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Microbial community analysis in rice paddy soils irrigated by acid mine drainage contaminated water

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Abstract Five rice paddy soils located in southwest China were selected for geochemical and microbial community analysis. These rice fields were irrigated with river water which was contaminated by Fe–S-rich acid mine drainage. Microbial communities were characterized by high-throughput sequencing, which showed 39 different phyla/groups in these samples. Among these phyla/groups, *Proteobacteria* was the most abundant phylum in all samples. *Chloroflexi*, *Acidobacteria*, *Nitrospirae*, and *Bacteroidetes* exhibited higher relative abundances than other phyla. A number of rare and candidate phyla were also detected. Moreover, canonical correspondence analysis suggested that pH, sulfate, and nitrate were significant factors that shaped the microbial community structure. In addition, a wide diversity of Fe- and S-related bacteria, such as GOUTA19, *Shewanella*, *Geobacter*, *Desulfobacca*, *Thiobacillus*, *Desulfobacterium*, and *Anaeromyxobacter*, might be responsible for biogeochemical Fe and S cycles in the tested rice paddy soils. Among the dominant genera, GOUTA19 and *Shewanella* were seldom detected in rice paddy soils.

Keywords Fe and S cycles · Illumina sequencing · Soil · Acid mine drainage · Fe- and S-related bacteria

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Introduction

Rice is one of the world's most important agronomic plants and is a stable food for many countries. Around 75 % of rice grows under flooded conditions. Rice paddy soil is subjected to periodic changes in oxic and anoxic conditions by repeating the flooding and drainage cycles. Before harvest, the fields are drained and reduced compounds are oxidized. After flooding, oxidant such as oxygen, nitrate, iron oxides, sulfate, and carbon dioxide are reduced sequentially based on the thermodynamic theory (Zehnder and Stumm 1988). All the reactions are carried out by microorganisms which use one of these compounds as an electron acceptor. Methane is the end product of degradation of organic matters by methanogens in rice paddies. Estimation of the globally methane annual emission rate from rice paddies ranges between 60 Tg (Prinn 1994) and 110 Tg (Cicerone and Oremland 1988), which is around 1/4 to 1/5 of total annual methane emission into the atmosphere (Prinn 1994; Cicerone and Oremland 1988; Liesack et al. 2000). Flooded rice paddies are an important source for atmospheric methane and therefore contributing to global warming.

Biotic iron and sulfate reduction played an important role by determining the onset of methanogenesis. This observation was first reported for competition between sulfate reducers and methanogens in sediments of Lake Mendota (Winfrey and Zeikus 1977) and was later validated in other anoxic environments (King 1984), including rice paddy soil (Achtnich et al. 1995a). Iron reduction may also suppress methanogenesis in rice paddies. For example, methane production was strongly inhibited when ferrihydrite was added to soil slurries. With increasing the amount of ferrihydrite added to the soil, methanogenesis was completely inhibited (Achtnich et al. 1995a). The competition for electron donors between iron and sulfate reducers and methanogens may be attributed to the inhibition of methanogenesis. Therefore, iron and sulfate

reduction has been suggested as an effective strategy to suppress of methane emission from rice paddies, especially in the cases that electron donors are limiting (Achtnich et al. 1995a). If we want to deeply characterize the microbial functions of the in situ iron and sulfate reduction, detailed information regarding the community structure is essential. To date, very little is known about the biogeochemical Fe and S cycling and the responsible organisms have yet to be conclusively identified in rice paddy soils. A comprehensive investigation of the microbial communities in rice paddy soils has been strongly expected.

For this purpose, we selected rice fields located in Guiyang in southwest China. There were numerous abandoned coal mines located in the upstream of the sampling sites. These abandoned coal mines produced a large amount of acid mine drainage (AMD) which has high concentrations of Fe and S. Specifically, the concentration of the total iron and sulfate in the acid mine waters could reach as high as 1000 mg/L and 6900 mg/L, respectively (Table S1). The AMD then flowed in to the downstream Youyu River and contaminated the water in Youyu River. AMD-contaminated water in Youyu River was used as the irrigation water to rice fields and introduced a relatively high amount of Fe and S to the rice fields. Therefore, the exogenously introduced Fe and S increased the abundance of Fe- and S-compounds in the paddy soil and made the paddy soil a good model to study microbial Fe and S biogeochemical cycling under in situ conditions. Here, an extensive survey of the microbial communities in the rice paddy soils was performed using high-throughput sequencing based on Illumina MiSeq platform. The overall goal of this study is to characterize the microbial communities in five rice paddy soils with an emphasis on the bacteria correlated with Fe and S cycling.

Materials and methods

Site information, sample description, and sample procedure

Five different rice paddy soils were obtained from five rice fields located in Guiyang city, the capital city of Guizhou province. Among them, four rice fields were irrigated by water from Youyu River (S1, S3–5) and one field was directly irrigated by the acid mine waters from abandoned Maochong coal mine (S6). Soils were sampled in October 2013 right after drainage but before harvest. Multiple sampling plots were randomly selected at each site 5 to 15 cm below the surface and were taken by a soil corer device. About 3 kg of soil from each rice filed was collected. Samples from each sampling plot were pooled and homogenized, and were immediately stored in a freezer at −20 °C until used for molecular analysis and 4 °C for soil physicochemical characterization.

