Microaerobic digestion of sewage sludge on an industrial-pilot scale: the efficiency of biogas desulphurisation under different configurations and the impact of O_2 on the microbial communities

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Abstract

Biogas produced in an industrial-pilot scale sewage sludge reactor ($5m^3$) was desulphurised by imposing microaerobic conditions. The H_2S removal efficiency was evaluated under various configurations: different mixing methods and O_2 injection points. Biogas was entirely desulphurised under all the configurations set, while the O_2 demand of the digester decreased over time. Although the H_2S removal seemed to occur in the headspace, S^0 (which was found to be the main oxidation product) was scarcely deposited there. O_2 did not have a significant impact on the digestion performance; the

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VS removal remained around 47%. Conversely, DGGE revealed that the higher O_2 transfer rate to the sludge maintained by biogas recirculation increased the microbial richness and evenness, and caused an important shift in the structure of the bacterial and the archaeal communities in the long term. All the archaeal genera identified (*Methanosaeta*, *Methanospirillum* and *Methanoculleus*) were present under both anaerobic and microaerobic conditions.

Keywords

Biogas desulphurization; DGGE; hydrogen sulfide; microaerobic digestion; microbial communities

1. Introduction

Anaerobic digestion (AD) is one of the oldest and most widely used processes for wastewater sludge stabilisation. It successfully reduces the quantity of solids, destroys pathogenic organisms, minimises odour problems, and produces an agricultural fertiliser, the digestate, and a renewable and versatile energy source, the biogas (Ward et al., 2008). Thus, AD optimises the costs of wastewater treatment plants (WWTPs) (Appels et al., 2008).

The biogas produced during AD is commonly used for heat and electricity production (Rasi et al., 2007) in relatively small and easy-to-manage industrial units (Antoni et al., 2007). A well-operated anaerobic reactor produces more energy (in the form of biogas) than that required on site, and the excess is usually sold to a local utility for domestic

use (Turovskiy and Mathai, 2006). Alternatively, it can be fed to the gas grid, or used as a fuel in combustion engines, cars, or fuel cells (Appels et al., 2008)

Biogas from sewage digesters usually contains around 55-65% of CH_4, 35-45% of CO_2, and 1% of N_2. Nevertheless, it typically also presents traces of H_2S and other S-containing compounds (Rasi et al., 2007). Besides its bad smell, H_2S is highly non-desirable in energy-recovery processes because it converts into SO_2 and H_2SO_4, which are highly corrosive, unhealthy and environmentally hazardous compounds (Abatzoglou and Boivin, 2009). Besides, H_2S itself is reactive with most metals (Appels et al. 2008); it attacks iron, copper, cement, etc (Noyola et al., 2006). As a result, the biogas quality in the different technologies for energy recovery is assessed as H_2S content.

The biogas generated during AD of sewage sludge can present H_2S concentrations above the technical limits recommended by the facility manufacturers; it can contain up to 10,000ppmv. Nonetheless, the usual H_2S concentration in the biogas produced in WWTPs is about 1,000ppmv. Therefore, with traditional boilers and internal combustion engines, in most cases the biogas can be used without any treatment, since the recommendations are that hydrogen sulphide content should not be more than 1,000 ppmv. Conversely, when the biogas is not used on site, the exhaustiveness required in the desulphurisation is much higher. The strictest limits of H_2S are set for fuel cells, where its concentration in the biogas must not exceed 0.1ppmv (Rasi et al., 2011).

The removal of H_2S from biogas can be carried out by biological and physico-chemical treatments. Besides being ecologically cleaner, the biological desulphurisation

techniques (the most notable of which are biofilters, biotrickling filters and bioscrubbers) are inexpensive in terms of capital outlay and operating costs (Burgess et al., 2001). Nevertheless, physico-chemical processes are the most common methods for biogas desulphurisation. Among them, the dosing of iron chloride to the digester is one of the most widespread solutions (Persson and Wellinger, 2006), especially for sewage sludge digesters, where H_2S concentrations of below 150ppmv can be achieved (Deublein and Steinhauser, 2008). However, it implies high costs due to large chemical consumption and iron sludge production (Abatzoglou and Boivin, 2009). The rest of the popular physico-chemical methods are applied in the gas stream and in the upgrading process, such as adsorption on activated carbon (Krischan et al., 2012) and water scrubbing (Appels et al., 2008), respectively.

As an alternative to internally and inexpensively desulphurise biogas, air/O_2 can be injected into the anaerobic reactor; thus, there is no hazardous reagent usage and chemical sludge production and H_2S levels of less than 50ppmv can be achieved (Díaz et al., 2010b). The basis of this method lies in the presence of sulphide-oxidising bacteria (SOB) in the feedstock (Weiland, 2010), which convert dissolved sulphide into S^0 and SO_4^{2-} (van der Zee et al., 2007). The ratio of $SO_4^{2-}/S^0_{produced}$ depends on the dissolved O_2 concentration; limited O_2 availability and high sulphide loads favour S^0 production (Fortuny et al., 2008). Moreover, under such conditions, SOB can effectively compete with the chemical sulphide oxidation (Robertson and Kuenen, 2006). In comparison to the other biological processes, microaeration/microoxygenation of “anaerobic” digesters has a smaller footprint, and entails lower capital outlay and only a minor modification of the process flowchart.

