Hindawi
Geofluids
Volume 2019, Article ID 7401304, 17 pages
https://doi.org/10.1155/2019/7401304

Research Article

High Diversity in Iron Cycling Microbial Communities in Acidic, Iron-Rich Water of the Pyhäsalmi Mine, Finland

Malin Bomberg, Jarno Mäkinen, Marja Salo, and Päivi Kinnunen

VTT Technical Research Centre of Finland Ltd., P.O. Box 1000 (Tietotie 2), FIN-02044 VTT, Finland

Correspondence should be addressed to Malin Bomberg; malin.bomberg@vtt.fi

Received 22 May 2018; Revised 31 August 2018; Accepted 27 September 2018; Published 10 March 2019

Academic Editor: Henrik Drake

Copyright © 2019 Malin Bomberg et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Microbial communities of iron-rich water in the Pyhäsalmi mine, Finland, were investigated with high-throughput amplicon sequencing and qPCR targeting bacteria, archaea, and fungi. In addition, the abundance of *Leptospirillum* and *Acidithiobacillus* was assessed with genus-specific qPCR assays, and enrichment cultures targeting aerobic ferrous iron oxidizers and ferric iron reducers were established. The acidic (pH 1.4–2.3) mine water collected from 240 m, 500 m, and 600 m depth from within the mine had a high microbial diversity consisting of 63-114 bacterial, 10-13 archaeal, and 104-117 fungal genera. The most abundant microorganisms in the mine water were typical acid mine drainage (AMD) taxa, such as acidophilic, iron-oxidizing *Leptospirillum*, *Acidiphilum*, *Acidithiobacillus*, *Ferrovum*, and *Thermoplasma*. The fungi belonged mostly to the phylum Ascomycetes, although a great part of the fungal sequences remained unclassified. The number of archaeal 16S rRNA genes in the mine water was between 0.3 and 1.2×10^7 copies mL^{-1} in the samples from 500 m and 600 m, but only 3.9×10^3 at 240 m and archaea were in general not enriched in cultures. The number of fungal 5.8S rRNA genes was high only in the mine water from 500 m and 600 m, where $0.2–3.4 \times 10^4$ spore equivalents mL^{-1} were detected. A high number of *Leptospirillum* 16S rRNA genes, $0.6–1.6 \times 10^{10}$ copies mL^{-1}, were detected at 500 m and 600 m depth and in cultures containing ferrous iron, showing the importance of iron oxidizers in this environment. The abundance of bacteria in general was between 10^3 and 10^6 16S rRNA gene copies mL^{-1}. Our results showed a high microbial diversity in the acid- and iron-impacted waters of the Pyhäsalmi mine, where *Leptospirillum* bacteria were especially prominent. These iron oxidizers are also the main nitrogen-fixing microorganisms in this ecosystem.

1. Introduction

Iron is the most abundant element on earth, and it is an essential part of the reactive centre of enzymes catalysing redox reactions, such as haemoglobin, cytochromes, catalases, and peroxidases. In addition to being part of prosteric groups of these enzymes, iron is used by many microorganisms as electron acceptor (ferric iron) or electron donor (ferrous iron). The bioavailability of iron generally increases with decreasing pH [1].

Pyrite is a major source of energy for iron-oxidizing autotrophic acidophilic microorganisms. Ferric iron can solubilize pyrite, thus releasing both ferrous iron and reduced sulphur compounds [2]. The increase in ferrous iron may increase the biological iron oxidation, which regenerates ferric iron, which again solubilizes more pyrite. The oxidation of the reduced sulphur compounds by sulphur-oxidizing microorganisms produces acidity. The increased acidity, again, keeps ferric iron soluble to solubilize more pyrite. This process leads to the formation of acid mine drainage (AMD) [3, 4].

Iron-cycling microorganisms are common inhabitants of acidic metal-rich mine environments. Acidophilic dissimilatory iron reducers are able to reduce oxygen and ferric iron facultatively [1]. When reducing ferric iron, autotrophic iron reducers utilize sulphur or hydrogen as electron donors, while heterotrophic species utilize organic carbon compounds. Organic carbon and nitrogen are generally limited in AMD ecosystems. Organic carbon and nitrogen stores of the ecosystem are replenished by abundant autotrophic microorganisms that couple ferrous iron and reduced sulphur oxidation with inorganic carbon and nitrogen fixation [5, 6]. However, released carbon compounds, such as lysates

from ruptured microbial cells and cell exudates released through metabolic activity, may be toxic to obligate autotrophic iron and sulphur oxidizers. Toxicity is removed when heterotrophic microorganisms consume organic carbon, simultaneously releasing carbon dioxide, which may be used by autotrophs.

Most of the research of AMD microbial communities has concentrated on bacteria and archaea. Méndez-García et al. [7] reported in their review that bacterial phyla generally present in AMD are Proteobacteria, Nitrospirae, Actinobacteria, Firmicutes, and Acidobacteria. The phyla Euryarchaeota of Archaea are often also present. However, microscopic fungi and other eukaryotes are often overlooked. Nevertheless, protists and algae have been detected in the extremely acidic waters of Rio Tinto, Spain, to an abundance of 60% of the biomass [8]. In dark underground mines, photosynthetic algae do not thrive, but fungi may function as important decomposers in the AMD environment. Here, fungi provide a wide range of organic carbon compounds for the heterotrophic community and carbon dioxide for the autotrophic community. Numerous species of yeasts and hyphal fungi have been isolated or detected by molecular methods from AMD-affected streams [8–12], but from dark, underground mine environments, reports on fungi are scarce.

In this study, we aimed to characterize the microbial communities of three different underground AMD ponds in the Pyhäsalmi mine, using high-throughput amplicon sequencing targeting the bacterial and archaeal 16S rRNA genes and the fungal ITS1 region. We aimed to characterize the culturable aerobic ferrous iron-oxidizing and ferric iron-reducing communities and identify important microbial interactions in the water and enrichment communities. Lastly, we strived to determine the ratio of bacteria, archaea, and fungi in the mine water and enrichment in order to gain more insight into the role of fungi in dark AMD ecosystems.

2. Materials and Methods

2.1. Study Site. The Pyhäsalmi mine is situated in North Ostrobothnia, and it is the oldest operating mine in Finland (soon in the closure phase) and the deepest metal mine in Europe, reaching a depth of over 1.4 km. It is a multimetal mine producing copper, zinc, and pyrite concentrates. The main minerals of the ore body are pyrite, pyrrhotite, chalcopyrite, and sphalerite [13]. The mining activity started in 1962 when the mine was first operated as an open cast pit until 1967, after which the underground mining started.

2.2. Sampling. Water samples were collected into acid-washed, sterile 1 L borosilicate bottles on November 17, 2016. Samples were collected from 3 different underground levels in the mine, 240 m, 500 m, and 600 m belowground (Figure 1). The sampling sites were located in inactive parts of the mine, and thus, the locations were not illuminated. The water at the sampling locations was running from the bedrock, although in P500m and P600m the water flow was slow and the samples were collected from small ponds that were connected to each other by a narrow stream. Temperature, oxidation reduction potential (ORP), and pH were measured on site using a Consort C5010 meter, with Van London pHoenix electrodes (ORP and pH: Ag/AgCl in 3 M KCl). Biomass was collected from sample water onto Sterivex filtration units within one day of sampling, after which the Sterivex filter units were placed in sterile 50 mL plastic test tubes and frozen at −25°C until DNA extraction. The sample volumes were 165 mL (P240m), 2 × 200 mL (P500m_A and P500m_B), and 2 × 35 mL (P600m_A and P600m_B).

2.3. Culturing of Iron Cycling Microorganisms. Sterile growth media for aerobic iron-oxidizing bacteria (IOB) and iron-reducing bacteria (IRB) were prepared. IOB were enriched using medium 180 (APH) and medium 176 optimized for the enrichment of *Acidithiobacillus ferrooxidans* and *Leptospirillum ferrooxidans*, respectively (http://culturecollection.vtt.fi/m/html?p=mel&id=180; http://culturecollection.vtt.fi/m/html?p=mel&id=176). Both media are designed for the autotrophic growth of IOB containing $8\,g\,L^{-1}$ (medium 180) or $20\,g\,L^{-1}$ (medium 176) ferrous iron, added as $FeSO_4$, and no added carbon sources. IRB were enriched on basal mineral medium (medium 180 without ferrous iron) containing ferric iron $4\,g\,L^{-1}$ added as $Fe_2(SO_4)_3$ with or without yeast extract (0.025% *w*/*v*). All media were sterilized by filtration through a 0.2 μm pore-size cellulose acetate filter (Corning) before use. Yeast extract was added as an autoclaved stock solution.

Enrichment cultures were prepared by mixing 5 mL medium and 0.5 mL sample water in 15 mL sterile plastic test tubes equipped with screw caps. Cultures of all samples were incubated at 30°C and 50°C on a shaker at 140 rpm. The enrichments were checked biweekly for presence of microbial cells by phase-contrast microscopy, change in colour of the medium (IOB), and change in pH and ORP using a pH and ORP meter (Hach). Cultures for which microbial growth was detected were recultivated with intervals of 2 to 4 weeks. During the consecutive cultivations, the enrichment P240m_176 ceased to grow or oxidize ferrous iron. The culture was deemed lost and omitted from the study.

2.4. DNA Extraction. The Sterivex filter units containing the biomass from the mine samples were thawed on ice immediately before the DNA extraction. All DNA extraction steps were performed in a laminar flow hood. The filtration units were cut open with flame-sterilized pliers, and the membrane filter was removed with sterile scalpels and tweezers and put into 5 mL test tubes containing glass beads (MoBio).

The DNA from the enrichment cultures was extracted from 10 mL culture after 3-5 consecutive cultivation rounds. The microbial biomass was first collected by centrifugation at 3200 g for 10 min using an Eppendorf 5810R table-top centrifuge, after the supernatant was removed leaving only approximately 100 μL supernatant in the test tubes. The microbial pellets were frozen at −20°C until DNA extraction.

The microbial DNA was extracted using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel) with the SL1 lysis buffer. For the membrane filters, the lysis occurred in 5 mL bead tubes, and for the pelleted enrichment biomass, the pellets were first dissolved in the remaining supernatant to which SL1 buffer was added. The suspension was subsequently

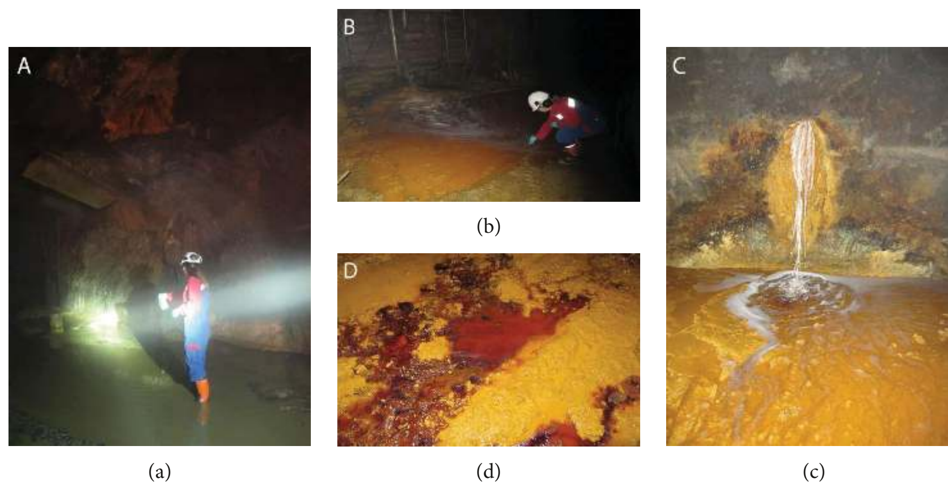


Figure 1: Collecting water samples in the Pyhäsalmi mine. (a) Collection of mine water dripping from the roof at the depth of 240 m belowground (sample P240m), (b) collection of water sample from the pond at depth 500 m belowground (sample P500m), and (c-d) sampling point at depth 600 m belowground (sample P600m).

transferred to NucleoSpin bead tubes for DNA extraction. The DNA extraction proceeded according to the manufacturer's protocol. The DNA was eluated into 50 μL SE buffer and stored at −20°C.

