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Early-stage response in anaerobic bioreactors due to high sulfate loads: Hydrogen sulfide yield and other organic volatile sulfur compounds as a sign of microbial community modifications

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ABSTRACT

In this work, the early-stage response of six lab-scale biogas bioreactors fed with different amounts of a sulfate-rich organic agro-industrial effluent was investigated. Biogas characterization, gas chromatography selective for sulfur compounds and high-throughput sequencing of 16S rRNA gene were performed. Hydrogen sulfide (H_2S) yield went from transient to steady state in ~ 2 weeks for all the studied conditions. In addition, volatile sulfur compounds (VSCs), like methanethiol (MeSH) and dimethyl sulfide (DMS), were generated at high sulfate loads. Changes were evidenced in the microbial community structures, with a higher abundance of genes involved in the dissimilatory sulfate-reduction pathway in high loaded sulfate bioreactors, as determined by PICRUSt analysis. Principal component analysis (PCA) and correlation analyses evidenced strong relationships between H_2S, VSCs and the microbial community. Sulfate-reducing bacteria (SRB) like *Desulfocarbo, Desulfocella* and *Desulfobacteraceae* might be possibly linked with methylation processes of H_2S.

1. Introduction

In terms of circular economy, agro-industrial effluents have been in the spotlight for a while. Many of their constituents are potential raw materials that can be easily transformed in building blocks by green chemistry techniques or fermentation bioprocesses. Building block molecules might be *in situ* intermediate byproducts or be further commercialized for a variety of applications. However, when these more profitable strategies are dismissed for different reasons (*e.g.,* costs scalability, markets, etc.), energy generation is the best option to still take an extra of all the stored chemical power. Additionally, there is a not less important benefit by avoiding large amounts of organic matter loads into the environment, which could cause water contamination and other impacts.

In such sense, the anaerobic digestion (AD) has been widely reported elsewhere, involving four sequential steps: hydrolysis, acidogenesis, syntrophic acetogenesis, and methanogenesis (Lv et al., 2010). Biogas produced in a controlled AD bioreactor contains CH_4 and CO_2 in variable ratios, but typically between 2:1 and 3:1 (Weiland, 2010). It can be used to replace fossil fuels in the generation of energy and heat, especially when it is upgraded or purified from CO_2 and trace gases (Kapoor et al., 2019).

However, there are some problems that may limit the application of AD in energy generation. One of the most well-known issues is the presence of sulfur at high concentrations in the substrates or feedstock, which produces sulfide because of its reduction in the digester environment. Many industries, such as pulp and chemical plants, pharmaceutical and food processing (vinasse, molasses, edible oil, etc.), generate wastewaters rich in sulfate (SO_4^{2-}) and organic matter (Khanal and Huang, 2003; Wu et al., 2018). When using AD to treat these wastewaters, the availability of sulfate as alternative electron acceptor stimulates the growth of sulfate-reducing bacteria (SRB), which employ H_2 and low molecular weight organics (VFAs, ethanol, and methanol) as electron donors to reduce sulfate to sulfide. Thus, SRBs compete with other prokaryotes for substrates at different levels in the degradation process (*i.e.*, fermentative, acetogenic and homoacetogenic bacteria, as

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Note: Low-resolution images were used to create this PDF. The original images will be used in the final composition.

Table 1
Inoculum (I) and substrates (BS and SS) characterization.

	COD (mg/l)	BOD (mg/l)	pH	Total S (mg/l)	Sulfide (mg/l)	Sulfate (mg/l)	TS (mg/l)	VS (mg/l)
I	1300	–	7.4	16	~4	49	29,800	13,100
BS	200,000	66,250	5.0	113	<1	338	56,683	38,478
SS	96,000	67,900	4.0	8419	<1	25,256	113,635	40,539

Table 2
Feeding composition of bioreactors R1 to R6 from zero-day.

Bioreactor	R1	R2	R3	R4	R5	R6
%vol of SS substrate	0%	10%	30%	50%	70%	100%
Total Vol. (ml/day)	30	31.6	35.5	40.4	47.1	62.4
BS Vol. (ml/day)	30	28.5	24.9	20.2	14.1	0
SS Vol. (ml/day)	0	3.1	10.6	20.2	33	62.4
Hydraulic Residence Time (days)	100	95	85	74	64	48
Sulfate load (mg/day)	10	88	276	517	838	1576

well as methanogenic archaea). Although many factors including pH, temperature, COD/SO_4^{2-} ratio and differential sulfide toxicity, may affect the outcome of the competition, thermodynamic and kinetic considerations generally favor SRBs in anaerobic environments in the presence of excess sulfate (Colleran et al., 1995). SRBs have a higher affinity for acetic acid, CO_2 and H_2 than methanogens; therefore, sulfate reduction typically compromises methane production in wastewater treatment systems (Wu et al., 2018).

In addition, H_2S in solution is mainly present as three species including unionized H_2S, ionized HS^- and S^{2-}, depending on the pH and temperature (APHA, 2017). High concentrations of free H_2S (unionized), which dominates at pH < 7, are toxic to methanogens as well as to other microorganisms. This toxicity is caused by its diffusion across the cell membrane of microorganisms, with further inhibition of their metabolic activity (Chen et al., 2008; Wu et al., 2018). When this happens, it begins with the accumulation of intermediates such as volatile fatty acids, followed by the consequent bioreactor acidification, and finally the failing of the chemical oxygen demand (COD) removal capacity (Chen et al., 2008).

There are many studies reporting sulfide control in the liquid sludge of the bioreactor or, in the biogas attempting to upgrade or purify it (Andersson et al., 2004). The first strategy is widely applied by means of the addition of ferric/ferrous cation (*e.g.*, iron chloride, iron hydroxide). One of its drawbacks is related to the dose regulation during the process and the increased sludge solids management (Cai et al., 2018; Zhang et al., 2013).

This article focused on the characterization of the early response of biogas anaerobic bioreactors to a sulfate-rich organic agro-industrial wastewater. These results illustrate the relationship between the sulfate load, the production of H_2S and other volatile sulfur compounds (VSCs) with strong correlations with the microbial community modification. The consequent analysis might contribute to a better understanding of AD of this type of effluents in order to optimize their treatments when transient to steady-state changes in terms of sulfate feeding take place in the bioreactor.

2. Materials and methods

2.1. Collection of inoculum and substrates

The anaerobic inoculum was obtained from a local biogas plant with two 3000 m^3 biodigesters each (SOLAMB S.R.L, Timbúes, Santa Fe, Argentina). This facility processes agro-industrial effluents of high COD values (usually more than 50,000 mg/l), producing biogas that is then transformed in thermal and electrical power.

