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RESEARCH ARTICLE

Long-term effects of increasing acidity on low-pH sulfate-reducing bioprocess and bacterial community

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Abstract An ethanol-fed, sulfate-reducing anaerobic baffled reactor was operated over a period of 260 days to assess the effects of sequentially more acidic conditions (pH 4.5–2.5) on sulfate reduction and bacterial community. Results showed that the reactor could reduce sulfate and generate alkalinity at progressively lower pH values of 4.5, 3.5, and 2.5 in a synthetic wastewater containing 2500 mg/L sulfate. About 93.9% of the influent sulfate was removed at a rate of 4691 mg/L/day, and the effluent pH was increased to 6.8 even when challenged with influent pH as low as 2.5. Illumina MiSeq sequencing revealed that a step decrease in influent pH from 4.5 to 2.5 resulted in noticeable decrease in the biodiversity inside the sulfidogenic reactor. Additionally, complete and incomplete organic oxidizers *Desulfobacter* and *Desulfovibrio* were observed to be the most dominant sulfate reducers at pH 2.5, sustaining the low-pH, high-rate sulfate removal and alkalinity generation.

Keywords Sulfate-reducing bioprocess · Wastewater treatment · Low pH · Illumina sequencing · Bacterial community · Anaerobic baffled reactor

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Introduction

Sulfate contaminants are often encountered in industrial wastewaters, which are generated in many processes, such as pulp and paper and rubber latex production, mining and metallurgical beneficiation, and leather tanning (Puhakka et al. 1990; Jarvis and Younger 2000). Considering the adverse effects of sulfate contamination on ecosystem health (Anthony 1999), the removal of sulfate from water media is essential for sustainable water resources management.

Microbial sulfate reduction has been heralded as a promising technology for the purification of sulfate-loaded industrial effluents for a number of decades, because of its efficiency, simplicity, and cost-effectiveness (Lens et al. 1998; Silva et al. 2002; Rodriguez et al. 2012). In this process, sulfate removal is based on the bioconversion of sulfate to sulfide by anaerobic respiration driven by sulfate-reducing bacteria (SRB) using organic electron donors or H_2 (Hao et al. 2014). The resulting sulfides can be readily purified by a diverse array of methods, such as adsorption in alkaline solution (Lens and Hulshoff Pol 2000), oxidation to elemental sulfur (as a reusable resource) (Sahinkaya et al. 2011; Klok et al. 2012), or formation of stable precipitates with heavy metals (Al-Tarazi et al. 2004).

Many factors affecting the efficacy of the sulfate-reducing process, such as hydraulic retention time (HRT), temperature, salinity, pH (mainly at neutral and moderately acidic conditions), chemical oxygen demand (COD)/SO_4^{2-} ratio, and sludge type (flocs/biofilm/aggregate), have been extensively investigated (Kaksonen et al. 2004; Chou et al. 2011; Montoya et al. 2013). These investigations demonstrated that sulfate-reducing activities thrive at circumneutral pH values of 5–8 but fail generally below pH 4.0, regardless of lab- or pilot-scale bioreactors (Kanyarat and Sumate 2008; Zhao et al. 2010; Rodriguez et al. 2012).

In recent years, many researchers proposed that several acidophilic or acid-tolerant SRB are able to withstand and even flourish in acidic environments where the pH ranges from 2 to 4, such as areas impacted by acid mine drainage (Senko et al. 2009; Sánchez-Andrea et al. 2013). This interesting finding inspired the use of specific SRB to treat acidic sulfate-rich wastewaters directly, without previous influent neutralization. Considerable effort has been exerted to operate low-pH sulfate-reducing systems (McCauley et al. 2009; Ňancucheo and Johnson 2012). For example, Johnson et al. (2006) developed a lab-scale in-line sulfate-reducing reactor with glycerol, acetic acid, and H_2 as energy sources to treat pH 3–4 liquors, in which organic substrates were completely oxidized to CO_2 by a syntrophic sulfidogenic consortium. This bioconversion process was improved by Bayrakdar et al. (2009) to treat pH 4.5–7.0 mine drainage containing sulfate (1000–2000 mg/L) and Zn (65–200 mg/L). They achieved sufficient simultaneous removal of sulfate and heavy metal (62–90% of SO_4^{2-} and 99% of Zn^{2+}) in a lactate-fed sulfate-reducing reactor. Meanwhile, some reports showed that low-pH sulfate reduction can be substantially reduced and even terminated once exposure to high levels of dissolved hydrogen sulfide or toxic metals (e.g., Cu, Cd, and Mo) occurred (Sánchez-Andrea et al. 2014), indicating that its implementation still has potential uncertainty.

pH value of many sulfate-rich industrial wastewaters is usually approximately 2–4 (Anthony 1999; Kanyarat and Sumate 2008). However, most of the previous studies on low-pH (pH < 4) sulfate reduction were conducted in only a short term (several weeks) and at constant pH levels (Zhao et al. 2010; Montoya et al. 2013; Sharma et al. 2014). These studies often avoided the effects of the fluctuation of process parameters (e.g., seeding sludge, continuous acid shock, and acid intensity variations). A detailed assessment of the long-term effects of changing pH, especially increasing acidity (that may be associated with variable real wastewater properties) on the sulfate-reducing bioprocess, has not been undertaken. Meanwhile, the response of microbial populations in the bioreactor to the acidity adjustment (sequentially lower pH) is also unclear. The culture-based methods and molecular-based detection techniques, such as denaturing gradient gel electrophoresis (DGGE), fluorescence in situ hybridization (FISH), and terminal restriction fragment length polymorphism (T-RFLP), have greatly extend our knowledge of the SRB community composition in some acidic media (Hiibel et al. 2008; Zhao et al. 2010; Liang et al. 2013; Montoya et al. 2013), but the true diversity and complete microbial structure can hardly be revealed using these conventional methods.

In the present study, an ethanol-fed sulfate-reducing anaerobic baffled reactor (ABR) was continuously operated for 260 days under sequentially more acidic conditions (pH of 4.5–2.5). The objectives of this study were (1) to evaluate the impact of a step decrease in influent pH on the robustness of the treatment system and (2) to investigate the diversity and relative proportions of bacterial constituents under varying acidity conditions by using a highly sensitive, high-throughput Illumina MiSeq sequencing method.

