

## Archaeal and bacterial communities in acid mine drainage from metal-rich abandoned tailing ponds, Tongling, China

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**Abstract:** To expand knowledge on microbial communities of various metal-rich levels of mine drainage environments in Anhui province, China, the archaeal and bacterial diversities were examined using a PCR-based cloning approach. Eight acid mine water samples were collected from five areas in Tongling. Phylogenetic analyses revealed that bacteria mainly fell into ten divisions, which were *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Deinococcus-Thermus*, *Nitrospira*, *Firmicutes*, *Actinobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Chloroflexi*. Archaea fell into three phylogenetic divisions, *Thermoplasma*, *Ferroplasma* and *Thermogymnomonas*. The unweighted pair group method with arithmetic mean (UPGMA) cluster analysis based on the microbial communities' compositions revealed that five samples shared similarity with the dominance of *Meiothermus* and *Thermomonas*. Two samples had the preponderant existence of *Acidithiobacillus* and *Leptospirillum*. The remaining sample owned higher microbial communities' diversity with the Shannon-Weaver  $H$  up to 2.91. Canonical correlation analysis (CCA) suggested that microbial community structures had great association with pH and the concentration of  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Fe}^{3+}$ ,  $\text{Cl}^-$ ,  $\text{SO}_4^{2-}$  in water.

**Key words:** acid mine drainage; microbial community; clone library; geochemical variables

### 1 Introduction

Acid mine drainage (AMD) water is a worldwide environmental problem caused by active and abandoned mines [1]. It has caused severe environmental problems, including contamination of rivers, streams and ground water. AMD arises when metal sulfide minerals, particularly pyrite ( $\text{FeS}_2$ ), are exposed to oxygen and water during the mining of metals and coals [2]. The overall procedure can be described as follows:  $\text{FeS}_2 + 14\text{Fe}^{3+} + 8\text{H}_2\text{O} \rightarrow 15\text{FeS}_2^{2+} + 2\text{SO}_4^{2-} + 16\text{H}^+$  [3], and the ongoing oxidation of sulfide minerals really depends on the regeneration of ferric iron. At pH values below 4, the rate of chemical oxidation of ferrous iron by  $\text{O}_2$  is negligible [4]; therefore, the activities of acidophilic iron-oxidizing microorganisms play a pivotal role in converting ferrous to ferric iron in acidic environment [1]. Due to the low pH of AMD, the solubility of transition metals is greater and so AMD often typically contains elevated concentrations of metals, including iron, aluminium, manganese and other toxic transition metals who present depending on the mineralogy of the

host rock [3].

Despite the acidic nature and elevated dissolved metal concentrations, the water still has the presence of microorganisms. Previous studies based on the diversity of microorganisms in AMD with extremely low pH (<2) showed that acidophilic bacteria mainly included a group of sulfur and/or iron-oxidizers, classifying into *Acidithiobacillus ferrooxidans*, *Acidithiobacillus thiooxidans*, *Leptospirillum ferrooxidans* and *Ferroplasma* spp. [4,5]. With the application of unculture-based molecular phylogenetic techniques, the understanding of the diversity of acidophilic microorganisms was accelerated, including *Legionella*, *Thiomanas* and *Gallionella* [6].

The distribution of acidophiles in AMD has close association with temperature, pH and metal concentrations [3]. In general, mesophilic acidophiles can be exclusively detected in AMD, though increased temperature makes the moderate thermophiles dominate in the microbial populations, such as *Bacillus* spp. and *Sulfobacillus acidophilus* [7]. Conversely, in many low-temperature AMD waters, *Acidithiobacillus ferrivorans*, the newly described psychrotolerant species, tend to

dominate over *A. ferrooxidans* and *L. ferrooxidans*. Another controlling factor of microbial populations is pH. *A. ferrooxidans* and *L. ferrooxidans*, as the typical iron-/sulfur-oxidizing microbes, dominate in extreme acidic environment (pH<2); the moderate acidophiles, such as *Thiomonas* and *Halothiobacillus*, dominate in moderately acidic AMD (pH 3–6) [1,8]. The low pH of AMD results in the solution of metals. The composition of AMD is also influenced by the concentrations of metals [3], such as Fe, Cu, and As. Besides, the low concentration of arsenic might facilitate a higher community proportion of *Acidithiobacillus* [9]. The composition of various tailing ponds may influence the dissolved elements in AMD. Previous studies showed that the microbial communities differed with the various tailing ponds. HAO et al [6] detected the dominance of chemoorganotrophic and photosynthetic metabolisms, such as *Acidiphilium*, based on the research of sulfide mine in Xiang Mountain, Anhui province. XIAO et al [10] showed that samples collected from Yunfu sulfide mine were rich in *Acidithiobacillus*, while waters from Yinshan lead–zinc mine were dominated in *Leptospirillum*; in addition, there was no detection of archaea in the study [10].

Although there have been some reports on the bacterial ecology of AMD, few detailed studies were on the various acidification levels of AMD involving different abandoned tailing ponds. The major objectives of this study were: to investigate the archaeal and bacterial communities of mine drainage from different mineral compositions of five main tailing ponds using a PCR-based cloning approach, and to explore the geochemical factors, resulting in community changes of mine drainage in tailing ponds.

## 2 Experimental

### 2.1 Sites description

Tongling, located in the southern bank of the middle and lower reaches of Yangtze River, has the subtropical monsoon climate, with an annual average temperature of 16.2 °C and the summer average temperature of 27.4 °C. On average, the frost-free period is 237–258 d, and rainfall is abundant with the mean annual precipitation of 1390 mm and mean annual humidity of 75%–81% [11,12].

