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Running title:

The patterns of bacterial community and relationships between SRB and hydrochemistry

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Notes

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Abstract

Microorganisms are the primary agents responsible for the modification, degradation and/or detoxification of pollutants, and thus play a major role in their natural attenuation; yet little is known about the structure and diversity of this subsurface community and how it correlates with groundwater hydrochemistry. In this study, denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP) allowed a comparative microbial community analysis of sulfate-contaminated groundwater samples from nine different wells in the region of Baogang rare earth tailings. Using real-time PCR, the abundance of total bacteria and the sulfate-reducing genes of *aprA* and *dsrB* were quantified. Statistical analyses showed a clear distinction of the microbial community diversity between the contaminated and uncontaminated samples with *Proteobacteria* being the most dominant members of the microbial community. SO_4^{2-} concentrations exerted a significant effect on the variation of the bacterial community ($P < 0.05$) with higher concentrations of sulfate reducing the microbial diversity (H' index), indicating that human activity (e.g. mining industries) was a possible factor disturbing the structure of the bacterial community. Real-time PCR analysis of the functional genes showed that the proportions of *dsrB* to total bacteria were 0.002% - 2.85% and the sulfate reducing bacteria (SRB) were predominant within the prokaryotic community in the groundwater. The uncontaminated groundwater with low sulfate concentration harbored higher abundance of SRB than that in the polluted samples, and no significant correlation was observed between sulfate concentrations and SRB abundances in this study suggesting other environmental factors possibly contributed to different distributions and abundances of SRB in the different sites. The results should facilitate expanded studies to identify robust microbe-environment interactions, and provide a strong foundation for qualitative exploration of the bacterial diversity in rare earth tailings groundwater that might ultimately be incorporated into the remediation of environmental contamination.

Keywords: Groundwater; Microbial community; Sulfate-reducing bacteria (SRB); DGGE; T-RFLP; Real-time PCR;

rare earth tailings

Introduction

Human activities were involved in the exploration of mining industries, which have caused many environmental problems owing to the lack of the treatment techniques. Acid mine drainage is one of the major and severe environmental problems in the mining industries. Sulfide minerals, mainly pyrite and pyrrhotite, which are often present in mine wastes and drainage systems, generate acidity when they are exposed to atmospheric oxygen and water in the presence of functional microorganisms (Oscar et al. 2009). Therefore, the resulting acid mine waters typically contain high concentrations of dissolved heavy metals and sulfate, with a high turbidity, and low pH (Evvie et al. 2009; Oscar et al. 2009). Owing to the lack of useful treatment techniques and proper liners, most of the untreated acid mine water in the drainage system may percolate into the peripheral subsurface in the form of leachate, which exerts a detrimental effect on nearby terrestrial and aquatic ecosystems. This causes lack of water necessary for households and industries, loss or killing of crops and even deterioration of human health caused by water-related problems (e.g., pollution by arsenic, benzene, trichloroethene and so on) (Roling et al. 2001, Kjeldsen et al. 2002; Mouser et al. 2010). It is estimated that metal and sulfate contaminated wastewater, produced by acid mine drainage and mineral processing, occurs at an estimated seventy percent of the world's mine sites, making it one of the mining industry's most significant environmental and financial liabilities (Evvie et al. 2009; Oscar et al. 2009). Therefore, the studies regarding to acid mine water and the resulting ecological effects should provide a solid foundation for the remediation of environmental contamination, in light of the potential damage these waters pose to sensitive eco-systems.

Rare earth elements (REEs) are important metallic raw materials for manufacturing many devices that people use every day. They prevail in computer, rechargeable batteries, catalytic converters, magnets, electric or hybrid vehicles and much more; and REEs are mainly smelted and refined from pyrite and pyrrhotite. Concentrated sulfuric acid roasting is the main technology for decomposing insoluble rare earth minerals (e.g. bastnaesite $LaFCO_3$ and xenotime YPO_4) to soluble sulfate of rare earth ($RE_2(SO_4)_3$), resulting in large-volume discharge of acid-containing (mainly in the form of sulfide or sulfate) effluent in the deposited tailings (Wang et al. 2010; Zhao et al. 2013). Baogang rare earth tailings is a typical effluent reservoir, which is used for holding the wastewater or slag produced by REEs refining from Baogang rare earth mine. It was previously reported that the sulfate concentrations in this site were far higher than that found in other potential contaminated groundwaters such as acid rock drainage where concentrations may range from 1000-2000 mg/L due to the leachate from tailings, wind power or precipitation (Wang et al. 2010). The plants and animals from the land surface have been adversely affected, with DNA damage, higher malonaldehyde (MDA) contents and increasing oxidative stress damage (Feifei et al. 2012; Wantong et al. 2014). Microbial organisms are the dominant members in the ecosystem and the relative distribution or patterns of microorganisms are strongly influenced by subsurface biogeochemical processes in the polluted aquifers (Wilfred et al. 2001). However, no systematic studies have been undertaken concerning the influence of physicochemical disturbances on microbial community and specific

functional groups in the polluted groundwater.

Microbially mediated reduction of pollutants offers a great potential to remediate contaminated groundwater *in situ* and the main natural microbial remediation mechanism has been determined in a contaminated aquifer (Chang et al. 2001; Kleikemper et al. 2002). However, comprehensive surveys of sulfate-polluted groundwater in relation to microbial communities and chemical characterization have been scarcely reported. Thorough knowledge of the structure and diversity of microbial populations in the groundwater across different spatial scales will help predict the potential for natural attenuation. With the rapid development of molecular biology, there are a number of techniques available to study bacterial diversity in subsurface environments. DNA-based molecular profiling tools, such as denaturing gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (T-RFLP), now allow rapid description of various microbial populations in groundwater ecosystems (De Vet et al. 2009; Geets et al. 2006; Vanni et al. 2012). Although the two techniques have their own limitations, for example, each band on DGGE may correspond to a microorganism at a population level of 1% or greater and multiple bands of T-RFLP may relate to the same group of microorganisms, the cooperation of DGGE and T-RFLP can be highly effective and low-cost in revealing bacterial community structure when compared with modern high throughput sequencing techniques (Camarinha-Silva et al. 2012; Siri et al. 2014; Sutton et al. 2009; Vanni et al. 2012). These techniques helped to provide a baseline for the bacterial community structure and were used in combination with various statistical techniques to evaluate relationships between groundwater chemistry and aquifer microbial properties.

The dissimilatory sulfate-reducing bacteria (SRB), as one of the environmentally ubiquitous microbial groups, are found over an extensive range of salt concentrations (such as sulfide) (Blazejak et al. 2011; Chang et al. 2001; Kleikemper et al. 2002). The SRB communities constitute a diverse group of prokaryotes that contribute to a variety of essential functions in many anaerobic environments. The functional groups participate in the carbon and sulfur cycles, and especially biodegradation of organic pollutants (such as aromatic hydrocarbons and petroleum-derived hydrocarbons) while using the sulfate ion as the terminal electron acceptor (Johnson and Hallberg, 2005; Dar et al., 2005). Despite their importance in deeply buried environments, the abundance and diversity of SRB in this environment is poorly understood. Most SRB are difficult to cultivate, and therefore the real-time PCR technique is widely used in quantifying the abundance of SRB in marine, soil and freshwater environments, which enables us to test the trends in relationships between SRB and the physicochemical environments (Blazejak et al. 2011; Liu et al. 2009). Dissimilatory sulfide reductase (encoded by the *dsrAB* gene), found in all known sulfate reducers, catalyzes the final reduction of sulfite to sulfide and therefore the *dsrAB* gene is a key functional marker for detecting SRB (Blazejak et al. 2011; Geets et al. 2006; Klein et al. 2001). Due to PCR bias or mismatches of the *dsrAB* of not yet discovered SRB with the available *dsrAB* primers, other important SRB might have been overlooked in environmental samples. For this reason, another independent SRB quantification method is useful to confirm the full quantitative coverage of SRB in environmental sample analyses, especially for the deep biosphere (Blazejak et al. 2011). A second functional gene *aprA* catalyzes the two-electron reduction of APS to sulfite and adenosine monophosphate (AMP). The *aprA* gene encodes

adenosine 5'-phosphosulfate (APS) reductase and has been thoroughly studied in quantifying genes involved in sulfate reduction in SRB (Meyer et al. 2007a).

