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Metagenomic analysis of a desulphurisation system used to treat biogas from vinasse methanisation

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HIGHLIGHTS

- A desulphurisation system treating biogas with high amounts of H_2S was studied.
- Metagenomic analysis of the biomass from the desulphurisation system was performed.
- The system showed a high H_2S RE; COD and DO were important parameters.
- Microbial community remained constant during the experiment.
- The abundance and diversity of SOB were affected by the sulphide loading rate.

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ABSTRACT

We investigated the response of microbial community to changes in H_2S loading rate in a microaerated desulphurisation system treating biogas from vinasse methanisation. H_2S removal efficiency was high, and both COD and DO seemed to be important parameters to biomass activity. DGGE analysis retrieved sequences of sulphide-oxidising bacteria (SOB), such as *Thioalkalimicrobium* sp. Deep sequencing analysis revealed that the microbial community was complex and remained constant throughout the experiment. Most sequences belonged to Firmicutes and Proteobacteria, and, to a lesser extent, Bacteroidetes, Chloroflexi, and Synergistetes. Despite the high sulphide removal efficiency, the abundance of the taxa of SOB was low, and was negatively affected by the high sulphide loading rate.

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1. Introduction

Vinasse is a by-product generated in large amounts in the process of ethanol production from sugarcane, approximately 13 $L_{vinasse}/L_{ethanol}$, on average (Salomon and Silva Lora, 2009). This value corresponds to 286 billion litres of vinasse only in Brazil's 2011/2012 season (MAPA, 2013). Vinasse has been widely applied to farming lands as fertilizer; however, this practice can pollute soil and water. An interesting alternative to the treatment of vinasse is anaerobic digestion, which allows the recovery of part of its energy content owing to the production of biogas. However, hydrogen sulphide (H_2S) is produced in significant amounts, reaching concentrations of approximately 30% v/v (Colturato, 2015). H_2S is a toxic and malodorous gas, which can inhibit microbial activity and lead to corrosion of biogas production structures (Krayzelova et al., 2014).

As alternative methods for removing H_2S from biogas (desulphurisation), there are physicochemical and biological processes. Currently, physicochemical processes are the most widely used; however, biological processes, based on the oxidation of H_2S by sulphide-oxidising bacteria (SOB), have some advantages, such as minimal requirement of chemical reagents and cost reduction

Abbreviations: BRT, Biological Regeneration Tower; COD, chemical oxygen demand; DGGE, denaturing gradient gel electrophoresis; DO, dissolved oxygen; ORP, oxidation–reduction potential; OTU, Operational Taxonomic Unit; RE_{H_2S}, H_2S removal efficiency; SOB, sulphide-oxidising bacteria; VAT, Venturi-like Absorption Tower; VLR_{H_2S}, H_2S volumetric loading rate.

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(Mannucci et al., 2012; Klok et al., 2012). Biological removal of sulphide in microaerobic conditions has been shown to be a promising alternative because of the high H_2S removal efficiency (RE_{H_2S}) and operational simplicity (Ramos et al., 2013). The process consists of a controlled introduction of atmospheric air or oxygen into a predominantly anaerobic system in order to promote the oxidation of H_2S to elemental sulphur or sulphate (Botheju and Bakke, 2011).

In this paper, we describe a desulphurisation system comprising a physicochemical unit as well as a biological reactor. The process is based on the absorption of H_2S in an alkaline solution in the first unit and the regeneration of hydroxyl ions in the second. These ions are important for maintaining the alkalinity necessary for H_2S absorption in the first step, and can be generated by biological sulphide oxidation (Klok et al., 2012). Since the biomass is crucial for the efficiency and maintenance of the process, the aim of this study was to investigate the microbial community composition and diversity in this system by denaturing gradient gel electrophoresis (DGGE) and deep sequencing metagenome analysis under different H_2S loading rates. To the best of our knowledge, metagenomic analysis of desulphurisation systems used for biogas treatment under high concentrations of H_2S has not been previously performed.

2. Methods

This study was conducted at a sugarcane mill in the City of Monte Belo, Minas Gerais State (Brazil), where a pilot-scale vinasse methanisation reactor coupled to the desulphurisation system was operated.

2.1. Apparatus description and monitoring

Part of the vinasse produced at a distillery plant ($4\ m^3\ d^{-1}$, on average), was treated in a pilot-scale anaerobic digester which had been in operation for nearly four years. The digester was developed by the Methanum Waste and Energy Company (Nova Lima, Minas Gerais, Brazil) and comprised of an $8.5\ m^3$ hybrid reactor operating with a mean organic loading rate of $15\ kg\ DQO\ m^{-3}\ d^{-1}$. The biogas produced in the digester, containing high amounts of H_2S, was stored in a gas container, and then directed to the desulphurisation system. Liquid effluent from the digester was also stored in a reservoir prior to final disposal. The desulphurisation system (Fig. 1) consisted of two units, a Venturi-like Absorption Tower (VAT) and a Biological Regeneration Tower (BRT). The first was manufactured in stainless steel and had a reaction volume of 1.2 L. The biogas and liquid phase of the system, taken from the digester effluent reservoir, were introduced through different points at the top of the VAT in a co-current flow. The Venturi channels generate a turbulence zone that optimizes the gas–liquid contact, allowing solubilisation and consequent removal of H_2S from biogas, which leaves the VAT desulphurised.

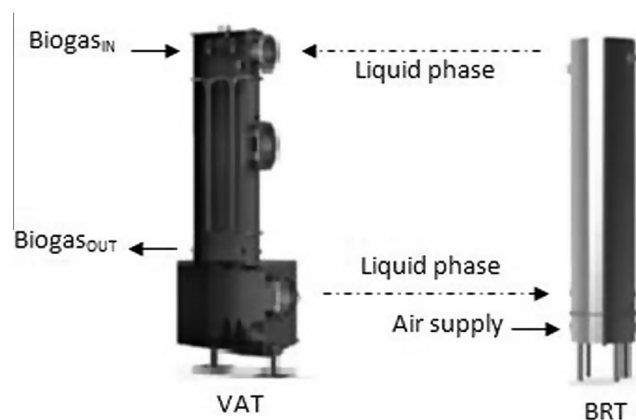

Fig. 1. Schematic configuration of the desulphurisation system. VAT: Venturi-like Absorption Tower; BRT: Biological Regeneration Tower.

