

Diversity of the Sediment Microbial Community in the Aha Watershed (Southwest China) in Response to Acid Mine Drainage Pollution Gradients

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Located in southwest China, the Aha watershed is continually contaminated by acid mine drainage (AMD) produced from upstream abandoned coal mines. The watershed is fed by creeks with elevated concentrations of aqueous Fe (total Fe > 1 g/liter) and SO_4^{2-} (>6 g/liter). AMD contamination gradually decreases throughout downstream rivers and reservoirs, creating an AMD pollution gradient which has led to a suite of biogeochemical processes along the watershed. In this study, sediment samples were collected along the AMD pollution sites for geochemical and microbial community analyses. High-throughput sequencing found various bacteria associated with microbial Fe and S cycling within the watershed and AMD-impacted creek. A large proportion of Fe- and S-metabolizing bacteria were detected in this watershed. The dominant Fe- and S-metabolizing bacteria were identified as microorganisms belonging to the genera *Metallibacterium*, *Aciditerrimonas*, *Halomonas*, *Shewanella*, *Ferrovum*, *Alicyclobacillus*, and *Syntrophobacter*. Among them, *Halomonas*, *Aciditerrimonas*, *Metallibacterium*, and *Shewanella* have previously only rarely been detected in AMD-contaminated environments. In addition, the microbial community structures changed along the watershed with different magnitudes of AMD pollution. Moreover, the canonical correspondence analysis suggested that temperature, pH, total Fe, sulfate, and redox potentials (E_h) were significant factors that structured the microbial community compositions along the Aha watershed.

Acid mine drainage (AMD) is one of the most environmentally threatening by-products of the mining industry. The chemical and/or microbial weathering of metal sulfide-rich rocks such as pyrite (FeS_2), sphalerite (ZnS), and galena (PbS) produces sulfuric acid, which leads to the formation of AMD. AMD typically has low pH and elevated concentrations of sulfate and metals, which severely impair water and soil quality (1, 2).

Despite the extreme toxicity and acidity, the AMD-associated environments harbor numerous acidophilic microorganisms (3, 4). Recently, molecular techniques have been extensively applied to study AMD microbiology, and a variety of acidophilic and metal-tolerant microorganisms have been identified (5). Prokaryotic microorganisms that are metabolically active in acidic niche boundaries (pH < 3) were found to be phylogenetically diversified, affiliated within the phyla *Proteobacteria*, *Firmicutes*, *Acitobacteria*, *Nitrospirae*, and *Acidobacteria* (5). Many of these acidophiles are capable of Fe and S cycling (e.g., oxidation and reduction of these elements in appropriate geochemical and microbial environments) and thus play an important role both in the formation of acid mine waters and in natural attenuation of AMD. However, our understanding of the roles of geochemical factors in shaping microbial community structure and the potential of microorganisms in natural attenuation of AMD and AMD-impacted watersheds is still limited.

The AMD of the Aha watershed in southwest China provides an ideal opportunity to study the interactions between microorganisms and AMD-impacted environments. The Aha watershed is located in the Southwest Coal Basin (SCB) in central Guizhou Province, which is one of the largest coal producers in China. Several abandoned coal mines are located upstream of the water-

shed. One of the abandoned coal mines, Dapo Coal Mine, has produced a large amount of acidic runoff (>7,000 m³/year; pH < 3) with high concentrations of Fe(II) (~1 g/liter) and sulfate (~7 g/liter). Dapo, along with other abandoned coal mines in this area, has led to an extensive input of iron and sulfate into the Youyu River, Aha Reservoir, and Xiaoche River (Fig. 1). Compared to the heavily contaminated creeks upstream, the downstream rivers and reservoirs were overall moderately to lightly contaminated. This watershed allows the investigation of the spatial distribution of microbial community composition along an AMD gradient. Furthermore, the contamination of the Aha Reservoir is of great concern since this reservoir is a source of drinking water for more than 500,000 people. In order to remediate these acidic mine waters, an onsite passive AMD treatment facility has been constructed in Dapo Creek, which will be operated in the near future (see Fig. S1

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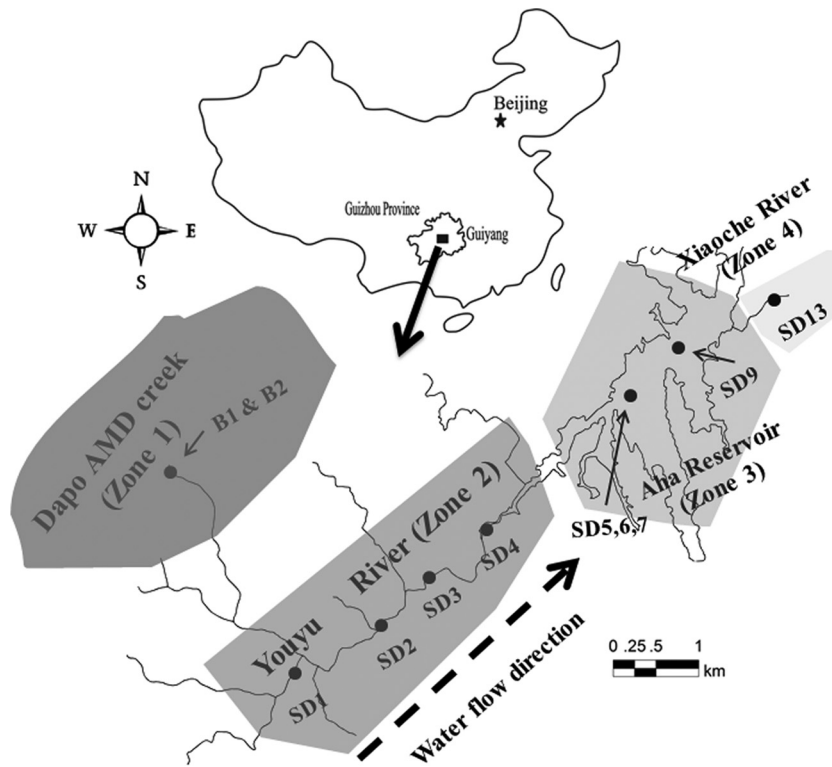


FIG 1 Location of the Aha watershed in China and the locations of 11 sampling sites in the Aha watershed. Please note that water flows from SD1 to SD13. The map was created using CorelDraw.

in the supplemental material). Therefore, a better understanding of the indigenous microbial diversity and its potential roles in natural attenuation of AMD will enable the development of molecular monitoring tools to evaluate and enhance bioremediation in this AMD treatment facility and other AMD-contaminated environments.