2.5. Optimization of Genus-Specific Primers for the Detection of Members of the Genus Acidithiobacillus. Bacteria belonging to the genus *Acidithiobacillus* are important constituents of many microbial bioleaching consortia and inhabitants of AMD-affected ecosystems. Thus, an assay for rapid estimation of the amount of *Acidithiobacillus* bacteria in such environments may be useful and we developed a qPCR assay targeting bacterial 16S rRNA genes belonging to the genus *Acidithiobacillus*. Primers targeting *Acidithiobacillus* 16S rRNA genes were designed in this study. We aligned full-length 16S rRNA gene sequences of representatives of all known *Acidithiobacillus* species obtained from the Ribosomal Database Project release 11 (RDP11) using ClustalW in Geneious Pro v 10.2.3 [14]. Primers of 19 to 24 nucleotides in length were designed with the Primer Design tool in Geneious Pro. The primers for *Acidithiobacillus* positioned on nucleotides 339-358 (forward) and 720–741 (reverse) enclosing a 402 bp section of the 16S rRNA gene covering the v4 and v5 variable regions of the 16S rRNA gene. The specificity of the primers was tested with the Probe Match tool in RDP. Subsequently, the primers were tested for specificity to the genus *Acidithiobacillus* by real-time PCR, using the LightCycler 480 and KAPA SYBR Fast qPCR Master Mix for Roche LightCycler 480. The reactions were performed in 10 μL triplicate reactions, and 10 pmol forward and reverse primers were used. The PCR programme was described for the bacterial qPCR, except that different annealing temperatures (58°C, 60°C, 62°C, and 64°C) were tested for the new primers. Altogether, 11 different bacterial strains, one commercial mock community DNA (ZymoBIOMICS™ Microbial Community Standard, Zymo Research Inc., Irvine, CA, USA), and three archaeal strains were used for preliminary optimization of the PCR conditions (Table 1). In addition, the bacterial strains were tested at a concentration of 0.5 ng DNA per 10 μL reactions with the *Acidithiobacillus* sp targeting primers and primers P1 and P2 in triplicate reactions using the amplification conditions described above and the annealing temperature of 64°C. The amplification result between the two primers pairs was compared, and a melting curve analysis was performed (Figures 2(a) and 2(b)).

2.6. Estimation of the Number of Different Microorganisms by Taxonomic and Genus-Specific Quantitative PCR. Quantitative PCR (qPCR) was used to estimate the concentration of bacterial and archaeal 16S rRNA genes and fungal 5.8S rRNA genes as a proxy for biomass in the mine water samples and enrichment cultures. In addition, we estimated the abundance of *Acidithiobacillus* sp. and *Leptospirillum* sp. using genus targeting qPCR. The bacterial community size was estimated using two parallel assays, one with primers P1 and P2 [15] amplifying a 200 bp fragment covering the variable v3 region and the other with primers S-D-Bact-0341-b-S-17/S-D--Bact-0785-a-A-21 [16], producing a 400 bp fragment covering the variable regions V3-V4 of the bacterial 16S rRNA gene. For archaea, an approximately 400 bp fragment of the 16S rRNA gene was amplified with primers A344F [17] and A744R, modified from [18]. The *Acidithiobacillus* abundance was targeted with primers Acthb_F and Acthb_R (this study) and the *Leptospirillum* bacteria using primers Lfp392F_mof and Lfp601R_mod (modified from [19] as described in [20]). The amplification was performed in a 10 μL reaction volume using the KAPA SYBR Fast qPCR Master Mix optimized for Roche LightCycler 480 (KAPA Biosystems, City, State, USA), 10 pmol each of the bacterial 16S rRNA, *Acidithiobacillus*- or *Leptospirillum*-specific primers, or 20 pmol each of the archaeal primers, and 1 μL of template DNA. The amplification programme consisted of an initial denaturation step at 95°C for 15 min, followed by 40 amplification cycles of 10 s at 95°C, 35 s at 57°C for bacterial and archaeal 16S rRNA genes, 64°C for *Acidithiobacillus* and 58°C for *Leptospirillum*-specific primers, and 30 s at 72°C, and a final elongation step of 3 min at 72°C and a melting curve analysis.

Table 1: The control strains used in the PCR tests for the genus-specific primers. All primers were tested on the same DNA preparates, i.e., the amount of DNA used in the universal bacterial and *Acidithiobacillus* sp. 16S rRNA gene targeting reactions is the same. However, DNA concentration may vary between preparates of different species.

Strain	Taxonomy (class; order)	Ct values P1/P2	Ct values *Acidithiobacillus*
Acidithiobacillus ferrooxidans	Acidithiobacilli; Acidithiobacillales	18.87	13.46
Methylosinus trichosporium	Alphaproteobacteria; Rhizobiales	15.16	21.87
Alcaligenes faecalis	Betaproteobacteria; Burkholderiales	20.26	30.57
Leptothrix discophora	Betaproteobacteria; Burkholderiales	15.79	9.81
Desulfovibrio desulfuricans	Deltaproteobacteria; Desulfovibrionales	20.53	25.27
Escherichia coli	Gammaproteobacteria; Enterobacteriales	20.10	23.66
Pseudomonas putida	Gammaproteobacteria; Pseudomonadales	10.58	7.14
Alcanivorax borkumensis	Gammaproteobacteria; Oceanospirillales	12.38	15.25
Leptospirillum ferrooxidans	Nitrospira; Nitrospirales	22.84	—
Sulfobacillus benefaciens DSM 19468	Bacilli; Bacillales	19.06	32.66
Clostridium ljungdahlii E-153486	Clostridia; Clostridiales	18.70	32.55
Mock community DNA#		7.21	17.51
Methanothermobacter thermautotrophicum DSM 1053*	Methanobacteria; Methanobacteriales	17.58	—
Sulfolobus solfataricus DSM 1616*	Thermoprotei; Sulfolobales	26.81	—
*Halobacterium salinarum**	Halobacteria; Halobacteriales	27.42	—

*Archaea; #mock community DNA (ZymoBIOMICS™ Microbial Community Standard, Zymo Research Inc., Irvine, CA, USA); – indicates that no amplification was detected.

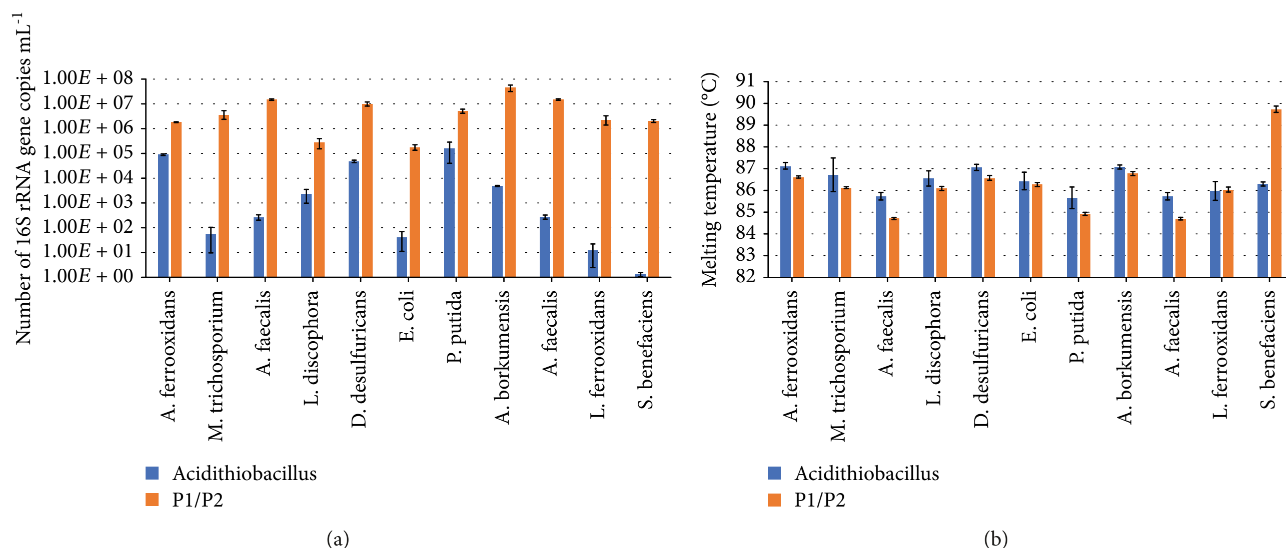


Figure 2: (a) The amplification of 16S rRNA genes with *Acidithiobacillus* targeting (blue) and general bacteria targeting P1 and P2 (orange) primers using 0.5 ng DNA per reaction and (b) the melting curve analysis of the amplified 16S rRNA gene fragments. All reactions were done in triplicate, and the error bars indicate standard deviation.

The fungal biomass was detected with a TaqMan probe assay targeting the 5.8 rRNA gene with primers 5.8F1 and 5.8R1 and a FAM-labeled probe 5.8P1 [21]. The amplification was performed in 10 μL reactions using the KAPA PROBE FAST qPCR Kit (KAPA Biosystems, USA), 500 nM of each primer, 200 nM probe, and 1 μL of template DNA. The amplification reaction consisted of enzyme activation at 95°C for 3 min and 40 cycles of 10 s at 95°C, 30 s at 62°C, and 1 s at 72°C. All qPCR runs were done using the LightCycler 480 instrument (Roche Diagnostics, Basel, Switzerland).

All qPCR reactions were conducted in triplicate, and each run included negative controls without added DNA template. Linear, tenfold dilution series of target DNA or genes were used as standards for the estimation of the amounts of specific target genes in the samples. The bacterial 16S rRNA

gene *Acidithiobacillus* and *Leptospirillum* standards consisted of a dilution series of plasmids containing the 16S rRNA gene of *E. coli* ATCC 31608, *Acidithiobacillus ferrooxidans* VTT E-991375, or *Leptospirillum ferrooxidans* VTT E-991377T, respectively, reaching concentrations from 10^2 to 10^8 copies per reaction. The archaeal amplification was compared to that of a dilution series of *Halobacterium salinarum* VTT E-103154T genomic DNA using a 10-fold dilution series from 10^0–10^7 cells per reaction. The fungal 5.8S rRNA gene numbers were compared to a dilution series of genomic DNA of *Aspergillus versicolor* VTT D-96667 spores with 10^0–10^5 spores per reaction. The pure cultures were obtained from the VTT Culture Collection (http://culturecollection.vtt.fi).

