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Bacterial and Archaeal Diversity in Sulfide-bearing Waste Rock at Faro Mine Complex, Yukon Territory, Canada

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Abstract

Acid mine/rock drainage (AMD/ARD) is generated by the microbially-accelerated oxidative dissolution of sulfide minerals in working and abandoned mine sites, and some natural environments. Iron-oxidizing microorganisms (IOM) regenerate the oxidant Fe^{3+}, while sulfur-oxidizing microorganisms (SOM) contribute to AMD/ARD by generating H_2SO_4 *via* the oxidation of elemental S and reduced inorganic sulfur compounds. Bacterial and archaeal diversity in 34 samples of sulfide-bearing waste rock recovered from three boreholes at the Faro Mine Complex (Yukon Territory, Canada; Pb/Zn production from 1969 to 1998) was investigated using high-throughput amplicon sequencing of 16S rRNA genes. A majority of the borehole pore water samples had circum-neutral or mildly alkaline pH (6.3 - 8.7), while some had low pH (3.3 - 5.2). Mean relative abundance of prokaryotic SOM/IOM accounted for 3.4% of the total amplicons. The acidophilic genera *Alicyclobacillus* and *Acidithiobacillus* were the most abundant sulfur- and iron-metabolizing prokaryotes detected, followed by neutrophilic and moderately acidophilic (non iron-oxidizing) SOM. Sulfate-reducing bacteria (SRB) were also detected, accounting for 0.6% of total reads. The presence of both acidophilic and neutrophilic prokaryotes catalyzing transformations of sulfur and iron in the same samples suggests development of microenvironments within the waste rock where dynamic biogeochemical transformations of these elements occur.

Keywords: mine waste, waste-rock dump, sulfur-oxidizers, iron-oxidizers, acid mine drainage.

1. Introduction

Open-pit metal mining generates large amounts of waste materials, including mill tailings and waste rock. Rock destined to end up as waste contains concentrations of target minerals that are too low for economical recovery, but is necessarily removed during open pit and underground mining operations along with higher grade ore materials. Disposal of mine tailings and waste rock can pose a serious threat to the environment, in particular to ground- and surface waters, in the

form of acid rock/mine drainage (ARD/AMD). ARD/AMD is formed when sulfidic minerals are oxidized on exposure to air and water, a process that is greatly accelerated in the presence of mineral-oxidizing microorganisms (Baker and Banfield, 2003; Johnson and Hallberg, 2003). The primary oxidant of sulfide minerals at very low pH (< 2.5) is often ferric iron, which is highly soluble in low-pH liquors. Prokaryotic microorganisms that regenerate ferric iron in acidic solutions are therefore of great significance to the continued oxidative dissolution of sulfidic minerals, such as pyrite (FeS_2). These include moderately and extremely acidophilic species of both bacteria and archaea (Dopson, 2016). Other (and sometimes the same) species that oxidize zero-valent sulfur, sulfide and sulfur oxy-anions contribute to ARD/AMD formation by generating sulfuric acid (Dopson and Johnson, 2012; Blowes *et al.* 2014). The mechanisms by which prokaryotes accelerate the oxidative dissolution of sulfide minerals have been described in detail (Vera *et al.* 2013). The microbiology of environments associated with ARD/AMD has been extensively reviewed (e.g. Baker and Banfield, 2003; Blowes *et al.* 2014).

Extremely acidophilic prokaryotes (Dopson, 2016) that are often found in large numbers in low pH environments (pH < 3) and which use Fe^{2+} and/or reduced forms of sulfur as electron donors include chemolitho- (and chemohetero-)trophic bacteria of the genera *Acidithiobacillus* and *Acidiferrobacter (*both *Proteobacteria)*, *Leptospirillum* (*Nitrospirae*), *Ferrimicrobium* (*Actinobacteria*), *Sulfobacillus* (*Firmicutes*), and also the archaea *Ferroplasma* and *Acidiplasma*. '*Acidibacillus'* spp. *(Firmicutes*) are extremely acidophilic iron-oxidizing and reducing obligate heterotrophs, some of which also oxidize sulfur, and strains of the mesophilic species '*Ab. ferrooxidans'* have been isolated from both acidic and neutral-pH waste rock samples from metal mines (Holanda *et al.* 2016). Most iron-oxidizing acidophilic prokaryotes that can use an alternative electron donor for ferrous iron are also able to reduce ferric iron in oxygen-depleted

(micro-)environments (Johnson *et al.* 2012). Moderately acidophilic (pH optima 3 to 5) sulfur-oxidizers isolated from mine environments include the obligately chemolithotrophic *Thiomonas* spp. (*Proteobacteria*; Battaglia-Brunet *et al.* 2006; Hallberg and Johnson, 2003) and *Sulfuriferula* (*Proteobacteria*; Jones *et al.* 2017). The genus *Alicyclobacillus* (*Firmicutes*) includes moderately and extremely acidophilic species (growth range between pH 2 and 6). Iron- and sulfur-metabolizing *Alicyclobacillus* species have been detected in AMD/ARD-impacted environments (Korehi *et al.* 2013; Guo *et al.* 2009), but the genus also contains obligately heterotrophic species that have been implicated in fruit juice spoilage (Huang *et al.* 2015). Various studies, focusing predominantly on tailings, have reported populations of acidophilic sulfur- (SOM) and iron-oxidizing (IOM) microorganisms of up to 10^8 cells g^{-1} (Southam and Beveridge, 1992; Benner *at al.*, 2000; Mendez *et al.* 2008; Fortin *et al.* 1996; Blowes *et al.* 1998). Neutrophilic SOM (e.g. *Thiobacillus thioparus; Proteobacteria*; Hutt *et al.* 2017) and IOM (e.g. *Gallionella* and *Sideroxydans* spp.; both *Proteobacteria*; reviewed by Dubinina and Sorokina, 2014) have also been detected in relatively high abundance in these materials (e.g. Blowes *et al.* 1998). Some investigations of bacterial and archaeal communities (BACs) in mine wastes using molecular techniques have reported known mineral bioleaching bacteria reaching tens of percent of total amplicons (e.g. Liu *et al.* 2014; Diaby *et al.* 2015; Kwon *et al.* 2015; Xiao *et al.* 2016; Bruneel *et al.* 2017).

