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Metal Mobilization by Iron- and Sulfur-Oxidizing Bacteria in a Multiple Extreme Mine Tailings in the Atacama Desert, Chile

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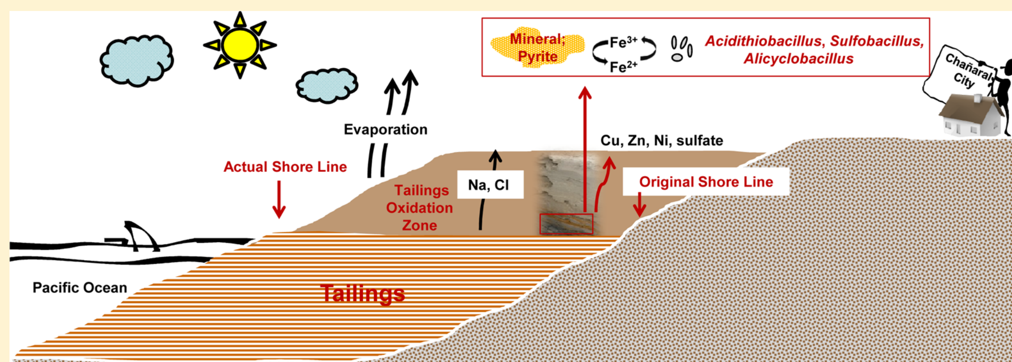
Supporting Information



ABSTRACT: The marine shore sulfidic mine tailings dump at the Chañaral Bay in the Atacama Desert, northern Chile, is characterized by extreme acidity, high salinity, and high heavy metals concentrations. Due to pyrite oxidation, metals (especially copper) are mobilized under acidic conditions and transported toward the tailings surface and precipitate as secondary minerals (Dold, *Environ. Sci. Technol.* **2006**, *40*, 752−758.). Depth profiles of total cell counts in this almost organic-carbon free multiple extreme environment showed variable numbers with up to 10^8 cells g^{-1} dry weight for 50 samples at four sites. Real-time PCR quantification and bacterial 16S rRNA gene diversity analysis via clone libraries revealed a dominance of *Bacteria* over *Archaea* and the frequent occurrence of the acidophilic iron(II)- and sulfur-oxidizing and iron(III)-reducing genera *Acidithiobacillus*, *Alicyclobacillus*, and *Sulfobacillus.* Acidophilic chemolithoautotrophic iron(II)-oxidizing bacteria were also frequently found via most-probable-number (MPN) cultivation. Halotolerant iron(II)-oxidizers in enrichment cultures were active at NaCl concentrations up to 1 M. Maximal microcalorimetrically determined pyrite oxidation rates coincided with maxima of the pyrite content, total cell counts, and MPN of iron(II)-oxidizers. These findings indicate that microbial pyrite oxidation and metal mobilization preferentially occur in distinct tailings layers at high salinity. Microorganisms for biomining with seawater salt concentrations obviously exist in nature.

INTRODUCTION

Extreme environments on Earth such as deep sediments and rocks, hot springs, acid mine drainages, or salt lakes have shown to harbor active and specialized microbial communities.[2−5] The hyperarid Atacama Desert is one of the driest deserts on Earth but is still a habitat for microorganisms.[6,7]

Microorganisms play a significant role in metallogenetic processes in dumps of mine waste rock and tailings from sulfide ore processing plants. Acidophilic iron(II)- and sulfur-oxidizing bacteria are responsible for the release of sulfuric acid and dissolved metals such as iron, copper, nickel, zinc, and arsenic, known as acid mine drainage from such dumps by catalyzing the oxidation of metal sulfides, mainly pyrite (or pyrrhotite).[8−19] Over a period of several years, an oxidized zone with depleted metal sulfide content, low pH, and enrichment of secondary minerals is developing above a not oxidized zone with unaltered material in the waste dump. Anaerobic iron(III)- and sulfate-reducing bacteria have been detected as well in several mine dumps enabling a complete biogeochemical iron- and sulfur-cycle, which is important for long-term (bio)-remediation.[8−17]

