Application of horizontal-flow anaerobic immobilized biomass reactor for bioremediation of acid mine drainage

R. P. Rodriguez, D. V. Vich, M. L. Garcia, M. B. A. Varesche and M. Zaiat

ABSTRACT

The production of low-pH effluent with sulfate and metals is one of the biggest environmental concerns in the mining industry. The biological process for sulfate reduction has the potential to become a low-cost solution that enables the recovery of interesting compounds. The present study analyzed such a process in a horizontal-flow anaerobic immobilized biomass (HAIB) reactor, employing ethanol as the carbon and energy source. Results showed that a maximal efficiency in the removal of sulfate and ethanol could only be obtained by reducing the applied sulfate load $(225.1 \pm 38\ g\ m^{-3}\ d^{-1})$. This strategy led to over 75% of chemical oxygen demand (COD) and sulfate removal. Among the COD/SO_4^{2-} studied ratios, 0.67 showed the most promising performance. The effluent's pH has naturally remained between 6.8 and 7.0 and the complete oxidation of the organic matter has been observed. Corrections of the influent pH or effluent recirculation did not show any significant effect on the COD and sulfate removal efficiency. Species closely related to strains of *Clostridium* sp. and species of *Acidaminobacter hydrogenomorfans* and *Fusibacter paucivorans* that can be related to the process of sulfate reduction were found in the HAIB reactors when the initial pH was 5 and the COD/SO_4^{2-} ratio increased to 1.0.

Key words | acid mine drainage, applied sulfate loading rate, COD/SO_4^{2-} ratio, horizontal-flow reactor, sulfate removal

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INTRODUCTION

Sulfate is widely involved in wastewaters discharged from various industries such as pharmaceutical, chemical units, and paper production (Zhang *et al.* 2013). Mine waters and industrial effluents may contain high sulfate and metal concentrations and pose significant disposal problems that require urgent solutions to avoid serious environmental contamination. One of the major environmental problems resulting from the mining industry is the production of acid mine drainage (AMD). Sources of acid drainage are primarily related to tailings and storage of sulfide-rich minerals. A widely quoted example occurs when pyrite (FeS_2) is spontaneously oxidized due to the presence of oxygen and water, producing sulfuric acid, which in turn dissolves metals present in the soil/rock (Oberholster *et al.* 2013). The main factors which determine the rate of AMD generation are pH, temperature, presence of oxygen, water and Fe^{3+}, the surface area of metal sulfide and bacterial activity (Akcil & Koldas 2006).

Conventional treatments for AMD are based on the chemical neutralization and precipitation of metal hydroxides. In order to increase pH, $Ca(OH)_2$ and CaO are usually added to react with the sulfate present in the acidic water, forming a $CaSO_4.2H_2O$ precipitate. The disadvantages of this process are the high cost of the chemical reagents, the low efficiency of the sulfate removal, and the production of a sludge that must be adequately disposed of (Garcia *et al.* 2001).

Several studies have suggested alternatives for the remediation of AMD (Neculita & Zagury 2008; Jiménez-Rodriguez *et al.* 2009; Bekmezci *et al.* 2011; Rodriguez *et al.*

2012). Sulfate-reducing bacteria (SRB) have been successfully applied on a process which removes metals and sulfate from AMD (Kieu *et al.* 2011; Xingyu *et al.* 2013). Under anaerobic conditions, SRB oxidize simple organic compounds by employing sulfate as an electron acceptor and producing sulfide (S^{2-}) and alkalinity. The anaerobic processes of sulfate removal have the relevant advantage of promoting heavy metal removal by precipitation (van Lier *et al.* 2001).

Jiménez-Rodríguez *et al.* (2009) evaluated the influence of pH on the removal of sulfate and metals from AMD and observed that it correlated to the influent's pH: at 3.5, most of the iron was removed, but Cu and Zn attained only partial removal; at 5.5, the metal removal increased considerably to 91.3% for Fe, 96.1% for Cu, 79.0% for Zn, and 99.0% for Al. On the other hand, a higher chemical oxygen demand (COD) and sulfate removal was achieved in a baffled reactor fed with synthetic AMD when the influent pH was 3.0. The same behavior was observed for the removal of metals (Bekmezci *et al.* 2011).

The reduction of the real AMD sulfate load seems to play an important role in the process. Xingyu *et al.* (2013) operated a low pH sulfidogenic upflow anaerobic sludge blanket (UASB) reactor with real, undiluted AMD, obtaining 38% of sulfate removal. When applying a diluted influent, an enhancement was observed with rates from 60 to 80%. Other studies have previously suggested this behavior. One in particular (Rodriguez *et al.* 2012) evaluated a bench-scale UASB reactor for treating real AMD. The reactor's performance was studied according to variations in the COD/SO_4^{2-} ratio and influent dilution. The use of diluted AMD resulted in 46.3% and 85.6% removal of the sulfate for COD/SO_4^{2-} ratios of 0.67 and 1.0, respectively. It is also worth mentioning that an increase in the effluent's pH (from 4.0 in the influent to 6.5) was observed, together with the predominance of the complete oxidation of the organic matter via sulfate reduction.

The horizontal-flow anaerobic immobilized biomass (HAIB) reactor has been successfully used for the treatment of sulfate-rich wastewater to remove organic matter and reduce sulfate (Silva *et al.* 2002). The effectiveness of this type of reactor was mainly related to its capacity to retain microorganisms, providing a high biomass concentration and cellular retention time, especially when polyurethane foam was used as support material. It has been shown (Silva *et al.* 2006) that the polyurethane foam offered better scaffolding conditions when compared to other support materials such as vegetal coal, low-density polyethylene, and alumina-based ceramics, facilitating a better adherence of SRB. However, this reactor had not been studied with regard to acidic sulfate-rich wastewaters.

In this work, the bioremediation of AMD employing the HAIB reactor is investigated. The performance of the process is evaluated with respect to the influence of the COD/SO_4^{2-} ratio, the initial influent pH, and the sulfate applied loading rate. The main criteria are the removal of sulfate and COD, while the main objective is to determine the feasibility of this system. Microbial community structures were examined, offering further support to the discussion. The following studies consist of a pioneering use of this type of reactor on the treatment of AMD related to biological sulfate reduction.

MATERIALS AND METHODS

Description of the HAIB reactor

Three bench-scale HAIB reactors were made of borosilicate glass, each measuring 99.8 cm in length and 5.04 cm in diameter (internal) (Figure 1). The reactors had a total volume of 1,991 mL, of which 1,735 mL was the working volume and 265 mL (13% of the total) was used for gas separation. A silicone tube was installed along the length of and at the top of the reactors to collect the gas generated during the operation of the reactors.