In this study, we used integrated geochemical and molecular biological analyses to understand the spatial distribution of microbial communities along the pollution gradients within the Aha watershed. Our aims were (i) to characterize the microbial community structure and composition within this watershed, (ii) to explore the correlation between microbial structure and different geochemical variables, and (iii) to identify the active organisms and their metabolic potentials for natural mitigation of the AMD contamination, especially for those involved in Fe and S cycling.

MATERIALS AND METHODS

Sample collection. The Aha watershed is located in southwest China at Guiyang, Guizhou Province (Fig. 1). Eleven samples were collected at different locations along the Aha watershed in September 2013. As illustrated by Fig. 1, samples B1 and B2 were collected from acid streamers of the mine portal (B1) and the orange sediment located 20 m downstream from mine portal (B2), respectively (see Fig. S1 in the supplemental material). Nine additional samples were collected from the downstream rivers and reservoir sediments. Specifically, samples SD1 to SD4 were collected from the Youyu River sediment, and sample SD13 was taken from Xiaoche River. The water depth ranged from 0.8 to 1.4 m for the Youyu River and was 0.6 m for the Xiaoche River. Approximately 300 g of sediment was collected per site. Samples from each site were pooled, homogenized, placed in sterile 50-ml tubes, and immediately stored at -20°C until further molecular analysis. Unlike river sediments, the Aha Reser-

voir has a sediment layer of more than 1 m. Thus, two sediment cores (cores 1 and 2), about 1 m in length, were taken from the reservoir using a gravity corer. The upper, middle, and bottom layers (SD5 to SD7) of core 1 and the bottom layer of core 2 (SD9) were used in all further molecular analyses.

Water chemistry analysis. The chemistry was analyzed for all water samples from each sampling site. About 5 liters of water was immediately filtered on each sampling site through a $0.45\text{-}\mu\text{m}$ sterile membrane (Jinjing, Shanghai, China). Measurements of redox potential (E_h), pH, salinity, conductivity, total dissolved solids, and temperature were carried out using a Hach ION156 portable multiparameter meter (Loveland, CO). Remaining water was preserved with ultrapure 37% HCl for prevention of Fe(II) oxidation and then stored at 4°C in darkness for subsequent determination of metal ions.

Mineralogical and element analyses. For inorganic geochemical analyses, bulk samples were dried at 105°C and thoroughly grated using a mortar and pestle before being passed through a 200-mesh sieve. Bulk chemical analyses of major elements were performed using X-ray fluorescence spectrometry (Axios-PW4400; PANalytical, Westborough, MA) at 40 kV and 95 mA, with a detection limit below 0.01%. In this analysis, 1 g of ground sample was combusted at 900°C for 2 h, and the difference in sample weight before and after combustion was reported as loss on ignition to quantify organic carbon in the sediment. The major elements were analyzed quantitatively after the fusion of 0.1 g of combusted sample with 3.6 g of dilithium tetraborate at $1,050^{\circ}\text{C}$ for 16 min.

Ferric and ferrous irons in water samples were measured by the spectrophotometric method (UV-9000s; METASH, Shanghai, China) with 1,10-phenanthroline at 510 nm (6, 7). Total sulfur was determined by a BaSO_4 -based turbidimetric method (8), and sulfate was measured by ion chromatography (ICS-90; Dionex, Sunnyvale, CA) (9). To measure pH in sediments, 10-g dry sediment samples were put into a 100-ml Erlenmeyer flask and mixed with 25 ml of distilled water (1:2.5 soil-water ratios). The

TABLE 1 Physicochemical parameters in the water samples taken from the Aha watershed^a

Sample	pH	Temp (°C)	E _h (mV)	TDS (g/liter)	Conductivity (mS/cm)	Salinity (‰)	Fe _{tot} (mg/liter)	Fe(II) (mg/liter)	SO ₄ ²⁻ (mg/liter)
B1	2.81	20.9	375.1	3.35	5.8	3.44	1,304	1,258	6,646
B2	2.81	25.4	419.2	3.19	6.19	3.27	1,273	971	6,838
SD1	8.1	15.5	20.8	0.251	0.422	0.25	0.015	ND	112
SD2	6.8	15.6	20.3	0.306	0.516	0.31	0.014	ND	219
SD3	8.12	15.7	35.3	0.347	0.581	0.35	0.035	ND	254
SD4	8.11	15.9	53	0.467	0.78	0.47	0.035	ND	412
SD5	7.59	18.9	60	0.269	0.487	0.27	0.015	ND	166
SD9	7.57	18.8	73.1	0.26	0.47	0.26	0.015	ND	151
SD13	7.44	17.5	-183.6	0.32	0.559	0.32	0.009	ND	157

^a Abbreviations: TDS, total dissolved solids; Fe_{tot}, total iron; Fe(II), ferrous; ND, not detected.

mixture was left to equilibrate for 20 min after shaking for 5 min. The pH was measured using a calibrated HQ30d pH meter (Hach, Loveland, CO). Total sulfur, soluble sulfur, and total organic carbon in sediments were measured using an elemental analyzer (vario MACRO cube; Elementar, Hanau, Germany) (8, 10).

DNA extraction, PCR amplification, and sequencing analysis. Eleven samples were selected for molecular analysis. The genomic DNA was extracted using the FastDNA spin kit (MP Biomedicals, Santa Ana, CA) by following the manufacturer's protocol. All DNA extracts were stored at -80°C until analyzed. The V4 region of the 16S rRNA gene was amplified using the 515f/806r primer set (11). 16S rRNA gene tag-encoded ultrahigh-throughput sequencing was carried out using the Illumina MiSeq platform at Novogene (Beijing, China).

Sequences were analyzed using Quantitative Insights Into Microbial Ecology (QIIME) software and the UPARSE pipeline (12). Default settings for Illumina processing in QIIME were used, and then the UPARSE pipeline was used to assign taxonomy to 97% similarity via the RDP classifier (13). Estimated species richness indicated with Chao1 and Shannon indexes for 11 libraries were calculated (14).