Chemical analysis

Soil samples from each site were homogenized by mixing and then stored in a refrigerator at 4 °C for further processing. The soils were air-dried for 48 h, and passed through a 2-mm sieve to remove leaves, plant roots, and gravel. To measure soil pH, 10 g dry soil samples were placed into a 100-ml Erlenmeyer flask and mixed with 25 ml distilled water (1:2.5 soil–water ratios). The mixture was left to equilibrate for 20 min after shaking for 5 min. The pH was measured using a calibrated HACH HQ30d pH meter (HACH, Loveland, USA). To measure nitrate and sulfate concentrations in soil, 10 g dry soil samples were placed into a 100-ml Erlenmeyer flask and mixed with 50 ml distilled water (1:5 soil–water ratios). The mixture was left to equilibrate for 4 h after shaking for 5 min. The supernatant was filtrated with 0.45-μm filter membrane after centrifuging at 2200×*g* for 10 min. Soil sulfate and nitrate concentrations were determined using the ion chromatography system (DIONEX ICS-1500, Sunnyvale, USA). Soil alkalinity was measured as the methods described previously (Shao et al. 1993). HCL-extractable Fe concentrations were measured as described previously (Komlos et al. 2007). One gram of soil was mixed with 50 ml 0.5 N HCl. After 22 h at room temperature, sample/HCl suspension was filtrated through 0.45 μm sterile membrane. Fe and Fe(II) were measured by a spectrophotometric method (UV-9000s, Shanghai METASH) with 1,10-phenanthroline at 510 nm (Tamura et al. 1974). Fe(III) was determined as the difference with Fe and Fe(II).

Mineralogical and element analyses

For the inorganic geochemical analysis, the bulk sample was dried at 105 °C and thoroughly ground using a mortar and pestle before passing a 200-mesh sieve. Bulk chemical analyses of major elements were performed using X-ray fluorescence spectrometry (PANalytical Axios-PW4400, Westborough, USA) at 40 kV and 95 mA. The detection limit was below 0.01 %. In this analysis, 1 g 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. The major elements were analyzed quantitatively after the fusion of 0.1 g combusted sample with 3.6 g dilithium tetraborate at 1050 °C for 16 min.

DNA extraction, PCR amplification, sequencing analysis, and statistical analysis

Soils from five rice fields (S1, S3, S4, S5, and S6) were chosen for molecular analysis. Total genomic DNA was extracted directly from these samples using FastDNA® spin kit (MP bio, Santa Ana, USA) following the manufacturer's protocol. DNA concentrations were then determined using a NanoDrop ND-

2000 UV–vis spectrophotometer (Thermo Scientific, Wilmington, USA). DNA was stored at −80 °C for subsequent analyses. Total genomic DNA was amplified using 515f/806r primer set that amplifies the V4 region of the 16S rDNA gene (Bergmann et al. 2011). The forward primer contains a 6-bp error-correcting barcode unique to each sample. DNA was amplified following the protocol described previously (Caporaso et al. 2011). 16S rRNA tag-encoded high-throughput sequencing was carried out in Illumina MiSeq platform at the Novogene (Beijing, China). The reads with an average length of 270 bp had been deposited into the NCBI short reads archive database with accession number SRP047111. Pairs of reads from the original DNA fragments were merged based on the method described previously (Magoč and Salzberg 2011). Sequencing reads were assigned to each sample according to the individual unique barcode. Sequences were analyzed with QIIME software package (Quantitative Insights Into Microbial Ecology) and UPARSE pipeline (Caporaso et al. 2010). The reads were first filtered by QIIME quality filters. Default settings for Illumina processing in QIIME were used. Then UPARSE pipeline was used to pick up operational taxonomic units (OTUs) at 97 % similarity. For each OTU, a representative sequence was selected and used to assign taxonomic composition by using the RDP classifier (Wang et al. 2007). Then, the estimated species richness was indicated with rarefaction analysis; Chao 1 and Shannon indexes for five libraries were determined as described previously (Schloss et al. 2009).

Statistical analysis

The similarity among microbial communities in different AMD samples was determined using UniFrac analysis. QIIME calculates both weighted and unweighted UniFrac. Principal coordinate analysis (PCoA) and unweighted pair group method with arithmetic mean (UPGMA) clustering were conducted by unweighted and weighted UniFrac based on the protocol published previously (Kuczynski et al. 2012). Canonical correspondence analysis (CCA) was performed to measure chemical properties that have the most significant influence on microbial communities. The significant correlations of the physiochemical parameters were examined by a Monte Carlo permutation. The triplot was generated by CANOCO 4.5 (Biometrics Wageningen, The Netherlands). The figures were generated by CanoDraw 4.0 (Biometrics Wageningen, The Netherlands).

Results

Environmental parameters

Five rice paddy soils were selected for physiochemical analysis. Soil pH, sulfate and nitrate concentrations, and alkalinity were measured as shown in Table 1. Four soils had pH values less than 7 except S4 which has a pH value of 7.25. Low pH values indicated that irrigative acid water might affect the soil pH. This phenomenon is more obvious in rice field S6 (pH= 4.02), which was irrigated directly with low pH acid mine waters. Sulfate concentration was relatively high in S5 and S6. S6 has the highest sulfate concentration as 4799 mg/kg soil. This observation could also be attributed to the effect of direct irrigation of high sulfate acid mine waters, indicating that exogenous sulfate might be accumulated in the rice fields. The nitrate concentration is relatively low in all rice paddy soils. The low nitrate concentration may be attributed to the leaching of nitrate (Cai et al. 1992; Katyal and Gadalla 1990) or denitrification (Xing et al. 2002). The mineral compositions were also measured from each sampling site. The concentrations of major elements were shown in Table 2. All samples had high concentration of SiO_2 and Al_2O_3, followed by Fe_2O_3. Among these major elements, the concentration of Fe_2O_3 has relatively lower value in S6 (7.76 %) but relatively higher value in S5 (13.16 %). The other soil samples has relative constant concentrations of Fe_2O_3, ranging from 10.21 to 11.65 %. It is noteworthy that irrigation of AMD contaminated water did not significantly increase the Fe_2O_3 concentration. This observation is more obvious in sample S6 which has the lowest Fe_2O_3 concentration, although S6 was irrigated with high Fe acid mine waters.