The Sulfate Substrate (SS) is a real wastewater effluent that was provided by another company that produces vegetable olein (*i.e.*, fatty acids mixture from chemical refining of various vegetable oils, mainly soybean and sunflower oils). These wastewaters contain high COD values (between 50,000 and 100,000 mg/l), which make them very attractive to be converted in thermal and/or electric energy in a biogas plant. The challenge to address is the very high sulfate concentration (usually between 10,000 and 25,000 mgSO_4^{2-}/l) as well as the acidic condition (pH between 2 and 4) (Table 1). Main organic substances in SS are mono-, di- and triglycerides, free glycerol, fatty acids and phospholipids.

On the other hand, a Base Substrate (BS) was used to prepare dilutions and blends of SS in order to set constant the volumetric Organic Load Rate (OLR) between lab-scale bioreactors. The BS is the same effluent (substrate) that normally feeds the biogas plant that provided the microbial inoculum (Table 1). The BS is a mixture of different wastewaters, mainly from the vegetable oil processing and biodiesel industry of the Argentinian central region; however, it does not contain significant concentrations of sulfates (< 350 mgSO_4^{2-}/l, Table 1). All samples were transported to the laboratory under refrigeration and protected from light and air. Chemical Oxygen Demand (COD), Biological Oxygen Demand (BOD), pH, Sulfides, Sulfates, Total Solids (TS) and Volatile Solids (VS) determinations were carried out according to APHA Standard Methods (APHA, 2017).

2.2. Experimental setup

The experiments were carried out in six lab-scale anaerobic digesters. These bioreactors were constructed using big boiling flasks sealed with rubber stoppers, with three hollow stainless-steel rods inserted through them. Two were used to feed the substrates and extract the digested sludge, respectively, by means of peristaltic pumps. The third one allowed the collection of the biogas in 5 l Tedlar® bags. Each unit operated under semi-continuous mode with constant agitation by magnetic stirrers that also provided the thermal regulation, and the additional assistance of a water bath to improve temperature homogeneity.

2.3. Bioreactor operation

Inoculum was loaded to fill 3 l of each bioreactor, leaving a headspace of about 1 l between the rest of the glass ball and its neck with the rubber stopper. Systems were set at 300 rpm and 31 ± 1 °C (mesophilic conditions). The feeding process and biogas measurements were done daily at the same hour for each bioreactor. Before feeding, the same quantity of digested sludge was extracted to keep constant the volume of liquid in the bioreactor. The volume of biogas, collected in each Tedlar® bag, was measured by liquid displacement method. Then, the relative composition of the generated biogas in terms of CH_4, CO_2, and H_2S, was analyzed using a portable instrument (Optima 7 Biogas Analyzer, MRU Instruments, USA). As the maximum concentration of H_2S that can be measured by this instrument is 2000 ppm, further dilutions with N_2 5.0 (Linde, Argentina) were performed by means of a high-volume gas tight syringe when necessary (Hamilton, USA). The syringe, nipples, and tubing used were made of PTFE.

During start-up and stabilization, all bioreactors were fed in the same fashion with the BS having a daily raised OLR from 0 up to 2000 mgCOD/day.l of liquid bioreactor in the course of the first 2 weeks. An additional week was then allowed for gas composition and volume stabilization. It should be said that the microbial inoculum was already adapted to this substrate (*i.e.*, BS) as well as to a similar OLR at the biogas facility plant.

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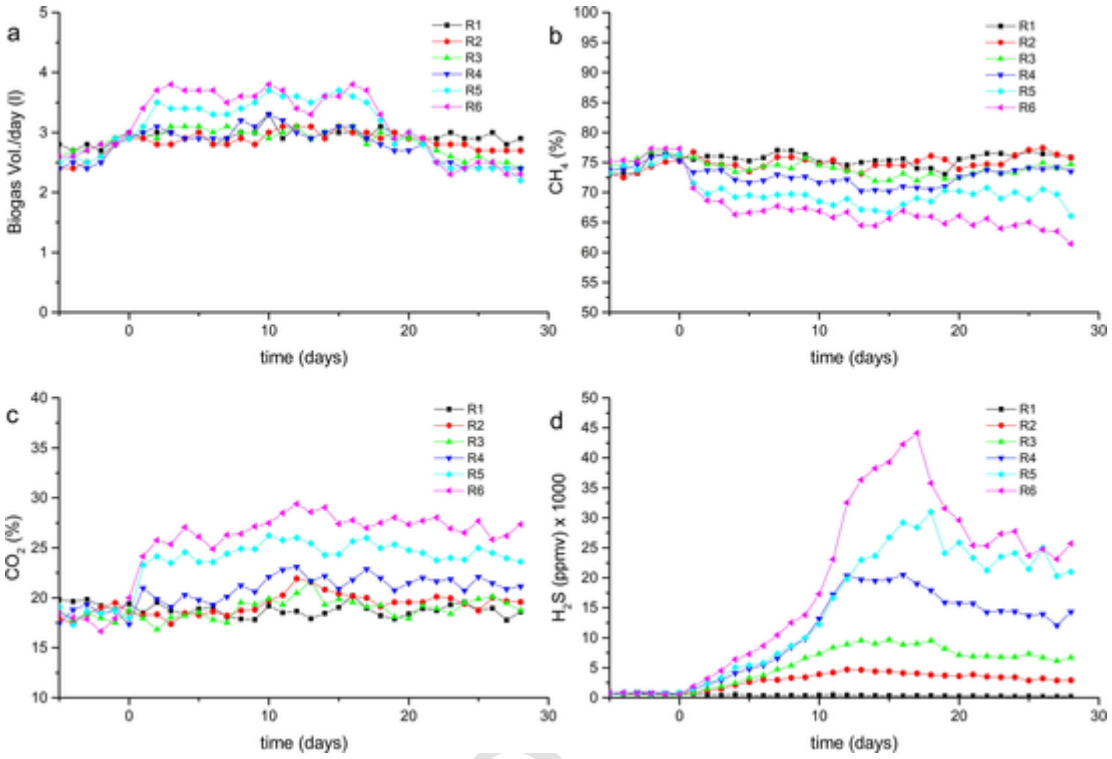

Fig. 1. Biogas volume generated per day (a) and biogas composition in terms of CH_4 (b), CO_2 (c) and H_2S (d), in each bioreactor. Zero-day corresponds to the beginning of feeding variation between bioreactors.

For an easier visualization of the results, zero-day corresponds to the beginning of variation of feeding composition between all bioreactors after start-up and stabilization. From this point onwards, bioreactors started to be fed with different SS/BS volumetric ratios. During all the experiment, the OLR was not changed (*i.e.*, 2000 mgCOD/day.l), while the %vol SS was set between 0 and 100% across the six units (Table 2).