Support for anaerobic sludge immobilization

Polyurethane foam with an apparent density of 23 kg m^{-3}, an average pore size of 543 ± 154 μm and a superficial area of 43.8 m^2 g^{-1} was used as a support for anaerobic sludge immobilization. The foam was cut to obtain cubes (cubic matrices) with sides of approximately 5 mm. Eighty percent of the total reactor volume was filled with polyurethane foam.

Inoculum and wastewater

The inoculum consisted of sludge from a UASB reactor applied to the treatment of poultry slaughterhouse wastewater. The AMD sample was obtained from the Osamu
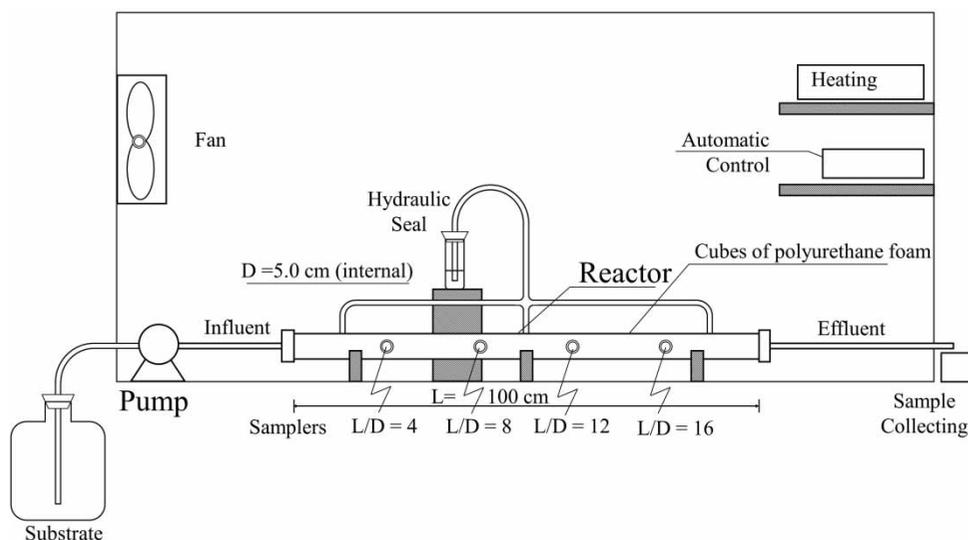
Figure 1 │ Schematic of the experimental set up with the HAIB reactor.

Utsumi Ore Treatment Unit of the National Nuclear Energy Commission in Caldas–Minas Gerais state, Brazil. The characteristics of the AMD are presented in Table 1.

Preparation of the fixed bed of the HAIB reactor

The fixed bed of the reactor was filled with cubic matrices of polyurethane foam containing immobilized sludge from the UASB reactor. The sludge was immobilized using the methodology described by Zaiat *et al.* (1994).

Physicochemical analyses

The analyses of COD, sulfate, and pH were conducted according to the methods described by the *Standard*

Table 1 │ Characterization of AMD from Osamu Utsumi Mine

		Metals	(mg L^{-1})
pH	3.0–4.0	Cr	0.01 ± 0.01
COD (mg L^{-1})	<0	Cu	0.01 ± 0.01
SO$_4^{2-}$ (mg L^{-1})	1,200–1,500	Ni	0.3 ± 0.01
S^{2-} (mg L^{-1})	ND	Cd	0.06 ± 0.02
Acetate (mg L^{-1})	<0	Mn	63.2 ± 19.0
		Fe	102.3 ± 32.0
		Zn	6.0 ± 4.0

ND: not detected.

Methods for the Examination of Water and Wastewater (2012). The analyses of acetic acid and ethanol were performed by gas chromatography (HP6890) with a flame ionization detector operated at 300 °C. The column used was HP-Innowax, which has the following characteristics: a length of 30 m, internal diameter of 0.25 mm, film thickness of 0.25 μm and phase ratio of 250. The injector temperature was maintained at 250 °C. The column oven was initially set at 100 °C for 3 min, after which the temperature was raised at a rate of 5 °C min^{-1} up to 180 °C and maintained at this temperature for 5 min. N$_2$ (33 ml min^{-1}), H$_2$ (30 ml min^{-1}), and synthetic air (300 mL min^{-1}) were used as carrier gas, flame gas, and makeup gas, respectively. The split/splitness ratio used was 1:20 (Moraes *et al.* 2000).
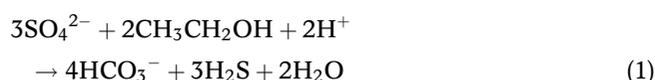
Operation of the HAIB reactor

Three reactors were operated under different operating conditions, as shown in Table 2. The hydraulic retention time was 24 hours, and ethanol was used as carbon and energy source in all tests.

The HAIB 1 reactor was operated under the condition denominated as control. The feeding pH was not adjusted and consisted of raw AMD with ethanol added as an organic source. During the first 30 days, progressive dilutions were made in the influent stream of HAIB 1 in an attempt to

Table 2 │ Phases conducted during the operation of HAIB reactors

Reactor	Phase	Time (days)	COD/SO$_4^{2-}$ ratio	pH correction	Dilution (1:4)	Recirculation
HAIB 1	I	27	0.67	no	no	no
	II	45	1.92	no	no	no
HAIB 2	I	120	0.67	yes	no	no
	II	132	0.67	no	yes	no
HAIB 3	I	130	0.67	no	yes	no
	II	150	1.0	no	yes	no
	III	80	1.0	no	no	yes

assist with the adaptation of the microbial community. After this period, dilution was suppressed and a COD/SO$_4^{2-}$ ratio of 0.67 (by mass) was set. This ratio was based on the stoichiometry of the sulfate reduction reaction when there is complete oxidation of the organic matter (Reaction 1). The COD and sulfate concentration in the influent stream of the HAIB 1 reactor in the first phase of operation (phase I) were 804 mg L^{-1} and 1,200 mg L^{-1}, respectively. Due to the observed results, after 70 days of operation, the COD/SO$_4^{2-}$ ratio was increased three-fold, to 1.92, in an attempt to achieve the total reduction of the sulfate present in the wastewater. This decision was made considering that the removal of sulfate was occurring through the incomplete dissimilatory oxidation of organic matter (Reaction 2). The concentration of COD in the influent stream in this second phase of operation (phase II) was 2,304 mg L^{-1}.

$$3SO_4^{2-} + 2CH_3CH_2OH + 2H^+$$
$$\rightarrow 4HCO_3^- + 3H_2S + 2H_2O \tag{1}$$

$$SO_4^{2-} + 2CH_3CH_2OH \rightarrow 2CH_3COO^- + H_2S + 2H_2O \tag{2}$$

The stoichiometric COD/SO$_4^{2-}$ ratio was maintained at 0.67 in the HAIB 2 reactor; however, the influent pH was adjusted from 3.0 to 5.0 using a 10 N NaOH solution for 130 days of operation (phase I). After this period, the reactor was fed with a diluted influent consisting of 75% tap water and 25% raw acid mine drainage (v/v) while maintaining the same COD/SO$_4^{2-}$ ratio (phase II).