The feasibility of sulphide oxidation during digestion under microaerobic conditions has been widely demonstrated; the process successfully competes for O_2 with other oxidative processes, and is faster than re-reduction of the oxidised sulphur species (van der Zee et al., 2007). Moreover, the presence of O_2 has been proved not to have a negative effect on either the organic matter removal, the biogas or the methane productivity (Fdz-Polanco et al., 2009; Díaz et al., 2010a; Díaz et al., 2010b). In fact, the full-scale experiments carried out by Jenicek et al. (2008, 2010) revealed that, besides efficiently desulphurising biogas, an intimate contact between the O_2 and the liquid phase can improve organic matter degradation, digestate quality and productivity of both biogas and CH_4 during municipal sludge digestion. This can arise from higher hydrolysis rates (Botheju et al., 2010; Lim and Wang, 2013), alleviation of sulphide toxicity (Jenicek et al., 2010; Zhou et al., 2007), and shifts in microbial activity and populations (Jenicek et al., 2011; Rodríguez et al., 2012; Tang et al., 2004; Zhou et al., 2007). Specifically, Zhou et al. (2007) observed that the rod-shaped methanogens almost disappeared and were replaced by cocci-shaped ones after imposing microaerobic conditions. Tang et al. (2004) reported a dramatic increase in the population size of *Methanoculleus* due to microaeration. In the meantime, they found a significant decrease in the population of *Methanosarcina*, which suggested that *Methanoculleus* sp. out-competed *Methanosarcina* sp. due to a higher tolerance for O_2.

The most profitable oxidative reactant for imposing microaerobic conditions during digestion is O_2 since air implies further biogas dilution (Díaz et al., 2010a; Jenicek et al. 2010). On the other hand, Díaz et al. (2010b) demonstrated that the most economical configuration for desulphurising biogas during the pilot-scale digestion of sewage

sludge consisted of O_2 supply to the headspace and liquid recirculation, which is consistent with the fact that the H_2S removal takes place in the gas space of the reactor (Ramos et al., 2012). However, further research is required in order to corroborate these findings on a larger scale. Hence, the main aims of this research were:

- to investigate the efficiency of the process of biogas desulphurisation in an industrial-pilot scale digester under different configurations (mixing method and concentrated O_2 dosing point)
- to study the impact of micro-oxygenation on the microbial communities

2. Materials and methods

2.1. Industrial-pilot scale reactor

The research was carried out in a continuous stirred tank reactor with $5m^3$ ($7m^3$ of total volume) located at the WWTP of Villalonquéjar (Burgos, Spain). Temperature (35ºC) was maintained by a water heat exchanger and monitored by probes (Fig. 1). Mixed sludge produced in the aforementioned municipal WWTP was continuously pumped to the digester by a screw pump. It presented a highly variable organic load, with a VS concentration ranging between 24.7 and 44.9g/L. As a result, its rheology was highly changeable, which in turn led to fluctuations in the feeding flow rate. Hydraulic retention time (HRT) was approximately 20d. The reactor's content was recirculated by a peristaltic pump at a rate of approximately $25m^3/d$. Depending on the operational period, a compressor provided $21m^3/d$ of biogas recirculation (Fig. 1). Microaerobic conditions were implemented from the period P2 to P8 using an O_2 concentrator (Table 1), which achieved a purity of 92-98%. O_2 was introduced intermittently (approximately every 10min). Fig. 1 shows the O_2 dosing point in each operational period.

Pressure was monitored by a sensor. Biogas production was quantified by a thermal mass flow meter, and its composition was determined by gas chromatography (GC) (CP-4900 Micro-GC) according to Díaz et al. (2010b) from the beginning of the study to the 87th day. From the 117th day, it was measured by a gas analyser (GA-3000) whose precision in determining CH_4, O_2 and H_2S was ±3%v/v, ±1%v/v, and ±5% of the result or 500ppm (the largest value), respectively. SCADA software was used for monitoring; data (pressure, temperature, biogas production and recirculation rate) were displayed and stored in real-time in a computer.

2.2. Experimental procedure

The reactor was inoculated with sludge from a full-scale anaerobic reactor located at the aforementioned WWTP. It was started-up under anaerobic conditions, using sludge recirculation as the mixing method. HRT was set at about 24d until the 50th day; thereafter, it was around 20d. At day 135 (day 0 in Fig. 2), it was considered that a stationary state had been achieved.

The study was divided into eight operational periods (Table 1): two anaerobic (P1 and P5) and six microaerobic (P2, P3, P4, P6, P7 and P8). In P1, P2, P7 and P8, the reactor's content was mixed by sludge recirculation (Fig. 1). In the rest of the experimental periods, the digester operated with both biogas and sludge recirculation.

In P2, O_2 was supplied to the gas space, just above the liquid interface, where the H_2S removal from biogas reportedly occurs (Abatzoglou and Boivin, 2009; Weiland, 2010), and the flow rate was gradually raised from approximately 12 to 34L/m^3/d. During P3,

the micro-oxygenation level was gradually lowered to 14L/m^3/d. In P4, the O_2 dosing point was changed; it was injected into the biogas recirculation at a constant flow rate (14L/m^3/d). Micro-oxygenation was interrupted in order to evaluate the H_2S production during P5. In P6, the configuration and the O_2 flow rate set in P4 were restored. During P7, the biogas recirculation was stopped, and O_2 was injected into the liquid phase, just above the midpoint of the digester. As shown in Table 1, the O_2 flow rate was gradually lowered from 14 to 5L/m^3/d. In the last experimental period (P8), the configuration applied during P2 was restored, while the micro-oxygenation level remained constant (5L/m^3/d).

2.3. Chemical analysis

The feed and the digested sludge were analysed in terms of total and soluble chemical oxygen demand (TCOD and SCOD), total and volatile solids (TS and VS), carbon (C) and sulphur (S), total kjeldahl nitrogen (TKN), N-NH_4^+, NO_2^- , NO_3^-, PO_4^{3-}, sulphide, SO_4^{2-}, and $S_2O_3^{2-}$. Specifically, NO_2^- , NO_3^-, PO_4^{3-} were analysed by UV-visible spectroscopy (APHA, 1998), SO_4^{2-} and $S_2O_3^{2-}$ concentrations were determined by ion chromatography (APHA, 1998) and high-performance liquid chromatography (van der Zee et al., 2007), respectively. Dissolved sulphide was analysed by potentiometric titration (APHA, 1998), and elemental analysis (C and S) were carried out by IR spectroscopy (LECO CS-225). The pH of the reactor was monitored by a pH-meter with a temperature probe.