Statistical analyses. The similarity of microbial communities among different sediment samples was determined using UniFrac. QIIME calculated both weighted and unweighted UniFrac. The unweighted pair group method with arithmetic mean (UPGMA) was conducted on weighted UniFrac (15). Canonical correspondence analysis (CCA) was performed to measure chemical properties that had the most significant influence on the microbial community structure. The significant correlations of the physicochemical parameters were examined by Monte Carlo permutation.

The triplot was generated by CANOCO 4.5 (Biometrics Wageningen, The Netherlands). Except as otherwise noted, the figures were generated by CanoDraw 4.0 (Biometrics Wageningen).

Illumina sequencing data accession number. The reads generated by ultrahigh-throughput sequencing were deposited into the NCBI Sequence Read Archive database under accession number [SRP044396](https://www.ncbi.nlm.nih.gov/sra/SRP044396).

RESULTS

Physicochemical characterization of collected samples. Eleven samples were collected from four geographical zones, including Dapo Creek (zone 1 for samples B1 and B2), the Youyu River (zone 2 for samples SD1 to SD4), the Aha Reservoir (zone 3 for SD5, SD6, SD7, and SD9), and the Xiaoche River (zone 4 for SD13). As shown in Tables 1 and 2, the physicochemical parameters of the water and sediments differed significantly in the samples collected from environments with different extents of AMD contamination. The pH was very low (<3) in Dapo Creek at zone 1 and increased in downstream rivers and reservoirs within zones 2 to 4, with pHs typically higher than 6.5. The total soluble Fe in the AMD-contaminated creek was much higher than that in zones 2, 3, and 4 due to the elevated solubility of iron compounds in strongly acidic environments. Most Fe(II) was rapidly oxidized to Fe(III) due to the increase in pH of the Youyu River. The increase in pH was accompanied by a significant decrease in soluble Fe in zones 2 to 4. In addition, the AMD effluents had high sulfate

TABLE 2 The pHs and concentrations of total nitrogen, total carbon, organic carbon, total hydrogen, and total and soluble sulfur in 11 sediment samples^a

Geographical zone	Sample	pH	Concn (g/kg) of:					
			TN	TC	TOC	TS	SS	H
Zone 1	B1	2.15	0.1974	1.2214	0.532	0.5821	0.555	0.2447
	B2	2.02	0.0216	0.1426	0.136	0.5875	0.563	0.186
Zone 2	SD1	7.76	0.022	0.9281	0.249	0.0606	0.051	0.0736
	SD2	7.39	0.0327	0.465	0.349	0.0417	0.029	0.1379
	SD3	7.84	0.0242	1.0477	0.855	0.1219	0.081	0.1188
	SD4	7.66	0.0267	1.063	0.77	0.1121	0.071	0.1236
Zone 3	SD5	7.4	0.0272	0.8025	0.262	0.0805	0.032	0.0949
	SD6	6.66	0.0218	0.8003	0.633	0.1879	0.151	0.1388
	SD7	6.48	0.0238	0.8687	0.675	0.1701	0.133	0.1442
	SD9	7.25	0.0298	0.4697	0.271	0.1002	0.083	0.1022
Zone 4	SD13	6.86	0.053	0.7769	0.565	0.4878	0.423	0.1453

^a Abbreviations: TN, total nitrogen; TC, total carbon; TOC, total organic carbon; TS, total sulfur; SS, soluble sulfur; H, total hydrogen.

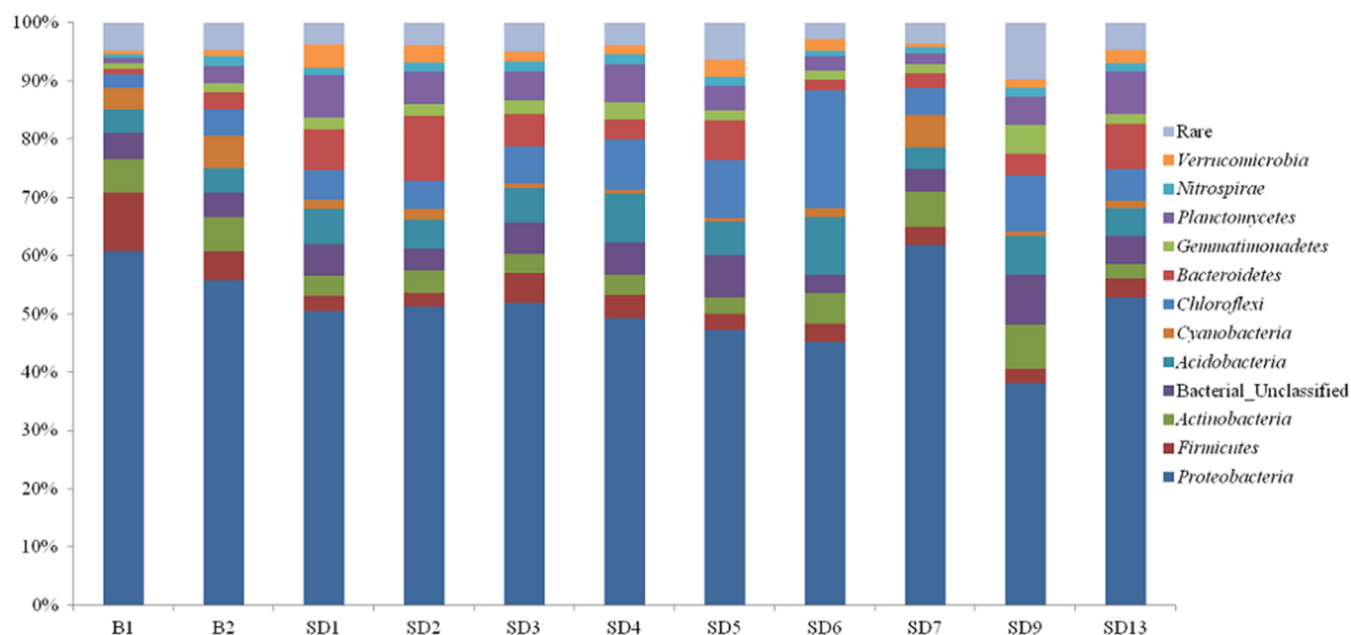


FIG 2 Taxonomic classification of bacterial reads retrieved from different watershed samples at phylum level using the RDP classifier. “Rare” indicates phyla with relative abundances of less than 0.5%.

concentrations (~ 7 g/liter). In contrast, concentrations of sulfate in downstream samples were orders of magnitude lower and ranged from 112 mg/liter to 412 mg/liter. This may be mainly due to dilution given that the volume of the Youyu River is much greater than that of AMD streams. The redox potentials were also high in the AMD-contaminated creek (375.1 to 445.2 mV) but decreased to less than 100 mV in downstream samples.