BRT, also manufactured in stainless steel, had a working volume of ~96 L, and was packed with a support medium made of 0.1 mm-thick and ~1 m-long nylon strings (blue in colour), to facilitate the retention and continuous contact of the biomass with the liquid phase. The colour of packing material in the BRT after 15 days of inoculation and biomass retention was gray. The liquid effluent from the VAT was directed to the BRT, to promote the regeneration of the hydroxyl radicals consumed in the previous step. This was achieved via oxidation of hydrogen bisulphide, which originated from H_2S solubilisation. Oxygen supply (air) and liquid entry were carried out through points located at the bottom of the reactor.

Gas flow was measured by using a gas metre (LAO Industry, G 1.6 and G 2.5). Temperature and CH_4, CO_2, H_2S and O_2 concentrations were monitored by using a gas analyzer (Landtec GEM™ 5000 Plus) for the desulphurisation system influent biogas and a Landtec GA™3000 for the effluent biogas. The H_2S volumetric loading rate (VLR_{H_2S}) was calculated based on the influent and effluent H_2S concentration, biogas flow rate, and the VAT reaction volume.

With respect to the liquid phase, temperature (maintained at ~30 °C), pH, oxidation–reduction potential (ORP) (combined transducers Endress+Hauser, CPS16D-1009/0) and dissolved oxygen (DO) (Endress+Hauser, COS61D-1077/0) were monitored at the BRT. Colloidal sulphur (S), chemical oxygen demand (COD), hydrogen bisulphide (HS^-), and sulphate (SO_4^{2-}) were measured from samples of the recirculation liquid at the BRT exit. The procedures comprised an adaptation of the AOAC 980.02 method for determining S, the use of reagents potassium dichromate for COD (CAT-21259.25, Hach), SulfaVer (CAT-21067-69, Hach) for SO_4^{2-}, and Sulfite 1 and 2 (CAT-1816-32/1817-32, Hach) for HS^-; all measured using a portable spectrophotometer (DR800, Hach). All transducers were coupled to an automation and monitoring system, which allowed data storage for later analysis.

2.2. Experimental design

This study was divided in six experimental phases, as summarised in Table 1. Before inoculation, biomass from the digester effluent reservoir (from where the inoculum was collected) was subjected to an acclimatisation stage as follows: diffused air was injected in the reservoir ($2\ m^3$ volume) for 15 days at a rate of $4.5\ m^{-3}\ h^{-1}$. Daily pulses of $15\ kg\ H_2S\ d^{-1}$ were applied for a period of 10 days. After this procedure, the biomass was transferred to the BRT.

1. *Start-up*: BRT was subjected to a controlled injection of air ($46.7\ m^3\ m^{-3}\ h^{-1}$) and liquid recirculation flow of $1\ m^3\ h^{-1}$. An initial VLR_{H_2S} of $3.4\ kg\ H_2S\ m^{-3}\ h^{-1}$ was applied and it was maintained until the system presented a H_2S removal efficiency (RE_{H_2S}) of 99%.
2. *Load increase*: VLR_{H_2S} progressively increased until a maximum of $14.6\ kg\ H_2S\ m^{-3}\ h^{-1}$. However, a decrease in RE_{H_2S} was observed, and the load rise was interrupted.
3. *Stationary period 1*: VLR_{H_2S} was decreased to restore RE_{H_2S} and maintained at $11.8\ kg\ H_2S\ m^{-3}\ h^{-1}$, on average.
4. *Shock loading:* To evaluate the robustness and resilience of the system, a shock loading of $31.2\ kg\ H_2S\ m^{-3}\ h^{-1}$ (mean value) was applied for two days.

Table 1
Mean values of physicochemical parameters and experimental phases in the desulphurisation system.

Experimental phase/parameter	Duration (days)	H_2S load ($kg\ m^{-3}\ h^{-1}$)	HS^- ($mg\ L^{-1}$)	SO_4^{2-} ($mg\ L^{-1}$)	COD ($mg\ L^{-1}$)	pH (BRT)	OD ($mg\ L^{-1}$)
(1) Start-up	0–4	3.4	1.0	52.0	3938	7.9	2.4
(2) Load Increase	5–12	9.9	3.6	383.0	3556	7.8	1.6
(3) Stationary period 1	13–56	11.8	2.7	1319.9	3610	7.5	2.5
(4) Shock loading	57 and 58	31.2	3.3	1775.0	4150	7.6	0.17
(5) Stabilisation	59–94	8.0	4.3	1954.7	4523	7.4	0.74
(6) Stationary period 2	95–105	12.5	0.7	2579.1	1951	7.2	1.42

Table 2
Identification of biomass samples.

Identification	Source	Experimental phase
INOC	Anaerobic digester effluent reservoir	Prior to the experiment
DIG	Anaerobic digester	Prior to inoculation
DISP-3	Dispersed biomass from the recirculation line	3 (stationary period 1)
ATTA-3	Attached biomass from the BRT	3 (stationary period 1)
DISP-4	Dispersed biomass from the recirculation line	4 (24 h after shock)
ATTA-4	Attached biomass from the BRT	4 (24 h after shock)
DISP-5	Dispersed biomass from the recirculation line	5 (stabilisation)
ATTA-5	Attached biomass from the BRT	5 (stabilisation)

5. *Stabilisation:* VLR_{H_2S} was reduced and then progressively increased in order to re-establish the stability conditions obtained in stationary period 1.
6. *Stationary period 2:* An average load of 12.5 kg $H_2S\ m^{-3}\ h^{-1}$ was maintained for a period of 10 days.

2.3. Sampling

During the experiment, six samples from the desulphurisation system were collected, from both biomass dispersed on the recirculating line at the BRT exit (in 500 mL duplicates) and biomass attached to nylon strings inside the equipment (in ~15 cm nylon strings triplicates). Samples from the anaerobic digester (2 L composed sample) and the effluent reservoir from where the inoculum was taken (500 mL in triplicates), were also analysed, totalling 8 samples, as shown in Table 2.

