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Exploring the performance limits of a sulfidogenic UASB during the long-term use of crude glycerol as electron donor

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ABSTRACT

SOx contained in flue gases and S-rich liquid effluents can be valorized to recover elemental sulfur in a two-stage bioscrubbing process. The reduction of sulfate to sulfide is the most crucial stage to be optimized. In this study, the long-term performance of an up-flow anaerobic sludge blanket (UASB) reactor using crude glycerol as electron donor was assessed. The UASB was operated for 400 days with different sulfate and organic loading rates (SLR and OLR, respectively) and a COD/S-SO$_4^{2-}$ ratio ranging from 3.8 g O$_2$ g$^{-1}$ S to 5.4 g O$_2$ g$^{-1}$ S. After inoculation with methanogenic, granular biomass, the competition between sulfate-reducing and methanogenic microorganisms determined to what extent dissolved sulfide and methane were produced. After the complete washout of methanogens, which was revealed by next-generation sequencing analysis, the highest S-EC was reached in the system. The highest average sulfate elimination capacity (S-EC=4.3 kg S m$^{-3}$d$^{-1}$) was obtained at a COD/S-SO$_4^{2-}$ ratio of 5.4 g O$_2$ g$^{-1}$ S and an OLR of 24.4 kg O$_2$ m$^{-3}$d$^{-1}$ with a sulfate removal efficiency of 94%. The conversion of influent COD to methane decreased from 12% to 2.5% as the SLR increased while a large fraction of acetate (35% of the initial COD) was accumulated. Our data indicate that crude glycerol can promote sulfidogenesis. However, the disappearance of methanogens in the long-term due to the outcompetition by sulfate reducing bacteria, lead to such large accumulation of acetate.
Keywords: crude glycerol, UASB, SRB, carbon sink, bioscrubber, SO₂ valorization

1. INTRODUCTION

Combustion of sulfur-containing fuels, such as coal, natural gas, peat, wood and oil, results in SO₂ formation mainly generated in the energetic and industrial sectors (Klimont et al., 2013). These emissions are usually treated through physical-chemical processes that are expensive and generate additional effluents requiring further processing and energy inputs (Srivastava and Jozewicz., 2001; Philip and Deshusses., 2003). As an example, aqueous slurries with high sulfite and sulfate content are generated from wet flue gas desulfurization (FGD) with sodium hydroxide. The development of environmentally friendly alternatives to valorize not only SO₂ from FGD but also S-rich liquid effluents is clearly needed. The two-stage bioscrubber concept described in Figure S1 (Supplementary Information section) is a potential alternative process to recover elemental sulfur from such gaseous effluents (Fernández et al., 2017). The process consists of a first scrubbing stage for SOx absorption in water at slightly alkaline pH, followed by two-stage biological process to obtain elemental sulfur. The biological process converts, firstly, sulfate to total dissolved sulfide (TDS) using an organic waste as C source and electron donor and, secondly, TDS to elemental sulfur through a partial oxidation performed under oxygen limiting conditions. Partial TDS oxidation can be also performed through autotrophic denitrification.

Sulfate to TDS reduction has been studied under a range of substrates such as sewage or methanol under a range of operating conditions including thermophilic processes (Qian et al., 2015; Jiang et al., 2013; Weijma et al., 2000). However, the sulfate reduction stage is still the one that requires further economic and technical improvements (Chen et al., 2014). Sulfate reduction, which is catalyzed by sulfate-reducing bacteria (SRB)
(Liamleam and Annachhatre, 2007), can be carried out with a large assortment of organic wastes and under different operating conditions. Recently, crude glycerol has been proposed as a competitive substrate to reduce high loads of sulfate to TDS in batch tests as well as during the start-up of an up-flow anaerobic sludge blanket reactor (UASB) (Mora et al., 2018). Crude glycerol is a waste organic effluent produced in the biodiesel industry with an exceptional COD concentration (≈800 g COD L$^{-1}$) that does not require any additional treatment before its use as carbon source. In most of the recent research, crude glycerol has been used as a suitable substrate for biogas production in anaerobic systems (Nakazawa et al., 2015) or as a co-substrate in anaerobic digestion to increase biogas production (Nghiem et al., 2014; Athanasoulia et al., 2014). Despite different approaches to reduce sulfate from S-rich streams have been investigated using pure glycerol (Santos et al., 2017) the potential of crude glycerol has been poorly explored in sulfidogenic reactors. In fact, to the best of our knowledge, assessment of the long-term performance of a UASB using crude glycerol as electron donor for sulfate reduction has not been addressed before.