2.4. Microbial analysis

In order to evaluate how the presence of O_2 affected the microbial community, four biomass samples (S1, S2, S3 and S4) were collected and stored immediately at -20ºC. Samples S1, S2, S3 and S4 were taken in P1 (18rd day), P2 (67 rd day), P3 (109th day) and P4 (165th day), respectively.

The V6-V8 and the V2-V3 regions of the bacterial and the archaeal 16S rRNA genes were amplified by polymerase chain reaction (PCR) using the universal bacterial primers 968-F-GC and 1401-R, and A 109(T)-F and 515-GC-R (Sigma- Aldrich, St. Louis, MO, USA), respectively (Rodríguez et al., 2012). The PCR mixture contained 1µL of each primer (10 ng µL-1 each primer), 25µL of BIOMIX ready-to-use 2× reaction mix (Bioline, Ecogen), PCR reaction buffer and deoxynucleotide triphosphates, 2µL of the extracted DNA, and Milli-Q water up to a final volume of 50 µL. PCR was performed in a iCycler Thermal Cycler (Bio Rad Laboratories, Inc.) applying the thermo-cycling program described by Rodríguez et al. (2012). DGGE analysis of the bacterial and archaeal amplicons were performed according to Rodríguez et al. (2012). DGGE profiles were compared using the 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). Similarity dendrograms were constructed by using UPGMA clustering with error resampling (500 resampling experiments).

Individual bands were excised from the archaeal DGGE gel. Both DNA extraction and purification of PCR products were carried out according to Rodríguez et al. (2012). The

taxonomic position of the sequences was obtained using the RDP classifier tool (80% confidence level) (Wang et al., 2007). Moreover, the sequences were compared with those included in GenBank by the BLAST search tool at the National Centre for Biotechnology Information (NCBI) website (McGinnis and Madden, 2004). Along with BLAST, DECIPHER was used as the chimera checking tool (Wright et al., 2012). Finally, the sequences were deposited in GenBank under accession numbers KJ402278-KJ402293.

3. Results and discussion

3.1. Biogas quality

3.1.1. H_2S and O_2

The average sulphide content of the biogas produced in P1 (under anaerobic conditions) was 0.49%v/v (Table 2). Microaerobic conditions were applied on the 29th day (period P2) by injecting around 12LO_2/m^3/d into the gas space, just above the liquid interface (by a single point) (Table 1). Under such a configuration, presumably the O_2 transfer rate to the sludge was significantly limited. Moreover, it is possible that O_2 could only be transferred to a limited area in the headspace (around the injection point). Within 24h, the H_2S concentration decreased to 0.35%v/v (Fig. 2a). It indeed remained at around that value until the 54th day, despite the gradual increase in the O_2 supply. Thereafter, at micro-oxygenation levels above 29L/m^3/d, the biogas sulphide content remained below 0.10%v/v. Specifically, from the 70th to the 74th day (at approximately 34LO_2/m^3/d), the average H_2S and O_2 concentration in biogas was 0.02 and 4.3%v/v, respectively. It is worth noting that at even the highest micro-oxygenation level, the biogas N_2 content did not exceed 0.1%v/v.

On the 74th day (period P3), the biogas recirculation was started, while the O_2 supply was maintained at approximately 34L/m^3/d (Table 1). As expected, the biogas O_2 content dropped significantly (to approximately 1.2%v/v), which was attributed to improved O_2 transfer due to higher contact between the gas and the liquid phase. The H_2S concentration in biogas also decreased, and stabilised at around zero. Hence, from the 77th to the 87th day, the O_2 flow rate was gradually reduced from approximately 31 to 19LO_2/m^3/d. This hardly affected the H_2S and the O_2 concentration in the biogas. Regarding the O_2 content in the biogas, it presumably remained constant due to lesser O_2 consumption in unidentified processes, which could in turn result from the lower gradient of O_2 in the liquid phase.

In contrast to Díaz et al. (2010b), who reported a decrease in dissolved sulphide in the digester from approximately 130mg/L to less than 40mg/L due to the implementation of biogas recirculation, the digestate sulphide content in P3 did not decrease significantly in relation to the previous operational periods. The feeding sulphide content was around 26mg/L during the whole study, while the sulphide concentration in the effluent in P1, P2 and P3 was 58, 63 and 50mg/L, respectively.

During P4, the O_2 was introduced in the biogas recirculation instead of in the gas space (Table 1). As expected, the H_2S and the O_2 concentration in the biogas remained almost constant (around zero). Regarding the O_2 concentration, it must be noted that the difference between the first and the last 12 days of P3 was due to the change in the measuring device (Fig. 2a).

In P5, the micro-oxygenation was stopped in order to evaluate the H_2S concentration under anaerobic conditions (Table 1). The biogas sulphide content remained around 0.25%v/v (Table 2). The considerable decrease in the H_2S concentration in relation to P1 was related to the sludge variability. At this point, it must be noted that, besides a variable organic load, the feeding presented a highly variable concentration of S. Specifically, the C and S content of the sewage sludge was in the range of 29-37 and 0.5-2.0%w/w, respectively.