Microbial community analysis

There were 392,394 valid reads from 5 samples after filtering low-quality reads and chimeras and trimming the adapters, barcodes, and primers. All valid reads were classified from phylum to genus according to the QIIME using default settings. The taxonomic distribution at phylum level was summarized in Fig. 1. These sequences were assigned to 39 phyla as demonstrated in Table S2. *Proteobacteria* was the most abundant phylum in all samples, accounting for 35.79 to 47.32 % of the total valid reads in all samples, with an average relative abundance of 42.03 %. *Chloroflexi* was the second most abundant phylum in all samples with an average relative abundance of 15.40 %. The other dominant phyla were *Acidobacteria* (6.36–8.89 %, averaging at 7.22 %), *Nitrospirae* (2.83–6.26 %, averaging at 4.71 %), *Bacteroidetes* (4.14–4.93 %, averaging at 4.52 %), *Verrucomicrobia* (2.78–5.29 %, averaging at 4.04 %), and *Planctomycetes* (1.61–4.53 %, averaging at 2.98 %).

At the class level, a wide range of classes were dominated. Based on the average relative abundance, the most abundant classes were *Anaerolineae*, *Deltaproteobacteria*, *Betaproteobacteria*, *Gammaproteobacteria*, and *Alphaproteobacteria*. At the order level, a total number of

Table 1 Physiochemical characteristics of the rice paddy soils

Sample	pH	Fe(II) (mg/kg soil)	Fe(III) (mg/kg soil)	SO_4^{2-} (mg/kg soil)	NO_3^{-1} (mg/kg soil)	Alkalinity (measured as CaO, mg/kg soil)
S1	5.63	957.1	11,225.1	516	10.6	18.1
S3	5.28	922.6	7062.0	1243	18.2	4.2
S4	7.25	643.9	9716.4	751	10.5	8.38
S5	5.55	733.8	13,708.8	3319	9.5	4.18
S6	4.02	993.8	10,028.0	4799	8.6	13.95

34 orders were dominant (>1 % in any soil sample). Based on the average relative abundance, *Syntrophobacterales* (5.48 %) was the most abundant order followed by *Nitrospirales* (4.7 %), *Rhizobiales* (3.91 %), and *Oceanospirillales* (3.89 %). In addition, GCA004, *Pedosphaerales*, *Burkholderiales*, *Bacteroidales*, *Anaerolineales*, *Desulfuromonadales*, *Myxococcales*, and *Hydrogenophilales* were the orders commonly shared by all paddy soils. At the family level, *Halomonadaceae*, *Thermodesulfovibrionaceae*, *Anaerolinacea*, *Solibacteraceae*, and *Syntrophaceae* were dominant (>1 %) in all soils. In addition, the dominance has a site-specific trend. For instance, *Hyphomicrobiaceae* and *Geobacteraceae* were more abundant in S3 but *Desulfobacteraceae* was more abundant in S6. Other families, such as *Rhodocyclaceae*, *Shewanellaceae*, *Syntrophobacteraceae*, *Gallionellaceae*, *Acetobacteraceae*, and *Acidobacteriaceae* were commonly detected in all soils.

Core genera

The most abundant genera within different samples were also determined as shown in Fig. 2. The most abundant genera included *Halomonas*, *Rhodoplanes*, *Thiobacillus*, *Shewanella*, GOUTA19, *Anaerolinea*, Candidatus *Solibacter*, and *Desulfobacca* with average relative abundances greater than 1 % and were dominant in at least three rice paddy soils. Other genera, such as *Desulfobacterium*, *Geobacter*, *Dechloromonas*, *Ochrobactrum*, *Gallionella*, *Acidovorax*, *Kaistobacter*, *Nitrospira*, *Aquicella*, *Sulfuricurvum*, *Anaeromyxobacter*, *Staphylococcus*, *Acinetobacter*, Candidatus *Koribacter*, *Syntrophobacter*, *Sphingomonas*, *Gemmata*, *Flavobacterium*, *Planctomyces*, *Chthonomonas*, and *Novosphingobium* were detected in all samples and were dominated in several samples.

Microbial community grouping and CCA analysis

The similarity among the microbial communities in the five rice paddy soils was evaluated using cluster analysis. As shown in Fig. 3, cluster analysis revealed that bacterial communities could be clustered into three groups. Group I contains samples S1, S5, and S6. Groups II and III contain only one sample, S3 and S4, respectively. This grouping was also confirmed by PCoA analysis (Fig. 4). This pattern indicated that microbial community structure may not be direct correlated with the source of irrigation water, as shown in group I which contained samples irrigated with water from Youyu River (moderate pH) and Maochong creek (low pH). Microbial community may be more correlated with indigenous environmental parameters. Therefore, CCA analysis was used to reveal how microbes can adapt to the changes of in situ physiochemical environments. A correlation between the important environmental parameters and microbial community was discerned by CCA analysis as shown in Fig. 5. Five environmental parameters and the dominant genera (>1 %) in each sample were selected to determine their correlation. The length of an environmental parameter arrow indicated the strength of the environmental parameter to the overall microbial communities. As such, pH, sulfate, and nitrate concentrations appears to be the most important environmental parameters.

