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The headspace of microaerobic reactors: Sulphide-oxidising population and the impact of cleaning on the efficiency of biogas desulphurisation

I. Ramos, R. Pérez, M. Fdz-Polanco *

Department of Chemical Engineering and Environmental Technology, Escuela de Ingenierías Industriales, Sede Dr. Mergelina, University of Valladolid, Dr. Mergelina s/n, 47011 Valladolid, Spain

HIGHLIGHTS

- Microaerobic conditions are applied in order to control the H_2S content of biogas.
- The S^0-rich deposits found in the HS of two microaerobic reactors are removed.
- H_2S-free biogas is rapidly achieved after cleaning the HS.
- A cleaning interval of less than 14 months ensures minimum micro-oxygenation cost.
- Moisture level determines the composition, richness and size of the SOB population.

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ABSTRACT

O_2-limiting/microaerobic conditions were applied in order to control the H_2S content of biogas. The S^0-rich deposits found all over the headspace of two pilot reactors (R1 and R2) as a result of operating under such conditions for 7 and 15 months (respectively) were sampled and removed. After restarting micro-oxygenation, H_2S-free biogas was rapidly obtained, and the O_2 demand of R2 decreased. This highlighted the need for a cleaning interval of less than 14 months in order to minimise the micro-oxygenation cost. The H_2S removed from R2 after approximately 1 month was recovered from its headspace as S^0, thus indicating that the biogas desulphurisation did not take place at the liquid interface. Denaturing gradient gel electrophoresis indicated that the composition, species richness and size of the sulphide-oxidising bacteria population depended on the location, and, more specifically, moisture availability, and indicated increasing species richness over time. Additionally, a possible succession was estimated.

1. Introduction

H_2S is a common biogas compound arising from the anaerobic digestion of proteins and S-containing compounds. It can exceed concentrations of 0.05% v/v and up to 2.0% v/v, which inevitably causes corrosion problems in combustion engines, and the release of SO_x in flue gases (Fortuny et al., 2008). Therefore, most manufacturers of combined heat and power installations recommend a biogas sulphide content of less than 0.01% or 0.03% v/v, depending on the equipment concerned (Peu et al., 2012).

H_2S can be controlled either at the source, by controlling the feedstock, at the end, by desulphurising the biogas in a later stage, or at process level, directly inside the anaerobic digester (Peu et al., 2012). The first solution is not realistic, and it is in fact the latter H_2S removal from biogas which is the most established method in practice, as it can be carried out during digestion or in an additional unit (Cirne et al., 2008). The most common end-of-pipe techniques for H_2S removal are based on physical–chemical processes. However, their high costs of both operation and by-product disposal have encouraged research and the application of biological processes (Park et al., 2011). Specifically, biological desulphurisation has been reported to be approximately 62% cheaper than chemical absorption (Burgess et al., 2001). Furthermore, it can achieve more complete removal due to the extremely high affinity of sulphide-oxidising bacteria (SOB) for the substrate (Kobayashi et al., 2012).

Abbreviations: DGGE, denaturing gradient gel electrophoresis; HS, headspace; PCR, polymerase chain reaction; SOB, sulphide-oxidising bacteria; TS, total solids.

* Corresponding author. Tel.: +34 983423166.
E-mail addresses: irisrc@iq.uva.es (I. Ramos), rebeca.perez@iq.uva.es (R. Pérez), maria@iq.uva.es (M. Fdz-Polanco).

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The most widespread biotechnologies for H_2S removal are biofilters, biotrickling filters and bioscrubbers, in which aerobic species of chemolitotrophic SOB oxidise the sulphide mainly to S^0 or SO_4^{2-}, depending on the O_2 availability (Tang et al., 2009). Besides requiring fourfold less O_2, the conversion of H_2S into S^0 is preferred over conversion to SO_4^{2-} due to the fact that S^0 is harmless and can be recovered from liquid streams and reused in bioleaching and agriculture (Kleinjan, 2005). Inside these biological systems, sulphide can be also chemically oxidised to $S_2O_3^{2-}$ (Lohwacharin and Annachhatre, 2010). Importantly, this oxidation mechanism can be catalysed by any metal ion present in the bioreactor (Kleinjan, 2005). As a result, SOB have to contend with chemical sulphide oxidation for O_2. According to Robertson and Kuenen (2006), they compete effectively at very low O_2 and sulphide concentrations. However, the contribution of the chemical mechanisms increases at high sulphide loads due to limitations in biological activity.

As an economically attractive alternative to employing additional units (that is, a process-level solution), H_2S can be removed from biogas simply by imposing microaerobic conditions in the anaerobic reactor. In Europe, this technique has been applied by injecting air directly into the headspace (HS) of the digester in order to maintain 4–6% of air in the biogas, and as a result, S^0 deposits have been found at the liquid interface and on other surfaces of the gas space (Abatzoglou and Boivin, 2009). This is partly consistent with the results of Díaz et al. (2010), who demonstrated that the desulphurisation process basically occurs in the HS independently of both the O_2 (or air) dosing point and the mixing method, but in this case neither S^0 nor SOB were found at the liquid interface . Similarly, Rodríguez et al. (2012) only identified representatives of this microbial group in the S^0-rich biomass attached to the HS, although micro-oxygenation was introduced from the bottom of the reactor.

It is essential to know how the increasing accumulation of S^0 in the headspace over time affects the O_2 transfer conditions and, therefore, the performance of the biogas desulphurisation, since this could lead to a reduction in the intervals of time at which the digester must be cleaned. Although the S^0 accumulation could also significantly reduce the volume of the gas space in microaerobic reactors in the long-term, Ramos et al. (2012) demonstrated that a biogas residence time of approximately 1 h sufficed to achieve H_2S removal efficiencies similar to those obtained at around 7 h. Díaz and Fdz-Polanco (2012) reported that the desulphurisation performance in a microaerobic digester treating sewage sludge was very similar just before HS cleaning and 30 h later, after almost 21 months intercalating anaerobic and microaerobic experiments. Moreover, they highlighted the rapidity with which the H_2S was removed from the biogas just after cleaning the HS, which suggested extremely high activity levels of SOB at the liquid interface and/or a great contribution by the chemical oxidation mechanisms. With regard to this, it must be noted that Ramos et al. (2012) provided evidence that this process is predominantly biological.