The microbial metal sulfide oxidation processes are also used for biomining, an industrial recovery of copper, cobalt, nickel, zinc, gold, and uranium via dump or heap bioleaching.[20,21] High concentrations of chloride ions inhibit the growth of acidophilic microorganisms used in biomining, a problem particularly relevant to Australian and Chilean biomining operations.[22] Few species of iron- and sulfur-oxidizing bacteria grow in saline, strongly acidic environments; however, copper ore bioleaching with halotolerant microorganisms has been demonstrated in the laboratory.[23] In the best case seawater should be used for

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biomining operations to save freshwater resources, but microbial metal sulfide oxidation at seawater salt concentrations and low pH has not been shown yet in the environment, such as sulfidic mine tailings.

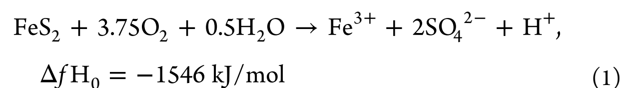
Microorganisms were found so far only in sulfidic mine waste rock and tailings at humid and semiarid conditions but not under the multiple extreme conditions of the Atacama Desert to the best of our knowledge.[8] The extreme acidic, metal-rich, and high-saline, sulfidic mine tailings dump at the Chañaral Bay in the Atacama Desert originated from copper ore processing (1938−1975) and was classified by the United Nations Environmental Programme (UNEP) in 1983 as one of the most serious contamination sources in the Pacific area. Tailings with an average pyrite content of 0.8 wt % were deposited into the bay, in total over 220 Mt. This resulted in a 10−15 m thick tailings dump covering about 4 km^2 and in a more than 1 km seaward displacement of the shoreline. The tailings material has been exposed to oxidation since 1975 which resulted in a 70−188 cm thick low-pH (2−4) oxidation zone. The redox potential (Eh) showed values of up to 600 mV, and NaCl concentrations of up to more than 1 M were measured in the pore water of the oxidation zone. Elevated pore water concentrations of up to more than 6000 mg/L sulfate, 2265 mg/L copper, 20 mg/L zinc, and 18 mg/L nickel were found. Evaporation-induced upward transport of metals led to metal enrichment at the tailings surface and secondary chlorides and/or sulfates precipitated (e.g., up to 2.4% Cu). The mainly water-soluble, secondary minerals have been transported by the wind, also toward the village of Chañaral (Supplementary Figure S1; ref 1). In this study an existence of microorganisms, their identification, and quantification as well as their impact on pyrite oxidation at high salinity and low pH relevant for copper mobilization have been explored.

■ METHODS

Tailings Sampling. In November 2008, 50 samples were taken down to a maximal depth of 105 cm from outcrop profiles at four sites of the tailings dump with sterile spatulas or spoons and filled in sterile 100 mL containers. Site CH1 was located at the southern part, sites CH11 and CH12 at the central part, and site CH14 at the northern part of the tailings dump. Site CH12 was located close to the sea shore (beach). The paste pH was measured in the field with an electrode after shaking of 5 g tailings material in 12.5 mL of 1 M KCl for 5 min. The samples were transported to the BGR geo-microbiology laboratory within a couple of days for further analyses. Immediately after arrival the samples were split in subsamples which were either instantly used for geochemical and microcalorimetric measurements and inoculation of media for cultivation, or fixed with formaldehyde for total cell counts and CARD-FISH, or frozen at −20 °C for later analyzes with DNA-based techniques.

Geochemistry and Mineralogy. Humidity was determined as weight difference after drying of 5 g tailings at 105 °C. The mineralogy was quantitatively analyzed by an Environmental Scanning Electron Microscope (ESEM, type FEI Quanta 600 FEG) coupled with an energy dispersive X-ray (EDX) detector (Apollo XL from Ametek Inc.) and in combination with the MLA software package (Mineral Liberation Analyzer, FEI). The investigated polished sections were measured by the XBSE method[24] with HV 25 Kv and 5.7 μm beam spot size. Total element analysis was done as previously described (XRF and LECO; ref 16).