Table 2 Major elemental concentrations in the AMD sediment samples from AHA watershed

Sample	SiO2 (%)	Al2O3 (%)	Fe2O3 (%)	MgO (%)	CaO (%)	Na2O (%)	K2O (%)	MnO (%)	P2O5 (%)	TiO2 (%)	LOI (%)	Total (%)
S1	54.5	14.46	11.65	1.15	0.668	0.446	1.342	0.058	0.2863	2.707	13.49	100.76
S3	49.48	13.61	10.21	0.96	0.759	0.185	1.314	0.0369	0.2885	2.005	21.45	100.3
S4	51.14	12.89	10.27	0.81	1.025	0.124	1.534	0.0651	0.3051	1.971	20.75	100.88
S5	49.31	12.7	13.16	0.73	0.886	0.13	1.11	0.0469	0.272	1.974	19.47	99.79
S6	48.19	15.22	7.76	0.81	0.631	0.398	1.346	0.0311	0.2522	2.554	22.59	99.78

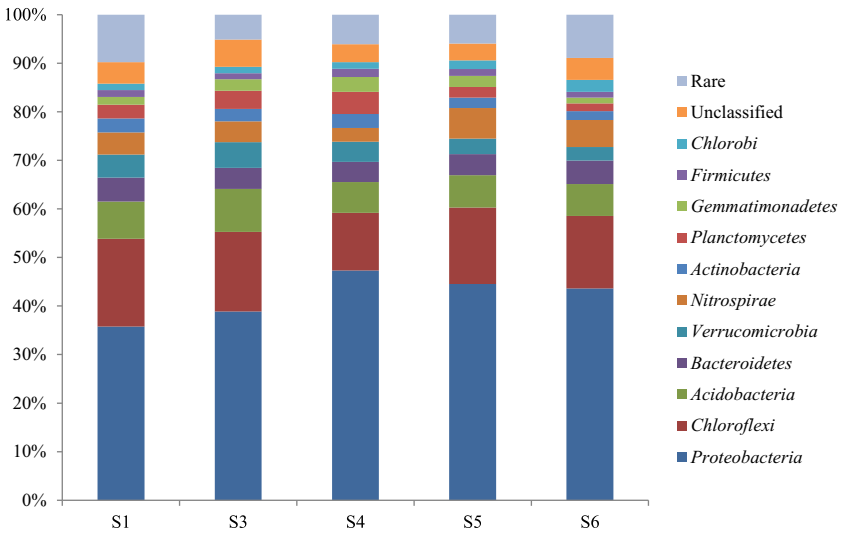

Fig. 1 Taxonomic classification of bacterial reads retrieved from different watershed samples at phylum level using RDP classifier

Discussion

Rice field soils represent one of the most important sources of atmospheric methane (Lelieveld et al. 1998; Wang et al. 2004). A comprehensive understanding of microbial community and biogeochemical cycling of C, N, S, and Fe is essential for mitigating methane production and sustaining soil fertility. However, investigations on the overall microbial communities, especially the microbial communities related to biogeochemical Fe and S cycling, were still scarce. In the current study, the rice fields irrigated with the AMD contaminated river water provided a good model to study the Fe- and S-

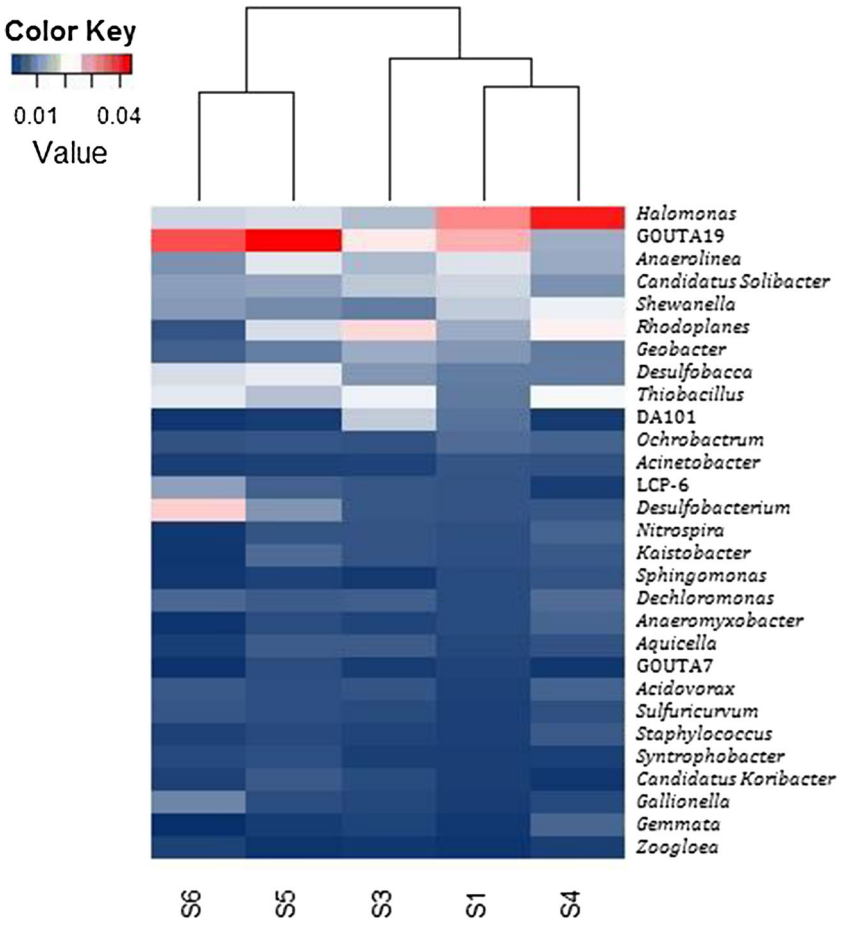

Fig. 2 Heatmap analysis of the dominant genera distribution of the five samples. The double hierarchical dendrogram shows the microbial distribution of the five samples. The relative values (0–1) for the microbial genera are depicted by the color intensity; the legend can be found at the top of the figure. The abundance is expressed as the value of the targeted sequences to the total high-quality sequences from each soil sample