2.7. Amplicon Library Preparation. Amplicon libraries for high-throughput sequencing with Ion Torrent PGM were prepared by PCR from the DNA samples. Bacterial 16S genes were amplified with primers S-D-Bact-0341-b-S-17/S-D--Bact-0785-a-A-21 [16], targeting the variable V3-V4 regions of the 16S rDNA gene, archaeal 16S genes with primers S-D-Arch-0349-a-S-17/S-D-Arch-0787-a-A-20 [22], targeting the V4 region of the gene, and fungal internal transcribed spacer (ITS) gene markers with primer pairs ITS1 and ITS2 targeting the fungal ITS1 region [23, 24]. PCR amplification was performed in parallel 25 μL reactions for every sample containing 1x MyTaq™ Red Mix (Bioline, London, UK), 20 pmol of each primer, up to 25 μL molecular-biology-grade water (Sigma), and 2 μL of template. The PCR programme consisted of an initial denaturation step at 95°C for 3 min, 35 cycles for bacteria and fungi, and 40 cycles for archaea of 15 s at 95°C, 15 s at 50°C, and 15 s at 72°C. A final elongation step of 30 s was performed at 72°C. The PCR products were verified with agarose gel electrophoresis. Amplicons were sent to Ion Torrent sequencing with PGM equipment (Bioser, Oulu, Finland), and amplicons were purified before sequencing by the staff at Bioser.

2.8. Amplicon Sequence Analysis. The Ion Torrent sequence data was analysed with mothur v. 1.39.5 [24]. Sequence reads were first subjected to quality control where raw sequence reads were subjected to quality trimming. Minimum sequence lengths were set to 250 for bacterial and archaeal sequences and 200 bp for fungal ITS sequences. A maximum of 8 homopolymers and 1 primer difference were allowed. Default settings were used with the exception of a lower qwindowaverage set to 25 to compensate for the Ion Torrent sequencing platform. Bacterial and archaeal sequences were aligned to the Silva seed alignment (v.132) [25] for clustering and distance matrix. The ITS sequences were clustered using the pairwise.seq command in mothur. Chimeric sequences were identified using the chimera.slayer in mothur and were subsequently removed. Pairwise distances with cutoff of 0.03 were calculated for bacterial and archaeal sequences. Classification of bacterial and archaeal sequences and operational taxonomic units (OTUs) was done with Silva version 132 taxonomy [25]. Pairwise distance for ITS sequences was calculated with a cutoff of 0.03, and sequences and OTUs were classified with UNITE ITS v. 6 dynamic taxonomy [26]. The make.biom command was used for constructing.-biom files for subsequent analyses in QIIME [27] and R [28]. Taxonomy summaries were visualized in QIIME (summarize_data_through_plots.py) command.

2.9. Statistical Analyses. Alphadiversity measures were calculated based on the absolute number of sequences per OTU using the phyloseq package in R [28, 29] and visualized using ggplot2. The alphadiversity measurements included the total number of observed OTUs, the estimated number of OTUs that could be detected if every individual OTU was detected (Chao1 OTU richness), and Shannon's diversity index H', which is a qualitative measurement of the number of species equivalents in a specific environment and the evenness (all species equivalents equally common) or unevenness (unequal representation of the different species equivalents) of the of the tested community. It describes the uncertainty of predicting the type of species equivalents sampled at random from a community. A high Shannon index indicates high uncertainty, which means a high number of equally common types, while a low Shannon index describes a community with only a low number of species equivalents with only few dominating types. The similarity of the archaeal, bacterial, and fungal communities between the different sample sites was tested by principal coordinate analysis (PCoA) using the Phyloseq package in R using the Bray-Curtis dissimilarity model. Eigenvalues for the variance explained by the PCoA dimensions were calculated on 999 random repeats.

Significant difference between the mean number of bacterial and archaeal 16S rRNA and fungal 5.8S rRNA gene copies per mL sample water was tested using one-way ANOVA, Tukey's Q test, and Kruskal-Wallis test using the PAST3 software [30].

2.10. Accession Numbers. The sequence data has been deposited in the European Nucleotides Archive (ENA) under accession number PRJEB26699.

3. Results

3.1. Samples. The temperature of the sample water varied between 14°C at 240 m depth and 19°C at 600 m depth (Table 2). The pH of the sample water was between 1.4 and 2.3, and the ORP measured at the time of sampling was 494–600 mV. P500m had the highest concentration of iron while P240m contained the highest concentration of sulphate, sulphur, Cu, and Mn (Table 2). Al, Zn, and Mg concentrations were especially high in the P240m and P500m samples. In general, P600m had the lowest concentrations of any measured chemical component. The concentration of other elements in the mine water is given in Table S1.

3.2. Enrichments. Iron-oxidizing and iron-reducing microbial communities were enriched on 4 different enrichment media, two with ferrous (media 180 and 176) and two with ferric (media Fe(III) and Fe(III)Ye) iron. The enrichments were regrown biweekly, at which time the pH and ORP of the growth medium was checked. The pH increased in all enrichments from 1.96 and 1.61 in the ferrous iron media

Table 2: The physicochemical parameters and the concentration of the most important elements and sulphate measured from the original mine water samples.

Measurement	Method	unit	P240m	P500m	P600m
pH	Electrode		1.4	2.2	2.3
ORP	Electrode	mV	494	520	600
T	Electrode	°C	14	17	19
Fe	ICP-OES	mg/L	713	1550	426
SO_4	IC	mg/L	58,000	31,000	5000
S	ICP-OES	mg/L	17,600	8910	1400
Al	ICP-MS	mg/L	2604	844	96
Zn	ICP-MS	mg/L	1993	1551	91
Mg	ICP-OES	mg/L	4620	2470	344
Cu	ICP-MS	mg/L	129	34	19
Mn	ICP-MS	mg/L	136	75	14

180 and 176 to 2.25 and 2.02 after two consecutive cultivations. The ORP of the ferrous iron culture media increased from 370 mV and 433 mV in media 180 and 176 to above 675 mV and 710 mV. In the ferric iron-containing media Fe(III) and Fe(III)Ye, the pH remained at 2.0 and 2.10 throughout the recultivations. The starting ORP of media Fe(III) and Fe(III)Ye was 659 mV and 645 mV, respectively. After two consecutive recultivations, the ORP rose to above 690 mV and 679 mV in cultures on media Fe(III) and Fe(III)Ye, respectively.

3.3. Optimization of Acidithiobacillus-Specific qPCR. The specificity of the newly designed *Acidithiobacillus* targeting primers were tested with in silico PCR using the RDP II probe match tool, and in practice by testing the primers in qPCR with 11 different bacterial strains, one mock community DNA mixture, and three archaeal strains (Table 1). In the *in silico* primer match performed using the RDP II probe match tool, the primer pair did not recognize any other bacterial or archaeal 16 rRNA genes than members of the genus *Acidithiobacillus*. In the qPCR, we compared the cycle threshold (Ct) values of the reactions using bacteria-specific primers P1 and P2 with annealing temperature 57°C and the *Acidithiobacillus* primers using annealing temperature 64°C. The Ct values for the negative control strains and mock community were in general substantially higher than the Ct values obtained for the target genus, with the exception for the betaproteobacterial *Leptothrix discophora* and gammaproteobacterial *Pseudomonas putida* (Table 1). In the optimization test with equal amounts of DNA (Figures 2(a) and 2(b)), the qPCR with primers Acthb_F and Acthb_R amplified *A. ferrooxidans*, *D. sulfuricans*, and *P. putida* 16S rRNA genes with relatively similar efficiency (Figure 2(a)). However, the melting curve analysis distinguished well between *A. ferrooxidans* and *P. putida*, as the melting temperature of the amplified fragment from *P. putida* was lower than that of *A. ferrooxidans* (Figure 2(b)). For *D. desulfuricans* the melting temperature was similar to that of *A. ferrooxidans*, but as *D. desulfuricans* predominates in anaerobic environment and *A. ferrooxidans* in aerobic environments, the primers were considered suitable to detect *Acidithiobacillus* sp in this study.

3.4. Number of Bacteria, Archaea, and Fungi. Leptospirillum 16S rRNA genes were in general the most abundantly detected bacterial 16S rRNA genes in the original mine water samples and the enrichments with a few exceptions (Figure 3). The highest number of *Leptospirillum* 16S rRNA genes reaching between 10^9 and 10^{10} copies mL^{-1} was detected from the original mine water samples P500m both replicate samples and P600m_B, from which *Leptospirillum* bacteria were also readily enriched on medium 176. In media Fe(III) and Fe(III)Ye, *Leptospirillum* were detected by qPCR, but they did not constitute the majority of the bacterial community in these enrichments. The abundance of *Acidithiobacillus* was below 10^3 mL^{-1} in all other enrichments except in enrichment P600m_176, where their number was 2.6×10^3 mL^{-1}. The number of archaea in the enrichments was lowest (1.4×10^3 mL^{-1}) in P240m_180 but reached an abundance of 4.4×10^5 mL^{-1} in P600m_176. In addition, in the medium 180 enrichment from P600m_A and B, medium 176 enrichments from P500m_A and B, and Fe(III) from P600m_A and B and Fe(III)Ye enrichments from all samples, the number of archaeal 16S rRNA gene copies was above 10^3 mL^{-1}. Fungi were not detected by qPCR in any of the enrichments.

The primers P1 and P2 used for the enumeration of bacteria in general did not detect *Leptospirillum* 16R rRNA genes. Thus, the 16S rRNA genes detected by these primers are henceforth considered "other bacteria." The number of 16S rRNA gene copies belonging to other lineages than *Leptospirillum* in the original mine water samples varied from 2.9×10^2 to 2.7×10^7 16S rRNA gene copies in P240m and P500m_B, respectively (Figure 3). The number of archaeal 16S rRNA genes varied from 4.0×10^2 to 1.2×10^7 16S rRNA gene copies mL^{-1} in P240m and P600m_B, respectively. Fungi could be enumerated only in 3 samples, namely, P500m_A, P500m_B, and P600m_B, where the number of fungal 5.8S rRNA genes varied between 1.6×10^3 mL^{-1} in P600m_B and 3.4×10^4 mL^{-1} in P500m_A. In the other samples and in the enrichment cultures, the abundance of fungi was below the detection limit of the qPCR assay.

3.5. Sequencing and Alphadiversity. The number of bacterial sequence reads detected in the samples varied from 552 sequences in P500m_Fe(III) to 9003 in P500m, with a mean number of 3196 (+/−554 standard error of mean, SEM) sequences per sample (Table 3). The mean number of bacterial OTUs and Chao1 estimated number of bacterial OTUs was 363 (+/− 39 SEM) and 621 (+/− 69), respectively, with the lowest numbers of 148 OTUs and 283 Chao1 estimated OTUs observed for P500m_Fe(III) and the highest numbers of 879 OTUs and 1487 Chao1 estimated OTUs detected in P600m. Shannon's diversity index H' was lowest, $H' = 2.7$, in the enrichment P600m_176 and highest, $H' = 5.3$, in P600m. P240m_176 did not produce any sequences.