Pyrite Oxidation Rates. The potential pyrite oxidation rate at atmospheric oxygen partial pressure was determined by microcalorimetry as described[12,14−16,25,26] because the reaction rate correlates with the heat output. A complete oxidation of pyrite to iron(III) and sulfate produces a reaction energy of −1546 kJ/mol:



$$FeS_2 + 3.75O_2 + 0.5H_2O \rightarrow Fe^{3+} + 2SO_4^{2-} + H^+, \quad \Delta f H_0 = -1546 \text{ kJ/mol} \tag{1}$$

The pyrite oxidation rate r (μg/kg/s) was calculated using the transformed reaction energy value of −1.546 kJ/mmol, the molecular mass of pyrite of 0.12 kg/mol, the measured heat output a (μW), and the sample weight w (g) by the following equation:

$$r\ (\mu g/kg/s) = 1/-1.546\ (mmol/kJ) \times 0.12\ (kg/mol) \times a\ (\mu W) \times 1/w\ (1/g) \tag{2}$$

After measuring the total rate (chemical plus biological), the bacteria were inactivated by heating to 60 °C for ca. 12 h, and the chemical rate was measured afterward. The inactivation of mesophilic pyrite-oxidizers was already described and checked via cultivation.[25] The biological rate was calculated as the difference of both measurements.

Cultivation. Microorganisms were detected and quantified by cultivation and molecular techniques as described previously.[16] The most-probable-number (MPN) cultivation technique was used to enumerate acidophilic chemolithoautotrophic iron(II)- and sulfur-oxidizing bacteria.[26] Aerobic acidophilic heterotrophs were enumerated on agar plates.[12] Acidophilic halotolerant iron(II)-oxidizers were enriched and cultivated in 100 mL Erlenmeyer flasks in 50 mL medium supplemented with 1 g L^{-1} Fe^{2+} as $FeSO_4$ and sodium chloride concentrations of 0.5 and 1 M at 30 °C on a rotary shaker with a rotation speed of 120 rpm.[26] In the porewater from the oxidized tailings NaCl concentrations of up to more than 1 M were measured,[1] thus our medium mimicked field salinity. Besides, the enrichment medium consisted of the following: 0.15 g/L $(NH_4)_2SO_4$, 0.05 g/L KCl, 0.5 g/L $MgSO_4 \times 7H_2O$, 0.05 g/L K_2HPO_4, and 0.01 g/L $Ca(NO_3)_2 \cdot 4H_2O$, adjusted to pH 3.5 with H_2SO_4 or NaOH. Water evaporation was compensated at regular intervals by addition of acidic deionized water during the experiments, and samples were taken at different time intervals. Growth was checked by phase contrast microscopy, the pH was measured, and the iron(II)-oxidation activity was monitored by regular Fe^{2+} and total Fe measurement by the o-phenanthroline colorimetric method.

Total Cell Counts and CARD-FISH. Total cell numbers were determined in formaldehyde-fixed samples by staining with SYBR Green II following two different protocols. On the one hand cells were counted directly in the samples;[16] on the other hand cells were detached from tailings particles before counting using a different protocol.[27] The highest number of the two counts is reported here since an overestimation of counts is unlikely. CARD-FISH analysis was carried out as previously done with formaldehyde fixed samples, and filters were hybridized for *Archaea* and *Bacteria* using probes ARCH915 or EUB338 I-III as a mixture.[3,16,28−30] As a negative hybridization control the probe NON338 was applied and cell signals were not detected. The formamide concentrations were 55% for all probes. DAPI was used for counterstaining.