When microaerobic conditions were restored with the same configuration as in P4 (period P6), the sulphide content of the biogas dropped to an average of 0.01%v/v, while the surplus of O_2 hardly rose (Fig. 2a). It must be mentioned that the peaks of H_2S concentration detected on the 223rd and 224th day could result from the increase in the biogas production recorded during those days. Once the biogas recirculation was stopped (period P7), the H_2S concentration decreased negligibly. Regarding the biogas O_2 content, it hardly changed, which could be related to the precision of the gas analyser; it was expected to rise due to lower O_2 transfer to the sludge. In fact, from the 235th day, although the O_2 supply was gradually lowered, the biogas composition remained almost constant in terms of both O_2 surplus and H_2S concentration. From the 250th day until the end of the study, the O_2 flow rate was maintained at around 5L/m^3/d.

In P8, the H_2S and O_2 concentrations in the biogas were almost equal to those in P7 (Table 2), which highlighted that the success of the biogas desulphurisation was independent of the O_2 transfer rate to the liquid phase. At this point, it should be mentioned that the objective of the configuration applied in P7 was to maintain a

successful O_2 transfer to the all the surfaces of the headspace. As a result, it was concluded that the biogas desulphurisation took place predominantly in the gas space.

Despite applying the same configuration, the molar ratio of O_2 supplied to H_2S produced which was required to achieve similar H_2S concentrations in the biogas in P2 was almost four times higher than in P8 (8 against 2v/v, respectively) (Table 1). Maybe the population size of SOB in the area of the headspace with sufficient O_2 availability during P2 (that around the O_2 injection point) did not suffice to achieve efficient biogas desulphurisation. Presumably the SOB population in the gas space increased over time due to the accessibility of both H_2S and O_2.

3.1.2. CH_4

The average CH_4 content of the biogas produced in P1 was 63.5%v/v (Table 2). During P2, while the surplus of O_2 in the biogas increased, the CH_4 concentration declined gradually; it was specifically 62.4%v/v on average (Fig. 2b). It should be noted that this oscillated between 61.7 and 60.9%v/v from the 87th day. Subsequently, in P3, due to the implementation of biogas recirculation and the consequently higher O_2 consumption in the reactor, the biogas CH_4 content rose, remaining around 62.7%v/v until the 67th day. Consequently, the decrease in the CH_4 concentration observed in P2 was attributed to dilution rather than aerobic oxidation of methanogenic substrate. In fact, the ratio of CH_4 to CO_2 was 1.8 in both P1 and P2 (Table 2). In P3, before the change in the measuring device of biogas composition, this variable was negligibly lower (1.7), which indicated that the additional O_2 was scarcely employed to oxidise soluble organic matter by aerobic respiration; this finding was confirmed in the following operational periods.

Nonetheless, it must be considered that slight changes in the biogas composition could arise from the variability of the feeding composition.

In P3, after the 117th day, due to the replacement of the measuring device, the CH_4 and O_2 concentrations were significantly lower, and the CH_4/CO_2 ratio remained around 1.5 (Fig. 2b). The average biogas CH_4 content in P3 from the aforementioned day was specifically 60.0%v/v. In the rest of the operational periods, including P4, P5 (anaerobic), P6, P7 and P8, it was 59.1, 58.8, 59.4, 60.1 and 59.2%v/v (respectively) (Table 2). Regarding the CH_4/CO_2 ratio, it varied negligibly, which along with the biogas production (Table 3) suggested that O_2 did not cause a significant impact on the reactor productivity, independently of its availability.

3.2. Digestion performance

Due to the continuous changes in the organic loading rate to the reactor arising from the variability of the feeding composition, the digestion performance (organic matter removal and biogas productivity) could not be estimated with the necessary precision in order to identify significant differences between the various operational periods, specifically, between P1, P2, P3 and P4, that is, the periods with a sufficiently long duration. With regard to this, it must be considered that the reactor was started-up and operated under the configuration set in P1 for 135 days.

The VS removal in P1, which was considered the baseline period, was 45% on average and, during P2, P3 and P4, this variable fluctuated around that value (Table 3). Likewise, neither the digestate quality deteriorated significantly nor the biogas

production or its CH_4 content decreased substantially during the different stages in relation to P1. Similarity to the CH_4 concentration in the biogas, the TS and VS content of the digestate and the biogas production both fluctuated within a narrow range all through the research (31.6-32.8g/L, 17.0-18.1g/L, and 0.86-1.08$m^3/m^3/d$, respectively). Moreover, these variables rose concurrently, which was consistent with the increasing VS content of the feeding (data not shown). These results are in turn consistent with those reported in literature, since even on an industrial scale, it has been broadly demonstrated that the presence of limited amounts of O_2 in digesters does not negatively affect digestion performance (Jenicek et al., 2008, 2010).

3.3. Sulphide oxidation products

According to the SO_4^{2-} and $S_2O_3^{2-}$ analysis, H_2S was mostly oxidised to S^0. $S_2O_3^{2-}$ was detected in negligible amounts in both the sewage and the digested sludge. The average SO_4^{2-} concentration in the feeding from P1 to P4 was between 141 (P4) to 238mg/L (P1), while the effluent SO_4^{2-} content remained between 24 (P1) and 29mg/L (P2). According to the H_2S production and the operational conditions, the expected increase in the digestate S content during the microaerobic periods in relation to P1 was also negligible. Moreover, even if it had been significant, it would hardly have been detectable due to the aforementioned variable S concentration in the feeding. Nevertheless, it is worth noting that the effluent S and C content of the digested sludge oscillated continuously between 0.7 and 2.0%w/w, and between 22 and 28%w/w, respectively.