Among these six study areas, there were five tailing ponds, Jinkouling, Tongguanshan, Shuimuchong Yangshanchong and Heishahe, discarded from 1980s to 2010s; the rest one was still under use, named Chaoshan gold mine [13]. Sample A was collected from Jinkouling tailing pond, which had been discarded for 20 years; and the bed rock was composed of pyrrhotite, pyrite, magnetite, hematite, limonite, siderite and a small

amount of chalcopyrite. Tongguanshan tailing pond was discarded in 1991, from which samples B and C were collected. Samples D and G were collected from Shuimuchong tailing pond and Chaoshan gold mine, respectively. There were four samples collected from Yangshanchong tailing pond, named E, F, H and I. This tailing pond began natural ecological restoration process after being discontinued in 1991. Tailing wasteland is mainly composed of stone powder and sandy texture, and the main mineral in tailings is calcium iron (aluminum) garnet, quartz, feldspar and pyroxene. Sample J was collected from Heishahe tailing pond, which has been discharged for more than 50 years. Due to the fluctuation of the Yangtze River, many naturally colonized plants grew here; moreover, oilseed rape and some other crops were planted by the local farmers, so tailing substrate was greatly improved [11].

### 2.2 Samples collection

A total of ten AMD samples were collected from five different tailing ponds in Tongling, Anhui Province, China, on February, 2012. The pH values of samples B (7.83) and J (6.08) went over 6.0, so they did not belong to the acidic waste water. They were excluded from the samples. For the eight remaining samples, approximately 10 L of water was collected from these tailing ponds, and then transported to the laboratory within 24 h. About 500 mL original water of each sample was taken out for geochemical analysis, and the remaining water of each sample was filtered through 0.22 μm hyper filtration membrane with vacuum pump, respectively. These filters were then immediately transferred to a tube and stored at –20 °C until they were used for analysis. The filtered water samples were for chemical analysis.

### 2.3 Geochemical analysis of water samples

Latitude and longitude of sampling sites were determined using a hand holding GPS. The measurements of  $\text{SO}_4^{2-}$ ,  $\text{Cl}^-$  and  $\text{PO}_4\text{-P}$  were carried by ion chromatography (ICS–1500),  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  by UV spectrophotometry.

Elemental analysis was carried out using inductively coupled plasma-atomic emission spectrometry (ICP-AES) (XSP Intrepid II, USA). Each sample was tested in the presence of 20 elements, Al, As, Ba, Be, Ca, Cd, Co, Cu, Fe, Hg, K, Li, Mg, Mn, Na, Ni, Pb, Se, Ti and Zn.

### 2.4 DNA extraction and purification

Each millipore filter was cut and placed into a 1.5 mL falcon tube, and then nucleic acids extraction was carried out according to the established procedures [13]. The crude DNA was purified by agarose gel electrophoresis (1%) and the Wizard DNA Clean-Up Kit

(Promega, Madison, Wis.). DNA was quantified by UV-visible spectrophotometry (Thermo, USA).

### 2.5 PCR and amplification of 16S rDNA

In the reactions, bacterial 16S rDNA genes were amplified with the primer set 27F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-CGGCTACCTTGTTACGACTT-3'). A PCR amplifier (Biometra, T-Gradient, Germany) was used to incubate reactions through an initial denaturation at 94 °C for 2 min, followed by 35 cycles at 94 °C for 40 s, 55 °C for 30 s, and 72 °C for 1 min, and completed with an extension period of 10 min at 72 °C. Products from the amplification reactions of expected size (about 1500 bp) were pooled and purified before ligation later.

PCR amplification of archaeal 16S rDNA genes was carried out following the PCR reactions described as above with archaea-specific primer set: Arch-21F(5'-TTCYGGTTGATCCYGCC RGA-3') and Arch-958R (5'-YCCGCGCTTGAMTCCAWTT-3') to yield 900 bp PCR products.

### 2.6 Cloning and sequencing

The purified PCR products were ligated to the pEASY-T3 cloning vector using a rapid ligation kit according to the instructions of the manufacturer (Transgen, Beijing, China), and then transformed to competent *Escherichia coli* cells (Transgen, Beijing, China). The transformed cells were plated onto Luria-Bertani agar plates in the presence of ampicillin. After about 14 h of incubating at 37 °C, about 150 white colonies were randomly selected from each library, followed by the sequencing of single clone (BGI, Beijing, PRC).

### 2.7 Phylogenetic analysis

The nucleotide sequences were manually checked for chimeras using Ribosomal Database Project II. Identified chimeric sequences were discarded. Operational taxonomic units (OTUs) were determined by neighbor-joining analysis of Mega 4.0, which shows the phylogenetic relationships of bacterial 16S rRNA gene sequences recovered from these AMD samples. The coverage of clone libraries was calculated using the equation:  $R_{cov}=1-n/N$ , where  $n$  is the number of clones that occurred only once and  $N$  is the total number of analyzed clones in each clone library [14].

### 2.8 Statistical methods

Canonical correspondence analysis (CCA) was performed to analyze the geochemical variables and community structure with CANOCO (Version 4.5, Biometris-Plant Research International, Netherlands) (Terbraak 1988). To evaluate the community diversity, Shannon-Weaver index ( $H$ ) was calculated using the

formula:  $H=-\sum(p_i)(\ln p_i)$ , where  $p_i$  is the relative abundance of the clone sequenced in each sample. Unweighted pair-group method with arithmetic means (UPGMA) was performed to reveal the distance relationship between sample by the archaeal and bacterial communities. Statistical product and service solution (SPSS) were conducted to reveal the correlation between geochemical variable and community structure.