Materials and methods

Experimental setup and operational condition

A laboratory-scale ABR with an 8-L working volume was used in this study (Fig. 1). The rectangular-shaped reactor was composed of internal vertical baffles alternately hanging and standing that serve as the upward/downward flow channels. These baffles divided the reactor into four equal-sized compartments. This configuration ensures effective mixing and promotes contact between wastewater and anaerobic sludge at the base at each upward flow.

Synthetic wastewater (pH of 4.5–2.5) containing 2500 mg/L sulfate, 4500 mg COD/L ethanol, and a fraction of nutrients from Postgate's medium C (50 mg/L KH_2PO_4, 100 mg/L NH_4Cl, 100 mg/L $FeCl_2$, and 50 mg/L yeast extract) was used for operational trials. Ethanol was chosen as carbon source due to its advantages of being relatively cheap and easily degradable. The pH of the wastewater was adjusted to the desired value using 1 M H_2SO_4. The wastewater sample was prepared daily to minimize influent characteristic changes over the duration of the experiments.

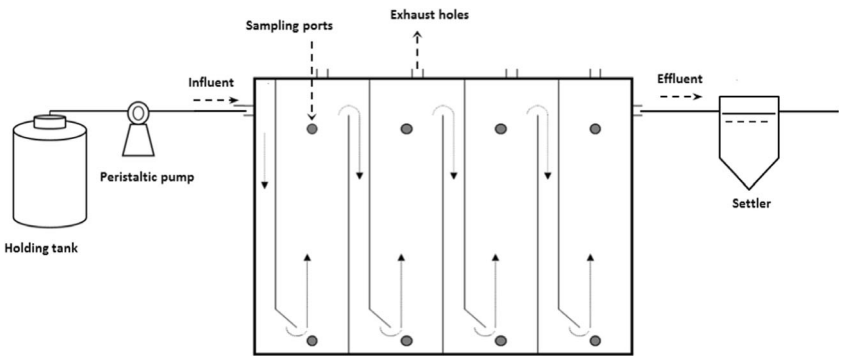

Fig. 1 Schematic of the laboratory anaerobic baffled reactor

The SRB seed sludge was collected from a pilot-scale bioreactor, which has been operated for 2 years, and had been treating mildly acidic wastewaters (pH 5 and 1500 mg/L sulfate). The ABR containing 6.8 L of the wastewater and 1.2 L of SRB sludge (3.2 g MLSS/L) was initially operated in batch mode for the first 5 days to obtain a steady anoxic condition. Once the solution turned into a turbid dark gray, continuous flow was started with a predetermined flow rate. After several weeks of the start-up phase, the ABR system tune-up was completed, and day 80 was selected as the starting point in this study.

The reactor operation at a fixed flow rate was sequentially divided into three experimental periods, namely, influent pH 4.5 (period I), pH 3.5 (period II), and pH 2.5 (period III). The progressively lower pH influent solution was fed after the effluent of each period reached a stable condition. The reactor was operated for 260 days at room temperature. During the experimental operation, SRB sludge was regularly wasted from the reactor to maintain a constant sludge retention time (SRT) of 20 days. In this experiment, an increased sampling frequency, instead of replicate, was employed as a way to attain a more efficient description. Reactor influent and effluent samples of each compartment were periodically measured for sulfate, sulfide, pH, alkalinity, COD, and volatile fatty acids (VFAs). The operational conditions of the reactor are listed in Table 1.

Chemical analysis

Sulfate was analyzed by the photometric turbid metric method (Kolmert et al. 2000). Sulfide was determined by using a spectrophotometer (Shimadzu UV-1601, Japan) according to the methylene blue method (Greenberg et al. 2005). VFAs (mainly acetate) were analyzed by using a gas chromatography (Agilent 6890, USA) according to the methods as described previously (Zhao et al. 2010). COD and alkalinity were measured according to the standard methods (Greenberg et al. 2005). Before COD measurement, samples were acidified with concentrated H_2SO_4 to below pH 2 and purged with N_2 gas for 5 min to remove H_2S. For the alkalinity measurement, samples were titrated by 0.1 N HCl to an end point pH of 4.5.

Table 1 Operational conditions of anaerobic baffled bioreactor

Parameter	Periods		
	I	II	III
Days	80–140	140–200	200–260
HRT (day)	2	2	2
Influent SO_4^{2-} content (mg/L)	2500	2500	2500
Influent ethanol content (mg COD/L)	4500	4500	4500
Influent pH	4.5	3.5	2.5

Microbial analysis

When the reactor could be deemed to have achieved its steady-state operation after running for >10 times HRT, sludge samples used for microbial analysis were periodically collected from the bottom of each compartment in the reactor on days 132, 192, and 252 and then were analyzed by a high-throughput Illumina MiSeq sequencing system to detect the bacterial community structure evolution resulting from acidic influent pH variations.

DNA extraction and PCR amplification

DNA extraction was conducted using the E.Z.N.A.® DNA Kit (Omega Bio-tek, Norcross, GA, USA) according to the manufacturer's protocols. The quantity and quality of the extracted DNA were assessed by NanoDrop spectrophotometry (Thermo Scientific, Waltham, MA, USA) and agarose gel electrophoresis. Considering the high biodiversity in sulfate-reducing reactor, the V4–V5 region of bacterial 16S ribosomal RNA (rRNA) gene, instead of dsrA gene specific for SRB, was amplified by PCR. PCR and sequencing were performed according to the protocols previously described (Caporaso et al. 2011). The thermocycling steps were as follows: 95 °C for 2 min, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a final extension step at 72 °C for 5 min using primers 515F (5′-barcode-GTGCCAGC MGCCGCGG-3′) and 907R (5′-CCGT CAATTCMTTTRAGTTT-3′), where barcode is an eight-base sequence unique to each sample. PCR was conducted in a 20 μL mixture containing 4 μL of 5× FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA.