After approximately 415 days of operation (240 days under microaerobic conditions), the ceiling of the reactor was removed. A relatively thick deposit with 19%w/w of C and 58%w/w of S was observed at the area of the walls surrounding the liquid phase. Presumably it formed due to the slight, unavoidable fluctuations in the liquid level and even sludge splashes. The rest of the headspace was covered with a really thin, dry layer with 6 and 86%w/w of C and S, respectively. The high degree of dryness observed in most of the surfaces of the gas space after the operation shutdown suggested that moisture availability could limit the growth of SOB in the headspace. This could be the cause of the low desulphurisation performance and relatively high O_2 demand maintained at the early stage of the microaerobic operation (Fig. 2a).

Considering that the biogas desulphurisation appeared to occur predominantly in the headspace (see 3.1.1), and, as noted above, H_2S was mostly converted into S^0, larger amounts of this compound were expect to be found all over the headspace. Presumably the S^0 formed there fell into the sludge, thereby leaving the reactor with the effluent. Since the main factor affecting the profitability of the implementation of microaerobic conditions in full-scale reactors is the required periodicity for cleaning the headspace (Díaz, 2011), this observation could be of utmost importance.

3.4. Microbial communities

3.4.1. Biodiversity

In order to evaluate the richness and evenness of species (diversity) in the reactor in P1 (sample S1), P2 (sample S2), P3 (sample S3), and P4 (sample S4) the Shannon-Wiener index (*H*) was calculated from both DGGE gels (Fig. 3a and b). This index typically

ranges from 1.5 to 3.5 (McDonald, 2003). All the samples showed a moderate archaeal diversity, with *H* ranging from 2.4 to 2.8 (Fig. 3a). The diversity indices calculated from the bacterial DGGE gel were in the range of 2.6 to 3.1, which highlighted a moderately high bacterial richness and evenness (Fig. 3b).

The archaeal diversity of the sample S3 (2.4) was negligibly lower than that of S2 and S1 (2.5), while S4 presented the highest *H* (2.8) (Fig. 3a). On the other hand, S1 and S4 exhibited the lowest and the highest bacterial diversity (2.6 and 3.1, respectively), while S2 and S3 presented the same *H* (2.9) (Fig. 3b). Therefore, the O_2 availability in the sludge maintained during P2 sufficed to cause a significant increase in the bacterial diversity. Likewise, the archaeal and the bacterial richness and evenness both rose in P4. This indicated that the increase in the O_2 transfer rate to the liquid phase arising from the implementation of biogas recirculation in P3 positively affected the microbial diversity in the long term (in period P4).

3.4.2. Similarity

The pair-wise similarity indices indicated a moderate-high correspondence between the microbial communities growing in the sludge at the different periods of the study (Fig. 4). The similarity coefficients between the archaeal communities fluctuated between 60 and 91% (Fig. 4a). Sample S1 and S2 presented the highest similarity, which suggested that the archaeal communities were only slightly affected by the injection of O_2 into the headspace. This was consistent with the presumably low O_2 transfer to the sludge maintained during P2. Likewise, a high similarity was also found between sample S3 and S4 (87%). By contrast, the similarity indices between the rest of the sample pairs

were lower than 70%. Therefore, the implementation of biogas recirculation in P3 and the resulting increase in the O_2 transfer rate to the sludge caused a significant impact on the structure of the archaeal communities.

The similarity coefficients between the bacterial communities fluctuated from 41 to 93% (Fig. 4b). The highest similarities were found between S1 and S2 (93%), S1 and S3 (91%), and S2 and S3 (90%). Conversely, S4 presented a similarity of between 41 and 53% with the rest of the samples. Hence, as in the archaeal communities, the bacterial communities were only slightly affected when O_2 was injected into the gas space. The increase in the O_2 transfer rate to the liquid phase during P3 also had a limited impact on them; however, in the long term (P4), it caused a significant change in the structure of the bacterial communities.

3.4.2. Archaeal DGGE dynamics

From the archaeal DGGE gel (Fig. 3a), sixteen bands were sequenced (Table 4). According to the RDP classifier (confidence threshold of at least 80%), all of them belonged to the Euryarchaeota phyla. Band 1 and 2 remained unclassified, and the rest were all ascribed to one class, Methanomicrobia, and two orders, Methanosarcinales (band 3-9) and Methanomicrobiales (band 10-16). According to the BLAST search tool, band 1 and 2 could belong to the Methanosarcinales order. The rest of the results obtained from this database were consistent with those given by the RDP classifier.

Most of the sequences were close to sequences of already-known methanogenic genera retrieved from reactors (Leclerc et al., 2004). The sequences affiliated to the

Methanosarcinales order were assigned to the *Methanosaeta* genus (Table 4), which contains acetotrophic methanogens capable of utilising acetate. On the other hand, from all the DGGE bands ascribed to the Methanomicrobiales order, six (10-15) were assigned to *Methanospirillum* sp., and band 16 was assigned to *Methanoculleus* sp.. These two genera have been reported to consist of hydrogenotrophic methanogens (Robertson and Kuenen, 2006).

All the genera identified were present in the four samples. Moreover, according to the bands intensity, O_2 increased the evenness of all these genera (Table 4). Therefore, in contrast to Tang et al. (2004), micro-oxygenation did not seem to cause a particular impact on any of the archaeal populations.

4. Conclusions

Biogas produced in an industrial-pilot reactor was efficiently desulphurised independently of the mixing method and the O_2 dosing point. The O_2 demand of the digester decreased with time. The H_2S removal seemed to occur in the headspace; however, S^0, which was found to be the main oxidation product, scarcely accumulated there. O_2 did not have a significant impact on digestion. In the long term, the relatively high transfer rate of O_2 to the sludge maintained by the biogas recirculation caused a significant change in the structure and a considerable increase in the richness and evenness of the microbial communities.