The Faro Mine Complex (62.36° N, 133.37° W) is located in south-central Yukon Territory, Canada. Once the most productive open pit Pb/Zn mine in the world, as well as a significant producer of Ag, the mine was opened in 1969, and abandoned in 1998. Currently it is the site of one of the most complex abandoned mine remediation projects in Canada. The Faro Mine Complex, covering 25 km^2, includes three main areas: (i) Faro open pit, mill buildings and

waste rock dump area, (ii) the Rose Creek Tailings Area, and (iii) the Vangorda Plateau. Mining and mineral processing at the mine generated ~70 million tonnes (Mt) of tailings and ~320 Mt of waste rock, both of which have the potential to act as source materials from which heavy metals and acid can be leached into the surrounding land and water. Waste rock recovered from the Faro open-pit excavation, including large amount of pyrite, sphalerite, galena, and trace amounts of other minerals (*i.e.,* pyrrhotite, and chalcopyrite), was disposed of in a series of dumps. Mineralogical examination suggested that significantly high amounts of partially oxidized sulfide-bearing minerals (such as pyrite, sphalerite and galena) are widely distributed on the surface and within the waste-rock dumps. Material for microbiological characterization used in this study was collected from drill cores of three boreholes in the Main Dump East and Main Dump West (both in area 'i', as described above; Figure 1). Together the Main Dump and the Intermediate Dump contain ~145 Mt of sulfide-bearing waste rock deposited from 1970's until 1990´s (RGC, 1996). In an attempt to minimize acid and metal contamination from the high-sulfide waste rock, calc-silicate and schist were interbedded at the later stage of waste-rock construction with the high-sulfide rock in two sulfide cells (Figure 1) deposited in the center of the waste-rock dumps (RGC, 1996).

The main aim of the present study was to provide an evaluation of the microbiology of waste rock at the Faro Mine Complex. The reported research contributes to the ongoing field-scale characterization within the Faro Waste Rock Project (University of Waterloo & University of Alberta, Canada). Understanding of the microbial processes at the site is especially important with regard to the future mine closure, remediation and long-term monitoring. The diversity of BACs was investigated, with a particular focus on identifying prokaryotes that are known to be involved in the biogeochemical cycling of iron and sulfur.

2. Materials and Methods

2.1. Sample Collection

Continuous core samples were collected in July 2017 (Bao *et al.*, 2020) from three boreholes (UW17-BH1, UW17-BH2, and UW17-BH3; depth of 60-80 m) in sulfide-bearing waste-rock dumps at the Faro Mine Complex (Figure 1). The waste-rock core samples were stored at -20 °C until they were opened, after which sub-samples were collected and stored in sterile 50 mL centrifuge tubes at -20 °C for later laboratory analyses. Geochemical parameters were analyzed on a large number of pore-water and mineralogical samples (Bao *et al*., 2020). Based on calculated neutralization potential ratios from solid-phase carbon and sulfur contents, and field measurements of gas-phase oxygen concentrations (and thus the mechanism of gas transport), three zones were defined within the waste-rock dump profiles (Bao *et al.*, 2020): a rapid oxygen supply zone (ROS; upper 20 – 30 m depth), a strong oxygen depletion zone (SOD), and a thermally-influenced zone (TI; ~ 10 – 20 m above the pre-mining surface). Pore-gas oxygen concentrations in the ROS zone were close to atmospheric values (17.5 ± 3.3 vol. %; mean ± s.d.), while in the SOD and TI zones these were as low as 7.8 ± 5.1 vol. %. Based on the segregation of the three chemical zones and pore-water geochemistry, 34 waste-rock sub-samples were selected throughout the waste-rock profiles in the three boreholes (Table 1), and subjected to high-throughput sequencing to characterize the microbiology of the (fine-grained) waste-rock matrix material.

2.2. Aqueous and Solid-phase Geochemistry

Aqueous geochemical parameters were determined from centrifuged pore-water samples (for the detailed procedure of pore-water extraction from fine-grained waste-rock samples see Bao *et al.*, 2020). Measurements of pH (using an Orion Ross Ultra combination pH electrode, coupled

to an Orion 3 Star pH/mV meter) were completed on filtered pore-water samples (through 0.45 µm PVDF membranes). Water samples were filtered (through 0.2 µm PVDF membranes for dissolved cations, and 0.45 µm PVDF membranes for dissolved anions) and stored at 4°C before chemical analyses. Cations were analyzed (samples preserved with HNO_3; pH <2) using combined inductively coupled plasma-optical emission spectrometry (ICP-OES ICAP 6000, Thermo Scientific; EPA Method 6010C, 2000) and inductively coupled plasma-mass spectrometry (ICP-MS X Series II, Thermo Scientific; EPA Method 6020A, 1998). Anion concentrations were determined by ion chromatography (Dionex IC-CO3 system; EPA Method 300.0, 1993). Pulverized waste rock samples were analyzed for total carbon and total sulfur contents using an ELTRA CS-2000 carbon/sulfur analyzer coupled with an induction furnace (CS800).

2.3. DNA Extraction, PCR Amplification and Illumina MiSeq Sequencing

DNA was extracted in duplicate from 34 spatially distributed sub-samples (collected from cores at depths summarized in Table 1), using DNeasy PowerSoil Kits (Qiagen Inc., Germany), and following the manufacturer's instructions. Extracted DNA was analyzed using a Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, USA) to check its quality (DNA concentration in all samples > 4.5 ng μL^{-1}), and stored at −20 °C prior to submission for Illumina MiSeq sequencing (Metagenom Bio Inc.; Toronto, Canada) using the modified universal primers 515F/806R (Walters *et al.* 2015) to amplify the V4 region of 16S rRNA genes.