Illumina MiSeq sequencing

After purification using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) and quantification using the QuantiFluor™-ST Fluorometer (Promega, Madison, WI USA), a mixture of amplicons was pooled in equimolar ratios and paired-end sequenced (2 × 250) on an Illumina MiSeq platform according to the standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (accession number: SRP070739).

Raw reads were strictly filtered using QIIME (version 1.8) according to a previous study to obtain effective reads (Bokulich et al. 2013). After quality control processes, a total of 197,836 sequences were obtained, with an average length of 395 bp and an average coverage of 16,486

sequences per sample. Then operational taxonomic units (OTUs) were clustered with a 97% similarity cutoff using the UPARSE 7.1 program (http://drive5.com/uparse/), and chimeric sequences were identified and removed using UCHIME. In order to calculate the alpha diversity, three metrics were generated in mothur for each sample: Chao1 metric, observed species metric, and Shannon index. Concerning the Chao1 estimator (http://www.mothur.org/wiki/Chao), it was calculated by the equation: $s_{\mathrm{chao1}} = s_{\mathrm{obs}} + n_1(n_1 - 1)/2(n_2 + 1)$ where S_{obs} is the observed number of species, n_1 is the number of OTUs with only one sequence, and n_2 is the number of OTUs with only two sequences. The Shannon index (http://www.mothur.org/wiki/Shannon) was calculated as follows: $H = -\sum_{i=1}^{S}(n_i/N)\ \ln(n_i/N)$, where S the number of observed OTUs, n_i is the number of individuals in OTU_i, and N is the total number of individuals in the community. These metrics were calculated in mothur (v.1.35.0) for each sample.

The phylogenetic affiliation of each 16S rRNA gene sequence was classified to the phylum and genus levels by the RDP classifier (http://rdp.cme.msu.edu/) against the SILVA (SSU115) 16S rRNA database with a confidence threshold of 70% (Amato et al. 2013). Meanwhile, the OTU level identification (with relative abundances of >2%) was also conducted to better understand the functions of dominant species. The representative sequences of the dominant OTUs were aligned with sequences listed in the GenBank database by Basic Local Alignment Search Tool (BLAST).

Results and discussion

Sulfate reduction and sulfide production

Figure 2 shows the sulfate reduced and sulfide produced in the ABR as incubation progressed at various influent pH values. The results indicated that the ethanol-fed reactor was capable of supporting sulfate reduction at pH values of 4.5, 3.5, and 2.5 in synthetic wastewater containing 2500 mg/L sulfate. The average sulfate reduction rates of 4656, 4743, and 4691 mg/L/day were obtained at pH values of 4.5, 3.5, and 2.5, respectively, which are similar to the values obtained previously in an ethanol-fed bioreactor (Kaksonen et al. 2004; Johnson et al. 2006). In addition, as the influent became more acidic, the percentage of sulfate removed in the front part of the reactor (the first three compartments) gradually decreased but that removed in the last compartment (C-4th) substantially increased. For example, 40.3, 20, and 12.1% of the influent sulfate were removed from the first compartment (C-1st) at pH values of 4.5, 3.5, and 2.5, respectively, compared with that of 27, 28.5, and 65% from the fourth compartment (C-4th), indicating an increased contribution of the last

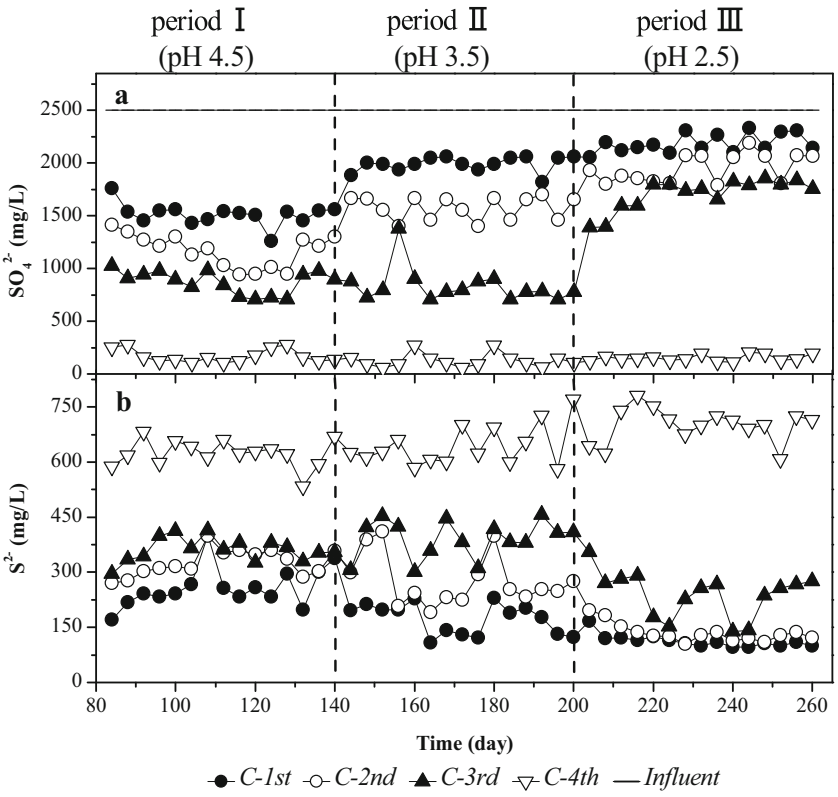

Fig. 2 Sulfate (**a**) and sulfide (**b**) concentrations in each compartment of the bioreactor under sequentially more acidic influent conditions. Period I: influent pH 4.5; period II: influent pH 3.5; period III: influent pH 2.5. *C-1st*: the first compartment of the reactor; *C-2nd*: the second compartment of the reactor; *C-3rd*: the third compartment of the reactor; *C-4th*: the last compartment of the reactor

compartment of the reactor to low-pH high-rate sulfate conversion. The reactor's compartmentalized configuration could be plausible explanations for this observation, because this special reactor can limit, to a great extent, the exposure of the biomass to adverse environmental conditions, such as low-pH shock (Bekmezci et al. 2011).