One of the main problems related to the start-up of a reactor for sulfate reduction with organic matter is the competition between SRB and methanogens. Since the inoculum is usually obtained from full-scale anaerobic digesters targeting methane production, the enrichment of sulfate reducing bacteria (SRB) becomes a decisive threat between sulfate reduction and methane production. SRB and methanogens competition for the common intermediates in the anaerobic degradation process has been widely reported, which results in a variable performance of the reactor. Then, the origin of the inoculum becomes critical as it contains diverse microbial populations leading to differences in initial activity and substrate adaptation (De Vrieze et al., 2015). Some variables that have been studied to assess this competition are COD to SO$_4^{2-}$ ratio (COD/S ratio), TOC/S ratio, organic
loading rates (OLR), sulfate loading rates (SLR) and the type of electron donor used to reduce sulfate (Pol et al., 1998). Most of them have been studied using different electron donors, such as glucose (O’Reilly and Colleran, 2006), lactate (Zhou et al., 2014), ethanol (Hu et al., 2015) and Volatile Fatty Acid (VFA) mixtures (acetate, propionate and butyrate) (Omil et al., 1996; Omil et al., 1998; Lens et al., 1998) but, there are no reports on the long-term operation using a substrate with a significant fraction of slowly hydrolysable carbon source such as that contained in crude glycerol. Despite the competition of SRB over methanogens has been widely described, the use of crude glycerol as carbon source implies the production of metabolites through its fermentation that may lead to microbial diversity changes that have not been yet explored. It remains uncertain if such competition may be beneficial or not to process performance.

Another important parameter in the start-up and long-term operation of UASB reactors for sulfate reduction is biomass granulation. Granular biomass provides a strong structure and good settling properties that contribute to high biomass retention, and stands up against possible shock and high loading rates (Liu and Tay., 2004). As demonstrated by De Vrieze et al. (2015), selecting an inoculum according to your objective is crucial for a robust operation. In the current study, granular sludge from methanogenic anaerobic digesters is used as inoculum in UASB bioreactors for sulfate reduction (Mora et al., 2018) considering that no granular SRB reactors are currently operated in the field. Long-term operation may lead to microbial diversity changes that could affect UASB performance during the long-term operation of such sulfidogenic reactors.

To better understand the limits and applicability of sulfate reduction, this study aimed at assessing 1) the limits of the process in terms of sulfate reducing capacities and 2) the long-term performance of a UASB for the treatment of synthetic sulfate-rich effluents to produce TDS using crude glycerol as electron donor. This study not only provides new
information regarding S-rich streams valorization but also assesses the use of crude
glycerol specifically for sulfate reduction through the analysis of C sinks to the main
bioprocess occurring in the system.

2. MATERIALS AND METHODS

2.1. Experimental setup
A jacketed glass-made UASB reactor of 2.5 L, with a granular sludge volume of 1L, was
used in this study. A detailed scheme of the UASB is presented in Figure 1. During the
operation, inlet pH ranged between 8.4 and 8.6 and temperature was controlled at 35ºC
by a thermostatic bath connected to the water jacket of the reactor (Figure 1). The
composition of the mineral medium was (g L⁻¹): K₂HPO₄ (3), NH₄Cl (0.2) dissolved in
tap water to add macro- and micronutrients and adjusted to pH=8.8-9.0 with NaOH (2
M). Because of the difficulty to control pH in a plug-flow type bioreactor, the pH in the
UASB was not controlled. However, the buffering capacity of the mineral medium
allowed maintaining the outlet pH above 7 along the whole operation of the reactor (data
not shown). Mineral medium was pumped at a flow rate of 0.5 L h⁻¹, once mixed with the
organic influent, from the bottom to the top of the UASB (up-flow velocity of 0.25 m h⁻¹).
The flow rate of the organic influent was set at 30 mL h⁻¹. Hence, crude glycerol was
diluted to adjust the inlet COD concentration. The hydraulic residence time (HRT),
calculated as that corresponding to the reaction volume only (sludge blanket), was 2h.
Biogas produced in the UASB was collected in a 5 L Tedlar bag (FlexFoil, SKC Inc.) to
monitor its composition and flow rate. Inlet and outlet flows were also sampled every
two/three days to analyze COD, S compounds (sulfate, thiosulfate and TDS) and VFA.

2.2 Operating conditions and short-term experiments
Granular sludge obtained from an anaerobic digester treating wastewater in a pulp and paper industry was used to inoculate the UASB reactor to reach an initial Volatile Suspended Solids (VSS) concentration of 28 g VSS L⁻¹. As shown in Table 1, the reactor was operated during 400 days at different sulfate inlet concentrations. Inlet sulfate concentrations ranging from 235±17 mg S-SO₄²⁻ L⁻¹ to 859±30 mg S-SO₄²⁻ L⁻¹ were fed by adding sodium sulfate to the mineral medium. Different SLR and OLR were tested during the long-term operation of the UASB in order to assess the sulfate reducing capacity of the system.