After transporting under refrigerated conditions, samples were transferred to 50 mL Falcon tubes, centrifuged (3.500 rpm, for 10 min), re-suspended in phosphate buffer saline (PBS) solution (1:10 dilution of a 70 mM Na_2HPO_4, 34 mM NaH_2PO_4, 1300 mM NaCl, pH 7.2), homogenized, centrifuged again and stored at −20 °C until use. Total DNA was extracted from samples with PowerSoil® DNA Isolation Kit (MOBIO Laboratories, USA) and quantified (Qubit, LifeTechnologies). DNA purity was measured in a spectrophotometer (Nanodrop 1000, Thermoscientific).

2.4. Denaturing gradient gel electrophoresis (DGGE)

DGGE was used to perform a preliminary analysis of the microbial community profile and to monitor the various experimental stages, as previously described. In order to prepare the extracted DNA for DGGE, a PCR was performed with universal primers for the V8 region of the 16S rRNA gene (1055F/1392R-GC), according to the methods of Ferris et al., 1996. PCR products from duplicates and triplicates of each sample were pooled together and subjected to electrophoresis on 2% agarose. Quantification of PCR products was performed using the software ImageJ (ThermoScientific). DNA samples (400 ng) from each pool were used for DGGE (Universal Mutation Dcode, Bio-Rad Laboratories) in an 8% polyacrylamide gel with 30–70% denaturing gradient for 17 h at 80 V.

The gel was stained with SybrGold (LifeTechnologies) and analysed with the BioNumerics 7.1 software (Applied Maths). Band profiles were compared using Dice similarity coefficient and the dendrogram was generated with UPGMA method, with 1% position tolerance.

Bands were excised, eluted in 50 μL of ultrapure water and incubated at 4 °C overnight. DNA was then re-amplified with the same primer pair (without GC-clamp) as described above. PCR products were purified (Wizard® SV Gel and PCR Clean-Up System, Promega) and quantified as described for the first PCR. Sequencing reactions were performed by Macrogen Inc. in a 3730XL sequencer. Sequences were then analysed in Geneious 8.04 software (Biomatters Ltd.) and compared to Ribosomal Database Project (RDP) and NCBI databases with RDP Classifier and BLASTn tools.

2.5. Deep sequencing metagenome analysis

To investigate the microbial diversity present in the desulphurisation system in a more comprehensive and detailed manner, a deep sequence metagenome analysis was carried out. The DNA extracted from duplicates and triplicates of each sample (six samples from the desulphurisation system) were pooled together and sent to Macrogen Inc. for library construction and sequencing on the MiSeq platform (Illumina).

The primers used in the amplification reaction were 515F (GTGNCAGCMGCCGCGGTAA) and 926R (CCGYCAATTYMTTTRAGTTT), which delimit the regions V4 and most of the V5 of the 16S rRNA gene of archaea and bacteria (Quince et al., 2011).

Bioinformatic analysis was carried out with the QIIME software package (Quantitative Insights Into Microbial Ecology) to process the libraries using a clustering strategy to which taxonomic classification was added (Caporaso et al., 2010). First, fastq sequence data were demultiplexed, and barcodes and primers were removed. A minimum average quality Phred score of 20 was set. UCHIME was used for error correction and chimera checking. 16S rRNA gene identification was performed by an Operational Taxonomic Unit (OTU) *de novo* picking approach. Sequence clusters were created via UCLUST (identity of 97%). Post-processing of the OTUs was performed by picking the optimal representative sequence centroid. Taxonomical assignment was performed against the Greengenes database (core reference alignment). A sub-sampling taxonomic classification by the lowest common ancestor was obtained using MEGAN (Huson et al., 2007). Simpson's reciprocal index and Shannon–Weaver diversity indices were calculated. Distances between samples were computed based on Bray–Curtis ecological index. Principal Coordinate Analysis (PCoA)

and Hierarchical Clustering (UPGMA tree) were performed using the MEGAN distance matrix. Raw sequences were deposited in the NCBI database (project accession number PRJNA293416).

3. Results and discussion

3.1. Physicochemical analysis

The mean values of physicochemical parameters measured at the desulphurisation system are summarised in Table 1. To exclude the possibility of chemical oxidation of H_2S, biogas was injected in the system prior to inoculation. No significant sulphide oxidation activity was observed in the absence of biomass (data not shown). The average H_2S concentration in the VAT influent biogas corresponded to approximately 22,000 ppmv, which is considerably higher than the frequently reported values of 10–10,000 ppmv (Abatzoglou and Boivin, 2009). Furthermore, the system presented a RE_{H_2S} greater than 99% for most of the experiment, as shown in Fig. 2.

At the start-up (phase 1), initiated at day 0, a mean VLR_{H_2S} of 3.4 kg H_2S m^{-3} h^{-1} was applied, and a RE_{H_2S} ranging from 88.5% to 99.6% was observed. The high sulphide RE_{H_2S} observed already in this step can be explained by the fact that the biomass used as inoculum was acclimatised prior to inoculation, as described previously.

During load increase (phase 2), VLR_{H_2S} was gradually increased to 14.6 kg H_2S m^{-3} h^{-1}. As RE_{H_2S} decreased, VLR_{H_2S} was set at 11.8 kg H_2S m^{-3} h^{-1} (mean value) in stationary period 1 (phase 3), when the RE_{H_2S} was 99.2% on average.

Subsequently, in shock loading (phase 4), a pronounced VLR_{H_2S} increase was induced to test the robustness and resilience of the system. The load of H_2S was increased to approximately 270% relative to the previous period, reaching a maximum of 35 kg H_2S m^{-3} h^{-1} and 31.2 kg H_2S m^{-3} h^{-1} on average. At this phase, RE_{H_2S} dropped to 77% and returned to stability (above 99%) only after day 80.