Conditions of HAIB 3's phase I were identical to those of HAIB 2's phase II (previously described). After 224 days of HAIB 3 operation, the COD/SO$_4^{2-}$ was increased to 1.0 to evaluate the influence of a higher electron donor

concentration on the efficiency of sulfate removal (phase II). Phase III began on the 365th day and, from that point on, the feed was substituted by undiluted AMD. However, an effluent recirculation system was placed in the reactor to maintain the same inflow sulfate concentration.

Molecular biology analysis

The evaluation of microbial community structure was performed based on fragments of the 16S rRNA gene, using the techniques of polymerase chain reaction (PCR), cloning, sequencing and the analysis of phylogeny. Samples were collected at the end of the operation of each reactor. All support material was removed from the reactor, homogenized and 5 g of a foam sample was taken for the biomass extraction. From this material approximately 1 g of biomass was extracted which was submitted to analysis. DNA was extracted according to the protocol of Melvin & Hobson (1994). The amplification of DNA fragments by PCR was performed using specific primers (27F and 1100R) for the domain bacteria (Table 3).

The cloning was performed with the pGEM Easy Vector System I, according to the manufacturer's specifications. The clones were grown in LB medium and were subjected to PCR with primers M13. PCR products were purified with the GFX Illustra kit (GE Healthcare) and were sequenced on an ABI Prism 310 automated sequencer (Applied Biosystems) at the Centro de Estudos do Genoma Humano at São Paulo University (http://www.genoma.ib.usp.br/). Obtained sequences were edited in the program DNAstar (Lasergene Sequence Analysis), aligned with the micro-database Greengenes NAST aligner (http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi) and analyzed

Table 3 │ Primers used in PCR reactions

Primer	Program of thermal cycler
M13 F: 5′-gta aaa cga cgg ccag-3′	94 °C–5 min
M13 R: 5′-cag gaa aca gct atg ac-3	(94 °C–1 min; 55 °C–1 min; 72 °C–1 min) × 25 cycles
	72 °C–7 min
	4 °C–∞
Bacteria	94 °C–2 min
27 F: 5′-aga gtt tga tcm tgg ctc ag-3′	(94 °C–1 min; 69 °C–30 s
1100 R: 5′-ggg ttg cgc tcg ttg-3′	72 °C–3 min) × 15 cycles
	(94 °C–1 min; 63 °C–30 s
	72 °C–3 min) × 20 cycles
	4 °C–∞

phylogenetically using ARB Cabinet Software (Ludwig *et al.* 2004).

Figure 2 │ Removal of COD and sulfate in the HAIB reactors. HAIB 1–Phase I: (COD/SO$_4^{2-}$: 0.67, pH correction: no, dilution: no) and Phase II (COD/SO$_4^{2-}$: 1.92, pH correction: no, dilution: no); HAIB 2–Phase I (COD/SO$_4^{2-}$: 0.67, pH correction: yes, dilution: no) and Phase II (COD/SO$_4^{2-}$: 0.67, pH correction: no, dilution: yes); HAIB 3–Phase I (COD/SO$_4^{2-}$: 0.67, pH correction: no, dilution: yes), Phase II (COD/SO$_4^{2-}$: 1.0, pH correction: no, dilution: yes) and Phase III (COD/SO$_4^{2-}$: 1.0, pH correction: no, dilution: no, recirculation: yes).

RESULTS AND DISCUSSION

Removal of sulfate and COD

The results obtained from the operation of the three HAIB reactors showed significant variations related to COD and sulfate removal (Figure 2).

Regarding HAIB 1 operation, some instability was observed at the beginning (from 0 to 32nd day), as is expected during the start-up phase. These systems require an adaptation period for the selection of biomass capable of self-sustaining under the conditions imposed by the wastewater (Nancucheo & Johnson 2014; Hu *et al.* 2015). After the start-up, during phase I, the average COD and sulfate removals were $32.1 \pm 6.1\%$ and $17.3 \pm 4.9\%$, respectively (uncertainties represent 1 standard deviation). At phase II, the system quickly reached a pseudo-steady state upon increasing the COD/SO$_4^{2-}$ ratio to 1.92. The average COD and sulfate removals for phase II were $25 \pm 12\%$ and $13.8 \pm 8.6\%$, respectively. Therefore, the increase of COD/SO$_4^{2-}$ did not have any meaningful effect on HAIB 1's performance. It is possible to state, however, that the stability of the system was indeed affected. Previous studies, however, indicated great influence of the COD/SO$_4^{2-}$ ratio in the removal of sulfate with more than 200% increase in sulfate removal when the ratio was changed from 0.67 to 2.0 (Velasco *et al.* 2008).

The inoculum chosen for the HAIBs' operation was a granular mesophilic with broad microbial diversity (Hirasawa *et al.* 2008). However, it seems that the conditions applied to the HAIB 1 reactor were not ideal for selecting SRB, at least during the observed period. This conclusion is based on the low sulfate and COD removals observed in both phases, which indicates that the whole microbial consortium was not able to adapt and be established. During phase I, the concentration of sulfide was 39.0 ± 10.4 mg L^{-1}, whereas it was 27.4 ± 13.9 mg L^{-1} during phase II, substantiating the occurrence of sulfate reduction. The initiation of phase II affected sulfate removal, and this was reflected in the concentration of sulfide in the effluent.

Regarding HAIB 2, phase I performance was unstable throughout its operational period when the pH influent was adjusted to 5.0 (the pH of AMD was close to 3.0–4.0).

The average COD and sulfate removal efficiency was $38 \pm 21\%$ and $18 \pm 16\%$, respectively. The increase in pH influent from 3.0 to 5.0–5.5 has been proposed by some authors as an effective way to promote an increase in sulfate removal (Jimenez-Rodriguéz *et al.* 2009; Zhang *et al.* 2013). However, the increase in the influent pH did not result in better sulfate removals in the horizontal reactor. HAIB 2 phase I was identical to HAIB 1 phase I, except for the pH correction in HAIB 2. Even when considering the sulfate loading rate removed (Table 4), the average values are similar to HAIB 1 and 2 (both at phase I). The sulfate loading rate removed by HAIB I (phase I) was $174.2 \pm 12 \text{ g m}^{-3} \text{ d}^{-1}$ whereas it was $201.5 \pm 27 \text{ g m}^{-3} \text{ d}^{-1}$ in HAIB 2 (phase I). However, pH adjustment to 5.0 improved the COD loading removed from $199.1 \pm 37 \text{ g m}^{-3} \text{ d}^{-1}$ in HAIB I (phase I) to $262.3 \pm 21 \text{ g m}^{-3} \text{ d}^{-1}$ in HAIB 2 (phase I). In this case, the higher pH allowed a more suitable environment for heterotrophic microbial groups using ethanol as carbon and energy source.