The operation was divided into 6 different periods according to the initial sulfate inlet concentrations and the COD/S ratio tested (Table 1). Period I focused on the UASB start-up to enrich the microbial community with SRB; Period II served to optimize the operation at the same inlet sulfate concentration set in Period I by providing a higher OLR; Periods III and IV were set to study the sulfate reducing activity at a moderate initial sulfate concentration; Period V served to explore the limits of the system by setting the highest sulfate inlet concentration and, finally, Period VI targeted the recovery of the initial UASB stability when the lowest SLR was set. Table 1 shows average operating conditions and standard deviations obtained from each operational period. SLR and OLR were calculated considering the reaction volume only.

During period VI, short-term assays were carried out during 60 h to assess the sulfate elimination capacity (S-EC) in the UASB reactor under variable loading rate conditions typically found in industrial activities. The experiment consisted of a stepwise decrease of the sulfate inlet concentration every 12 h (from 450 mg S L⁻¹ to 120 mg S L⁻¹). The COD/S was also varied since the OLR remained constant during the short-term experimental assays. At each concentration tested, effluent was collected to measure the
concentration of sulfate, TDS and VFA. In addition, sulfate concentration in the influent
was also measured every 12 hours.

2.3. Analytical methods

Sulfate (SO$_4^{2-}$) and thiosulfate (S$_2$O$_3^{2-}$) concentrations were analyzed by ion
chromatography with conductivity detection using a Dionex ICS-2000 equipment with
an Ultimate 3000 Autosampler Column Compartment, and an IonPac AS18 column
(ThermoScientific, USA). COD was measured using COD kits and a photometer
(Lovibond®).

VFA concentration were measured by gas chromatography (7820-A, Agilent
Technologies) equipped with a DB-FFA column and using a flame ionization detector
(FID) with helium as carrier gas. Prior to VFA analyses, samples were prepared following
the procedure described in Baeza et al. (2017) which consisted of pipetting 0.8 mL of
filtered samples together with 0.2 mL of a preserving solution (which also contained
hexanoic acid as the internal standard) in a glass vial of 1.5 mL. The VFA species
analyzed included acetic, propionic, butyric, isobutyric and valeric acids. Only acetic and
propionic acids were detected in significant amounts. All samples were filtered at 0.22
µm (Millipore, USA).

A sulfide selective electrode (VWR International Eurolab, S.L) connected to a benchtop
meter (Symphony, VWR) was used for the off-line measurement of TDS concentration.

Prior to their measurement, samples were diluted and preserved in sulfide antioxidant
buffer (SAOB). The SAOB composition was (g L$^{-1}$): ascorbic acid (35) and EDTA (67)
dissolved in NaOH (2M).

CH$_4$, CO$_2$ and H$_2$S in the biogas produced were analyzed by gas chromatography (7820-
A, Agilent Technologies, USA). The volume of the gas produced in the UASB reactor
was calculated following the Gas Bag Method (GBM) as presented in Ambler and Logan
This method is based on 1) measuring the initial composition of the collected gas in the bag, 2) adding a known volume of tracer gas (CO\textsubscript{2} in this case) in order to produce an appreciable change in the area of the CO\textsubscript{2} peak in the GC chromatogram and 3) analyzing the new composition after the injection. The average methane flowrate was calculated based on the volume of gas collected along variable time periods in which biogas was accumulated in the sampling bag located on top of the UASB and the methane concentration in the gas bag.

2.4. Illumina sequencing analysis

Microbial diversity analysis was performed using next-generation sequencing. Genomic DNA was extracted from samples of the inoculum and on day 190 of the UASB operation by applying the protocol of MoBio PowerSoil™ DNA extraction kit (MoBio Laboratories, USA). The quantity and quality of the extracted DNA were evaluated by using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific, USA). DNA metabarcoding analysis was performed on an Illumina MiSeq platform by AllGenetics & Biology SL (A Coruña, Spain). For library preparation, a fragment of the bacterial 16S V4-V5 ribosomal RNA gene of around 400 bp was amplified using the primers 515F (5' GTG CCA GCM GCC GCG GTA A 3') and 909R (5' CCG TCA ATT YHT TTR AGT 3').

3. RESULTS

3.1 Long-term performance of the UASB and short-term experiments

The UASB performance was evaluated during 400 days of continuous operation in terms of sulfate removal efficiency (S-RE), COD removal efficiency (COD-RE) and sulfate and COD elimination capacities (S-EC and COD-EC, respectively) using crude glycerol as carbon source. Table 2 shows the results obtained from the long-term UASB operation as
averages and standard deviations of all data acquired in each period. Monitoring results of sulfur species are shown in Figure 2 while COD measurements together with the average flowrate of methane and the concentration of each VFA monitored are presented in Figure 3.

