Influence of crude glycerol load and pH shocks on the granulation and microbial diversity of a sulfidogenic Upflow Anaerobic Sludge Blanket reactor

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ABSTRACT

Bioscrubbers are an environmental-friendly alternative to valorize SO_2 contained in flue gases to obtain elemental sulfur as final value-added product. The bottleneck of a SO_2 bioscrubber relies on the heterotrophic reduction of the absorbed SO_2 to obtain sulfide. In this study, the performance and stability of a sulfidogenic Upflow Anaerobic Sludge Blanket reactor (UASB) using crude glycerol was investigated during 6 months of operation under variable organic loading rates. The UASB presented a maximum elimination capacity and a sulfate removal efficiency of 110 mg $S\text{-}SO_4^{2-}$ L^{-1} h^{-1} and 100%, respectively, when the chemical oxygen demand to sulfate ratio (COD/S-Sulfate) was 8.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$. The intermediate compounds identified from crude glycerol degradation were mainly propionic and acetic acid, which varied along the sludge bed together with the pH. Microbial diversity analyses of the sulfidogenic granular sludge showed that the most abundant sulfate reducing genera were *Desulfovibrio* spp. and *Desulfobulbus* spp. *Methanosaeta* and other fermentative/acidogenic microorganisms were also found in significant amounts. Particle size distribution analyses showed that biogas production allowed the granulation of the sulfidogenic sludge, which had an average particle size ranging from 729.3 μm (lower part of the bed) to 391.7 μm (upper part of the test). A short-term pH shock caused a detrimental effect over the system performance due to degranulation. Concomitantly, biogas production was interrupted and acetic acid was accumulated also causing a significant impact on microbial diversity. Unclassified *Clostridiales*, *Desulfovibrio* spp. and *Desulfobulbus* spp. showed a higher resistance to pH shocks.

Keywords: Crude glycerol, sludge bed stratification, SO_2 valorization, sulfidogenesis, SRB, UASB

1. INTRODUCTION

Combustion of sulfur-containing fuels results in SO_2 formation, which cause detrimental impacts on human health and the environment such as respiratory illness and acid rain, respectively, among others (Srivastava and Jozewicz, 2001). During the last decades, the SO_2 release to the atmosphere in Europe has been limited by different directives and decreased due to the implementation of treatment systems. However, anthropogenic worldwide emissions of SO_2 in 2011 were around 100 Tg mainly generated in the energetic and industrial sectors (Klimont et al., 2013). The treatment of SO_2 emissions from flue gases performed through physical-chemical treatments such as absorption are expensive and generate effluents that require further processing as limestone forced oxidation or furnace sorbent injection (Srivastava and Jozewicz, 2001; Philip and Deshusses, 2003).

Biological processes offer advantages with respect to physical-chemical ones such as reduced operational costs and wastes generation. The circular economy culture is also driving biological processes development towards waste valorization (Scherson and Criddle, 2014). Some authors have proposed alternatives for the valorization of SO_2 from flue gases. However, many of them pose significant drawbacks such as the use of expensive carbon sources for sulfate reduction (such as glucose, volatile fatty acids or alcohols), the use of immobilized biomass for sulfur production (hindering elemental sulfur recovery) or the use of iron or nitrate for sulfide oxidation (increased operational costs) (Gasiorek, 1994; Philip and Deshusses, 2003; Qian et al., 2015). Considering that elemental sulfur is a valuable compound actually obtained from non-renewable sources, the two-step bioscrubbing process was envisaged as a promising alternative to valorize SO_2 from flue gases as elemental sulfur if a valuable waste organic source such as crude glycerol is used (Mora et al., 2016). The two-step bioscrubbing process consists of a first absorption stage with a slightly alkaline solution followed by a heterotrophic Upflow Anaerobic Sludge Blanket (UASB) reactor for the reduction of sulfate to sulfide in series with downstream bioreactor for partial oxidation of sulfide to elemental sulfur (see Figure S1 in Supplementary Material).