All the sediment samples taken from zones 2, 3, and 4 showed high concentrations of SiO_2 and relatively high concentrations of Al_2O_3 (see Table S1 in the supplemental material). In comparison, Fe_2O_3 was not distributed evenly within the watershed and was predominant in two samples from the AMD-contaminated creek. Among samples from the other zones, however, only sample SD2, which was taken adjacent to the abandoned mines, exhibited high Fe_2O_3 abundance (43.72%). Fe_2O_3 abundance was particularly low in samples SD1 and SD9. Therefore, these sediments were probably not contaminated or only lightly contaminated by AMD. The relatively high Fe_2O_3 concentrations in the sediment samples from Aha Reservoir (SD6 and SD7) indicated a long-term accumulation of iron compounds from acid mine waters.

As shown in Table 2, other geochemical and physiochemical parameters (e.g., total nitrogen, total carbon [TC], total organic carbon [TOC], total hydrogen, and total and soluble sulfur) exhibited different trends across samples. The entire watershed demonstrated low concentrations of total nitrogen. The total and organic carbon was relatively high in Youyu River but was low in sample B2. Total and soluble sulfur was high in sediments of Dapo Creek and the Xiaoche River but was relatively low in sediments from the Youyu River and Aha Reservoir. One possible explanation for the high soluble sulfur in sediments from the Xiaoche River is that a pipeline was connected between the Xiaoche River and the bottom of the Aha Reservoir. Slurry at the bottom of the Aha Reservoir was discharged into the Xiaoche River. Hence, the Xiaoche River may have accumulated large amounts of sulfur-containing compounds from the Aha Reservoir.

General analyses of the Illumina-derived data set. After filtering of the low-quality reads and chimeras and trimming of the adapters, as well as bar codes and primers, a total of 691,040 valid reads (average read length, 275 bp) were identified from 11 Illumina sequencing libraries. Nonparametric indicators of community diversity (Chao1 and Shannon indexes) revealed that the diversity of communities was not uniform throughout the watershed (see Table S2 in the supplemental material). The lightly and moderately contaminated sediments, SD1 and SD3, had the richest diversity, with operational taxonomic unit (OTU) numbers of 5,402 and 5,197, respectively, followed by the sediment sample from the Youyu River (SD4; OTU number of 5,130) and the upper layer sediment sample of the Aha Reservoir (SD5; OTU number of 5,109). In comparison, the two acidic sediment samples, B1 and B2, from an AMD-polluted area displayed considerably less richness, with OTU numbers of 4,059 and 2,951, respectively.

The most abundant prokaryotic organisms in all samples phylogenetically belonged to the *Proteobacteria* phylum, accounting for 38 to 62% of the total valid reads in all libraries (Fig. 2; see also Table S3 in the supplemental material). Within the *Proteobacteria*, *Betaproteobacteria* was the most abundant class in eight libraries, accounting for 9% to 35% of the total reads, while *Gammaproteobacteria* was the most abundant class in the libraries constructed from B2 (30%) and SD7 (35%). Frequently, pollution-related changes in the relative abundances of other phyla were observed. For example, *Cyanobacteria* and *Firmicutes* occurred at higher abundances in the libraries from Dapo Creek than in those from downstream samples. However, *Chloroflexi* showed relatively higher abundances in several libraries from the sediment samples taken from the Aha Reservoir but was less abundant in the upstream AMD-contaminated creek. Other phyla, such as *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, and *Nitrospirae*, that are most often encountered in AMD worldwide were detected in the libraries retrieved from all 11 samples. In addition to bacteria, two archaeal phyla were also detected. *Crenarchaeota* was detected in

