Performance and bacterial diversity of bioreactors used for simultaneous removal of sulfide, solids and organic matter from UASB reactor effluents

L. S. Azevedo, I. M. P. Castro, C. D. Leal, J. C. Araújo and C. A. L. Chernicharo

ABSTRACT

Two bioreactors were investigated as an alternative to post-treatment of effluent from an upflow anaerobic sludge blanket (UASB) reactor treating domestic sewage, with an aim of oxidizing sulfide into elemental sulfur, and removal of solid and organic material. The bioreactors were operated at different hydraulic retention times (HRTs) (6, 4, and 2 h) and in the presence or absence (control) of packing material (polypropylene rings). Greater sulfide removal efficiencies – 75% (control reactor) and 92% (packed reactor) – were achieved in both reactors for an HRT of 6 h. Higher organic matter (COD) and solid (TSS) removal levels were observed in the packed reactor, which produced effluent with low COD (100 mg $CODL^{-1}$) and TSS concentrations (30 mg $TSSL^{-1}$). Denaturing gradient gel electrophoresis results revealed that a metabolically diverse bacterial community was present in both bioreactors, with sequences related to heterotrophic bacteria, sulfur bacteria (*Thiocapsa, Sulfurimonas* sp., *Chlorobaculum* sp., *Chromatiales* and *Sulfuricellales*), phototrophic purple non-sulfur bacteria (*Rhodopseudomonas*, *Rhodocyclus* sp.) and cyanobacteria. The packed reactor presented higher extracellular sulfur formation and potential for elemental sulfur recovery was seen. Higher efficiencies related to the packed reactor were attributed to the presence of packing material and higher cell retention time. The studied bioreactors seemed to be a simple and low-cost alternative for the post-treatment of anaerobic effluent.

Key words | bioreactors, post-treatment, sulfide oxidation, sulfur bacteria, UASB reactor

L. S. Azevedo (corresponding author)
I. M. P. Castro
C. D. Leal
J. C. Araújo
C. A. L. Chernicharo
Department of Sanitary and Environmental Engineering,
Federal University of Minas Gerais,
Av. Antônio Carlos 6.627, Campus Pampulha,
31.270-901, Belo Horizonte, MG,
Brazil
E-mail: *lariza.azevedo@engenharia.ufjf.br*

INTRODUCTION

Upflow anaerobic sludge blanket (UASB) reactors have been widely adopted for sewage treatment in many warm climate countries. Despite several advantages attributed to the system, some limitations still remain, such as odour and the need for a post-treatment unit for the removal of additional organic matter and nutrients (i.e. nitrogen, phosphorus and sulfur), in order to reduce the discharge impact of effluents on receiving waters (Chernicharo *et al.* 2015). Odour from anaerobic-based sewage treatment plants is mainly related to the release of dissolved sulfide (H_2S) present in the bulk liquid, especially in outlet structures where turbulent conditions prevail (Souza *et al.* 2012). Along with its characteristic offensive odour, H_2S has other negative effects, such as toxicity, corrosiveness and flammability (USEPA 2010). Moreover, H_2S may inhibit the metabolism of microorganisms involved in the process of organic matter degradation, as well as potentialize the corrosion of metal and concrete structures within and near the reactors (Speece 1996; Chernicharo *et al.* 2015).

Strategies for sulfide control involve either inhibition of H_2S production, or elimination of the produced gas (Garcia de Lomas *et al.* 2005). Sulfide oxidation may occur by biological or chemical pathways; however, the biological process has major advantages, such as low cost and high efficiency, achieving an effluent sulfide concentration of less than 1 mg L^{-1} (Henshaw *et al.* 1998). Biological sulfide oxidation in liquid or gas streams may occur through the activity of phototrophic or chemolithotrophic bacteria, under aerobic, anoxic or anaerobic conditions.

doi: 10.2166/wst.2018.403

Sulfide-oxidizing bacteria are grouped according to the sulfur compound used, colour and environmental conditions. Aerobic sulfide oxidation is performed by colourless sulfur bacteria, while anaerobic oxidation occurs by means of anoxygenic photosynthesis, from the activity of green and purple bacteria that use light as their energy source in anoxic environments (Madigan *et al.* 2010). Although the advantages of the biological process have been reported, scale-up of bioreactors is not straight forward, due to the requirement to allow light entrance (Janssen *et al.* 1999).

Previous studies have investigated the biological oxidation of sulfide to elemental sulfur (Vannini *et al.* 2008; Fajardo *et al.* 2012; Liu *et al.* 2015); however, information regarding the removal of dissolved sulfide using real anaerobic effluent – and the bacteria naturally present in it – is scarce. The possibility of using microorganisms naturally occurring on the surface of the settler compartment of UASB reactors to remove sulfide from anaerobic effluent has only recently been investigated by our research group (Garcia *et al.* 2015, 2017).

In this context, we consider that further studies evaluating low-cost biotechnological methods for sulfide removal, using real wastewater, are still required, as well as systems that incorporate both nutrient removal and overall quality improvement of anaerobic effluent. Garcia *et al.* (2017) investigated two low-cost bioreactors exposed to sunlight (packed with polypropylene – R1 and with sponge – R2) at three different hydraulic retention times (HRTs – 24 h, 12 h and 6 h) for the abatement of sulfide from the effluent of a UASB reactor treating domestic wastewater by means of anoxygenic photosynthesis performed by sulfur bacteria. The authors obtained higher sulfide removal (90%) for an HRT of 12 h for both bioreactors, and the microbial diversity was investigated in depth, showing the presence of sulfur bacteria, such as those from the *Chlorobium* and *Chromatium* genera. However, shorter HRTs were not tested, and the overall performance of the bioreactors regarding the removal of chemical oxygen demand (COD) and solids was not investigated. Therefore, in the present study, two bioreactors were investigated as alternatives for the post-treatment of effluent from a UASB reactor treating domestic sewage, to determine the potential of these bioreactors regarding sulfide oxidation to elemental sulfur – and also regarding complementary solids and organic matter removal.

Sulfur compounds were monitored, with an aim of determining oxidation pathways and major products. Partial sulfide oxidation to elemental sulfur was of particular interest, due the possibility of elemental sulfur recovery. Parameters such as solids and organic matter were evaluated, in order to determine overall performance of the designed system. The bioreactors were designed with a shape similar to the settler compartment of a UASB reactor and operated under realistic conditions. Therefore, results obtained in this work may be used to optimize operational reactor conditions, to favour elemental sulfur formation and to improve overall bioreactor performance. The main goal of testing the lowest HRT was to verify the possibility of inserting the unit into the upper part of the UASB reactor, eliminating the need for adding an external post-treatment system for such purposes.

METHODS

Experimental set-up

The experiments were performed in a system that comprised a pilot-scale UASB reactor, built in fiberglass (340 L), followed by two Plexiglas bioreactors (30 L), operating in parallel, designed to perform oxidation of dissolved sulfide from the UASB reactor effluent. The bioreactors were designed with flow direction and configuration similar to the settler compartment of UASB reactors, with cone-shaped bottoms (h = 20 cm) and cylindrical chambers (h = 30 cm), as shown in Figure 1(a). The Plexiglas reactors were identical, except for the absence (R1-control), or presence (R2-packed), of packing material (Figure 1(b)).

