichments in a ferrous iron medium used widely to cultivate iron-oxidizing autotrophic acidophiles (pH=1.9–2.2) (Golyshina et al., 2016), recovering up to 42% Muribaculaceae 16 S rRNA gene sequences (Fig. S4). Additionally, some reported and as yet uncultivated species of Muribaculaceae occur most frequently in non-host environments, while genomic analysis has pointed to traits useful for survival in nutrient-limited environments, including nitrogen utilization via sources such as cyanate (OCN^-) and various strategies for carbohydrate metabolism (Ormerod et al., 2016;

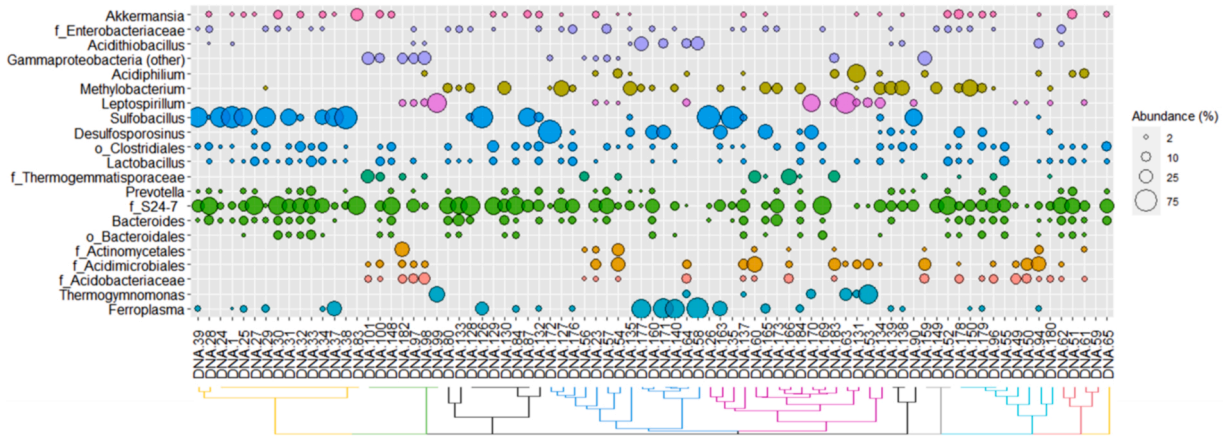

Fig. 2. Abundance of individual taxa in individual Mynydd Parys mineral samples. Shown are those taxa present at an overall abundance of at least 1%. Taxa are represented by bubbles, the size of which is representative of their abundance (%) in the sample. Where taxonomy could not be resolved to genus-level using the SILVA database, family (f) or order (o) identity is provided. 'Gammaproteobacteria (other)' could not be taxonomically resolved further. The family S24–7 is also known as Muribaculaceae. Samples are organised based on hierarchial clustering (hclust) by sample mineralogy (Table S2), where the 10 coloured clusters shown (left to right) contain samples composed entirely of or are largely dominated (%) by the following minerals: Melanterite (yellow), Amorphous (green), Pyrite (black), Quartz and Muscovite (dark blue), Quartz (pink), Calcite (black), Goethite (green), Aluminite (light blue), Gypsum (red) and unidentified mineralogy (yellow).

Lagkouvardos et al., 2019). Compared to other families within the order Bacteroidales, Muribaculaceae are deficient in heat shock proteins (Jiménez et al., 2019), which could explain why they are not reported among microbial communities in extensively-studied, higher temperature acidic mine environments such as the Richmond Mine at Iron Mountain (CA, USA) (Aliaga Goltsman et al., 2015). Much is yet unknown regarding the ecology and metabolic potentials of this family, and indeed other relatively abundant taxa recovered in Mynydd Parys that have previously been associated with gut microbiomes. As such, their current strong association with mammalian host environments may be merely a result of a research bias towards gut microbiome studies, and does not preclude the possibility that uncharacterised genera, species or strains exist that are suited to thriving in environmental mineral biofilms. The comparatively low sequence abundance of soil-associated taxa among samples, which should otherwise greatly outnumber 'gut taxa' were infiltration and persistence of external DNA a significant concern, supports the theory that the Muribaculaceae have colonized and not merely infiltrated the subterranean chambers within Mydydd Parys. The absence of Muribaculaceae sequences from wetlands above ground at Mynydd Parys is also noted, despite Bacteroidetes being a dominant phylum in libraries (Aguinaga et al., 2018).