### 2.9 Nucleotide sequence accession number

Sequences have been submitted to GenBank with accession numbers as follows: KC537424-KC537737, KC620584-KC620676, KC620745- KC620899 and KC749063-KC749417.

## 3 Results

### 3.1 Physico-chemical characteristics of samples

The samples were all collected from the exposed pit outdoor, and the temperatures of them were all about 10 °C. The pH values of the eight sites were not substantially different from each other in the range of 2–4. The pH values of samples A, D, G, I were under 2.5, and the colors of these samples were dark red, especially for sample D, with the strongest acidity (pH=2.04) and the deepest color (brown). The samples all contained high levels of  $\text{SO}_4^{2-}$ ,  $\text{Ca}^{2+}$ ,  $\text{Fe}^{3+}$  and  $\text{Mg}^{2+}$ ; and the concentration of  $\text{Ca}^{2+}$  was even higher than that of  $\text{Fe}^{3+}$ , maybe resulting from the surrounding tailings (Table 1). Besides, sample D had the highest concentrations of  $\text{SO}_4^{2-}$ , nitrate and electrical conductivity (EC). The ionic contents of samples A, G and I were also generally higher than other samples, and the ion concentrations varied widely. As shown in Table 1, sample C had the highest concentration of Mg, while sample E had the highest  $\text{NH}_4\text{-N}$ , sample F had the highest  $\text{PO}_4^{3-}\text{-P}$ . Meanwhile, other ion concentrations also fell in a wide range. As to the rest ions, Co, Cd, Ba, Li, Hg levels were ignorable.

### 3.2 Analysis of 16S rDNA cloning libraries

The PCR products of 16S rDNA gene with the expected size (bacteria 1500bp and archaea 900bp) were successfully amplified from community genome DNA of the samples. After TA cloning, a total of 856 positive colonies (603 for bacteria, 253 for archaea) were obtained from the eight AMD samples.

In order to test whether the analyzed clones could represent the bacteria community in each sample, the coverages of all clone libraries were calculated (Table 2). All samples had the coverage over 90%, which suggested that the obtained data could represent the real communities of bacteria. Number of OTUs in each sample is quite different from each other, with a range of

**Table 1** Physico-chemical characteristics of collected AMD samples

Sample	pH	COD/ (mg·L <sup>-1</sup> )	ρ(Cl <sup>-</sup> )/ (mg·L <sup>-1</sup> )	ρ(PO <sub>4</sub> <sup>3-</sup> -P)/ (mg·L <sup>-1</sup> )	ρ(SO <sub>4</sub> <sup>2-</sup> )/ (g·L <sup>-1</sup> )	ρ(NH <sub>4</sub> -N)/ (mg·L <sup>-1</sup> )	ρ(NO <sub>3</sub> -N)/ (mg·L <sup>-1</sup> )	EC/ (ms·cm <sup>-1</sup> )	ρ(Ca <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Fe <sup>3+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Mg <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Al <sup>3+</sup> )/ (mg·L <sup>-1</sup> )
A	2.65	104.04	17.86	7.14	2.343	2.27	17.23	3.63	228.90	73.88	35.15	5.37
C	3.37	60.26	3.20	5.13	2.297	0.96	1.42	3.35	253.70	47.47	82.73	20.40
D	2.11	63.04	2.60	5.00	6.272	1.72	126.26	5.89	111.10	121.20	26.16	9.84
E	2.70	156.33	8.65	4.90	1.841	4.81	33.04	3.11	186.50	17.23	6.31	11.49
F	3.48	143.84	9.61	8.76	0.833	3.74	27.03	1.81	165.00	8.48	4.20	5.02
G	2.33	28.14	3.67	4.89	3.865	3.28	34.46	4.58	138.60	62.81	28.76	33.71
H	2.54	155.78	2.24	4.90	2.651	2.00	43.57	3.59	357.50	108.10	16.44	5.23
I	2.40	156.97	4.82	4.89	2.982	4.23	38.86	3.85	423.30	151.80	17.25	23.66

Sample	ρ(Na <sup>+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Zn <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Mn <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Cu <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Pb <sup>2+</sup> )/ (mg·L <sup>-1</sup> )	ρ(K <sup>+</sup> )/ (mg·L <sup>-1</sup> )	ρ(As <sup>3+</sup> )/ (mg·L <sup>-1</sup> )	ρ(Co <sup>2+</sup> )/ (μg·L <sup>-1</sup> )	ρ(Cd <sup>2+</sup> )/ (μg·L <sup>-1</sup> )	ρ(Ba <sup>2+</sup> )/ (μg·L <sup>-1</sup> )	ρ(Li <sup>+</sup> )/ (μg·L <sup>-1</sup> )	ρ(Hg <sup>2+</sup> )/ (μg·L <sup>-1</sup> )
A	8.00	1.90	3.53	2.25	0.04	1.40	0.71	95.00	38.00	16.00	8.00	10.00
C	1.96	1.41	6.16	1.45	1.62	3.37	–	229.00	–	8.00	17.00	9.00
D	0.59	0.37	0.70	2.23	4.55	0.30	–	121.00	–	9.00	9.00	23.00
E	7.77	3.11	1.68	2.88	1.78	0.67	0.10	4.00	17.00	12.00	10.00	9.00
F	14.73	1.52	0.91	2.80	1.64	1.05	0.02	24.00	9.00	19.00	8.00	15.00
G	1.37	11.40	4.20	4.48	3.11	0.35	0.57	89.00	7.00	9.00	27.00	7.00
H	0.56	5.61	2.61	2.24	1.94	0.45	2.92	33.00	48.00	6.00	14.00	13.00
I	5.75	10.94	3.47	6.44	0.07	0.51	1.56	91.00	83.00	11.00	27.00	8.00