Sequence data were analyzed using the Mothur program v.1.39.5, updated: 3/20/2017 (Schloss *et al.* 2009), and the Mothur MiSeq Standard Operating Procedure (Kozich *et al.* 2013; https://www.mothur.org/wiki/MiSeq_SOP) from 12/12/2017. Seven out of a total of 34 samples which contained fewer than 10,000 sequences were removed, after which the duplicate samples

were merged. Chimeric sequences were discarded based on predictions by *vsearch* using the Silva database for 16S rRNA gene sequences (Release 128 for Mothur, downloaded 12/12/2017) as a reference. Sequences were clustered into OTUs at a 97% similarity level by a de novo picking method. Taxonomic annotation of individual OTUs was based on Mothur-formatted version of the RDP training set (version 16 from February 2016). Several taxons (unknown, mitochondria, eukaryotes) were not considered for further data analysis.

Good's coverage, generated using Mothur, was used to assess how representative the samples were of the environments from which they were taken. β-diversity was investigated using weighted UniFrac (Lozupone and Knight, 2005), and visualized using 3D non-metric multidimensional scaling (3D-NMDS). The analysis of molecular variance (AMOVA), was used to determine whether the diversity within a group was greater than their pooled diversity. The homogeneity of molecular variance (HOMOVA) was used to assess whether the variation in samples within one group differed from the variation in samples within other groups. Relative abundances of sulfate-reducing bacteria (SRB), iron-oxidizing (IOM), and sulfur-oxidizing (SOM) microorganisms were obtained by screening the taxonomy file for prokaryotic genera (or in a few instances higher taxa when identification to the genus level was not possible) containing at least one species with the investigated metabolic trait.

3. Results

3.1. Overview of Aqueous and Solid-phase Geochemistry

Waste rock collected from UW17 boreholes at the Faro Mine Complex has been characterized in detail within a broader initiative (Faro Waste Rock Project). Selected geochemical data of solid-phase (and corresponding centrifuged pore-water) samples used for microbiological

analysis are listed in Supplemental Table S1. Most samples used for microbiological analyses (Tables 1, S1) were of circum-neutral or moderately alkaline pH, and several samples were acidic (pH 3.3 to 5.2; marked with asterisks in Table 1). Total dissolved iron concentrations closely correlated with pH values; very low Fe concentrations (< 0.1 g L^{-1}) were observed in samples that had pH values >6, while elevated concentrations (up to 2.3 g L^{-1}) were detected in the acidic samples. Dissolved sulfate concentrations ranged from 1.4 to 109 g L^{-1}, and increased with decreasing pH (R = 0.653). The total sulfur content of the waste rock ranged from 0.07 to 23.3 wt.% S (3.64 ± 5.95 wt.%, mean value ± s.d.; n = 33). The total carbon content was 0.86 ± 0.39 wt.% (mean value ± s.d.; n = 33).

Mean aqueous concentrations of transition metals in acidic samples (11.6 Cd, 18.9 Co, 0.3 Cr, 29.4 Cu, 629 Fe, 820 Mn, 19.3 Ni and 6,218 Zn; all in mg L^{-1}) were at least one order of magnitude greater than those in samples with pH values > 6 (0.5 Cd, 3.3 Co, <0.01 Cr, 0.1 Cu, 0.3 Fe, 106 Mn, 3.2 Ni, and 202 Zn; all in mg L^{-1}), reflecting the lower solubility of most of these metals at circum-neutral (and higher) pH. However, the differences between metal concentrations in samples of different pH were significant ($P > 0.05$) for Cd, Co, Ni and Zn. Aqueous concentrations of arsenic ranging from below detection to 1.8 mg L^{-1} were observed in the samples. Aluminum concentrations varied greatly (from 0.01 to 8,750 mg L^{-1}), and were strongly pH related up to pH 7.3.

3.2. Overview of 16S rRNA Gene Sequence Data Statistics

Duplicates of each sample were sequenced in the same sequencing run. All average values described in this section refer to pooled samples. In total 3,436,576 raw sequence reads were obtained, with an average of 99,305 ± 15,378 reads per pooled sample. About 6.7% of sequences were flagged as chimeric, and another 27% of reads were lost during quality trimming. A total

effective sequence number was 2,270,017, with 66,765 ± 11,196 reads per sample, and an average of 107 ± 10 OTUs (97% sequence similarity) per library. Good's coverage (calculated for an OTU definition of 0.03) ranged from 82.0 to 99.2%. Sequence data statistics are summarized in Supplemental Table S2.

3.3. Entire Bacterial and Archaeal Communities

Proteobacteria were the most abundant phylum in most samples (ranging from 17.1 to 60.3% of total reads; mean 39% of the total reads), followed by *Actinobacteria* (4.9 to 43.1; 18%), *Firmicutes* (2.2 to 37.1; 16%), *Bacteroidetes* (1.0 to 14.7; 7%) and *Acidobacteria* (0 to 19.9; 4%; Figure 2). The phyla *Proteobacteria* and *Firmicutes* contain many species that catalyze the dissimilatory oxido-reduction of iron and/or sulfur (such as *Acidithiobacillus, Sulfobacillus* and *Alicyclobacillus* spp.). Both *Actinobacteria* and *Bacteroidetes*, phyla are widely distributed in the environment, and are often found in mine wastes (Fernandes *et al.* 2018; Mendez-Garcia *et al.* 2015; Bond *et al.* 2000). The phylum *Actinobacteria* also contains iron-metabolizing acidophiles (e.g. *Acidimicrobium* and *Ferrimicrobium*). *Acidobacteria* is a diverse phylum and also includes some acidophilic species, though these are not known to catalyze dissimilatory transformations of iron or sulfur. Proportions of unclassified bacteria were relatively low across the waste-rock samples (ranging from 0.6 to 33.9% of total reads); their mean relative abundance on the genus level accounted for 13% of the total amplicons. Archaeal DNA constituted for only 0.8% of the total amplified 16S rRNA genes.