As shown in Table 2, the UASB operation was divided into six periods. During the UASB start-up, sulfate inlet concentration and OLR were maintained at $235\pm17 \text{ mg } \text{S-SO}_4^{2-} \text{ L}^{-1}$ and $12.0\pm2.1 \text{ kg } \text{O}_2 \text{ m}^3\text{d}^{-1}$, respectively. As can be observed in Figure 2, sulfate reduction started almost immediately after inoculation and increased steadily during period I. During period II (days 99-115) the OLR was stepwise increased since the organic matter was limiting in Period I for the complete reduction of sulfate. An S-RE up to 99% with an almost complete removal of the COD (Figure 3A) was obtained at the end of this period. Consequently, the SLR and OLR were increased in Period III, day 115 to 197, by doubling the inlet sulfate and COD concentrations in order to reach a higher sulfate reduction capacity in the system while maintaining the COD/S ratio around $5 \text{ g } \text{O}_2 \text{ g}^{-1} \text{ S}$, which was found to provide the best results in terms of sulfate and COD removal efficiencies during period II. Even if almost complete sulfate removal (S-RE up to 94%) was found during period III, there was a progressive VFA accumulation coupled to a decrease of the COD-RE (Figure 3B).

Despite the S and C loads were not changed, a fourth period was defined because the actual crude glycerol (glycerol 1) was replaced by a new batch from the supplier at the beginning of period IV. The new crude glycerol (glycerol 2) contained 35% less water, an average COD of $900 \text{ g } \text{O}_2 \text{ L}^{-1}$ ($640 \text{ g } \text{C}_3\text{H}_8\text{O}_3 \text{ L}^{-1}$) and a lower $\text{BOD}_5/\text{COD}$ ratio (Table SI-3 in Supplementary Information). Lower average S-REs were found with the new crude glycerol (Table 2) despite some progressive acclimation of functional bacteria to the crude glycerol towards the end of period IV (S-RE above 85%). Consequently, the
SLR was increased in Period V (days 238-288) to verify the maximum treatment capacity of the reactor. In Period V the lowest COD/S ratio was tested despite the system was already overloaded. VFAs accumulated to reaching their maximum concentrations (Figure 3B) as described in section 3.2. During Period VI the UASB operated during 112 days under the conditions tested during period III-IV to recover the initial stability of the system.

In addition, short-term experiments were performed. For that purpose, different sulfate inlet concentrations were tested at the end of period VI (days 360-370) to verify system robustness to face quickly variable inlet loads during the operation. Figure 4 shows sulfate and sulfide concentration profiles as well as the corresponding S-RE and S-EC obtained during the short-term assays. As can be observed in Figure 4, the sulfate RE was almost doubled for the lowest sulfate concentration tested (120 mg S-SO$_4^{2-}$ L$^{-1}$) compared to the initial situation before the short-term experiment.

3.2 Organic matter sink: sulfate reduction and biogas and VFA production

Even if traces of other VFA were measured from the biodegradation of crude glycerol, only acetate and propionate were predominant and therefore considered for further analysis (Figure 3B). During periods I and II, inlet COD was completely consumed and no VFA were detected in the effluent while some CH$_4$ was produced and recovered as part of the gas phase. From period III onwards, when an average OLR of 25 kg O$_2$ m$^{-3}$d$^{-1}$ was fed (Table 1), the effluent contained mainly acetate. As can be observed in Figure 3A, this increase in acetate coincided with a decrease in CH$_4$ production, which ceased 75 days after the beginning of period III. During period V, the maximum concentration of acetate in the reactor was reached (1000 mg acetate L$^{-1}$), which progressively decreased
until the end of the operation when acetate concentrations below 340 mg L\(^{-1}\) were detected.

The conversion of COD resulting from each operating period was also assessed in terms of CH\(_4\) composition in biogas, VFA concentrations in the effluent (acetate and propionate), and COD used for sulfate reduction (Table 3). The COD balance was calculated based on measurements of inlet and outlet COD and VFAs and corresponding methane production. According to the methane composition in biogas, the biogas flowrate and the TDS and residual COD in the effluent, COD conversion proportions along the different periods were obtained according to processes stoichiometry (see Supplementary Information for calculation details). Table 3 shows that along Periods I and II around 11% of the inlet COD was directed to methane production while almost no VFA accumulated in the reactor. However, between 33 and 41% of the influent COD ended up in acetic acid from Period III until the end of the operation while between 3 and 6% of the inlet COD was converted to propionic acid from Period III onwards. Concomitantly, the COD fraction converted to methane had the opposite behavior and was around 0% from Period III onwards, which was taken into account as a way of reporting the percentage of electrons utilized by methanogens. The potential use of COD for sulfate reduction was more stable along the operation even if a progressive deterioration could be detected that accounted for a 26.5% less of organic matter calculated for this purpose comparing the last and the first periods. The rest of COD was assumed to be used for growth and CO\(_2\) formation, although it could not be accurately quantified.