The set-up was installed at the Centre for Research and Training in Sanitation UFMG/COPASA, located at the Arrudas STP (Belo Horizonte, Brazil, coordinates 19°53′42″ S and 43°52′42″ W, altitude 800 m). A sample of raw sewage was submitted to preliminary treatment for solids and grit removal, and then the UASB reactor was fed with an aliquot of the wastewater. The main characteristics of the typical urban wastewater were: pH – 7.5; COD – 515.7 mg L^{-1}; S^{2-} – 0.5 mgL^{-1}; SO_4^{2-} – 32 mgL^{-1}, as shown in Table 1.

The bioreactors were monitored over 7 months under specific operational conditions, according to the tested HRTs of 6 h, 4 h and 2 h. Throughout, monitoring of redox potential, temperature and pH inside the bioreactors was conducted, giving values between −68 mV and −110 mV (standard hydrogen electrode), 21.4–26.6 °C and 6.8–7.1, respectively. From the values observed for pH and temperature, it was possible to estimate that about 50% of the sulfide in the UASB reactor effluent was present in non-ionized form (Chernicharo 2007).

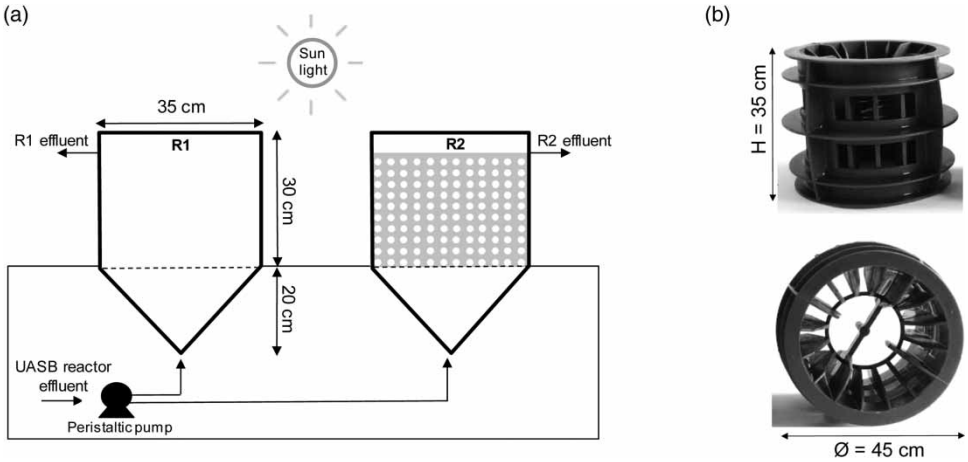

Figure 1 | Schematic representation and dimensions of (a) bioreactors and (b) packing material adopted in this work.

Table 1 | Composition of raw sewage influent to the UASB reactor

Parameters	Average	Median	Standard deviation	Maximum	Minimum
pH	7.5	7.5	0.2	8.0	7.2
Total COD ($mgCODL^{-1}$)	542.9	515.7	181.5	895.7	309.0
Sulfide ($mgS^{2-}L^{-1}$)	0.6	0.5	0.5	1.8	0.0
Sulfate ($mgSO_4^{2-}L^{-1}$)	32.3	32.0	5.3	46.5	24.5

At the end of each phase, the reactors were emptied and the packing material replaced. Aiming to retain the polypropylene rings used as packing material (Ø 45 mm, h = 35 mm, 86 m^2/m^3 specific surface – Bio Project, Brazil), as illustrated in Figure 1(b), R2-packed was equipped with a perforated stainless-steel basket. The adoption of packing material was intended to provide higher retention of microorganisms naturally present in the UASB reactor effluent, once the bioreactors were not inoculated.

Physicochemical analysis

Sulfate (SO_4^{2-}), total suspended solids (TSS) and chemical organic matter (COD) were measured according to *Standard Methods for the Examination of Water and Wastewater* (APHA 2012). Sulfide was measured according to Plas *et al.* (1992), and elemental sulfur (S^0) was measured by extracting with chloroform and analysis with high performance liquid chromatography (HPLC), using a PRP-1, reverse phase, HPLC column (dimensions: 15 cm-L × 4.1 mm-ID), as described by Henshaw *et al.* (1998). Analyses were performed twice a week, except for TSS and S^0, which were analyzed weekly. At the end of each phase, samples from the microbial layer (attached: propylene rings and non-attached: scum layer) and sludge (bottom of the reactor) were collected, aiming at the determination of elemental sulfur accumulation for all tested HRTs.

To extract elemental sulfur from biomass and sludge, the cells were mechanically lysed using a mini-beadbeater apparatus (Biospec, San Gabriel, China), containing 0.1 mm glass beads (Sigma, Brazil), followed by extraction with chloroform. The mass distribution of sulfur compounds was determined by considering the volume of treated effluent, the mass of biomass and sludge produced in each operational phase, as well as the corresponding mass of each sulfur fraction formed.

Analysis of the bacterial community

The morphology of sulfur bacteria, and the formation of elemental sulfur, were observed by monitoring the scum layer and attached biomass of the bioreactors by optical microscopy, using a binocular Olympus microscope equipped with an Olympus DP70 camera.

At the end of each phase, samples from the microbial layer (attached and non-attached biomass) and sludge

(bottom of the reactors) were collected, to allow the comparison of bacterial diversity for all tested HRTs. Cells were taken from several polypropylene rings located in different heights, to provide representative samples. Samples were centrifuged three times (4,000 rpm for 10 minutes), applying a phosphate buffer saline solution (1 × PBS, NaCl, Na_2HPO_4, NaH_2PO_4, pH = 7.2–7.4). The supernatant was discharged and the pellets were stored at −20 °C. DNA was extracted using a PowerSoil DNA Isolation Kit (MO BIO Laboratories, USA). Polymerase chain reaction and denaturing gradient gel electrophoresis (PCR-DGGE) were performed using the primer set 1055F and 1392R, with a GC clamp, according to the method of Ferris *et al.* (1996). DGGE was performed at 60 °C in 0.5 × TAE buffer at 80 V for 16.5 h, using a DCode system (Bio-Rad Universal Mutation Detection, (Hercules, CA, USA), comprising 8% polyacrylamide gel, with a 50–65% gradient of urea formamide denaturant. Gels were stained with SYBR Gold solution (Life Technologies) and visualized under UV transillumination.

Specific gel bands were excised, re-amplified, purified, and sequenced using, a genomic service (Macrogen Inc., Seoul, Korea). Sequences were compared with that from the Ribosomal Database Project (https://rdp.cme.msu.edu/classifier/classifier.jsp) and the National Center for Biotechnology Information (NCBI) database. DGGE patterns were analysed using BioNumerics software 6.6, and the Shannon index (Gafan *et al.* 2005) was employed to assess bacterial biodiversity in the biomass and sludge, under the tested conditions.

RESULTS AND DISCUSSION

Biological sulfide oxidation

Dissolved sulfide and elemental sulfur concentrations in the effluent of the UASB reactor and bioreactors are shown in Figure 2. Values observed for dissolved sulfide in UASB reactor effluent, 5–11 $mgS^{2-}L^{-1}$, were similar to those obtained by Souza *et al.* (2012), of 7–11 $mgS^{2-}L^{-1}$, and higher than values determined by Garcia *et al.* (2017), of 2–3 $mgS^{2-}L^{-1}$, both of which were studies involving anaerobic reactors treating domestic sewage from the same contribution region.