**Table 2** Analyses of bacterial 16S rDNA clones libraries constructed for AMD from Tongling

Sample	Number of clone	OTUs	Coverage/ %	Shannon-weaver index <i>H</i>
A	93	20	92.47	2.36
C	71	6	98.59	1.21
D	72	10	95.83	1.17
E	73	12	95.89	1.95
F	72	6	97.22	1.07
G	68	10	91.18	1.49
H	71	5	98.59	1.16
I	83	12	93.98	1.39

**Table 3** Analyses of archaeal 16S rDNA clones libraries constructed for AMD from Tongling

Sample	Number of clone	OTUs	Coverage/ %	Shannon-weaver index <i>H</i>
A	31	2	100.00	0.24
C	27	3	100.00	0.67
D	30	3	96.67	0.68
E	32	4	100.00	1.16
F	31	4	100.00	1.22
G	35	3	100.00	1.12
H	35	3	100.00	0.57
I	32	4	93.75	0.89

5–20. Sample A had the most OTUs (27), sample E (12) and sample I (12) the second, sample C (6), sample F (6) next, sample H (5) was the lowest. This suggested that there contained less kinds of bacteria in samples C, F and H.

There were at least 27 archaeal cloning sequences obtained for each sample, but only four patterns of OTUs were found, and not all samples contained the four OTUs (Table 3). From the coverage, it could be shown that the number of clones tested in the experiment was sufficient to detect the level of archaeal community diversity within these eight samples.

### 3.3 Phylogenetic analysis of bacterial sequences

Phylogenetic analysis was established by a bootstrap neighbor-joining method, and the 16S rRNA gene sequences of the bacterial library fell into ten phylogenetic divisions: *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Deinococcus-Thermus*, *Nitrospira*, *Firmicutes*, *Actinobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Chloroflexi* and some unknown bacterial clusters (Table 4).

The *Gammaproteobacteria* group was ubiquitous in these eight samples. For samples A, D, I, the group

**Table 4** Relative abundance of bacterial clones affiliated with each major group

Sample	Relative abundance/%				
	<i>Betaproteobacteria</i>	<i>Gammaproteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Deinococcus-Thermus</i>	<i>Nitrospira</i>
A	54.84	29.03	1.08	–	5.38
C	4.23	45.07	11.27	39.44	–
D	–	72.22	9.72	–	9.72
E	4.11	46.58	9.59	27.40	5.48
F	2.78	40.28	9.72	47.22	–
G	1.47	39.71	20.59	36.76	–
H	1.41	45.07	14.08	39.44	–
I	6.02	45.78	–	–	42.17

Sample	Relative abundance/%					
	<i>Firmicutes</i>	<i>Actinobacteria</i>	<i>Deltaproteobacteria</i>	<i>Bacteroidetes</i>	<i>Chloroflexi</i>	Others
A	–	3.23	–	6.45	–	–
C	–	–	–	–	–	–
D	4.17	2.78	–	–	1.39	–
E	1.37	–	5.48	–	–	–
F	–	–	–	–	–	–
G	–	–	–	–	–	1.47
H	–	–	–	–	–	–
I	2.41	–	3.61	–	–	–

was mainly classified by *Acidithiobacillus* with the proportion of 17.20% (A), 70.83% (D) and 40.96% (I) of total bacterial clones. Sample A still contained the genus of *Halothiobacillaceae* and *Legionella*. For samples C, E, F, G, H, *Gammaproteobacteria* was almost composed of *Thermomonas* (45.07%, 31.51%, 40.28%, 35.29% and 43.66%). *Betaproteobacteria* was detected in samples but sample D, with the dominant in sample A. The group was mainly constituted of *Massilia*, *Herminiimonas* (4.30% and 5.38%) in the family of *Oxalobacteraceae*; *Polaromonas* (5.38%) in *Comamonadaceae*; *Thiobacillus* and *Sulfuricella* (8.60% and 20.43%) in *Hydrogenophilaceae* for sample A. *Alphaproteobacteria* widely distributed in the samples except sample I. For sample D, the group was almost composed of *Rhodobacter* (5.56%), *Phyllobacteriaceae*. Samples C, E, F, G and H had the proportions of 7.04%, 4.11%, 9.72%, 14.71%, 8.45%, respectively.

The genus of *Meiothermus*, in the family of *Deinococcus-Thermus*, had the largest proportions in samples C, E, F, G and H (39.44%, 27.40%, 47.22%, 36.76% and 39.44%), but it is not detected in other samples. *Leptospirillum* in the family of *Nitrospira* appeared in samples A, D, E and I, and gained superiority especially in sample I (42.17%). *Firmicutes* was detected in samples D, E and I with no significant advantage. *Actinobacteria* was found in samples A and D, the remaining *Deltaproteobacteria* was detected in samples E and I; *Bacteroidetes*, *Chloroflexi* only appeared in samples A and D, respectively.

The phylogenetic diversity was evaluated for each sample from the clone data by Shannon-Weaver index  $H$ . The results showed that sample A ( $H=2.36$ ) had the preeminent diversity, followed by sample E ( $H=1.95$ ), while sample F ( $H=1.07$ ) possessed the lowest  $H$ , which was less than half of sample A (Table 3). The remaining five samples approximately shared almost the same diversity, in the range of 1.16–1.49.