2.4. Analytical calculation of sulfur (S) loaded and released in the biogas

The cumulated S loaded to each bioreactor was calculated from the sulfate load rate (Table 2), considering sulfides in BS and SS negligible (Table 1). The sulfur released in the biogas was computed by calculating and adding the mass of S from each measurement day. Ideal gas condition was considered (Eq. (1)).

$$\begin{aligned} massS &= MW_S.n_{H_2S_g} \\ &= MW_S.\frac{P.V_{H_2S_g}}{R.T} \\ &= MW_S.\frac{P.V_{Biogas}.[H_2S_g]}{R.T} \end{aligned} \quad (1)$$

where P = $1.013.10^5$ Pa (1 atm), T = 298 K (25 °C), MW_S = 32.06 $g.mol^{-1}$, and R = 8.314 $J.K^{-1}.mol^{-1}$. *n* is the number of moles (*i.e.*, 1 mol of S per mol of H_2S). Brackets indicate volumetric concentration.

2.5. Gas chromatography

A Varian CP–3800 Gas Chromatograph with a Pulsed Flame Photometric Detector (GC-PFPD) with sulfur filter was employed to analyze the presence of H_2S but mainly the presence of other VSCs. The determinations were made on day #26, when bioreactors had already reached almost constant biogas parameters. Measurements of biogas samples were done during the first hour after sampling and were diluted with N_2 5.0 (Linde, Argentina) as needed. In addition, two split settings were used for running samples: the higher (1:100) to quantify the H_2S to avoid peak saturation, and the lower (1:10) to observe the VSCs present in low concentrations. Analytes were separated using a 60-m CP-5 CB (fused-silica 0.53 mm I.D., 5 μm film thickness, Chrompack, Sweden). The gas samples were injected using a VICI Valco 6 ports gas sampling valve with air actuator. The valve temperature was held at 120 °C and the split/splitless injector was held at 200 °C. Helium (He 5.0; Linde, Argentina) was used as carrier gas at a constant flowrate of 2 ml/min. The column oven was temperature programmed from 40 °C to 200 °C at 15 °C/min after 1 min initial hold and 5 min final hold. The detector temperature was held at 250 °C, photomultiplier voltage was 570 V, gate delay was 6 msec and gate width 20 msec. Gas flow rates to the detector were: H_2 5.0, 13.4 ml/min; Air1 4.0, 9.7 ml/min; Air2 4.0, 17.3 ml/min (Linde, Argentina). Peak identifications were done by running the following standards: H_2S, carbonyl sulfide (COS), MeSH, ethanethiol (EtSH) and DMS in N_2 gas balance cylinders (Linde, Argentina) and carbon disulfide (CS_2), isopropanethiol (iPrSH), thiophene and diethyl sulfide (DEtS) in liquid analytical grade (Sigma-Aldrich, Argentina). Quantification of identified compounds were done using the standards of H_2S (4.5 ± 0.5 ppm) and DMS (14.7 ± 0.3 ppm) (Linde, Argentina). Samples and standards were injected and measured in triplicate.

2.6. DNA extraction, sequencing, and taxonomic assignment

Biomass samples for DNA extraction and microbiological analysis were collected from the bioreactors on day #26, following the same criteria above mentioned for GC analysis. Samples were centrifuged at 1000 *g* for 10 min to eliminate solid residues from the medium and DNA extractions were performed according to Shan *et al.* with some modifications (Shan et al., 2008). Briefly, 1.5 ml of sludge sample were washed three times with sterile distilled water by centrifugation (5000 *g* for 5 min) and then suspended in 492 μl of TENP buffer (50 mM Tris-HCl pH 8.0, 20 mM EDTA, 100 mM NaCl and 0.01 g/ml polyvinylpyrrolidone) by pipetting. Cell lysis was performed by adding

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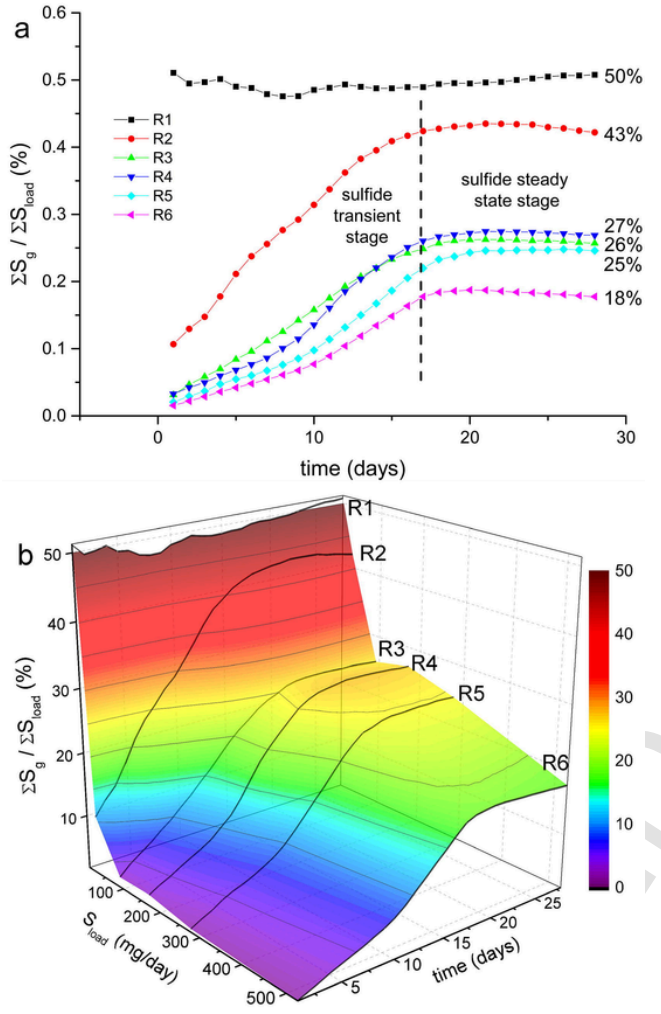

Fig. 2. Ratio between the total cumulated sulfur released in the biogas (ΣS_g) vs. the total cumulated sulfur loaded to the system (ΣS_{load}) (a). Surface 3D-plot including S_{load} rate (b).

Table 3
Identification of VSCs peaks by GC-PFPD.

RT (min)	Compound	Chemical Formula
8.33	Hydrogen Sulfide	H_2S
9.5	Methanethiol	CH_3SH or MeSH
10.42	Ethanethiol	CH_3CH_2SH or EtSH
10.64	Dimethyl sulfide	CH_3SCH_3 or DMS
11.14	Isopropanethiol	$(CH_3)_2CHSH$ or iPrSH
12.8	Diethyl sulfide	$(C_2H_5)_2S$ or DEtS

SDS at a final concentration of 1.8 % (w/v) followed by incubation in a 65 °C water bath for 1 h. Then, 100 µl of 5 M NaCl and 80 µl of 10 % (w/v) CTAB in 0.7 M NaCl were added and the samples were incubated for 10 min at 65 °C. Samples were then mixed with an equal volume of chloroform:isoamyl alcohol (24:1, v/v) and centrifuged at 13,000 *g* for 5 min at room temperature. The aqueous phase was then successively subjected to extractions with equal volumes of phenol:chloroform:isoamyl alcohol (25:24:1, v/v) and chloroform:isoamyl alcohol (24:1, v/v). DNA was finally precipitated with 1 vol of isopropanol at −20 °C for 20 min. The pellet of crude nucleic acids was obtained by centrifugation at 13,000 g for 30 min at room temperature, washed with cold 70 % (v/v) ethanol, and resuspended in sterile milliQ water. 16S rRNA gene PCR amplification and sequencing were conducted by an external service (Macrogen, Korea). The V3-V4 region of prokaryotic 16S rRNA gene was amplified with primers Bakt_341F (5′-CCTACGGGNGGCWGCAG3′) and Bakt_805R (5′-GACTACHVGGGTATCTAATCC-3′), and the amplicon library was sequenced on Illumina MiSeq platform (Illumina, Inc.). Biodiversity analysis and phylogenetic classification was performed using the QUIIME™ platform. Metagenome functional content was predicted from the 16S rRNA gene data by Phylogenetic Investigation of Communities by Reconstruction of Unobserved States (PICRUSt) 1.0.0 (Langille et al., 2013).