An adaptation period was commonly considered to be essential for the SRB to adjust to the decrease in pH (Silva et al. 2002; Hiibel et al. 2008), which was also evident in this study and was reflected by the immediate decrease in sulfide production concentration and effluent pH after the influent pH was decreased. Moreover, a longer period of adaptation was required to obtain a more acidic influent solution. Take the C-4th for instance, after 8 days of continuous flow with pH 3.5 influent (Fig. 2, day 148), its effluent pH was 7.1 and sulfide concentration was 628 mg/L. When pH 2.5 influent was introduced on day 201, the effluent pH and sulfide concentration decreased significantly and 16 days were required before significant sulfate reduction occurred again, increasing the effluent pH to 6.8 and producing 750 mg/L sulfide (Fig. 2, day 216).

Following the removal of sulfate, sulfide was gradually produced in the reactor. As shown in Fig. 2b, sulfide production data were basically consistent with the corresponding sulfate removal. As high as 580–750 mg/L sulfide concentrations were detected in periods I–III, but any inhibitory effect of sulfide on the treatment efficiency was not recorded. These excess sulfides in the effluent need to be carefully disposed of. In fact, sulfide, under oxygen-limited conditions, can be safely converted into elemental sulfur by sulfide-oxidizing bacteria. Bekmezci et al. (2011) achieved simultaneous sulfate reduction and sulfide oxidation in a single bioreactor, in which 32–74% of sulfide producing from the front part of ABR was oxidized to elemental sulfur (S^0) in the aeration-limited last compartment of the reactor. The experimental results of Klok et al. (2012) emphasized the importance of accurate control of oxygen-limiting level (an O_2/H_2S mole ratio <1) for the S^0-forming process. Our recent experiments observed that bacteria from the genus *Bacillus*, *Pseudomonas*, and *Azoarcus* were significantly involved in the sulfide-oxidizing bioprocess (unpublished data).

The comparison between the amounts of sulfate reduced and sulfide produced in the effluent indicated that dissolved sulfide levels were considerably underestimated based on the stoichiometry of sulfate reduction. This result could be attributed to three factors. The first factor possibly contributing to the observed effect was the formation of metal sulfide precipitates. During the sulfate bioconversion process, the amount of sulfide lost from metal precipitates accounted for ~7.6% of the total sulfur mass (Fang et al. 2012). The second factor possibly contributing to the observed effect could be the loss of volatile H_2S (under acidic conditions) from the solution. The third possible factor could be the observation that the inner part of the poly(methyl methacrylate) panel was dark gray, implying that a certain amount of volatile H_2S diffused into the walls of the reactor and leading to the further loss of dissolved sulfide in the solution.

COD oxidation and acetate production

When easy degradable ethanol is fed as the COD substrate for the sulfate reduction, the main VFA product is acetate as shown in Eqs. (1) and (2). From the variations in COD and acetate concentrations (Fig. 3), we noted a part of acetate remaining in the final effluent. It could be attributed to the fact that the initial high-influent COD/SO_4^{2-} ratio used in this study caused only trace levels of sulfate (<180 mg/L) remaining, and, thus, the acetate could hardly be oxidized completely. In addition, it was observed that a large amount of ethanol was oxidized in the first three compartments of the ABR and acetate oxidation mainly occurred in the last compartment. Especially in period III (influent pH 2.5), there was more than 49% COD removal in compartment 4, indicating the presence of significant acetate oxidation in this part. Our subsequent Illumina sequencing indeed detected a high proportion of acetate-oxidizing SRB from the genus *Desulfobacter* in the pH 2.5 reactor.

$$2CH_3CH_2OH + SO_4^{2-} \rightarrow 2CH_3COO^- + H_2S + 2H_2O \quad (1)$$

$$2CH_3COO^- + 2SO_4^{2-} \rightarrow 4HCO_3^- + 2HS^- \quad (2)$$

Alkalinity production and pH variation

pH and alkalinity are considered to be the important factors affecting the sulfate-reducing bioprocess (Lens et al. 1998; Hao et al. 2014; Sánchez-Andrea et al. 2014). Figure 4 shows that the data of pH and alkalinity were consistent with the changes in other operational parameters, e.g., sulfate conversion and COD oxidation. At increasingly acidic influent conditions, acid neutralization (alkalinity production) was mainly achieved in the last compartment of the ABR. This finding supports the speculation that acetate oxidation mainly occurred in the last compartment because sulfidogenic oxidation of ethanol to acetate cannot produce alkalinity and only acetate oxidation produces sufficient alkalinity. At influent pH 2.5, the ultimate reactor effluent pH was 6.8 and the alkalinity was 1818 mg $CaCO_3$/L, respectively.

Based on the experimental data on ABR performance, we can conclude that influent acidity adjustment (from pH 4.5 to 2.5) did not substantially interfere with the microbial sulfate conversion, as it was not reflected by any decline in the final effluent pH and sulfate removal. As the influent became more acidic, the last compartment of the ABR played an increasingly important role in maintaining treatment efficiency. The