After a dilution in the influent stream (HAIB 2 phase II), an increase in the removal efficiency of COD (to $65.0 \pm 7.4\%$) and sulfate ($40.6 \pm 7.5\%$ became evident. McCauley *et al.* (2009) suggested that better removal efficiencies were obtained in downstream reactors, when certain conditions of low sulfate loading rate ($0.03 \text{ kg L}^{-1} \text{ d}^{-1}$) and alkalinity producing compounds were applied. However, COD and sulfate loading removed were low in this phase ($119.8 \pm 24 \text{ g COD m}^{-3} \text{ d}^{-1}$ and $89.3 \pm 10 \text{ g SO}_4^{2-} \text{ m}^{-3} \text{ d}^{-1}$) due to the lower loading applied. The average sulfide effluent was $30.9 \pm 19.5 \text{ mg L}^{-1}$ and $44.7 \pm 18.3 \text{ mg L}^{-1}$ in phases I and II, respectively, and could be related to the increase in sulfate removal in phase II.

When comparing HAIB 2, phase I and II, the dilution of the influent was a more efficient way to improve the reactors' performance with respect to percentage removal. The lower sulfate load in the influent may still be regarded as the most influential parameter to culture adaptation in this type of reactor. Based on this observation, HAIB 3 was fed with diluted AMD from day 1. Although instabilities were dominant during the start-up period, COD and sulfate removal already showed promising rates. Stability was reached on the 90th day from when phase I started (at the end of the 180th day of operation). During phase I, the average COD and sulfate removal efficiencies were $76 \pm 13\%$ and $72.8 \pm 9.0\%$, respectively. This result means that the sulfate global daily removal is of the same order as the undiluted case, $163.9 \pm 28 \text{ g m}^{-3} \text{ d}^{-1}$. Furthermore, HAIB 3 is not only more stable (presenting less variability in the period, when compared to HAIB 1 and 2) but also the removal efficiencies are quite similar, which could be attributed to the thriving of SRB in the consortium. Thus, a real improvement was observed when a low sulfate load feed is employed from the beginning, both on stability and on adaptation.

In an attempt to confirm the results from phase I, the amount of electron donors was increased, leading to a COD/SO_4^{2-} ratio of 1.0 (phase II). The expectation was that if the system was SRB dominated, the removal rates would still be correlated with the stoichiometric relation between COD and sulfate. For this period, the average COD and sulfate removal was $148.2 \pm 25 \text{ g m}^{-3} \text{ d}^{-1}$ and $166.4 \pm 18 \text{ g m}^{-3} \text{ d}^{-1}$, respectively. As expected, the sulfate removal remained unchanged, indicating that when SRB are active and dominant over the other microorganisms, an excess of COD should not improve the sulfate reduction (Velasco *et al.* 2008). The presence of an adapted biomass was essential for achieving efficiencies of 95% in the treatment of AMD in fluidized-bed reactor (FBR), as reported by Sahinkaya *et al.* (2013). Moreover, the sulfate removal

Table 4 | Organic loading rate applied (OLR_a) and removed (OLR_r) and sulfate loading rate applied (SLR_a) and removed (SLR_r)

Reactor	Phase	Samples	OLR_a (g m^{-3} d^{-1})	OLR_r (g m^{-3} d^{-1})	SLR_a (g m^{-3} d^{-1})	SLR_r (g m^{-3} d^{-1})
HAIB 1	I	11	620.2 ± 116	199.1 ± 37	$1,007.0 \pm 70$	174.2 ± 12
	II	22	$1,592.0 \pm 117$	398.0 ± 29	966.0 ± 82.5	133.3 ± 11
HAIB 2	I	23	694.0 ± 56	262.3 ± 21	$1,095.2 \pm 148$	201.5 ± 27
	II	25	184.3 ± 37	119.8 ± 24	221.7 ± 26	89.3 ± 10
HAIB 3	I	17	204.1 ± 41	154.5 ± 31	225.1 ± 38	163.9 ± 28
	II	43	220.6 ± 27	148.2 ± 25	223.4 ± 24	166.4 ± 18
	III	10	500.9 ± 47	137.2 ± 13	522.3 ± 170	78.3 ± 26

efficiency can be significantly influenced by the concentration of metals present in the environment. High concentrations of metals can result in decreases in SRB metabolism (Sani et al. 2001). Thus, the dilution of the influent decreased the metals load applied to the biomass and may have contributed to the better performance of HAIB 3.

In fact, a comparison of the absolute values of the influent COD for phase I and phase II, both fed with the same sulfate concentration, reveals that an average removal of 75% of sulfate would correspond to 150 mg L^{-1} of COD removal. This means that even with an excess of energy and organic matter, other species could not compete with the SRB dominance in such conditions.

In phase III, the reactor was fed with raw AMD, and effluent recirculation was added with a recirculation ratio of 3.0 to provide influent dilution. However, the reactor collapsed, and there was an instantaneous decrease in COD removal efficiency to an average of $27.4 \pm 5.7\%$ (137.2 ± 13 g m^{-3} d^{-1}) and sulfate removal to $15.0 \pm 8.8\%$ (78.3 ± 26 g m^{-3} d^{-1}). The change from a plug-flow-like hydrodynamic system to one closer to a completely stirred reactor (data not shown) may have altered the distribution of the bacterial populations settled along the front part of the HAIB 3. However, it is more likely that the reactor collapse was caused by the exposure of the microorganisms to intermediate products, mainly sulfide, derived from the treated effluent.

HAIB 3 had the best performance, with a sulfate loading rate removed close to 170 g m^{-3} d^{-1} in phase I and II (Table 5). Previous studies at neutral pH achieved similar results (120–170 g m^{-3} d^{-1}) using a complete stirred tank reactor (CSTR) and packed-bed reactors (Chang et al. 2000; Moosa et al. 2002; Waybrant et al. 2002). In the present work, the reduction of the sulfate loading rate was crucial

for obtaining satisfactory results. However, better results were observed when starting with a low sulfate loading rate (HAIB 3) than when reducing this loading rate later in the operational period (HAIB 2). Baskaran & Nemati (2006) proposed this same correlation using a packed-bed reactor. In their study, the overall sulfate conversion reduced from 100% to around 50% when the sulfate loading rate was higher than 200 g m^{-3} d^{-1}. In this study, 75% of sulfate removal was achieved when the sulfate loading rate was 223.4 ± 24 g m^{-3} d^{-1}.