2.7. Statistical analysis

In this study, most of the analyses were performed in triplicate and the data were reported as mean ± SD (standard deviation). Statistical analyses were performed using the SigmaStat 3.5 program (Systat Software Inc., USA). Following the assessment of data normality and homogeneity of variances, the ANOVA test was used to compare the collected data between bioreactors. Tukey's post-hoc test was applied when the differences in the measured values were different ($p < 0.05$). Dissimilarities between SRB and methanogenic communities (at genus level), and other principal parameters such as CH_4, CO_2, H_2S and VSCs were analyzed through principal component analysis (PCA). PCA analysis was carried out using "prcomp" function from "stats" package of R software ver. 4.0.5 (R Foundation for Statistical Computing, Vienna, Austria) (Borcard et al., 2011). Spearman correlation coefficients were computed also in R (ver. 4.0.5) environment to appraise the relationship between parameters. Data was visualized using the R packages "ggplot2" version 3.3.5 and "corrplot" version 0.9.

3. Results and discussion

3.1. Biogas production and composition

Fig. 1 shows the daily measurement of biogas production and composition from the six lab-scale bioreactors. As can be observed, CH_4 generated by R1-R6 exhibited average values of 75.59% ± 1.17, 75.53% ± 1.07, 73.37% ± 0.89, 72.71% ± 1.38, 69.28% ± 1.27 and 64.76% ± 1.44, respectively. On the other hand, average CO_2 contents of R1-R6 were as follows: 18.65% ± 0.60, 19.70% ± 0.93, 19.22% ± 0.91, 21.44% ± 0.95, 24.72% ± 0.83 and 27.29% ± 1.05. Statistical analysis of these data revealed significant differences ($p < 0.001$) among all bioreactors, except between R1 and R2.

As it can be seen, from zero-day onwards, bioreactors R5 and R6 showed a clear step increase in the volume of generated biogas that became quite stable during the first ~ 17 days. This behavior agreed with a higher amount of CO_2, as evidenced by the analysis of CH_4 and CO_2 concentrations (Fig. 1b and 1c, respectively), and the subsequent partial volume calculation for each gas (data not shown).

It should be noted that pH remained almost constant for all bioreactors, with the following mean values: $pH_{R1} = 7.29 \pm 0.03$, $pH_{R2} = 7.26 \pm 0.05$, $pH_{R3} = 7.31 \pm 0.16$, $pH_{R4} = 7.34 \pm 0.15$, $pH_{R5} = 7.47 \pm 0.18$, and $pH_{R6} = 7.26 \pm 0.12$. Hence, the higher CO_2 detected in R5 and R6 could be associated with differences in substrate composition between SS and BS, since there was not an evident breaking of the buffer capacity of each unit. As reported elsewhere, different kinds of substrates produce biogas with variable CH_4/CO_2 ratios (Weiland, 2010).

On the other hand, the consequence of sulfate load was clearly observed through the gradual increase of H_2S released in the biogas from zero-day onwards in each bioreactor (Fig. 1d). R1 (bioreactor kept as a control reference) showed background values around 450 ppmv, which remained almost constant during the analyzed period as well as the volume and CH_4/CO_2 ratio (Fig. 1a-c). This suggests a quite good overlap-

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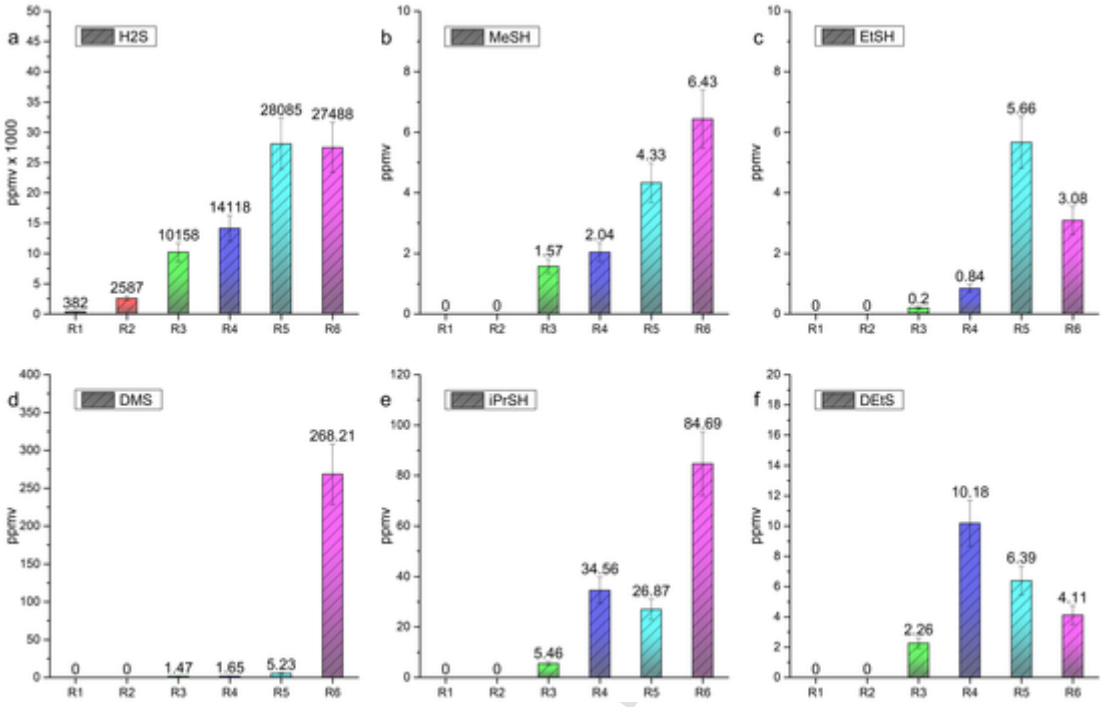

Fig. 3. Concentration (ppmv) of H_2S and VSCs in biogas samples on day #26 (see e-supplementary materials and Table 3), as determined by GC-PFPD.

ping conditioning -for all bioreactors- of the inoculum, from its source (at the biogas facility plant) to the laboratory.