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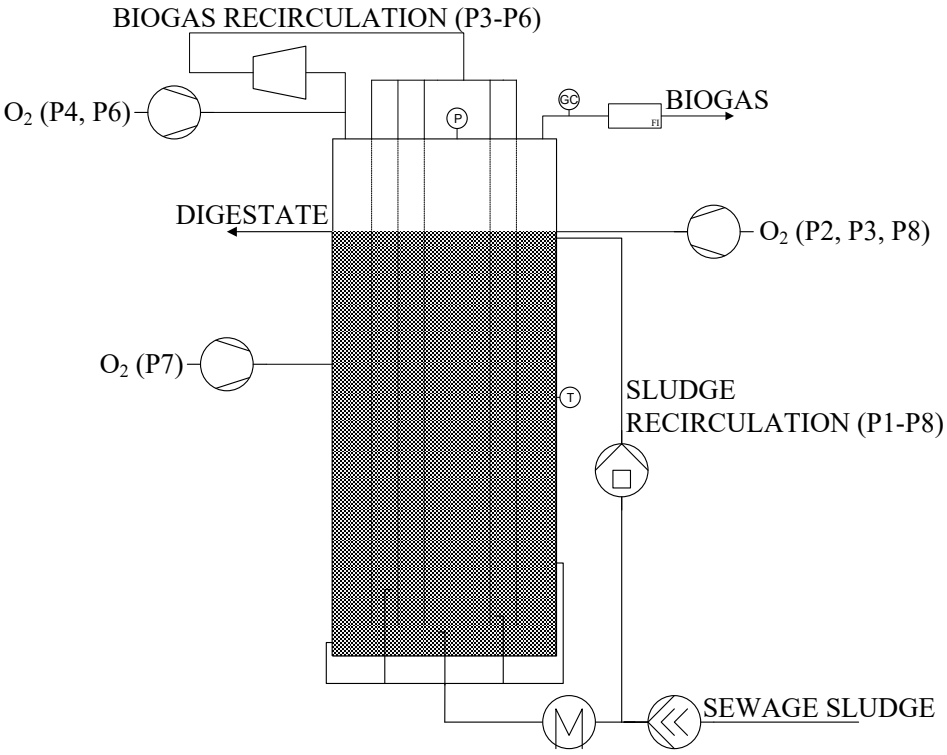


Fig. 1. Digester diagram. Operational periods (P1-P8) are indicated in brackets.

Table 1. Sequence of variations applied to the digester.

Period	Conditions	Recirculation	O_2 dosing point	O_2 flow rate ($L/m^3/d$)
P1	Anaerobic	Sludge	-	-
P2	Microaerobic	Sludge	Headspace	12-34 (increasing)
P3	Microaerobic	Sludge and biogas	Headspace	34-14 (decreasing)
P4	Microaerobic	Sludge and biogas	Biogas recirculation	14
P5	Anaerobic	Sludge and biogas	-	-
P6	Microaerobic	Sludge and biogas	Biogas recirculation	14
P7	Microaerobic	Sludge	Liquid phase	14-5 (decreasing)
P8	Microaerobic	Sludge	Headspace	5

Table 2. Biogas quality.

Period	O_2 (%v/v)	H_2S (%v/v)	CH_4 (%v/v)	CH_4/CO_2
P1	0.0	0.49	63.5	1.8
P2	1.7	0.21	62.4	1.8
P3	0.9	0.01	62.0	1.7
P4	0.1	0.02	59.1	1.5
P5	0.0	0.25	58.8	1.5
P6	0.1	0.01	59.4	1.5
P7	0.0	0.00	60.1	1.5
P8	0.0	0.00	59.2	1.4