Beside the sulfate removal, the effluent pH was also indicative of success in reducing the sulfate load rate applied. Most of the known SRB are neutrophilic, and for this reason, bioreactors have been operating with a previously neutralized influent (Jimenez-Rodriguéz et al. 2009; Zhang et al. 2013). However, acidophilic reactors present a more suitable technology when pilot scale is desired in the biological processes of AMD treatment. For SRB, thermodynamical calculations show that the Gibbs free energy of sulfate reduction is higher at a lower pH, resulting in more energy gain (Sánchez-Andrea et al. 2014), and previous studies have demonstrated the feasibility of sulphidogenic acidophilic reactors (Rodriguez et al. 2012; Nancucheo & Johnson 2014).

The COD/SO_4^{2-} parameter also did not significantly affect the HAIB reactors' performance, and the best results for the removal of sulfate and COD were obtained at the stoichiometric COD/SO_4^{2-} ratio of 0.67.

The average effluent sulfide concentrations in HAIB 3 were 32.0 ± 17.0, 75.8 ± 19.3, and 29.8 ± 23.9 mg l^{-1} for phases I, II, and III, respectively. The concentrations of sulfide were approximately 80 mg L^{-1} at the end of phase II, and part of this was returned to the reactor with the recirculation of treated effluent and likely contributed to the

Table 5 | Mean values of pH, COD removal, effluent acetic acid, sulfate removal, and effluent sulfide

Reactor	Phase	pH effluent	COD removal (%)	Effluent acetic acid (mg l^{-1})	SO_4^{2-} removal (%)	Sulfide (mg L^{-1})
HAIB 1	I	4.3	32.1	208.0	17.3	39.0
	II	4.1	25.0	10.0	13.8	27.4
HAIB 2	I	7.0	37.8	240.0	18.4	30.9
	II	5.8	65.0	11.0	40.6	44.7
HAIB 3	I	7.0	75.7	3.0	72.8	32.0
	II	6.8	67.2	7.0	74.5	75.8
	III	4.6	27.4	143.0	15.0	29.8

HAIB 3 collapse in phase III. Hydrogen sulfide can affect the functioning of coenzymes and denature proteins by precipitating metals in the active sites of both elements; it can also affect the assimilation of sulfur and the intracellular pH. Additionally, hydrogen sulfide can decrease the bioavailability of metals essential to bacterial metabolism (Lens *et al.* 1998). The toxicity of sulfide to SRB has been shown to be closely associated with the bacterial species present. Maillacheruvu & Parkin (1996) studied propionate- and acetate-oxidizing SRB and hydrogenotrophic methanogens and reported that the acetotrophics were much more sensitive to sulfide toxicity. In contrast, Kaksonen *et al.* (2004a) described that ethanol oxidation was more affected than acetate oxidation by the presence of sulfide.

Acetic acid accumulation occurred primarily under reactor conditions of greater instability (HAIB 1 phase I, HAIB 2 phase I, and HAIB 3 phase III). This evidence was also observed by Nancucheo & Johnson (2014) operating an acidophilic up-flow biofilm reactor. The results indicated that when sulfate removal became stable after a pH influent decrease to 3.0, acetic acid concentrations in the effluent reduced from 10 mM to less than 5 mM. High effluent concentrations of acetic acid indicated partial ethanol oxidation, whereas low (or negligible) acetic acid effluent concentrations indicated complete ethanol oxidation. In this work, effluent acetic acid concentrations were directly associated with the sulfate loading rates: the smaller the applied sulfate loading rates, the smaller the acetic acid effluent concentrations.

Metal removal

Metal removal was evaluated only in HAIB 2 and 3 since both showed better performance related to sulfate removal. Iron, zinc, and nickel were analyzed, as they were at slightly higher concentrations in acid mine water, except for manganese (Table 6). Nickel removal was 28–48% and we cannot establish a better situation, since the built-in analysis error was high due to the low concentration of this element in the influent (0.3 mg L^{-1}). On the other hand, zinc removal was higher in phase I of HAIB 2, while it remained similar in phase II and phases I and II of HAIB 3. The higher efficiency of phase I (HAIB 2) must be due to the pH adjustment made in the influent of this reactor. It is possible that in the influent a partial precipitation of this element occurred. Considering a

**Table 6** | Effluent pH and metal removal performed in the horizontal-flow anaerobic immobilized reactors 2 and 3

HAIB	2		3	
	I	II	II	II
Effluent pH	7.0	5.8	7.0	6.8
Ni removal (%)	48 ± 10	30 ± 9	28 ± 9	30 ± 9
Zn removal (%)	90 ± 8	69 ± 7	58 ± 5	60 ± 5

mean zinc removal of 60%, the effluent concentration still was above the concentration of 1.0 mg L^{-1}, considered strongly inhibitory to anaerobic systems (Parkin & Owen 1986). However, metals could be stimulatory or inhibitory and this effect is determined by total metal concentration, chemical forms, pH, and redox potential (Chen *et al.* 2008). Additionally, many studies (Lin 1993; Wong & Cheung 1995) have determined inhibitory metals' concentrations for the methanogenic or acidogenic processes, since they are characterized by the most sensitive steps of the process. In this work it was not assessed whether zinc could have acted as an inhibitory element to the sulfidogenic process.

The bacterial community structures of HAIB reactors

The microbial bacterial community structures in the HAIB 1, HAIB 2, and HAIB 3 reactors were represented by 50, 50, and 100 clones of the domain bacteria, respectively. The analysis of HAIB reactor clones was performed by comparing their 16S rRNA partial gene sequences to those available in the GenBank database (Figure 3). Operational taxonomic units (OTUs) were compiled using the Dotur program (Schloss & Handelsman 2005) from clones' DNA sequences with greater than 97% similarity. In this study, clones were obtained with sequences smaller than 400 base pairs; some correlations related to the sulfate removal process were only possible in reactors HAIB 2 and 3 (Figure 3).