In this study, the main objective was to better understand the structure and diversity of the bacterial community associated with the sulfate-contaminated groundwater near the rare earth (RE) tailings using DGGE and T-RFLP. A real-time PCR assay specific for the abundances of total bacteria and SRB was carried out to obtain the profile of the proportion of bacteria that contribute to sulfur redox cycling and to evaluate the importance of SRB in the natural attenuation of sulfate. Furthermore, the correlation of bacterial abundances with the pollutants was also investigated in this work. In summary, this study has the overarching goal of providing a solid foundation for more detailed explorations of bacterial diversity and relationship between SRB abundance and environmental parameters in sulfate-polluted groundwater.

Materials and methods

Study site, sample collection and chemical analysis

Baogang rare earth tailings pond (a total area of about 12 km^2) is located in the southwest of Baotou city, China, along the west side of the Kundulun River (containing water year-round) and the east side of Gerhard Gate Ditch (a seasonal river) (Fig. S1). Both rivers flow southward along the opposite side of the tailings pond. Based on the hydrogeological parameters (data not shown), the groundwater flows from northeast to southwest. The nine groundwater wells in different sites were drilled at a water depth of about 25 m in July 2013 along the groundwater flow path. The wells were labeled as GW-1, GW-2, GW-3, GW-4, GW-5, GW-6, GW-7, GW-8 and GW-9 in this study. One uncontaminated control sample was taken from the GW-5 well (Fig. S1), which was outside the downstream flow from the pond. GW-9 well is located furthest from the tailings but adjacent to local pig farms.

All groundwater samples (single sample of 2 L from each site) were collected using a submersible peristaltic pump (Boshan, China) at a rate of 8 L min^{-1} from a depth of 15 m below the ground surface. The pH and temperature of the *in-situ* groundwater ranged from 6.98-7.88 and 11.1°C -13 °C, respectively (Table 1). All water samples were collected in July 2013, placed into individual sterilized plastic barrel and kept cool in ice bags. For each site, three subsamples were merged into one sample. Each sample was mixed uniformly to achieve high representativeness before collecting cells. Microbial cells were collected by filtering through a 0.22 μm millipore membrane (50 mm, Saiyintan, China) using a vacuum manifold (Lichen, SHZ-D (Ⅲ), China) with six coupling filters (Hannuo, MS-6, China). All filters were stored at -20 °C prior to DNA extraction.

Water sub-samples were immediately acidified with concentrated HCl to prevent precipitation of metals, and stored in completely filled polyethylene bottles (500 mL). Permangannate indexes, representing the contents of dissolved organics, were measured by the titration method. In the acidified samples, the major dissolved cations were determined

by inductively coupled plasma atomic emission spectroscopy (ICP-AES, Optima 7000DV, PerkinElmer, USA), while fluorinion, nitrate and sulfate were determined on untreated subsamples by ion chromatography (DIONEX-500, USA). Dissolved ammonium and nitrite were determined colorimetrically according to the study reported by Gorra et al. (2012).

DNA extraction

Frozen 0.22 μm filters (equal to the volume of 600 mL for each water sample) were cut aseptically into small pieces (~1 cm^2). Genomic DNA was extracted using the FastDNA Spin Kit for Soil (MP Biomedicals, USA) according to manufacturer's instructions. The DNA pellet was resuspended in 100 μL of sterile DNase-treated water (Invitrogen, USA) and the quantity of extracted DNA was evaluated using QuantiFluords DNA System (Promega, USA) with a spectrophotometer (SpectraMax M5, USA). Quantified DNA was stored at -20 °C until required for molecular analysis.

PCR amplification

PCR amplifications were performed in a DNA Thermal Cycler (Mastercycler gradient, Eppendorf). For PCR-DGGE, total bacterial 16S rRNA genes were amplified directly from the quantified DNA using the bacterial universal primers 341F-GC (with the GC clamp) and 517R (Table S1) (Cho et al. 2003). The PCR reaction mixtures consisted of 1 μL of primers (10 μM), 1 μL of DNA (20 ng/μL), 25 μL of 2 × *Premix ExTaq*™ polymerase (1.25 U/25 μL, Takara, Japan), 0.5 μL bovine serum albumin (20 mg/mL, BSA, Takara, Japan) and sterile H_2O up to a final volume of 50 μL. The reaction mixtures were preheated at 95 °C for 5 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 15 s and 72°C for 15 s, with an additional 72 °C for 5 min as a final extension step. The amplification products were purified using the universal DNA Purification Kit (Tiangen Biotech, China). Prior to DGGE analysis, the presence of the expected 167 bp PCR product was confirmed by agarose gel electrophoresis.

DGGE analysis

DGGE was performed using a DCode Universal Mutation Detection System (Bio-Rad, USA) with a gradient of denaturant (7 M urea and 40% deionized formamide) from 45% to 55%. The DGGE was conducted and bands of interest were cut as described by Cho et al. (2003). All nucleotide sequences were compared with GenBank entries, using BLASTn to select reference sequences and obtain preliminary phylogenetic affiliation (Kan et al., 2014). The resulting sequences were aligned using MEGA 6 with sequences retrieved from the GenBank database. A phylogenetic tree was constructed using maximum likelihood analysis, maximum parsimony and neighbor joining analysis under the default parameters and all distance trees were bootstrapped 1,000 times. All the 16S rRNA gene sequences (in total 25 sequences) were deposited in the DDBJ (DNA Data Bank of Japan, http://www.ddbj.nig.ac.jp/) database under the accession numbers LC038196-LC038219.

T-RFLP fingerprinting

Approximately 1,500-bp region of bacterial 16S rRNA gene was amplified using single fluorescently labeled bacterial-specific oligonucleotide primers: 27F (FAM-labeled) and 1492R (Table S1). The PCR reaction mixture was prepared as previously described except different primers were used. For each sample, reactions were performed in triplicate and the PCR products were pooled. The PCR procedure consisted of an initial denaturation step of 5 min at 94 °C followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min with a final extension at 72 °C for 7 min. The PCR products were purified as the above described. The purified PCR products were digested with restriction endonuclease *Msp* I (New England Biolabs, USA) at 37 °C for 2 h followed by an inactivation step at 65 °C for 10 min. Fragment analysis was conducted by Capillary electrophoresis on an ABI 3730 DNA analyzer (PE Biosystems) with parameters set to exclude fragments shorter than 50-bp or larger than 550-bp and those under 40 fluorescence units. Fragments were binned into T-RFs at a spacing of 1.0 ± 0.2 bp standard deviation. The relative abundance of each peak was calculated and expressed as a fraction of relative abundance = peak area/∑ peak areas of sample X (Fahy et al. 2005). Fragments with relative abundance values less than 1% were discarded.