Crude glycerol is a chemical oxygen demand (COD) rich by-product produced in large quantities from the soap and biodiesel manufacturing processes (Dinkel et al., 2010a). In the literature there are some studies assessing the chemical and/or biological conversion of crude glycerol into more valuable products (Yazdani and Gonzalez, 2007). Anaerobic

digestion of crude glycerol to produce biogas is the most popular or well-known alternative to obtain energy from this attractive waste (Lopez et al., 2009). Santos et al. (2018) recently reported that the use of crude glycerol promotes sulfate-reducing communities growth while Mora et al. (2018) also demonstrated that crude glycerol is a powerful organic source to promote sulfidogenesis under certain conditions. Crude glycerol use in sulfidogenic reactors has been described for recovering precious or valuable metals, for immobilizing heavy/toxic metals or simply to study and get knowledge about the competition between methanogens and sulfate reducers (Dinkel et al., 2010b; Bertolino et al., 2014; Santos et al., 2017). On the contrary, the use of crude glycerol in sulfidogenic UASB reactors towards elemental sulfur recovery is scarce.

The limiting step in SO_2 bioscrubbing is the heterotrophic reduction of sulfate to sulfide (Mora et al., 2016). The presence of organic matter promotes the methanogenic activity and the production of biogas (Thirugnanasambandham et al., 2014; Sridhar et al., 2016; Thirugnanasambandham et al., 2016; Thirugnanasambandham 2017), which is really positive to enhance and maintain the granulation of the sludge due to the shear stress that biogas circulation generates (Liu and Tay, 2002). However, the generation of biogas in sulfidogenic reactors is not advisable since sulfide strips out from the liquid to the gas phase resulting in generation of a highly corrosive biogas. Then, the production of biogas has to be minimized by increasing the COD selectivity towards sulfate reduction. In general, literature focuses on the enhancement of methane production avoiding sulfidogenesis or at least the presence of the produced sulfide in the biogas. Many studies have reported the start-up and operation of sulfidogenic UASB reactors (Alphenaar et al., 1993; Jing et al., 2013). Nonetheless, there is a deficiency of studies related with sulfidogenesis promotion against methane production and a few target the reduction of sulfate in UASB reactors with crude glycerol under switching conditions such as variable carbon loads and pH shocks. There is even less information about the bed stratification in terms of bioreactions progress, granulation and microbial diversity. This sort of research is particularly needed since granular sludge is often used, not only because it allows enhancing the distribution of the liquid phase and the settling capability of sludge but because the granulation of sulfate reducing bacteria at low up-flow velocities is not feasible.

The aim of the present study was to assess the performance and stability of a sulfidogenic UASB, inoculated with methanogenic granular sludge, under different conditions in terms of sludge and liquid phases stratification in the sludge blanket, microbial diversity

distribution and sulfur mass balance. The SO_2 absorption stage and the partial oxidation of sulfide into elemental sulfur, which are part of the two-step bioscrubber concept, were out of the scope of the present study.

2. MATERIALS AND METHODS

2.1 Experimental setup for sulfidogenesis promotion in a UASB reactor

The experimental setup used in this study to promote sulfidogenesis from sulfate rich effluents is presented in Figure 1. It consisted of a Upflow Anaerobic Sludge Blanket (UASB) type reactor made of glass with a reaction volume of 1L and a total volume of 2L. An UASB reactor configuration was selected since UASBs are well-known, versatile, work with high biomass densities and face well product inhibitions, overall leading to larger volumetric productivities compared to other reactor configurations. The reactor was divided in two different sections, i.e. a lower section (riser) containing the sludge blanket or biological bed, with an internal diameter (D) of 0.05 m, and the upper section acting as biomass, liquid and gas separator (D=0.11 m). Mineral medium was pumped from bottom to top of the reactor. The composition of the mineral medium was (g L^{-1}): NH_4Cl (0.5), K_2HPO_4 (3.5) and Na_2SO_4 (1) dissolved in tap water and adjusted to pH=8.8-9.0 with NaOH (2 M). A total flow rate of 0.5 L h^{-1} was pumped into the system (up-flow velocity of 0.25 m h^{-1}), which consisted of a mixture of the mineral medium and crude glycerol (ecoMotion S.A., Spain). The flow rate of the influent was set at 1.2 mL h^{-1}. Hence, crude glycerol was diluted to adjust the inlet COD concentration. The hydraulic residence time (HRT) considering the reaction volume (riser) was 2h. T and pH in the UASB reactor were not controlled but monitored. Inlet pH ranged from 8.8 to 9.0. A 5 L Tedlar bag (SKC Inc.) was used to collect and analyze the composition and flow of the biogas produced. Inlet and outlet flows were also sampled every two to three days to analyze COD, sulfur species and volatile fatty acids (VFA).