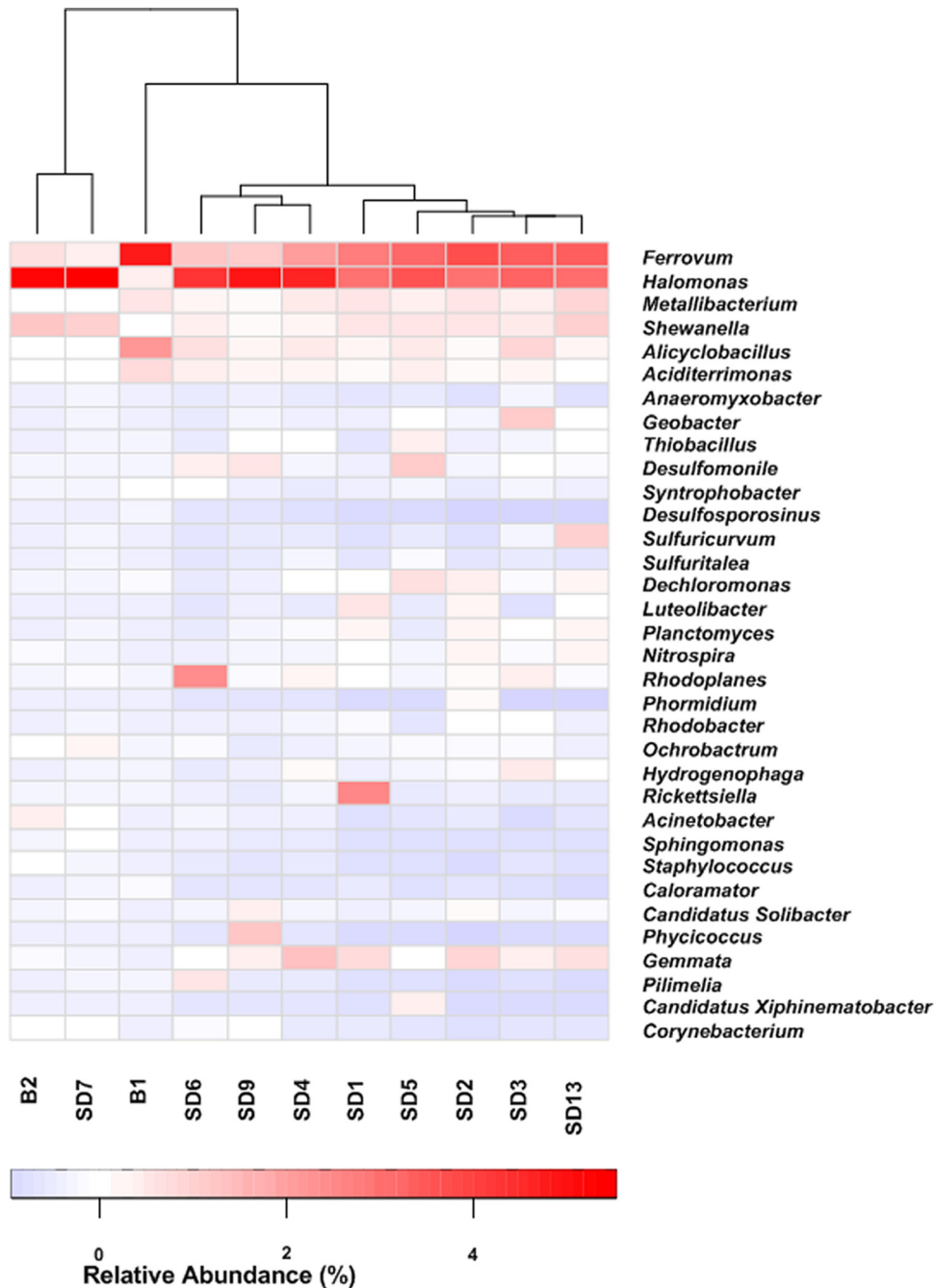


FIG 3 Heat map analysis of the distribution of dominant phylotypes in the 11 samples. The double hierarchical dendrogram shows the microbial distribution of the 11 samples. The relative percentages for the microbial genera are depicted by the color intensity; the color key is at the bottom.

all samples, with relative abundances ranging from 0.02% to 2%. *Euryarchaeota* was detected in eight samples but not in sample B1, SD2, or SD6.

At the genus level, the microbial compositions also differed significantly across the four zones (Fig. 3; see also Table S4 in the supplemental material). Among the genera identified, *Ferrovum* showed relatively high abundance (>1%) in all the libraries, especially in the library retrieved from B1 (>13%). *Halomonas* was another genus with relative abundances over 2% in all the sequencing libraries. Other genera only demonstrated relatively

high abundances in part of the samples. For instance, the sequences assigned to *Shewanella* were found at high abundances in libraries from B2 (4%) and SD7 (4%). The sequences related to *Alicyclobacillus* displayed high relative abundance in B1 (7%) but an abundance lower than 2% in all the other libraries. Detailed information about the relative abundances of dominant genera in each sample is provided in the heat map (Fig. 3) and Table S4.

Differences between 11 microbial communities were demonstrated from the 16S rRNA amplicon data. The UPGMA tree (Fig. 4) indicated that sequencing libraries from five river sediment

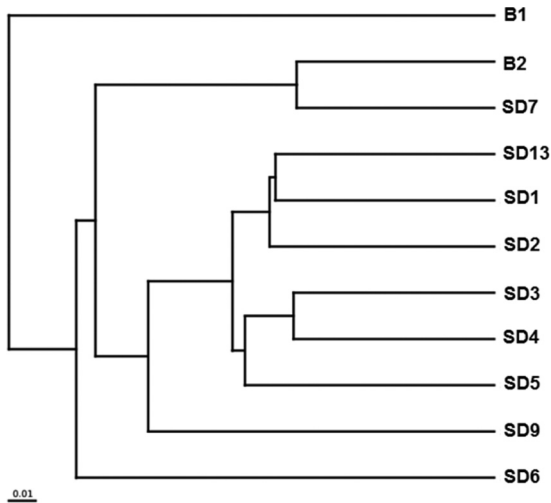


FIG 4 UPGMA tree showing clusters of microbial communities based on weighted UniFrac with 100% support at all nodes.

samples (SD1 to SD4 and SD13) and one upper layer sediment of Aha Reservoir (SD5) were clustered. However, the microbial community in sample B1 was distinct from those in the downstream rivers and reservoir. The general taxonomic patterns in Fig. 4 were mainly driven by differences in the abundances of major taxonomic groups. Some genera, such as *Ferrovum*, *Halomonas*, *Alicyclobacillus*, and *Shewanella*, were observed throughout the watershed, but the abundances of these bacteria were generally higher in the heavily AMD-contaminated area (Fig. 3). In contrast, some bacteria, including members of the genera *Aciditerrimonas*, *Syntrophobacter*, and *Desulfosporosinus*, accounted for relatively higher proportions in the libraries retrieved from heavily AMD-contaminated areas, suggesting that low pH may have stimulated these bacteria. In contrast, *Desulfomonile*, *Hydrogenophaga*, *Geobacter*, *Sulfuricurvum*, *Dechloromonas*, and *Thiobacillus* species appeared to be more abundant in less contaminated areas.

Relationship between microbial community and the environment. The CCA that showed the relationship between environmental variables and microbial communities was conducted to examine correlations between genera with a relative abundance greater than 1% in at least one Illumina sequencing library and selected geochemical parameters (pH, temperature, E_h , SO_4^{2-} , total Fe in each water sample, and concentrations of TC and TOC in each sediment sample). CCA ordination (Fig. 5) showed that specific geochemical conditions shaped the variations in microbial community compositions. CCA axis 1 and TC had a strong positive correlation. CCA axis 2 was positively correlated with pH and negatively correlated with temperature, E_h , sulfate, and total Fe. The relatively small magnitude of the TOC vector indicated that TOC was not as strongly correlated to community composition as other tested factors. *Syntrophobacter*, *Aciditerrimonas*, *Ferrovum*, *Alicyclobacillus*, and *Desulfosporosinus* were positively correlated with higher total aqueous Fe and sulfate concentrations and negatively correlated with pH, which may suggest that AMD environments may facilitate the growth of these bacteria. However, *Geobacter* and *Desulfomonile* were positively correlated with pH, indicating that these bacteria favor environments with higher pHs.