Real-time PCR assays

PCR measurements were performed in triplicate using SYBR *Premix Ex Taq*™ Ⅱ (TIi RNaseH Plus, Takara, Japan) with the real-time fluorescent quantitative PCR (Roche, Switzerland). Abundances of total bacteria were determined by real-time PCR assay of 16S rRNA gene using a published real-time PCR protocol and primer pairs of 341F and 517R (Cho et al. 2003; Kan et al. 2014). The dissimilatory sulfite reductase gene *dsrB* of SRB was quantified with the primer set of DRRp 2060F and DSR4R as described by Geets et al. (2006). To quantify the gene *aprA*, the primers APS1F and APS4R were used and are listed in Table S1. The total real-time PCR reaction mixture volume was 20 μL and consisted of 10 μL SYBR *Premix Ex Taq*™ II (2 × Conc., Takara, Japan), 1 μL of BSA (20 mg/mL), 0.8 μL of each primer (10 μM) and 2 μL of diluted extracted DNA (10 ng/μL) as template. The real-time PCR assays of *dsrB* and *aprA* were carried out under the following reaction conditions: 2 min at 95°C for initial denaturation; 40 cycles of 30 s at 95°C, 30 s at 60°C, and 30 s at 72°C. All real-time PCR reactions were performed in triplicate.

Melting curve analysis was used to check the specificity of real-time PCR products. Real-time PCR amplification of the standards was achieved using genes of 16S rRNA, *dsrB* and *aprA* that were incorporated into plasmids as inserts after the combined molecular mass of the plasmids been determined by spectrophotometric measurement at 260 nm. The results were analyzed using the software LightCycler 480. Based on the slope of the standard curve, the amplification efficiency was calculated using the formula: $E = (10^{-1/slope} - 1) \times 100\%$. According to this formula, an efficiency of 100% means a doubling of the product in each cycle and the data with the amplifications efficiency beyond the range (90% −110%) were discarded. The copy numbers in the samples were calculated through comparison with the threshold cycle values of the standard curve, taking into account the dilution of DNA and the liquid volume of the samples. A basic assumption was made that there were 3.6 copies of 16S rRNA gene in each cell and only one copy of *dsrB* gene per

SRB cell, the proportion of sulfate reducer to the total bacterial community was calculated according to Liu et. al (2009).

Statistical analysis

A phylogenetic tree was constructed from the DGGE data using UPGMA (unweighted pair-group method with arithmetic averages) with MAGE 6.0 software (http://www.megasoftware.net/). The T-RFLP patterns were analysed by principal component analysis (PCA) on both of chemical parameters and microbial community using R (version 3.1.2, http://www.r-project.org/). Canonical correspondence analysis (CCA) was conducted to analyse the relationship between environmental physio-chemical parameters and bacterial community. All groundwater properties were $\log_2$ (x+1) transformed for standardization. The Shannon index (H' index) was used to define the bacterial community diversity, and it was calculated based on the data of the number of T-RFs in the contaminated groundwater using R. In order to analyse the real-time PCR data, copy numbers were $\log_{10}$-transformed to normalize the values and statistical analysis was conducted using Origin 9.0. Pearson correlation analysis of microbial abundances (log data of gene copies and relative abundance) with chemical variables was performed using software SPSS 19.0 (IBM, USA). Here, relative abundance was defined using the formula: R = $\log_{10}$ (targeted gene copies)/$\log_{10}$ (16S rRNA gene copies). In statistical analysis, samples that differed by $P < 0.05$ were described as being significantly different.

Results

Chemical characteristics of groundwater

All nine wells were slightly alkaline, pH ranged from 7.47 to 7.88, with the exception of GW-1 which had a pH of 6.98. Sulfate concentrations decreased from 4444 mg/L to 2.54 mg/L corresponding to wells GW-2, GW-4, GW-3, GW-6 and GW-7 in the order along the advective groundwater transport flow (from northeast to southwest) (Table 1). The GW-9 well, which was farthest away from the tailings pond but nearby to husbandry farms, contained far more sulfate (584 mg/L) than the control GW-5 well (16.1 mg/L). Permanganate index partly represent the contents of organic materials, ranging from 1.4 mg/L to 3.2 mg/L. It was observed that the highest organic concentrations were detected in the GW-9 well and the lowest concentrations of organics were found in the GW-1 well. As for cations (Na^+, Mg^{2+} and Ca^{2+}), there was a similar decreasing trend as observed with sulfate. Nitrogen, mainly in the form of dissolved ammonium (from 0.364 mg/L to 57.26 mg/L) was in relatively low concentrations in the groundwater at the different wells. The quantity of nitrate was below the detection limit with the exception of the well GW-1 (5.1 mg/L).

Analysis of bacterial community structure by PCR-DGGE analysis

Microbial communities in groundwater were profiled by DGGE of amplified 16S rRNA gene fragments. The profiles of the bacterial communities were complex, and a minimum of eleven bands was observed for each of the samples (Fig. 1). More complex DGGE profiles (about 15 visible bands for each sample) were obtained from the control GW-5 with the

lower concentration of sulfate (16.1 mg/L) and the GW-9 well near pig farms. The lowest bacterial community diversity with only eleven bands was observed at GW-2, which was detected with highest concentrations of sulfate (4,444 mg/L) and nitrite (4.91 mg/L). The sample from GW-7 well with the least sulfate (only 2.54 mg/L), showed a profile with a relatively limited bacterial community (about twelve visible bands). Nevertheless, the sample from GW-1 well with the highest nitrate concentrations showed more than fifteen bacterial bands.

Sequencing of the 16S rRNA gene-based DGGE bands revealed that most of bacterial strains were affiliated with members of the phylum *Proteobacteria* (*Alphaproteobacteria* [GW9-B3]; *Betaproteobacteria* [GW1-B9, GW3-B10, GW5-B14, GW8-B5, GW8-B13 and GW9-B21]; *Gammaproteobacteria* [GW5-B23, GW8-B19, GW9-B2, GW9-B7 and GW9-B20]; *Epsilonproteobacteria* [GW6-B24 and GW9-B26]) and some uncultured bacteria (Table 2 and Table S2). The majority of these gene sequences had close matches with bacteria isolated from contaminated aquatic environments (groundwater, river or urban water, sediments or biofilms). There was only one sequence (GW9-B3, *Novosphingobium* sp. IAR13, KF053364, 100% identity), identified and affiliated into the order of *alpha proteobacteria*, which was capable of degradation of hydrocarbon. Sulfur-oxidizing bacteria (SOB) were detected and had the maximum match with three bacterial sequences , including GW1-B9 (*Thiobacillus thioparus*, *Betaproteobacteria*, 99% identity), GW6-B24 (*Sulfurimonas denitrificans* DSM, *Epsilonproteobacteria*, 97% identity) and GW9-B26 (*Sulfurimonas autotrophica* DSM, *Epsilonproteobacteria*, 99% identity); and these corresponding samples were found with relatively higher concentrations of sulfate (2,645 mg/L, 332 mg/L and 584 mg/L, respectively). In the control GW-5 with the low sulfate concentration, sulfur-oxidizing bacteria were not observed. It was evident that a large proportion of sequences related to microorganisms that were capable of degrading hydrocarbon (i.e., GW3-B12, GW5-B11, GW9-B3, GW9-B7 and GW9-B21) or transforming nitrogen (i.e., GW1-B25, GW9-B2, GW9-B6 and GW9-B8). Band B5, which is specifically present at the low SO_4^{2-}-enriched groundwater of GW-8, was affiliated with *Hydrogenophaga* sp. XT-N8 (98% identity), while B25, closely related to *Comamonas* sp. AP5s2-M2b (98% identity), was only retrieved from the relatively higher SO_4^{2-}-enriched groundwater of GW-1. The presence of *Salmonella* sp. XJ134-1212-NF1 (KF828874, 99% identity, B23) was only observed in the control GW-5 well. In addition, it seemed that nearly all the samples from the contaminated area had the sequence B12 that was closely affiliated with *Acidovorax* sp. IW-204 (99% identity). It is a facultative anaerobe, which makes ATP by aerobic respiration if oxygen is present, but is capable of switching to fermentation or anaerobic respiration if oxygen is absent (Garrity et al. 2005). The facultative properties of *Acidovorax* possibly explain why it could survive in either environment. None of 16S rRNA gene sequences from the groundwater showed any affiliation to SRB.