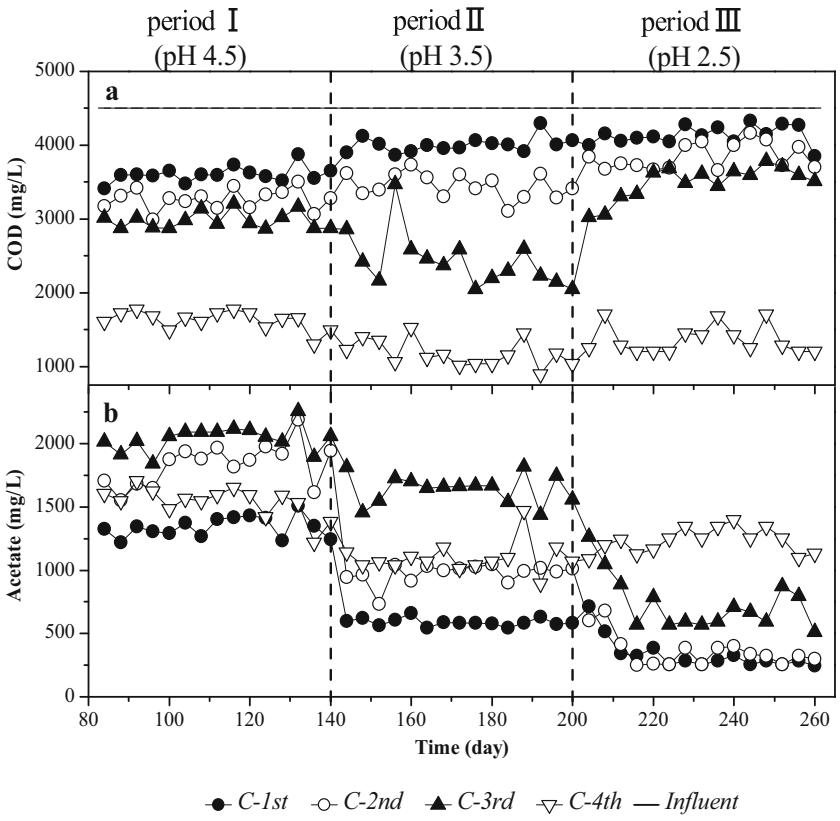

Fig. 3 COD (**a**) and acetate (**b**) levels in each compartment of the bioreactor under increasingly acidic influent conditions. Period I: influent pH 4.5; period II: influent pH 3.5; period III: influent pH 2.5. *C-1st*: the first compartment of the reactor; *C-2nd*: the second compartment of the reactor; *C-3rd*: the third compartment of the reactor; *C-4th*: the last compartment of the reactor

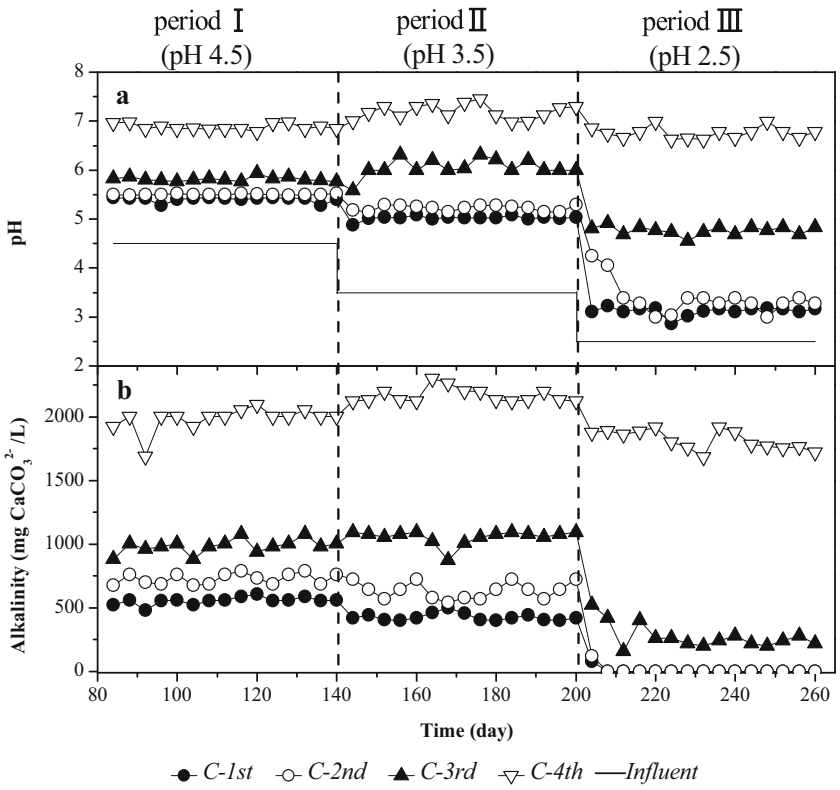

Fig. 4 Variations in pH (**a**) and alkalinity (**b**) in each compartment of the bioreactor under increasingly acidic influent conditions. Period I: influent pH 4.5; period II: influent pH 3.5; period III: influent pH 2.5. *C-1st*: the first compartment of the reactor; *C-2nd*: the second compartment of the reactor; *C-3rd*: the third compartment of the reactor; *C-4th*: the last compartment of the reactor

ABR has compartmentalized microenvironments and also provides effective retention of SRB sludge organisms (Kanyarat and Sumate 2008; Bekmezci et al. 2011), which might represent a favorable condition for being resilient to the operational factors' shock. The average performance obtained in this study compare favorably with that of other treatment systems designed for the cleanup of acidic sulfate-polluted wastewaters, in terms of sulfate removal, acid shock intensity, and experimental duration period (Table S1) (Gallegos-Garcia et al. 2009; Jong and Parry 2006; Montoya et al. 2013; Silva et al. 2002; Zhao et al. 2010).

Microbial diversity and phylogenetic analysis

Sulfate reduction is essentially microbiologically driven, and gaining insight into the diversity and composition of the microbial community may contribute to better understanding of reactor performance. Given that Illumina sequencing can generate a large amount of DNA data through a massively parallel sequencing-by-synthesis method, thousands of OTUs can be identified to investigate the microbial diversity (Loman et al. 2012; Zhou et al. 2015). To our knowledge, to date, little information on the diversity and evolution of the population of SRB resulting from the variations under sequentially more acidic conditions (especially detected by Illumina MiSeq sequencing method) are available.

Illumina sequencing indicated that the microbial richness decreased with the increase in influent acidity, which was reflected by a sharp decrease in the total number of OTUs estimated by the Chao1 estimator from period I 472 to period III 309 (Table 2). This observation is consistent with previously published literature (Liang et al. 2013; Montoya et al. 2013). It is generally assumed that the pH directly affects the activity of different microorganisms and acidic systems harbor a low biodiversity. However, it was worthwhile to note that the decline of biodiversity did not adversely affect the reactor performance as over 90% of sulfate was removed and effluent pH was always ~6.8 throughout the entire pH 2.5 experimental period (period III). The Shannon index is indicative of the evenness of the species in a microbial community. Considering the fact that Shannon index of period I (3.05–3.71) was much larger than that of period III (2.32–3.35), it could be inferred that the enriched OTUs at moderately acidic conditions were distributed more evenly than those at highly acidic condition.