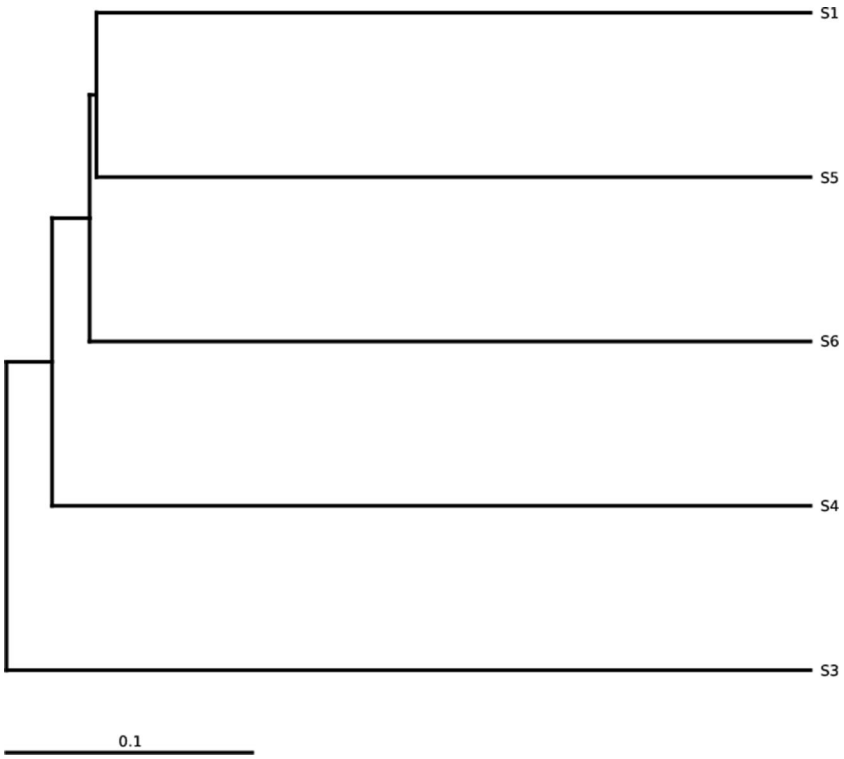

Fig. 3 UniFrac UPGMA cluster of microbial communities associated with different soil samples from different sampling locations. The figure was constructed on the basis of Illumina sequencing data

related bacteria in the rice paddy soil. The sampling time in the current study was representative for active Fe and S biogeochemical cycling. Firstly, we sampled these soils right after the end of the anoxic period. The anaerobic environments favored the growth and proliferation of anaerobic bacteria such as Fe- and S-related bacteria and methanogens. Secondly, summer is the monsoon in Guiyang. Elevated rainfalls and higher temperatures accelerated the weathering of the pyrite in abandoned coal mines and increased Fe and S concentrations in irrigation water. After irrigation with Fe- and S-rich water for a whole monsoon period, rice fields had accumulated sufficient Fe- and S-compounds that were able to stimulate the enrichment of Fe- and S-related bacteria.

Correlation between environmental parameters and microbial community

Analyzing the dynamic changes of microbial communities with geochemical factors will reveal the correlation between environmental parameters and microbial community. Sulfate

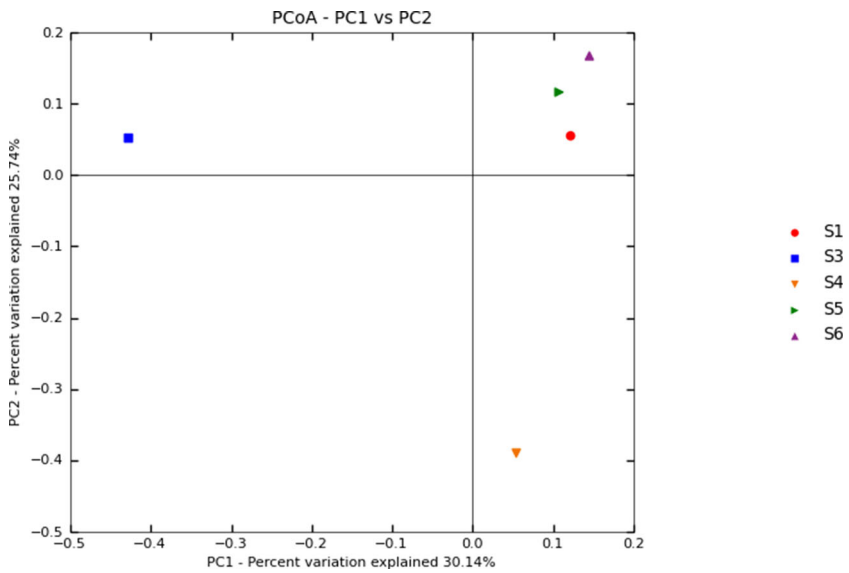

Fig. 4 Principal coordinate analysis (PCoA) plot based on the 16S rRNA sequencing genes from five samples. The scatter plot is of principal coordinate 1 (PC1) vs principal coordinate 2(PC2). The percentages are the percentage of variation explained by the components