Sulfide median concentration in the UASB reactor effluent for the 2 h HRT, 11.3 $mgS^{2-}L^{-1}$, was higher than that obtained previously (5.4 $mgS^{2-}L^{-1}$ and 6.6 $mgS^{2-}L^{-1}$), justifying the use of removal efficiencies instead of absolute concentration values. Higher sulfide removal was observed for both bioreactors for an HRT of 6 h – 75% for R1-control, and 92% for R2-packed.

Garcia *et al.* (2017) achieved 50–65% sulfide removal when evaluating an HRT of 6 h. The lower efficiency could be related to the median sulfide concentration in the UASB reactor effluent, 2 $mgS^{2-}L^{-1}$, which was 63% lower than the efficiency determined in this study – 5.4 $mgS^{2-}L^{-1}$. In the sequential sampling program, sulfide effluent concentrations for the bioreactors were 1.2 $mgS^{2-}L^{-1}$, 2.0 $mgS^{2-}L^{-1}$ and 4.9 $mgS^{2-}L^{-1}$ for R1-control, and 0.5 $mgS^{2-}L^{-1}$, 3.1 $mgS^{2-}L^{-1}$ and 3.4 $mgS^{2-}L^{-1}$, for R2-packed, for HRTs of 6 h, 4 h and 2 h, respectively.

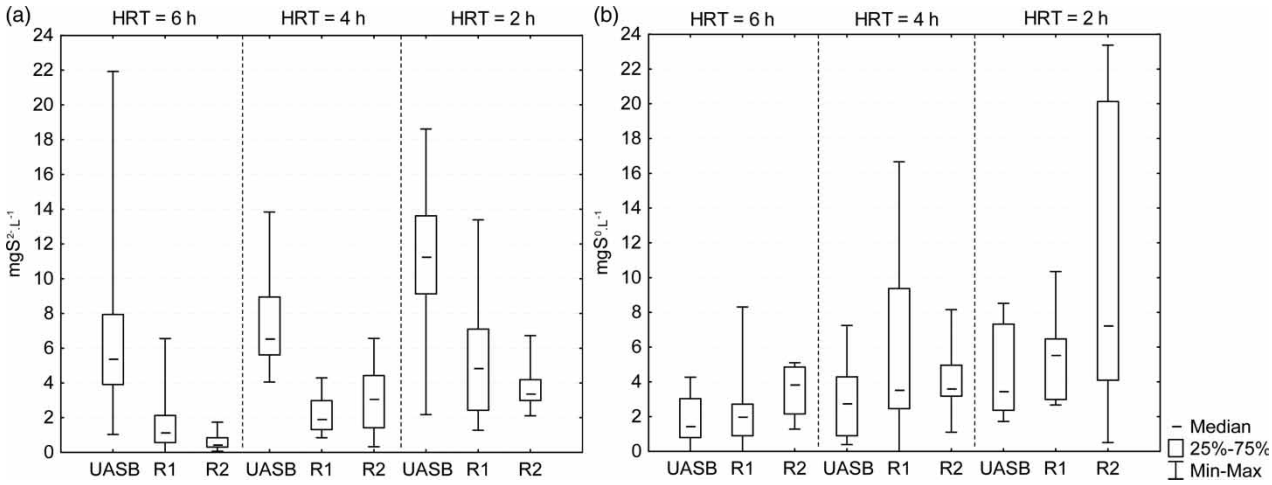

Figure 2 | (a) Dissolved sulfide and (b) elemental sulfur concentrations in the effluent of the UASB reactor and phototrophic bioreactors. R1: control reactor and R2: packed reactor (polypropylene rings).

Among studied conditions, higher sulfate concentrations were observed in R1-control effluent, pointing out that the absence of packing material favoured complete sulfide oxidation to sulfate, with median concentrations of 40.2 $mgSO_4^{2-}L^{-1}$, 27.0 $mgSO_4^{2-}L^{-1}$ and 28.1 $mgSO_4^{2-}L^{-1}$ for 6 h, 4 h and 2 h of HRT, respectively. Although R2-packed effluent showed higher sulfide oxidation under 6 h and 2 h HRT conditions, the same did not occur regarding sulfate formation, with median concentrations of 28.0 $mgSO_4^{2-}L^{-1}$ and 24.1 $mgSO_4^{2-}L^{-1}$, possibly due to the greater formation of elemental sulfur being facilitated by the use of packing material.

Since biologically-produced, elemental sulfur has a white or pale yellow colour (Janssen *et al.* 1999), the formation of this compound was observed from the white precipitate present in the effluent of the bioreactors, and confirmed by HPLC. The white precipitate observed in the R2-packed effluent corresponding to extracellular sulfur is shown in Figure 3(a) and 3(b), whereas intracellular-stored sulfur is shown in Figure 3(c).

Although no significant differences were observed (Wilcoxon's matched pairs test, $\alpha = 5\%$), regarding elemental sulfur formation, higher concentrations were detected in R2-packed effluent for all tested HRTs, even when higher sulfide removal efficiency was associated with R1-control (see Figure 2(b)). Elemental sulfur concentrations in the R1-control effluent were 2.0 $mgS^0\,L^{-1}$, 3.6 $mgS^0\,L^{-1}$ and 5.6 $mgS^0\,L^{-1}$, while higher concentrations were observed in the R2-packed effluent, 3.8 $mgS^0\,L^{-1}$, 3.6 $mgS^0\,L^{-1}$ and 7.3 $mgS^0\,L^{-1}$, for HRTs of 6 h, 4 h and 2 h, respectively.

According to the Pourbaix Diagram (Rickard & Luther 2007), the pH and ORP conditions observed inside the bioreactors indicated that the formation of sulfate would be chemically favoured in the bioreactors, rather than elemental sulfur. Therefore, these conditions indicate that the elemental sulfur formed was due to biological conversions, while the amount of sulfate was possibly formed from both chemical and biological conversions. DNA sequences for sulfur oxidizing bacteria from different genera, such as *Thiocapsa* and *Chlorobaculum*, were detected, and confirmed these results (as presented later in this paper).

Since the highest levels of sulfur formation were related to R2-packed, it is appropriate to assume that the presence of polypropylene rings provided longer cell retention time, and favoured elemental sulfur formation. From Figure 2(b), it can be seen that elemental sulfur was produced in the UASB reactor, probably in the settling compartment. Green- and purple-coloured biomass was observed, indicating the presence of naturally-occurring phototrophic sulfur bacteria in the UASB reactor effluent. Therefore, noting that sunlight was present in the upper part of the reactor, in the uncovered settling compartment, it could be assumed that the elemental sulfur quantified in the UASB reactor effluent was related to sulfur bacteria activity, located in the reactor scum layer, naturally formed, and maintained on the surface of the settler compartment of the UASB reactor.