In this study, PCR-DGGE analysis was simply used to target bacteria using the universal primer pairs 341F-515R (Table S1), but a dominant archaeal sequence belonging to an unknown member from the methanogenic prokaryote enrichment culture (B18, 100% identity) was detected in the sample of GW-1. The enrichment culture originated from the microbial communities associated with hexadecane degradation under hypothermia methanogenic condition. There were no more relevant references reporting which order the methanogenic prokaryote clustered with. Hierarchical cluster analysis of

DGGE-band sequences revealed six distinct groups were clustered and are denoted, revealing a signification proportion of *Proteobacteria* in the phylogenetic tree (Fig. 2). In addition, the *Epsilonproteobacteria* group consisted of GW6-B24 (97% identity) and GW9-B26 (99% identity) was found in marine sediments, showing affinity to the sequences of the family *Sulfurimonas*. The sequences identified in GW1-B25 (98% identity), GW3-B10 (100% identity), GW3-B12 (99% identity), GW5-B4 (100% identity) and GW9-B6 (100% identity) which were similar to each other, appeared to be clustered into a group.

T-RFLP analysis

Fig S2 showed T-RFLP patterns of microbial composition, and highlighted that, bacterial diversity varied significantly among all the investigated wells. Correlation analysis showed that all the H' indexes were significantly correlated with NH_4^+ ($P < 0.05$) and F^- ($P < 0.05$), respectively (Table 3). Among the top 18 OTUs, control sample GW-5 showed relatively fewer T-RFs than other polluted samples, consisting of two main T-RFs (F4 and F6), while H' index of GW-5 (11.38) was highest except for GW-4 (11.67) (Table 4). The diversity of bacterial community in GW-7 was relatively lower with H' index of 10.93, which was similar to the result observed in DGGE profile. To further evaluate the differences between control and polluted samples, PCA was conducted using bacterial community profiles and 6 PCs were needed to describe the large majority (~97%) of data set variance (Fig. 3a). PC1 and PC2 accounted for 36.1% and 21.81% of the variance, respectively, indicating that the two dimensions gave a good representation of the community data. Three samples GW-6, GW-7 and GW-8 with relatively less sulfate concentrations formed a tight cluster; GW-2 and GW-9 clustered together far away from all the other samples. For two contaminated samples nearby the tailings pond, GW-3 and GW-4 were separated distantly from the control sample along PC2.

To investigate the main environmental factors affecting bacterial community structures, PCA of geochemical properties was carried out, followed by CCA associating geochemical characteristics with bacterial population profiles. PCA biplot showed GW-3, GW-4 and GW-6 formed a cluster, while GW-5, GW-7 and GW-8 grouped together to form another cluster (Fig. 3b). Despite there being no distinct differences among GW-5, GW-7 and GW-8 in the geochemical environments (Fig. 3b), the structure of bacterial community of the control GW-5 was different from any other polluted samples (Fig. 3a). Similarly, although microbiomes from GW1 and GW2 were present in the similar chemical environments (Fig. 3b), the microbial community showed distinctly different patterns that clustered in separate groups (Fig. 3a).

The relative positions of the samples were observed as a function of both the bacterial T-RFLP profiles and the geochemical data by CCA (Fig. 4). In the study, environmental geochemical characteristics, as constrained variables, explained 100% of the bacterial structural dissimilarity among all of the samples. As in the PCA analysis of microbial community (Fig. 3a), the control sample was separated from all the other samples; however, the four samples GW-2, GW-3, GW-4 and GW-9 formed a cluster, while GW-1 was an outlier in the bi-plot. Based on the variance test

significance and envfit function with 999 Monte Carlo permutations, four environmental factors, including SO_4^{2-} ($P < 0.05$), Mg^{2+} ($P < 0.01$), Na^+ ($P < 0.05$) and Ca^{2+} ($P < 0.05$), contributed significantly to the shift in bacterial community composition.

Quantification of the functional genes *aprA* and *dsrB* of SRB and 16S rRNA of total bacteria in groundwater samples

Standard curves for real-time PCR were obtained by preparing 10-fold dilutions of three plasmids containing 16S rRNA, *aprA* and *dsrB* genes with the primers shown in Table S2. All the standard curves showed a linear range between $10^2$and 10^8 copies, with a slope of -3.329, -3.337 and -3.35 for 16S rRNA, *aprA* and *dsrB* genes, respectively. The calculated PCR efficiencies for the 16S rRNA, *aprA* and *dsrB* assays were 99.7%, 99.4% and 98.8%, separately. Fig. 5 showed the profile of DNA copy numbers of the functional genes *aprA* and *dsrB* present in sulfate-reducers and the genes of 16S rRNA present in the total bacterial population. In each of the groundwater samples, at the depth of 15 m, the copy numbers of the 16S rRNA gene exceeded those of the two functional genes. The abundance of the *aprA* gene was relatively higher than that of the *dsrB* gene across all the samples. From this study, it appeared to be that the changing profiles of copy numbers of both of functional genes, *aprA* and *dsrB*, were almost similar in most of samples with the exception of the wells GW-1 and GW-9. By assuming 3.6 copies of 16S rRNA gene per cell and only one copy of *dsrAB* gene per SRB cell, the proportion of sulfate reducers to total bacteria was calculated (Liu et al. 2009). The copy numbers of the functional gene *dsrB* comprised between 0.002% - 2.85% of the 16S rRNA gene copy numbers of the total bacteria in the initial groundwater samples.

No significant differences were detected in the bacterial abundance (16S rRNA gene copy numbers) among the nine different wells, even though there were different concentrations of SO_4^{2-} (Fig. 5). There were up to 10^{11} 16S rRNA gene copy numbers L^{-1} in most of the samples. Sulfate-reducing bacteria numbered between 10^5 and 10^9 *aprA* gene copies L^{-1} with the highest gene copy number (1.86×10^9) in GW-1 well. In contrast to *aprA*, a distinctly different abundance was found with *dsrB*, the other gene characterizing SRB, where there were 10^1 to 10^7 copies L^{-1}. There were significantly more *dsrB* copy numbers (1.08×10^7) in the control GW-5 well than those in any other wells. In contrast, *dsrB* abundance was close to the detection limit of real-time PCR method (only one order of magnitude, 3.09×10^1 copies) in the well GW-9 well with a sulfate concentration of 584 mg/L.