In contrast, as it could be expected in a non-steady state response, R2 to R6 bioreactors showed an increase in released H_2S related to their own sulfate constant load rate (see Table 2). Quite remarkable is that all units developed a kind of maximum of H_2S generation between day #13 and #17, from where concentrations started to decrease and partially stabilized. An explanation to this behavior could be an adaptation of microbial SRB growth to each constant load rate of sulfate. This becomes evident when plotting in Fig. 2a the ratio between the total cumulated sulfur (S) released in the biogas vs. the total cumulated S loaded to the system day after day. As it can be observed, from the beginning to the end of the experiment, R1 converts around 50% of the loaded S in gasified H_2S. Furthermore, although it is out of the aim of this work to discuss a detailed mass balance and the gas–liquid sulfide transfer, it is noteworthy that extra loaded S to R2-R6 systems undergo 2 separate stages: a first transient-like, and a second one by achieving a steady state (in terms of S); both governed by 3 different and sequential steps:

i) A constant load rate of SO_4^{2-} activates a biological response by stimulating the growth of SRB, which start to reduce it to sulfides by using this anion as an electron acceptor in anaerobic respiration (Muyzer and Stams, 2008; Zhang et al., 2013).

ii) H_2S yield in aqueous media is instantly subjected to a dissociation equilibrium HS^-_{liq}/H_2S_{liq} (Yongsiri et al., 2004), which depends mainly on pH and temperature (conductivity has a minor significance). Eq. (2) allows to calculate the molar ratio between both species at constant temperature. The pK_{a1} is ~ 6.99 at 25 °C (APHA, 2017), hence at pH = 7, they are equally represented (1:1).

$$\log \frac{[HS^-_{liq}]}{[H_2S_{liq}]} = pH - pK_{a1} \quad (2)$$

Nevertheless, for example, at an average pH of ~ 7.3, HS^-_{liq}/H_2S_{liq} ratio changes to ~ 2 (*i.e.*, the ~ 66% of sulfide in solution is in its ionized form). Besides, rising the temperature to 31 °C (*i.e.*, bioreactor temperature) has an additional shift of pK_{a1} to slightly lower values, around ~ 6.86 (Millero, 1986), in favor of HS^-_{liq}, giving a corrected HS^-_{liq}/H_2S_{liq} ratio close to ~ 2.75 (*i.e.*, now the ~ 74% of sulfide is ionized). This highlights the importance to consider, under similar conditions, the resulting risk of an acidification by other causes (*i.e.*, COD overload). A small decrease in pH could cause a sudden increase in the concentration, and then the inhibition effect, of the unionized form.

iii) The amount of H_2S_{liq} will define the equilibrium with the H_2S in the biogas through Henry's law. This law describes, at a given temperature, the partitioning of a volatile compound between the water (liquid) phase and the air (gas) phase. Pragmatically, total sulfide is used as analytical reference of the sulfide level (Yongsiri et al., 2004).

Despite this description, during the 1st stage (*i.e.*, the transient-like) the different slopes of the ratio ΣS_g / ΣS_{load} curves from R1 to R6 observed in Fig. 2a, might be dominated by the increased load rates of sulfates over the biological response capacity. Remarkably, all bioreactors achieved the 2nd stage simultaneously when these ratios became steady although different. Furthermore, analysis of Fig. 2b seems to indicate that at medium load rates of sulfates (250 – 850 $mgSO_4^{2-}$/day), bioreactors achieved a load-independent capability of sulfate conversion (*i.e.*, released S in biogas as H_2S is around 25% of the loaded S amount from R3 to R5). However, at higher loads (R6, above 1500 $mgSO_4^{2-}$/day), self-inhibition issues could be possibly acting due to the accumulation of H_2S_{liq} (even though this form represents only ~ 25% of total sulfide), as well as other causes, such as microbial competition for the same substrates. This effect might also be related to the drop of biogas volume observed in R6 from day #17 onwards (Fig. 1a), despite CH_4 and CO_2 remained almost constant.

3.2. GC analysis and detection of volatile sulfur compounds (VSCs)

To elucidate the presence of sulfur-containing molecules other than H_2S during sulfate reduction, GC-PFPD analyses were performed in biogas samples of each bioreactor on day #26, when sulfide steady state had already been reached (see Fig. 2a).

The first eluted compound, at a retention time (RT) of 8.33 min, was identified as H_2S. Additionally, with much smaller signal intensities or areas, a variety of other VSCs eluted at RT 9.5 min, 10.42 min, 10.64 min, 11.14 min and 12.8 min (see e-supplementary materials).

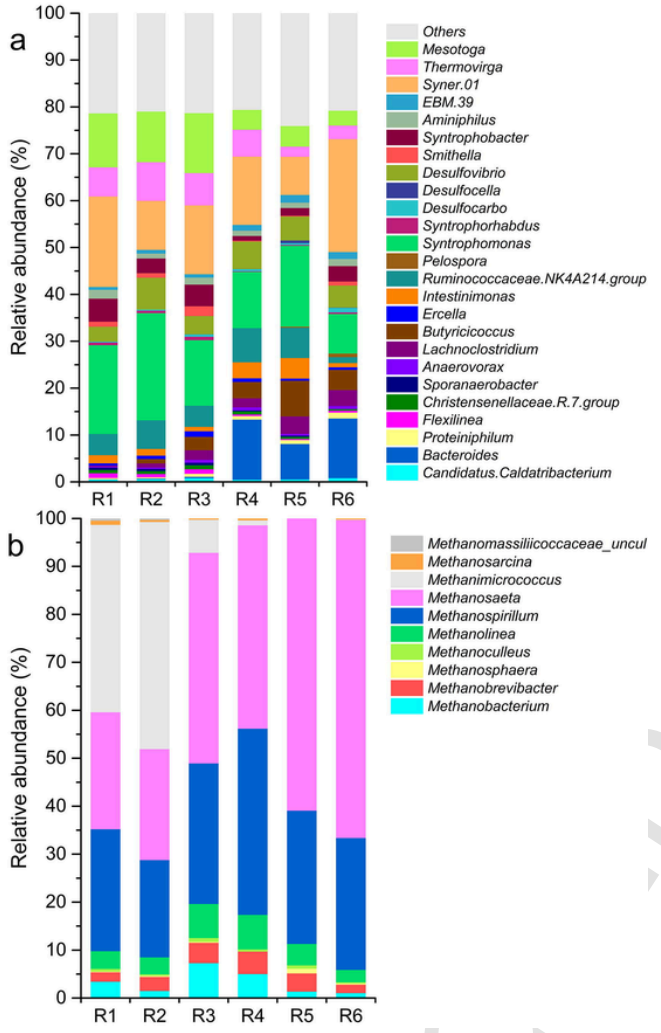

Fig. 4. Taxonomic classification of bacterial (a) and archaeal (b) communities for each bioreactor at genus level on day #26. Sequences that accounted for <0.5% of the population were grouped into the category "Others".