Phylogenetic analysis at the phylum level shows that in total nine phyla were identified detected in periods I–III (Fig. 5a). *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* were observed to be the predominant phyla. The total of these three groups accounted for 76.7 (period I), 86.5 (period II), and 92.1% (period III) of the total reads. In fact, most SRB previously discovered belong to the phyla *Proteobacteria* and *Firmicutes* (Senko et al. 2009).

Further phylogenetic analysis based on the genus and OTU levels allowed us to verify the function evolution of the bacterial community. The results showed that under mildly acidic conditions (periods I and II), the dominant genera (relative abundance of >5%) include *Desulfovibrio*, *Desulfobacteraceae*, *Enterococcus*, *Acetobacter*, and unclassified groups (Fig. 5b). The OTU identifications indicated that an overwhelming OTU 222 in periods I and II (with relatively high abundance of more than 25%) was most similar to *Desulfovibrio* sp. clone SR_FBR_E38 (Table S2 and Fig. S1), which commonly inhabited in extreme low-pH environments, e.g., metallurgical wastewater, sulfuric geysers, as well as river sediments impacted by acid mine drainage (AMD) (Liang et al. 2013; Montoya et al. 2013; Sánchez-Andrea et al. 2013). SRB belonging to the species *Desulfovibrio* sp. are known to oxidize their substrates incompletely to acetate (i.e., incomplete oxidizing SRB), which will sometimes create high-effluent COD concentration in those ethanol-fed sulfate-reducing bioreactors (Kaksonen et al. 2004; Johnson et al. 2006).

When the influent became extremely acidic (period III), the bacterial population composition showed significant changes, particularly in the last compartment, compared with periods I and II (Fig. 5b, Table S2 and Fig. S1). More specifically, the incomplete oxidizing SRB *Desulfovibrio* sp. (OTU 222) still dominated. Another important species, OTU 268, was also highly enriched, accounting for >19% of the total observed

Table 2 Richness and diversity estimators of the bacteria phylotypes under increasingly acidic operational conditions

	Period I (pH 4.5)				Period II (pH 3.5)				Period III (pH 2.5)			
	C-1st	C-2nd	C-3rd	C-4th	C-1st	C-2nd	C-3rd	C-4th	C-1st	C-2nd	C-3rd	C-4th
OUT	284	394	358	400	315	389	326	459	211	177	206	283
Chao1[a]	414	493	475	506	428	450	453	529	253	282	335	368
Shannon[b]	3.05	3.68	3.67	3.71	2.98	3.61	3.13	3.64	2.78	2.32	2.56	3.35
Coverage[c]	0.987	0.988	0.986	0.99	0.996	0.994	0.992	0.997	0.996	0.997	0.99	0.994

[a] Chao1 richness estimator: the total number of OTUs estimated by infinite sampling. A higher number indicates higher richness

[b] Shannon index: an index to characterize species diversity. A higher value represents more diversity

[c] Good's coverage: estimated probability that the next read will belong to an OTU that has already been found

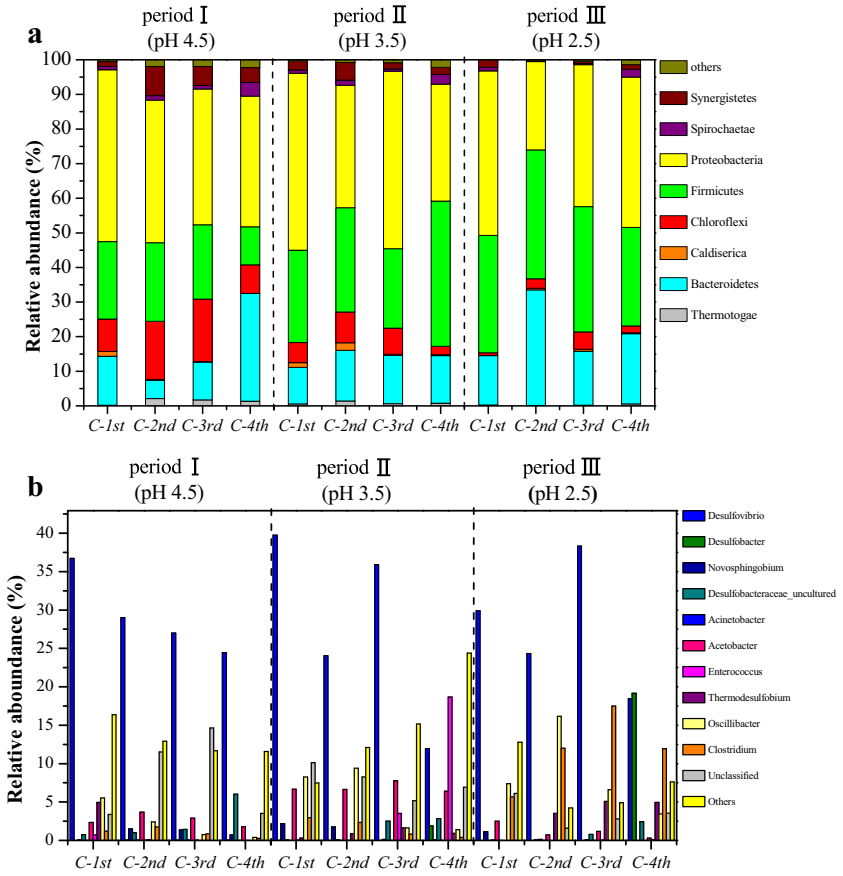

Fig. 5 The relative abundances of the predominant phylogenetic groups of the reactor samples from Periods I, II, and III at the phylum level (**a**) and genus level (**b**). Relative abundance is defined as the number of sequences affiliated with that taxon divided by the total number of sequences per sample (%). Phyla and genera making up <1% of the total composition are defined as *others*