### 3.4 Phylogenetic analysis of archaeal sequences

Archaeal lineages reported from AMD environments were restricted to the *Thermoplasmatales* and *Sulfolobales* [15]. In our study, the archaeal clones were tested and all belonged to *Thermoplasmatales*, with no appearance of *Sulfolobales*. Archaea detected in the ten samples fell into three known phylogenetic divisions, *Thermoplasma*, *Ferroplasma*, *Thermogymnomonas* and a genus unclassified. The difference between each other was the percentage of archaea communities. For samples E, F and G, *Thermoplasma* (E:56.25%; F:51.61%; G:48.57%) occupied an important advantage over *Ferroplasma* (E:18.75%; F:16.13%; G:34.29%). For the rest five samples A, C, D, H and I, *Ferroplasma* (A: 93.55%; C: 77.78%; D: 73.33%; H: 82.86%; I: 59.38%) was several times. *Thermoplasma* (A: 6.45%; C: 14.81%; D: 23.33%; H: 11.43%; I: 34.38%). Samples E, F, H and I had *Thermogymnomonas* with the relative abundance of 12.50%, 19.35%, 5.71% and 3.13%, respectively (Table 5).

**Table 5** Relative abundance of archaeal clones affiliated with each major group

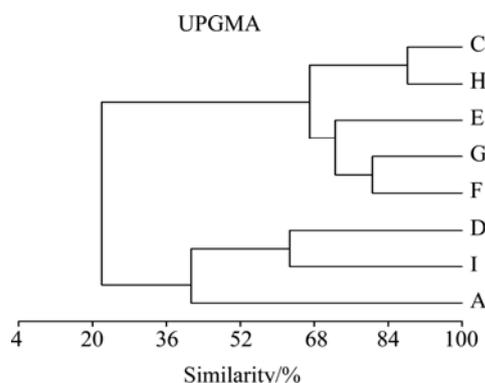
Sample	Relative abundance/%			
	<i>Ferroplasma</i>	<i>Thermoplasma</i>	<i>Thermogymnomonas</i>	Unclassified
A	93.55	6.45	–	–
C	77.78	14.81	–	7.41
D	73.33	23.33	–	3.33
E	18.75	56.25	12.5	12.5
F	16.13	51.61	19.35	12.9
G	34.29	48.57	–	17.14
H	82.86	11.43	5.71	–
I	59.38	34.38	3.13	3.13

The diversity index of archaea was generally low for these eight samples, and the highest diversity index reached 1.22 only, which showed that the archaeal communities of acidic waste water were quite limited. Especially for sample A, there were only two OTUs detected, with the diversity index of 0.24, which was the lowest value in all. And the rest samples were almost limited (Table 3).

### 3.5 Statistical analysis

#### 3.5.1 UPGMA cluster analysis of clone libraries

UPGMA cluster analysis based on the microbial communities' compositions including bacteria and archaea in the eight samples was used to reveal the microbial communities' relationship among them. This suggested that samples C, E, F, G and H had more similarity in microbial community structure among the eight samples (Fig. 1). The percentage of similarity reached between 66.98% and 88.24% for the predominance of *Thermomonas* and *Meiothermus*. Besides, samples D and I had 62.67% similarity with the rich *Acidithiobacillus* and *Leptospirillum*. There was a varied quite different from others for the abundance of microbial communities' diversity.

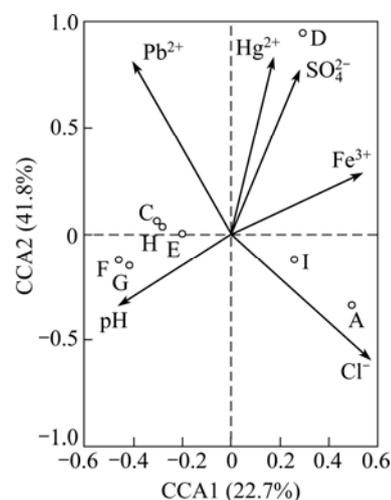


**Fig. 1** UPGMA cluster analysis based on microbial communities' compositions of water samples

#### 3.5.2 Relations between phylogenetic microbial community and geochemical variables

To determine the key geochemical variables shaping microbial community structure, canonical

correspondence analysis (CCA) was performed with the phylogenetic microbial communities of OTUs and each geochemical variable; at last, there were six significant correlation geochemical variables (Fig. 2). The result showed that geochemical variables influenced distribution of samples, especially the pH and the contents of  $\text{Hg}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{Cl}^-$ ,  $\text{Fe}^{3+}$  and  $\text{SO}_4^{2-}$ .  $\text{Fe}^{3+}$  and pH made more contribution to the distribution of microorganisms. The distribution of samples D and I might have close association with the contents of  $\text{Cl}^-$  and  $\text{SO}_4^{2-}$ . The complexity of the microbial communities of samples C, F, G and H might be due to the effects of many geochemical variables.



**Fig. 2** CCA based on microbial communities' compositions and some significant correlation geochemical variables

## 4 Discussion

AMD is an extremely acidic environment with low pH and high levels of metal concentrations [16]. The investigation on this extreme environment can not only help us to understand the community structures of different acid mines relative to environment pollution, but also provide useful information about bioleaching. In this work, the bacterial community structures of these tailing ponds were identified. Moreover, multivariate

analysis was performed to analyze the relationship between the geochemical variables and microbial communities.

The geochemical property of AMDs is heterogeneous, and due to the low pH of AMD, the solubility of transition metals is great, containing elevated concentrations of metals [3]. In these studied samples, the contents of S, Ca, Fe, Mg, Al, Na, Zn, Mn, Cu elements were very high, which was consistent with the previous reports [17]. AMDs have different characters in geochemical backgrounds (i.e., element composition in bedrock, and wall rock), which depend upon the location of the mining site, thus the element concentration of the AMDs varies in different locations all over the world [8]. For these eight samples, the content of S, Ca, Fe was much higher than others, especially for Ca element, which might result from the composition of bedrock. In addition, AMD may also be affected by other minerals, such as silicates, which will lead to an increase in the dissolved extents of Na, K, Mg, Ca, Si, and Al [8]. In this study, the samples were obtained from five main areas with the pH 2.0–3.5 at the temperature around 10 °C, and the samples were collected in the exposed pit outdoor.