Influence of HRT and presence of packing material on elemental sulfur formation

The amount of elemental sulfur produced within 30 days, for all tested HRTs, as well as its respective fractions (biomass, sludge and effluent), are shown in Table 2. It was observed from these results that about 95% of total produced elemental sulfur was present in the effluent of the bioreactors (liquid phase). The higher proportion of elemental sulfur

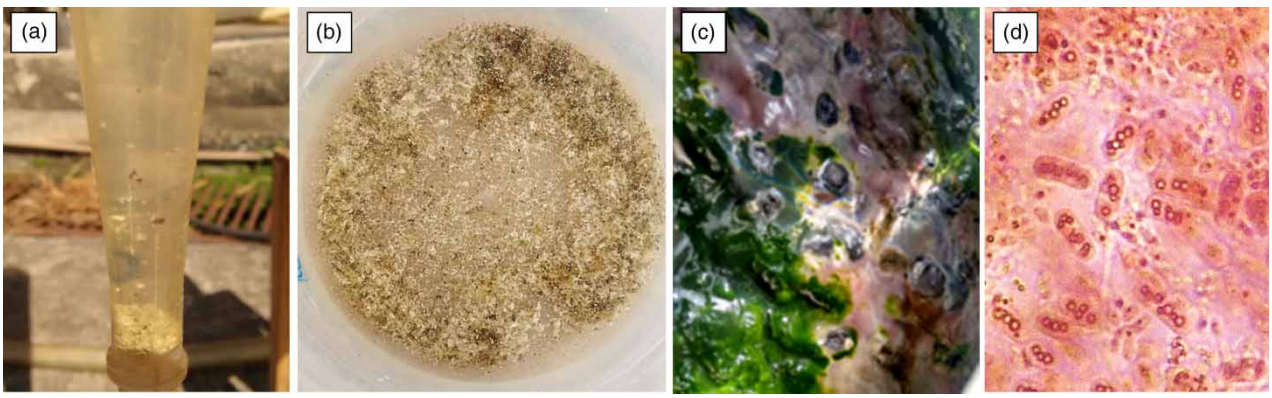

Figure 3 | (a) Settling test of R2 - packed effluent (HRT = 6 h) after 30 minutes, with white solids at the bottom of the Imhoff cone (b) white precipitate present in the R2-packed effluent (c) picture shows the appearance of a green and purple microbial layer; (d) microscopic image of purple bacteria in R2-packed biomass (HRT = 4 h), emphasizing intracellular sulfur.

Table 2 | Mass of elemental sulfur accumulated in the bioreactors (normalized to 30 days of operation)

	S^{a}_{Sludge} (g)	$S^{a}_{Biomass}$ (g)	$S^{b}_{Effluent}$ (g)	S^{0}_{total} (g)	$S^{0}_{Effluent}/S^{0}_{total}$ (%)
R1-control 6 h	0.2 (0.0)	0.1 (0.0)	7.2 (8.2)	7.5	96
R2-packed 6 h	0.6 (0.1)	0.6 (0.0)	13.8 (5.1)	15.0	92
R1-control 4 h	0.9 (0.0)	0.1 (0,0)	19.3 (31.7)	20.3	95
R2-packed 4 h	0.8 (0.0)	0.1 (0.1)	19.5 (11.8)	20.3	96
R1-control 2 h	0.7 (0.4)	–	60.0 (27.1)	60.7	99
R2-packed 2 h	0.8 (2.3)	–	78.6 (88.2)	79.4	99

[a]Median results obtained for the samples collected at the end of each operational phase.
[b]Results obtained from the median of 10 samples. In parentheses: standard deviation.

in the effluent, rather than in the biomass, even under low HRT conditions, is of particular interest, since the process of extraction and recovery would be simpler. Segregation of sulfur from sludge/biomass would not be required (Madigan *et al.* 2010), but only segregation of the white precipitate from the liquid phase. Sulfur recovery may be worthwhile, as it is a valuable product, used in different industrial processes such as for the production of sulfuric acid, fertilizers and fungicides (Vannini *et al.* 2008; Klok *et al.* 2012).

By assessing the total amount of elemental sulfur produced in the bioreactors, it is possible to observe that greater sulfur formation was associated with lower HRT, probably due to the increase of the influent flow rate entering the bioreactors, and the corresponding increment of the influent sulfide load. However, by comparing the accumulated sulfur and the rate S^0produced/S^{2-}influent, as shown in Table 3, it is possible to observe that, although the higher amount of elemental sulfur was determined for the lowest tested HRTs, less elemental sulfur was produced in proportion to the sulfide load influent to the reactor. For both bioreactors, the rate S^0 produced/S^{2-} influent increased with reduction of the HRT, with notable rates observed for the HRTs of 6 h and 2 h – 47% and 33%, respectively.

Several studies have evaluated formation of elemental sulfur for high HRT conditions, between 12 and 48 h (Reyes-Avila *et al.* 2004; Beristain-Cardoso *et al.* 2011; Fajardo *et al.* 2012; Liu *et al.* 2015). In this context, it is possible to assume that the influence of the sulfide load overlapped the influence of the HRT, thus favouring the formation of elemental sulfur, even under conditions of low HRT (2 h). Regarding the influence of packing material, equal or higher formation of elemental sulfur was observed for R2-packed when compared to the amount obtained for R1-control, for all tested HRTs. Similar formation of elemental sulfur was observed for the HRT of 4 h, for both bioreactors. Although not significantly different (Wilcoxon's matched pairs test, $\alpha = 5\%$), R2-packed provided greater formation of elemental sulfur – 50% and 25% greater than values observed for R1-control for 6 h and 2 h of HRT, respectively.

Overall performance of the system

Overall performance of the system investigated in this study was evaluated using the parameters of COD and TSS, with the results shown in Figure 4. Regarding COD, higher median removal efficiency was observed for R2-packed – about 40% for HRTs of 6 h and 4 h – while about 28% removal efficiency was observed to R1-control for an HRT of 4 h.

R1-control median effluent COD concentrations were 113.2 mgCODL^{-1}, 122.3 mgCODL^{-1} and 120.7 mgCODL^{-1}, whereas lower concentrations were observed to R2-packed – 91.5 mgCODL^{-1}, 96.0 mgCODL^{-1} and 104.4 mgCODL^{-1} – for HRTs of 6 h, 4 h and 2 h, respectively.

Table 3 | Sulfide loads applied to the bioreactors, median sulfide removal efficiencies and rate of produced elemental sulfur and influent sulfide throughout the monitoring period

			$S^{0}_{produced}/S^{2-}_{influent}$ (%)			
Reactor	**Sulfide load (gS^{2-}m^{-3}d^{-1})**	**Sulfide removal (%)**	**[a]Sludge**	**[a]Biomass**	**[b]Effluent**	**Total**
R1-control – 6 h	22	78	0.8	0.7	10.1	11.6
R2-packed – 6 h		92	3.1	0.3	43.7	47.1
R1-control – 4 h	40	70	2.5	0.2	12.2	14.9
R2- packed – 4 h		53	2.1	0.3	12.6	15.0
R1-control – 2 h	135	57	0.5	0.0	18.0	18.5
R2- packed – 2 h		70	0.6	0.0	33.3	33.3

[a]Median results obtained for the samples collected at the end of each operational phase.
[b]Results obtained from the median of 10 samples.