### 3.3 Illumina sequencing analysis and bacterial community assessment

The scope of the microbial analysis was not to describe the evolution of the microbial diversity along the UASB operation but to provide further data to explain the switch from methane production to non-methane production conditions from a microbial perspective.
Thus, the bacterial community through Illumina analysis of the 16S rRNA gene was applied to compare the methanogenic granular sludge used as inoculum with the biomass developed after 190 days of the UASB operation when no methane production was observed. Results obtained from the microbial analysis are presented in Figure 5. *Deltaproteobacteria* and *Methanomicrobia* were the main classes detected in the inoculum with a relative abundance of 20% and 16% respectively (Figure 5). *Clostridia* was the third class in order of abundance (13.5%). After operating the UASB for 190 days, *Deltaproteobacteria* increased their relative abundance to 49% in the sludge bed sample, clearly the most abundant class of the total reads; while *Methanomicrobia* decreased to 0% without detecting any other methanogenic microorganism. *Clostridia* was the second class in order of abundance (12.15%), followed by *Gammaproteobacteria* and *Bacteroidia* (11 and 7.3% of total reads, respectively).

As the operation proceeded, in the biomass community of day 190 (Tables S1 and S2; Supplementary Information), *Desulfovibrio* was the most abundant OTU at genus level, with a 35.3% of total retrieved sequences. Oude Elferink et al. (1994) reported that *Desulfovibrio* had higher affinity for sulfate and higher growth rate than other SRB genera such as *Desulfobulbus* and *Desulfobacter*. Interestingly, *Desulfatirhabdium* sp. accounted for 2% of total operational taxonomic units at genus level which has been described as butyrate-oxidizing bacteria (Balk et al., 2008). In the case of *Proteobacteria* that were not SRB, the highest relative proportion of microorganisms belonged to the *Enterobacteriaceae* family (11%).

4. DISCUSSION

4.1 Startup of a sulfate reducing UASB reactor and influence of inoculum

In practice, start-up of full-scale UASB reactors for sulfate reduction is handicapped because of the lack of reactors from where inocula with a high density of SRB can be
withdrawn. Few works have reported the start-up of sulfidogenic reactors with inocula that have not been adapted to sulfidogenic conditions (García-Solares et al., 2014; Omil et al., 1998). Inoculation with methanogenic sludge from widespread, full-scale mesophilic anaerobic digesters is the most common alternative and, probably, the only alternative in practice at full-scale. The evolution of the UASB performance observed in Figure 2 shows that stable sulfate removal efficiencies higher than 80% were achieved just one month after the continuous operation of the UASB reactor initially inoculated with granular sludge from an anaerobic digester treating wastewater in a pulp and paper industry. The inoculum was not pre-adapted but sulfate reduction started almost from the beginning of the operation since sulfate was present in the pulp and paper industry and, consequently, sulfate-reducing bacteria. This is in agreement with Roest et al. (2005), who stated that anaerobic digesters sludge from paper mill industries are suitable for providing an appropriate process culture to promote sulfidogenesis.

Compared to previous works, such short and efficient start-up was remarkable considering the source of the inoculum used. As examples, Gonçalves et al. (2005) needed over 6 months to bioactivate an UASB to obtain anaerobic sulfidogenic sludge able to degrade 400 mg SO$_4^{2-}$ L$^{-1}$ using molasses as C source, while Bertolino et al. (2015) needed over 200 days to enrich granular sludge from an UASB treating domestic wastewater during the treatment of 2 g SO$_4^{2-}$ L$^{-1}$ influent with pure glycerol. In our work, the granular sludge used as inoculum was mainly methanogenic, which was confirmed through Illumina sequencing. *Methanosaeta* and *Methanobacterium* were the most abundant methanogens at genus level in the inoculum, what was expected as they are the most characteristic archaeal sequences found in anaerobic digesters (Leclerc et al., 2004), while a reduced amount of SRB were found. Figure 3A shows that the maximum flow of methane was produced during period I and II due to the influence of the inoculum. During
period I (OLR of 12.0±2.1 kg O₂ m⁻³ d⁻¹) and II (OLR of 15.8±4.6 kg O₂ m⁻³ d⁻¹), the average organic matter consumption was 86% and 89% respectively. COD concentrations were below 100 mg O₂ L⁻¹ in the effluent, which probably corresponded to the less biodegradable matter contained in crude glycerol considering that the anaerobic biodegradability of crude glycerol due to presence of such inhibitory impurities has been reported to be between 65-85% (Viana et al., 2012). Furthermore, 10% of the oxidized organic matter was used for methane production during period I (Table 3). Similarly, during period II, 11% of the transferred electrons were utilized for methane production.