2.2 Start-up and operating conditions of the process

The UASB was inoculated with 50 g of settled granular sludge from an anaerobic digester treating wastewater in a paper recycling company. The operation of the UASB reactor lasted for more than 6 months. Operating conditions at start-up were set at a COD/S-Sulfate ratio of 2.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$ since it has been proven that a COD limitation allows the development of sulfidogenic bacteria while higher COD/S-Sulfate ratios enhance a fast development of fermentative, acidogenic and methanogenic microorganisms that

compete with sulfidogenic heterotrophic bacteria (Choi and Rim, 1991; Hu et al., 2015). Sulfate inlet concentration was set at 220 mg S-SO_4^{2-} L^{-1} while crude glycerol was fed at four different organic loading rates (OLR), which led to operate under five different COD/S-Sulfate ratios (Table 1). A 24h pH shock was provoked on day 120. After resumption, a lower HRT was obtained during the last operation period (140 d to 200 d) since part of the sludge was progressively lost during the previous period (days 120 to 140) obtaining a lower reaction volume (blanket) in the UASB. A minimum of 30 days between each load change were established.

2.3 Analytical methods

Sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) concentrations were analyzed off-line by ion chromatography using suppressed conductivity detection (ICS-2000, Dionex Corporation). Chemical Oxygen Demand (COD) was analyzed off-line using COD commercial kits (LCK314 and LCK1414, Hach LTD) and a spectrophotometer (DR2800, Hach). A SenTix® 82 probe (WTW) connected to a benchtop meter (Inolab® Multi 740, WTW) was used for routine pH measurements. Sulfide concentration was analyzed off-line with a sulfide selective electrode connected to a benchtop meter (Symphony, VWR) after conditioning the samples with sulfide antioxidant buffer (SAOB). The SAOB composition was (g L^{-1}): ascorbic acid (35) and EDTA (67) dissolved in NaOH (2M). VFA were analyzed by gas chromatography (7820A, 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).

CH_4, CO_2 and H_2S were analyzed by gas chromatography (HP 5890, Hewlett Packard) equipped with a Porapack Q column and a thermal conductivity detector (TCD) with helium as carrier gas. The volume of the gas produced in the UASB reactor was analyzed following the Gas Bag Method (GBM) presented in Ambler and Logan (2011) using gas chromatography (7820A, Agilent Technologies) equipped with an HP-Mole Sieve column, a thermal conductivity detector (TCD) and using argon as the carrier gas. The GBM procedure consisted basically of measuring the initial composition of the collected gas in the bag, adding a known volume of tracer gas (nitrogen gas in this case) and analyzing the new composition. From these two analyses (before and after the tracer injection) the initial volume of gas could be calculated from mass balances (Baeza et al., 2017).

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169 **2.4 Granular sludge bed stratification and sequencing analysis**

170 The UASB performance was also assessed at different bed heights (5, 25 and 44 cm).
171 Granulation of the anaerobic granular from the liquid phase was studied on days 0, 10,
172 60, 110 and 200 after startup while the supernatant of the samples was also analyzed for
173 sulfide, sulfate, COD and VFAs on days 110 and 200. To this aim, samples were
174 centrifuged at 8000 rpm during 2 min. The particle size distribution (PSD) was measured
175 by a laser particle size analysis system (Malvern MasterSizer Series 2600, Malvern
176 instruments Ltd., UK). Analysis of inorganic elements in the granules was also performed
177 to study the composition of the granular sludge and the formation of inorganic
178 precipitates. An Inductively Coupled Plasma-Mass Spectrometry (ICP-MS 7500 CE,
179 Agilent) was used. Previously, samples were washed and centrifuged twice (8000 rpm, 2
180 mins), dried at 105ºC during 24h and digested with nitrohydrochloric acid in a microwave
181 digester (Ultrawave, Milestone). Total solids and volatile solids concentrations in the bed
182 were also analyzed at different heights following the standard method of analysis (APHA,
183 2005). Microbial diversity was assessed on days 0, 110 and 200 by extracting and
184 sequencing the deoxyribonucleic acid (DNA) following the procedure described in Mora
185 et al. (2018).