Likewise, it is of utmost importance to know how SOB grow in the HS to optimise the efficiency of H_2S removal from biogas in microaerobic reactors. However, only Kobayashi et al. (2012) have provided valuable information in this area. They showed that both cell density and bacterial activity in the HS were much higher in the areas nearest the liquid phase, which was attributable to an increased availability of water and nutrients.

Based on the points outlined above, the main objectives of this study were:

- To evaluate the impact of HS cleaning on the efficiency of biogas desulphurisation.
- To investigate where exactly the biogas desulphurisation takes place in the HS.
- To characterise and locate the SOB population that is removing H_2S during sewage sludge digestion.
- To approach the temporal differences in the SOB population.

2. Methods

2.1. Pilot plant scale reactors

Research was carried out in two continuous stirred tank reactors (R1 and R2) with 200 L (250 L of total volume) treating sewage sludge with a variable organic and sulphur load at 19d of hydraulic retention time. A diagram of the digesters is shown in Fig. 1. Temperature (35 °C) was monitored by probes and was regulated by electric resistors surrounding their walls, which were in turn covered with insulation. Mixing was carried outat approximately 50 L/h by peristaltic pumps. Microaerobic conditions were implemented by making a single-point injection of pure O_2 into the HS using mass flow controllers. Biogas composition was determined by gas chromatography (Díaz et al., 2010), and its production was measured volumetrically.

2.2. Digestion monitoring

Digestion performance was assessed by measuring total and soluble chemical oxygen demand (COD), total solids (TS), volatile solids (VS), volatile fatty acids, total kjeldahl nitrogen and ammonia according to APHA (1998). Total dissolved sulphide and SO_4^{2-} concentrations were measured by the potentiometric and the chromatographic method, respectively (APHA, 1998). $S_2O_3^{2-}$ was measured by high liquid performance chromatography according to the procedure described by van der Zee et al. (2007). A LECO CS-225 was utilised to determine elemental composition in terms of S and C.

2.3. Experimental procedure

The operational sequence is schematised in Fig. 2. The HS of R2 was cleaned at $t = 0$ (Fig. 2); however this reactor was operated for several months before beginning this research under the aforementioned conditions. Seven months afterwards (at $t = 8$), R1 was started up with sludge from R2. Thereafter, both digesters operated in parallel and under the above conditions. Until $t = 15$, they basically operated under microaerobic conditions; micro-oxygenation was rarely interrupted.

At $t = 15$, the ceiling of both reactors was removed. Six samples (A–F) were taken from different points of the HS for TS, elemental, and microbial analysis (Fig. 3a). As indicated in Fig. 2, A1–F1 were retrieved from R1, and A2–F2 belonged to R2. The A and B samples were taken from the walls (the lowest and the upper area, respectively), the C samples were taken from the ceiling, the D and E samples were taken from the dip tube (the upper and the lowest area, respectively), and the F samples were taken from the liquid interface (Fig. 3a). After sampling, all the surfaces were cleaned, and the liquid interface (approximately 250 mm of sludge from the surface) was removed.

Once sealed, the digesters were operated under anaerobic conditions for 1 month (Fig. 2).

Micro-oxygenation was restarted at $t = 16$. At $t = 17$, R2 was uncovered again, and the S^0-rich deposits accumulated in the HS were retrieved separately according to Fig. 3a. As shown in Fig. 2, those samples were called A3–F3. After drying them, they were weighed and characterised in terms of S and C percentages in order to estimate the amount of S^0 deposited.

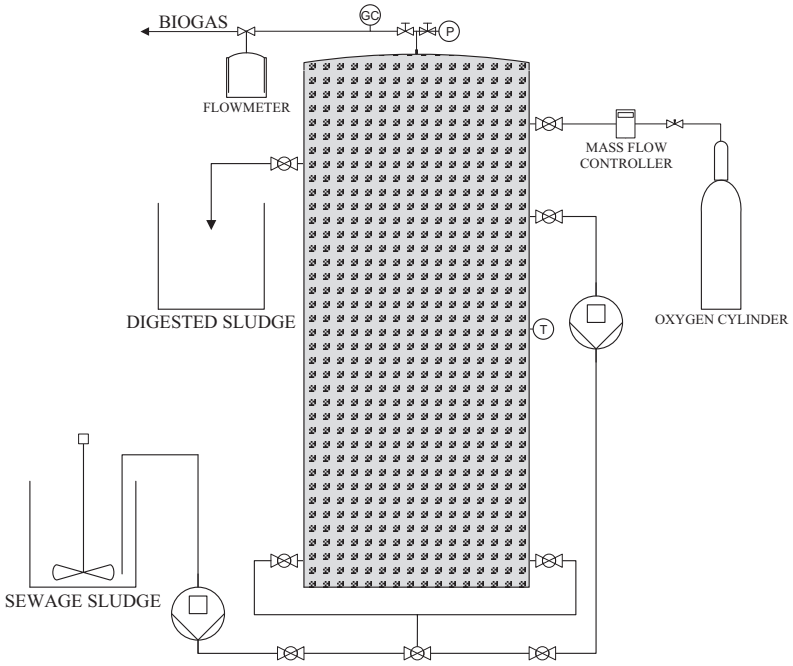

Fig. 1. Digesters diagram.

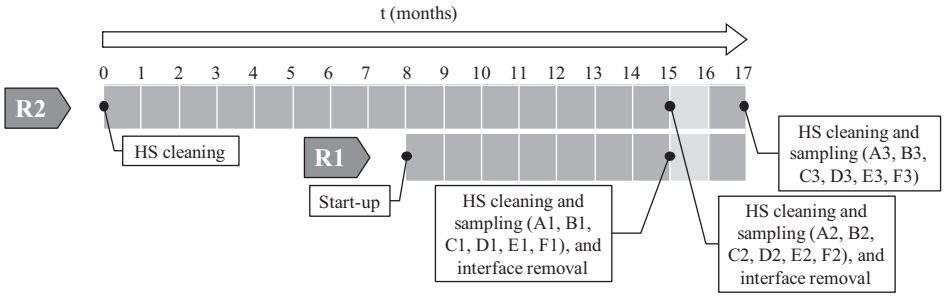

Fig. 2. Operational sequence. ■: microaerobic period; ■: anaerobic period.