The number of fungal ITS sequence reads detected in the samples varied from 1874 sequences in P240m_Fe(III)Ye to

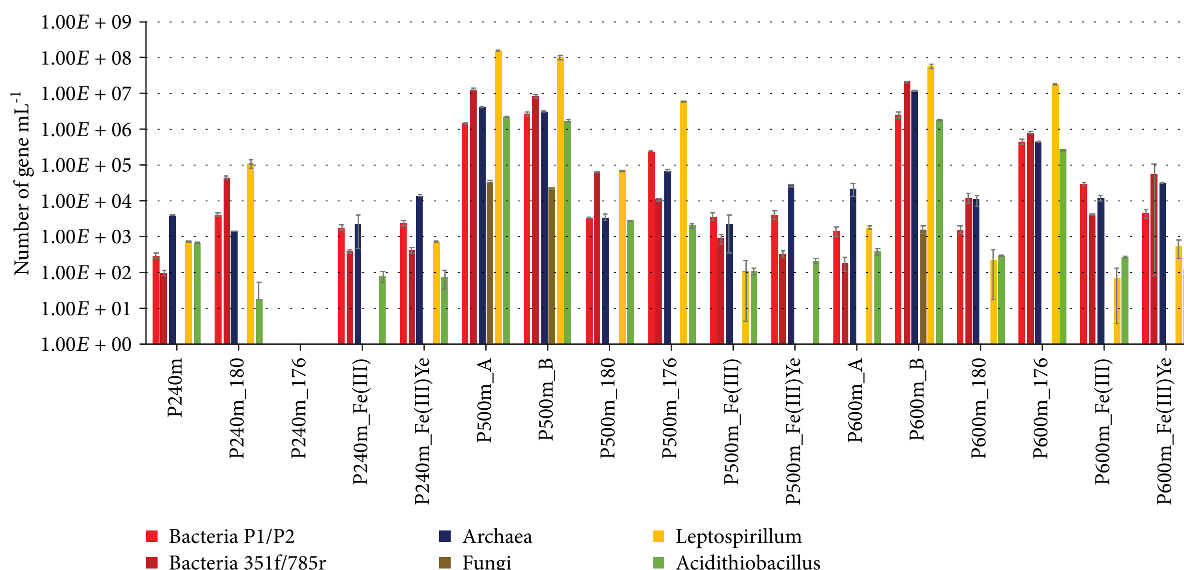


Figure 3: The number of bacterial (bright red – primers P1/P2, dark red – primers 358f/785r) and archaeal (blue) 16S rRNA genes, fungal 5.8S rRNA genes (brown), and the number of bacterial 16S rRNA genes belonging to *Leptospirillum* (yellow) and *Acidithiobacillus* (green) mL^{-1} in the sample water and enrichment cultures. The efficiencies of the qPCR assays (in order from left to right) were 2.01 (slope −3.29), 2.10 (slope −3.08), 1.65 (slope −4.71), 1.88 (slope −3.66), 1.96 (slope −3.2), and 1.77 (slope −4.02).

7544 in P600m, with a mean number of 4077 (+/−388) sequences per sample (Table 3). The mean number of fungal ITS OTUs and Chao1 estimated number of fungal OTUs was 1368 (+/− 108) and 2955 (+/− 157), respectively, with the lowest numbers of 768 OTUs and 1866 Chao1 estimated OTUs observed for P240m and the highest numbers of 2319 OTUs and 4146 Chao1 estimated OTUs detected in P240m_Fe(III). Shannon's diversity index H' was lowest, $H' = 4.8$, in the enrichment P240m and highest, $H' = 7.3$, in P240m_Fe(III). Less than 100 ITS sequences were obtained from one of the replicate P240m and P600m samples, and these samples were left out of the alphadiversity calculations.

Archaeal 16S rRNA gene sequences were generally only obtained from 5 of the 17 samples, i.e., P500m (both replicate samples), one replicate of the P600m samples, P600m_176 and P600m_Fe(III)Ye. In these samples, the number of sequences, OTUs and Chao1 estimated OTUs varied between 3035 and 8001, 141 and 376, and 271 and 586 per sample, respectively. The H' index varied between 2.0 and 3.6. From the other samples, 204 sequences were obtained from P500m_176 and less than 40 sequences from the rest of the samples, which leads us to exclude these samples from further analyses concerning the archaea.

3.6. Bacteria. Altogether, 271 different bacterial genera were detected in the samples and enrichments, of which 70 genera were detected at relative abundances of at least 1% of the bacterial sequence reads in any of the examined samples (Table S2). This group of core genera contributed with 91.7% to 98.6% of the total number of bacterial sequence reads detected in the samples. The bacterial communities in the original mine water samples varied from the bacterial communities that were enriched and fell to the lower middle and left parts of the PCoA plot (Figure 4(a)). The enrichments on medium 176, which had the highest concentration of ferrous iron, from P500m and P600m fell close together in the upper left corner of the plot, far away from the rest of the enriched communities.

Sequences of the iron-oxidizing *Leptospirillum* were detected in all samples and enrichments and was the main bacterial genus present in the P500m samples as well as in the P500m enrichments containing Fe(II) (Figure 5). In these samples, the *Leptospirillum* sequences contributed with 48–84% of the bacterial sequences. *Leptospirillum* bacteria were also clearly enriched (84.5% of the bacterial sequence reads) from P600m in the 176 medium (Figure 5, Table S2) although *Leptospirillum* contributed with only 8.6–24.6% of the bacterial sequence reads identified from the P600m original sample and enrichment on medium 180. In the P240m samples, *Leptospirillum* contributed with 18.3–30.7% of the bacterial sequence reads in the original mine water and was slightly enriched (45.0%) on medium 180. The microbial community grown on medium 176 from P240m did not thrive and was lost after a few consecutive cultivations. In the enrichment cultures with Fe(III), *Leptospirillum* contributed with 4.0–8.0%, 5.1–5.7%, and 2.6–4.8% of the bacterial sequences in P240m, P500m, and P600m, respectively.

Iron-oxidizing *Acidithiobacillus* species were also present in all samples and enrichments (except P240m_176) at relative abundances of between 1.9% and 6.7% but were prominent (27.2–34.5%) only in P240m. *Marinobacter* species were detected at relative abundances of 2.5 to 10.6% in all samples and enrichments, with the highest relative abundances enriched in medium Fe(III) from P240m and P500m and medium 180 from P600m.

Table 3: The number of sequences, OTUs, Chao1 estimated number of OTUs, Shannon's diversity index, and number of identified genera of the bacteria, fungi, and archaea found in the original mine water samples and enrichments. The values in grey indicate that only low numbers of sequence reads were obtained and the data was excluded from the analyses. The sample name abbreviations are as follows; P = Pyhäsalmi; 240 m, 500 m, 600 m = depth; A, B = sample replicate A or B; 176 = ferrous iron-containing medium 176; 180 = ferrous iron-containing medium 180; Fe(III) = ferric iron containing growth medium; Ye = Yeast extract containing growth medium.

Samples	Number of sequences	Number of OTUs	Estimated number of OTUs (Chao1)	Shannon's diversity index	Number of genera
Bacteria					
P240m_A	1653	309	475	4,8	63
P240m_B	2279	324	520	4,6	70
P240m_176					
P240m180	2986	342	538	4,0	90
P240m_Fe(III)	787	261	514	4,6	75
P240m_Fe(III)Ye	2250	307	519	4,0	84
P500m_A	9003	599	1070	4,0	104
P500m_B	5098	468	877	4,1	80
P500m_176	6452	342	530	2,8	77
P500m180	2291	303	482	4,0	78
P500m_Fe(III)	552	148	283	3,4	50
P500m_Fe(III)Ye	1375	273	492	3,8	78
P600m_A	1842	339	660	4,3	70
P600m_B	6002	879	1487	5,3	114
P600m_176	4723	296	460	2,7	84
P600m180	2259	337	603	4,5	78
P600m_Fe(III)	2795	303	514	3,0	72
P600m_Fe(III)Ye	1987	334	538	4,4	89
Fungi					
P240m_A	22	17	47	3	
P240m_B	4355	768	1866	5	104
P240m_176					
P240m180	6113	1641	3367	6	111
P240m_Fe(III)	5764	2319	4146	7	120
P240m_Fe(III)Ye	1878	889	2352	6	103
P500m_A	3712	1566	3129	7	114
P500m_B	4174	1690	3551	7	117
P500m_176	3049	1270	3001	7	114
P500m180	3868	1508	3170	7	110
P500m_Fe(III)	2199	890	2813	6	109
P500m_Fe(III)Ye	4978	1410	2735	6	117
P600m_A	100	82	520	4	
P600m_B	7544	1871	3791	6	114
P600m_176	3040	1227	2837	6	111
P600m180	3666	1100	2163	6	109
P600m_Fe(III)	3537	1379	2888	7	104
P600m_Fe(III)Ye	3271	991	2513	6	110
Archaea					
P240m_A	2	2	3	1	
P240m_B	18	10	13	2	
P240m_176					
P240m180	11	3	3	1	

Table 3: Continued.

Samples	Number of sequences	Number of OTUs	Estimated number of OTUs (Chao1)	Shannon's diversity index	Number of genera
P240m_Fe(III)					
P240m_Fe(III)Ye	36	11	14	2	
P500m_A	8001	376	535	4	10
P500m_B	7228	347	586	4	10
P500m_176	204	34	76	2	
P500m180	1	1	1	0	
P500m_Fe(III)					
P500m_Fe(III)Ye					
P600m_A	28	14	32	2	
P600m_B	4499	206	379	3	13
P600m_176	3035	141	308	2	10
P600m180	4	4	10	1	

Ferrimicrobium species were present in all samples and enrichments and were especially prominent in the original water sample and the enrichments from P500m, where their relative abundance was between 1.0 and 3.5% of the bacterial sequences.

Aciditrix and *Ferrovum* were present at relative abundances of below 1% in most samples and enrichments, but in the A replicate sample of P600m, the relative abundances of these two genera was 13.6% and 40.8%, respectively. In contrast, in replicate B of P600m, *Metallibacterium* contributed with 28.8%. In replicate A of P600m, *Metallibacterium* contributed with 1.6% of the bacterial sequences. In all other samples, the *Metallibacterium* relative abundance was below 1%, with the exception of P240m_Fe(III) (1.1%) and P500m replicate B (1.8%).

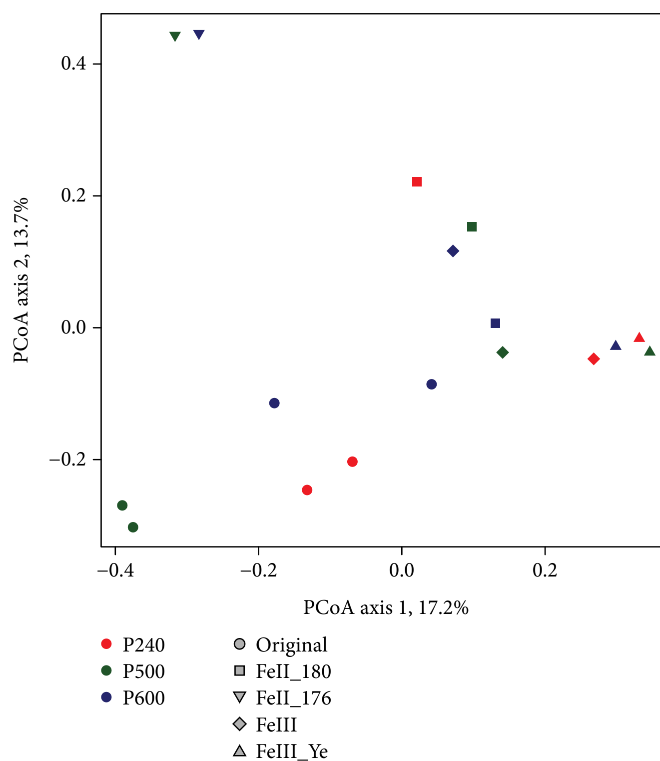
The relative abundance of known iron-reducing bacteria was lower than that of the iron oxidizers but were nonetheless found in all samples (Figure 5, Table S2). Heterotrophic, iron-reducing *Acidibacter* were present at relative abundances of 0.1–4.5% in all samples. Species belonging to the Clostridia *sensu stricto* group 1 were detected from all samples at relative abundances of less than 1% in all samples but were enriched in medium 180 and Fe(III)Ye from P600m at relative abundances of 11.6% and 7.3%, respectively.