Despite such methanogenic activity, Figure 2 shows that a stable operation in terms of sulfate reduction was reached by the end of period II with almost complete removal of sulfate and COD (S-RE of 96.5% and COD-RE of 89.3%). Despite methane production, results confirmed that organic substrates were available for sulfate reduction and that microbial communities underwent a fast and gradual acclimation to their environment. Our work demonstrates that using methanogenic granular sludge from a paper and pulp industry leads to a fast start-up of sulfidogenic UASBs when moderate inlet sulfate concentrations of 235±17 mg S-SO₄²⁻ L⁻¹ are treated using crude glycerol as electron donor at C/S ratio of 3.8 g O₂ g⁻¹ S.

4.2 Shifts in the organic matter sink

The sink of the organic matter can shift drastically due to the evolution of the microbial population, which influences the performance of the UASB reactor. During Period III, a high S-RE was reached after few days of operation which allowed obtaining a maximum S-EC of 6.6 kg S-SO₄²⁻ m⁻³ d⁻¹ (273 g S-SO₄²⁻ m⁻³ h⁻¹) from this period (Period III). Compared to previous periods, the COD-RE dropped drastically (Figure 3A) and an accumulation of VFA was observed (Figure 3B), which indicated that a steady, almost complete sulfate reduction could be reached at a SLR of 4.6 kg S-SO₄²⁻ m⁻³ d⁻¹ and COD/S
As previously described by Pol et al. (1998), when a sulfate-rich wastewater is fed into an anaerobic reactor, organic matter will be removed both via methanogenesis and sulfate reduction and when methanogenesis becomes suppressed then a gradual decrease in the organic matter conversion (COD removal) is observed, which was corroborated herein with crude glycerol instead. During this third period (OLR of 24.4 kg COD m$^{-3}$ d$^{-1}$), the average organic matter consumption was 38% while only 2.3% of the COD removed ended in methane production when the COD/S was increased to 5.4 g O$_2$ g S$^{-1}$ (Tables 2 and 3). Taking into account that the reported cellular yield for acidogenic bacteria (0.14-0.17 gVSS/gCOD) is five times higher than that of acetogenic bacteria (0.025-0.051 gVSS/gCOD) or methanogenic archaea (0.01-0.054 gVSS/gCOD) (Pavlostathis and Giraldo-Gomez, 1991) glycerol will be readily available for acidogenic bacteria, and the limiting step will be the methanogenesis.

During the anaerobic digestion of glycerol, some organic acids (acetic, propionic, butyric, valeric and others), produced by fermentative acidogenic bacteria, cannot be consumed by methanogenic archaea at the same rate at which they are produced (Viana et al., 2012). The accumulation of VFA indicated that the slowly growing methanogens could not sufficiently and rapidly metabolize the intermediate products from VFA producers (acidogenic and acetogenic populations). Since acetate is mainly converted by methanogens and no methanogens were found in the sludge sample from day 190 (Figure 5), increasing concentrations of acetic acid were found in the reactor between period III and V. This is in agreement with the production of methane measured from the gas phase (Figure 3A) and with some authors statements (Harada et al., 1994; Omil et al., 1998), who pointed out that the predominance of SRB over methanogens in sulfate-rich streams is only achieved after long-term operation (more than 100 days) in UASB reactors. As reported with other electron donors (Dar et al., 2008; Raskin and Rittmann, 1996), SRB
also out-competed methanogens using crude glycerol. However, more research is needed to understand to which extent is this competition beneficial or, if losing completely the presence of methanogens at such a low up-flow velocity would imply losing S-EC due to other problems. Potentially, diffusional limitations and bed stratification may appear due to the lack of gas bubbles moving upward.

4.3 Long-term UASB performance and microbial diversity changes

High sulfate reduction efficiencies together with VFA accumulation were also observed by Bertolino et al. (2012). From period III onwards, the acetate concentration remained in the 400-1100 mg O\textsubscript{2} L\textsuperscript{-1} range. Although the S-RE was significant in Period III (80%), it progressively decreased to below 80% (even below 50% in periods V and VI). Despite some SRB are able to oxidize acetate to CO\textsubscript{2} (Widdel and Pfennig, 1982; Szewzyk and Pfennig, 1987; Muyzer and Stams, 2008), only incomplete oxidizers were detected in the 190-day sample (Supplementary Information). Consequently, promoting acetate-oxidizing SRB may be an alternative to increase the sulfate reduction concomitantly producing a less C loaded effluent.