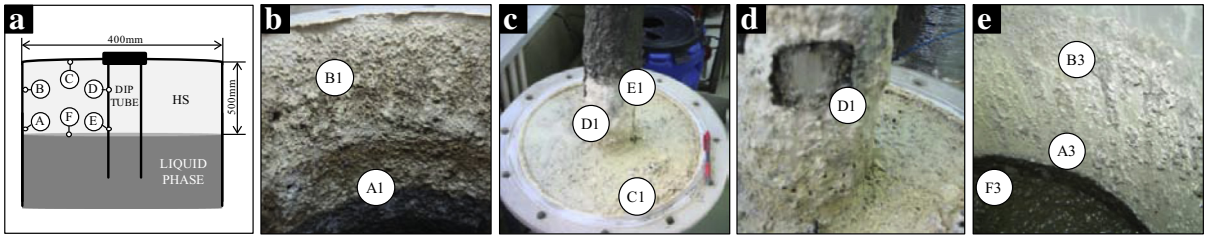

Fig. 3. HS diagram and samples codes (a). Walls of the HS (b), ceiling and dip tube (c and d) of R1 at $t = 15$. Walls of the HS and liquid interface of R2 at $t = 17$ (e).

2.4. Bacterial analysis

The bacterial community established in the HS of the reactors at $t = 15$ was characterized by denaturing gradient gel electrophoresis (DGGE) analysis. Samples were stored at −20 °C. Extraction of genomic DNA, polymerase chain reaction (PCR) amplification and DGGE analysis were performed according to Lebrero et al. (2013).

The DGGE profiles were processed by GelCompar IITM software (Applied Maths BVBA, Sint-Martens-Latem, Belgium). The Shannon–Wiener diversity index (H) of every sample and the pair-wise similarity coefficient were both calculated according to Lebrero et al. (2013). The desired bands were excised from the gels according to the procedure described by Lebrero et al. (2011). The taxonomic position of the sequenced DGGE bands was obtained by the RDP classifier tool at a confidence level of 50% (Wang et al., 2007). Moreover, the closest matches to every band were obtained from the Blast search tool at the National Centre for Biotechnology Information (McGinnis and Madden, 2004). Sequences alignment (ClustalW) and phylogenetic analysis were performed using the MEGA software (version 6.0). The phylogenetic trees were

constructed using the neighbor-joining method (1000-fold bootstrap analysis). The sequences were deposited in the GenBank database under accession numbers KF148033–KF148052.

3. Results and discussion

3.1. Deposition of S^0

3.1.1. Medium-long term operation

Fig. 3b–d show the state of different surfaces of the HS of R1 at $t = 8$ (Fig. 2). As in R2, S^0 was present all over the HS; however, it was not observed at the liquid interface of either of the two digesters. The S content of the samples F1 and F2 (Fig. 3a) were indeed negligible in comparison with most of the rest of the samples (Table 1). In this regard, it must be noted that although the digesters were continuously recirculated, an inefficient mixing was maintained in the highest area of the liquid phase due to the fact that both the recirculation and the effluent streams left the reactor below the interface level (Fig. 1). This explains the great difference in TS content between F1 and F2 (which both contained approximately 60 g/kg) and the respective effluents of R1 and R2 (containing around 19 g/kg). Therefore, if H_2S had been oxidised there, presumably some S^0 would have been observed at the liquid interface.

The S^0-rich deposits covering the walls of R2 were thicker than in R1, which was consistent with the operation time. By contrast, the S^0-rich deposits that accumulated on the ceiling and the dip tube of R2 were inexplicably thinner than in R1. The aspect of the walls and the dip tube of R1 was similar to that of the walls of R2 in terms of the proportion of S^0 (yellow part) to digested sludge (black part) against height. Obviously, the lowest parts of the HS were more frequently touched by sludge as a result of droplets and even momentary liquid level rises; hence the stratification pattern shown in Fig. 3b. This was indeed analytically proved; in general, the shorter the distance from the liquid surface, the higher the C content and the lower the S percentage (Table 1).

Regarding the dip tube in R2, it inexplicably did not present the aforementioned stratification pattern. In fact, although both samples D2 and B2 were taken at approximately the same height, D2 had a significantly lower S content, and its C concentration was approximately the double. Conversely, C1 and D1 had more similar S and C contents (90% and 8% w/w compared with 82% and 11% w/w, respectively). At this point, it should be highlighted that the digestate and the S^0 were intercalated in the different deposits (Fig. 3d); nevertheless, and in contrast to Kobayashi et al. (2012), no specific stratification pattern was identified.

The different moisture levels maintained in both HS corresponded only partly to the above reasoning, that is, the larger the distance from the liquid phase, the lower the moisture content, or equivalently, the higher the level of dryness. The dryness of the samples is expressed as TS content in Table 1. The TS concentration in the samples taken from the walls and the ceiling of both reactors was fairly similar, and it was in turn considerably higher than in their respective dip tubes. Presumably, the moisture and sludge reaching these surfaces gradually dried. Regarding the relatively high moisture content in the samples taken from the ceiling (where the sludge made hardly, if any, contact), this was attributed to water condensation since this area was less well insulated than the walls.

3.1.2. Short-term operation

The total amount of H_2S removed from $t = 16$ to 17 was estimated according to the daily biogas production and the H_2S concentration recorded just before $t = 16$ (under anaerobic conditions) (Fig. 2). Considering the weight and the elemental composition of the different S^0-rich deposits (A3–E3 in Table 1), all the H_2S removed during that period (approximately 26 g) was deposited equivalently in the form of S^0 on both the walls (excluding the highest area, as shown in Fig. 3e) and the ceiling. Namely, it was specifically accumulated where TS concentration was higher at $t = 15$. A negligible amount of S^0 was recovered from the dip tube. Furthermore, S^0 was not observed at the liquid interface. In fact, as at $t = 15$, comparison of the S concentration in F3 and in the effluent pointed to negligible (if any) H_2S oxidation in this area. Consequently, nutrient accessibility did not seem to be a limiting factor for biogas desulphurisation; it presumably decreased with the increasing distance from the liquid interface. Moisture level could indeed be the key factor for the process, which could be in turn related to the O_2 availability, since dryness may increase O_2 transfer.