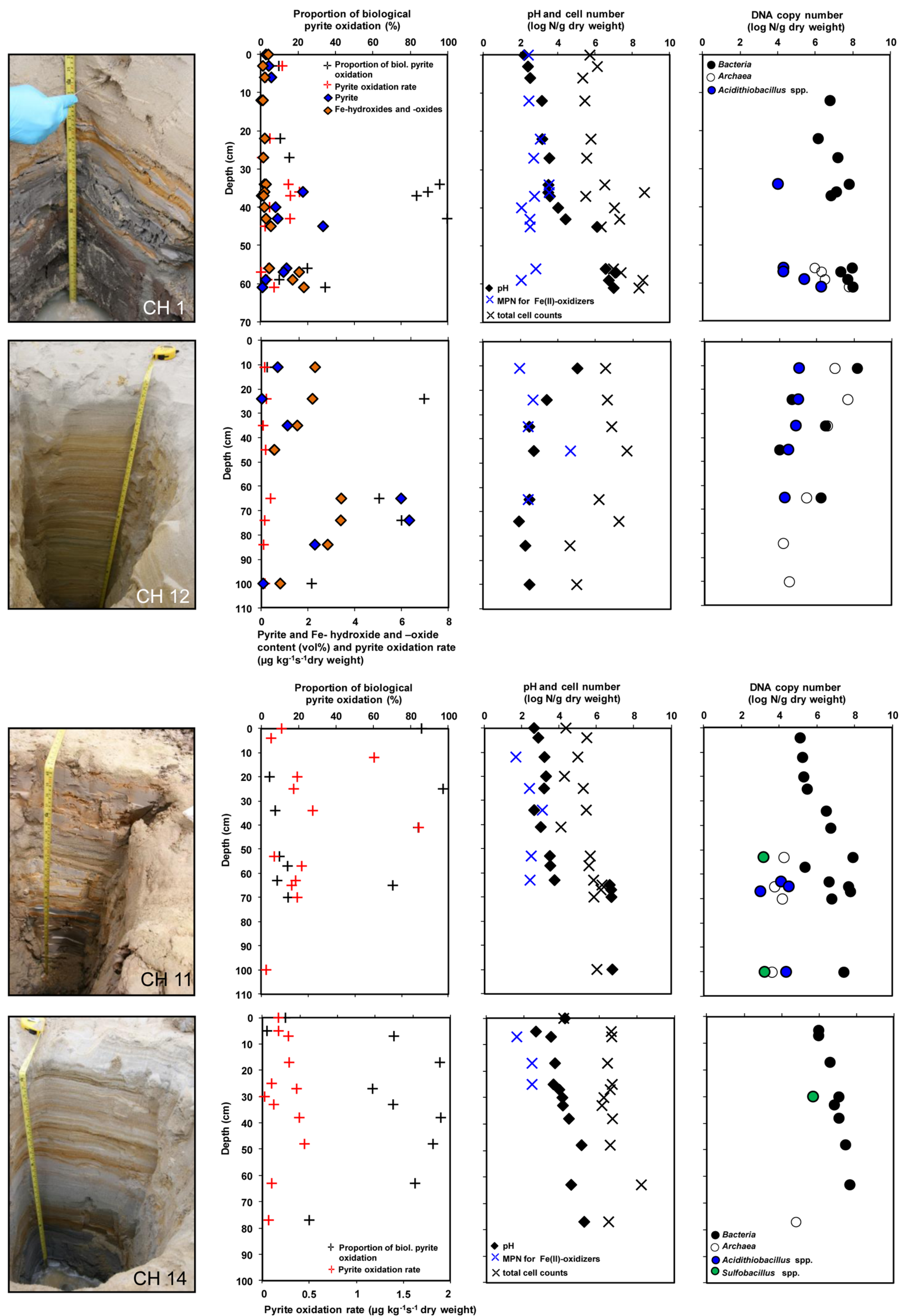


Figure 1. Pictures of outcrop profiles and depth dependent quantitative mineralogy, pyrite oxidation activity, and quantitative microbial community analysis for the sampling sites of the high-saline, sulfidic mine tailings dump at Chañaral, Atacama Desert, northern Chile. From top to bottom data for the sites are shown in the order CH1, CH12, CH11, and CH14.