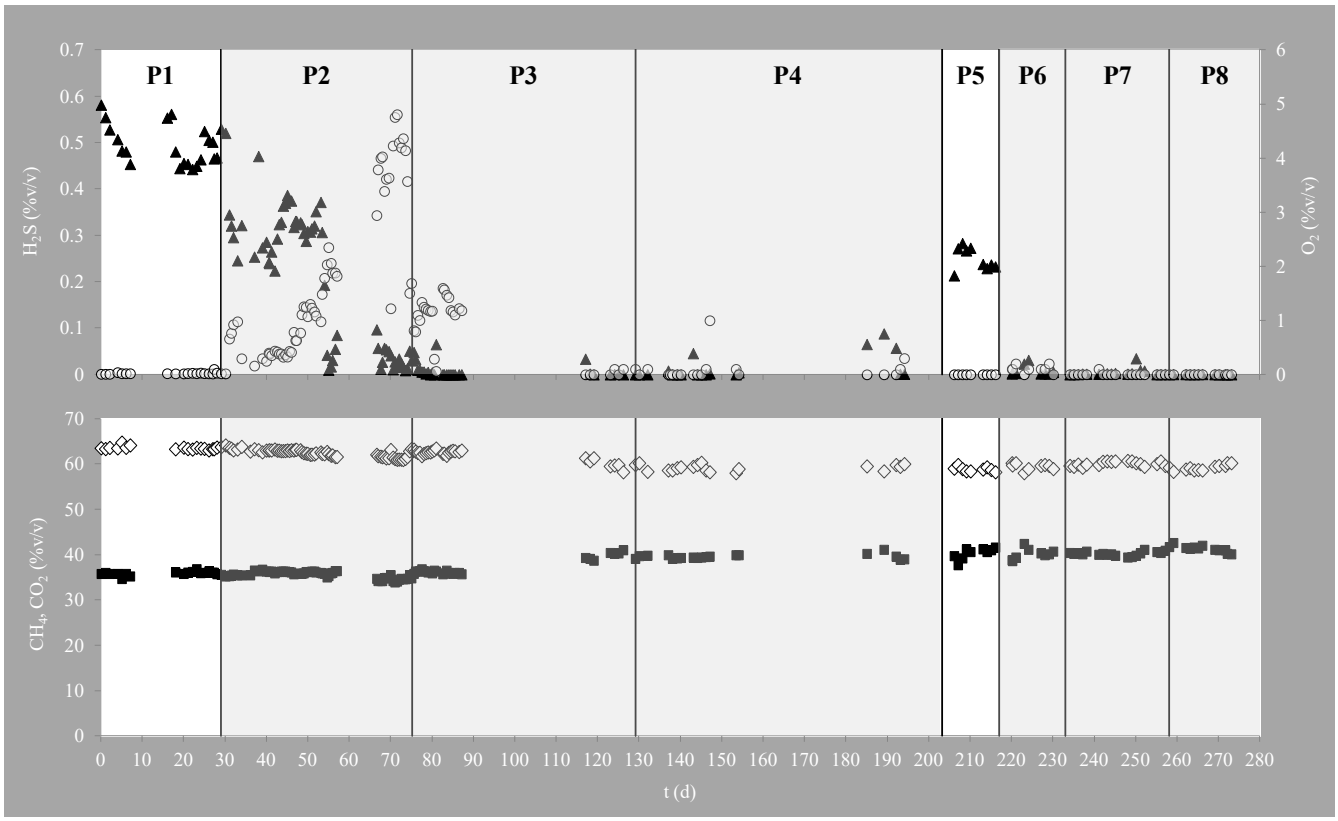


Fig. 2. Concentration of H_2S (▲) and O_2 (○) (a), and CH_4 (◊) and CO_2 (■) (b).

Anaerobic periods are represented in white.

Table 3. Biogas production, digestate quality and VS removal.

	P1	P2	P3	P4	P5	P6	P7	P8
Biogas production ($m^3/m^3/d$)	0.86	0.92	0.94	0.97	1.01	1.05	1.08	1.05
TS (g/L)	31.6	31.9	32.8	32.8	31.1	29.5	29.8	30.0
VS (g/L)	17.0	17.4	18.1	17.8	17.0	17.3	17.4	17.6
VS removal (%)	45	46	44	46	48	47	49	47

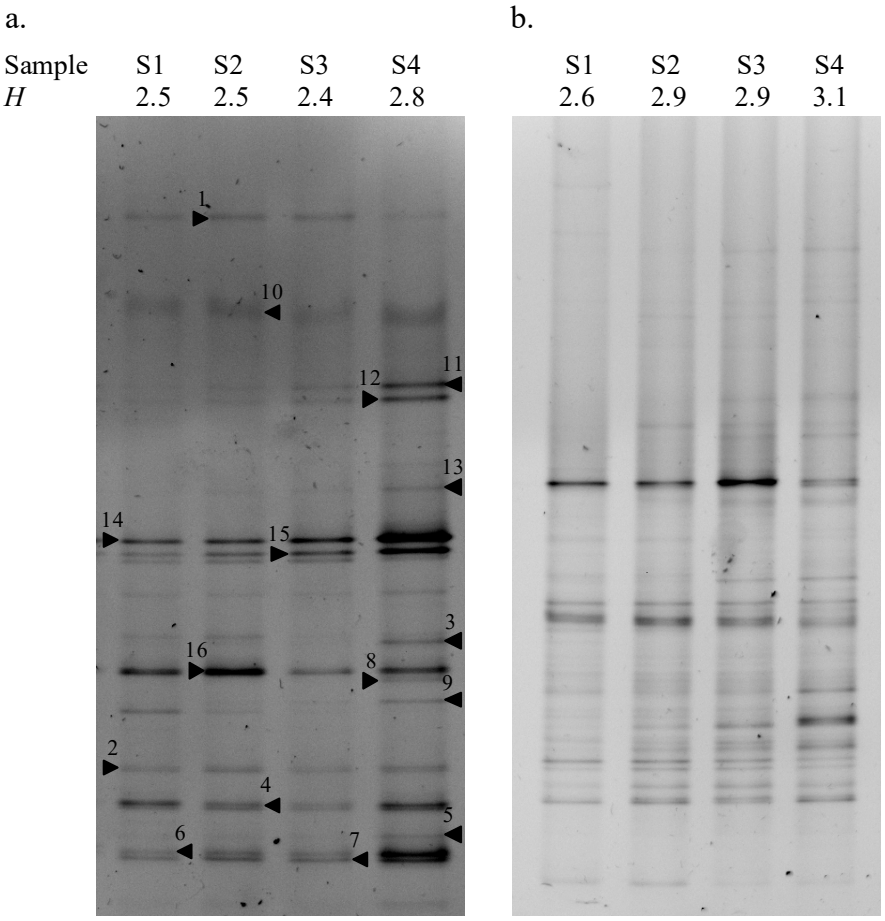
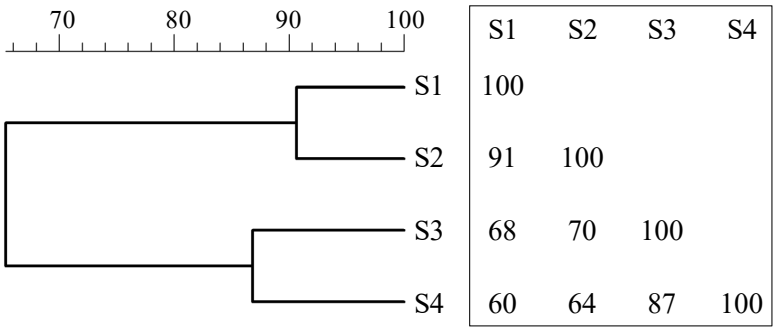


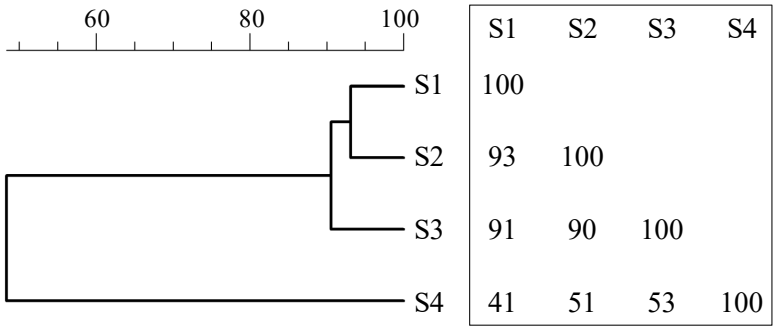
Fig. 3. Archaeal (a) and bacterial (b) DGGE profiles of the 16S rRNA amplicons of the samples S1-S4 with their respective diversity indices.

a.