3.2. Recovery after cleaning

As noted, microaerobic conditions were restored at $t = 16$ (Fig. 2). The O_2 flow rate was frequently adjusted according to the evolution of the O_2 and the H_2S concentrations in the biogas (Fig. 4a and b); the objective was to achieve the minimum amount of O_2 leaving the reactor and at least a 97% H_2S removal efficiency. In R1, a biogas flow rate of 201NL/d containing approximately 0.27% v/v of H_2S was entirely desulphurised within 6 h (Fig. 4a). By then, the biogas O_2 content was 0.08% v/v, and the average molar ratio of O_2 supplied to H_2S produced was approximately 2.0. Nonetheless, this relationship was further reduced during the following days; as shown in Table 2, an O_2/H_2S molar ratio of about 0.9 was achieved. As a result, the O_2 content of the biogas decreased to 0.02%, which implied that only 3% of the O_2 supplied left the digester (Table 2). Assuming that all the H_2S oxidised was converted into S^0 due to the limited O_2 availability, it was estimated that around 54% of the O_2 injected was consumed in H_2S removal from biogas, and 43% was employed in other oxidative processes.

In R2, the biogas production at $t = 16$ was similar to that inR1 (194NL/d). However, the H_2S concentration recorded under anaerobic conditions was significantly higher (0.37% v/v); hence the higher O_2 flow rate reached (Fig. 4b). Nevertheless, the response of R2 to O_2 injection was slower than that of R1; the O_2/H_2S molar ratio after 10 h of micro-oxygenation was 7.6, while the H_2S removal efficiency was only 65%. However, the next day, the biogas was entirely desulphurised and, therefore, the O_2 supply to R2 was reduced. Equivalent efficiencies were eventually achieved in both digesters; an O_2/H_2S molar ratio of approximately 1.0 was

Table 1
Elemental composition of the samples retrieved from the HS of the reactors.

	R1						R2												
Time	$t = 15$						$t = 15$						$t = 17$						
Sample	A1	B1	C1	D1	E1	F1	A2	B2	C2	D2	E2	F2	A3	B3	C3	D3	E3	F3	Effluent
TS (g/kg)	296	279	297	150	114	63	425	387	371	167	126	57	–	–	–	–	–	–	14
S (% w/w)	26	90	89	82	1	<1	10	84	97	62	3	<1	28	90	85	85	7	<1	<1
C (% w/w)	22	8	5	11	20	26	25	6	2	13	8	28	24	8	12	12	26	30	27

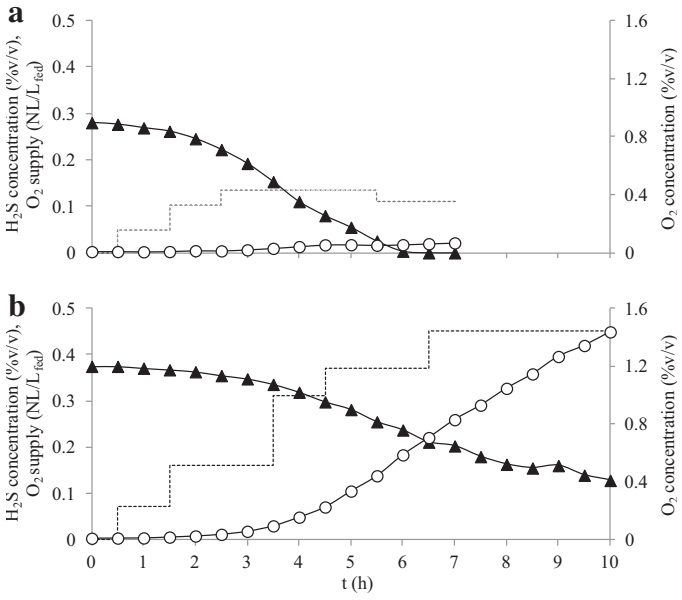

Fig. 4. O_2 flow rate (discontinuous line), and concentrations of H_2S (▲) and O_2 (○) in the biogas from R1 and R2 at $t = 16$.

achieved, and approximately 47% and 45% of the O_2 supplied was consumed in the H_2S removal from biogas and in unidentified processes, respectively (Table 2).

Although faster in R1, the rapidity with which the biogas was desulphurised in both reactors at $t = 16$ suggested an important contribution of the chemical mechanisms of sulphide oxidation. Although the proportion of H_2S removed by each of the mechanisms (chemical and biological) could not be estimated, at least at that point the biological oxidation rate was considered to be negligible. Due to the tasks carried out at $t = 15$ (Fig. 2), the presence of a significant population of SOB in the HS or at the liquid interface at $t = 16$ were ruled out. It should be taken into account that the absence of O_2 (electron acceptor) during the preceding anaerobic period (from $t = 15$ to 16) prevented SOB growth.

3.3. Desulphurisation performance over time

The impact of the operation time on the process of biogas desulphurisation was evaluated by comparing the H_2S removal efficiency achieved just before and after cleaning the HS in R1 and R2 (Fig. 2). At $t = 14$, 100% and 97% of the H_2S produced in R1 and R2, respectively, was oxidised (Table 2). The performance of R2 was more unstable than in the same period in R1, however, due to the variability of the feed sludge, this could not be unequivocally attributed to the longer operation time. At $t = 16$, the H_2S removal efficiency in R1 and R2 remained stable at around 100% and 99%, respectively.

The efficiency of O_2 usage in R2 increased substantially after cleaning the HS, and a similar yield was observed in R1 (Table 2). In both reactors, the amount of O_2 consumed in unidentified processes at $t = 16$ was slightly lower than at $t = 14$, probably as a result of the removal of the O_2-using microorganisms growing on the HS and at the liquid interface (Fig. 2). Considering the inefficient mixing conditions maintained in this area, it is possible that uniform mixing would have reduced the O_2 demand of the digesters. Besides, the amount of O_2 leaving the digester decreased, especially in R2, where it declined by 19%, while in R1, a decrease of only 6% was estimated. This suggested improved O_2 transfer in R2, and highlighted the need for a cleaning interval of less than 14 months in order to minimise the micro-oxygenation cost.

3.4. Composition and structure of the microbial communities

3.4.1. Bacterial diversity

In general, the HS of both digesters showed a high species evenness and richness at $t = 15$; H typically ranges from 1.5 to 3.5 (McDonald, 2003). In R1, H ranged from 3.2 to 3.6 (Fig. 5a), while it varied between 2.5 and 3.5 in R2 (Fig. 5b). The diversity indices of the samples A2–C2 were considerably higher than those of the samples D2–F2. Moreover, C1 and C2 presented the highest and the lowest H, respectively. With regard to this, it should be mentioned that the samples taken from the ceilings were expected to present significantly less H, even lower than in C2, due to the large distance from the liquid phase and the presumably deficiency in nutrients availability. Hence, taking into account that A2–C2 presented the highest TS concentrations in relation to the rest of the samples (including those taken from R1), it was hypothesised that the moisture levels maintained on the walls and the ceiling of R2 limited the bacterial community diversity (Table 1). Maybe the O_2 transfer was highest there, which certainly could prevent the growth of a wide group of microorganisms (those with no or low tolerance to O_2).