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Real-Time PCR. DNA was extracted from 0.5 g of a frozen tailings sample following a modified Fast DNA Spin Kit for Soil (Bio 101) protocol.[31] This protocol has shown to exhibit the highest DNA copy numbers from tailings samples among several others tested. DNA extracts from blank tubes (no sample added) were used as negative control in the extraction procedure. Extracted DNA was amplified by qPCR using the device ABI Prism 7000 (Applied Biosystems) and master mixes from the companies Applied Biosystems, Eurogentec, or Invitrogen. Each DNA extract was measured in triplicate. The copy numbers of the 16S rRNA gene were quantified for *Archaea*[32] and *Bacteria*[33] based on the TaqMan chemistry. The specific bacterial 16S rRNA genes of *Acidithiobacillus* spp.,[34] *Leptospirillum* spp.,[35] and *Sulfobacillus* spp.,[35] and the functional *dsr*A gene of sulfate-reducers[36] were also quantified using qPCR with SYBR Green I chemistry. After each qPCR, melting curves were measured. The primer specificity for the specific qPCR assays was confirmed by sequence alignment in databases (Blast, Ribosomal Database Project). The detection limits for qPCR analyses were 10^3 16S rRNA gene copies g^{-1} dry weight (dw) for the assays specific for *Bacteria* and *Acidithiobacillus* spp., 10^2 copies g^{-1} dw for the assays specific for *Leptospirillum* spp. and *dsrA*, and 10^1 copies g^{-1} dw for the assays specific for *Archaea* and *Sulfobacillus* spp..

Microbial Diversity. The amplification of 16S rRNA genes from *Bacteria* was performed by PCR with the universal bacterial primers GM3F (5′-AGAGTTTGATCMTGGC-′3, position 8 to 24) and GM4R (5′-TACCTTGTTACGACTT-′3, position 1492 to 1507).[37] PCR mix was prepared from Thermo Scientific 2xMasterMix (final concentration: 75 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 0.2 mM each of dNTP, 0.5 μM each of primer, 0.652 U ThermoPrime Taq DNA Polymerase, 100 ng/μL BSA) and a 2 μL template of extracted DNA (see above) in a total reaction of volume 50 μL. Negative controls without template were used as a contamination check. Reaction mixtures were held at 95 °C for 2 min followed by 35 cycles of 94 °C for 30 s, 52 °C for 30 s, and 72 °C for 90 s, with a final extension step of 5 min at 72 °C. Products of PCR reactions were cloned and sequenced by the company Microsynth (Switzerland). Overlapping sequencing from both sides of the 16S rRNA genes was performed. Contigs were constructed with the software Geneious Pro 5.4 and checked for chimera with UCHIME.[37] In total 800 sequences with more than 1300 bp were obtained (Supplementary Table S4). Obtained sequences were aligned with the SILVA Incremental Aligner (SINA[38]) and the SILVA_108NR database and curated by hand with the ARB software package (v.5.4). This alignment was used in the Mothur v 1.29 program to build operational taxonomic units (OTU, 97% similarity) and calculate coverage and diversity indices. One sequence from each OTU harboring at least 5 sequences was picked as a representative and imported to the SILVA_108NR template tree[39] using the ARB program suite.[40] An additional 10 sequences for each OTU-representative were selected based on the phylogenetic affiliation (min identity 5%, in total 300) in the SILVA_108NR. Selected reference sequences together with the OTU-representatives were used for tree construction using maximum likelihood algorithm (RAxML) with GTRGAMMA as rate distribution model and the general bacteria filter provided in ARB. The rRNA gene sequences obtained in this study were submitted to the European Nucleotide Archive with the accession numbers HF558531-HF558644.