According to the UPGMA cluster analysis, the microbial communities' compositions could be divided into three parts, including the samples rich in iron-/sulfur-oxidizing microorganisms, such as samples D and I; the samples rich in moderate acidophiles (*Meiothermus*, *Thermomonas*), such as samples C, E, F, G and H [18]; and sample A without dominant species but with abundant diversity.

#### 4.1 Communities of putative iron-/sulfur-oxidizing bacterial in related samples

For these eight samples, four samples had the existence of putative iron-/sulfur-oxidizing bacteria, and the percentage of them in each sample is enumerated in Table 6. The results showed that bacteria participated in the process of iron-/sulfur-oxidizing fell into the following seven genera, with a significant advantage in samples D and I, with the proportion up to 86.11% and 84.34% respectively. Members of the genus *Acidithiobacillus* and *Leptospirillum* were ubiquitous in the following four samples, and the rest genus was not dominant.

*Acidithiobacillus* spp. is widely considered to be the microorganism that controls the generation rate of AMD and *Acidithiobacillus ferrooxidans* has been used as model microbe in bioleaching system [19]. *Acidithiobacillus thiooxidans* which can oxidize only sulfur and *A. ferrooxidans* which can oxidize sulfur and reduce inorganic sulfur compounds in addition to ferrous iron[3], were both detected in our samples. The proportion of *Acidithiobacillus* in each sample was 17.20% for sample A, 70.83% for sample D, 10.96% for sample E and 40.96% for sample I. The largest proportion of clones affiliated with *Acidithiobacillus* (70.83%) was detected in sample D, which showed that this site might be the most suitable for the growth of the organisms among these samples. Previous researches reported that *Acidithiobacillus* spp. was very sensitive to arsenic ions, especially arsenic ion (III) [18]. The concentration of element arsenic in sample D was not detected, and the pH was the lowest among all samples. *Acidithiobacillus* occupied a higher community proportion which might have been facilitated by the low arsenic concentration and low pH. For sample I, the percentage of *Acidithiobacillus* was the second, and the concentration of element arsenic was the highest among these five samples (1.56 mg/L). Compared with others, such as the arsenic concentration of 2.5–29 mg/L reported by XIAO et al [10], the arsenic concentration might not reach the inhibition extent. From the result of SPSS, the dominance of *Acidithiobacillus* had significant association with the concentrations of  $Fe^{3+}$ ,  $NO_3^-$  and  $SO_4^{2-}$  and EC value.

Members of the genus *Leptospirillum* were also detected in the four samples, but the percentage was very different, with the largest occupation in sample I (42.17%), and the rest was identically lower. It should be attributed to the concentration of iron element and pH values of four samples. Previous studies with respect to *Leptospirillum* spp. suggest that these organisms could only use reduced iron as energy resource and occupy a greater percentage of the community structure at low pH [20]. The concentration of iron element was 73.9 mg/L for sample A, 121.2 mg/L for sample D, 17.2 mg/L for sample E, 151.8 mg/L for sample I, and the pH values were 2.6, 2.04, 2.67, 2.4, respectively. Sample D had the highest concentration of element iron and a relatively low pH, which may result in the high percentage of

**Table 6** Percentage of putative iron-oxidizing or sulfur-oxidizing bacteria in four relative samples

Sample	Percentage/%							Total
	<i>Acidithiobacillus</i>	<i>Leptospirillum</i>	<i>Sulfuricella</i>	<i>Thiobacillus</i>	<i>Aciditerrimonas</i>	<i>Sulfobacillus</i>	<i>Ferribacterium</i>	
A	17.20	5.38	1.08	8.60	–	–	1.08	33.33
D	70.83	9.72	–	–	2.78	2.78	–	86.11
E	10.96	5.48	–	–	–	–	–	16.44
I	40.96	42.17	1.20	–	–	–	–	84.34

*Leptospirillum*. Previous studies based on metal ion resistance showed that *Leptospirillum* spp. exhibited lower tolerance to  $\text{Cu}^{2+}$  and  $\text{Co}^{2+}$  [21,22], which was the highest in sample I, but compared with other studies [9], that was scores of times low. The statistical product and service solutions (SPSS) based on the percentage of *Leptospirillum* and geochemical variables revealed that the concentrations of  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$  were influence factors.

The rest five genera always occupied in sample A, which had the highest diversity among all samples; however, the percentage of iron-oxidizing or sulfur-oxidizing bacteria was low. It may be due to the low concentration of toxic metal ions.

#### 4.2 Communities of other predominant bacteria in related samples

Except for the samples rich in iron-oxidizing or sulfur-oxidizing bacteria, there were still five samples occupied with two moderate acidophilic genus including *Thermomonas* and *Meiothermus*, and the proportion of them in the following samples reached 58.91%–87.50% (Table 7). Previous reports based on the bacterial communities of AMD had rarely detected these two genera [23,19,10].