3.4.2. Analysis of the DGGE profiles

Overall, the pair-wise similarity indices indicated a low-moderate correspondence between the bacterial communities growing inside each HS at $t = 15$ (Table 3). In R1, the highest similarity coefficients were found between samples A, B and C (68–72%), and between D, E and F (58–64%). C1 presented a similarity of 48–54% with D1–F1, while the rest of the samples presented low coefficients (13–28%). The samples with similar TS content were found to have the highest similarity. Hence, taking into account Table 1, it was the moisture level that determined the bacterial community. In fact, although the similarity indices calculated for R2 did not present so much variability, they roughly led to this conclusion. The similarity coefficients between A2 and C2 (59%), B2 and C2 (66%), and E2 and F2 (84%) were the highest.

Table 2
Assessment of the desulphurisation performance before and after $t = 15$.

Reactor	Time	Biogas production (NL/d)	H_2S^{AN} (%v/v)	O_2/H_2S (mol/mol)	H_2S^{MA} (%v/v)	O_2^{MA} (%v/v)	O_2 to S^0 (%)	O_2 in biogas (%)	O_2 other processes (%)
R1	$t = 14$	176	0.55	1.3	0.00	0.05	36	9	55
R1	$t = 16$	173	0.53	0.9	0.00	0.02	54	3	43
R2	$t = 14$	139	0.48	2.5	0.02	0.20	20	27	53
R2	$t = 16$	141	0.48	1.0	0.00	0.05	47	8	45

[AN] anaerobic conditions.
[MA] microaerobic conditions.

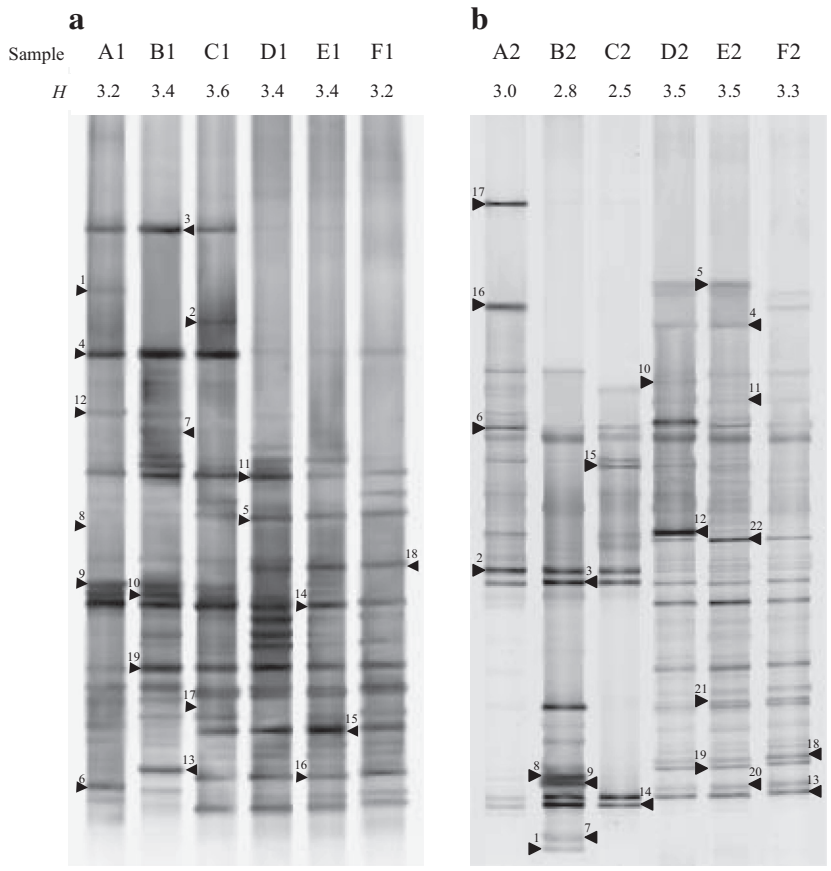

Fig. 5. Bacterial DGGE profiles of the 16S rRNA amplicons of the samples retrieved from the HS of R1 (a) and R2 (b) at $t = 15$ with their respective diversity indices.

Table 3
Similarity indices (%R1/%R2) between the samples taken from the HS of the reactors at $t = 15$.

Sample	B	C	D	E	F
A	68/28	68/59	13/39	16/25	16/17
B	–	72/66	28/25	21/30	18/47
C	–	–	54/51	43/37	48/35
D	–	–	–	64/54	65/39
E	–	–	–	–	58/84

As shown in Table 3, the pair-wise similarity indices of A1 and B1 (68%) and D1 and F1 (65%) were considerably higher than those of A2 and B2 (28%) and D2 and F2 (39%). Conversely, the similarity between A and D, B and F, and E and F was between 26% and 29% lower in R1. The rest of the sample pairs presented relatively low differences (1–13%).

3.4.3. DGGE analysis

According to the RDP classifier tool, from the 19 bands sequenced from the DGGE gel of R1 (Fig. 5a), 6 and 7 and were assigned to the Proteobacteria and Firmicutes phylum, respectively (Table 4). In addition, the phyla Actinobacteria (3 bands) and Verrucomicrobia (1 bands) were identified. It is worth noting that 2 bands could not be classified. From the bacterial DGGE profile of R2 (Fig. 5b), 8, 9 and 5 bands were placed within the Proteobacteria, Firmicutes, and Actinobacteria phylum, respectively (Table 5). Only 1 band remained unclassified. Fig. 6a and b depict the phylogenetic relationships between the bands sequenced from the DGGE gel of R1 and R2 (respectively) and their closest relatives in GenBank (obtained by the Blast search tool).

The RDP classifier tool affiliated all the SOB genera found in R1 (DGGE bands 3, 4 and 6 in Fig. 5a) to the Proteobacteria phylum. They were members of two families: Epsilonproteobacteria (*Arcobacter* sp., *Sulfuricurvum* sp.) and Gammaproteobacteria (*Acidithiobacillus* sp.). Hence, at least three SOB species grew in the HS of R1 (Table 4). Both *Arcobacter* sp. and *Acidithiobacillus* sp. were found by Kobayashi et al. (2012) growing on the HS of a microaerobic digester. *Sulfuricurvum* was indicated by Kodama and Watanabe (2004) as a chemolithoautotrophic and sulphur-oxidising genus capable of thriving under microaerobic and anaerobic conditions. Nonetheless, the Blast search tool also identified the genus *Alyciclobacillus* (band 13, within the Firmicutes phylum); it indicated a similarity of up to 100% with the specie reported in the study of Díaz et al. (2010). It must be highlighted that *Alyciclobacillus* was the only SOB genus found in F1.