From Fig. 3, it can be observed that the shock loading (phase 4) was followed by an increase in S, HS^- and COD. The first third of the stabilisation (phase 5) was characterised by oscillations in the RE_{H_2S} and sulphide build-up, even after the VLR_{H_2S} was reduced. A pronounced increase in HS^- concentration (maximum of 20.5 mg L^{-1}) was observed between days 61 and 70. Since high concentrations of sulphide can be toxic to microorganisms, dilution of the recirculation liquid, with water only, was performed on day 71. HS^- concentration dropped to 3 mg L^{-1} approximately, and 10 days later, RE_{H_2S} was restored.

The COD at the BRT exit during shock loading (phase 4) and the first third of stabilisation (until day 70) ranged from 4100 to 8500 mg L^{-1} (Fig. 3). This significant increase in COD, apparently due to a reduction in biomass activity and system imbalance, was concomitant to a decrease in DO (from 2.5 mg L^{-1} on average to nearly 0), since organic matter oxidation probably caused consumption of all the available O_2. It is possible that these conditions (COD increase and DO reduction) have favoured the development of heterotrophic anaerobic microorganisms, capable of surviving in a sulphide-rich environment. When the stability was restored, DO was higher than 1 mg L^{-1}, and this value was fixed as the lower threshold for DO in the BRT effluent, in order to avoid deficit and to maintain the sulphide oxidation efficiency.

At the final third of the stabilisation (phase 5) and during the stationary period 2 (phase 6), the VLR_{H_2S} was gradually increased and finally stabilised at 12.5 kg H_2S m^{-3} h^{-1}, on average. With this VLR_{H_2S}, which was slightly higher than that applied during the stationary period 1 (phase 3), it was possible to maintain a RE_{H_2S} of 99.4–99.7%.

From Fig. 3, it can be observed that after day 71, HS^- decrease was concomitant to SO_4^{2-} production. Some bacteria may also oxidize sulphide to colloidal sulphur (S), which can be recovered and used in different industrial processes. However, except for the initial third of stabilisation, when an increase was observed, the mean value of S concentration remained low, and it was not possible to retrieve it.

3.2. Microbial diversity

Sequencing on the MiSeq platform generated an average of 1,036,268 reads per sample. After processing, this number decreased to approximately 472,476, with an average length of

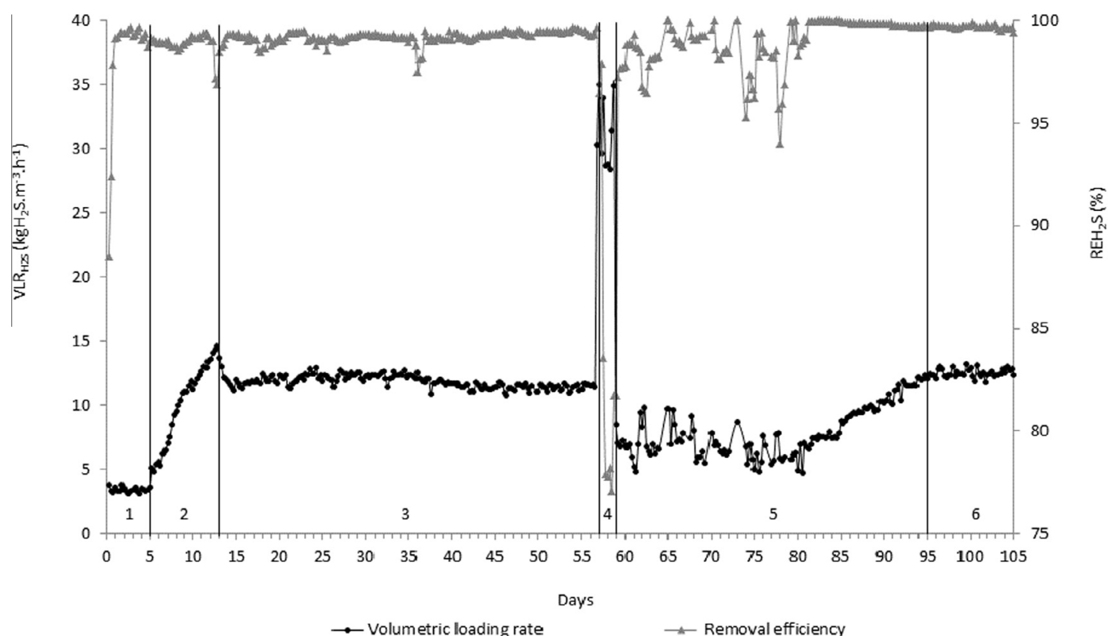

Fig. 2. H_2S volumetric loading rate × H_2S removal efficiency. The numbers in the graph area indicate the corresponding experimental phase. 1: start-up, 2: load increase, 3: stationary period 1, 4: shock loading, 5: stabilisation, 6: stationary period 2.