**Table 7** Percentage of other predominant bacteria in five relative samples

Sample	Percentage/%		
	<i>Thermomonas</i>	<i>Meiothermus</i>	Total
C	45.07	39.44	84.51
E	31.51	27.40	58.91
F	40.28	47.22	87.50
G	35.29	36.76	72.06
H	43.66	39.44	83.10

The *Thermomonas* organism has an optimum growth temperature of 37–50 °C and is distantly related to the species of the *Xanthomonas* [23]. For these five samples, this organism owed great advantage with the proportion of 31.51%–45.07%. However, a large amount of *Thermomonas* sp. existed at 25°C during the cultivation of aerobic granular sludge [19]. *Thermomonas fusca* which was aerobic and Gram-negative had been detected in the the nitrifying sludge microbial community and reported in the denitrification process, and also proved to have high nitrification rate at temperature of 10 °C [24]. KIM et al [25] discovered the existence of *Thermomonas haemolytica* in mesophilic sludge. *T. haemolytica* was said to grow in the temperature range of 18–50 °C and can produce acetate and propionate from carbohydrate fermentation [26]. For our samples, *Thermomonas* was

not clearly classified into species, but high  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  concentrations in sample showed that this organism had close relationship with denitrification and nitrification process.

*Meiothermus* demonstrated another dominance of samples C, E, F, G and H with the percentage 27.40%–47.22%. This organism has the common characteristics described as non-sporeforming moderate thermophiles with optimum growth near neutral pH and capable of growth by aerobic oxidation of sugars, sugar alcohols, and some polymers [27]. It is moderately thermophilic and is capable of starch depolymerization and reduction of  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and some *Thermus* strains are known to reduce metals [28]. For samples C, E, F, G and H, the more or less  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  may have close relationship with the *Thermomonas* and *Meiothermus*; the rest microbial community was quite few. In this study, temperature was only 4–10 °C, and pH ranged between 2.31 and 3.81, which were quite different from the former studies. It can be speculated that this environment was also suitable for *Meiothermus* to survive, thereby it is possible to reduce  $\text{NO}_3^-$  to  $\text{NO}_2^-$  and reduce metals.

#### 4.3 Communities of abundant diversity in related samples

Shannon-Weaver index  $H$  was evaluated for the phylogenetic diversity of each sample. The results showed that sample A ( $H=2.36$ ) had the preeminent diversity. The specific communities can be seen in Table 8.

For sample A, the acidity was almost the same with samples D and I, but the community was extremely plentiful. Except for the composition of putative iron-oxidizing or sulfur-oxidizing bacteria, there still was more other bacteria, such as *Herminiimonas*, *Polaromonas* and *Halothiobacillaceae*. *Herminiimonas arsenicoxydans*, which has been isolated from the activated sludge of industrial plant contaminated with heavy metals [29]. *H. arsenicoxydans* was said to be able to resist multiple toxic elements, especially arsenic by oxidizing the more toxic As (III) into the less toxic inorganic form As (V) [30]. *Polaromonas* sp. strain JS666, a  $\beta$ -proteobacterium, is shown so far to degrade a wide variety of xenobiotic compounds, including the recalcitrant groundwater contaminant cis-1, 2-dichloroethene (cDCE). Resting cells of this strain can also transform vinyl chloride (VC) and trichloroethene (TCE) [31]. For sample A,  $\text{Cl}^-$  was predominantly rich, with the concentration up to 17.86 mg/L. It can be concluded that the existence of *Polaromonas* had close relationship with  $\text{Cl}^-$ . The appearance of *Halothiobacillaceae*, an obligate heterotrophs, had been

**Table 8** Bacterial communities of three samples with abundant diversity

Phylum	Class	Order	Family	Genus	Clone number of sample A
<i>Proteobacteria</i>	<i>Betaproteobacteria</i>	<i>Burkholderiales</i>	<i>Oxalobacteraceae</i>	<i>Massilia</i>	4
				<i>Janthinobacterium</i>	1
				<i>Herminiimonas</i>	5
				Unclassified	2
				<i>Limnobacter</i>	1
			<i>Comamonadaceae</i>	<i>Albidiferax</i>	2
				<i>Polaromonas</i>	5
			<i>Alcaligenaceae</i>	<i>Achromobacter</i>	1
				Unclassified	1
				<i>Rhodocyclus</i>	<i>Rhodocyclusaceae</i>
	<i>Gammaproteobacteria</i>	<i>Hydrogenophilales</i>	<i>Hydrogenophilaceae</i>	Unclassified	1
				<i>Thiobacillus</i>	8
				<i>Sulfuricella</i>	1
				Unclassified	18
				<i>Acidithiobacillus</i>	16
		<i>Xanthomonadales</i>	<i>Xanthomonadaceae</i>	<i>Lysobacter</i>	1
				<i>Thermomonas</i>	1
				<i>Legionella</i>	3
		<i>Legionellales</i>	<i>Legionellaceae</i>		
				<i>Chromatiales</i>	<i>Halothiobacillaceae</i>
<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Phyllobacteriaceae</i>	Unclassified	2	
			<i>Phyllobacterium</i>	1	
<i>Nitrospira</i>	<i>Nitrospira</i>	<i>Nitrospirales</i>	<i>Nitrospiraceae</i>	<i>Leptospirillum</i>	5
<i>Actinobacteria</i>	<i>Actinobacteridae</i>	<i>Actinomycetales</i>	<i>Microbacteriaceae</i>	<i>Leifsonia</i>	1
<i>Bacteroidetes</i>	<i>Flavobacteria</i>	<i>Flavobacteriales</i>	<i>Flavobacteriaceae</i>	<i>Flavobacterium</i>	2
	<i>Sphingobacteria</i>	<i>Sphingobacteriales</i>	<i>Sphingobacteriaceae</i>	<i>Pedobacter</i>	2
			<i>Cyclobacteriaceae</i>	<i>Algoriphagus</i>	2
<i>TM7</i>					1
Unclassified					1

called moderate acidophiles [18], and was previously considered to grow by sulfur oxidation only, which was latterly isolated from AMD as iron-oxidizing bacteria in moderately acidic (~pH 4) solid media [1].