The majority of clones (95%) of HAIB 1 were grouped in the phylum Proteobacteria, class Gammaproteobacteria, and 55% of them were closely related to Acidithiobacillales. The HAIB 1 OTUs 1, 2, 5, 6, 12, 13, and 15 matched microorganisms of the genus *Acidithiobacillus* and ranged from 88 to 98% DNA similarity. The extreme conditions of AMD result in a homogeneous environment with a well-adapted community based on iron and sulfur metabolism,
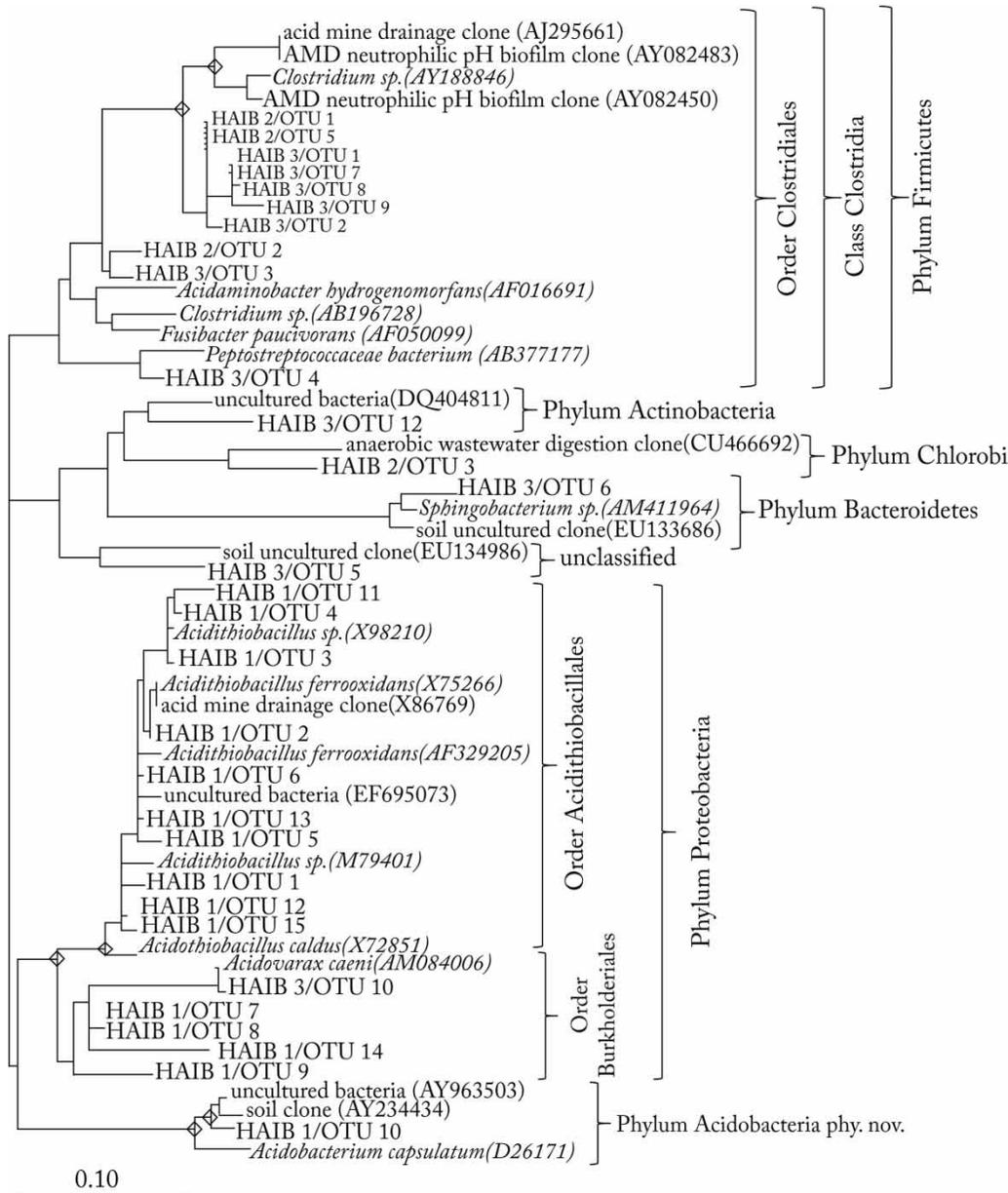
Figure 3 | ARB parsimony-interactive phylogenetic tree containing clones related to the members of *Firmicutes* and *Proteobacteria* phyla, constructed from a maximum likelihood existing tree. The bar represents 10% DNA sequence divergence.

such as members of *Leptospirillum* and *Acidithiobacillus* (Sáchez-Andrea *et al.* 2014). The presence of these species may indicate that in the HAIB 1 reactor it was not possible to establish a fully anaerobic environment with low redox potential. This fact could explain the low sulfate removal observed. Garcia-Moyano *et al.* (2007) studied the microbial diversity of AMD of Rio Tinto (Spain) (sample pH values between 2 and 3), and the library of clones was composed

of the species *Acidithiobacillus ferrooxidans*, *Leptospirillum ferrooxidans* and *Acidiphilium* spp. SRB were not detected in the biomass of the HAIB 1 reactor, which means this population could be at very low concentrations in this reactor, probably because that AMD does not provide the proper environment for stimulating SRB growth. It is therefore unlikely that sulfate removal will occur in natural mining environments unless very specific conditions are met.

HAIB 2 showed dominance (96%) of clones of the phylum Firmicutes, all of the class Clostridia and order Clostridiales. Like the HAIB 2 reactor, the HAIB 3 reactor was represented by clones of the phylum Firmicutes (94%). Selenska-Pobell (2002) reported a strain of *Clostridium* sp. found in piles of tailings from uranium mines. *Clostridium* has a large phenotypic diversity, and its representatives seem to have the ability to act under different metabolic conditions. Some studies have revealed new species of the genus *Clostridium* that carry out sulfate reduction. Hernández-Eugenio *et al.* (2002) identified a new species of SRB in UASB granules and proposed the name *Clostridium thiosulfatireducens* sp. nov. Ma *et al.* (2008) identified a species of SRB highly phylogenetically related to the genus *Clostridium*, with the temporary name *Clostridium bifermentans* H1. Sallam & Steinbüchel (2009) proposed a new species, named *Clostridium sulfidogenesis* sp. nov., that was able to reduce sulfate and thiosulfate under mesophilic conditions. This species was isolated from sediment ponds. Many representatives of the Clostridia group have been detected in AMD remediation processes. The organisms are specialized in sugar fermentation, but their presence in reactors fed with easily degradable substrates (ethanol, lactate, or acetate) also suggests their active role in SRB communities (Castro *et al.* 2000; Kaksonen *et al.* 2004b; Hiibel *et al.* 2011; Sánchez-Andrea *et al.* 2014).

HAIB 2 OTU 2 and HAIB 3 OTU 3 also matched a strain of *Clostridium* sp. This strain also has 100% DNA identity with the species *Acidaminobacter hydrogenomorfans*, a bacterium isolated by Stams & Hansen (1984) from reactors containing mixed cultures of methanogenic archaea and SRB. These OTUs also showed close identity with *Fusibacter paucivorans*, a new species described by Ravot *et al.* (1999) as reducing bacteria that are able to metabolize thiosulfate and different sources of organic matter, and that produce acetate, CO_2 and H_2.