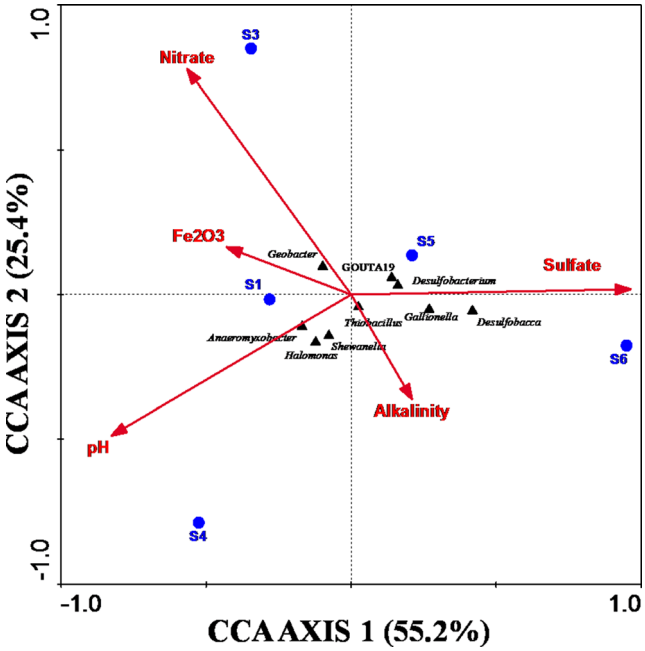

Fig. 5 Canonical correspondence analysis (*CCA*) of 16S rRNA gene data and environmental parameters. *Arrows* indicate the direction and magnitude of environmental parameters associated with bacterial community structure

is positively correlated with CCA axis 1 and is strongly and significantly linked to the overall microbial community. As shown in Fig. 5, microbial communities in S5 and S6 were positively correlated with sulfate. Sulfate concentrations varied significantly in different rice paddy soils. For example, sulfate concentrations were 3319 and 4799 mg/kg soil in S5 and S6, respectively, while it was only 516, 1243, and 756 mg/kg soil in S1, S3, and S4. The elevated sulfate concentrations in S5 and S6 would influence the overall microbial communities through controlling the distribution of SRB. This observation is in accordance with the fact that a relatively high proportion of SRB were more inclined to be present in soils with elevated sulfate concentrations (S5 and S6). For instance, *Desulfobacterium* and *Desulfobacca*, which were positively correlated with sulfate as shown in Fig. 5, were dominant in S5 and S6 but not dominant in S1, S3, and S4. Therefore, it is fair to propose that sulfate played an active role in shaping the indigenous microbial communities.

Nitrate is also well-recognized as an important parameter for shaping the microbial community structure. Specifically, nitrate was positively correlated with the microbial community in S3. Rice paddy soils have been reported as good habitats for dynamic nitrate reduction (Achtnich et al. 1995a; Chidthaisong and Conrad 2000; Takai and Kamura 1966). Nitrate was also frequently reported to be able to inhibit methanogenesis in rice paddy soils (Klüber and Conrad 1998; Roy and Conrad 1999; Van Bodegom and Stams 1999). As derived from CCA analysis, N cycling is considered as another important geochemical process in addition to Fe and S cycling. It is noteworthy that the nitrate concentration is relatively low in the current study, suggesting that even a low nitrate concentration may have the potential to shape the microbial communities in rice paddy soils.

pH appears to be one of the most important environmental parameters. It is widely accepted that pH has a significant effect on the overall diversity and composition of microbial communities in a range of terrestrial and aquatic environments (Kuang et al. 2013; Lauber et al. 2009; Nicol et al. 2008; Wang et al. 2012). Any significant deviation of pH would impose stress on single-celled organisms because the intracellular pH of most microorganisms is usually within 1 pH unit of neutral (Fierer and Jackson 2006). There are several environmental parameters such as nutrient availability and cationic metal solubility that are often correlated with soil pH (Brady and Weil 1996). The differences of these factors may also drive the observed changes in microbial community composition.

Microbial community structure

Anaerobic processes such as denitrification, iron reduction, sulfate reduction, and methanogenesis are the terminal steps in the degradation of organic matters in rice paddy soil. The active anaerobic respiratory processes depend on the availability of electron acceptors. The irrigation water provided exogenous ferric iron and sulfate for iron reduction and sulfate reduction. The microbial community analysis exhibited that the dominant organisms (with an average relative abundance greater than 1 %) in the rice paddy soil included *Halomonas*, GOUTA19 (family: *Thermodesulfovibrionaceae*), *Anaerolinea*, Candidatus *Solibacter*, *Rhodoplanes*, *Shewanella*, *Desulfobacca*, *Thiobacillus*, and *Geobacter*. A large number of dominant genera could be classified with the bacteria related with microbial Fe and S cycling, indicating dynamic Fe and S cycling in the rice paddy soils.

Fe- and S-related bacteria

In rice paddy soils, iron-reducing bacteria (FeRB) are of great concern because of their capability to inhibit methanogenesis. Due to the alternation between oxic and anoxic conditions and the abundance of iron in paddy soils, biotic iron reduction is prevalent and perceived as a critical biogeochemical process in flooded rice paddy soils (Ding et al. 2014). Numerous FeRB have been isolated, characterized, and identified from rice paddy soils by multiple molecular methods (Ding et al. 2014; Hori et al. 2009; Wang et al. 2009). However, comprehensive studies of the iron-reducing microbial community in paddy soils have been limited.