Table 3 summarizes the identification of each eluted compound after running the corresponding standards.

On the other hand, Fig. 3 depicts the calculated concentration of each VSC in biogas samples. H_2S values were consistent with the portable detector ones measured the same day (see Fig. 1d). Furthermore, VSCs other than H_2S appeared from R3 to R6 when H_2S had exceeded ~ 10,000 ppmv. As these measurements belong to the 2nd stage (*i.e.*, the steady state), the detectable presence of these organic VSCs could be related to medium and high sulfate load rates, which push the biological conversion to H_2S and its methylation at the top capability under these experimental conditions.

MeSH formation is reported to be strongly correlated with H_2S concentrations, acting as a methyl acceptor in the degradation of organic matter in sediments. Methoxylated aromatic compounds have been shown to be good methyl donors by this sulfide-mediated o-demethylation, resulting mainly in MeSH and DMS (Drotar et al., 1987; Lomans et al., 2002). This is in agreement with the similar uptrends observed for MeSH and H_2S production from R3 to R6 (Fig. 3a and 3b); moreover, it is remarkable the dramatic increase of DMS in R6. Besides these compounds, Fig. 3 shows that other methylated or ethylated VSCs like EtSH, iPrSH or DEtSH are produced due to microbial activity during bioreactors operation under anaerobic conditions, as was also previously reported by Andersson et al. (Andersson et al., 2004).

4. Metagenomics analysis

4.1. Characterization of microbial community structures

In order to address the relationship between the biochemical reduction of sulfates in sulfides (*e.g.*, H_2S and VSCs) and microbial communities' modifications at the six lab-scale bioreactors, high-throughput sequencing analysis was performed on day #26. A total of 290,943 high-quality partial 16S rRNA gene sequences, trimmed to 280 bp, were obtained with an average number of 48,490 reads per sample. Rarefaction curves were performed to assess the representativeness of the libraries. In all the samples the rarefaction curve reached the plateau before 5000 reads, indicating adequate sequencing depths. Results related to the diversity and evenness of community distribution are summarized in e-supplementary materials. Shannon and Pielou indexes were both very similar for the six bioreactors suggesting that the alpha microbial diversity was not affected by the sulfate loads.

Dynamics of the microbial communities and their relative abundances at phylum level are reported in e-supplementary materials. All bioreactors showed a similar microbial community structure consisting of six main bacterial phyla: *Firmicutes* (30.0–39.6% of the total sequence reads), *Synergistetes* (14.4–29.4%) *Proteobacteria* (8.0–12.5%), *Bacteroidetes* (1.3–14.9%), *Thermotogae* (2.9–12.1%) and *Chloroflexi* (2.4–4.8%). Among the minor communities, *Atribacteria* was the dominant phylum (0.7–1.3%). Microorganisms affiliated with *Synergistetes*, *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* have been proposed to compose a core group of phylotypes common to most anaerobic digesters, whereas the others would be shared among a few digesters or specific phylotypes adapted to the treated effluent (Rivière et al., 2009). Archaea domain, represented by the phylum *Euryarchaeota*, constituted 13.5% of the total number of obtained sequences in R1, but exhibited a gradual decrease towards reactor R5 (relative abundance of 3.4%), then increasing to 9.7% in R6. The decline in the archaeal abundance, as well as in CH_4 production between bioreactors at day #26 (Fig. 1.b), could be the consequence of several factors, including the competition of methanogenic archaea with SRB for the same substrates and the higher sensitivity of methanogens to H_2S toxicity (Cai et al., 2021).

Analysis at the taxonomy of genus level revealed the presence of 25 bacterial genera with relative abundances ≥ 0.5% in at least one of the samples (Fig. 4a). *Syner.01* (8.2–24.1%) and *Syntrophomonas* (8.4–22.9%) were the most abundant genera, followed by *Mesotoga* (3.2–12.8%), *Thermovirga* (2.2–8.2%), *Syntrophobacter* (1.0–4.9%), the *Rumminococcaceae*.NK4A214 group (1.2–7.3%) and *Desulfovibrio* (3.2–6.8%). *Syner.01* and *Thermovirga* are *Synergistetes*-affiliated genera with the ability to degrade amino acids, probably providing short-chain fatty acids and sulfate to methanogens and SRB in anaerobic digesters (Rivière et al., 2009). *Mesotoga* spp. are fermentative bacteria commonly detected in anaerobic methanogenic environments (Nesbø et al., 2010), where they may grow in syntrophic association with hydrogenotrophic SRB (Fadhlaoui et al., 2018). Members of the *Ruminococcaceae* family are known to degrade carbohydrates and produce H_2 and short-chain fatty acids as fermentation products (Ntaikou et al., 2008). Most members of *Syntrophomonas* and *Syntrophobacter* are syntrophic fatty acid oxidizing bacteria that play an essential role in the conversion of short-chain fatty acids such as butyrate and propionate to methanogenic precursors (acetate, H_2, and formate) (Müller et al., 2010).

Regarding the archaeal community, the overall diversity was far lower than that of bacteria, being all the sequences affiliated with 10 archaeal genera (Fig. 4b). Among them, *Methanosaeta* (23.1–66.3%), *Methanospirillum* (20.3–38.8%) and *Methanimicrococcus* (0–47.4%) were the dominant genera, followed by *Methanolinea* (2.6–7.1%), *Methanobacterium* (1.0–7.3%) and *Methanobrevibacter* (1.7–4.7%). *Methanosaeta* is an obligate acetoclastic methanogen, while the others belong to the hydrogenotrophic methanogen species. Mixotrophic