Based on the significant difference of spatial distribution among the groundwater wells, the Pearson correlation test was used to evaluate how microbial abundance (log data of the gene copies numbers) related to variations in groundwater chemistry. Our data indicated that the abundance of SRB did not appear to positively correlate with the chemical composition of groundwater (Table 3). However, significant and negative correlation was observed between relative abundance of *dsrB* and Na^+ concentrations ($P < 0.05$). There was no significant correlation between the organic concentrations (permanganate index) and the microbial community.

Discussion

Although REEs have many important applications in modern, so-called green technology, including, electric or hybrid vehicles and wind turbines, mining, refining, and recycling of REEs have serious environmental consequences if not properly managed. Toxic acids are required during the refining process of REEs and improper handling of these substances can result in extensive environmental damages. In this study, the groundwater in the surrounding area of Baogang REEs tailings pond was established to be contaminated by sulfate associated with the leachate from the tailings pond (Wang et al. 2010). The SO_4^{2-} concentrations in the polluted groundwater (up to 4444 mg/L) were far greater than the regulatory value (250 mg/L) provided by World Health Organization (Guidelines for Drinking Water Quality, 2nd Ed), posing a serious threat to human health. It was expected that the bacterial structure and composition would change in response to geochemical conditions (i.e., nutrients level, redox potential, chemicals, etc.) (Flynn et al. 2013; Yeung et al. 2013). In this study, it was revealed that groundwater with different concentrations of sulfate harbored distinct bacterial communities, which indicated spatial heterogeneity of bacterial assembly was observed possibly due to the geochemical conditions. CCA analysis revealed SO_4^{2-} concentrations explained a large and significant proportion of the variation in the bacterial community ($P < 0.05$) and microbial community diversity (H' index) was distinctly affected by the change of geochemical niches (NH_4^+ and F^-). For the control GW-5 well, there was a higher bacterial diversity with more bands and higher H' index than the polluted samples, indicating that human activities (e.g. mining industries) was a probable factor disturbing the structure of the bacterial community and reducing the microbial diversity (Sirisena et al. 2014). It was also indicated that the microbial ecosystem in the sulfate-polluted groundwater had been compromised due to the loss of microbial diversity (Cho et al. 2003). The GW-9 well revealed the most DGGE bands, which might mean that higher organic matter (3.2 mg/L) from the nearby pig farms entered the groundwater and became a source of electron donors (Cho et al. 2003).

In this study, target-based 16S rRNA gene DGGE and T-RFLP approaches provided good profiles and adequately elaborated information of the bacterial species. *Proteobacteria* was the major taxonomic group in the observed in groundwater. This observation is consistent with other reports that have also stated *Proteobacteria* to predominate in aquifers, sediments and rivers (Grebler et al. 2009; Lear et al. 2007; Sutton et al. 2009). In the landfill leachate-polluted aquifers, the families of *Geobacteraceae* and *Desulfobacteriaceae*, which were affiliated to the class *Delta Proteobacteria*, were commonly found to make a strong contribution to microbial community and play an important role in iron reduction and sulfate reduction, separately (Wilfred et al. 2001; Xiujuan et al. 2009). However, the class *Delta Proteobacteria* acting in sulfate reduction was not detected in this study. It was presumed that *Geobacteraceae* and *Desulfobacteriaceae* tended to grow in the sediment or seawater environments (Wilfred et al. 2001; Xiujuan et al. 2009). In addition, since DGGE analysis had shown that for a band to form, the sequence must at least form 1% of the total population. In case of a diverse population, the bands were perhaps too faint to discern, which partly explained the different *Delta Proteobacteria* distribution patterns that were achieved from leachate-polluted aquifers and the

sulfate-polluted groundwater (Cho et al. 2003). The groundwater environment was an anaerobic habitat for microbial organisms, which was confirmed by the form of nitrogen. In the sulfate-polluted groundwater, nitrogen were primarily represented by NH_4^+-N, and oxidized N forms (NO_3^- and NO_2^-) were relatively low or even less than detection limit, indicating that anoxic zones were available in groundwater (Flynn et al. 2013; Gorra et al. 2012). Sulfur-oxidizing and nitrogen-reducing microorganisms, including GW1-B9 (*Thiobacillus thioparus*), GW6-B24 (*Sulfurimonas denitrificans* DSM) and GW9-B26 (*Sulfurimonas autotrophica* DSM) were found in the oxygen limited environment. It was presumed that even when redox conditions are unfavorable, several types of redox reaction-performing microorganisms were usually present at the same location (Takai et al. 2006; Ludvigsenet al. 1999).

Remarkably, one of the dominant bands in GW-1 well (B18) was clearly related to methanogenic enrichment culture. This also suggested that the presence of anaerobic microorganisms with the potential of consuming a wide range of C1 compounds, such as carbon dioxide, formic acid, methanol and methanethiol (Luton et al. 2002). It was presumed that there was an indication of decay and many bacteria were destroyed by the unfavorable conditions (high sulfate concentration) enabling the growth of methanogens on organic nutrients under anaerobic conditions. Sequences related to potential denitrifiers, the family *Comamonadaceae* (GW3-B12, GW9-B21, GW9-B6 and GW1-B25), were encountered in the polluted groundwater. Therefore, the consistent presence of methanogens (B18) and denitrifiers (B25) in GW-1 well demonstrated the possible presence of microorganisms performing denitrifying anaerobic methane oxidation (DAMO) or sulphate anaerobic methane oxidation (SAMO) (Hoshino et al. 2005; Li et al. 2009; Mechichi et al. 2003; Torrentó et al. 2011).

Although SRB was not detected in all samples by DGGE, real-time PCR results proved the popular existence of SRB by the quantitative copy numbers. In the samples GW-1 and GW-9, percentages of SRB were relatively lower than those in the control GW-5 well when determined with the genes of *aprA* and *dsrB*, suggesting SRB ecology was very complex (Purdy et al. 2002). There could be many reasons for this. It was possible that the different distributions and abundances of SRB were due to environmental factors contributing to distinguishing the different sites (Purdy et al. 2002; Pester et al. 2012). The electron acceptors and donors utilized by SRB were the vital contributors for the abundances and distributions of SRB. For example, SRB coupled sulfate dissimilation with heterotrophic carbon degradation or carbon dioxide fixation, which was important anaerobic degradation pathway for organic matter (Pester et al. 2012; Steve et al. 2004). In a previous study, > 8 mM dissolved organic carbon (DOC) exhibited a sulfate reduction rate of 3.2 mmol SO_4^{-2} (L sediment)$^{-1}$ day^{-1} (Steve H. Harris Jr., 2004). In salt marshes, acetate, a major substrate for sulfate reduction, supported 10% of the sulfate reduction (Hines et al. 1994). Julie et al. found diversity and abundance of *dsrAB* gene differed between the two mudflats with different salinity and sulfate concentrations and the distribution profile of SRB was related to the salinity and the sulfate concentration (Julie et al. 2007). The increased chloride concentrations may have caused an additional impact on the diversity and distribution of the SRB community, which has a metabolism that is highly dependent on habitat (Xiujuan et al. 2009).