According to the RDP classifier, five genera of SOB grew in the HS of R2. Nonetheless, the genus *Alyciclobacillus* was also identified by the Blast search tool. Along with the four SOB genera found in R1, *Acinetobacter* sp. (bands 10–12 in Fig. 5b) and *Rhodococcus* sp. (band 20), which are representatives of the Proteobacteria and Actinobacteria phyla, respectively, were found (Table 5). *Acinetobacter* sp. was reported by Omri et al. (2011) to be instrumental in desulphurising the air stream in a biofiltration system. Zhang et al. (2009) utilised a strain of *Rhodococcus* sp. to successfully remove H_2S in a biotrickling filter. It should be highlighted that *Rhodococcus* sp., *Acinetobacter* sp. and *Acidithiobacillus* sp. were found in F2.

In R1, the highest species richness of SOB was concentrated on the walls and the ceiling; only the genera *Arcobacter* and *Alicyclobacillus* were identified in the samples taken from the dip tube and the liquid interface, respectively (Table 4). Additionally, the

Table 4
Taxonomic placement of the bacterial DGGE bands of the gel obtained from the samples taken from R1 at $t = 15$ according to the RDP classifier at 50% of confidence level, and closest relatives in GenBank obtained by the Blast search tool showing sequence similarity and environments from which they were retrieved. "×" indicates presence of the band in the sample; high-intensity bands are depicted with "××".

Taxonomic placement	Band no. (accession no.)	A1	B1	C1	D1	E1	F1	Closest relatives in Blast (accession no.)	Similarity (%)	Source of origin
Phylum Proteobacteria	1 (KC306914)	×						Uncultured bacterium (AB286499)	95	Activated sludge
Class Epsilonproteobacteria										
Order *Campylobacterales*	2 (KF130769)			××				Uncultured epsilon proteobacterium (DQ295695)	99	Floating microbial mat in sulfidic groundwater, Movile Cave
								Uncultured bacterium (AB286499)	98	Activated sludge
Family Campylobacteraceae										
Genus *Arcobacter*[a]	3 (KC306915)	××	××	××	×	×		Uncultured *Arcobacter* sp. (HQ392829)	99	Headspace of a digester of sewage sludge under microaerobic conditions
								Uncultured *Arcobacter* sp. (HQ392823)	99	Headspace of a digester of sewage sludge under microaerobic conditions
Family Helicobacteraceae										
Genus *Sulfuricurvum*[a]	4 (KC306917)	××	××	××				Uncultured bacterium (AB286499)	99	Activated sludge
								Uncultured bacterium (EU662592)	97	Floating microbial mat from sulfidic water
Class Deltaproteobacteria										
Order Syntrophobacterales										
Family Syntrophaceae										
Genus *Syntrophus*	5 (KC130770)	×	×	×	×	×	×	Uncultured Deltaproteobacteria bacterium (CU926874)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
								Uncultured bacterium (JQ085713)	99	Anaerobic digester
Class Gammaproteobacteria										
Order Acidithiobacillales										
Family Acidithiobacillaceae										
Genus *Acidithiobacillus*[a]	6 (KC130771)	××	××	×				*Thiobacillus* sp. (AJ459802)	99	Culture collection
								Acidithiobacillus sp. (FJ915156)	99	Culture collection
Phylum Firmicutes										
Class Clostridia										
Order Clostridiales	7 (KC306921)		×	×	×	×		Uncultured bacterium (FJ978625)	96	Feces
Family Syntrophomonadaceae										
Genus *Thermohydrogenium*	8 (KC130772)	×	×	×				Uncultured bacterium (GQ259594)	95	Bioreactor
	9 (KC306923)	××	××	×		×		Uncultured bacterium (GQ259594)	96	Bioreactor
	10 (KC306924)	××	××	××	×		×	Uncultured bacterium (GQ259594)	96	Bioreactor
Family Peptostreptococcaceae										
Genus *Clostridium XI*	11 (KC306922)	××	××	××	××	×	×	Uncultured soil bacterium (JX489929)	99	Soil
								Uncultured bacterium (FJ660495)	99	Activated sludge
Family Lachnospiraceae	12 (KC306925)	×	×	×		×		*Clostridium* sp. (GU247219)	93	Waste water of a pesticides firm
Class Bacilli										
Order Bacillales[a]	13 (KC306927)	×	××	×	×	×	×	Uncultured *Alicyclobacillus* sp. (HQ392831)	100	Headspace of a digester of sewage sludge under microaerobic conditions
								Bacillus solfatarensis (AY518549)	98	Culture collection
Phylum Actinobacteria										
Class Actinobacteria										
Subclass Actinobacteridae										
Order Actinomycetales										
Suborder Corynebacterineae										
Family Dietziaceae										
Genus *Dietzia*	14 (KC306928)	××	××	××	×	×	×	*Dietzia* sp. (FJ529029)	95	Excess sludge of municipal wastewater treatment plant
Family Mycobacteriaceae										
Genus *Mycobacterium*	15 (KF130773)			××	××	××	××	Uncultured bacterium (EU677397)	97	Soil
Family Nocardiaceae										

(*continued on next page*)

Table 4 (*continued*)

Taxonomic placement	Band no. (accession no.)	A1	B1	C1	D1	E1	F1	Closest relatives in Blast (accession no.)	Similarity (%)	Source of origin
Genus *Gordonia*	16 (KF130774)			××	××	××	××	Gordonia hirsuta (NR_026297)	99	Biofilter of an animal rendering plant
								Uncultured bacterium (CU925412)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
Phylum Verrucomicrobia										
Class Optitutae										
Order Optitutales										
Family Optitaceae										
Genus *Alterococcus*	17 (KC306929)		×	×		×	×	Uncultured bacterium (FN985251)	99	Long-term biogas completely stirred tank reactor
								Uncultured Verrucomicrobia bacterium (CU918353)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
Unclassified bacteria	18 (KC306930)				×	×	××	Uncultured bacterium (FN985598)	99	Long-term biogas completely stirred tank reactor
								Uncultured bacterium (AB175392)	99	A mesophilic anaerobic BSA digester
	19 (KC306931)	×	××	××	××	×	××	Uncultured Firmicutes bacterium (CU923016)	97	Mesophilic anaerobic digester which treats municipal wastewater sludge

[a] Putative SOB.