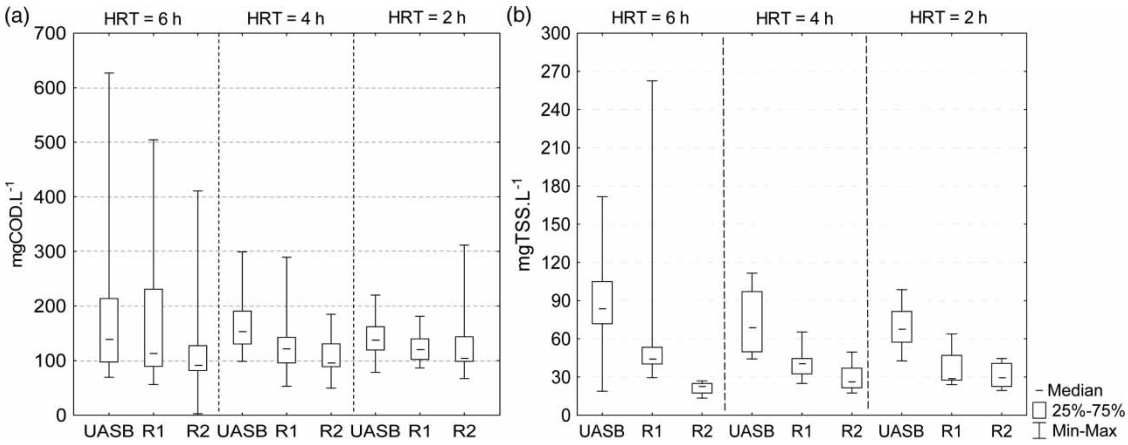

Figure 4 | Concentrations of (a) COD and (b) TSS present in UASB and bioreactor effluents under HRTs of 6 h, 4 h and 2 h.

Considering TSS, significantly lower concentrations (Wilcoxon's matched pairs test, $\alpha = 5\%$) were observed in the R2-packed effluent for HRTs of 6 h and 4 h, 22.8 mg $TSSL^{-1}$ and 26.4 mg $TSSL^{-1}$ respectively, while 40.5 mg $TSSL^{-1}$ and 44.7 mg $TSSL^{-1}$ respectively, were observed in the R1-control effluent. It is assumed that the higher flow associated with a 2 h HRT favoured the loss of biomass attached to the polypropylene rings, and that the presence of packing material under conditions of low HRT (2 h) did not increase the solids retention capacity of the bioreactors, as the difference between the two bioreactors was not significant – about 29 $mgTSSL^{-1}$ for both bioreactors (Wilcoxon's matched pairs test, $\alpha = 5\%$).

Nevertheless, higher COD removal efficiencies and TSS retention were observed in the R2-packed, which was able to produce a final effluent with low COD and TSS concentrations – close to 100 $mgCODL^{-1}$ and 30 $mgTSSL^{-1}$, respectively.

Effect of packing material on bacterial diversity

Bacterial community diversity for biomass and sludge samples was evaluated at the end of each phase by PCR-DGGE, a qualitative technique that, in principle, detects the dominant members of a bacterial community (Van Elsas & Boersma 2011). The comparison of DGGE profiles (Figure 5(a)) was performed by use of the dendrogram (Figure 5(b)), which illustrated the similarity between the profiles. DNA extraction of R2-packed biomass for the HRT of 4 h was not successful, and, for this reason, it was not included in the following stages.

DGGE profiles showed that the bacterial community in the bioreactors varied with different operational conditions. Three major and distinct clusters were identified, according to the tested HRTs (showing only 57–71% similarity). Bacterial profiles were also very different between the reactors, with the exception for the HRT of 6 h, in which biomass samples showed 74.3% similarity. Therefore, the dendrogram indicated possible differentiation, due to the presence of the packing material and HRT reduction.

Biomass and sludge samples taken from R2-packed were more divergent from each other (showing 46% similarity), compared to the samples taken from R1-control at the same HRT of 2 h (showing 71% similarity). The difference between the profiles of the reactors suggests that the presence of the packing material in R2-packed, and absence in R1-control, affected both sludge and biomass bacterial communities. The Shannon diversity index values were slightly different between samples, ranging from 1.05 to 1.29 for samples of R1-control sludge and R2-packed biomass, for HRTs of 6 h and 2 h, respectively. This indicated that microbial diversity did not vary widely between the reactors, or among samples (sludge and biomass), although the dendrogram indicated a possible differentiation as a consequence of the presence of the support material and HRT reduction (as discussed further ahead).

Bacterial community in the bioreactors determined by sequencing

DGGE results showed the presence of a metabolically diverse bacterial community, with sequences related to heterotrophic bacteria, sulfur bacteria, phototrophic non-sulfur