Notwithstanding the abundance of the Muribaculaceae, cosmopolitan acidic mine genera, particularly *Leptospirillum*, *Acidithiobacillus*, *Sulfobacillus*, *Desulfosporosinus* and *Ferroplasma*, represented up to ~98% of sequences within Mynydd Parys mineral samples (Table 2). The contribution of such taxa to entire communities was highly variable however, and ~25% of samples did not definitively contain taxa previously associated with extremely acidic, metal-rich environments, although many OTUs were unidentifiable to a taxonomic level where any association or lack thereof could be inferred (Fig. 1).

Previous research of natural communities in acidic mine and AMD environments (as opposed to those environments undergoing remediation treatment) has typically revealed microbial communities dominated by one or a few typical co-dominant taxa, accounting for 50% or more of the community, while "satellite species" vary in number (Quatrini and Johnson, 2018). In Richmond Mine a transcriptome study of eight samples revealed a total of 159 OTUs, > 85% of which were affiliated with the phylum Nitrospira and the *Leptospirillum* genus in particular (Aliaga Goltsman et al., 2015). Dominance of *Leptospirillum* and apparent low taxonomic diversity was also observed in earlier Richmond Mine studies, where fluorescence in-situ hybridization (FISH) revealed that *Leptospirillum* comprised up to 98% of microbial cells (Wilmes et al., 2009), whilst an even earlier clone-based analysis identified two bacterial and three archaeal lineages in an AMD biofilm community (Tyson et al., 2004). In the present study, samples dominated by known taxa of extreme acidophiles largely followed this trend of dominance by one or few acidophilic taxa. With one exception, communities containing less than ten abundant OTUs were dominated by known acidophilic taxa (>64%), while as the number of abundant OTUs increased above twenty, the contribution of known acidic mine taxa tended to be substantially reduced (Table 2).

3.3. *Mineralogical influence on microbial community composition*

While the subterranean temperature at Mynydd Parys remains relatively constant throughout the year (8–9 °C) (Johnson, 2012), the fact that sampling was performed over three seasons suggested the possibility that variability in microbial community structure in the sub-surface was influenced by above-ground seasonal factors, such as infiltration of nutrients and microorganisms. A comparison of community structure with source mineralogy, factoring in seasonality, revealed statistically significant ($P < 0.05$) correlations at both phylum- and OTU-level for the major primary (pyrite and quartz) and secondary (pisanite and melanterite) minerals (Table 1; Fig. 3). Additionally, significant OTU-level correlations occurred for amorphous samples ($P = 0.042$, $R^2 = 0.016$) and those containing hydronium jarosite ($P = 0.020$, $R^2 = 0.019$). All the above primary and secondary minerals also had a significant ($P < 0.05$) influence on communities if the seasonal factor was ignored (phylum- and OTU-level; not shown), implying that season had only a minor effect.

A selective effect of substratum mineralogy on microbial communities i.e. a mineralosphere effect (Uroz et al., 2015), has been established in various, less-extreme terrestrial environments, including soil (Carson et al., 2009; Uroz et al., 2012; Ahmed et al., 2017), large rocks (Hutchens, 2009; Kelly et al., 2010), tombstones (Brewer and Fierer, 2018), caves (Cuezva et al., 2012; Riquelme et al., 2015), and most recently has been shown to influence biofilm behaviour via preferential colonisation of minerals (Casar et al., 2021). However, the current study reports the potential for a mineralosphere effect in an extremely acidic, low-temperature, subterranean mine environment. Despite the general hostile environmental conditions within the abandoned mine, in particular the low pH (~2–2.5) and elevated concentrations of potentially toxic soluble transition metals in the infiltrating water and pools (Coupland and Johnson, 2004), minerals may represent refuges for