RESULTS AND DISCUSSION

The extreme acidic, metal-rich, and high-saline, sulfidic mine tailings dump at the Chañaral Bay in the Atacama Desert with copper enrichment at the surface is the most extreme mine tailings studied so far. The copper enrichment zone is particularly interesting for mining of this secondary copper resource. In November 2008, 50 samples were taken from different depths of the oxidation zone above the seawater level at four sites of the Chañaral mine tailings. The geochemistry and mineralogy of these sites was previously studied in particular.[1] Here, in addition the mineralogy has quantitatively been analyzed by scanning electron microscopy − mineral liberation analysis (SEM-MLA, Figure 1 and Supplementary Table S1) together with total element analysis (XRF and LECO, Supplementary Table S2). Organic carbon was below the detection limit of 0.01% at all sites (besides at about 60 cm depth of site CH1 where a buried alluvion (soil material) and no tailings material was sampled). Thus the substrate for heterotrophic microorganisms usually found in extreme environments is absent in the tailings. According to the quantitative mineralogical analysis, pyrite is the main substrate for chemolithoautotrophic microorganisms. In Figure 1, depth profiles of pyrite and its oxidation product iron hydroxides and oxides are shown together with pyrite oxidation rates as well as the proportion of the biological versus the chemical pyrite oxidation rate determined by microcalorimetric measurements. A coincidence of the maxima of the pyrite content and the pyrite oxidation rate was found. The pyrite maxima at ∼35−45 cm depth for CH1 and ∼60−80 cm depth for CH12 coincided with a high proportion of biological pyrite oxidation indicating current oxidation activity there.

Microorganisms were detected and quantified by cultivation as well as molecular techniques.[16] Depth profiles of cell numbers together with pH-values and pictures of the four sampled depth profiles are shown in Figure 1. The pictures of all sites show alternating layers of gray and brown colors reflecting the deposition history of the tailings as well as a variable precipitation of iron hydroxides and oxides as secondary minerals due to mainly pyrite oxidation. Living acidophilic chemolithoautotrophic iron(II)-oxidizing microorganisms able to oxidize pyrite were detected via cultivation in liquid media in variable most probable numbers (MPN) at all sites in half of the samples (25 of 50 samples). The highest MPN numbers were detected in tailings layers with a pH 2−4. Acidophilic chemolithoautotrophic sulfur-oxidizing microorganisms and acidophilic heterotrophs were scarcely detected via cultivation (6 of 50 and 5 of 50 samples, respectively, data not shown). Thus the MPN cultivation approach likely missed most of these organisms. The heterotrophs presumably exist from little organic carbon released from the chemolithotrophs as previously shown for sulfidic mine waste.[8,12] Total cell counts determined by counting under a fluorescence microscope after DNA-staining with SYBR Green occurred in orders of magnitude higher numbers than the iron(II)-oxidizers. This means that the MPN cultivation approach covered only a minor part of the microbial community. However, the maxima of pyrite content and pyrite oxidation activity coincided with the maxima of total cell counts and MPN numbers of acidophilic iron(II)-oxidizers. This proposes that pyrite is oxidized by the microorganisms in these tailings layers.

In order to proof that iron(II) is indeed microbiologically oxidized at high salinity, enrichment cultures at salt