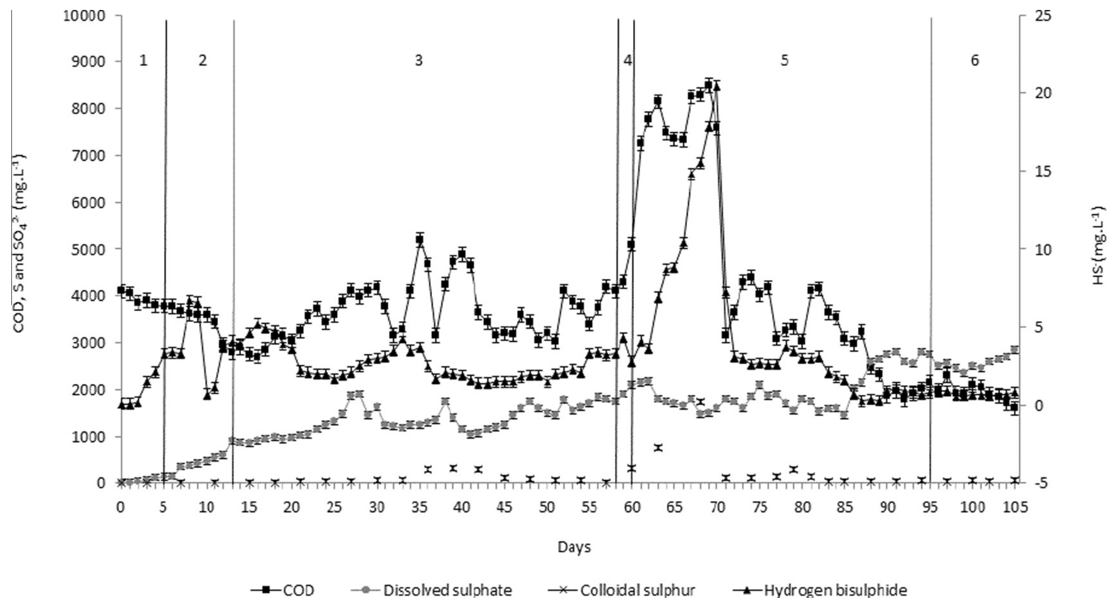

Fig. 3. Concentrations of SO_4^{2-}, HS^-, S, and COD (with standard error bars) on the recirculation line at BRT exit over time. The numbers in the graph area indicate the corresponding experimental phase. 1: start-up, 2: load increase, 3: stationary period 1, 4: shock loading, 5: stabilisation, 6: stationary period 2.

Table 3
Alpha-diversity estimators and number of OTUs from MiSeq data analysis.

Sample	Shannon–Weaver	Simpson's	Number of OTUs
INOC	4.751	13.467	8295
DIG	4.840	14.270	7625
DISP-3	4.578	12.907	8336
ATTA-3	4.928	13.987	9319
DISP-4	4.923	13.953	9198
ATTA-4	5.059	14.294	9280
DISP-5	5.107	14.440	9317
ATTA-5	4.976	13.978	6760

240 bp. Table 3 shows the values of Shannon–Weaver and Simpson's reciprocal index, as well as the number of OTUs identified in each sample. Once the diversity estimators based on DGGE band profile could be greatly influenced by non-ideal migration of bands, the indices calculated from deep sequencing results were considered ideal for diversity analysis.

Both indices (Shannon–Weaver and Simpson's) indicated that the microbial community in the system was diverse, and that it did not vary considerably between the experimental phases. Some fluctuation below the significance level was observed (Kruskal–Wallis test was performed to compare the diversity indices between samples with 5% of significance on R platform).

DGGE sequenced bands and dendrogram are shown in Fig. 4. Two general clusters can be observed, which share 72.7% similarity. The lower part of the dendrogram consists of samples ATTA-5 and DISP-5, and apparently, there was more divergence of these as compared to the samples prior to shock loading. In addition, samples of dispersed and attached biomass in each experimental phase had high similarity with each other (above 80%). This result was expected, since the packing material is in contact with the recirculating liquid, despite the possible existence of specific groups in each site. In this way, according to DGGE, the microbial community did not appear to have varied widely between phases, although the dendrogram indicates a possible differentiation as a consequence of shock loading. These results are partially in accordance with sequencing analysis, as shown in Fig. 5. Samples INOC and DIG were closer to each other, whereas the other samples from BRT showed higher similarity among themselves. The exception is ATTA-5 (stabilisation), which was more distant from the rest of the BRT samples, including DISP-5, collected at the same time. It is possible that the high amount of sulphide applied during the shock loading, followed by an increase in COD and a reduction in DO, have influenced on the microbial community dynamics (favouring some groups over others, for instance, Firmicutes increased in abundance, as shown in Fig. 6), which is reflected in the deep sequencing results as a larger distance between ATTA-5 and the other samples. The higher proximity of DISP-5 with the others could be attributable to the fact that DNA from dead cells remained for some time in the recirculation line and was present in higher concentration at the time of sampling.

3.3. Predominant microbial populations

Sequences from DGGE bands (Fig. 4) were compared with NCBI and RDP databases; the results are summarised in Table 4. There was a significant presence of Proteobacteria (6 from 7 sequences), specifically Gammaproteobacteria. This class includes many well-studied sulphide oxidizers such as *Halothiobacillus* and *Chromatium*. In addition, there were sequences classified as Betaproteobacteria and Deltaproteobacteria, and also as Chloroflexi. Bands 3 and 5, both belonging to the genus *Thioalkalimicrobium* (Gammaproteobacteria), represent organisms that could play an important role in the system since they are bacteria capable of oxidising sulphide and thiosulphate (Sorokin et al., 2001). The correspondent bands were detected in all samples, which may indicate the constant presence of these bacteria, even after the shock loading.

With respect to deep sequencing data, the phylum Euryarchaeota (Archaea) corresponded to 2.4% of the total number of sequences (this value is an average of the relative abundances of the taxon in all samples). This phylum is composed of the methanogenic prokaryotes commonly found in anaerobic systems. As expected, archaea species were present in the digester sample (7.7%), but also in a small proportion in the other samples. However, in the stabilisation (phase 5), the relative abundance of this group was the lowest (below 0.5%), possibly indicating that shock loading led to the suppression of these microorganisms.

The major phyla of bacteria were Firmicutes (53.9%), Proteobacteria (13.5%), Bacteroidetes (7.5%), Chloroflexi (4.1%), and Synergistetes (4%). Together, these five phyla accounted for approximately