Major genera (or higher taxa when identification to the genus level was not possible) are shown in Supplemental Table S3. Minor genera (< 0.5% of total amplicons) were grouped together, and their sum accounted for 33.2% of total reads. Prokaryotes that dominated Faro Mine

core samples use many different types of metabolic strategies and have been observed in a variety of different habitats. Some members of the order *Actinomycetales* (4.8% of total reads) are generally anaerobic decomposers found in healthy soil, metabolizing a wide array of substrates (e.g. chitin and cellulose). *Oxalobacteraceae* (4.3% of total reads) and other *Betaproteobacteria* (4.2%) include strict aerobes, strict anaerobes, and also nitrogen-fixing species. The chemoorganotrophic *Corynebacterium* spp. (4.0% of total reads) are aerobic or facultatively anaerobic bacteria commonly occurring in nature in soil, water, plants, food products, and also animals. *Duganella* spp. (3.7%) are aerobic chemoorganotrophs that have been isolated from sewage, polluted waters, forest and agricultural soil. In total, DNA of 605 genera were detected in the core samples. Members of these genera were common soil bacteria (e.g. *Actinobacteria, Arthrobacter*), plant symbionts (*Rhizobium*), animal pathogens (*Burkholderia, Clostridia, Enterobacteriaceae*), and other bacteria (and to a much lesser extent also archaea) possessing a broad variety of metabolic traits.

No significant differences (AMOVA, $P > 0.05$) in β-diversity (determined by weighted UniFrac) were found among the three boreholes (not shown). A 3D-NMDS plot (based on weighted UniFrac) in Figure 3 shows the comparison of BACs within the three oxidation zones (Table 1; described in more detail in Bao *et al.*, 2020). AMOVA test showed no significant differences ($P > 0.05$) between the centres of the clouds representing each zone within the waste rock profiles. HOMOVA test indicated insignificant differences ($P > 0.05$) in biodiversity variation in samples collected from each zone.

Relationships between BACs (on the genus level) and selected geochemical parameters (pH, dissolved sulfate, dissolved total iron, and solid-phase sulfur content) were investigated using NMDS plots of Bray-Curtis similarity matrixes (not shown). Few correlations were observed,

which might be explained by a synergetic effect of a number of geochemical parameters on BACs in waste rock.

3.4. Iron- and Sulfur-metabolizing Genera

Figure 4 shows the relative abundances of prokaryotic microorganisms involved in dissimilatory oxido-reduction of sulfur and iron in samples collected from waste-rock dumps at the Faro Mine Complex. Physiological characteristics of the sulfur- and iron-metabolizing genera detected are summarized in Table 2.

The total abundance of SOM/IOM varied greatly across both samples and borehole profiles, indicating the presence of diverse BACs, composition of which were affected by a variety of physicochemical factors. Genera that oxidize sulfur and/or iron accounted for 3.4% of the total reads (range 0 to 15.7% across the core samples), of which 2.08% are genera that include species that oxidize both substrates, 0.31% IOM, and 0.99% SOM. *Alicyclobacillus* and *Acidithiobacillus* spp. were the most abundant SOM/IOM; their average proportions of the total amplicons in all samples reached 0.99 and 0.85% of total reads, respectively. The neutrophilic genus *Thiobacillus* was the most numerous (non iron-oxidizing) SOM, detected with a mean abundance of 0.49% of total reads, followed by moderately acidophilic *Sulfuriferula* (0.32%) and *Thiomonas* spp. (0.07%). In contrast, extremely acidophilic (non iron-oxidizing) SOM accounted for < 0.03 % of total reads, though this could be an underestimate as several species of *Acidithiobacillus* also have this trait. The most abundant acidophilic (non sulfur-oxidising) IOM, the *Acidimicrobiales,* constituted for 0.2% of the total DNA amplicons. The mean sum of SRB constituted for 0.59% of the total reads, ranging from 0 to 3.83% across the core samples. *Desulfobulbus* and *Desulfovibrio* were the most abundant sulfate-reducing genera, accounting for 0.15 and 0.09% of total amplicons, respectively.

4. Discussion

Bacteria inhabiting mine-impacted environments, including those that are acidic, have been found to be members of the phyla *Proteobacteria, Nitrospirae, Actinobacteria, Firmicutes*, and *Acidobacteria* (Mendez-Garcia *et al.* 2015). The Faro Mine waste rock samples were dominated by all of these phyla, with the exception of *Nitrospirae*. Members of these phyla include chemolitho-autotrophic and chemolitho-heterotrophic species that catalyze the dissimilatory oxidation of ferrous iron and reduced sulfur (and sometimes of both). Interpretation of the sequencing results is complicated by: (i) the material deposited in waste-rock dumps is highly heterogeneous; (ii) many genera include species that differ in their abilities to oxidize (or reduce) iron, and to oxidize sulfur (Table 2); (iii) high-throughput amplicon sequencing of the 16S rRNA genes allowed detection only at the genus level, due to limited amplicon lengths. To interpret the results of this study, genera containing at least one species catalyzing the respective dissimilatory reaction were categorized as SOM/IOM, SOM, IOM, and SRB.

The mean abundance of sulfur- and iron-oxidizers in the Faro Mine waste rock samples accounted for 3.37% of the total reads, which is a significantly lower abundance than reported elsewhere. However, most studies of the microbiology of mine wastes that have used high-throughput sequencing have focused on mill tailings which are potentially much more reactive materials in terms of their potential for acid generation. Within these, the predominance of typical mineral-leaching bacteria (such as *Acidithiobacillus, Leptospirillum, Sulfobacillus* or *Ferroplasma*) have been detected, in relative abundances of tens of percent (e.g. Liu *et al.* 2014; Diaby *et al.* 2015; Kwon *et al.* 2015; Xiao *et al.* 2016; Bruneel *et al.* 2017). The overall abundance of SOM/IOM in this study might be slightly underestimated. The geochemical environment within the Faro Mine waste rock has characteristics previously identified as a suitable environment for

'*Ab. ferrooxidans*' (Holanda *et al.* 2016), though the presence of this *Firmicute* could not be confirmed as sequences are not yet present in the reference file for taxonomic identification.