OTUs. This is a significant result because the OTU 268 had a similarity of 99% with *Desulfobacter postgatei*, and members of *D. postgatei* could completely oxidize acidified intermediates to CO_2 (i.e., complete oxidizing SRB) and were previously detected in AMD bioremediation systems (Widdel 1992). The prevalence of these two complete and incomplete organic oxidizers in period III is beneficial for the stability of reactor performance, because this avoided the acetate inhibition to the SRB growth at pH 2.5. In a pioneering study of low-pH sulfate reduction carried out by Kimura et al. (2006), a Gram-negative acidophile isolate PFBC (an *Alphaproteobacteria*) was proven to be responsible for the degradation of acetic acid produced by SRB. They chose glycerol as the substrate and controlled the pH at 3.8–4.2. However, in our reactor, only a few *Acetobacterium* genus could be classified into *Alphaproteobacteria*, and *Alphaproteobacteria* did not play a dominant role in acetic acid degradation. This difference could be highly related to varying experimental conditions. It is well documented that during sulfate-reducing process, the running pH directly affects the activity of different microorganisms and the type of substrate has a major influence on the complexity of the communities (Sánchez-Andrea et al. 2011; Hao et al. 2014). Meanwhile, we observed that the OTU 152 was detected with high relative abundances (5–17.5%) in period III, which was closely related to *Clostridium* sp. This species was commonly considered as a hydrolytic and fermentative bacterium, which was involved in the breakdown of complex organic materials and provided SRB with easily utilized carbon and energy sources such as volatile fatty acids (Hiibel et al. 2008; Zhao et al. 2010; Sánchez-Andrea et al. 2014). The prevalence of *Clostridium* sp. in the low-pH reactor indicated its role in the oxidation of ethanol into acetate and better adaptation to extremely acidic environments. In fact, so far, the detailed acid tolerance mechanisms of acidophilic SRB still are not fully understood. In most cases, the sulfate-reducing activity at extremely low pH is mainly explained by the existence of special microniches of elevated pH around these bacteria (Koschorreck 2008; Sánchez-Andrea et al. 2014). Several microelectrode investigations showed the existence of pH gradients in SRB sludge aggregates. Additionally, some acidophilic or acid-tolerant SRB can form spores, a property which is favorable for their survival in low-pH environments (Widdel 1992). In further studies, it is of great significance to try to isolate these specially acidophilic or acid-tolerant SRB (e.g., *Desulfovibrio* and *Desulfobacter*) and examine their potential roles in the treatment of highly acidic real wastewaters rich in sulfate.

On the other hand, it has to be pointed out that in period III, a high proportion of unclassified group (2–11.5%) was found together with SRB in the reactor. The presence of this group

suggests its potential active role in the reactor, but, thus far, its physiological properties are not clear. Perhaps there is such a possibility that the synergistic effects of various microbial populations, where the presence of one microbial population promotes the growth of other specific types, jointly mediate low-pH high-rate sulfate reduction.

Conclusions

The ethanol-fed, sulfate-reducing anaerobic baffled reactor can be used for the treatment of artificially synthetic low-pH sulfate-loaded wastewaters. The reactor was able to support significant sulfate reduction and efficient acid neutralization even when challenged with influent pH as low as 2.5. A decrease in influent pH resulted in noticeable decrease in the microbial diversity inside the reactor. At extremely acidic conditions (pH 2.5), the presence of high proportions of incomplete oxidizing *Desulfovibrio* sp. and complete oxidizing *Desulfobacter* sp. sustained the low-pH, high-rate sulfate-reducing activity. These results suggested that this treatment system developed in this study may cope with variable field conditions that are particularly associated with additional acidic fluxes. The next step of studies should be carried out to evaluate its possibility in the purification of real sulfate-rich industrial wastewaters.

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References

Al-Tarazi M, Heesink ABM, Versteeg GF (2004) Precipitation of metal sulphides using gaseous hydrogen sulphide: mathematical modeling. Chem Eng Sci 59:567–579

Amato KR, Yeoman CJ, Kent A, Righini N, Carbonero F, Estrada A, Gaskins HR, Stumpf RM, Yildirim S, Torralba M, Gillis M, Wilson BA, Nelson KE, White BA, Leigh SR (2013) Habitat degradation impacts black howler monkey (*Alouatta pigra*) gastrointestinal microbiomes. ISME J 7:1344–1353

Anthony MK (1999) Ecology of streams contaminated by acid mine drainage near Reefton, South Island. M.Sc thesis, University of Canterbury, New Zealand

Bayrakdar A, Sahinkaya E, Gungor M, Uyanik S, Atasoy AD (2009) Performance of sulfidogenic anaerobic baffled reactor (ABR) treating acidic and zinc-containing wastewater. Bioresour Technol 100:4354–4360

Bekmezci OK, Ucar D, Kaksonen AH, Sahinkaya E (2011) Sulfidogenic biotreatment of synthetic acid mine drainage and sulfide oxidation in anaerobic baffled reactor. J Hazard Mater 189:670–676

Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG (2013) Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. Nat Methods 10:57–59

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, Fierer N, Knight R (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci 108:4516–4522

Chou HH, Huang JS, Chen SK, Lee MC (2011) Process kinetics of an expanded granular sludge bed reactor treating sulfate-containing wastewater. Chem Eng J 170:233–240

Fang D, Zhang RC, Liu X, Zhou LX (2012) Selective recovery of soil-borne metal contaminants through integrated solubilization by biogenic sulfuric acid and precipitation by biogenic sulfide. J Hazard Mater 219:119–126

Gallegos-Garcia M, Celis LB, Rangel-Méndez R, Razo-Flores E (2009) Precipitation and recovery of metal sulfides from metal containing acidic wastewater in a sulfidogenic down-flow fluidized bed reactor. Biotechnol Bioeng 102:91–99

Greenberg AE, Clesceri LS, Eaton AD (2005) Standard methods for the examination of water and wastewater, 21st edn. American Public Health Association, Washington DC

Hao TW, Xiang PY, Mackey HR, Chi K, Lu H, Chui HK, van Loosdrecht MCM, Chen GH (2014) A review of biological sulfate conversions in wastewater treatment. Water Res 65:1–21