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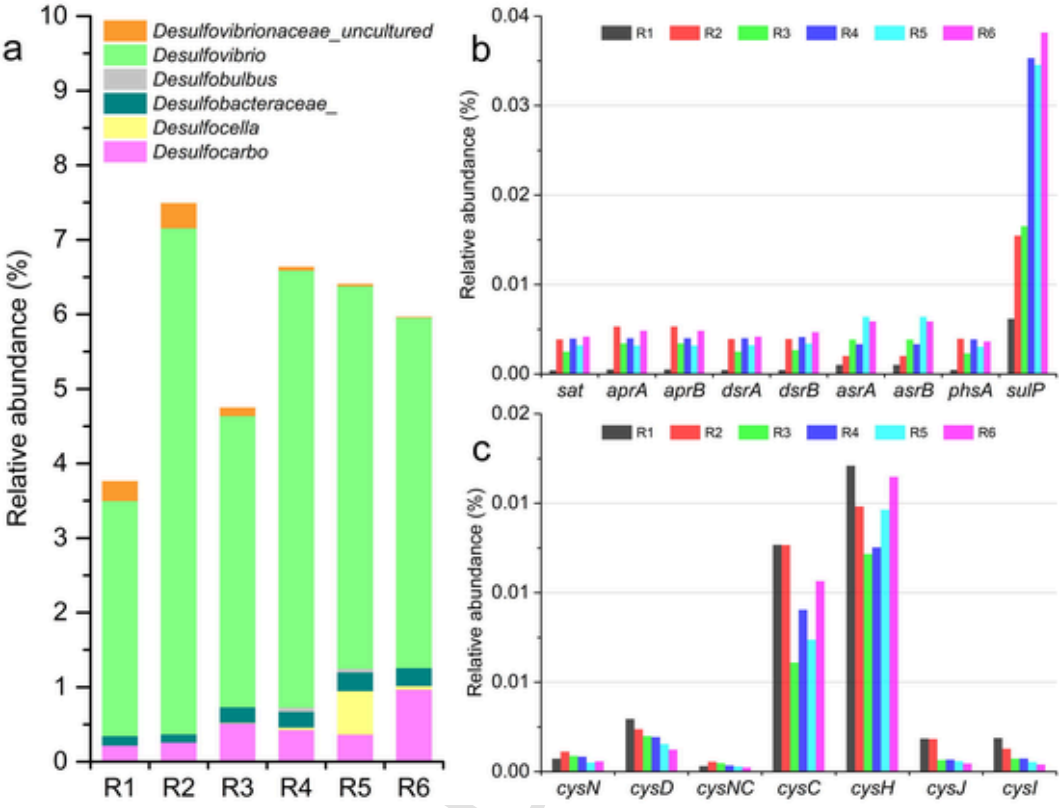

Fig. 5. Taxonomic classification of SRB community at genus level (a). Abundance of sulfur-related functional genes associated with dissimilatory (b) and assimilatory (c) sulfate-reduction pathways, identified by PICRUSt analysis.

Methanosarcina genus was identified in bioreactor R1 with 0.8% relative abundance but decreased in the other five bioreactors, probably due to the higher sensitivity of *Methanosarcina* to sulfide toxicity compared to obligate hydrogenotrophic methanogens (Oliveira et al., 2020).

Overall, microbial community found in all the samples contained microorganisms with biochemical capacities related to the four phases of the anaerobic digestion process (Lv et al., 2010). Differences between communities were mostly found in the proportions of some fermentative bacteria like *Mesotoga* and *Thermovirga*, whose relative abundances noticeably decreased in bioreactors R4-R6. However, these changes would be offset by the increased abundance of other bacterial genera with similar predicted functions, such as *Bacteroides* and *Butyricicoccus*, thus sustaining the stable operation of the bioreactors.

In the archaeal community, *Methanimicrococcus*, which predominated in R1 and R2 (39.1% and 47%, respectively), decreased in R3 and R4 (6.9% and 1.0%) and was almost undetectable in R5 and R6. In contrast, *Methanosaeta* accounted for 24% of the archaeal OTUs in bioreactors R1 and R2 and raised to reach a relative abundance of 66% in R6, being the dominant archaeal genera in that sulfate load condition. This noticeable increment of the acetoclastic methanogen population could be due to potential syntrophic associations with hydrogenotrophic microorganisms in the consortium. Syntrophic acetate oxidation involves the oxidation of acetate to CO_2 by an acetoclastic microorganism with the generation of H_2 as reducing equivalents, and the concomitant scavenging of the produced H_2 by an hydrogenotrophic partner. This process has been well documented for *Methanosaeta* spp. in association with hydrogenotrophic sulfate reducers such as *Desulfovibrio* spp. and, in fact, syntrophic acetate oxidation coupled to sulfate reduction through interspecies H_2 transfer has greater energy yield than acetoclastic methanogenesis (Ozuolmez et al., 2015).

Given the key functional role of SRB in the sulfidogenic anaerobic process, the dynamic of the SRB population was further examined. Even though SRB were detected at low levels (< 8% abundance), a slight increase in the SRB population was observed in all sulfate-fed bioreactors with respect to R1 (Fig. 5a), showing a rapid adaptation with the consequent rise in H_2S production (see Fig. 1d). All identified SRB belonged to the delta subdivision of Proteobacteria and most of the sequences were affiliated with *Desulfovibrio* (78.9–90.7%), an incomplete oxidizing SRB genus that can use H_2, organic acids and alcohols as electron donors for sulfate reduction (Heidelberg et al., 2004). *Desulfovibrio* spp. were reported to have the highest affinity to sulfate among SRB (Muyzer and Stams, 2008), which could be related with their higher and almost constant relative abundance among all bioreactors, including R1.

As shown in Fig. 5a, the other sequences identified at the taxonomy of genus level belonged to the *Desulfobulbus*, *Desulfocella,* and *Desulfocarbo* genera, the last two being enriched in the higher sulfate load bioreactors (R5 and R6, respectively). *Desulfocarbo´s* relative abundance was 0.19% in R1 and 0.87% in R6, meanwhile *Desulfocella* was not detected in R1 and reached 0.56% in R5. *Desulfocella halophila*, the only known species of the genus, is a halophilic SRB that grows with fatty acids, butyrate, alanine, and pyruvate as electron donors (Brandt et al., 1999). The only identified member of the *Desulfocarbo* genus (*D. indianensis*) can grow autotrophically with H_2 as electron donor and heterotrophically on numerous organic substrates, which are completely oxidized to CO_2 (Thuy and Picardal, 2015). Collectively, both complete and incomplete oxidizing SRB exhibited higher relative abundances in the bioreactors with higher sulfate loads.

4.2. Analysis of predicted potential sulfur enzymes

Bioinformatic PICRUSt tool was applied to predict the content of functional enzymes of sulfur metabolism from the 16S rRNA gene libraries of bioreactors R1-R6. Several genes encoding sulfur-related enzymes were detected in the microbial communities, including those in-

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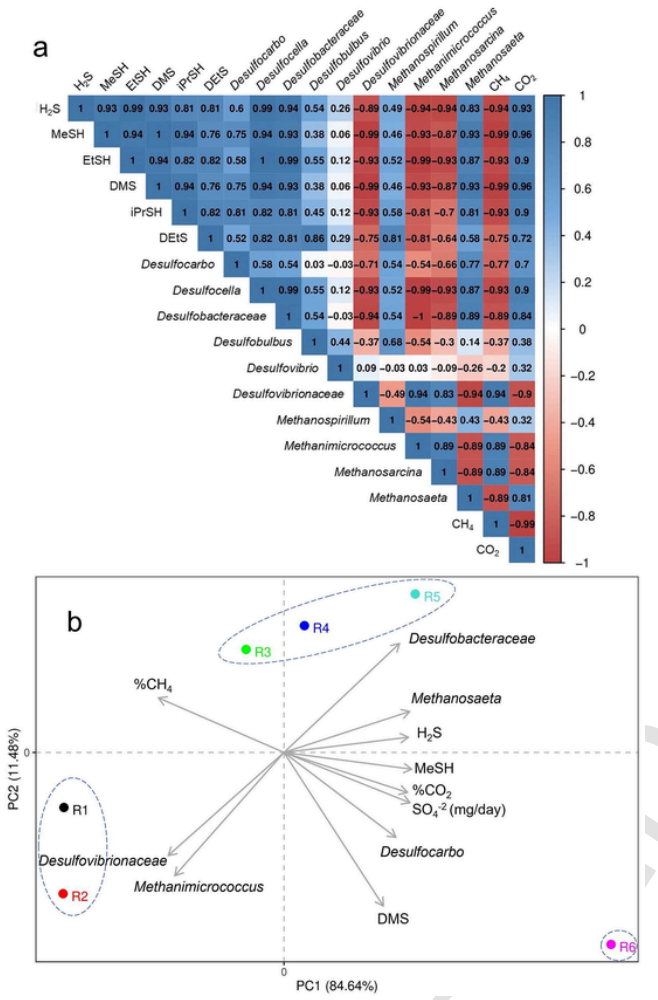