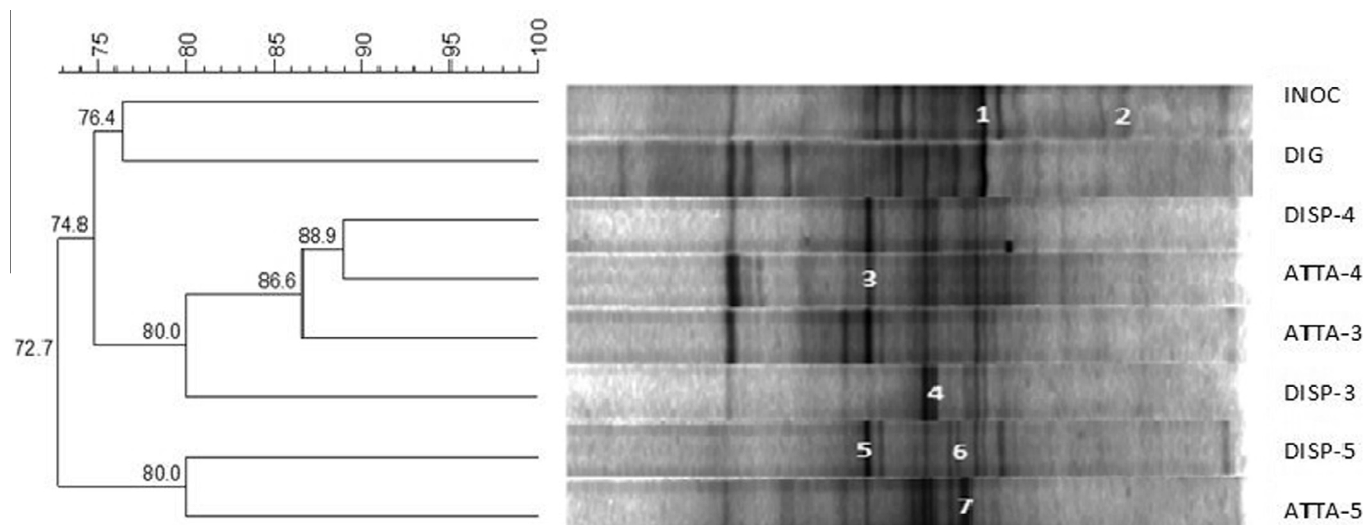

Fig. 4. DGGE sequenced bands and dendrogram. The numbers on the gel picture correspond to band identification and those on the dendrogram nodes, to the similarity level. Anaerobic digester effluent reservoir; DIG: digester; DISP-3/ATTA-3: respectively, dispersed and attached biomass in stationary period 1 (phase 3); DISP-4 e ATTA-4: respectively, dispersed and attached biomass after shock loading (phase 4); DISP-5/ATTA-5: respectively, dispersed and attached biomass in the end of stabilisation (phase 5).

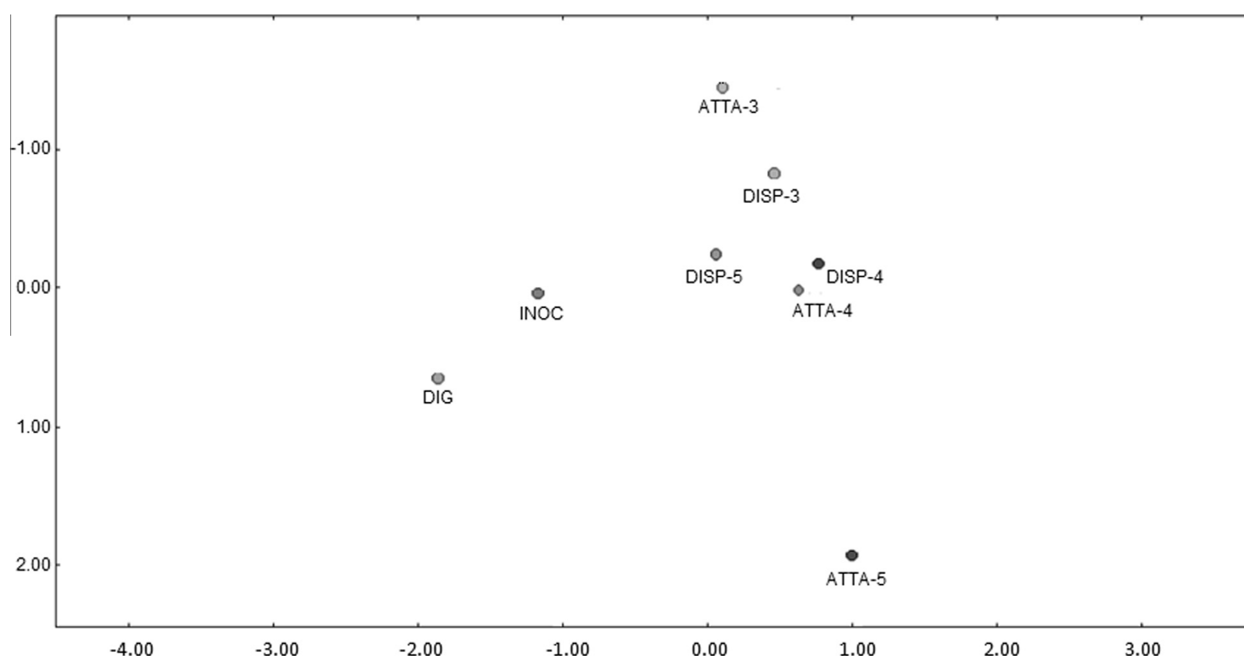

Fig. 5. Principal Coordinate Analysis (PCoA) of taxonomy profile, PC1 vs PC2, stress = 0.47, ecological index Bray–Curtis. INOC: anaerobic digester effluent reservoir; DIG: digester; DISP-3/ATTA-3: respectively, dispersed and attached biomass in stationary period 1 (phase 3); DISP-4 e ATTA-4: respectively, dispersed and attached biomass after shock loading (phase 4); DISP-5/ATTA-5: respectively, dispersed and attached biomass in the end of stabilisation (phase 5).

83% of the diversity, and they were present in all experimental phases, as shown in Fig. 6. OTUs with relative abundance below 2% (in each sample) were grouped as "Others", which included, on average, the phyla Thermotogae (0.68%), Planctomycetes (0.58%), WWE1 (0.30%), WPS-2 (0.12%), Caldiserica (0.11%), NKB19 (0.03%), Crenarchaeota (0.02%), Deferribacteres (0.02%), Acidobacteria (0.01%), Chlorobi (0.01%), Fibrobacteres (0.01%), Lentisphaerae (0.01%) and WS1 (0.01%). NKB19, WPS-2, WS1 and WWE1, like OP9 (which appears in Fig. 6), are candidate phyla composed of yet-uncultivated lineages.

Some microbial groups identified by sequencing of the bands obtained by DGGE (*Thioalkalimicrobium* sp. and *Oligella* sp.), which detects, in principle, the dominant organisms in a community, were observed at a very low proportion or were not identified by MiSeq. The primer pair used for DGGE amplified the bacterial V8 hypervariable region, whereas that used for metagenome sequencing targeted the V4–V5 regions of the 16S rRNA gene of both bacteria and archaea. Furthermore, the absence of *Thioalkalimicrobium* and *Oligella* from sequencing results could be attributed to their classification as unidentified sequences.