Most of the COD used for methane production lead to acetate and propionate accumulation from Period III onwards (Table 3) together with an evolution of the microbial diversity (Figure 5). Deltaproteobacteria was the most abundant class after 190 days of operation. Many genera such as Desulfovibrio, Desulfbacter and Desulfuromonas belong to this class and play a fundamental role in the sulfur cycle. Only 4% of reads were not identified at class level, compared to the 31% of reads not identified in the inoculum sample. This result indicated that the microbial community specialized in more specific functions and that populations were selected according to operating conditions. In the presence of sulfate, SRB usually out-compete methanogens, which only dominate in a low-sulfate environment (Oude Elferink et al., 1994).
As acclimation proceeded under the high TDS concentration reached during the operation of the reactor, methanogens were completely washed out. In general, sulfate-reducing bacteria can grow with a much wider substrate range than methanogens (Muyzer and Stams, 2008). Consequently, methanogenic communities require syntrophic associations, which are not essential in sulfate reducing environments (Janssen et al., 2009). Several SRB are able to use glycerol as an electron donor and some *Desulfovibrio* species have been reported to grow with glycerol (Stams et al., 1985; Kremer and Hansen, 1987; Esnault et al., 1988). As reported by Hu et al. (2015) and Lens et al. (1998) the complete or incomplete oxidation of organic substrates accomplished by some species of SRB will depend on the COD/\(\text{SO}_4^{2-}\) ratio in the influent. However, in this study, it is reasonable to conclude that SRB always performed incomplete oxidation at the ratios tested. The disappearance of methanogens and the concomitant accumulation of acetate in the system suggested that methanogens were probably the only microorganisms consuming acetate at observable rates.

It remains an open question how acetate oxidation can be stimulated in order to improve the reactor performance. Despite some promising attempts have been made (Lens et al., 1998), further development of strategies for augmentation of acetotrophic SRB are warranted to increase the sulfidogenic capacity of the process. A strong association between two acidophiles, a sulfate reducing bacterium and a non-sulfate reducing bacterium is proposed by Kimura et al. (2006) but further improvements of the operation are still required. Also, despite VFA accumulation has been regarded as a sign of process failure in anaerobic digestion, VFA accumulation can be seen as an opportunity in sulfate-reducing UASBs since VFA have important biotechnological potential as these carboxylates can be used as substrates for production of biofuels and bioplastics, or in other bioprocesses. The loss of organic matter from the UASB reactor is economically
undesirable since the reducing power supplied with glycerol is only partly used. In addition, further resources must be used to treat the excess COD from the anaerobic reactor. In the sulfur recovery process depicted in Figure S1, a reduction-oxidation bioprocess was proposed. Then, the COD in the effluent could be treated in the CSTR reactor for the partial oxidation of sulfide to elemental sulfur although an extra consumption of oxygen to treat COD would be required. Consequently, optimization in the use of the electron donor is warranted.

4.4 Potential process limitations

Inhibitory substances are often found to be the main cause of anaerobic reactor disturbance and failure as they cause an adverse shift in the microbial population or inhibition of bacterial growth (Chen et al., 2008). Inhibition of anaerobic digestion is usually diagnosed by a decrease of the steady-state rate of methane gas production and accumulation of organic acids (Kroeker et al., 1979), which was found in the long-term operation of the UASB. The organic acid and methane forming microorganisms differ widely in terms of physiology, growth kinetics, and sensitivity to environmental conditions (Pohland and Ghosh, 1971). At pHs below 7.0, most carboxyl groups are undissociated, thus they pass freely through the membrane and can inhibit the growth of many bacteria. Uncharged molecules such as acetic acid may be inhibitory because they diffuse across the cell membrane and act as an uncoupler (Ghose and Wiken, 1955), whereas acetate ion is not permeant. Inhibition concentrations of $4.68 \cdot 10^{-3}$ mg free acetic acid L$^{-1}$ (pH=7.5) have been reported to block acetoclastic methanogenesis (Fukuzaki et al., 1990). Despite the buffer used, the pH in the reactor varied from 8.4-8.7 at the inlet to 6.7-7.5 at the outlet due to VFAs accumulation, and particularly acetic acid. Considering the $pK_a$ of acetic acid (4.76) and the concentrations of acetate found in the early stages of period III (around 375 mg acetate L$^{-1}$), concentrations above 0.23 mg free
acetic acid L\(^{-1}\) found in the UASB could have led to significant methanogens inhibition.

In addition, free H\(_2\)S concentrations leading to 50% inhibition of methanogenesis of 250 mg S L\(^{-1}\) in the pH range 6.4–7.2 and 90 mgS L\(^{-1}\) at pH = 7.8–8.0 have been reported (Koster et al., 1986), indicating that methanogens were also inhibited by sulfide accumulation in the early stages of Period III. Overall, TDS and acetate accumulation lead to a fast decrease of the methanogenic activity in Period III. No methane production from period IV until the end of the operation indicated that methanogenic communities were more susceptible to dissolved sulfide concentration than SRB as was also observed in Jing et al. (2013).