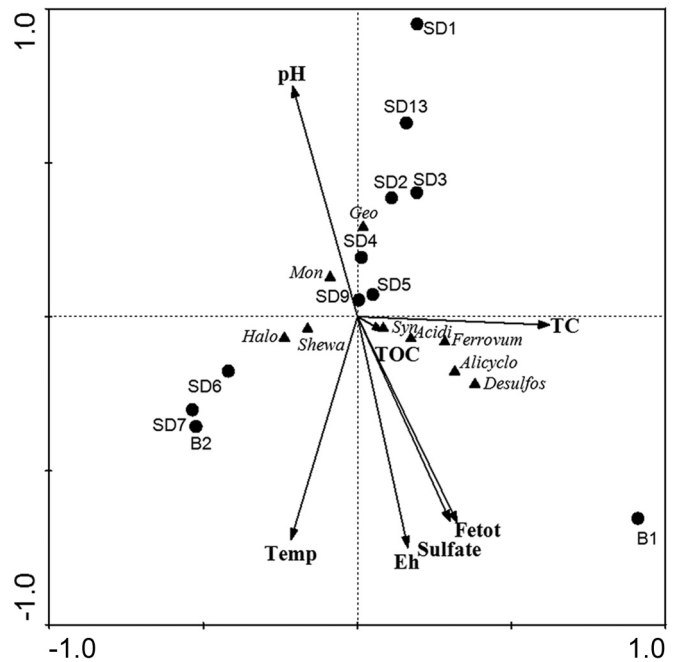


FIG 5 Ordination diagrams from canonical correspondence analysis (CCA) of bacterial abundances and geochemical parameters. Response variables of bacterial abundances are indicated by triangles. Arrows indicate the direction and magnitude of geochemical parameters associated with bacterial community structures. Each sample is represented by colored circles according to sampling zones. Abbreviations: Temp, temperature; Fetot, total Fe; TC, total carbon in the sediments; TOC, total organic carbon in the sediments. Bacterial abbreviations: *Halo*, *Halomonas*; *Shewa*, *Shewanella*; *Mon*, *Desulfomonile*; *Geo*, *Geobacter*; *Syn*, *Syntrophobacter*; *Acidi*, *Aciditerrimonas*; *Alicyclo*, *Alicyclobacillus*; *Desulfos*, *Desulfosporosinus*.

DISCUSSION

Spatial variations in physiochemical data. Based on physiochemical analyses, the sites studied in the present work were grouped within three major conditions, each with a varied extent of AMD contamination. Dapo Creek exhibited the greatest extent of AMD pollution, while the downstream Youyu River was moderately polluted. The Aha Reservoir and Xiaoche River were identified as lightly AMD-contaminated areas. In previous research, environmental gradients have been observed in other AMD-contaminated watersheds, such as the Amous River region in southern France (16), the Odiel River basin in southwest Spain (17), the abandoned Coval da M6 mine in Portugal (18), and the Molonglo River in Australia (19). Similar to these AMD-contaminated environments, the Aha watershed exhibited a transition in physicochemical water quality from the upstream Dapo Creek [low in pH but dominated with Fe(II)] to the downstream rivers and reservoir (moderate-pH and low-Fe environments).

Geochemical analyses of different iron and sulfur species along the Aha watershed suggested dynamic Fe- and S-related geochemical processes. The rapid oxidation of Fe(II) and subsequent precipitation of Fe(III) at neutral pH removed a large amount of dissolved Fe from the water body. However, precipitated Fe compounds accumulated in sediments, as demonstrated by the relatively high Fe_2O_3 concentrations in downstream sediments. Fe_2O_3 may have been utilized by microorganisms as the electron acceptor and therefore contributed to biogeochemical Fe cycling in the

watershed habitats. Similar to decreasing Fe(II) concentrations along the watershed, sulfate concentrations were also lower in the downstream water body. This may have been due to dilution from uncontaminated river water.

Environmental data that shape the microbial community composition. Our analyses revealed that microbial communities were patterned along the AMD pollution gradients. Statistical analyses suggested that microbial diversity in the heavily polluted upstream area was distinct from that in the downstream rivers and reservoir. Specifically, samples (e.g., B1 and B2) from the heavily AMD-polluted zone had a lower predicted microbial diversity than the less contaminated areas, which could be attributed to the harsh contaminated environments in zone 1. The environmental conditions, such as low pH and high Fe and sulfate concentrations, acted as environmental stressor for microbial adaptation. CCA was performed to discern how microbial communities adapt to AMD-contaminated environments. Total Fe and sulfate were the major environmental factors that affected the microbial community, as indicated by CCA (Fig. 5). With respect to AMD-contaminated environments, iron and sulfur compounds might have influenced the overall microbial communities primarily by controlling the distribution of Fe- and S-metabolizing microorganisms. This agrees with the observation that Fe-metabolizing bacteria were more frequently detected in libraries from samples in the AMD-contaminated creek. Meanwhile, sulfate-reducing bacteria (SRB) within the orders *Desulfobacterales*, *Desulfuromonadales*, *Syntrophobacterales*, *Clostridiales*, and *Desulfovibrionales*, which exhibited increased relative abundances in downstream rivers, may contribute to decreases in sulfate concentration in downstream rivers and reservoirs.

CCA indicated a significant relationship between overall community composition and pH. The strong selective pressure with extremely acidic conditions in zone 1 determined which microorganisms could survive. In downstream zones where pH was moderate, however, increased amounts of neutrophiles were present and therefore changed the overall microbial community. This observation is consistent with the previously published studies on AMD. For example, Kuang et al. (20) recently explored the phylogenetic differentiation among 59 microbial communities from different AMD sites across southeast China, finding that microbial communities were largely correlated with pH. In another study, pH was correlated with overall microbial communities in the Rio Tinto, which is an extremely acidic, Fe-rich location (21). Microbial communities were also significantly altered by pH in other Fe-S-rich environments, such as in black shale weathering profiles (22) and rice paddy soils which were irrigated using AMD-contaminated water (23). It is suggested that pH influences microbial survival directly and/or through control of ancillary environmental parameters that are closely related to soil pH, such as nutrient availability and cationic metal solubility (24).