3.4. Possible functions of dominant bacterial populations

The major bacterial phyla, detected in the present study, were Firmicutes and Proteobacteria (67.4% on average), Bacteroidetes (7.5%), Chloroflexi (4.1%) and Synergistetes (4%). Previous studies

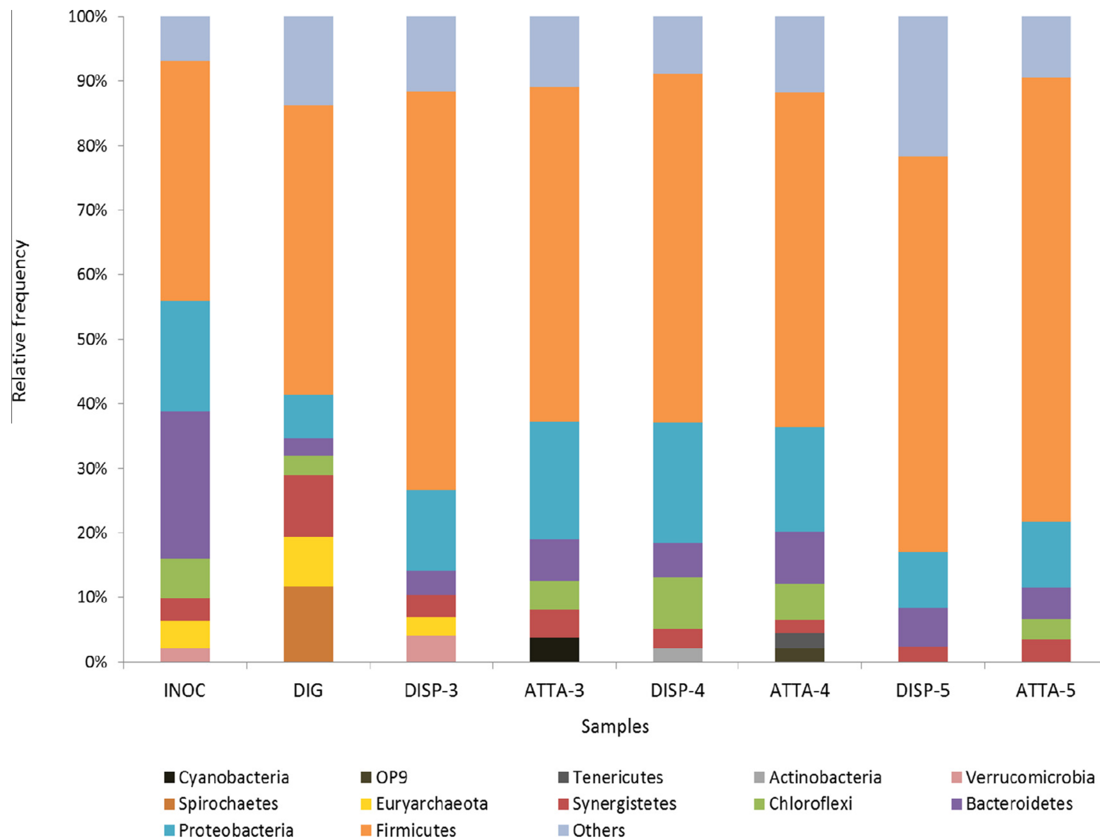

Fig. 6. Microbial community at phylum level. OTUs with less than 2% of abundance (in each sample) were included in the group "Others" to improve data visualisation. INOC: anaerobic digester effluent reservoir; DIG: digester; DISP-3/ATTA-3: respectively, dispersed and attached biomass in stationary period 1 (phase 3); DISP-4 e ATTA-4: respectively, dispersed and attached biomass after shock loading (phase 4); DISP-5/ATTA-5: respectively, dispersed and attached biomass in the end of stabilisation (phase 5).

Table 4
Identification of DGGE band sequences.

Band	RDP Classifier	BLASTn	Accession number	Identity (%)
1	Family *Anaerolineaceae*	Uncultured *Chloroflexi*	CU917991	100
2	*Desulfomicrobium* sp.	*Desulfomicrobium* sp.	JN828421	97.9
3	*Thioalkalimicrobium* sp.	*Thioalkalimicrobium* sp.	GU735085	97.2
4	*Acinetobacter* sp.	*Acinetobacter soli*	KJ806407	99.3
5	*Thioalkalimicrobium* sp.	*Thioalkalimicrobium* sp.	GU735085	97.2
6	Order *Chromatiales*	*Pseudomonas* sp.	KJ950456	96.8
7	*Oligella* sp.	*O. ureolytica*	CU927589	99.7

in biogas plants and anaerobic reactors have reported similar results (Luo and Angelidaki, 2014; Sundberg et al., 2013; Nelson et al., 2011). Although the analysis of the anaerobic reactor was not the focus of the present study, the biomass present in the desulphurisation system came, ultimately, from the vinasse digester. Thus, it was expected that the groups present therein were found in some proportion in the other samples, as shown in Fig. 6.

Firmicutes contains bacteria capable of fermenting various organic substrates and forming spores, among others. These bacteria were abundant in both the digester and the inoculum reservoir samples, and in all samples from the desulphurisation system (Fig. 6). The vast majority of OTUs was classified as Clostridia (52.4%), which contains bacteria capable of degrading proteins, lipids, and complex carbohydrates. The class Clostridia is often observed in anaerobic reactors (Kröber et al., 2009; Sundberg et al., 2013; Luo and Angelidaki, 2014) and also includes some genera of bacteria able to reduce SO_4^{2-} (such as *Desulfotomaculum* and *Pelotomaculum*) (Madigan et al., 2010). Since the concentrations of SO_4^{2-} and organic matter in the desulphurisation system were high, sulphate-reducing bacteria (SRB) could proliferate and thereby generate H_2S. In this sense, it is possible that undesired sulphide generation in the liquid phase could be significant. However, as can be seen in Fig. 3, after the initial third of the stabilisation (phase 5), the content of HS^- remained low, whereas the concentration of SO_4^{2-} increased. Therefore, we can conclude that after stabilisation, no undesired sulphide production occurred, and that the dominant process in the system was the oxidation of reduced sulphur compounds.