Table 5
Taxonomic placement of the bacterial DGGE bands of the gel obtained from the samples taken from R2 at $t = 15$ according to the RDP classifier at 50% of confidence level, and closest relatives in GenBank obtained by the Blast search tool showing sequence similarity and environments from which they were retrieved. "×" indicates presence of the band in the sample; high-intensity bands are depicted with "××".

Taxonomic placement	Band no. (accession no.)	A2	B2	C2	D2	E2	F2	Closest relatives in Blast (accession no.)	Similarity (%)	Source of origin
Phylum Firmicutes										
Class Clostridia										
Order Clostridiales	1 (KF148033)		×					Uncultured bacterium (JF937217)	99	Anaerobic fluidized bed reactor treating vinasse
Family Syntrophomonadaceae										
Genus *Thermohydrogenium*	2 (KF148034)	××	××	××	×	×	×	Uncultured bacterium (GQ259594)	95	Bioreactor
	3 (KF148035)	××	××	××	××	×	××	Uncultured bacterium (GQ259594)	96	Bioreactor
Family Lachnospiraceae	4 (KF148036)				×	××		Uncultured bacterium (CR933122)	99	Every municipal wastewater treatment plant
								Uncultured bacterium (JX627844)	99	Membrane bioreactor treating acetone, toluene, limonene and hexane
Genus *Hespellia*	5 (KF148037)				×	××		Uncultured bacterium (CR933122)	99	Every municipal wastewater treatment plant
								Uncultured bacterium (JX627844)	99	Membrane bioreactor treating acetone, toluene, limonene and hexane
Family Peptostreptococcaceae										
Genus *Clostridium XI*	6 (KF148038)	××	×	×	××	××	×	Uncultured soil bacterium (JX489929)	99	Soil
								Uncultured bacterium (GQ480145)	99	Activated sludge from wastewater treatment plant
Family Clotridiales_incertae sedis III	7 (KF148039)		×					Uncultured bacterium (JF937217)	100	Anaerobic fluidized bed reactor treating vinasse
Class Bacilli										
Order Bacillales[a]	8 (KF148040)		×					Uncultured *Alicyclobacillus* sp. (HQ392831)	99	Headspace of a digester of sewage sludge under

Table 5 (*continued*)

Taxonomic placement	Band no. (accession no.)	A2	B2	C2	D2	E2	F2	Closest relatives in Blast (accession no.)	Similarity (%)	Source of origin
										microaerobic conditions
								Bacillus solfatarensis (AY518549)	98	Culture collection
	9 (KF148041)		××					Uncultured *Alicyclobacillus* sp. (HQ392831)	100	Headspace of a digester of sewage sludge under microaerobic conditions
								Bacillus solfatarensis (AY518549)	98	Culture collection
Phylum Proteobacteria										
Class Gammaproteobacteria										
Order Pseudomonadales										
Family Moraxellaceae										
Genus *Acinetobacter*[a]	10 (KC306918)	×			×			Acinetobacter johnsonii (NR_044975)	95	Culture collection
	11 (KF148042)	×			×	×	×	Acinetobacter johnsonii (NR_044975)	97	Culture collection
	12 (KC306919)	××		××	××	×	×	Acinetobacter johnsonii (NR_044975)	99	Culture collection
								Uncultured bacterium (JX040380)	99	Wastewater
								Uncultured *Acinetobacter* sp. (JN679102)	99	Membrane bioreactor
Order Acidithiobacillales										
Family Acidithiobacillaceae										
Genus Acidithiobacillus[a]	13 (KF148043)	×	××	××	××	××	××	*Thiobacillus* sp. (AJ459802)	99	Culture collection
								Uncultured *Acidithiobacillus* sp. (EF612419)	98	Mine tailings
								Uncultured bacterium (JQ906816)	97	Hydrogen sulfide biofilter
	14 (KF148044)	×	××	××				*Thiobacillus* sp. (AJ459802)	99	Culture collection
								Uncultured bacterium (JQ906816)	97	Hydrogen sulfide biofilter
Order Enterobacteriales										
Family Enterobacteriaceae										
Genus *Raoultella*	15 (KF148045)	×		××	××	×	×	Uncultured bacterium (JF689907)	97	MFC anode biofilm
								Enterobacteriaceae bacterium (HQ259701)	97	Activated sludge
Class Epsilonproteobacteria										
Order Campylobacterales										
Family Helicobacteraceae										
Genus *Sulfuricurvum*[a]	16 (KF148046)	××						Uncultured epsilon proteobacterium (DQ295695)	99	Floating microbial mat in sulfidic groundwater, Movile Cave
								Uncultured bacterium (AB248647)	99	A mesophilic anaerobic butyrate degrading reactor
Family Campylobacteraceae										
Genus *Arcobacter*[a]	17 (KF148047)	××	×	×				Uncultured *Arcobacter* sp. (HQ392829)	100	Headspace of a digester of sewage sludge under microaerobic conditions
								Uncultured *Arcobacter* sp. (HQ392823)	99	Headspace of a digester of sewage sludge under microaerobic conditions
Phylum Actinobacteria										
Class Actinobacteria										
Subclass Actinobacteridae										
Order Actinomycetales										
Suborder Corynebacterineae										
Family Nocardiaceae										
Genus *Gordonia*	18 (KF148048)				×	×	××	Uncultured Actinobacteria bacterium (CU925412)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
								Gordonia hirsuta (NR_026297)	98	Biofilter of an animal rendering plant
	19 (KF148049)		××		××	××	××	Uncultured Actinobacteria bacterium (CU925412)	98	Mesophilic anaerobic digester which treats municipal wastewater sludge
								Gordonia hirsuta (NR_026297)	98	Biofilter of an animal rendering plant

(*continued on next page*)

Table 5 (*continued*)

Taxonomic placement	Band no. (accession no.)	A2	B2	C2	D2	E2	F2	Closest relatives in Blast (accession no.)	Similarity (%)	Source of origin
Family Nocardiaceae										
Genus *Rhodococcus*[a]	20 (KF148050)				×	××	××	*Rhodococcus* sp. (AJ007001)	99	Compost biofilter
								Rhodococcus sp. (FR690460)	98	Sludge of a bioreactor
Family Mycobacteriaceae										
Genus Mycobacterium	21 (KF148051)	×			×	××	××	Uncultured bacterium (EU677397)	99	Soil
								Uncultured bacterium (JX627819)	98	Membrane bioreactor treating acetone, toluene, limonene and hexane
Unclassified bacteria	22 (KF148052)				××	××	××	Uncultured bacterium (FN985598)	99	Long-term biogas completely stirred tank reactor
								Uncultured bacterium (AB175392)	99	A mesophilic anaerobic BSA digester

[a] Putative SOB.