The T-RFLP technique is susceptible to the same drawback as DGGE because different species may generate T-RFs with the same length (Abdo et al. 2006; Sirisenaa et al. 2014). Nevertheless, CCA analysis with the data of T-RFs and chemical parameters provided evidence that bacterial diversity dramatically differed between uncontaminated samples and those impacted by the mine tailing pond leachate. Thus, it can be stated that bacterial community composition was related to hydrochemical changes resulting from human activities. This enables us to evaluate the relationship between the contaminated groundwater environment and the microorganisms present within this environment. Previous studies have reported that the bacterial diversity of groundwater is mainly related to groundwater redox-sensitive substances such as Fe, Mn, NO_3-N, NH_4-N and SO_4-S (Roling et al. 2001). In this study, SO_4^{2-} significantly contributed to the variance of bacterial community in the sulfate-contaminated groundwater ($P < 0.05$). PCA analysis with the data of T-RFs also revealed the samples with the similar amount of SO_4^{2-} clustered together. This might indicate that bacterial diversity was mainly influenced by SO_4^{2-} concentration of groundwater, and conversely, the groundwater microbial communities mediate SO_4^{2-} reactions while obtaining energy for survival. Moreover, to keep potential balance, cation ions such as Na^+, Mg^{2+} and Ca^{2+} were dissolved in the groundwater and likewise, influenced the bacterial diversity (Xiujuan et al. 2009).

In contrast to terrestrial environments, the prokaryotic DNA in groundwater has been rarely quantified. Real-time PCR analysis in this study has indicated bacterial abundances to range from 3.23×10^{10} to 4.24×10^{11} copies L^{-1} groundwater. In contrast to the previously studies, the bacterial abundance was an average ~1-2 orders of magnitude higher in groundwater (L^{-1}) compared with marine sediments (cm^{-3}) (Axel et al. 2006; Cindy et al. 2006). There was no correlation between abundance of bacteria and SO_4^{2-} concentration, potentially indicating these detected microorganisms could be found in any groundwater environment irrespective of SO_4^{2-} concentrations, and that possibly the bacterial abundance in each of the wells was influenced by the other physico-chemical properties (eg. Fe or Mn concentrations) (Grebler al. 2009). Compared with groundwater, higher *dsrB* copy numbers have been detected in paddy soil, freshwater and marine sediments environments (Leloup et al. 2007; Stubner et al. 2004). Stubner *et al.* (2004) reported $2\sim4 \times 10^8$ copies g^{-1} dry soil was detected in rice field soil. In the estuarine sediment, it was revealed that SRB abundances ranged from 0.2×10^8 to 5.7×10^8 copies (Leloup et al. 2007). The observed proportions of *dsrB* to total bacteria were 0.002% - 2.85%, indicating that SRB was a major group of the prokaryotic community in the groundwater, but were also lower than the previously reported in marine sediments (0.5%-1%) (Klein et al. 2001; Inagaki et al. 2006). There was a possible interpretation that the detected *dsrB* was not extracted from living cells but was a fraction of fossil DNA, adsorbed to sediment particles as discussed previously (Axel et al. 2006; Inagaki et al. 2006; Riedinger et al. 2010; Schippers et al. 2005). However, the percentage range in the groundwater was much greater than the range reported by Anna Blazejak et al. (2011) where marine sediments samples were analysed (0.5%-1%). For the gene *aprA*, it was found that the copy numbers were more than 10^5 L^{-1} groundwater and the *aprA* abundances were more than the *dsrB* abundances. Usually the copy numbers of these two genes have been found to be very close to one another in the marine sediments, but the *dsrB* and *aprA* abundances in groundwater were unequal and varied with a similar trend across all

443 groundwater wells except in GW-1 well and GW-9 well (Blazejak et al. 2011). A possible explanation is that primers
444 designed for the amplification of a fragment of the *aprA* gene allowed the positive *aprA* amplification in
445 sulfate-reducing as well as sulfur-oxidizing bacteria (Hügler et al. 2010; Meyer et al. 2007b). Different organic matter
446 availability may partially explain the different gene copy numbers of the two genes in the two wells (Blazejak et al.
447 2011).
448 In summary, our study demonstrates a remarkable diversity of bacteria based on analysis of DGGE and T-RFLP. The
449 compositions of bacterial communities displayed distinct spatial variation at the class level. *Proteobacteria* were the
450 most dominant phyla of the total bacterial 16S rRNA gene sequences. CCA analysis indicated sulphate concentrations to
451 explain a large percentage of variation of the bacterial community ($P < 0.05$). . Real-time PCR analysis of the functional
452 genes revealed that SRB are the dominant groups of within the prokaryotic community in the groundwater.
453 Uncontaminated groundwater with lower sulfate concentration harboured higher abundance of SRB than polluted
454 samples, but no significant correlation between sulfate concentrations and SRB abundances was observed.
455 Environmental factors possibly contributed to different distributions and abundances of SRB in the different sites. In
456 addition, sulfur-oxidizing microbes were detected in the groundwater, partially contributing to sulfur metabolism. Future
457 work is needed to characterize the associations between environmental conditions and SRB/SOB communities and
458 obtain a better understanding of biotic and abiotic effects on functional dynamics.
459
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Figure and Table Legends

Figure 1. DGGE profile of 16S rRNA gene fragments using DNA from different groundwater wells as templates. Numbered bands were excised and sequenced (SI Table S1).

Figure 2. Phylogenetic tree of bacterial 16S rRNA genes cloned from groundwater. The tree was obtained using Maximum Likelihood method. Bootstrap values were 1,000 replicates and bootstrap numbers are shown for branches with > 50% bootstrap support.

Figure 3. Principal component analysis (PCA) conducted on (a) 16S rRNA gene bacterial community composition and (b) groundwater hydrochemistry collected from SO_4^{2-}-polluted groundwater.

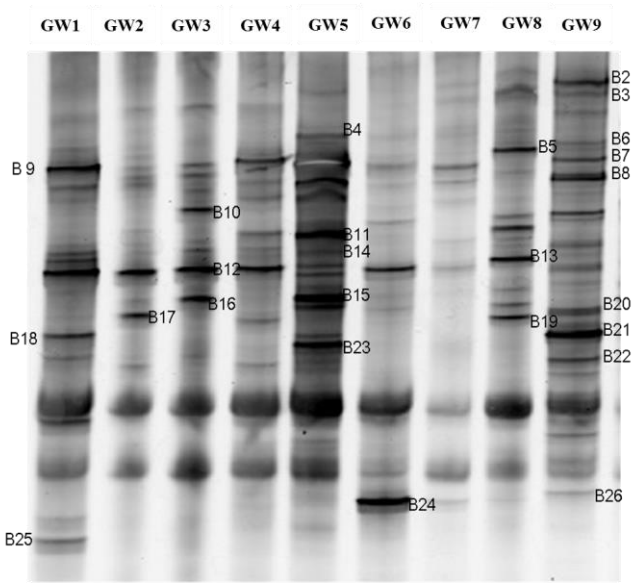
Figure 4. Canonical correspondence analysis (CCA) of T-RFs data and environmental factors. Arrows stand for the direction and magnitude of environmental factors associated with bacterial community structure in different samples.

Figure 5. DNA copy numbers of the numbers of the functional genes *dsrB* and *aprA* as marker for sulfate-reducing bacteria (SRB) and the 16S rRNA gene of total bacteria.

Table 1. Geochemical characteristics of tailings groundwater

Table 2. Pearson correlation analysis between gene abundance and physicochemical parameters.