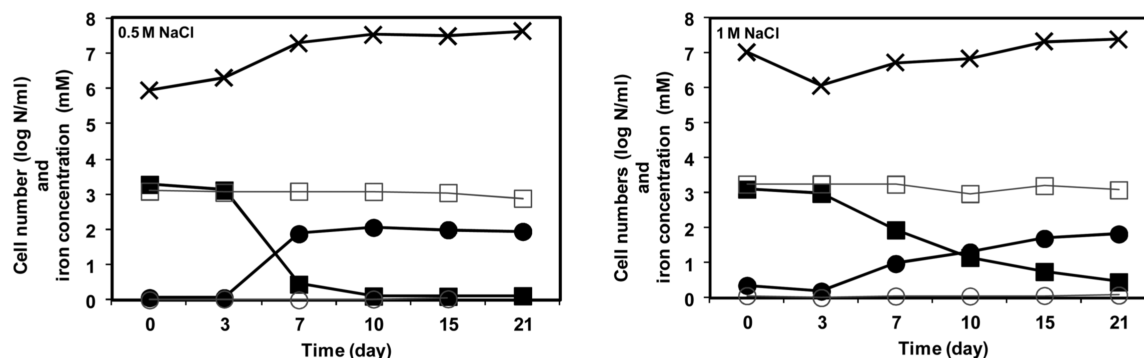

Figure 2. Growth of halotolerant, acidophilic, chemolithoautotrophic Fe(II)-oxidizing bacteria at NaCl concentrations of 0.5 and 1 M. Cell numbers (X), Fe(II) concentration (■,□) and Fe(III) concentration (●,○). Full and open symbols represent enrichment cultures and chemical controls, respectively.

concentrations of 0.5 and 1 M NaCl were set up. The mixed enrichment cultures have shown to be stable in several transfers to fresh medium. A slow growth was observed in a period of three weeks and iron(II)-oxidation was measurable only in the presence of bacteria as shown for one transfer of the most active culture (Figure 2). This culture was initially inoculated with tailings from site CH1 at about 35 cm depth, the tailings layer with the highest pyrite oxidation rates and total cell counts (Figure 1). Most of the known acidophilic iron(II)-oxidizing microorganisms are inhibited by chloride ions.[22] To the best of our knowledge this is the first report of a halotolerant, acidophilic iron(II)-oxidizing culture able to oxidize iron(II) at the high salt concentration of 1 M NaCl, higher than in a previous study with the halotolerant iron(II)-oxidizer *Thiobacillus prosperus* which oxidized iron(II) in the presence of up to 5% w/v NaCl concentration.[23,41] Phylogenetic analysis based on 16S rRNA gene isolation and sequencing revealed an affiliation of the halotolerant bacteria in our stable, most active enrichment culture to *Sulfobacillus* spp. and *Acidiphilium* spp. (Supplementary Tables S3 and S4).

Also, living bacteria were detected by catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) in samples from the mine tailings selected based on the brightness of the signals (Figure S2). CARD-FISH signals were not quantified due to the low number of bright cell signals in many samples; here the data just confirm that living *Bacteria* exist in the multiple extreme mine tailings.

A molecular quantification of microorganisms using quantitative real-time PCR (qPCR) assays allowed a comparative quantitative microbial community analysis in the Chañaral tailings dump (Figure 1). At all sampling sites, the 16S rRNA gene copy numbers (10^4–10^8 copies g^{-1} dry weight) of the domain *Bacteria* were higher than those of the domain *Archaea* which were detected in fewer samples (36 of 50 and 15 of 50 samples, respectively). In other mine tailings most detected genera belong to the domain *Bacteria*.[8] The *Archaea* are mainly thermophiles,[8] but high temperatures have not been observed in the Chañaral tailings dump. Thus, *Archaea* are obviously of minor importance in this multiple extreme environment.

To further explore the quantitative bacterial community composition relevant for mine tailings, additionally, iron(II)- and sulfur-oxidizing bacteria and anaerobic iron(III)- and sulfate-reducing bacteria were analyzed via specific qPCR assays.[16] The acidophilic iron(II)- and sulfur-oxidizing and iron(III)-reducing genera *Acidithiobacillus* (gram-negative) and *Sulfobacillus* (gram-positive) were detected in three and two sampling sites, respectively, in specific tailings layers with up to 10^5 16S rRNA gene copies g^{-1} dry weight (Figure 1) The acidophilic Fe(II)-oxidizing genus *Leptospirillum* was below detection limit of 10^2 16S rRNA gene copies g^{-1} dry weight. A dominance of *Acidithiobacillus* over *Sulfobacillus* and *Leptospirillum* was also previously found for three other mine tailings sites.[16] Sulfate-reducers were mainly detectable in the buried alluvion of site CH1 where neutral pH and little organic carbon prevailed (4 of 50 samples, data not shown). Thus, sulfate-reducers do not play a relevant role in these extreme tailings as reported for other tailings.[16,17]