Acidiphilum species were especially common in P240m water samples and enrichments, contributing with 5.2–8.4% of the bacterial community in the original water sample of P240m. In P500m, the *Acidiphilum* sequences remained below 1% of the bacterial 16S rRNA gene sequence pool, but in P600m their contribution was between 3 and 4%. Nevertheless, these bacteria were enriched in the different media from all three water samples to a relative abundance of 23.6% and 8.9% in medium 180 from P240m and P500m, respectively, and 77.3% in medium Fe(III) from P600m.

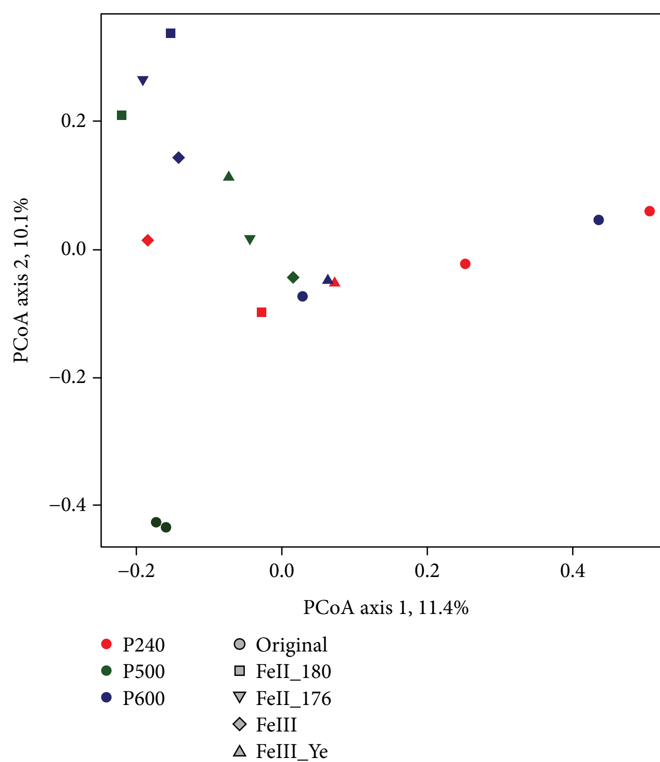
Other putative iron-reducing bacteria, such as species belonging to the order Corynebacteriales were enriched on Fe(III) from P500m to a relative abundance of 2.4% of the bacterial sequence reads.

3.7. Fungi. Altogether, 109 fungal genera were detected in total from the mine water samples and enrichments. Of these, 39 genera contributed with at least 1% of the fungal ITS sequence reads in at least one sample and between 87.1 and 98.0% of the total number of fungal sequence reads in the samples (Figure 6, Table S3). Between 10.6 and 68.7% of the identified fungal sequences belonged to Ascomycota, 2.4–47.7% to Basidiomycota, and 0.1–13.9% to Chytridiomycota, while 11.4 and 80.0% of the fungal sequences in each sample could not be classified to any known fungal groups. On the OTU level, the fungal communities of the original mine water and enrichments differed from each other (Figure 4(b)). In the PCoA plot, the fungal communities of P500m and P600m fell to the middle right of the plot, the P240m community to the bottom left corner, and the enriched communities to the middle and upper left parts of the plot. The proportion of unclassified fungi was greatest in the original water samples. Ascomycetes fungi belonging to the genus *Davidella* were enriched in most of the cultures from all original samples to a relative abundance of 46.0% in P500m_180 and 22.2% in P600m_180 and P600m_176. *Malassezia*, a member of the phylum Basidiomycota, were present in all samples and enrichments but were especially enriched in P240m_Fe(III) (41.3%), P500m_176 and P500m_Fe(III) (22.1% and 15.0%, respectively), and P600m_Fe(III) (6.2%). Nevertheless, a genus of so far unclassified *Malasseziales* fungi was enriched to a high relative abundance of 15.1% and 9.2% in the P600m ferrous iron enrichments. Interestingly, *Cryptococcus* yeasts of the phylum Basidiomycota were highly enriched in the medium 180 from P240m and P600m, where they reached a relative abundance of more than 17% of the fungal sequence reads. In addition, *Cryptococcus* was also enriched to a relative abundance of 33.4% in P600m_Fe(III).

3.8. Archaea. Archaeal sequences were obtained from samples P500m (both replicates) and P600m (replicate sample B) and from enrichments P600m_176 and P600m_Fe(III)Ye. In the other samples and enrichments, the proportion of



(a)



(b)

Figure 4: Principal coordinates analysis (PCoA) of the (a) bacterial and (b) fungal communities detected in the original mine water (circle) and enriched in medium 180 (square), medium 176 (downwards triangle), Fe(III) (diamond), and Fe(III)Ye (triangle). The colours indicate which mine water the sample originated from; red – P240m, green – P500m, and blue – P600m.

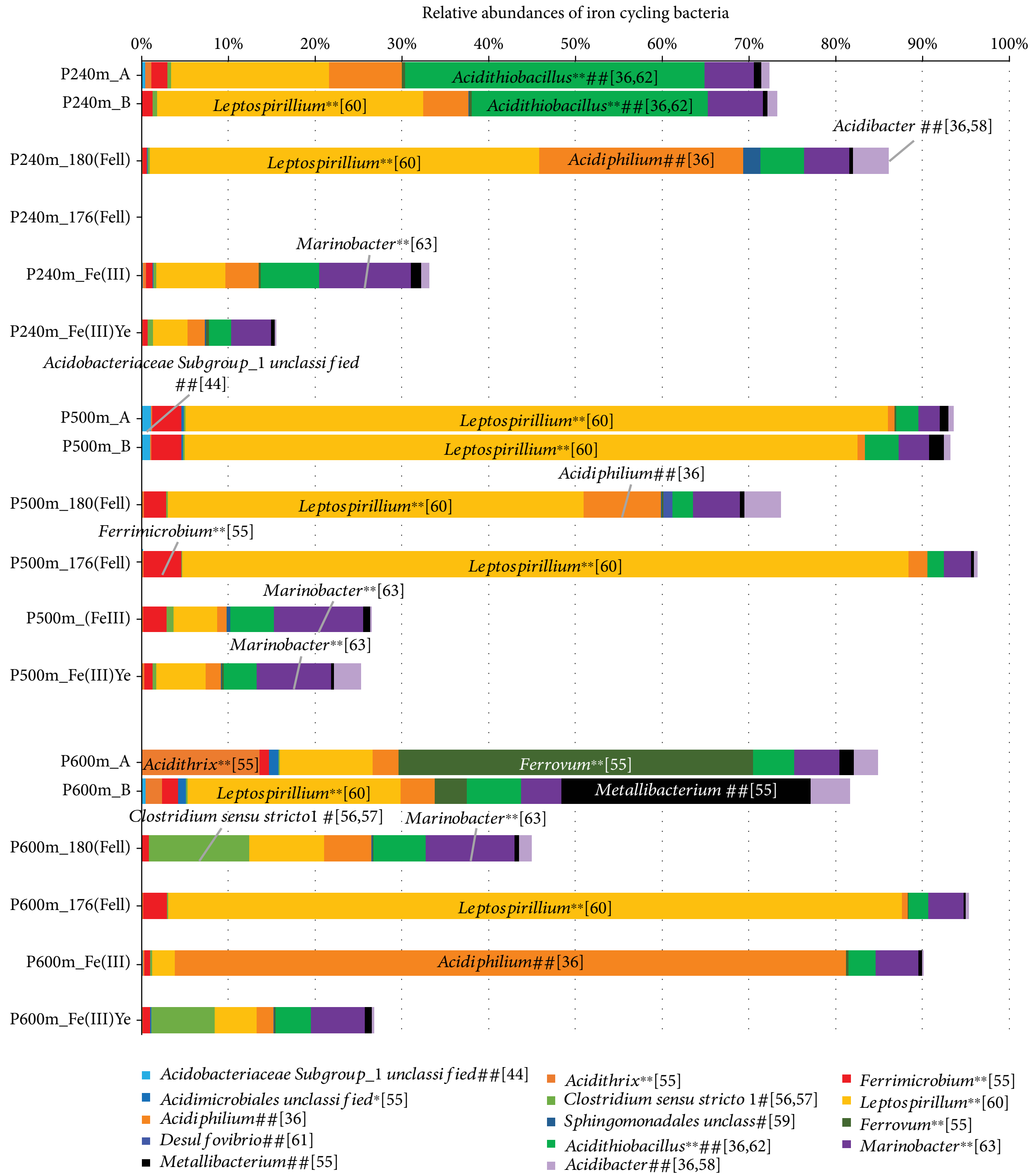


Figure 5: Relative abundances of iron cycling bacteria in the mine water samples and enrichments. ** indicates iron oxidation. *Iron oxidizers identified within this taxonomic group. ##Iron reducer. #Iron reducers identified within this taxonomic group, according to literature [36, 44, 56–63]. The complete bacterial diversity is found in Table S2.

archaea was too low for proper detection. Nevertheless, the archaea that were detected belonged to the genus *Thermoplasma* (88.1%-97.8% of the archaeal sequence reads) (Figure 7). In the original P500m and P600m samples, the uncultured Thermoplasmatales group BSLdp215 was also present at relative abundances of approximately 6.0% of the archaeal community in P500m and 2.0% in P600m, but below 1% in the enrichments. Terrestrial Miscellaneous Euryarchaeotal Group (TMEG) archaea were detected at low relative abundance, 2.4%, in P600m and Parvarchaeota Candidate genus *Micrarchaeum* at almost 3.0% in P500m.