Table 3. Bacterial diversity of microbial community in groundwater based T-RFLP data



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678 **Fig. 1 DGGE profile of 16S rRNA gene fragments using DNA from different groundwater wells as templates.**

679 **Numbered bands were excised and sequenced (Table 2 and SI Table S1).**

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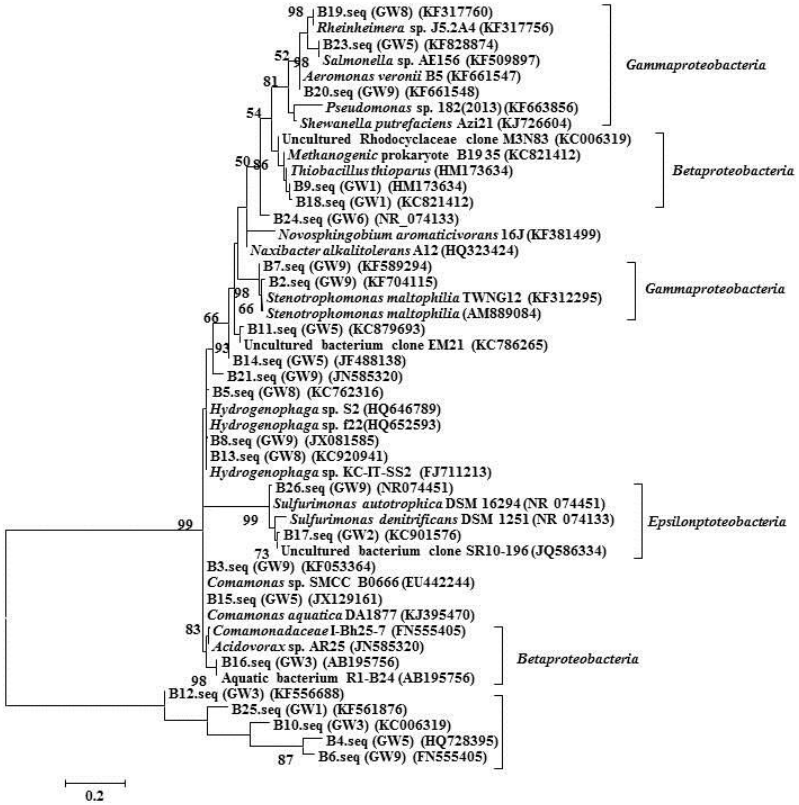
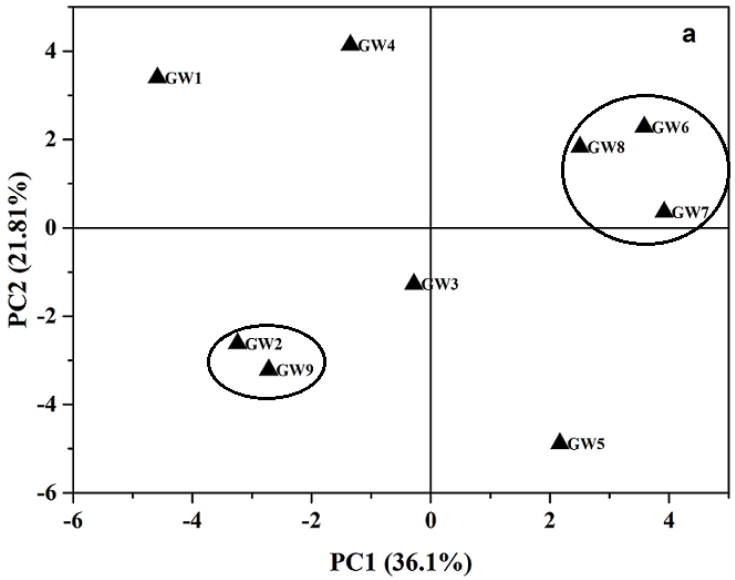


Fig. 2 Phylogenetic tree of bacterial 16S rRNA genes cloned from groundwater. The tree was obtained using Maximum Likelihood method. Bootstrap values were 1,000 replicates and bootstrap numbers are shown for branches with > 50% bootstrap support.



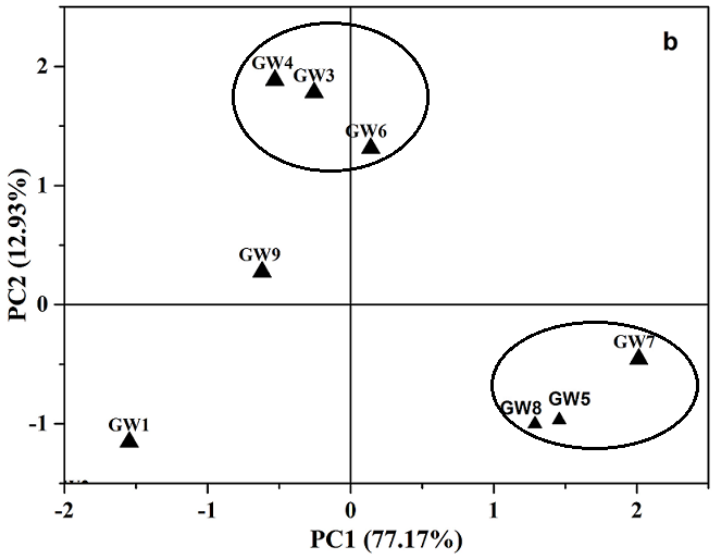


Fig. 3 Principal component analysis (PCA) conducted on (a) 16S rRNA gene bacterial community composition and (b) groundwater hydrochemistry collected from SO_4^{2-}-polluted groundwater.

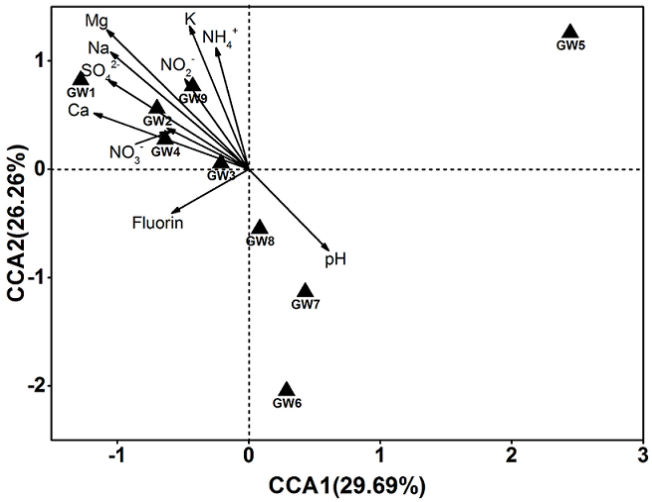


Fig. 4 Canonical correspondence analysis (CCA) of T-RFs data and environmental factors. Arrows stand for the direction and magnitude of environmental factors associated with bacterial community structure in different samples.