	S1	S2	S3	S4
S1	100			
S2	91	100		
S3	68	70	100	
S4	60	64	87	100

b.



	S1	S2	S3	S4
S1	100			
S2	93	100		
S3	91	90	100	
S4	41	51	53	100

Fig 4. Archaeal (a) and bacterial (b) similarity dendrogram (UPGMA clustering) and matrix with error resampling (500 resampling experiments).

Table 4: RDP classification of the archaeal DGGE bands sequenced with at least 80% of confidence level, and corresponding closest relatives in Genbank obtained by the BLAST search tool with their similarity percentages, and environments from which they were retrieved. Intensity < 35 = ×, 35 ≤ intensity ≥ 80 = ××, intensity > 80 = ×××.

Taxonomic placement (confidence threshold ≥ 80%)	Band	S1	S2	S3	S4	Closest relatives (accession nº in Genbank)	Similarity (%)	Source of origin
Phylum Euryarchaeota	1 (KJ402278)	×	×	×	×	Uncultured archaeon (CU917025)	98	Mesophilic anaerobic digester which treats municipal wastewater sludge
						Uncultured *Methanosaeta* sp. (KC769082)	97	Active sludge
	2 (KJ402279)	×	×	×	×	Uncultured archaeon (HM639839)	98	Activated sludge wastewater treatment plant
						Uncultured Methanosarcinaceae (AJ879026)	97	Rice rhizosphere
Class Methanomicrobia								
Order Methanosarcinales								
Family Methanosaetaceae								
Genus *Methanosaeta*	3 (KJ402280)				×	Uncultured archaeon (KC412610)	99	Lab-scale anaerobic digester of agricultural waste material
						Uncultured *Methanosaeta* sp. (HQ290282)	98	Anaerobic digester fed with distillers grains
	4 (KJ402281)	××	××	×	××	Uncultured archaeon (FJ222220)	99	Biogas plant supplied with cattle liquid manure, cattle dung, maize silage, gras silage, grains
						Methanosaeta concilii (AB679168)	97	Culture collection
	5 (KJ402282)	×	×	×	×	Uncultured archaeon (EU636895)	99	Biogas plant (mesophilic CSTR) supplied with cattle liquid manure and maize silage
						Methanosaeta concilii (AB679168)	97	Culture collection
	6 (KJ402283)	×	××	×	×××	Uncultured archaeon (EU926764)	99	Anaerobic biogas reactor fed with core silage
						Methanosaeta concilii (AB679168)	97	Culture collection
	7 (KJ402284)	×	××	×	××	Uncultured archaeon (KC412572)	99	Lab-scale anaerobic digester
						Methanosaeta concilii (AB679168)	97	Culture collection
	8 (KJ402285)				×	Uncultured archaeon (HM639803)	97	Activated sludge wastewater treatment plant
						Uncultured archaeon (AJ576227)	97	Landfill leachate
	9 (KJ402286)		×	×	×	Uncultured archaeon (KC412593)	97	Lab-scale anaerobic digester
						Uncultured archaeon (EU926764)	97	Anaerobic biogas reactor fed with core silage
Order Methanomicrobiales								
Family Methanospirillaceae								

Genus *Methanospirillum*	10 (KJ402287)	×	×	×	×	Uncultured euryarchaeote (AB175351)	99	Mesophilic anaerobic BSA digester
						Uncultured Methanomicrobiales (CU917420)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	11 (KJ402288)	×	×	×	××	Uncultured Methanomicrobiales (CU916501)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
						Uncultured *Methanospirillum* sp. (HQ290290)	98	Anaerobic digester fed with distillers grains
	12 (KJ402289)	×	×	×	××	Uncultured euryarchaeote (AB248620)	99	Mesophilic anaerobic butyrate degrading reactor
						Uncultured Methanomicrobiales (CU916636)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	13 (KJ402290)			×	×	Uncultured euryarchaeote (AB175351)	99	Mesophilic anaerobic BSA digester
						Uncultured Methanomicrobiales (JX023174)	99	Sludge samples of anaerobic digesters treating sewage sludge or mixture of sewage sludge and food waste
	14 (KJ402291)	××	××	×××	×××	Uncultured euryarchaeote (AB248620)	99	Mesophilic anaerobic butyrate degrading reactor
						Uncultured Methanomicrobiales (CU915986)	99	Mesophilic anaerobic digester which treats municipal wastewater sludge
	15 (KJ402292)	×	××	××	×××	Uncultured euryarchaeote (AB175351)	99	Mesophilic anaerobic BSA digester
						Uncultured Methanomicrobiales (JX023211)	99	Sludge samples of anaerobic digesters treating sewage sludge or mixture of sewage sludge and food waste
Family Methanomicrobiaceae								
Genus *Methanoculleus*	16 (KJ402293)	××	×××	××	×××	Uncultured *Methanoculleus* sp. (JN052756)	99	Anaerobic digestion of animal waste
						Methanoculleus marisnigri (NR_074174)	98	Culture collection