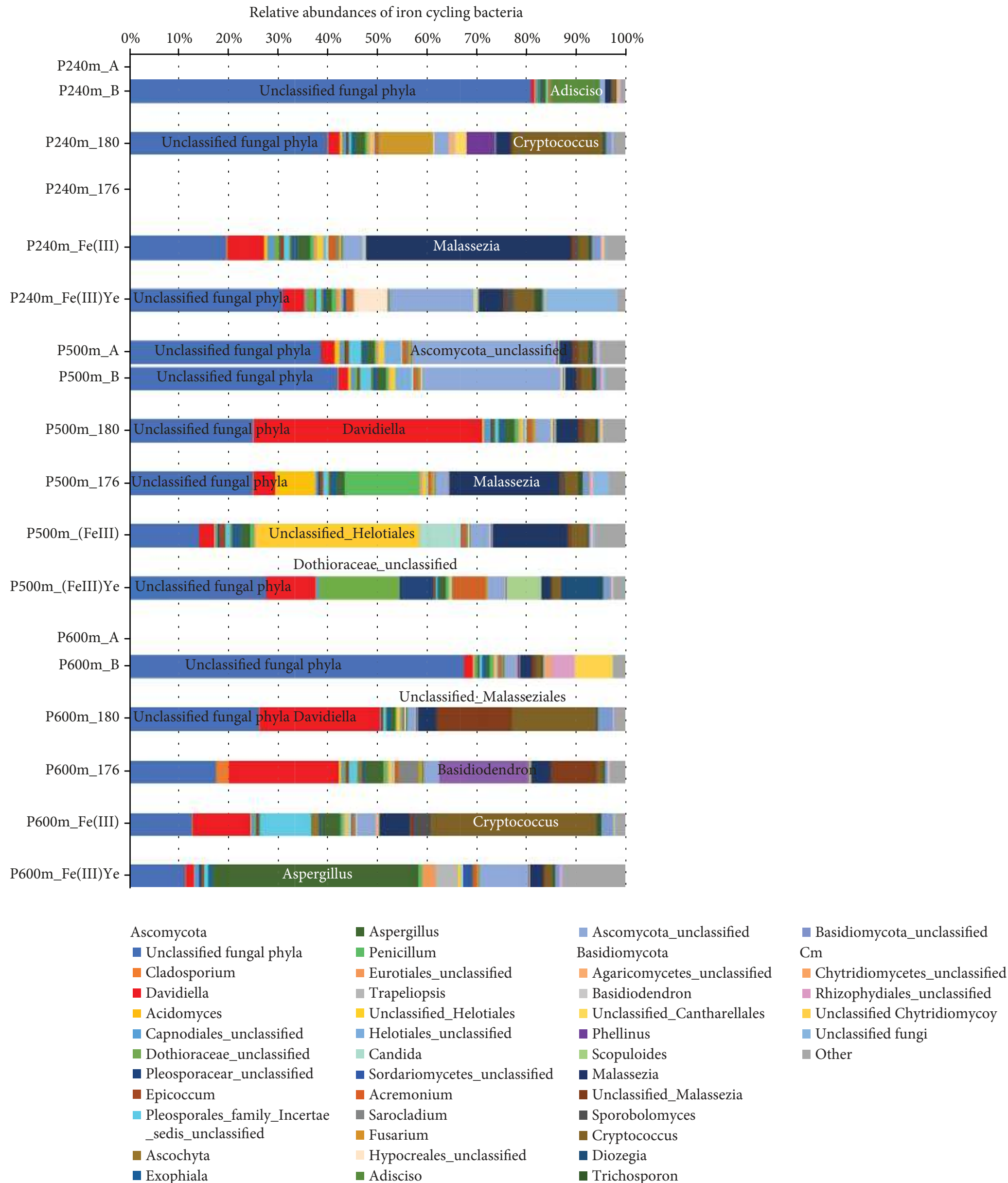


Figure 6: Relative abundance of fungal genera detected in the original water samples and the enrichment cultures. Fungal genera with relative abundances of less than 0.1% of the sequence reads are grouped into the category "Other." The fungal phyla are indicated to the right of the legend and Cm stands for Chytridiomycota.

4. Discussion

The Pyhäsalmi mine close to the city of Oulu, Finland, is the deepest active metal mine in Europe. The fluids discharging and collecting in different basins inside the mine are generally acidic and contain high amounts of iron (and other metals) and sulphate. In contrast, the undisturbed deep subsurface environments of the Fennoscandian Shield are in general anoxic, highly reducing, and have an alkaline pH (e.g., [31, 32]). For example, anoxic water from deep boreholes in the Pyhäsalmi mine reaching depths of 2–2.5 km below land surface had a pH of >8.5 and a Fe content of <0.0025 mM [32], while anoxic water from the Outokumpu deep scientific drill hole have been shown to have a pH of >8.5 and an iron <0.5 μM [31]. The microbial communities detected in the undisturbed, anoxic bedrock are generally low in abundance and consist of anaerobic types that are capable of fermentation or anaerobic respiration on sulphate or ferric iron or belong to methanogenic archaeal taxa [33, 34]. In the studied mine environment, the water is oxic and as the newly exposed and fresh rock surface (consisting mainly of sulphides like pyrite) come into contact with oxygen and water, they start to weather and iron and sulphates are released from the rock leading to a significant drop in the pH of water. The availability of reduced iron and oxygen in the mine water radically changes the microbial communities developing in these water bodies, and in Pyhäsalmi, these microbial communities

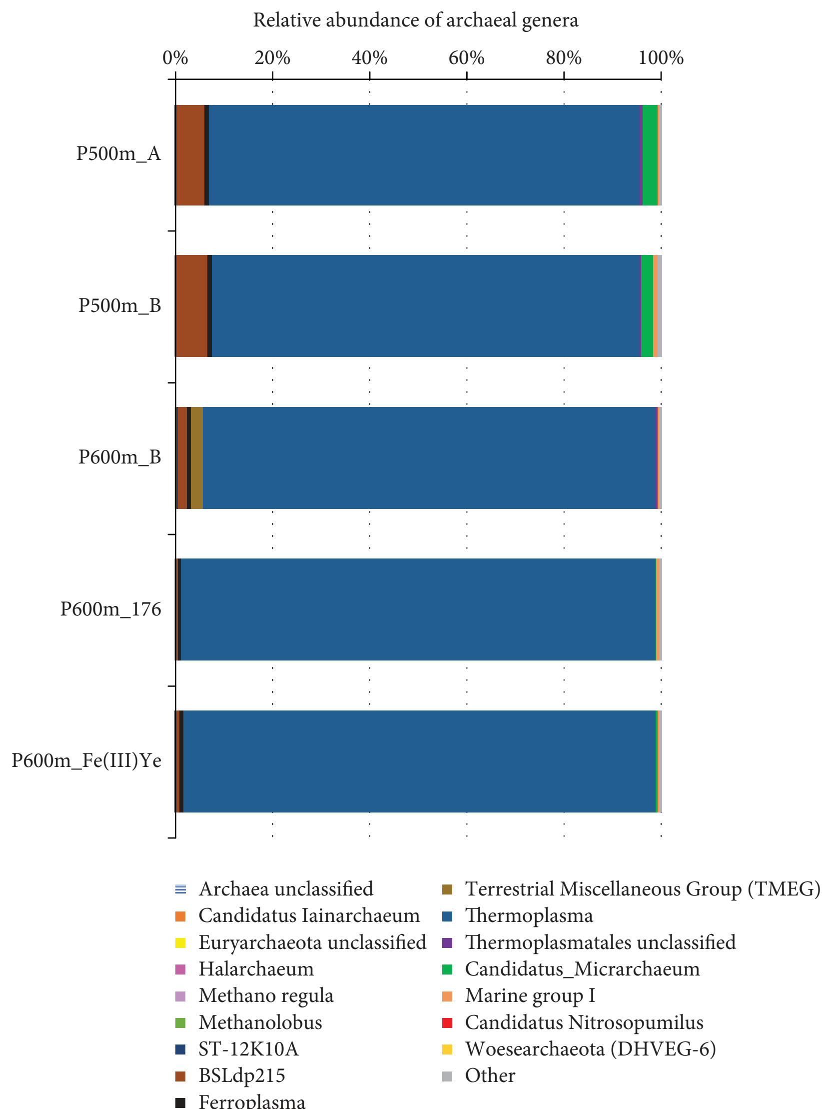


Figure 7: The relative abundance of archaeal 16S rRNA gene sequence reads in the samples and enrichment cultures from 500 m and 600 m belowground. No archaeal sequence reads were detected from the other samples or enrichment cultures.

consist largely of acidophilic iron- and sulphur-oxidizing microorganisms. These microbes thrive in oxygenated environments and are absent in anoxic, alkaline groundwater. In addition, the activity of these acidophiles increases the AMD effect because they increase acidity, which in turn dissolves the rock.

In the Pyhäsalmi mine, we found an unexpected high bacterial and fungal diversity in the acidic water collected from different depth levels of the mine, which is in contrast with, e.g., the low diversity found in the Richmond mine [35]. The number of bacterial genera was between 63 and 114 in the original Pyhäsalmi mine water samples, while the fungal genus richness was between 104 and 117 genera in the original samples. High diversity of the bacterial communities have been shown in other AMD-affected drainage from, e.g., a polymetallic ore mine, tailings impoundment and pyrite mine from China [36], and coal mine drainage in Svalbard [37]. Nevertheless, the AMD communities are often dominated by a few species, such as the *Ferrovum*, *Gallionella*-like bacteria, and *Sideroxydans* species [38], as well as species of *Acidithiobacillus*, *Leptospirillum*, and *Acidiphilum* [36, 37]. This is in accordance with our results, where *Acidithiobacillus* and *Leptospirillum* dominated in P240m water, *Leptospirillum* dominated in P500m, and *Ferrovum* and *Metallibacter* were the dominating bacterial genera in P600m. In addition, we detected heterotrophic *Acidiphilum* spp. in all samples and enrichments. Liu et al. [35] also showed that the microbial diversity of the Shuimuchong copper tailing impoundment in China was high, but that the diversity correlated strongly with pH, with the lowest diversity occurring in the samples with the lowest pH.

Jones et al. [39] discovered that *Gallionella*-like microorganisms dominated in $pH > 3$ when the concentration of Fe(II) was >4 mM, while *Acidithiobacillus* spp. were present only when pH was below 3 and Fe(II) less than 4 mM. *Ferrovum* spp. were found in environments where pH was below 3

and Fe(II) > 4 mM. In our study, all pH values were below 3 and all iron concentrations were above 4 mM, although we did not distinguish between Fe(II) and Fe(III). Nevertheless, the pH of our samples was too low for the *Gallionella*, which explains the absence of this iron oxidizer in our samples.

The prokaryotic communities of two different mine water samples from 630 m and 970 m below land surface in the Pyhäsalmi mine have been characterized previously [40]. Bacterial community characterization was performed with T-RFLP, and the authors reported a total of 10 T-RFs from the samples. The dominating bacteria belonged to iron-oxidizing *Acidithiobacillus*, *Ferrovum*, and *Leptospirillum* species. Our results are in agreement with Kay et al. [40], as these were the dominating bacterial groups found with high-throughput amplicon sequencing. In addition, the iron oxidation fluidized-bed reactor run for over one year and inoculated with mine drainage waters from Pyhäsalmi mine was dominated by bacteria belonging to the genus *Leptospirillum* in the studies of Kinnunen and Puhakka [41]. Less than 1% of the microbial community in their study was related to the archaea including *Ferroplasma* sp. We also detected more than one order of magnitude more bacterial genera present at low relative abundances in the original mine water samples, indicating a much higher metabolic diversity than previously thought. Nitrogen fixation in AMD ecosystems is performed by a few keystone taxa, such as *Leptospirillum ferrodiazotrophum* and *L. ferrooxidans* [5, 6], *Acidithiobacillus ferrooxidans* [42], and *Ferrovum myxofaciens* [43], and the removal of these could collapse the whole community.

Iron-oxidizing bacteria, especially the autotrophic *Acidithiobacillus*, *Leptospirillum*, and *Ferrovum*, were the most abundant bacterial groups detected in the mine water samples. These bacteria, in addition to oxidizing ferrous iron and reduced sulphur compounds, may be the main microorganisms causing the low pH (and AMD) in the mine water. We could not classify the sequences to species level, but due to the high relative abundances of these bacteria in specific water samples, they may be the key species driving the AMD communities through continuous iron and sulphur oxidation coupled with carbon and nitrogen fixation.

In oxygen-limited conditions, many acidophiles, such as members of the *Acidithiobacillus* (*At. ferrooxidans*, *At. ferrivorans*, and *At. ferrireducens*) can use ferric iron as electron acceptor instead of oxygen [1]. Heterotrophic *Acidiphilum*, *Metallibacterium*, *Acidibacter*, and *Acidobacterium* are also known ferric iron reducers. These bacteria were more or less abundant in all water samples and enrichments. However, *Acidiphilum* spp. were especially abundant in the Fe(III) enrichment from sample P600m, while *Metallibacterium* inhabited the original P600m mine water and unclassified Acidobacteriaceae bacteria were detected at low relative abundances in all original mine water samples. In accordance with Kay et al. [40], *Acidiphilum* was detected in the Pyhäsalmi mine water at relative abundance of 3-4%. *Acidibacter* spp. were also among the 70 most abundant bacterial genera detected in this study but were detected at >1% relative abundance only in P500m. Another bacterium capable of oxidizing and reducing iron [44] is *Ferrimicrobium*, which were present in all samples and enrichments to a relative abundance of up to 4.5%.