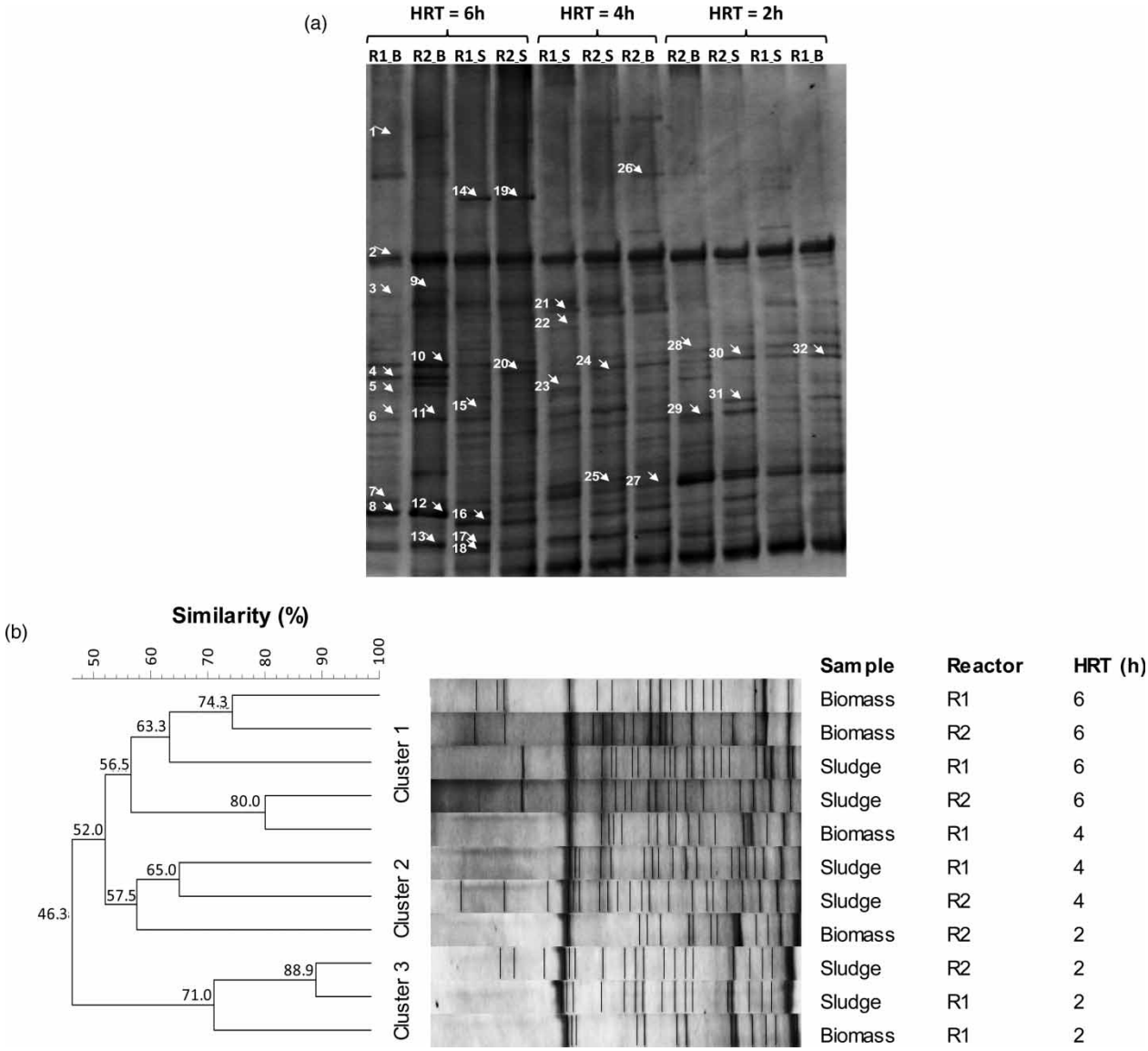

Figure 5 | Bacterial community analysis using DGGE. (a) The DGGE profile of the bacterial community in biomass (B) and sludge (S) sampled at the end of the operational phases from reactors 1 and 2. (b) Dendrogram based on the DGGE profiles of the bacterial community at HRTs of 6 h, 4 h and 2 h.

bacteria and cyanobacteria (Table 4). DNA sequences similar to the *Anaerolineaceae* family from the *Chloroflexi* phylum (probably *Anaerolinea* genus) were identified in the sludge of R2-packed (6 h and 2 h HRT) and R1-control (4 h). This genus consists of fermentative hydrolytic bacteria, responsible for the degradation of carbohydrates and present in anaerobic reactors, with low ammonium concentrations (Si *et al.* 2016).

The purple, non-sulfur bacteria of the genera *Rhodopseudomonas* and *Rhodocyclus* were identified in the biomass of R1-control (6 h HRT) and R2-packed (2 h HRT), and in the sludge of R1-control (4 h HRT) (Table 4). A sequence related to *Rhodopseudomonas palustris,* a phototrophic bacterium with versatile metabolism and capable of organic matter degradation (Madigan *et al.* 2010), was identified in the biomass of R2-packed (2 h HRT). Members of *Rhodopseudomonas* have been reported as being able to oxidize sulfide to elemental sulfur or sulfate (Neutzling *et al.* 1985; Maier 2000), thus suggesting participation of this genus in the sulfide oxidation observed in the reactors.

Many of the DNA sequences detected were related to cyanobacteria (such as *Oscillatoriales*, *Planktothrix mougeotii*, *Limnothrix planktonica, Chroococcales*). DNA

Table 4 | Identification of DGGE profiles from RDP Classifier and Blastn

Bands	RDP Classifier	Blastn	Similarity[a] (%)	Accession number
1	***Campylobacterales***	***Sulfurimonas*** sp.	**89**	**NR_074451.1**
2	*Cyanobacteria*	*Spirulina* sp.	89	NR_125712.1
3	*Oscillatoriophycideae*	*Moorea* sp.	82	NR_116539.1
4	*Rhodocyclus* sp.	*Rhodocyclus tenuis*	98	NR_025839.1
5	***Sulfuricellales***	***Sulfuricella*** sp.	**90**	**NR_121695.1**
6,15	*Oscillatoriales*	*Planktothrix mougeotii*	97	NR_112129.1
7	***Rhodopseudomonas*** sp.	***Rhodopseudomonas*** sp.	**94**	**NR_024971.1**
8	*Chroococcales*	*Microcystis* sp.	92	NR_074314.1
9	*Flavobacteriaceae*	*Chryseobacterium* sp.	95	NR_113951.1
10	*Pseudanabaenaceae*	*Limnothrix* sp.	97	KF246506.1
11	*Oscillatoriales*	*Planktothrix mougeotii*	98	NR_112129.1
12	*Chroococcales*	*Microcystis* sp.	92	NR_074314.1
13	*Pseudanabaenaceae*	*Limnothrix planktonica*	99	KP726241.1
14	*Cyanobacteria*	Uncultured *Cyanobacteria*	91	HQ044431.1
16	*Comamonadaceae*	*Diaphorobacter polyhydroxybutyrativorans*	98	NR_137222.1
17	*Pseudanabaenaceae*	*Limnothrix planktonica*	99	KP726241.1
18	***Chromatiales***	***Thiocapsa*** sp.	**90**	**NR_115810.1**
19	*Cyanobacteria*	Uncultured *Cyanobacteria*	91	HQ044431.1
20, 24, 30, 32	*Anaerolineaceae*	*Anaerolinea* sp.	90	NR_074383.1
21	***Chlorobaculum*** sp.	***Chlorobaculum*** sp.	**95**	**NR_104865.1**
22	*Flavobacteriaceae*	*Chryseobacterium* sp.	96	NR_116343.1
23	*Neisseriales*	*Chromobacterium* sp.	91	NR_136426.1
25	***Rhodopseudomonas***	***Rhodopseudomonas thermotolerans***	**98**	**NR_108528.1**
26	*Cyanobacteria*	Uncultured *Cyanobacteria*	99	HQ041600.1
27	*Syntrophobacterales*	Uncultured *Syntrophorhabdaceae*	92	JX505362.1
28	*Bradyrhizobiaceae*	*Rhodopseudomonas* sp.	94	KX944463.1
29	***Rhodopseudomonas***	***Rhodopseudomonas palustris***	**97**	**NR_112912.1**
31	*Gulbenkiania* sp.	*Gulbenkiania mobilis*	99	NR_042548.1

[a]The percentage represents the similarity between the DGGE profile bands and the most similar sequences in GenBank. Words in bold indicate DNA bands related to sulfur cycle bacteria.

sequences related to *Spirulina* genus, a strong band present in all samples from both reactors, were related to filamentous cyanobacteria. Phototrophic sulfur bacteria and cyanobacteria may coexist, due to sulfur bacteria oxygen tolerance (Stal 1995).

Sequences related to sulfur bacteria of the genera *Thiocapsa, Chlorobaculum, Sulfurimonas* and *Sulfuricella* were identified in the sludge and biomass of R1-control (6 h HRT) (Table 4). Some species within *Sulfuricella* genus can oxidize elemental sulfur and thiosulfate to sulfate as sole energy sources (Kojima & Fukui 2010). Purple sulfur bacteria from *Thiocapsa* genus perform anoxygenic photosynthesis, using H_2S as an electron donor, to produce intracellular elemental sulfur. The identification of purple bacteria and intracellular sulfur (Figure 3(c)), confirmed that sulfur bacteria occurred at different HRTs.

Chlorobaculum, green sulfur bacteria, were identified in the biomass of R1-control for an HRT of 4 h. The main advantage attributed to green bacteria is related to extracellular elemental sulfur formation (Madigan *et al.* 2010). Although the genus *Chlorobaculum* was identified specifically in one band from R1-control, the occurrence of extracellular elemental sulfur in the bioreactors effluent was verified throughout the monitoring period. Once the

evaluated samples showed bands at the same height, and both bioreactors operated under similar conditions (sulfide load and sunlight presence) and purple and green colours were observed throughout the monitoring period (see Figure 3(c)), it could be inferred that phototrophic sulfur bacteria occurred under all the conditions that were tested.