There was also a significant and strong correlation between overall microbial community and temperature. Temperature has been previously reported as an important factor in shaping microbial community composition in AMD (16, 25). For example, Volant et al. used CCA and found that temperature was one of the primary environmental factors that shape the composition of bacterial communities along an acid drainage stream (16). Similarly, in the present study, temperature was strongly and significantly linked to bacterial community variance, especially to *Halomonas* and *Shewanella* populations (Fig. 5).

Biogeochemical element cycling in the watershed. Although microorganisms have not been isolated in this study, it may be possible to predict their metabolic capabilities based on those of phylogenetically related microorganisms, since closely related microorganisms tend to have similar physiological traits (26). The majority of the most abundant genera identified in this study are known to be involved in Fe and S cycling, indicating dynamic biogeochemical Fe and S cycling in the watershed as reported previously (5, 27).

Biogeochemical Fe cycling. *Ferrovum* and *Alicyclobacillus* were the commonly detected acidophilic Fe-oxidizing bacteria (FeOB) in AMD (5). *Ferrovum* spp. are aerobic, acidophilic, and autotrophic FeOB (28) and have been shown to be the predominant organisms in many AMD-impacted systems (20). Previous research reported that “*Ferrovum myxofaciens*” is known to produce copious amounts of extracellular polymeric substances (EPS), which are the major components of “acid streamers” (28). This agrees with our finding that *Ferrovum* was in highest relative abundance in the library retrieved from site B1 (acid streamers as shown in Fig. S1 in the supplemental material). *Alicyclobacillus* also demonstrated high abundances in many libraries. Most described *Alicyclobacillus* species are acidophilic, soil-dwelling *Firmicutes*. Isolates of these bacteria grow at a pH range of 2.0 to 6.0 (29). Prior studies that isolated these bacteria from mining areas showed that they were able to oxidize ferrous iron and reduced sulfur (30–32). In addition, many *Alicyclobacillus* species are able to grow using organic carbon sources (33, 34) and could grow in oxygen-free media using ferric iron as a terminal electron acceptor (35). *Halomonas* was abundant in all 11 libraries, with the highest relative abundances in libraries from Dapo Creek. Previous research has isolated a novel chemolithoautotrophic FeOB closely related to *Halomonas* from low-temperature weathering habitats in deep-sea hydrothermal areas (36, 37). Interestingly, recent studies have detected *Halomonas*-related bacteria in AMD environments (38, 39). However, the role of *Halomonas* species in AMD environments requires further investigation.

Acidithiobacillus, *Thiobacillus*, and *Leptospirillum* have frequently been detected in AMD environments (4, 40), but these FeOB showed relatively low abundances in the current study (see Table S4 in the supplemental material). *Leptospirillum* populations are often reported to be dominated in low-pH AMD environments (e.g., pH < 2) (41, 42) but are minor community members or absent in AMD with pHs above 2.5 (43, 44). The pH in Dapo Creek (pH > 2.5) may have been too high to facilitate the proliferation of *Leptospirillum*. *Acidithiobacillus* and *Thiobacillus* are reported as the dominant FeOB in AMD with pH values similar to those in the current study (3, 45). It is possible that other environmental factors may have affected the abundance of *Acidithiobacillus* and *Thiobacillus*. *Gallionella* is an FeOB commonly detected in neutral environments (46–48); however, it exhibited very low abundance in neutral downstream samples from this study. Other FeOB, including *Aciditerrimonas*, *Thermomonas*, and *Aquabacterium*, were also detected at lower abundances in the current study.

A wide variety of known Fe-reducing bacteria (FeRB) were detected in the watershed, especially in Dapo Creek. *Shewanella*-related sequences demonstrated relatively high abundances in libraries retrieved from most samples, especially in B2 and SD7. *Shewanella* species in the *Gammaproteobacteria* are well known for their capability of Fe(III) reduction. *Shewanella* species have been