To further explore the microbial diversity in the tailings dump, the 16S rRNA genes of *Bacteria* from the samples from the sites CH1 and CH12 were amplified by PCR. Products were obtained for seven samples, cloned, and subsequently sequenced. The results are shown in Figure 3 and the Supplementary Tables S3, S4, and S5. In total 114 operational taxonomic units (OTUs, potentially representing bacterial species) were detected. Site 1 clone libraries from the depth interval 34–57 cm showed high values for recovery between 88 and 98% which correlated with the low number of observed OTUs. Relative abundant sequences were closely related to strains of the acidophilic iron(II)- and sulfur-oxidizing and iron(III)-reducing *Alicyclobacillus* spp. (Firmicutes, closely related to *Sulfobacillus*) and *Acidithiobacillus ferrooxidans* (Gammaproteobacteria) in agreement with the qPCR data and previous tailings studies.[8]

In addition, moderate acidophilic sulfur-oxidizing *Halothiobacillus* spp., *Thiohalomonas* spp., *Thiomicrospira* spp., and Bacteriodetes were often found in the clone library data. These particular bacterial groups have not been reported to occur in other mine tailings;[8] however, moderate acidophilic sulfur-oxidizing bacteria are regularly found in sulfidic mine waste at slightly acidic or neutral pH, where due to chemical pyrite oxidation sulfur compound intermediates are delivered as substrates for these microorganisms.[9,12,15]

In the buried alluvion of site CH1 (~60 cm depth) where, with neutral pH and little organic carbon availability, less extreme conditions prevailed, the microbial diversity was much higher, and unknown representatives of the phyla Alphaproteobacteria, Gammaproteobacteria (including *Acidithiobacillus ferrooxidans* probably introduced from upper horizons), Deltaproteobacteria (presumably sulfate-reducers according to qPCR data), Bacteriodetes, Nitrospira, Firmicutes, and Actino-

Figure 3. Maximum-likelihood phylogenetic tree of abundant 16S rRNA gene sequences isolated from selected samples from the multiple extreme sulfidic mine tailings dump at Chañaral. Represented in bold is one representative sequence from each defined OTU which contained at least five assigned sequences. The scale bar indicates the number of substitutions per site.

bacteria were found. Clone libraries from these depths showed lower values for coverage between 73 and 78%.

This study has shown that the multiple extreme arid, high-saline, metal-rich, and almost organic carbon-free mine tailings are populated by prokaryotes dominated by halotolerant, acidophilic, iron- and sulfur-oxidizing chemolithoautotrophic bacteria. The maximum abundance of these bacteria coincided with a high proportion of biological pyrite oxidation, maximal pyrite oxidation rates as well as a high pyrite content in distinct layers. These findings indicate that pyrite oxidation is driven by microbial activity in the extreme mine tailings. Due to microbial pyrite oxidation sulfuric acid in generated, and metals (mainly copper) are mobilized under acidic conditions which is reflected by the elevated metal and sulfate concentrations in the pore water.[1] Driven by evaporation the ions are transported toward the tailings surface where metal precipitation occurs in the form of secondary chlorides and/or sulfates.[1] The metal enrichment in distinct tailings horizons is potentially relevant for copper mining from mine waste. One processing option is biomining using halotolerant, acidophilic, pyrite-oxidizing chemolithoautotrophic bacteria.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Supplementary Figures S1−S3, Supplementary Tables S1−S5, and references. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

A.S. and B.D. designed the study and performed sample collection. H.K. carried out the microbiological investigations. M.B. contributed to phylogenetic analyses. M.A.S. performed SEM-MLA analyses. H.K. and A.S. wrote the manuscript.

Notes

The authors declare no competing financial interests.

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