Heterotrophic microorganisms may facilitate the growth of autotrophic iron oxidizers by removing organic material, such as decomposing biomass and cell exudates [7]. *Acidiphilium acidophilum* grows heterotrophically together with *Acidithiobacillus ferrooxidans*, promoting the growth of *At. ferrooxidans* by removing organic compounds that may be toxic to the autotrophic iron oxidizer [45]. *Acidiphilum* species have also been shown to grow in cocultures with *Ferrovum*, where the *Acidiphilum* oxidizes, e.g., organic acids that are toxic to the obligately autotrophic *Ferrovum* [46]. By oxidizing organic carbon compounds, the heterotrophs also supply the autotrophs with carbon dioxide, which further boosts the growth of autotrophic communities.

Only little is so far known about fungi in acid mine environments. Fungi are generally found in various environments covering a wide pH range from at least pH 1 to pH 11 [47]. They have, for example, been found in deep terrestrial bedrock environments [48–51], in the deep (2 km below land surface) boreholes of the Pyhäsalmi mine [32], in the extremely acidic and metal-rich Rio Tinto [8], and other AMD-affected streams [9–11, 52]. A striking feature of the fungal communities in the extremely acidic environments is their high diversity, which was also detected in the present study, although Baker et al. [52] reported a low number of eukaryotic species in extremely acidic (pH < 0.9) runoff in the Richmond underground mine (USA). In our study, fungi were detected by end-point PCR and sequencing from almost all samples and enrichments. However, the culturing conditions used in our tests were not optimized for fungal growth, and although they survived consecutive recultivations, their abundances remained below the limit of detection in the qPCR assay. Nevertheless, use of organic carbon and nitrogen compounds in the cultivations could increase their community sixes in the enrichments.

Although a great part of the detected fungi remained without a proper taxonomical assignment in this study, the high number of OTUs and their abundance suggests an important role for the fungi in acidic mine environments. Low pH environments may favour fungal growth, and the fungi can act as decomposers contributing to the general carbon and nitrogen cycling in these environments [7]. They may also be directly involved in reduction of ferric iron and sulphur [53]. Fungi are known to weather minerals by mechanical or biochemical means, i.e., by invasive growth into cracks or zones of weakness in the crystal lattice and/or along grain boundaries in the rock and releasing organic acids (oxalic acid, citric acid, etc.), enzymes and metal chelating agents that dissolve the rock (reviewed by [53]). Fungi may produce mucilage, secrete oligosaccharides, and otherwise influence the organic carbon load of the environment. By increasing the concentration of organic carbon compounds, regulating the concentration of released carbon dioxide, nitrogen, and phosphorus compounds, the fungi may be able to control the whole AMD ecosystem. By secreting carboxylic acids with strong chelating properties, the fungi can aggressively attack mineral surfaces. For example, oxalic acid can leach, e.g., iron by forming oxalate complexes

with this metal. Nevertheless, these phenomena have mostly been detected and studied in environments with more moderate pH. However, as poorly studied and highly diverse and abundant the fungal communities appear to be, also in AMD ecosystems, their role in the formation or control of AMD may be greater than previously thought. In addition, in agreement to Baker et al. [52], the fungi found in the acidic water of the Pyhäsalmi mine mostly belonged to clades of the Ascomycota and Basidiomycota, although the detected genera were not the same.

In accordance with Kay et al. [40], we also detected archaea in the original water samples, but in general, they did not grow in the selected culture media. The archaeal diversity was low and consisted almost exclusively of *Thermoplasma*, which have been reported to be heterotrophic, sulphur reducers [54]. These archaea are closely related to the iron-oxidizing *Ferroplasma* but have not been found to oxidize iron. Nevertheless, *Thermoplasma* are frequently detected in AMD environments [55], where they may have an important role in the carbon cycle and degradation of senescent iron-oxidizing biofilms.

5. Conclusions

The microbial communities inhabiting drainage waters at different depths within the Pyhäsalmi mine had high diversity, consisting of bacteria, archaea, and fungi. Iron-oxidizing bacterium *Leptospirillum* was especially prominent in the mine water samples, but also other iron and sulphur cycling taxa were present. Microbial consortia containing aerobic ferrous iron oxidizers and ferric iron reducers were enriched. These microbial consortia may in the future be used for example in industrial bioleaching applications employing acidophilic iron and sulphur cycling microorganisms.

Data Availability

The chemical and biological (qPCR) data measurements used to support the findings in this study are included within the article. The sequence data has been deposited in the European Nucleotides Archive (ENA) under accession number PRJEB26699.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Acknowledgments

The Pyhäsalmi mine staff and First Quantum Minerals Ltd. are acknowledged for providing the opportunity to collect samples in the mine. Veera Partanen, Johanna Lukin, and Tuula Kuurila are acknowledged for excellent laboratory assistance and handling of the enrichment cultures. This project was funded by the Academy of Finland (Funding decision No. 306079).

Supplementary Materials

Table S1: the composition of elements in the original mine water. The most important compounds are shown in Table 2. Table S2: relative abundance of bacterial genera present in the different samples and enrichment cultures. All genera present at higher than 0.1% relative abundance in at least one sample or enrichment are shown. The genera present at less than 0.1% relative abundance in all samples or enrichments are included in the "Other bacteria" group at the end of the table. Table S3: relative abundance of fungal genera present in the different samples and enrichment cultures. All genera present at higher than 0.1% relative abundance in at least one sample or enrichment are shown. The genera present at less than 0.1% relative abundance in all samples or enrichments are included in the "Other fungi" group at the end of the table. *(Supplementary Materials)*

References

[1] D. B. Johnson, T. Kanao, and S. Hedrich, "Redox transformations of iron at extremely low pH: fundamental and applied aspects," *Frontiers in Microbiology*, vol. 3, 2012.

[2] T. Rohwerder, T. Gehrke, K. Kinzler, and W. Sand, "Bioleaching review (part A): Progress in bioleaching: fundamentals and mechanisms of bacterial metal sulfide oxidation," *Applied Microbiology and Biotechnology*, vol. 63, no. 3, pp. 239–248, 2003.

[3] W. Salomons, "Environmental impact of metals derived from mining activities: processes, predictions, prevention," *Journal of Geochemical Exploration*, vol. 52, no. 1-2, pp. 5–23, 1995.

[4] D. B. Johnson and K. B. Hallberg, "Acid mine drainage remediation options: a review," *Science of the Total Environment*, vol. 338, no. 1-2, pp. 3–14, 2005.

[5] V. Parro and M. Moreno-Paz, "Gene function analysis in environmental isolates: the *nif* regulon of the strict iron oxidizing bacterium *Leptospirillum ferrooxidans*," *Proceedings of the National Academy of Sciences*, vol. 100, no. 13, pp. 7883–7888, 2003.

[6] G. W. Tyson, I. Lo, B. J. Baker, E. E. Allen, P. Hugenholtz, and J. F. Banfield, "Genome-directed isolation of the key nitrogen fixer *Leptospirillum ferrodiazotrophum* sp. nov. from an acidophilic microbial community," *Applied and Environmental Microbiology*, vol. 71, no. 10, pp. 6319–6324, 2005.

[7] C. Méndez-García, A. I. Peláez, V. Mesa, J. Sánchez, O. V. Golyshina, and M. Ferrer, "Microbial diversity and metabolic networks in acid mine drainage habitats," *Frontiers in Microbiology*, vol. 6, p. 475, 2015.

[8] L. A. Amaral-Zettler, "Eukaryotic diversity at pH extremes," *Frontiers in Microbiology*, vol. 3, p. 441, 2013.

[9] W. Bridge Cooke, "Fungi in and near streams carrying acid mine drainage," *Ohio Journal of Science*, vol. 76, pp. 231–240, 1976.

[10] A. L. Mills, "Acid mine waste drainage: microbial impact on the recovery of soil and water ecosystems," in *Soil Reclamation Process: Microbiological Analysis and Applications*, R. L. Tate and D. A. Klein, Eds., pp. 35–74, CRC Press, 1985.

[11] B. J. Baker, M. A. Lutz, S. C. Dawson, P. L. Bond, and J. F. Banfield, "Metabolically active eukaryotic communities in extremely acidic mine drainage," *Applied and Environmental Microbiology*, vol. 70, no. 10, pp. 6264–6271, 2004.

[12] B. K. Das, A. Roy, M. Koschorreck, S. M. Mandal, K. Wendt-Potthoff, and J. Bhattacharya, "Occurrence and role of algae and fungi in acid mine drainage environment with special reference to metals and sulfate immobilization," *Water Research*, vol. 43, no. 4, pp. 883–894, 2009.

[13] H. Toropainen and P. Heikkinen, *Mineralogical and Chemical Composition of the Tailings and Waste Rock Samples of Pyhäsalmi, Hitura, Talvivaara and Ihalainen Ore Deposits*, Geological Survey of Finland, Archival documentation S49/0000/2006/3, 2006.

[14] M. Kearse, R. Moir, A. Wilson et al., "Geneious basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data," *Bioinformatics*, vol. 28, no. 12, pp. 1647–1649, 2012.

[15] G. Muyzer, E. C. De Waal, and A. G. Uitterlinden, "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA," *Applied and Environmental Microbiology*, vol. 59, no. 3, pp. 695–700, 1993.

[16] D. P. Herlemann, M. Labrenz, K. Jürgens, S. Bertilsson, J. J. Waniek, and A. F. Andersson, "Transitions in bacterial communities along the 2000 km salinity gradient of the Baltic Sea," *The ISME Journal*, vol. 5, no. 10, pp. 1571–1579, 2011.

[17] N. Bano, S. Ruffin, B. Ransom, and J. T. Hollibaugh, "Phylogenetic composition of Arctic Ocean archaeal assemblages and comparison with Antarctic assemblages," *Applied and Environmental Microbiology*, vol. 70, no. 2, pp. 781–789, 2004.

[18] S. M. Barns, R. E. Fundyga, M. W. Jeffries, and N. R. Pace, "Remarkable archaeal diversity detected in a Yellowstone National Park hot spring environment," *Proceedings of the National Academy of Sciences*, vol. 91, no. 5, pp. 1609–1613, 1994.

[19] S. Hedrich, A. G. Guézennec, M. Charron, A. Schippers, and C. Joulian, "Quantitative monitoring of microbial species during bioleaching of a copper concentrate," *Frontiers in Microbiology*, vol. 7, p. 2044, 2016.

[20] M. Bomberg, H. Miettinen, M. Wahlström et al., "Post operation inactivation of acidophilic bioleaching microorganisms using natural chloride-rich mine water," *Hydrometallurgy*, vol. 180, pp. 236–245, 2018.

[21] R. Haugland and S. Vesper, "Method of identifying and quantifying specific fungi and bacteria," US Patent 6,387,652, 2002.

[22] A. Klindworth, E. Pruesse, T. Schweer et al., "Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies," *Nucleic Acids Research*, vol. 41, no. 1, pp. e1–e1, 2013.

[23] T. J. White, T. Bruns, S. J. W. T. Lee, and J. L. Taylor, "Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics," *PCR Protocols*, vol. 18, no. 1, pp. 315–322, 1990.

[24] P. D. Schloss, S. L. Westcott, T. Ryabin et al., "Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities," *Applied and Environmental Microbiology*, vol. 75, no. 23, pp. 7537–7541, 2009.