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187 **3. RESULTS**

188 **3.1 Performance of the sulfidogenic UASB**

189 Figure 2 shows the profiles obtained during the monitoring of the main species involved
190 in the process. As can be observed in Figure 2A, after 15 days of operation the removal
191 efficiency (RE) of sulfate (S-RE) was 42.1%, which corresponded to an elimination
192 capacity of 55.8 mg $S\text{-}SO_4^{2-}$ L^{-1} h^{-1}. From this point until day 50, the average S-RE was
193 34.3±3.5% while the sulfur imbalance was around 10% due to the incomplete recovery
194 of sulfate as sulfide (Figure 2D). Regarding crude glycerol, the average RE of COD
195 (COD-RE) along this first period of 50 days was 76.9±7.0%. Another important aspect
196 observed from the first performance period was that the organic matter degraded was
197 mainly destined to reduce sulfate, nevertheless, almost 25% of the organic matter was
198 converted into methane (a methane production of 21.2±6.7 mL CH_4 h^{-1} was obtained with
199 a purity up to 95-99%). As can be observed in Figure 2C, VFA were not present in the
200 effluent indicating that VFA were produced and completely utilized to reduce sulfate and

to produce methane. In Table 2 the summary of S-RE and COD-RE resulting from all the UASB operation periods is presented.

During the second period (50 – 90 days) COD inlet concentration was increased (Table 1) to reach a theoretical COD/S-Sulfate ratio of 7.0 g O_2 g^{-1} $S\text{-}SO_4^{2-}$. The previous ratio tested (2.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$) was not high enough to completely reduce sulfate to sulfide although was adequate to avoid a high methanogenic activity. The sulfur imbalance in this second operation period was more than 20% (Figure 2D), while the H_2S content in the gas was less than 5% of the inlet sulfate (data not shown). The OLR set during this second period of operation (804±134 mg O_2 L^{-1} h^{-1}) allowed reaching S-RE and COD-RE of 83.0±12.8% and 80.8±5.2%. Methane production increased up to 100 mL h^{-1}, corresponding to 38.6% of the degraded COD, and the biogas produced had a CH_4 content of 89.8±4.0% (data not shown). As can be also observed in Figure 2C (second operation period from day 50 to 90), there was an instability of biogas production. At this point, biomass accumulated into the gas collector did not allow the gas produced to flow to the Tedlar bag, thus causing an inefficient separation of the gas phase in the upper part of the reactor.

During the following operating period (90 - 110 days) the COD/S-Sulfate ratio set (8.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$) allowed obtaining a successful S-RE. An average S-RE of 97.1±2.9% was reached (Figure 2A and Table 2). Compared to the previous operating period, the COD-RE and the COD elimination capacity (COD-EC) dropped drastically (from around 645 mg O_2 L^{-1} h^{-1} to around 415 mg O_2 L^{-1} h^{-1}) which caused an increase of the COD concentration in the effluent. A better collection of the biogas was obtained through the addition of nitrogen pulses in the mineral medium inlet. At the end of this period, the stratification study of the bed was also performed (day 110) to elucidate the processes occurring along the sludge bed.

A COD/S-Sulfate ratio of 4.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$ was set from day 110 until day 140 to reduce the excess of organic matter in the effluent of the reactor. The inlet glycerol concentration was reduced from 1891±370 mg O_2 L^{-1} to 1026±90 mg O_2 L^{-1} to obtain a COD load (524±47 mg O_2 L^{-1} h^{-1}) slightly higher than the COD-EC of the previous period (415 mg O_2 L^{-1} h^{-1}). The reduction of the inlet COD/S-Sulfate affected negatively the UASB performance and caused the increase of sulfate and COD concentrations in the effluent up to 55 mg $S\text{-}SO_4^{2-}$ L^{-1} and 636 mg O_2 L^{-1}, respectively. Moreover, from day 120 to day 121 a pH shock of 24h was provoked. The value of the pH in the mineral medium was set to pH=12.5. Consequently, the S-RE and COD-RE decreased down to

57.6±13.5% and 35.3±6.6%, respectively. The pH shock caused the sludge blanket migration and rupture due to the sludge degranulation (Figure S2 in Supplementary Material). Consequently, the biomass rising caused a loss of 30% of sludge bed volume during this operating period. Because of the bed instability, 100 mL of activated carbon were added on day 140 in the lower part of the UASB to procure a better liquid distribution. After the addition of activated carbon, S-RE was recovered and sulfide was produced with a minimal percentage in the S imbalance (Figure 2D). However, preferential paths were created again in the upper part of the bed leading to a S-RE increase the last 25 days of operation.

3.2 Assessment of the sludge bed stratification

Operating conditions set in the reactor (particularly the up-flow velocity) caused the stratification of the sludge bed, which naturally happens in plug-flow reactors such as UASB reactors. This phenomenon creates a set of layers with different particle size, microbial diversity and chemical compounds distribution along the sludge bed that were analyzed to assess the process performance at different reactor heights.