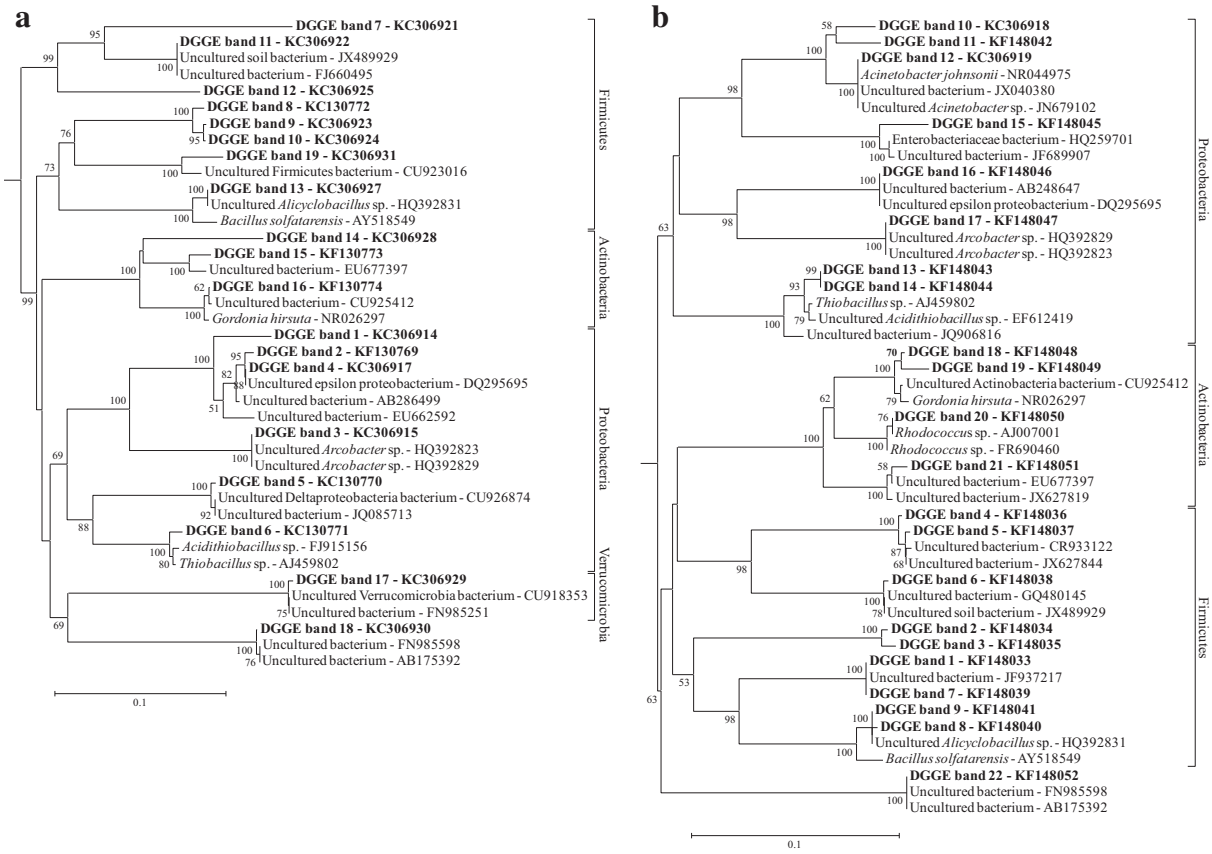

Fig. 6. Bacterial phylogenetic tree based on neighbour-joining analysis of 16S rRNA sequences from the HS of R1 (a) and R2 (b) (in boldface) and their closest relatives (similarity ⩾97%) in GenBank obtained by the Blast search tool. Accession numbers are indicated. Numbers on the nodes indicate bootstrap values of 50% and higher (1000 replicates). The scale bar indicates 10% sequence difference.

intensity of the bands representing SOB in the lanes of the samples D1, E1 and F1 was substantially lower than in A1, B1 and C1 (Fig. 5a), which suggested that the size of the sulphide-oxisiding population was significantly larger in the walls and the ceiling.

According to Tables 4 and 5, the species richness of SOB at the different locations in the HS was higher in R2, which could be at least partially related to the longer operation time. Sample A2 presented the highest SOB species richness; two or three SOB genera were found in the rest of the samples taken from R2 (Table 5). It must be noted that the genus *Rhodococcus* was found only in D2–F2, whereas *Alicyclobacillus* sp. was present only in A2–C2. Furthermore, although the difference between the samples was not as significant as in R1, the intensity of the bands representing SOB in A2 and C2 was higher than in the rest of the samples (Fig. 5b). Therefore, the moisture level seemed to determine the composition, species richness, and size of the SOB population, which is indeed consistent with the previous observations.

Obviously, the growing conditions in both HS changed over time as a result of H_2S oxidation. Along with the availability of water and O_2, pH, trophic property, and the ability to utilise different S-compounds probably conditioned the order of appearance of the SOB species in the HS. So, although both reactors operated normally under O_2-limiting conditions in order to completely convert H_2S into S^0, some $S_2O_3^{2-}$ and SO_4^{2-} could also be formed as a result

of occasional increases in sulphide load or in O_2 availability, respectively, especially in R2, which operated for longer. Hence, presumably the genera of SOB found in R1 was more accurate in representing the population carrying out the H_2S oxidation at the early stage of the microaerobic operation than those identified in R2. Namely, it was possible to estimate a succession of SOB.

4. Conclusion

Biogas desulphurisation took place in the HS of both reactors, excluding the liquid interface. A cleaning interval of less than 14 months was found to be necessary in order to minimise the micro-oxygenation cost. Once microaerobic conditions were restored after the HS cleaning, all the H_2S was rapidly removed from the biogas, which suggested chemical oxidation. The moisture level determined the composition, species richness and size of the SOB population at the various locations within the HS.

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