CONCLUSIONS

The present work provides a better understanding of the biological sulfate reduction of AMD in a HAIB reactor. It was shown that the reduction of sulfate applied load is the most relevant parameter in determining the HAIB's reactor efficiency. From several studied parameters, only a 75% reduction in sulfate load has enabled a sulfidogenic environment and, by applying this condition from the beginning, a meaningful enhancement was observed, both in the efficiency of removal and on the stability of the system. Under these optimal conditions, the COD and sulfate removal reached 75.7% (154.5 ± 31 g m^{-3} d^{-1}) and 72.8% (163.9 ± 28 g m^{-3} d^{-1}), respectively, and the effluent pH was observed to be between 6.8 and 7.0. Once the sulfidogenic process was fully established, the stoichiometric COD/SO_4^{2-} ratio of 0.67 has been proven to be enough to sustain it. Other parameters such as adjustments in the initial pH (from 3.0 to 5.0) or recirculation of the effluent liquid phase (to promote the dilution of the influent) provided no satisfactory results on any reactor. Nickel and zinc removal were 28%–48% and 58%–90%, respectively, probably by biogenic sulfide precipitation. A predominance of phylum Firmicutes representatives was found in the reactor-fed diluted drainage. These species are closely related to strains of *Clostridium* sp. and species of *Acidaminobacter hydrogenomorfans* and *Fusibacter paucivorans*, organisms that have been reported as possible sulfate reduction bacteria.

REFERENCES

Akcil, A. & Koldas, S. 2006 Acid mine drainage (AMD): causes, treatment and case studies. *J. Clean. Prod.* **14**, 1139–1145.

Baskaran, V. & Nemati, M. 2006 Anaerobic reduction of sulfate in immobilized cell bioreactors, using a microbial culture originated from an oil reservoir. *Biochem. Eng. J.* **31**, 148–159.

Bekmezci, O. K., Ucar, D., Kaksonen, A. H. & Sahinkaya, E. 2011 Sulfidogenic biotreatment of synthetic acid mine drainage and sulfide oxidation in anaerobic baffled reactor. *J. Hazard. Mater.* **189**, 670–676.

Castro, H. F., Williams, N. H. & Ogram, A. 2000 Phylogeny of sulfate-reducing bacteria. *FEMS Microbiol. Ecol.* **31**, 1–9.

Chang, I., Shin, P. & Kim, B. 2000 Biological treatment of acid mine drainage under sulphate-reducing conditions with solid waste materials as substrate. *Water Res.* **34**, 1269–1277.

Chen, Y., Cheng, J. J. & Creamer, K. S. 2008 Inhibition of anaerobic digestion process: a review. *Bioresour. Technol.* **99**, 4044–4064.

Garcia, C., Moreno, D. A., Ballester, A., Blàzquez, M. L. & Gonzàlez, F. 2001 Bioremediation of an industrial acid mine water by metal-tolerant sulphate-reducing bacteria. *Miner. Eng.* **14** (9), 997–1008.

Garcia-Moyano, A., Gonzalez-Toril, E., Aguilera, A. & Amils, R. 2007 Prokaryotic community composition and ecology of floating macroscopic filaments from an extreme acidic environment, Rio Tinto (SW, Spain). *Syst. Appl. Microbiol.* **30** (8), 601–614.

Hernandéz-Eugenio, G., Fardeau, M.-L. & Cayol, J.-C. 2002 Clostridium thiosulfatireducens sp. nov., a proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from an upflow anaerobic sludge blanket (UASB) reactor. *Int. J. Syst. Evol. Micr.* **52**, 1461–1468.

Hiibel, S. R., Pereyra, L. P., Breazeal, M. V. R., Reisman, D. J., Reardon, K. F. & Pruden, A. 2011 Effect of organic substrate on the microbial community structure in pilot-scale sulfate-reducing biochemical reactors treating mine drainage. *Environ. Eng. Sci.* **28**, 563–572.

Hirasawa, J. S., Sarti, A., Del Aguila, N. K. S. & Varesche, M. B. A. 2008 Application of molecular techniques to evaluate the methanogenic Archaea and anaerobic bacteria in the presence of oxygen with different COD: sulfate ratios in a UASB reactor. *Anaerobe* **14**, 209–218.

Hu, Y., Jing, Z., Sudo, Y., Niu, Q., Du, J., Wu, J. & Li, Y. 2015 Effect of influent COD/SO42- ratios on UASB treatment of a synthetic sulfate-containing wastewater. *Chemosphere* **130**, 24–33.

Jiménez-Rodríguez, A. M., Durán-Barrantes, M. M., Borja, R., Sánchez, M., Colmenarejo, M. F. & Raposo, F. 2009 Heavy metals removal from acid mine drainage water using biogenic hydrogen sulphide and effluent anaerobic treatment: effect of pH. *J. Hazard. Mater.* **165**, 759–765.

Kaksonen, A., Franzmann, P. & Puhakka, J. 2004a Effects of hydraulic retention time and sulfide toxicity on ethanol and acetate oxidation in sulfate-reducing metal-precipitating fluidized-bed reactor. *Biotechnol. Bioeng.* **86** (3), 322–343.

Kaksonen, A., Plumb, J. J., Franzmann, P. D. & Puhakka, J. 2004b Simple organic electron donors support diverse sulfate-reducing communities in fluidized-bed reactors treating acidic metal- and sulfate-containing wastewater. *FEMS Microbiol. Ecol.* **47**, 279–289.

Kieu, H. T. Q., Müller, E. & Horn, H. 2011 Heavy metal removal in anaerobic semicontinuous stirred tank reactors by a consortium of sulfate-reducing bacteria. *Water Res.* **45** (13), 3863–3870.

Lens, P. N. L., Visser, A., Janssen, A. J. H., Hulshoff Pol, L. W. & Lettinga, G. 1998 Biotechnological treatment of sulfate-rich wastewaters. *Crit. Rev. Env. Sci. Tec.* **28** (1), 41–88.

Lin, C. Y. 1993 Effect of heavy metals on acidogenesis in anaerobic digestion. *Water Res.* **27**, 147–152.

Ludwig, W., Strunk, O., Westram, R. & Richter, L. 2004 ARB: a software environment for sequence data. *Nucleic Acids Res.* **32** (4), 1363–1371.

Ma, F., Wei, L., Wang, L. & Chang, C.-C. 2008 Isolation and identification of the sulphate-reducing bacteria strain H1 and its function for hydrolysed polyacrylamide degradation. *Int. J. Biotech.* **10** (1), 55–63.

Maillacheruvu, T. Y. & Parkin, G. F. 1996 Kinetics of growth, substrate utilization and sulfide toxicity for propionate, acetate, and hydrogen utilizers in anaerobic systems. *Water Environ. Res.* **68** (7), 1099–1106.