Many of the genera include species that can also reduce ferric iron to ferrous (Johnson *et al.* 2012). Dissimilatory ferric iron reduction would be anticipated to dominate over iron oxidation in anoxic regions of the waste-rock dumps. SRB (*Firmicutes, Proteobacteria*) can also reduce ferric iron indirectly via their production of hydrogen sulfide, but some can also reduce iron directly through enzymatic mechanisms (Lovley *et al.* 1993). Reduction of ferric iron can also occur under oxic conditions; *Acidiphilium* isolates have been reported to reduce both soluble and solid-phase ferric iron even in the presence of oxygen (Johnson and Bridge, 2002). Relatively small concentrations of dissolved iron (mean 0.32 mg L^{-1}) were detected in the circum-neutral (and mildly alkaline) samples from Faro. Abiotic oxidation of ferrous iron is rapid under circum-neutral pH (King *et al*. 1995) which could help explain the low concentrations of ferrous iron and generally low abundance of iron-oxidizers in these samples. The only detected neutrophilic IOM, *Sideroxydans*, accounted for 0.05% of the total reads. However, the abundance of IOM was not significantly greater ($P > 0.05$) in acidic samples, despite elevated concentrations of soluble Fe (mean 629 mg L^{-1}) in the latter. *Leptospirillum* was the only genus detected that oxidizes iron, but does not oxidize sulfur or reduce ferric iron. The bacterium was detected in only four Faro microbiology samples, and the mean relative abundance equaled as low as 0.05% of the total reads. Because all Faro pore water samples corresponding to waste rock samples analyzed in this study had paste pH > 3.3, ferrous iron would have been anticipated to be the dominant ionic form present in solution, with ferric iron predominantly present in various solid phases, such as ferrihydrite and goethite. Soluble forms of iron are preferred electron donors and acceptors for both iron-oxidizers and iron-reducers (Weber *et al.* 2006). Due to coupling of microbial iron oxidation and iron

reduction, even low concentrations of iron can be recycled in rapid microscale cycling, and can thus have a significant impact on AMD/ARD generation.

Prokaryotes detected in the waste rock samples display considerable diversity in their carbon metabolisms. While some species are strict autotrophs (e.g. *Acidithiobacillus* and *Leptospirillum* spp.), others are capable of utilizing organic carbon (e.g. *Acidiphilium, Alicyclobacillus* and *Sulfobacillus*). In most mine-waste environments autotrophic iron- and sulfur-oxidizing species are the primary–producers, providing organic carbon used as growth substrates by heterotrophic species. Autotrophic species release organic compounds from active and dead cells, some of which (e.g. aliphatic acids) can inhibit their growth, and co-existence with heterotrophic species, which catabolize labile organic compounds, can eliminate this potential impedance. A significant portion (~72%) of the sulfur- and iron-metabolizing bacteria and archaea in Faro waste rock samples were heterotrophic/mixotrophic (including *Alicyclobacillus*, *Thiobacillus*, *Acidimicrobium*, *Acidiphilium, Sulfobacillus, Ferroplasma, Sulfurisoma,* and all SRB). Many members of the BACs in the waste rock samples can utilize organic carbon compounds, not only the prokaryotes involved in redox transformations of iron and sulfur redox transformations, mentioned previously. For instance, heterotrophic members of the phyla *Actinobacteria* and *Bacteroidetes,* which have also been detected in other mine-impacted environments (Mendez-Garcia *et al.* 2015). Strict autotrophs also were present in the core samples. *Acidithiobacillus* was the second most numerous sulfur- and iron-metabolizing genus (with 0.85% of total reads). The results indicate that active, microbially-mediated carbon cycling can occur in waste rock, despite low organic carbon content.

Waste-rock dumps are highly heterogeneous, which explains why few correlations between geochemical data and BACs in samples collected at the Faro Mine Complex were

observed. Strong positive correlations were, however, observed between the abundance of SOM/IOM and oxygen levels (reported by Bao *et al.*, 2020). As anticipated, abundance of SRB negatively correlated with oxygen levels. However, occurrence of SRB, together with increased sulfate concentrations (Bao *et al.*, 2020), were detected in the SOD zone, indicating the development of anoxic microenvironments in the waste rock within the zone. Elevated abundances of SOM/IOM were observed at the base of UW17-BH3, possibly due to thermally-driven advective transport of O_2. Also, elevated abundance of SOM/IOM and low abundance of SRB were observed near the bottom of UW17-BH2, where an oxygenated zone was detected (Bao *et al.*, 2020).

Other physicochemical parameters impact BACs in mine wastes. The extremely low relative abundance of archaeal 16S rRNA genes in the DNA isolated from waste rock collected at the Faro Mine Complex was ascribed to low temperatures and pH unsuitable for their metabolism and growth. The vast majority of archaea that are known to grow in sulfide mineral-rich environments have pH optima from 1 to 3 (Golyshina *et al.* 2016), values significantly lower than bulk pH measured in the Faro waste rock samples. Temperature throughout the three UW17 boreholes ranged from ~10 to 20 °C, except for the surface portion of the waste rock where greater temperature fluctuations were observed (Bao *et al.*, 2020). These low temperatures are much below growth optima of archaea known to be involved in iron- and sulfur-cycling.

Bacterial populations were also likely to be affected by the low temperature; Sand *et al.* (1992) reported that during leaching experiments at 14°C *Leptospirillum* grew slowly and *Acidithiobacillus* dominated, which is consistent with observations in this study. Due to heat generation during exothermic sulfide oxidation reaction, formation of regions with temperatures closer to growth optima of SOM/IOM can be expected, which would further promote mineral

weathering in these spots. Despite the circum-neutral pH of most of the Faro Mine waste rock samples, acidophilic SOM/IOM were detected in significant relative abundance in most of them. A significant proportion of these organisms belonged to extreme acidophiles (with pH optima $\leq$ 3), even though pH of even the most acidic samples (pH ~3.3) was on the high end of growth ranges of these prokaryotes.