Despite the high concentrations of acetate found in the reactor during period III, IV and V (400-1100 mg O\(_2\) L\(^{-1}\)), sulfate reduction proceeded at high S-RE during period III, indicating that SRB were not affected by acetic acid. Similarly to methanogens, free H\(_2\)S may inhibit SRB. However, inhibitory free H\(_2\)S concentration in literature are often contradictory and confusing probably due to the difference in anaerobic inocula used, the susceptibility of anaerobes and the experimental methods and conditions tested in each study, and particularly the pH in the bioreactor. Anaerobic treatment of sulfate-rich wastewater proceeds successfully at COD/sulfate ratios lower than 10 g COD g\(^{-1}\) SO\(_4^{2-}\) when precautions are taken to prevent sulfide toxicity (Pol et al., 1998). The TDS during the operation reached 460 mg S L\(^{-1}\) by day 240 (Period V). Reis et al. (1992) found that more than 547 mg H\(_2\)S L\(^{-1}\) can completely inhibit SRB activity at pH 6.2, whereas at pH 9.0, dissolved H\(_2\)S is mainly in the form of HS\(^-\), which does not penetrate into cells easily (Mora-Naranjo et al., 2003) and therefore would not have a strong inhibitory effect over SRB. As Reis et al. (1992) observed, sulfate uptake decreased when sulfide concentration in the medium increased, and increased again when it was removed from the medium, which pointed out that sulfide is a reversible inhibitor of SRB. A pH range of 6.7-6.8 at
the outlet of the reactor points out at a reduction of the potential maximum SRB rates due
to inhibition of SRB by hydrogen sulfide.

4.5 Overall performance of the sulfidogenic UASB

Sulfidogenesis was achieved through adaptation of granular sludge with important
methanogenic activity, using electrons derived from substrate towards sulfate reduction.
While adapting methanogenic granular sludge to sulfate reduction is one of the most
common and widespread procedures to engineer microbial sulfate reduction (García-
Solares et al., 2014), the stability of the system during long-term operations is still a cause
of concern. During period V, SLR was maintained during 50 days but the high sulfate
inlet resulted in a sulfate-reduction failure. The system was overloaded and its maximum
capacity, 6.5 kg S m\(^{-3}\) d\(^{-1}\), was reached after 165 days of operation at a SLR of 6.7 kg S-
SO\(_4^{2-}\) m\(^{-3}\) d\(^{-1}\) and a COD/S of 5.6 g O\(_2\) g\(^{-1}\) S. Overall, the performance of the UASB is
comparable to that obtained by Bijmans et al. (2008) (9.7 kg S m\(^{-3}\) d\(^{-1}\)) using formate,
which is more biodegradable than crude glycerol. Higher S-ECs were found compared to
Boshoff et al. (2004) (600 mg SO\(_4^{2-}\) L\(^{-1}\) d\(^{-1}\)) who used tannery effluent as carbon source.
After a long-term operation of 360 days, short-term SLR assays (Figure 4) were
performed to study the capability of the UASB to reduce sulfate at such a low HRT (2h)
and under dynamic conditions with non limiting COD availability. The UASB adapted
well to transient load changes and, more interestingly, recovered to the initial load
exhibiting a 25% higher S-RE compared to that before the short-term experiment.
However, it remains to be investigated why such temporary load decrease resulted
apparently beneficial for the UASB performance considering that the same sulfide
concentration was found before and after the stepwise decrease of the inlet sulfate
concentration. Based on a sulfur balance, a larger C/S ratio during the short-term
experiment (up to 19.1 g COD g S\(^{-1}\)) could have led to an increase in the production of
organosulfur compounds. Overall, a sulfur balance of 85-95% along the UASB operation was obtained based on inlet and outlet sulfate (S-SO$_4^{2-}$) concentrations and produced TDS (Figure 1). Such imbalance was attributed to other organosulfur compounds such as dimethyl sulfide (DMS) or dimethyl disulfide (DMDS) amongst others that were qualitatively detected in the effluent of the UASB (see Table SI-4 in Supplementary Information).

4. CONCLUSIONS

Long-term operation of a sulfidogenic UASB reactor can be successfully achieved using crude glycerol as carbon source at low up-flow velocities. It was demonstrated that at OLR above 24 kg O$_2$ m$^{-3}$d$^{-1}$ and SLR of 4.6 kg S-SO$_4^{2-}$m$^{-3}$d$^{-1}$ VFA were accumulated. The TDS concentration increase together with VFA accumulation were potential inhibitors of methanogenic activity and when methane production decreased, glycerol was converted mainly to acetic acid and propionic acid. It was not only the COD/S-SO$_4^{2-}$ ratio, but a sum and combination of factors along the operation that determined the competition between SRB and methanogenic archaea. However, further batch activity tests are warranted to properly validate the results obtained herein.

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