McCauley, C., O'Sullivan, A., Weber, P., Milke, M. & Trumm, D. 2009 Sulfate reduction and implications for metal removal in bioreactors from iron and aluminum dominated acid mine drainage. *Water Res.* **43** (4), 961–970.

Melvin, A. N. W. T. & Hobson, P. P. 1994 Identification of anaerobic digester bacteria using polymerase chain reaction method. *Bioresource. Technol.* **47**, 73–80.

Moosa, S., Nemati, M. & Harrison, S. T. L. 2002 A kinetic study on anaerobic reduction of sulphate. Part I: effect of sulphate concentration. *Chem. Eng. Sci.* **57**, 2773–2780.

Moraes, E. M., Adorno, M. A. T., Zaiat, M. & Foresti, E. 2000 Determinação de ácidos voláteis por cromatografia gasosa em efluentes de reatores anaeróbios tratando resíduos líquidos e sólidos. In: *Proceedings of Latin American Workshop and Symposium on Anaerobic Digestion (DAAL)*, Recife-Pernambuco, Brazil. 2, pp. 235–238.

Ñancucheo, J. & Johnson, D. B. 2014 Removal of sulfate from extremely acidic mine waters using low pH sulfidogenic bioreactors. *Hydrometallurgy* **150**, 222–226.

Neculita, C. M. & Zagury, G. J. 2008 Biological treatment of highly contaminated acid mine drainage in batch reactors: long-term treatment and reactive mixture characterization. *J. Hazard. Mater.* **157**, 358–366.

Oberholster, P. J., Genthe, B., Hobbs, P., Cheng, P. H., de Klerk, A. R. & Botha, A. M. 2013 An ecotoxicological screening tool to prioritise acid mine drainage impacted streams for future restoration. *Environ. Pollut.* **176**, 244–253.

Parkin, G. F. & Owen, W. F. 1986 Fundamentals of anaerobic digestion on wastewater sludges. *J. Environ. Eng.* **112**, 867–920.

Ravot, G., Magot, M., Fardeau, M. L. & Patel, B. K. 1999 *Fusibacter paucivorans gen. nov.*, sp. nov., an anaerobic, thiosulfate-reducing bacterium from an oil-producing well. *Int. J. Syst. Bacteriol.* **49**, 1141–1147.

Rodriguez, R. P., Oliveira, G. H. D., Raimundi, I. M. & 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., Dursun, N., Ozkaya, B. & Kaksonen, A. K. 2013 Use of landfill leachate as a carbon source in a sulfidogenic fluidized-bed reactor for the treatment of synthetic acid mine drainage. *Miner Eng.* **48**, 56–60.

Sallam, A. & Steinbüchel, A. 2009 *Clostridium sulfidigenes sp.* nov., a mesophilic, proteolytic, thiosulfate- and sulfur-reducing bacterium isolated from pond sediment. *Int. J. Syst. Evol. Micr.* **59** (7), 1661–1665.

Sánchez-Andrea, I., Sanza, J. L., Bijmans, M. F. M. & Stams, A. J. M. 2014 Sulfate reduction at low pH to remediate acid mine drainage. *J. Hazard. Mater.* **269**, 98–109.

Sani, R. K., Peyton, B. M. & Brown, L. T. 2001 Copper-induced inhibition of growth of Desulfovibrio desulfuricans G20: assessment of its toxicity and correlation with those of zinc and lead. *Appl. Env. Microbiol.* **67** (10), 4765–4772.

Schloss, P. D. & Handelsman, J. 2005 Introducing DOTUR, a computer program for defining operational taxonomic units and estimating species richness. *Appl. Env. Microbiol.* **71** (3), 1501–1506.

Selenska-Pobell, S. 2002 Diversity and activity of bacteria in uranium mining waste piles. In: *Radioactivity in the Environment*, Vol. 2 (M. J. Keith-Roach & F. R. Lievens, eds). Elsevier Science, Oxford, UK, pp. 225–254.

Silva, A. J., Varesche, M. B. A., Foresti, E. & Zaiat, M. 2002 Sulphate removal from industrial wastewater using a packed-bed anaerobic reactor. *Process. Biochem.* **37**, 927–935.

Silva, A. J., Hirasawa, J. S., Varesche, M. B., Foresti, E. & Zaiat, M. 2006 Evaluation of support materials for the immobilization of sulfate-reducing bacteria and methanogenic archaea. *Anaerobe* **12**, 93–98.

Stams, A. J. M. & Hansen, T. A. 1984 Fermentation of glutamate and other compounds by *Acidaminobacter hydrogenoformans gen. nov.* sp. nov., an obligate anaerobe isolated from black mud. Studies with pure cultures and mixed cultures with sulfate-reducing and methanogenic bacteria. *Arch. Microbiol.* **137**, 329–337.

Standard Methods for the Examination of Water and Wastewater 2012 22nd edn. American Public Health Association/ American Water Works Association/Water Environment Federation, Washington, DC, USA.

van Lier, J. B., Tilche, A., Ahring, B. K., Macarie, H., Moletta, R., Dohanyos, M., Hulshoff Pol, L. W., Lens, P. & Verstraete, W. 2001 New perspectives in anaerobic digestion. *Water Sci. Technol.* **43** (1), 1–18.

Velasco, A., Ramírez, M., Volke-Sepúlveda, T., González-Sánchez, A. & Revahd, S. 2008 Evaluation of feed COD/sulfate ratio as a control criterion for the biological hydrogen sulfide production and lead precipitation. *J. Hazard. Mater.* **151**, 407–413.

Waybrant, K., Ptacek, C. & Blowes, D. 2002 Treatment of mine drainage using permeable reactive barriers: column experiments. *Environ. Sci. Technol.* **36**, 1349–1356.

Wong, M. H. & Cheung, Y. H. 1995 Gas production and digestion efficiency of sewage sludge containing elevated toxic metals. *Bioresour. Technol.* **54**, 261–268.

Xingyu, L., Gang, Z., Xiaoqiang, W., Laichang, Z., Jiankang, W., Renman, R. & Dianzuo, W. 2013 A novel low pH sulfidogenic bioreactor using activated sludge as carbon source to treat acid mine drainage (AMD) and recovery metal sulfides: pilot scale study. *Miner. Eng.* **48**, 51–55.

Zaiat, M., Cabral, A. K. A. & Foresti, E. 1994 Cell wash-out and external mass transfer resistance in horizontal-flow anaerobic immobilized sludge (HAIS) reactor. *Rev. Bras. Eng.–Caderno de Engenharia Química* **11**, 33–42.

Zhang, J., Zhang, Y., Chang, J., Quan, X. & Li, Q. 2013 Biological sulfate reduction in the acidogenic phase of anaerobic digestion under dissimilatory Fe (III)–reducing conditions. *Water Res.* **47**, 2033–2040.