From the DGGE profile (Figure 5(a)), it is possible to observe that some DNA bands were affected by HRT reduction, as they decreased in intensity, or almost disappeared from the gel. It is worth mentioning, in the evaluation of lower HRT, that it not only reduced the available time for biological conversions and facilitated higher biomass release due to the increase of the up flow velocity, but also resulted in increasing organic and sulfide loads, since real anaerobic effluent was used in the present study.

Bands related to *Cyanobacteria* (bands 2, 14, 19), *Sulfurimonas* sp. (band 1), *Sulfuricella* sp. (band 5) and *Rhodocyclus* sp. (band 4) were negatively affected by HRT reduction. Others, such as *Rhodopseudomonas* sp. (band 28) and *Syntrophobacterales* (band 27), increased in intensity, and therefore were positively affected by HRT reduction. Some DGGE bands (20, 24, 30 and 32) related to *Anaerolineaceae* were present in all samples, independent of the HRT.

From the DNA sequences (Table 4) and the DGGE profile (Figure 5(a)), it is possible to observe that longer HRTs favoured the occurrence of different genera of sulfur bacteria. *Sulfurimonas* sp. (band 1) was identified in samples at 6 h HRT, and *Sulfuricella* sp. (band 5) and *Chlorobaculum* sp. were identified in samples at 6 h or 4 h HRT, whilst the only DNA sequence observed under both 6 h and 2 h HRT conditions was *Thiocapsa* sp. In this scenario, it is possible to presume that reducing the HRT (from 6 h to 2 h), and increasing the sulfide load by six times (from 22 to 135 $gSm^{-3}d^{-1}$), resulted in lower bacterial diversity. However, the lower sulfur bacteria diversity did not expressively affect the performance of the bioreactor packed with polypropylene rings, since 70% sulfide removal was observed under these conditions.

Regarding the fermentative hydrolytic bacteria, bands related to *Anaerolineaceae* were present in all samples, and bands related to *Rhodopseudomonas* sp. were present under 4 h HRT conditions – and were more intense at an HRT of 2 h, suggesting that a higher organic load positively affected the occurrence of these bacteria, and the increased sulfide load did not inhibit them. Since the presence of *Rhodopseudomonas* could be associated to the degradation of organic compounds, COD analysis corroborated the assumption that higher organic load favoured these bands, as the removal efficiency did not widely vary with HRT reduction from 6 h to 2 h, or organic load increase (3 times), as shown in Figure 4(a).

H_2S may cause inhibition of microorganism metabolism – for instance, the sulfide concentrations reported in the literature for inhibition of methanogens varied from 50 to 250 mgH_2SL^{-1} at pH 7 and 8 (Oleskiewicz *et al.* 1989; O'Flaherty *et al.* 1998). It is possible to assume, however, that the sulfide loading rate applied in the present study (22 $gS^{2-}m^{-3}d^{-1}$ at HRT of 6 h and 135 $gS^{2-}m^{-3}d^{-1}$ at HRT of 2 h loads) and sulfide concentrations (which varied from 5 to 11 $mgS^{2-}L^{-1}$) did not inhibit bacteria associated with organic matter degradation nor biological sulfide oxidation, since DNA sequences similar to those of sulfur and fermentative hydrolytic bacteria were detected in the bioreactors. Moreover, several studies have demonstrated the occurrence of biological oxidation under conditions of high loads of sulfide (Krishnakumar *et al.* 2005; Lohwacharin & Annachhatre 2010; Liu *et al.* 2015).

Since some Cyanobacteria can perform anoxygenic photosynthesis by oxidizing sulfide to sulfur at sulfide concentrations above 0.2 mM (Cohen *et al.* 1986; Friedrich 1998), and since bands associated to cyanobacteria were observed in all monitored phases, it is possible to assume that the occurrence of cyanobacteria – even under low HRT conditions – may be associated with the higher sulfide load.

In summary, bacterial diversity revealed by DGGE suggested that phototrophic purple and green sulfur bacteria and sulfur-oxidizing bacteria, combined with cyanobacteria, might be involved in the biological sulfide oxidation observed in the reactors. In addition, the results showed that the bacterial community was affected by HRT reduction and, consequently, sulfide load increase, suggesting that these parameters played a key role in altering the dynamic of the bacterial community, and in shaping its structure.

CONCLUSIONS

The results obtained in this study demonstrated that the bioreactors were able to improve the quality of the effluent from the UASB reactor, in terms of suspended solids removal/retention, COD removal and biological sulfide oxidation. Higher efficiencies were observed for the packed reactor, likely due to the presence of the packing material and higher cell retention time. Although greater elemental sulfur production was obtained for the lowest HRT (2 h), higher sulfide removal efficiencies were obtained for the

HRT of 6 h (R1-control: 78% and R2-packed: 92%), possibly because greater diversity of sulfur bacteria genera was detected at 6 h HRT. Higher COD removal efficiencies and TSS retention were observed in the R2-packed reactor, which was able to produce a final effluent with low COD and TSS concentrations of 100 $mgCODL^{-1}$ and 30 $mgTSSL^{-1}$, respectively. Elemental sulfur formation and the potential for sulfur recovery were observed, especially in the effluent of the bioreactors, demonstrating the feasibility of applying the bioreactors as a post-treatment alternative for sulfide oxidation.

Further studies are necessary in order to facilitate the scale up of this technology – in terms of the necessary light requirements and optimization of elemental sulfur formation and recovery methods – all with the purpose of improving the sustainability of sewage treatment plants.

Bacterial communities in the bioreactors were diverse, and changed in response to the HRTs and to the organic and sulfide loads. Sulfur bacteria were detected and identified, demonstrating the occurrence of biological sulfide oxidation in the reactors.

ACKNOWLEDGEMENTS

The authors would like to acknowledge the support obtained from the following Brazilian institutions: Conselho Nacional de Desenvolvimento Científico e Tecnológico – CNPq; Coordenação de Aperfeiçoamento de Pessoal de Nível Superior – CAPES; Financiadora de Estudos e Projetos – FINEP; Fundação de Amparo à Pesquisa do Estado de Minas Gerais – FAPEMIG; Instituto Nacional de Ciência e Tecnologia em Estações Sustentáveis de Tratamento de Esgoto – INCT ETEs Sustentáveis (INCT Sustainable Sewage Treatment Plants).

We also acknowledge the support from the sanitation company of Minas Gerais – COPASA.

REFERENCES

APHA 2012 *Standard Methods for the Examination of Water and Wastewater*, 22nd edn. American Public Health Association, Washington, DC, USA, 1496 p.

Beristain-Cardoso, R., Gómez, J. & Méndez-Pampín, R. 2011 Sulfide and ammonium oxidation, acetate mineralization by denitrification in a multipurpose UASB reactor. *Bioresource Technology* **102**, 2549–2554.

Chernicharo, C. A. L. 2007 *Biological Wastewater Treatment Series – Volume Four: Anaerobic Reactors*. IWA Publishing, London, UK, 188 p.

Chernicharo, C. A. L., Van Lier, J. B., Noyola, A. & Ribeiro, T. B. 2015 Anaerobic sewage treatment: state of the art, constraints and challenges. *Environ. Sci. Biotechnol.* **14**, 649–679.