Although a certain degree of caution with sequence data interpretation is needed, several general points can be inferred from the microbiological data regarding the microbiology of waste rock: (i) sulfate-reducing bacteria were detected in the same samples as mineral-oxidizing prokaryotes, indicating dynamic redox transformations of sulfur and iron in the waste rock; (ii) the co-existence of acidophilic sulfur- and iron-metabolizing and neutrophilic prokaryotes, indicating the existence of sulfide bio-oxidation hotspots within the waste rock, with pH and temperatures suitable for the activities of sulfur- and iron-metabolizing acidophiles. These local regions are not always possible to detect in the waste-rock pile using paste pH measurements or geochemical analysis, but high-throughput sequencing, which is an extremely sensitive biomolecular analysis, can detect acidophiles that catalyze the oxidative dissolution of sulfide minerals, and therefore the potential for AMD/ARD generation.

5. Conclusion

The Faro Mine Complex is one of the largest abandoned mine remediation projects in the world. Waste rock deposited at the site has been observed to generate acid mine/rock drainage (of pH as low as 1.4). Low relative abundance of prokaryotes catalyzing dissimilatory oxidation reduction of sulfur and iron, yielding 3.4% of the total reads, indicated low sulfide oxidation rates in the waste rock. However, waste-rock dumps are heterogeneous systems, and although most of

the Faro Mine pore-water samples were of circum-neutral pH, several of them were acidic. Both acidophilic and neutrophilic sulfur- and iron-metabolizing genera were detected in the waste rock samples, regardless their pH. The presence of genera in the waste-rock dumps at the Faro Mine complex, that are known to accelerate the oxidative dissolution of sulfide minerals, and thereby generate acidic, metal-rich effluents, should influence the future management strategy at the site. Storage conditions should be such that they limit the activity of these bacteria while being more conducive to those, like SRB, that can mitigate the release of dissolved metals and sulfate.

Data availability: The sequence data that support the findings of this study are openly available in ENA at http://www.ebi.ac.uk/ena/data/view/PRJEB34502, reference number PRJEB34502.

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Abbreviations:

AMD	acid mine drainage
AMOVA	analysis of molecular variance
ARD	acid rock drainage
BAC	bacterial and archaeal community
BH	borehole
HOMOVA	homogeneity of molecular variance
IOM	iron-oxidizing microorganisms
Mt	million tonnes

NMDS	non-metric multidimensional scaling
OTU	operational taxonomic unit
ROS	rapid oxygen supply zone
SOD	strong oxygen depletion zone
SOM	sulfur-oxidizing microorganisms
SRB	sulfate-reducing bacteria
TI	thermally-influenced zone

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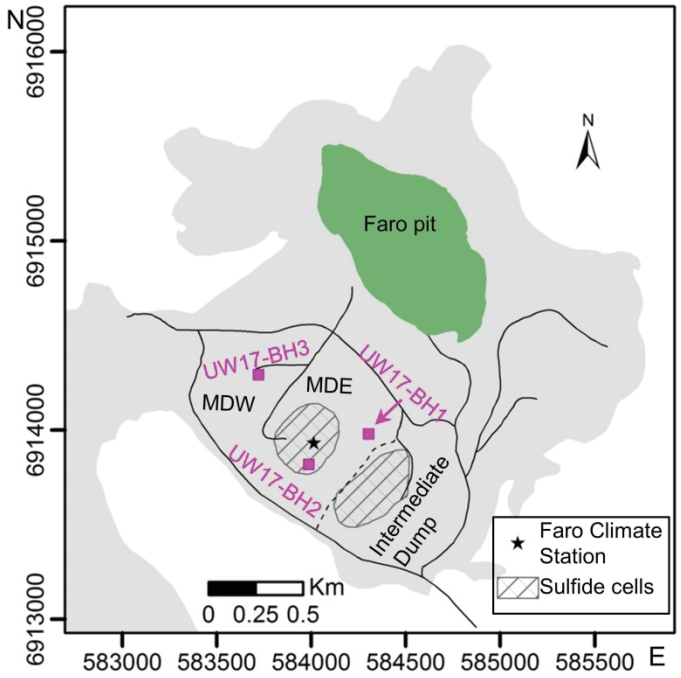

Figure 1. Site schematic of the Faro Mine Complex showing the Main Dump West (MDW), Main Dump East (MDE), and Intermediate Dump. Core samples for microbiological analysis described in this study were collected from three boreholes (UW17-BH1, UW17-BH2, and UW17-BH3) within the Main Dump East and Main Dump West. Legend: grey area refers to areas impacted by mine wastes; hatched areas refer to sulfide cells. (Modified from Bao *et al.* in submittal).