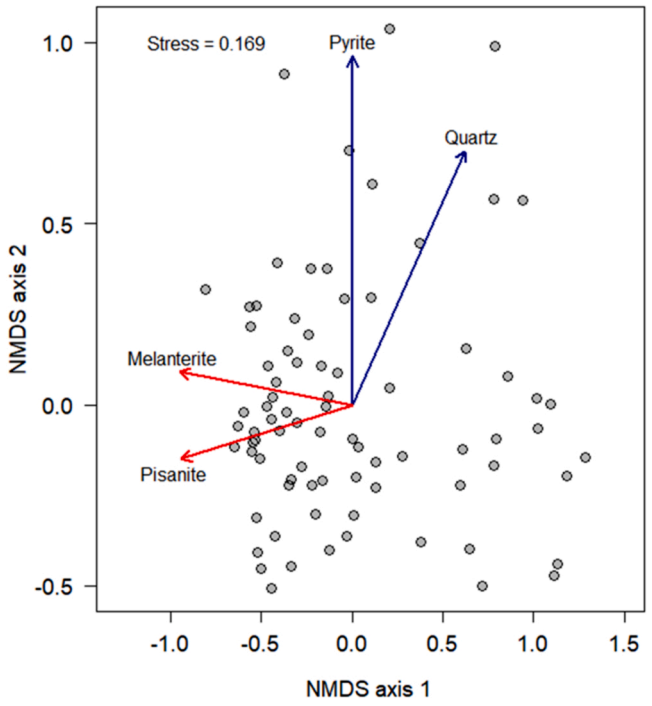

Fig. 3. Phylum-level nonmetric multidimensional scaling (NMDS) ordination of Bray-Curtis similarity indices of individual microbial communities showing significant mineral effects. Individual sample ordination is shown while vectors indicate significant mineral influences for primary (blue) and secondary (red) minerals. At phylum-level, pisanite ($P = 0.029$, $R2 = 0.03054$), melanterite ($P = 0.012$, $R2 = 0.03427$), pyrite ($P = 0.038$, $R2 = 0.02691$), quartz ($P = 0.034$, $R2 = 0.02827$). At OTU-level pisanite ($P = 0.025$, $R2 = 0.01808$), melanterite ($P = 0.010$, $R2 = 0.02001$), pyrite ($P = 0.001$, $R2 = 0.02996$), quartz ($P = 0.014$, $R2 = 0.01993$).

microbial colonisation where microscale conditions are conducive to the survival of both typical and atypical acidic mine taxa (Ahmed and Holmstrom, 2015; Dhami et al., 2018). Depending on their intrinsic factors such as geochemistry, microstructure, degree of weathering (biotic and abiotic) and microscale pH, minerals vary in their potential to provide a source of nutrients (both toxic and non-toxic), a physical surface for microbial attachment and biofilm formation (Jones and Bennett, 2017) and niches of differing pH (Coupland and Johnson, 2004).

The nonmetric multidimensional scaling (NMDS; stress=0.169) plot in Fig. 3 shows the results of a multivariate analysis of the entire prokaryotic populations (on the phylum level) in the Parys Mine mineral samples. The greater the distance between any two points in the ordination the greater the relative dissimilarity between the compositions of the two prokaryotic communities (considering the abundance of each phylum). Significance of the effect of the mineral type on the composition of the microbiome colonizing the samples is indicated in Fig. 3, with the arrow length being proportional to the degree of correlation.

While it is usually relatively easy to test for correlation of microbial populations in AMD environments with geochemical parameters, such efforts were complicated in the current study by the small size of the samples and particularly their nature (notably the hydration of secondary minerals making them soluble in water), resulting in an inability to determine pore water chemistry and pH measurements. The findings presented in this study focus specifically on the influence of mineralogy on the microbial community structure in solid mine samples, rather than the commonly researched influence of geochemical parameters on the composition of microbial populations in AMD (Méndez-García et al., 2015; Huang et al., 2016, 2021; Teng et al., 2017) or solid mine waste samples (e.g. Pakostova et al., 2020). Nevertheless, based on the obtained data, we speculate that the availability of electron donor(s) and pH changes on the surface of the minerals (resulting from iron and sulfur oxidation, as well as biomineralization and mineral weathering processes) were likely the factors that most affected the composition of the microbial communities closely associated with the mineral surfaces.