In the current study, high-throughput sequencing provided a chance to extensively study the phylogenetic diversity of FeRB (Table 3). *Shewanella* demonstrated high relative

Table 3 Major Fe- and S-related bacteria identified from five rice paddy soil

Taxonomic group	S1	S3	S4	S5	S6	Microbial metabolism	Reference
GOUTA19	2.895	2.436	1.395	4.387	3.724	Heterotrophic, anaerobic, sulfate reduction	(Lopes et al. 2014)
Shewanella	1.687	0.888	2.075	1.040	1.182	Facultative, halotolerant, Fe-reducing	(Lies et al. 2005)
Geobacter	1.154	1.365	0.863	0.906	0.609	Anaerobic, Fe-and sulfate reduction	(Lovley et al. 1993)
Desulfobacca	0.893	1.157	0.880	2.035	1.883	Heterotrophic, anaerobic, sulfate reduction	(Elferink et al. 1999)
Thiobacillus	0.845	2.103	2.154	1.588	1.992	Halotolerant, acidophilic Fe and S oxidation	(Tuovinen and Kelly 1973)
Desulfobacterium	0.464	0.497	0.500	1.132	2.656	Heterotrophic, anaerobic, sulfate reduction	(Bak and Widdel 1986)
Anaeromyxobacter	0.378	0.317	0.629	0.393	0.155	Facultative anaerobic, Fe reduction	(Treude et al. 2003)
Sulfuricurvum	0.266	0.388	0.436	0.408	0.487	Facultative anaerobic, chemolithoautotrophic, S oxidation	(Kodama and Watanabe 2004)
Syntrophobacter	0.254	0.244	0.261	0.421	0.360	Heterotrophic, anaerobic, sulfate reduction	(Harmsen et al. 1998)
Gallionella	0.231	0.348	0.373	0.396	0.969	Autotrophic, Fe oxidation	(Hallbeck and Pedersen 1991)
Clostridium	0.119	0.081	0.081	0.155	0.122	Heterotrophic, anaerobic, sulfate reduction	(Akagi and Campbell 1962)
Leptospirillum	0.094	0.084	0.094	0.056	0.114	Obligate aerobic, autotrophic, acidophilic, Fe oxidation	(Schrenk et al. 1998)
Desulfomonile	0.063	0.061	0.038	0.107	0.112	Heterotrophic, anaerobic, sulfate reduction	(DeWeerd et al. 1990)
Desulfococcus	0.061	0.084	0.112	0.162	0.079	Heterotrophic, anaerobic, sulfate reduction	(Imhoff-Stuckle and Pfennig 1983)

abundance in all five soils. *Shewanella* populations are well known FeRB and were frequently identified from aquatic environments such as river estuary (Skerratt et al. 2002) and marine sediments (Toffin et al. 2004; Zhao et al. 2005), but *Shewanella* spp. have never been identified in the rice paddy soil previously. In another study, we characterized the indigenous microbial community in Youyu River which is the irrigation water for four rice fields; *Shewanella* was predominated in some locations of Youyu River (data not published). We proposed that the dominance of the *Shewanella* might be introduced from the irrigation water.

Geobacter was the second most abundant FeRB with an average relative abundance of 0.98 %. *Geobacter* populations are well known for their capability to reduce metals (Lovley et al. 2004). In subsurface where Fe (III) is abundant and available for microorganisms, *Geobacter* populations have often been demonstrated as the most abundant microorganisms responsible for iron reduction (Lovley et al. 2004). Unlike *Shewanella*, *Geobacter* spp. were frequently detected in rice paddy soil in previous studies. Hori et al. (2009) used RNA-based stable isotope probing (RNA-SIP) to identify *Geobacter* as the predominant microbial population that incorporated ^{13}C-labeled acetate under iron-reducing environment. More recently, Ding et al. (2014) also utilized SIP to reveal that *Geobacter* spp. were the most abundant putative iron reducers in nitrogen-fertilized paddy soils. All these observations suggested that *Geobacter* may play a dynamic role in iron reduction in paddy soils.

Anaeromyxobacter is another microorganism that has been frequently identified in rice paddy soil. For example, Hori et al. (2009) also identified *Anaeromyxobacter* as the major FeRB in the same SIP investigation. Ding et al. (2014) detected a high relative abundance of *Anaeromyxobacter* in both nitrogen-fertilized and non-fertilized paddy soils as derived from pyrosequencing. However, relative abundances of *Anaeromyxobacter* were only accounted for 0.15 to 0.63 % among all five soils as derived from Illumina sequencing. Other FeRB, such as *Acidiphilium* and *Geothrix*, only showed very low abundances in the tested paddy soils. These results suggested that *Shewanella* and *Geobacter* might play a more important role than other FeRB in iron reduction in the tested rice paddy soils.

The high-throughput sequencing analysis revealed that many sulfate-reducing bacteria existed in the paddy soils (Table 3). Significant rates of sulfate reduction have been measured in rice field soils in previous studies (Scheid and Stubner 2001; Wind and Conrad 1997). It was reported that active S biogeochemical cycling occurred in habitats with oxic/anoxic interfaces (Ouattara and Jacq 1992; Wind and Conrad 1995; Wind et al. 1999). In rice paddy soil, the highest sulfate reduction rates and the enrichment of sulfate reducers were often found in or near oxygenated zones (Fründ and Cohen 1992). In the present study, the S-rich irrigation water and the indigenous sulfate provided sufficient sulfate for SRB in the rice fields. Sulfate reducers, such as GOUTA19 (*Thermodesulfovibrionaceae*), *Desulfobacca*, *Desulfobacterium*, and *Syntrophobacter* were detected in all soil samples. GOUTA19 (family: *Thermodesulfovibrionaceae*) was the most abundant sulfate reducer, especially in soil S5 and S6 that had higher sulfate concentrations. *Thermodesulfovibrionaceae* is a newly proposed family. Bacteria belonging to the family *Thermodesulfovibrionaceae* were seldom identified in rice filed soils. Recently, GOUTA19

(*Thermodesulfovibrionaceae*) was found in alfalfa-rice rotation system as revealed by pyrosequencing (Lopes et al. 2014). The predominance of *Thermodesulfovibrionaceae*-related bacteria in rice fields indicated that this phylotype might play an important role in sulfate reduction in paddy soils. *Desulfobacca* and *Desulfobacterium* both showed average relative abundances of more than 1 % in five soils. *Desulfobacca* and *Desulfobacterium* were both frequently identified in paddy soils such as rice paddy soils of southern China (Liu et al. 2009), rice field bulk and rhizosphere soil (Stubner 2004), and rice fields subject to long-fertilization practice (Ahn et al. 2012). Other sulfate reducers, such as *Desulfomonile*, *Desulfococcus*, *Desulfobulbus*, *Desulfosporomusa*, *Desulfovibrio*, *Desulfocapsa*, *Desulfomicrobium*, and *Desulfosporosinus*, were detected in rice paddy soils but with relatively lower abundances. The identification of a large number and wide diversity of sulfate reducing bacteria in the paddy soils indicated a dynamic S cycling in the tested rice fields.