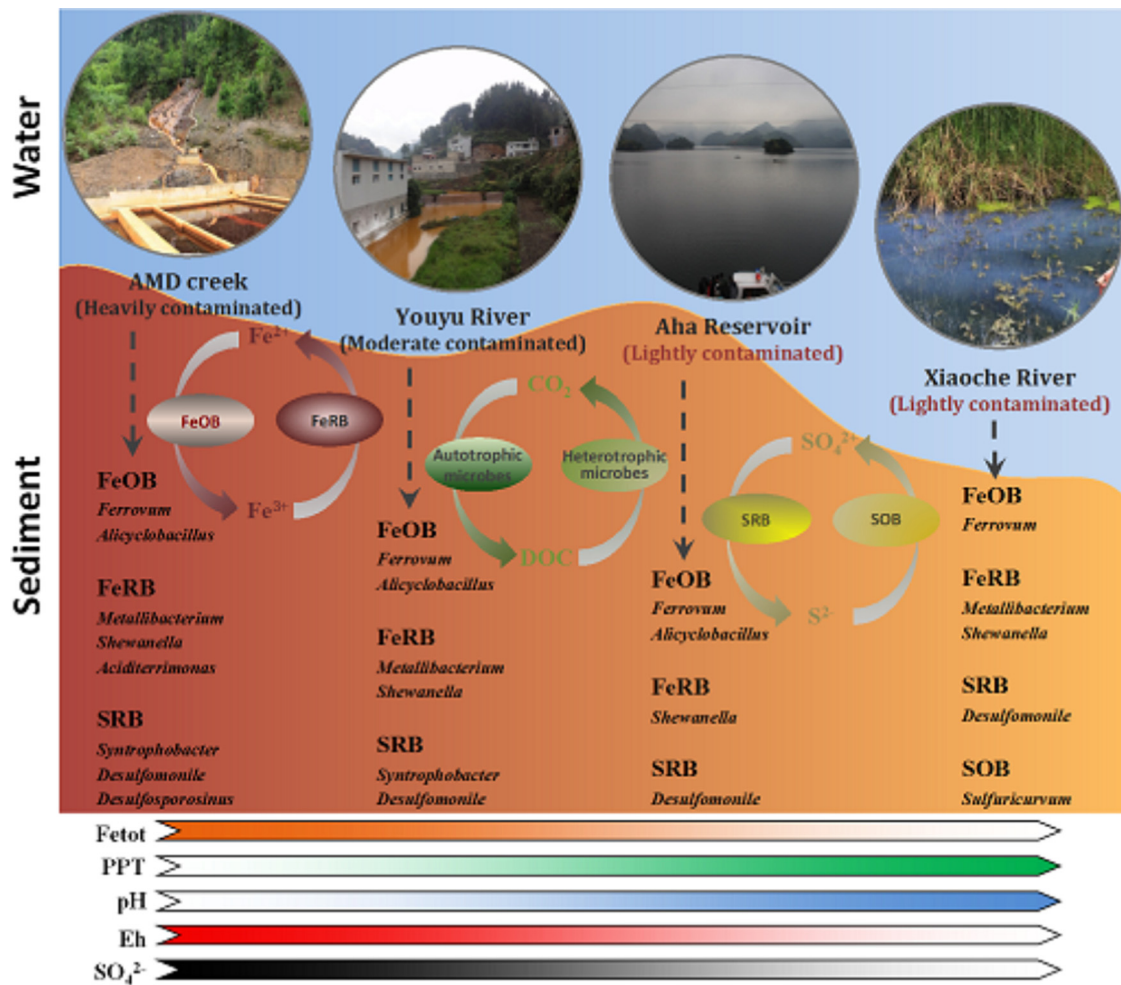


FIG 6 Geomicrobiological model of the Aha watershed profile. The model shows the roles of the major S- and Fe-related bacteria identified in the current study. The color of the arrows indicates the extent of the physiochemical parameters or geochemical process. A transition from saturated to transparent colors in a color ribbon indicates a decrease in the corresponding environmental factor from zone 1 to zone 4 and vice versa. Abbreviations: FeOB, Fe(II)-oxidizing bacteria; FeRB, Fe(III)-reducing bacteria; SOB, sulfur-oxidizing bacteria; SRB, sulfate-reducing bacteria; Fetot, total Fe; PPT, precipitation.

frequently isolated from marine environments (49–51). Our study suggested that *Shewanella* species were able to thrive, and may even play an important functional role, in Fe(III) reduction in AMD-impacted settings. The high abundances of *Shewanella* species in all sediment samples indicated that *Shewanella* were present in a wide range of pHs. *Metallobacterium*-related sequences were frequently detected in libraries derived from Dapo Creek and some downstream samples. The genus *Metallobacterium* is comprised of acidophilic microorganisms capable of heterotrophic or lithotrophic Fe(III) reduction (52, 53). In an increasing number of recent studies, *Metallobacterium* populations have been detected in high-Fe environments (53, 54). These observations may be attributed to their metabolic capability in iron reduction. *Aciditerrimonas* is another potential FeRB showing high abundances in the libraries retrieved from Dapo Creek, especially in sample B1. *Aciditerrimonas* contains thermophilic Fe(III)-reducing bacteria (55). Our current knowledge on this genus remains limited, especially concerning AMD-contaminated environments. Another important FeRB, *Geobacter*, was present in low abundances in libraries from the AMD creek but in higher

abundances in libraries from the Youyu River, which may suggest that acidic environments may not favor this important FeRB.

Biogeochemical S cycling. Sulfate reduction has a central role in AMD bioremediation and leads to the consumption of protons, increases the pH, and then generates sulfide as the reaction products to precipitate heavy metals (27). In contrast to the case with dissimilatory iron reduction, few acidophilic bacteria and archaea that can reduce sulfate have been described. The optimum growth conditions for SRB were found at pHs ranging from 5.0 to 9.0 (56, 57). In the present study, the low pH and high redox potentials in Dapo Creek may not have allowed for a beneficial habitat for SRB. However, many SRB were detected in acidic sediments in this study. *Syntrophobacter* and *Desulfosporosinus*, two genera containing many species involved in sulfate reduction (58–61), showed high relative abundances in libraries from Dapo Creek. Both *Syntrophobacter* and *Desulfosporosinus* have regularly been detected in AMD waters (62–65). It is noteworthy that *Desulfosporosinus* species are able to reduce various metals, such as Fe(III), Mn(IV), and U(VI), in acidic solutions (pH 4.2), indicating a potential role of *Desulfosporosinus* species for biological treatment of AMD (66).

The presence of various SRB indicated that SRB may have been able to cope with extremely harsh environments and survive under acidic conditions.

Biogeochemical C cycling. It has been reported that most subsurface AMD sites receive minimum carbon input from external sources (5). However, sediment B1 demonstrated a high proportion of TC and TOC content. Consistent with high concentrations of TC in AMD-contaminated creeks, *Comamonadaceae*, which are metabolically versatile and can use a wide range of organic compounds as carbon sources (60, 67), were one of the most dominant families in Dapo Creek. A broad range of other heterotrophic bacteria were also detected in zone 1, indicating dynamic C cycling in acidic AMD environments.

Interaction among microorganisms with various physiological traits may be critical in maintaining the metabolic diversity in AMD environments. For example, acidophilic autotrophs (e.g., *Ferrovum*) are able to produce organic matter which might later be used by heterotrophic organisms. The utilization of organic matter, which is toxic to autotrophs, could conversely benefit the growth of autotrophs (5).

Conclusion. In the present study, we aimed to more accurately characterize microbial communities in an AMD-impacted watershed by high-throughput sequencing. Our results indicated that a number of Fe- and S-metabolizing bacteria exhibited high abundances in the AMD-contaminated watershed. Furthermore, dominant functional groups varied along the AMD pollution gradients (Fig. 6). The spatial distribution of microbial communities in AMD pollution gradients facilitates the understanding of the metabolic capacities of indigenous microorganisms. Such knowledge may lead to the development of approaches for remediation of AMD contamination. It has been reported that bioreactor systems using acidophilic and sulfidogenic bacteria that are indigenous to mine-impacted environments could remove iron and precipitate other metals from mine waters (68, 69). These studies suggested that iron was removed by microbial iron oxidation and stable metal sulfides were selectively precipitated by acidophilic SRB (68, 69). Therefore, the phylogenetically divergent lineages coexisting in the Aha watershed may have the potential for *in situ* natural attenuation of AMD pollution. Future work should explore the significance of these phylotypes in natural attenuation of AMD at this and other AMD sites.

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