Pyrite provides electron donors (sulfur and iron) to acidophilic chemolithotrophs, which as discussed above, comprised significant proportions of the entire prokaryotic communities. The Parys mineral samples were collected from exposed (although wet) surfaces, it is therefore presumed that the sulfide oxidation occurred both by atmospheric oxygen (Eq. 1) and Fe^{3+} (Eq. 2), the latter being generally considered a primary oxidant of sulfide minerals under acidic conditions.

$$FeS_2 + 7/2\ O_2 + H_2O \rightarrow Fe^{2+} + 2\ SO_4^{2-} + 2\ H^+ \quad (1)$$

$$FeS_2 + 14\ Fe^{3+} + 8\ H_2O \rightarrow 15\ Fe^{2+} + 2\ SO_4^{\ 2-} + 16\ H^+ \quad (2)$$

Fe^{2+} released from pyrite may be re-oxidized, with the reaction rate greatly accelerated by the actions of iron-oxidizers (Eq. 3):

$$Fe^{2+} + 1/4\ O_2 + H^+ \leftrightarrow Fe^{3+} + 1/2\ H_2O \quad (3)$$

The precipitation of Fe(III) oxyhydroxides (e.g., ferrihydrite $Fe(OH)_3$) may occur (Eq. 4):

$$Fe^{3+} + 3\ H_2O \leftrightarrow Fe(OH)_3 + 3\ H^+ \quad (4)$$

All the above processes (Eqs. 1–4) affect the extent of proton release during pyrite oxidation, but the overall process reactions result in net acidity (Blowes et al., 2003). Melanterite and pisanite are both hydrated metal sulfates formed during the oxidation of pyrite and can be further oxidized to Fe^{3+} sulfates (and form goethite, lepidocrocite, or jarosite, depending on acidity and humidity). The transformations of these unstable secondary minerals can affect both substrate availability for microorganisms and pH on the mineral surface. Quartz, on the other hand, does not directly support the growth of acidophilic chemolithotrophs (which would result in pH changes on the mineral surface), due to the absence of iron and sulfur in its structure. Quartz is generally more stable than most other rock-forming minerals and constitutes the most common "gangue mineral" in ore deposits.

It is likely that sulfide oxidation processes (together with associated biomineralization and weathering processes) dominate on mineral surfaces in acidic mine environments such as Mynydd Parys, resulting in the creation of microenvironments which are specific to a mineral type and greatly shape the microbial communities present (which in return catalyse many of the biogeochemical transformations). The magnitude of mineral type influence in Mynydd Parys is demonstrated by the clear correlations between mineralogy and prokaryotic communities, which were (i) detected on as high a taxonomic level as phylum, and (ii) in samples most of which comprised of several minerals. The above indicates a major microbial diversity across the analysed samples and supports the speculations that specific microniches are formed on each mineral type.

The biogeochemical transformations involved in acidic mine environments are still not sufficiently described and further research is needed to fully elucidate the relationship between the geochemistry, mineralogy and microbiology of these diverse environments. The present findings demonstrate the magnitude of the influence that the presence of specific minerals have on the associated microbiomes, thus contributing to the general knowledge about the complex biogeochemical processes occurring in abandoned mines.

4. Conclusion

Our data support the hypothesis that different mineral substrata in the underground chambers within the abandoned Mynydd Parys copper mines facilitates the colonization of distinct microbial communities.

These findings not only imply a possible role for the mineralosphere in driving microbial biodiversity in extremely acidic mine environments, but also pose further questions regarding mechanisms by which taxa normally associated with disparate environments may survive in hostile acidic mine environments. Future microbiological investigations of attached communities within such environments need to consider substratum mineralogy in their experimental design if a comprehensive understanding of microbial community development is to be achieved.

CRediT authorship contribution statement

Laura C. Kelly: Conceptualization, Methodology, Software, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – reviewing and editing, Visualization, Supervision, Project administration, Funding acquisition. **Damian W. Rivett**: Formal analysis, Data curation, Writing – original draft, Visualization. **Eva Pakostova**: Writing – reviewing and editing. **Simon Creer**: Resources, Writing – original draft. **Tom Cotterell**: Resources, Formal analysis. **D. Barrie Johnson**: Resources, Writing – original draft, Supervision.

Data availability

Illumina sequence data used in this study have been deposited in the European Nucleotide Archive (ENA) under accession number PRJEB35982.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.micres.2022.127257.

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