[25] C. Quast, E. Pruesse, P. Yilmaz et al., "The SILVA ribosomal RNA gene database project: improved data processing and web-based tools," *Nucleic Acids Research*, vol. 41, no. D1, pp. D590–D596, 2012.

[26] U. Kõljalg, R. H. Nilsson, K. Abarenkov et al., "Towards a unified paradigm for sequence-based identification of fungi," *Molecular Ecology*, vol. 22, no. 21, pp. 5271–5277, 2013.

[27] J. G. Caporaso, J. Kuczynski, J. Stombaugh et al., "QIIME allows analysis of high-throughput community sequencing data," *Nature Methods*, vol. 7, no. 5, pp. 335-336, 2010.

[28] R Development Core Team, "R: A language and environment for statistical computing," 2013, http://www.r-project.org/.

[29] P. J. McMurdie and S. Holmes, "Shiny-phyloseq: web application for interactive microbiome analysis with provenance tracking," *Bioinformatics*, vol. 31, no. 2, pp. 282-283, 2015.

[30] Ø. Hammer, D. A. T. Harper, and P. D. Ryan, *PAST-Palaeontological Statistics, ver. 1.89. Paleontological Museum*, University of Oslo, Noruega, 2008, http://folk.uio.no/ohammer/past/index.html.

[31] L. Purkamo, M. Bomberg, R. Kietäväinen et al., "Microbial co-occurrence patterns in deep Precambrian bedrock fracture fluids," *Biogeosciences*, vol. 13, no. 10, pp. 3091–3108, 2016.

[32] H. Miettinen, R. Kietäväinen, E. Sohlberg, M. Numminen, L. Ahonen, and M. Itävaara, "Microbiome composition and geochemical characteristics of deep subsurface high-pressure environment, Pyhäsalmi mine Finland," *Frontiers in Microbiology*, vol. 6, 2015.

[33] M. Bomberg, T. Lamminmäki, and M. Itävaara, "Microbial communities and their predicted metabolic characteristics in deep fracture groundwaters of the crystalline bedrock at Olkiluoto, Finland," *Biogeosciences*, vol. 13, no. 21, pp. 6031–6047, 2016.

[34] L. Purkamo, R. Kietäväinen, H. Miettinen et al., "Diversity and functionality of archaeal, bacterial and fungal communities in deep Archaean bedrock groundwater," *FEMS Microbiology Ecology*, vol. 94, no. 8, article fiy116, 2018.

[35] J. Liu, Z. S. Hua, L. X. Chen et al., "Correlating microbial diversity patterns with geochemistry in an extreme and heterogeneous environment of mine tailings," *Applied and Environmental Microbiology*, vol. 80, no. 12, pp. 3677–3686, 2014.

[36] L. X. Chen, M. Hu, L. N. Huang et al., "Comparative metagenomic and metatranscriptomic analyses of microbial communities in acid mine drainage," *The ISME Journal*, vol. 9, no. 7, pp. 1579–1592, 2015.

[37] A. García-Moyano, A. Austnes, A. Lanzén, E. González-Toril, Á. Aguilera, and L. Øvreås, "Novel and unexpected microbial diversity in acid mine drainage in svalbard (78 N), revealed by culture-independent approaches," *Microorganisms*, vol. 3, no. 4, pp. 667–694, 2015.

[38] M. Mühling, A. Poehlein, A. Stuhr, M. Voitel, R. Daniel, and M. Schlömann, "Reconstruction of the metabolic potential of acidophilic *Sideroxydans* strains from the metagenome of an microaerophilic enrichment culture of acidophilic iron-oxidizing bacteria from a pilot plant for the treatment of acid mine drainage reveals metabolic versatility and adaptation to life at low pH," *Frontiers in Microbiology*, vol. 7, p. 2082, 2016.

[39] D. S. Jones, C. Kohl, C. Grettenberger, L. N. Larson, W. D. Burgos, and J. L. Macalady, "Geochemical niches of iron-oxidizing acidophiles in acidic coal mine drainage," *Applied and Environmental Microbiology*, vol. 81, no. 4, pp. 1242–1250, 2015.

[40] C. M. Kay, A. Haanela, and D. B. Johnson, "Microorganisms in subterranean acidic waters within Europe's deepest metal

mine," *Research in Microbiology*, vol. 165, no. 9, pp. 705–712, 2014.

[41] P. Kinnunen and J. Puhakka, "High-rate ferric sulfate generation by a *Leptospirillum ferriphilum*-dominated biofilm and the role of jarosite in biomass retainment in a fluidized-bed reactor," *Biotechnology and Bioengineering*, vol. 85, no. 7, pp. 697–705, 2004.

[42] J. Valdés, I. Pedroso, R. Quatrini et al., "Acidithiobacillus ferrooxidans metabolism: from genome sequence to industrial applications," *BMC Genomics*, vol. 9, no. 1, p. 597, 2008.

[43] D. B. Johnson, K. B. Hallberg, and S. Hedrich, "Uncovering a microbial enigma: isolation and characterization of the streamer-generating, iron-oxidizing, acidophilic bacterium "Ferrovum myxofaciens"," *Applied and Environmental Microbiology*, vol. 80, no. 2, pp. 672–680, 2014.

[44] K. Coupland and D. B. Johnson, "Evidence that the potential for dissimilatory ferric iron reduction is widespread among acidophilic heterotrophic bacteria," *FEMS Microbiology Letters*, vol. 279, no. 1, pp. 30–35, 2008.

[45] H. Liu, H. Yin, Y. Dai et al., "The co-culture of *Acidithiobacillus ferrooxidans* and *Acidiphilium acidophilum* enhances the growth, iron oxidation, and CO_2 fixation," *Archives of Microbiology*, vol. 193, no. 12, pp. 857–866, 2011.

[46] S. R. Ullrich, A. Poehlein, S. Voget et al., "Permanent draft genome sequence of *Acidiphilium* sp. JA12-A1," *Standards in Genomic Sciences*, vol. 10, no. 1, p. 56, 2015.

[47] S. Gross and E. I. Robbins, "Acidophilic and acid-tolerant fungi and yeasts," *Hydrobiologia*, vol. 433, no. 1/3, pp. 91–109, 2000.

[48] E. Sohlberg, M. Bomberg, H. Miettinen et al., "Revealing the unexplored fungal communities in deep groundwater of crystalline bedrock fracture zones in Olkiluoto, Finland," *Frontiers in Microbiology*, vol. 6, 2015.

[49] H. Drake and M. Ivarsson, "The role of anaerobic fungi in fundamental biogeochemical cycles in the deep biosphere," *Fungal Biology Reviews*, vol. 32, no. 1, pp. 20–25, 2018.

[50] H. Drake, M. Ivarsson, S. Bengtson et al., "Anaerobic consortia of fungi and sulfate reducing bacteria in deep granite fractures," *Nature Communications*, vol. 8, no. 1, p. 55, 2017.

[51] M. Ivarsson, S. Bengtson, H. Drake, and W. Francis, "Fungi in deep subsurface environments," *Advances in Applied Microbiology*, vol. 102, pp. 83–116, 2018.

[52] B. J. Baker, G. W. Tyson, L. Goosherst, and J. F. Banfield, "Insights into the diversity of eukaryotes in acid mine drainage biofilm communities," *Applied and Environmental Microbiology*, vol. 75, no. 7, pp. 2192–2199, 2009.

[53] J. C. Ottow and A. von Klopotek, "Enzymatic reduction of iron oxide by fungi," *Applied Microbiology*, vol. 18, no. 1, pp. 41–43, 1969.

[54] A. Segerer, T. A. Langworthy, and K. O. Stetter, "Thermoplasma acidophilum and Thermoplasma volcanium sp. nov. from solfatara fields," *Systematic and Applied Microbiology*, vol. 10, no. 2, pp. 161–171, 1988.

[55] L. X. Chen, L. N. Huang, C. Méndez-García et al., "Microbial communities, processes and functions in acid mine drainage ecosystems," *Current Opinion in Biotechnology*, vol. 38, pp. 150–158, 2016.

[56] P. S. Dobbin, J. P. Carter, C. GarcÃ-a-Salamanca San Juan, M. Hobe, A. K. Powell, and D. J. Richardson, "Dissimilatory Fe (III) reduction by *Clostridium beijerinckii* isolated from freshwater sediment using Fe (III) maltol enrichment," *FEMS Microbiology Letters*, vol. 176, no. 1, pp. 131–138, 1999.

[57] H. S. Park, B. H. Kim, H. S. Kim et al., "A novel electrochemically active and Fe (III)-reducing bacterium phylogenetically related to *Clostridium butyricum* isolated from a microbial fuel cell," *Anaerobe*, vol. 7, no. 6, pp. 297–306, 2001.

[58] C. Falagán and D. B. Johnson, "*Acidibacter ferrireducens* gen. nov., sp. nov.: an acidophilic ferric iron-reducing gammaproteobacterium," *Extremophiles*, vol. 18, no. 6, pp. 1067–1073, 2014.

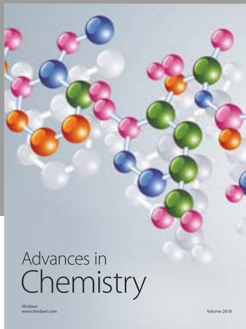
[59] J. Ding, Y. Zhang, X. Quan, and S. Chen, "Anaerobic biodecolorization of AO7 by a newly isolated Fe(III)-reducing *bacterium Sphingomonas* strain DJ," *Journal of Chemical Technology & Biotechnology*, vol. 90, no. 1, pp. 158–165, 2015.

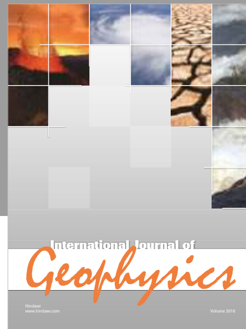
[60] M. L. Coleman, D. B. Hedrick, D. R. Lovley, D. C. White, and K. Pye, "Reduction of Fe (III) in sediments by sulphate-reducing bacteria," *Nature*, vol. 361, no. 6411, pp. 436–438, 1993.

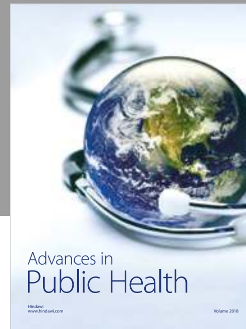
[61] D. R. Lovley, E. E. Roden, E. J. P. Phillips, and J. C. Woodward, "Enzymatic iron and uranium reduction by sulfate-reducing bacteria," *Marine Geology*, vol. 113, no. 1-2, pp. 41–53, 1993.

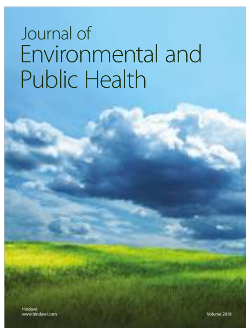
[62] D. B. Johnson and C. Falagán, "*Acidithiobacillus ferriphilus* sp. nov., a facultatively anaerobic iron- and sulfur-metabolizing extreme acidophile," *International Journal of Systematic and Evolutionary Microbiology*, vol. 66, no. 1, pp. 206–211, 2016.

[63] B. M. Bonis and J. A. Gralnick, "Marinobacter subterrani, a genetically tractable neutrophilic Fe (II)-oxidizing strain isolated from the Soudan Iron mine," *Frontiers in Microbiology*, vol. 6, p. 719, 2015.









Hindawi

Submit your manuscripts at
www.hindawi.com