Cohen, Y., Jorgensen, B. B., Revsbech, N. P. & Poplawski, R. 1986 Adaptation to hydrogen sulfide of oxygenic and anoxygenic photosynthesis among Cyanobacteria. *Appl. Environ. Microbiol.* **51**, 398–407.

Fajardo, C., Corral-mosquera, A., Campos, J. L. & Méndez, R. 2012 Autotrophic denitrification with sulphide in a sequencing batch reactor. *Journal of Environmental Management* **113**, 552–556.

Ferris, M. J., Muyzer, G. & Ward, D. M. 1996 Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl. Environ. Microb.* **62**, 340–346.

Friedrich, C. G. 1998 Physiology and genetics of sulfur-oxidizing bacteria. *Adv. Microb. Physiol.* **39**, 235–289.

Gafan, G. P., Lucas, V. S., Roberts, G. J., Petrie, A., Wilson, M. & Spratt, D. A. 2005 Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *J. Clin. Microbiol.* **43**, 3972–3978.

Garcia, G. P. P., Diniz, R. C. O., Bicalho, S. K., Franco, V. A., Gontijo, E. M. O., Toscano, R. A., Canhestro, K. O., Santos, M., Carmo, A. L., Lobato, L. C., Brandt, E. M., Chernicharo, C. A. L. & Araujo, J. C. 2015 Biological sulphide removal from anaerobically treated domestic sewage: reactor performance and microbial community dynamics. *Environ. Technol.* **36**, 1–26.

Garcia, G. P. P., Diniz, R. C. O., Bicalho, S. K., Franco, V., Pereira, A. D., Brandt, E. F., Etchebehere, C., Chernicharo, C. A. L. & Araujo, J. C. 2017 Microbial community and sulphur behaviour in phototrophic reactors treating UASB effluent under different operational conditions. *International Biodeterioration and Biodegradation* **119**, 486–498.

Garcia de Lomas, J., Corzo, A., Gonzalez, J. M., Andrades, J. A., Iglesias, E. & Montero, M. J. 2005 Nitrate promotes biological oxidation of sulfide in wastewater: experiment at plant-scale. *Biotechnology and Bioengineering* **93** (4), 801–811.

Henshaw, P. F., Bewtra, J. K. & Biswas, N. 1998 Hydrogen sulphide conversion to elemental sulphur in a suspended-growth continuous stirred tank reactor using *Chlorobium limicola*. *Water Research* **32** (6), 1769–1778.

Janssen, A. J. H., Lettinga, G. & Keiser, A. 1999 Removal of hydrogen sulfide from wastewater and waste gases by biological conversion to elemental sulfur. Colloidal and interfacial aspects of biologically produced sulphur particles. *Colloids and Surfaces A: Physicochemical and Engineering Aspects* **151**, 389–397.

Klok, J. B. M., Van Den Bosch, P. L. F., Buisman, C. J., Stams, A. J. M., Keesman, K. J. & Janssen, A. J. H. 2012 Pathways of sulfide oxidation by haloalkaliphilic bacteria in limited-oxygen gas lift bioreactors. *Environmental Science and Technology* **46**, 7581–7586.

Kojima, H. & Fukui, M. 2010 *Sulfuricella denitrificans* gen. nov., sp. nov., a sulfur-oxidizing autotroph isolated from a freshwater lake. *Int. J. Syst. Evol. Microbiol.* **60**, 2862–2866.

Krishnakumar, B., Majumdar, S., Manilal, V. B. & Haridas, A. 2005 Treatment of sulphide containing wastewater with sulphur recovery in a novel reverse fluidized loop reactor (RFLR). *Water Research* **39**, 639–647.

Liu, C., Zhao, D., Yan, L., Wang, A., Gu, Y. & Lee, D. 2015 Elemental sulfur formation and nitrogen removal from wastewaters by autotrophic denitrifiers and anammox bacteria. *Bioresource Technology* **191**, 332–336.

Lohwacharin, J. & Annachhatre, A. P. 2010 Biological sulfide oxidation in an airlift bioreactor. *Bioresource Technology* **101**, 2114–2120.

Madigan, M. T., Martinko, J. M. & Parker, J. 2010 *Brock Biology of Microorganisms*, 13th edn. Benjamin Cummings/Pearson Education, San Francisco, CA, USA, 1155 p.

Maier, R. M. 2000 Sulfur cycle. In: *Environmental Microbiology* (R. M. Maier, I. L. Pepper & C. P. Gerba, eds). Academic Press, San Diego, CA, USA, pp. 319–345.

Neutzling, O., Pfleider, C. & Truper, H. G. 1985 Dissimilatory sulphur metabolism in phototrophic 'non-sulphur' bacteria. *Microbiology* **131**, 791–798.

O'Flaherty, V., Mahony, T., O'Kennedy, R. & Colleran, E. 1998 Effect of pH on growth kinetics and sulphide toxicity thresholds of a range of methanogenic, syntrophic and sulphate-reducing bacteria. *Process Biochem.* **33**, 555–569.

Oleskiewicz, J. A., Marstaller, T. & McCartney, D. M. 1989 Effects of pH on sulphide toxicity to anaerobic processes. *Environ. Technol. Lett.* **10**, 815–822.

Plas, C., Harant, H., Danner, H., Jelinek, E., Wimmer, K., Holubar, P. & Braun, R. 1992 Ratio of biological and chemical oxidation during the aerobic elimination of sulphide by colourless sulphur bacteria. *Applied Microbiology and Biotechnology* **36** (6), 817–822.

Reyes-Avila, J., Razo-Flores, E. & Gomez, J. 2004 Simultaneous biological removal of nitrogen, carbon and sulfur by denitrification. *Water Research* **38**, 3313–3321.

Rickard, D. & Luther, G. W. 2007 Chemistry of iron sulfides. *Chem. Rev.* **107**, 514–562.

Si, B., Liu, Z., Zhang, Y., Li, J., Shen, R., Zhu, Z. & Xing, X. 2016 Towards biohythane production from biomass: influence of operational stage on anaerobic fermentation and microbial community. *International Journal of Hydrogen Energ.* **41** (7), 4429–4438.

Souza, C. L., Chernicharo, C. A. L. & Melo, G. C. B. 2012 Methane and hydrogen sulfide emissions in UASB reactors treating domestic wastewater. *Water Science & Technology* **65** (7), 1229–1237.

Speece, R. E. 1996 *Anaerobic Biotechnology for Industrial Wastewaters*. Archae Press, Nashville, 416 p.

Stal, L. J. 1995 Physiological ecology of cyanobacteria in microbial mats and other communities. *New Phytol.* **131**, 1–32.

United States Environmental Protection Agency 2010 *Acute Exposure Guideline Levels for Selected Airborne Chemicals*. Washington, DC, USA.

Van Elsas, J. D. & Boersma, F. G. H. 2011 A review of molecular methods to study the microbiota of soil and the mycosphere. *European Journal of Soil Biology* **47**, 77–87.

Vannini, C., Munz, G., Mori, G., Lubello, C., Verni, F. & Petroni, G. 2008 Sulphide oxidation to elemental sulphur in a membrane bioreactor: performance and characterization of the selected microbial sulphur- oxidizing community. *Systematic and Applied Microbiology* **31**, 461–473.

First received 23 April 2018; accepted in revised form 4 September 2018. Available online 17 September 2018