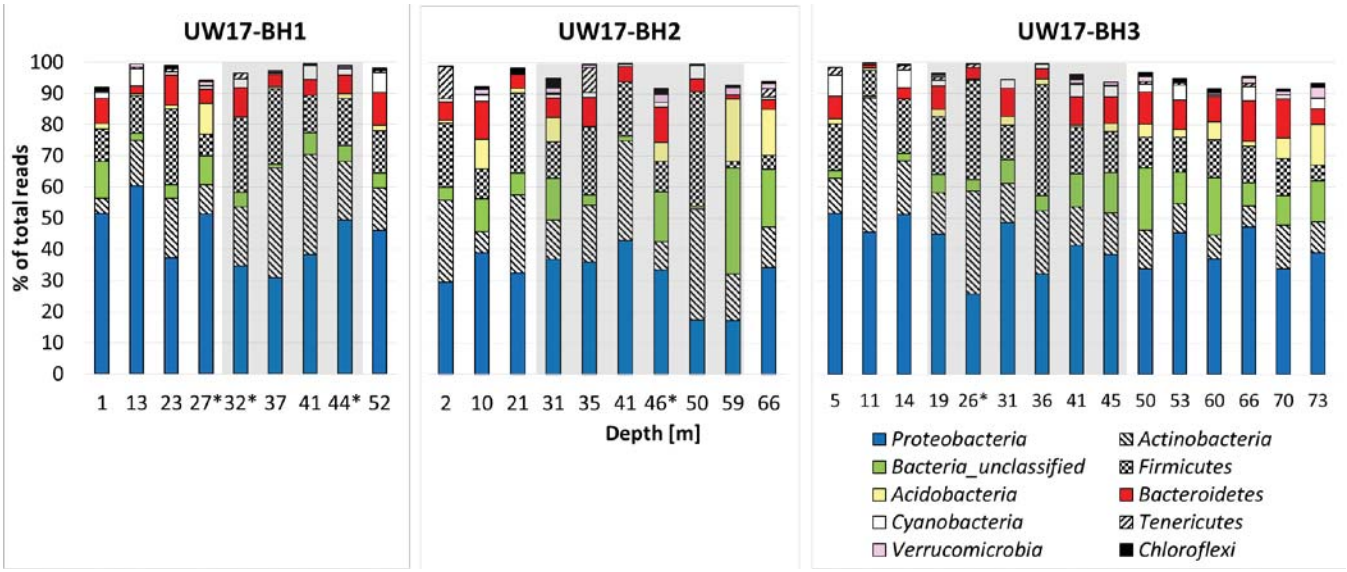

Figure 2. Proportions of total reads of major phyla (cut off = 1%) at three boreholes (UW17-BH1, UW17-BH2, UW17-BH3) at the Faro Mine Complex. Strong oxygen depletion zone is delineated by grey areas. Samples referred to as 'acidic' in this report are marked with asterisks.

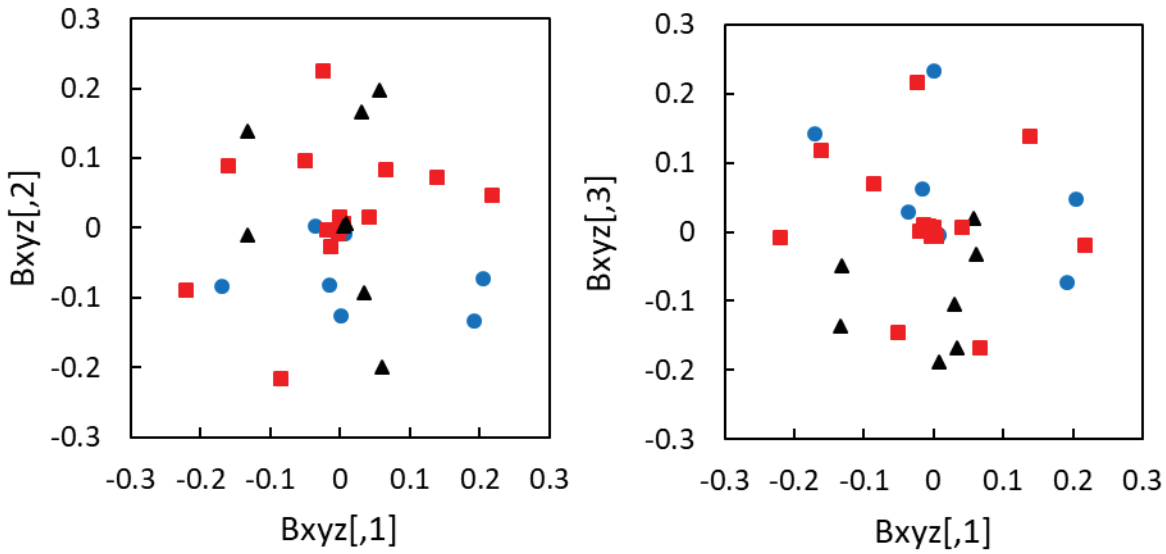

Figure 3. 3D-NMDS plot (stress=0.245) of weighted UniFrac used to investigate BACs in the (●) rapid oxygen supply (ROS), (■) strong oxygen depletion (SOD), and (▲) thermally-influenced (TI) zones in waste rock samples recovered from the three boreholes in the Main Dump at the Faro Mine Complex. The distance between any two points represents the difference between those two communities.