The dominance of iron-oxidizing bacteria (FeOB) and sulfur-oxidizing bacteria (SOB) here was in consistent with findings in previous research, which reported that Fe and S oxidation took place at the interface between oxygenated rhizosphere and anoxic bulk soil (Brune et al. 2000). The FeOB and SOB may utilize the oxygen penetrating to the subsurface soil or the oxygen transported via aerenchyma system. The oxygen then may be used for the oxidation of reduced compounds, such as ammonia, ferrous iron, sulfide, and methane, thus regenerating electron acceptors for anaerobic bacteria. Sulfur oxidizing bacteria (SOB), such as *Thiobacillus*, *Sulfuricurvum*, were detected in all soils. *Thiobacillus* contains acidophilic, aerobic, and disulfide-oxidizing species, and they can use reduced S and Fe as sole energy sources. For example, *Thiobacillus ferrooxidans*, a gram-negative bacterium, could gain energy for growth and maintenance from the oxidation of ferrous iron or reduced sulfur compounds (Jensen and Webb 1995). *Sulfuricurvum* contained some species that are facultatively anaerobic, chemolithoautotrophic, sulfur-oxidizing bacteria (Kodama and Watanabe 2004). Other SOB were detected but only show a low relative abundance. These SOB included *Sulfuritalea*, *Sulfurospirillum*, and *Sulfurimonas*. It is noteworthy that some FeOB were also identified. *Gallionella* were identified in all soils with a relatively high abundance. Members of the genus *Gallionella* were important FeOB due to their capability to autotrophic Fe oxidation (Hallbeck and Pedersen 1991; Hanert 1981).

Potential inhibition of methanogenesis

Several methanogens were detected in five rice paddy soils. In contrast to the enrichment of a number of Fe- and S-related bacteria, methanogens demonstrated low abundance in all five rice paddy soils. The most abundant class of methanogens was *Methanomicrobia*, accounting for only 0.04 % in total classified sequences, a very minor part of the microbial communities in rice paddy soils. The low abundance of identified methanogens indicated a possible inhibition of methanogenesis by competitors such as nitrate, sulfate, and iron reducers. The inhibition of methanogenesis by SRB and FeRB were reported elsewhere (Abram and Nedwell 1978; Bodegom et al. 2004; Achtnich et al. 1995a). The inhibition of methanogenesis was explained by competition between sulfate reducers and methanogens for H_2 (Abram and Nedwell 1978; Achtnich et al. 1995b). Addition of ferrihydrite also resulted in incomplete inhibition of methanogenesis by competition for transferring H_2 between FeRB and methanogens (Achtnich et al. 1995b).

Other dominant bacteria

Some other bacteria, never correlated with Fe and S cycling before, were dominated in all soils. These bacteria included *Halomonas* and *Rhodoplanes*. *Halomonas* represented a group of salt-tolerant bacteria (Mata et al. 2002; Mormile et al. 1999; Vreeland et al. 1980), including some denitrifying species (Mormile et al. 1999) and some iron-oxidizing species (Kaye et al. 2011). However, *Halomonas* spp. have never been detected in rice paddy soil. The role of *Halomonas* in rice fields is still ambiguous and need further investigation. *Rhodoplanes* consisted of some species isolated from brackish paddy soil (Lakshmi et al. 2009), rhizosphere soil of paddy (Srinivas and Ch 2014). In addition, some members of *Rhodoplanes* were classified as purple non-sulfur bacterium (Hiraishi and Ueda 1994; Oda et al. 2002; Okamura et al. 2009). The purple non-sulfur bacteria (PNSB) have been isolated and utilized for applications in the areas of environmental protection and agriculture because they are capable of photoautotroph and photoheterotroph growth under anaerobic light conditions, as well as chemolithotrophic growth under aerobic dark conditions (Kim et al. 2004; Nunkaew et al. 2012). PNSB have been considered to be one of the natural biofertilizers as they can fix nitrogen (Harada et al. 2005) and produce indole-3-acetic acid (IAA) and 5-aminolevulinic (ALA) (Koh and Song 2007). Therefore, the dominance of *Rhodoplanes* may have a beneficial ecological function to enhance the soil fertility.

In summary, we applied physiochemical analysis, high-throughput sequencing, and statistical analysis to characterize the microbial community in five rice fields irrigated by AMD-contaminated water. The combination of geochemical data and microbial community analysis provided knowledge of microbial community structure and the key microbial players in anoxic rice paddy soils. A number of Fe- and S-related bacteria such as phylotypes closely related to the genera GOUTA19, *Shewanella*, *Geobacter*, *Desulfobacca*, *Thiobacillus*, *Desulfobacterium*, and *Anaeromyxobacter* were

identified and dominant in rice fields. Among the dominant genera, GOUTA19 and *Shewanella* were seldom detected in paddy soils, indicating the flooded ecosystem may harbor functional microorganisms more than expected. Overall, one significant implication of these results was that these Fe- and S-related bacteria were widely distributed and were mainly responsible for Fe and S biogeochemical cycling in paddy soils. These bacteria may also have the potential to inhibit methanogenesis.

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