Fig. 6. Correlation map showing relationships between pairs of parameters or variables (a), and PCA plot (b), which points out the weight of sulfur stress in bioreactors response variables and microbial community modifications.

volved in the dissimilatory sulfate-reduction pathway (*sat*, encoding sulfate adenylyltransferase; *aprA* and *aprB*, encoding adenylylsulfate reductase subunits A and B; *dsrA* and *dsrB*, encoding dissimilatory sulfite reductase beta subunit), the *asrA/asrB* genes encoding anaerobic sulfite reductase subunits, the thiosulfate reductase gene *phsA*, and the *sulP* gene of the sulfate permease family. As shown in Fig. 5b, these 9 functional genes exhibited higher abundances in the sulfate-fed bioreactors R2-R6 in comparison to R1, in agreement with the higher SRB population detected. On the other hand, genes encoding enzymes specifically involved in the assimilatory sulfate-reduction pathway including *cysNC* (encoding bifunctional enzyme CysN/CysC), *cysN* (sulfate adenylyltransferase subunit 1), *cysD* (sulfate adenylyltransferase subunit 2), *cysC* (adenylylsulfate kinase), *cysH* (phosphoadenosine phosphosulfate reductase), *cysJ* (sulfite reductase (NADPH) flavoprotein alpha-component) and *cysI* (sulfite reductase (NADPH) hemoprotein beta-component) showed relative abundances in R2-R6 similar to or lower than those observed in R1 (Fig. 5c). These results suggest that dissimilatory sulfate-reduction pathway is favored in reactors R2-R6, where sulfate is being used to provide energy for bacterial growth with concomitant sulfide generation (Rückert, 2016).

Finally, Lomans et al. describe the general mechanisms for the production and degradation of MeSH and DMS in AD environments (Lomans et al., 2002). Slurry incubations at anaerobic conditions indicated that methylation of sulfide and MeSH produce MeSH and DMS, respectively. Regardless of the continuous formation of VSCs, degradation attributed to the activity of certain bacteria and methanogenic archaea might avoid higher concentrations of such compounds. More recently, Carrión et al. (Carrión et al., 2015), showed that in terrestrial or freshwater ecosystems, the pathway of microbial methylation of H_2S to MeSH and then to over-methylated VSCs (*i.e.*, DMS) can also occur in aerobic bacteria. The *mddA* gene has been reported to encode a methyltransferase that methylates MeSH and generates DMS. However, in this work PICRUSt analysis did not predict the presence of *mddA* gene from the 16S rRNA gene libraries of bioreactors R1-R6. This could be related to the presumption that in anaerobic conditions other genes with no homology to *mddA* encode the methyltransferases involved in these reactions.

4.3. Correlation analysis and PCA

Fig. 6a shows the correlation plot where H_2S among other VSCs, as well as CH_4 and CO_2 behaviors were correlated among them and with SRB and methanogenic main genera. It is quite remarkable the strong positive and negative correlations between all categories. For example, correlation coefficients between H_2S, MeSH, EtSH and DMS and the SRB genera *Desulfocella* and *Desulfobacteraceae* have values above 0.93. In addition, the strong positive correlation between *Methanosaeta* and SRBs supports the above described acetoclastic (archaeal) - hydrogenotrophic (sulfate reducers) syntrophic association. On the other hand, *Methanimicrococcus* confirms to be the most sensitive genera to sulfide yield, meanwhile *Desulfovibrio*, the most abundant SRB (Fig. 5a), does not seem to be significantly affected by the parameters and microbial genera analyzed in Fig. 6a.

Additionally, PCA plot depicted in Fig. 6b points out the weight of sulfur, both in the input stress (*e.g.*, sulfate load rate) and in the sulfide outputs (*e.g.*, H_2S and VSCs), as well as the microbial community modifications. Remarkably, both coordinates, PC1 and PC2, explain more than 95% of dissimilarities or variance between bioreactors. The six bioreactors were clustered into three groups: i) reactors R1 and R2, with slight differences; ii) R3-R5; with a clear projection from the first group to the top-right quadrant; and iii) R6, at the bottom-right quadrant, clearly related to *Desulfocarbo* and DMS.

Overall, results obtained in the present work demonstrate a strong correlation between

H_2S and other VSCs with SBR genera of low relative abundances in the microbial community and represent a first step in the characterization of the transient to steady-state of bioreactors fed with sulfate-rich wastewaters. A deeper understanding of these dynamics will be useful for the optimization of current treatment strategies of these wastewaters for biogas production.

5. Conclusions

In this work six lab-scale biogas bioreactors were fed with different amounts of a sulfate-rich agro-industrial effluent. Notably, H_2S yield went from transient to steady state in ~ 2 weeks in all bioreactors. Between 250 and 850 $mgSO_4^{2-}$/day, bioreactors achieved a load-independent capability of sulfate conversion of ~ 25% of total loaded sulfur. Above 1500 $mgSO_4^{2-}$/day, operational parameters showed signs of system deterioration over the evaluated period. VSCs were observed when H_2S exceeded 10,000 ppmv, being MeSH, EtSH and DMS strongly correlated with SRBs as *Desulfocella, Desulfobacteraceae* and *Desulfocarbo*. An increased abundance of dissimilatory sulfate reduction enzymes was predicted by PICRUSt analysis.

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CRediT authorship contribution statement

Camila Olivera: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Writing – original draft. **María Laura Tondo:** Conceptualization, Methodology, Investigation, Validation, Writing – original draft, Writing – review & editing. **Valentina Girardi:** Investigation, Formal analysis. **Lucía Fattobene:** Investigation. **María Sol Herrero:** Investigation, Writing – original draft, Writing – review & editing. **Leonardo Martín Pérez:** Formal analysis, Writing – review & editing. **Lucas Matías Salvatierra:** Conceptualization, Methodology, Formal analysis, Writing – original draft, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.126947.

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