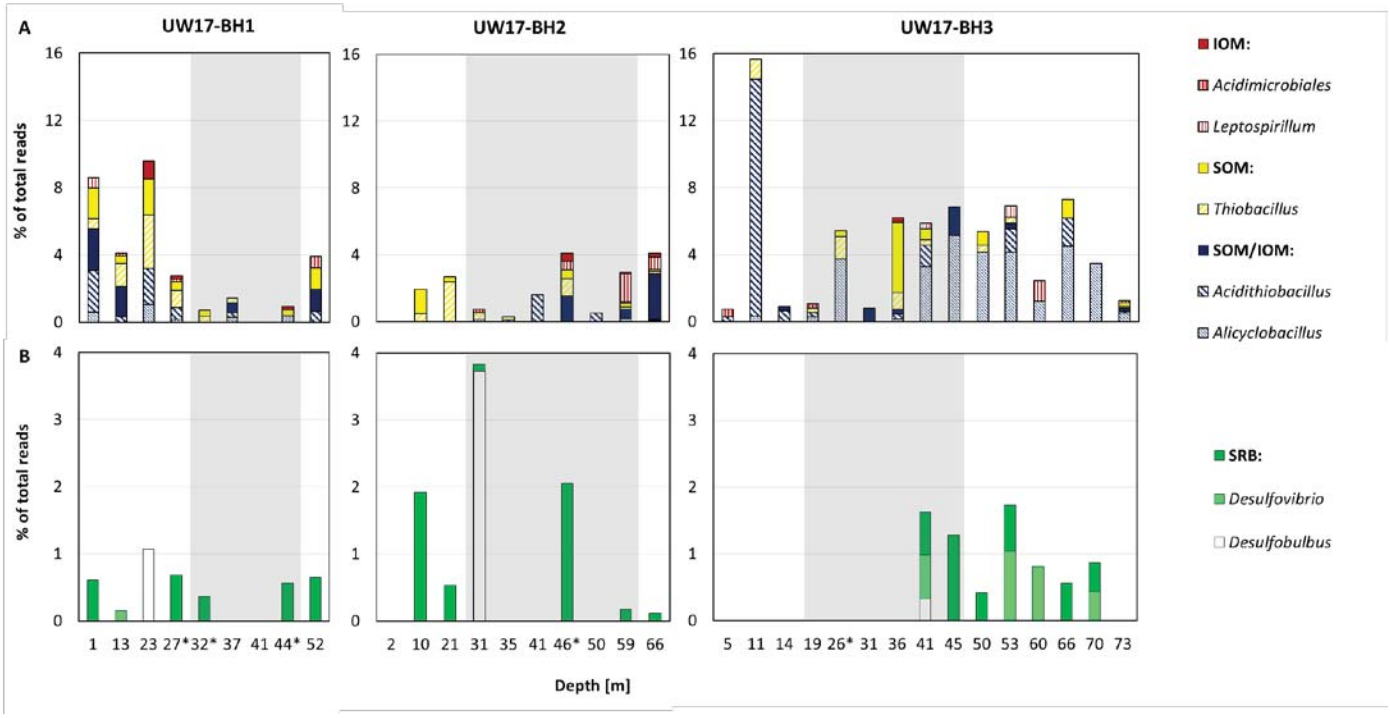

Figure 4. Proportions of total reads of (A) sulfur- and/or iron-oxidizing, and (B) sulfate- and/or sulfur-reducing bacteria and archaea in waste rock collected from the UW17-BH1, UW17-BH2, UW17-BH3 boreholes at the Faro Mine Complex. Legend: IOM = iron oxidizers; SOM = sulfur oxidizers; SOM/IOM = sulfur- and iron-oxidizers; SRB = sulfur- and/or sulfate-reducers. Strong oxygen depletion zone is delineated by grey areas. Samples referred to as 'acidic' in this report are marked with asterisks.

Table 1. Depths at which waste rock samples collected from three boreholes (UW17-BH1, UW17-BH2, and UW17-BH3) at the Faro Mine Complex were analyzed for microbial diversity, using high-throughput amplicon sequencing of the 16S rRNA genes. Legend: ROS = rapid oxygen supply, SOD = strong oxygen depletion, and TI = thermally-influenced zones. Bracketed numbers are the pH values of pore water samples. Samples referred to as 'acidic' in this report are marked with asterisks, and the remaining samples were grouped together as circum-neutral or mildly alkaline.

Depth [m]		
UW17-BH1	**UW17-BH2**	**UW17-BH3**
1.0 (ROS; 8.0)	1.5 (ROS; 8.3)	4.9 (ROS; 8.0)
12.8 (ROS; 7.7)	9.8 (ROS; 8.2)	11.0 (ROS; 8.3)
22.6 (ROS; n.a.[1])	21.3 (ROS; 8.7)	13.7 (ROS; 8.2)
27.4 (ROS; 4.5)*	31.1 (SOD; 8.5)	18.9 (SOD; 7.5)
31.7 (SOD; 3.7)*	35.1 (SOD; 8.6)	26.2 (SOD; 5.2)*
36.9 (SOD; 7.3)	40.8 (SOD; 6.3)	31.4 (SOD; n.a.[1])
40.5 (SOD; n.a.[1])	46.3 (SOD; 3.9)*	35.7 (SOD; 6.7)
43.9 (SOD; 3.3)*	50.3 (SOD; 7.6)	40.8 (SOD; 6.6)
51.5 (TI; 7.8)	59.4 (SOD; 7.4)	44.5 (SOD; 7.7)
-	65.8 (TI; n.a.[1])	50.0 (TI; n.a.[1])
-	-	53.3 (TI; 8.6)
-	-	59.7 (TI; n.a.[1])
-	-	65.5 (TI; 8.0)
-	-	69.8 (TI; 6.6)
-	-	73.2 (TI; 6.6)

[1]not available due to low water content

Table 2. Metabolic traits of genera detected in waste rock samples at the Faro Mine Complex that are known to catalyze the dissimilatory oxido-reduction of iron and sulfur (Quatrini and Johnson, 2016). '+' indicates that at least one species of the genus has been reported to catalyze the dissimilatory reaction referred to. EA=extremely acidophilic, MA=moderately acidophilic, N=neutrophilic. Higher taxa that could not be identified on the genus level are marked with asterisks.

Genus	Mean % of total reads	pH response	Sulfur oxidation	Iron oxidation	Sulfate reduction	Iron reduction[1]
Alicyclobacillus	0.99	EA & MA	+	+		+
Acidithiobacillus	0.85	EA	+	+		+
Thiobacillus	0.49	N	+			
Sulfuriferula	0.32	MA (& N)	+			
Acidiferrobacter	0.21	EA	+	+		+
*Acidimicrobiales**	0.18	EA		+		+
Desulfobulbus	0.15	N			+	+
Desulfovibrio	0.09	N & MA			+	+
Desulfuromonas	0.07	N			+	+
Thiomonas	0.07	MA	+			
Desulfosporosinus	0.06	N & MA			+	+
Desulfitobacterium	0.06	N & MA			+	+
Leptospirillum	0.05	EA		+		
Sideroxydans	0.05	N (& MA)	+	+		
Acidiphilium	0.03	EA	+			+
Sulfobacillus	0.03	EA	+	+		+
*Desulfobulbaceae**	0.03	N			+	+
Sulfuricurvum	0.03	N	+			
Sulfurimonas	0.02	N	+			
*Desulfobacteraceae**	0.02	N			+	+
*Desulfuromonadaceae**	0.02	N			+	+
*Acidimicrobiaceae**	0.02	EA		+		+
Desulfatiglans	0.02	N			+	+
Desulfofustis	0.02	N			+	+
*Desulfovibrionales**	0.01	N & MA			+	+
Desulfoprunum	0.01	N			+	+
Desulfomonile	0.01	N & MA			+	+
Sulfuricella	0.01	N	+			

Sulfurisoma	<0.01	N	+			
Desulfatiferula	<0.01	N			+	+
*Ferroplasmaceae**	<0.01	EA		+		+
*Alicyclobacillaceae**	<0.01	EA	+	+		+
Desulfocapsa	<0.01	N			+	+

[1]both direct and indirect