Hiibel SR, Pereyra LP, Inman LY, Tischer A, Reisman DJ, Reardon KF, Pruden A (2008) Microbial community analysis of two field-scale sulfate-reducing bioreactors treating mine drainage. Environ Microbiol 10:2087–2097

Jarvis AP, Younger PL (2000) Broadening the scope of mine water environmental impact assessment: a UK perspective. Environ Impact Assess 20:85–96

Johnson DB, Sen AM, Kimura S, Rowe OF, Hallberg KB (2006) Novel biosulfidogenic system for selective recovery of metals from acidic leach liquors and waste streams. Miner Process Extr Metall 115:19–24

Jong T, Parry DL (2006) Microbial sulfate reduction under sequentially acidic conditions in an upflow anaerobic packed bed bioreactor. Water Res 40:2561–2571

Kaksonen AH, Franzmann PD, Puhakka JA (2004) Effects of hydraulic retention time and sulfide toxicity on ethanol and acetate oxidation in sulfate-reducing metal-precipitating fluidized-bed reactor. Biotechnol Bioeng 86:332–343

Kanyarat S, Sumate C (2008) Effects of pH adjustment by parawood ash and effluent recycle ratio on the performance of anaerobic baffled reactors treating high sulfate wastewater. Bioresour Technol 99: 8987–8994

Kimura S, Hallberg KB, Johnson DB (2006) Sulfidogenesis in low pH (3.8–4.2) media by a mixed population of acidophilic bacteria. Biodegradation 17:159–167

Klok JBM, van den Bosch PLF, Buisman CJN, Stams AJM, Keesman KJ, Janssen AJH (2012) Pathways of sulfide oxidation by haloalkaliphilic bacteria in limited-oxygen gas lift bioreactors. Environ Sci Technol 46:7581–7586

Kolmert A, Wikstrom P, Hallberg KB (2000) A fast and simple turbidimetric method for the determination of sulfate in sulfate-reducing bacteria cultures. J Microbiol Meth 41:179–184

Koschorreck M (2008) Microbial sulfate reduction at a low pH. FEMS Microbiol Ecol 64:329–342

Lens PNL, Hulshoff Pol LW (2000) Environmental technologies to treat sulfur pollution: principles and engineering. IWA Publishing Inc, London

Lens PNL, Visser A, Janssen AJH, Hulshoff Pol LW, Lettinga G (1998) Biotechnological treatment of sulfate-rich wastewaters. Crit Rev Env Sci Tec 28(1):41–88

Liang F, Xiao Y, Zhao F (2013) Effect of pH on sulfate removal from wastewater using a bioelectrochemical system. Chem Eng J 218: 147–153

Loman NJ, Misra RV, Dallman TJ, Constantinidou C, Gharbia SE (2012) Performance comparison of benchtop high-throughput sequencing platforms. Nat Biotechnol 30:434–439

McCauley CA, O'Sullivan AD, Milke MW, Weber PA, Trumm DA (2009) Sulfate and metal removal in bioreactors treating acid mine drainage dominated with iron and aluminum. Water Res 43:961–970

Montoya L, Celis LB, Gallegos-García M, Razo-Flores E, Alpuche-Solís ÁG (2013) Consortium diversity of a sulfate-reducing biofilm developed at acidic pH influent conditions in a down-flow fluidized bed reactor. Eng Life Sci 13:302–311

Ňancucheo I, Johnson DB (2012) Selective removal of transition metals from acidic mine waters by novel consortia of acidophilic sulfidogenic bacteria. Microb Biotechnol 5:34–44

Puhakka JA, Salkinoja-Salonen M, Ferguson JF, Benjamin MM (1990) Carbon flow in acetotrophic enrichment cultures from pulp mill effluent treatment. Water Res 24:515–519

Rodriguez RP, Oliveira GHD, Raimundi IM, Zaiat M (2012) Assessment of a UASB reactor for the removal of sulfate from acid mine water. Int Biodeter Biodegr 74:48–53

Sahinkaya E, Hasar H, Kaksonen AH, Rittmann BE (2011) Performance of a sulfide-oxidizing, sulfur-producing membrane biofilm reactor treating sulfide-containing bioreactor effluent. Environ Sci Technol 45:4080–4087

Sánchez-Andrea I, Rodriguez N, Amils R, Sanz JL (2011) Microbial diversity in anaerobic sediments at Rio Tinto, a naturally acidic environment with a high heavy metal content. Appl Environ Microbiol 77:6085–6093

Sánchez-Andrea I, Sanz JL, Bijmans MFM, Stams AJM (2014) Sulfate reduction at low pH to remediate acid mine drainage. J Hazard Mater 269:98–109

Sánchez-Andrea I, Stams AJM, Amils R, Sanz JL (2013) Enrichment and isolation of acidophilic sulfate-reducing bacteria from Tinto River sediments. Env Microbiol Rep 5:672–678

Senko JM, Zhang G, McDonough JT, Bruns MA, Burgos WD (2009) Metal reduction at low pH by a *Desulfosporosinus* species: implications for the biological treatment of acidic mine drainage. Geomicrobiol J 26:71–82

Sharma K, Derlon N, Hu SH, Yuan ZG (2014) Modeling the pH effect on sulfidogenesis in anaerobic sewer biofilm. Water Res 49:175–185

Silva AJ, Varesche MB, Foresti E, Zaiat M (2002) Sulphate removal from industrial wastewater using a packed-bed anaerobic reactor. Process Biochem 37:927–935

Widdel F (1992) The genus *Desulfotomaculum*. In: Balows A, Triiper HG, Dworkin M, Harder W, Schleifer KH (eds) The prokaryotes. Springer-Verlag, New York, pp. 1792–1799

Zhao YG, Li XW, Wang JC, Bai J, Tian WJ (2010) Performance of a sulfidogenic bioreactor and bacterial community shifts under different alkalinity levels. Bioresour Technol 101:9190–9196

Zhou JM, Zhou XM, Li YG, Xing JM (2015) Bacterial communities in haloalkaliphilic sulfate-reducing bioreactors under different electron donors revealed by 16S rRNA MiSeq sequencing. J Hazard Mater 295:176–184