#### 4.4 Archaeal communities' diversity

Archaea were detected in all eight samples; and they were classified into three known genus: *Ferroplasma*, *Thermoplasma*, *Thermogymnomonas* and a genus unclassified, all of which belong to *Thermoplasmatales* order. According to previous research, all the archaea in this order are heterotrophic except *Ferroplasma*, which has the ability to gain energy during the oxido-reduction of iron. All the archaea in *Thermoplasmatales* order tend to be thermophilic, especially for *Thermoplasma* and *Thermogymnomonas*, while with the exception of *Ferroplasma*.

The genus of *Ferroplasma* is dominant in the most extreme environment of lower pH and higher ionic

strength [9], including some mesophilic iron (II)-oxidizing archaea, such as *F. thermophilum*, *Ferroplasma acidiphilium* and *Ferroplasma acidarmanus* [9]. It was reported that *F. thermophilum* was often flourishing at extremely low pH (lower than 1.4), high concentration of total iron, ferrous iron and other heavy metals [1]. According to ZHANG et al [32], during the bioleaching of chalcopyrite, *F. thermophilum* L1 was capable of chemomixotrophic growth on ferrous iron and organic matters; moreover, it could relieve toxicity of organic matters to autotrophic bacteria. *F. acidiphilum*, described as a strictly chemolithoautotrophic microbe, can oxidize  $Fe^{2+}$  from  $Fe_2SO_4$  and pyrite  $FeS_2$ . It is the only described archaeon capable of growth at the relatively low temperatures commonly encountered in AMD. Compared with *F. thermophilum*, the use of organic compounds as carbon sources has not been reported thus far [33]. The *Ferroplasma* genus detected in our samples was not

classified into species, but it was important for the iron-/sulfur-oxidation.

Archaea in *Thermoplasma* and *Thermogymnomonas* were detected in extremely thermoacidophilic environment, such as burning coal refuse pile in USA and volcanic solfataric field in Italy, with the growth temperature of 33–67 °C, pH 0.5–4 [34]. The detection of them in the eight samples showed that the acidic water was not “cold” based on the archaeal communities and the growth temperature for *Thermoplasma* and *Thermogymnomonas* was not limited. The existence of heterotrophic archaea in *Thermoplasma* and *Thermogymnomonas* may create a more suitable environment for the growth of the iron-oxidizers by removing organic compounds that can be toxic to the autotroph [3]. Samples E and F were abundant in *Thermoplasma* and *Thermogymnomonas*, with the percentages of 68.75% and 70.96%, respectively. It may have close relationship with the organic carbon, for the COD of these two samples being relatively high, reaching 156.3 mg/L and 143.8 mg/L accordingly.

## 5 Conclusions

1) The UPGMA cluster analysis based on the microbial communities' compositions revealed that these eight samples were divided into three parts, including the samples rich in iron-/sulfur-oxidizing microorganisms; samples rich in moderate acidophiles (*Meiothermus*, *Thermomonas*); and sample without dominant species but with abundant diversity.

2) The acidophilic microorganism existing in the mine drainage had close relationship with the surrounding environment. The dominance of *Acidithiobacillus* spp. had close relationship with the concentration of  $\text{Fe}^{3+}$ ,  $\text{NO}_3^-$ ,  $\text{SO}_4^{2-}$  and EC value, *Leptospirillum* spp. was significantly associated with the concentrations of  $\text{Cu}^{2+}$  and  $\text{Fe}^{3+}$ .

3) Except for *A. ferrooxidans*, *A. thiooxidans*, and *L. ferrooxidans* dominating in AMD, there still was some thermophilic microorganism, such as *Meiothermus*, *Thermomonas* and *Thermoplasmatales* at the low pH, while the influence factor causing the abundance of *Meiothermus* and *Thermomonas* was not clear. It might be affected synthetically by pH, ion concentration, especially by high  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  concentrations.

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## 铜陵重金属富集的尾矿废弃地 酸性废水中细菌及古菌的多样性

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**摘要:** 为了加深对安徽省铜陵市不同重金属富集程度的尾矿附近酸性废水中微生物多样性的了解, 运用 PCR-16S rDNA 克隆文库技术对酸性废水中细菌及古菌的群落结构进行研究。在铜陵的 5 个样点采集了 8 个水样。系统发育分析结果表明, 酸性废水中的细菌主要是 *Betaproteobacteria*, *Gammaproteobacteria*, *Alphaproteobacteria*, *Deinococcus-Thermus*, *Nitrospira*, *Firmicutes*, *Actinobacteria*, *Deltaproteobacteria*, *Bacteroidetes*, *Chloroflexi*, 古菌是 *Thermoplasma*, *Ferroplasma* 以及 *Thermogymnomonas*。根据细菌和古菌的组成对样品进行聚类分析, 结果表明, 5 个样品由于优势菌株 *Thermomonas* 和 *Meiothermus* 拥有较高的相似性。2 个样品由于 *Acidithiobacillus* 和 *Leptospirillum* 占主导也拥有较高的相似性。剩余的样品多样性较高, 拥有最高的香浓-微纳指数 2.91。典型相关分析 CCA 的结果表明微生物的多样性组成与理化因子有密切的关系, 如水的 pH 以及  $Hg^{2+}$ 、 $Pb^{2+}$ 、 $Cl^-$ 、 $SO_4^{2-}$ 、 $Fe^{3+}$  的含量。

**关键词:** 酸性矿业废水; 微生物结构组成; 克隆文库; 理化因子