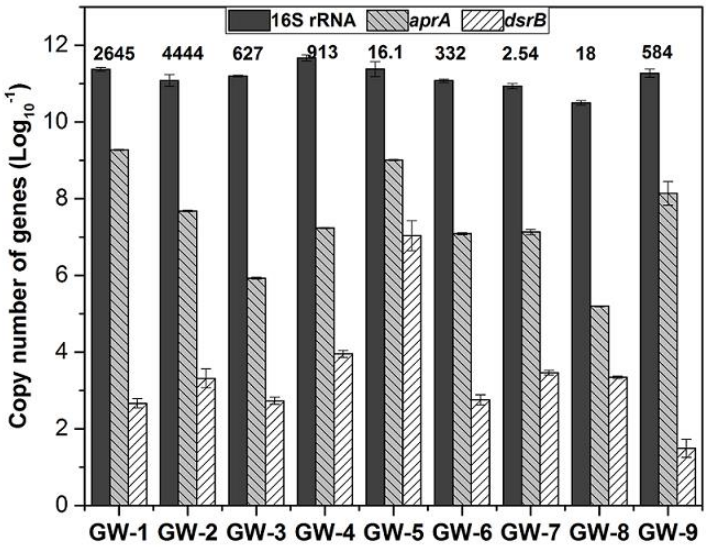


Fig. 5 DNA copy numbers of the numbers of the functional genes *dsrB* and *aprA* as marker for sulfate-reducing bacteria (SRB) and the 16S rRNA gene of total bacteria. The numbers above the columns represent the concentrations of sulfate (mg/L).

Table 1 Geochemical characteristics of tailings groundwater.

	F^{-a}	NO_3^{-a}	NO_2^{-a}	SO_4^{2-a}	NH_4^{+a}	Na^{+a}	K^{+a}	Mg^{2+a}	Ca^{2+a}	T (℃)	pH	Permangannate index[a]
GW-1	0.86	5.1	0.078	2645	36.79	618	17.2	548	535	11.4	6.98	1.4
GW-2	0.49	-[b]	4.91	4444	57.26	802	40.4	797	643	11.4	7.47	2.0
GW-3	0.74	-	0.009	627	1.333	342	5.51	102	132	10.2	7.59	1.9
GW-4	1.81	-	0.003	913	0.905	302	8.85	180	219	11.1	7.73	2.1
GW-5	0.47	-	0.003	16.1	10.36	69.2	11.9	47.7	15	12.3	7.66	2.7
GW-6	0.79	-	0.011	332	2.057	168	7.02	68	90.2	13	7.69	1.9
GW-7	0.72	-	0.008	2.54	3.357	62.1	6.95	33.6	28	10.6	7.7	1.6
GW-8	0.84	-	0.003	18	9.526	103	15.1	54	21.1	1	7.83	2.8
GW-9	0.57	-	0.003	584	0.364	1885	22.6	263	56.7	1	7.88	3.2

[a] Dosage unit of these chemical parameters is mg/L.

[b] Data is less than the detection limit.

783 **Table 2 The predominant 16S rRNA sequences recovered from groundwaters and their closest phylogenetic affiliations.**

DGGE band	Isolation environment of nearest sequence match	% Sequence similarity	Phylogenetic affiliation	Closest described species using BLASTN (accession number)	% Sequence similarity
B2 (GW9)	Qinghai-Tibet Plateau	99	Gammaproteobacteria	*Stenotrophomonas maltophilia* DX-R3 (KF704115)	99
B3 (GW9)	nodules	100	Alphaproteobacteria	*Novosphingobium* sp. IAR13 (KF053364)	100
B4 (GW5)	biofilm on artificial substrates	100	unclassified	Bacterium M5 (2011) (HQ728395)	100
B5 (GW8)	lake water	98	Betaproteobacteria	*Hydrogenophaga* sp. XT-N8 (KC762316)	98
B7 (GW9)	effluent treatment plant	99	Gammaproteobacteria	*Stenotrophomonas maltophilia* TERI L1 (KF589294)	99
B9 (GW1)	biofilm attached on the top of pure sulfur carrier packed bed reactor	99	Betaproteobacteria	*Thiobacillus thioparus* (HM173634)	99
B10 (GW3)	estuary in middle of river	99	Betaproteobacteria		100
B12 (GW3)	eutrophic urban river water	99	unclassified	*Acidovorax* sp. IW-204 (KF556688)	99
B13 (GW8)	sediment of reed	100	Betaproteobacteria	Hydrogenophagaatypica strain DT34-12 (KC920941)	100
B14 (GW5)	Milwaukee harbor	96	Betaproteobacteria	Beta proteobacterium SCGC AAA206-G21 (JF488138)	95
B19 (GW8)	Cuatro Cienegas pond water	100	Gammaproteobacteria	*Rheinheimera* sp. J4.1B7 (KF317760)	100
B20 (GW9), B22 (GW9)	gold fish	100	Gammaproteobacteria	*Aeromonas veronii* B7 (KF661548)	100
B21 (GW9)	full-scale drinking water treatment plant green sand filter media	98	Betaproteobacteria	*Acidovorax* sp. AR25 (JN585320)	97
B24 (GW6)	Shipballast tank specimens	98	Epsilonproteobacteria	*Sulfurimonas denitrificans* DSM 1251 (NR_074133)	97
B25 (GW1)	marine sediment	99	unclassified	*Comamonas* sp. AP5s2-M2b (KF561876)	98
B26 (GW9)	seawater	99	Epsilonproteobacteria	*Sulfurimonasautotrophica* DSM 16294 (NR074451)	99

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Table 3 Pearson correlation analysis between gene abundance and physicochemical parameters.

	dsrB	*aprA*	16S	SO_4^{2-}	NO_3^-	NO_2^-	NH_4^+	F^-	Na^+	K^+	Mg^{2+}	Ca^{2+}	pH	*dsrB*/16S	*aprA*/16S	H' index
dsrB	1															
aprA	0.264	1														
16S	0.191	0.638	1													
SO_4^{2-}	-0.449	0.257	0.480	1												
NO_3^-	-0.190	0.352	-0.021	0.136	1											
NO_2^-	-0.279	0.199	-0.014	0.508	-0.237	1										
NH_4^+	0.090	0.445	-0.168	0.280	0.493	0.551	1									
F^-	-0.088	-0.227	0.367	0.166	0.094	-0.445	-0.488	1								
Na^+	-0.645	0.278	0.354	**0.837****	0.084	0.665	0.253	-0.047	1							
K^+	-0.128	0.356	-0.070	0.430	0.141	**0.837****	**0.811****	-0.385	0.584	1						
Mg^{2+}	-0.400	0.417	0.379	**0.898****	0.299	**0.695****	0.582	0.006	**0.862****	**0.722***	1					
Ca^{2+}	-0.408	0.244	0.409	**0.907 ****	0.312	0.448	0.371	0.246	0.663	0.364	**0.872****	1				
pH	0.050	-0.520	-0.275	-0.488	-0.793	-0.026	-0.609	0.029	-0.237	-0.211	-0.563	-0.669	1			
dsrB/16S	**0.998****	0.222	0.122	-0.487	-0.187	-0.281	0.105	-0.113	**-0.677***	-0.124	-0.430	-0.441	0.068	1		
aprA/16S	0.252	**0.993****	0.542	0.208	0.379	0.228	0.508	-0.311	0.251	0.398	0.397	0.208	-0.523	0.217	1	
H' index	-0.003	-0.643	0.054	-0.139	-0.150	-0.590	**-0.683***	**0.700***	-0.269	-0.655	-0.361	-0.110	0.267	-0.006	**-0.716***	1

** There is significant correlation at the level ($P < 0.01$);

* There is significant correlation at the level ($P < 0.05$).

Table 4 Bacterial diversity of microbial community in groundwater based T-RFLP data.

Sample name	H' index
GW-1	11.37
GW-2	11.08
GW-3	11.20
GW-4	11.67
GW-5	11.38
GW-6	11.08
GW-7	10.93
GW-8	10.50
GW-9	11.27