Within the order Clostridiales (44.7%), the most abundant genus was *Syntrophomonas* (3.8% of the total) that contains bacteria capable of fermenting fatty acids in syntrophy with microorganisms that use H_2 and formate (Sieber et al., 2010). Since vinasse is rich in organic acids – previous studies have reported volatile fatty acid concentrations in the order of 19 g L^{-1} in sugarcane molasses (Fuess and Garcia, 2014) – such microbial consortia could benefit significantly from its degradation. *Syntrophomonas* was detected in all samples, along with *Coprococcus*, *Sedimentibacter*, and *Clostridium* (3.8%, 3.4%, 3.0%, and 2.2%, respectively, on average), all of which are capable of fermenting different substrates.

Table 5
Identified genera related to sulphide oxidation in the desulphurisation system from MiSeq data analysis.

SOB-related genera	Relative frequency on BRT samples (%)					
	DISP-3	ATTA-3	DISP-4	ATTA-4	DISP-5	ATTA-5
Paracoccus	0.00	2.31	0.01	0.02	0.01	0.00
Acinetobacter	0.61	0.22	0.10	0.37	0.20	0.03
Pseudomonas	0.17	1.05	0.08	0.26	0.21	0.23
Thiomicrospira	0.01	0.67	0.03	0.03	0.22	0.01
Arcobacter	0.02	0.14	1.38	0.51	0.11	0.04
Sulfuricurvum	0.00	0.00	0.01	0.59	0.00	0.00
Thioalkalimicrobium	–	–	–	–	–	–
Total	0.81	4.39	1.61	1.78	0.75	0.31

Within the phylum Proteobacteria, representatives of all classes were found, but the most abundant were Deltaproteobacteria (4.8% of total), Betaproteobacteria (4.1%) and Gammaproteobacteria (3.6%). Identified Proteobacteria genera showed, in general, a low relative frequency. *Desulfomicrobium* sp. (0.9%), a well-studied SRB (also identified by DGGE), was the most prevalent.

Bacteroidetes, the third most abundant phylum in inoculum sample, is composed of bacteria that are involved in the hydrolysis and acidogenesis steps of anaerobic digestion; a positive correlation between hydrolytic activity and percentage of these bacteria in anaerobic digesters has already been described (Regueiro et al., 2012). Synergistetes, which was present in a greater proportion in the digester sample, consists of strict anaerobes that are capable of degrading protein-rich substrates (Vartoukian et al., 2007; Jumas-Bilak et al., 2009).

Chloroflexi, previously identified with the green non-sulphur bacteria, is still relatively little studied, and includes bacteria with diversified metabolism (Hug et al., 2013). Most of the reads classified as Chloroflexi (4.1% of total) corresponded to the class Anaerolineae, which was also detected by DGGE. This class is composed of anaerobic and heterotrophic bacteria reported in different environments, including anaerobic reactors and sulphur springs; however, there is no definitive evidence that these microorganisms are able to metabolise sulphur compounds (Yamada et al., 2006; Youssef et al., 2012).

In general, the results indicate the presence of a metabolically diverse microbial community in the BRT with sequences related to hydrolytic/fermenting bacteria, acidogenic and acetogenic bacteria. Sequences related to bacterial groups potentially involved in the sulphur cycle (such as the genus *Thioalkalimicrobium*, *Desulfomicrobium*, among others) have been identified as well. Previous studies, González-Sánchez and Revah (2007), Baquerizo et al. (2013), successfully used an alkaliphilic bacterial consortium to promote sulphide and thiosulphate oxidation, respectively. Interestingly, the system investigated in this study showed a capacity of self-buffering (unpublished data). This aspect is very important, because once the microorganisms themselves help to maintain the pH of the medium, this microbial consortium might be used for the treatment of other sulphide-rich effluents, with lower costs and waste generation.

In summary, many of the identified microorganisms were related to the anaerobic digestion process. Since the biomass came from the vinasse digester and the COD in the desulfurization system remained high throughout the experiment (Table 1), this result was expected. However, a high RE_{H_2S} was also observed and the taxa related to the sulphide oxidation process were identified and are discussed below.

3.5. Sulphide-oxidising bacteria

The major groups of green and purple sulphur bacteria were detected in very low abundance; consequently, sulphide oxidation was probably carried out mainly by colourless sulphur bacteria. However, important genera commonly associated with this group, such as *Thiobacillus*, *Beggiatoa*, and *Halothiobacillus*, were not observed. Table 5 shows the identified SOB genera, which included *Paracoccus*, *Acinetobacter*, *Pseudomonas*, *Thiomicrospira*, *Arcobacter*, *Sulfuricurvum*, and *Thioalkalimicrobium* (only by DGGE). Furthermore, orders Thiotrichales and Chromatiales and families Alcaligenaceae and Pseudomonadaceae, which comprise bacteria capable of oxidising reduced sulphur compounds, were observed. The presence of these SOB genera were reported before in other desulphurisation systems (Maestre et al., 2010; Luo et al., 2013; Ramos et al., 2014). The results from Table 5 also showed that the abundance and diversity of SOB sequences decreased after sulphide shock load, and some genera persisted in the system whereas others disappeared. Thus, despite the high sulphide removal efficiency showed by the desulphurisation system, the abundance and diversity of SOB were negatively affected by the high sulphide loading rate. Since many OTUs were not classified to the genus level, it is possible that the relative abundance of SOB in the system was much higher than what was observed.

4. Conclusions

The microbial communities in the digester and desulphurisation system shared similarities, remaining constant between experimental phases and sample source. This stability could be positive, since RE_{H_2S} remained high for most of the time. The most prevalent phylum was Firmicutes, whereas the identified SOB belonged to Proteobacteria. Despite the evidences of high sulphide oxidation activity, the relative abundance of related taxa was low, and was affected by the sulphide shock loading. Detection of *Thioalkalimicrobium* sp. suggests the existence of important SOB among unidentified reads. Besides high HS^- concentration, COD and DO may have had a significant influence on the microbial community structure and system efficiency.

Conflict of interest

All authors declare that they have no conflict of interest.

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