3.2.1 Granular size distribution along the bed height

Figure 3 shows the particle size distribution at different bed heights at each sampling time compared to that of the inoculum. The PSD 10 days after the start-up of the process revealed that the granules size of the inoculum was maintained in the first part of the bed (5 cm). However, granulation was almost lost at H=25 (half reactor, Figure S2A) since half of the particles had an average diameter lower than 215 μm (i.e. D(0.5)=215 μm), which is around the limit to consider a biomass aggregate as a granule (200 μm).

Granulation recovered in all bed heights between days 60 and 110 coincident with the increase of the OLR and subsequent production of biogas (Figure 2C). The PSD revealed that half of the granules measured at H=5 cm had a diameter lower than 729 μm while at H=25 cm and H=44 cm the D(0.5) was below 392 μm. However, after the pH shock (days 120 and 121) the granulation was completely lost and many operational problems occurred until the end of the operation, as migration events of the bed (Figure S2B) or the creation of preferential paths due to the rupture of the bed (Figure S2C).

3.2.2 Stratification of chemical species along the sludge blanket

Figure 4 shows the profiles of sulfate, sulfide, VFA and pH along the sludge bed on days 110 (Figure 4A) and 200 (Figure 4B). After 110 days of operation, when S-RE was close to 100%, sulfate and sulfide profiles indicated that sulfidogenesis was not occurring in the lower part of the UASB since sulfate was not degraded at H<5cm. In contrast, crude glycerol was rapidly hydrolyzed and mainly converted into propionic acid and acetic acid. The pH also decreased concomitantly with VFA production. From H=25cm upward, acetic acid was consumed while the propionic acid concentration was maintained. The concentration of VFA in the effluent of the UASB was over 700 mg O_2 L^{-1} and sulfate was not completely recovered as sulfide. At this point the sulfur imbalance was 38%.

After 200 days of operation (Figure 4B), stratification of the bed had a completely different pattern from that obtained when the operation of the UASB did not present any problem (Figure 4A). In this case, an incomplete reduction of sulfate was observed while the S imbalance obtained was lower than 10%. Interestingly, the VFAs accumulated switched with respect to the previous operation period from propionic acid to acetic (predominant) and butyric acid.

3.2.3 Microbial diversity developed in the sufidogenic UASB

The inoculum, the sludge bed after 110 days of operation at 3 bed heights and a single sample at the end of the operation were analyzed for their microbial diversity. Figure 5 shows the percentages of main genera identified in the samples. Most of the genera identified in the inoculum were mainly fermentative bacteria and acidogenic bacteria such as *Syntrophobacter* (9.6%), *Clostridium* (3.3%), *Smithella* (3.0%), *Bacteroides* (2.7%), *Pseudomonas* (1.1%), *Saccharofermentans* (1.0%) besides Unclassified Clostridia (6.6%), Unclassified Anaeroliniales (2.0%) or the phylum Unclassified Firmicutes (1.8%). *Methanosaeta* (15.9%) and *Methanobacterium* (1.0%) were the main methanogenic microorganisms identified. These genera of archaea and bacteria are typically found in sludge from anaerobic digesters (Ariesyady et al., 2007). On the contrary, the SRB identified genus *Desulfobulbus* represented only 1.1% of the culture, which can grow using organic matter to produce acetate and/or using H_2 as electron donor. It must be mentioned that also Unclassified Syntrophobacterales (4.3%) have been described to reduce sulfate (Liu et al., 1999), which would enlarge the relative percentage of SRB in the inoculum.

After 110 days of operation, the microbial diversity changed completely. A decrease in the diversity from 22 to 16 identified genera compared to the inoculum was found. The

genus *Anaerosinus, Citrobacter, Eubacterium, Klebsiella, Dysgonomonas* and *Trichococcus* were the most abundant genera along the sludge bed of the UASB on day 110. The abundances of Unclassified *Clostridiales* and also *Methanosaeta* were higher than 5% in all bed heights. With regard to sulfate reducers, *Desulfovibrio* was the most abundant genus ranging from 6.4% (upper part of the sludge bed) to 2.9% (lower part of the sludge bed). *Desulfobulbus* was also found with abundances lower than 1.5%. After the reduction of the organic load and the pH shock (120 days), the genera found in the UASB were mainly *Desulfobulbus* (19.8%), *Desulfovibrio* (8.5%) and Unclassified *Clostridiales* (45.2%).

4. DISCUSSION

4.1 Assessment of the sulfidogenic UASB performance

Figure 2A shows that complete sulfate reduction was only achieved in the third operating period (days 90 to 110 of operation) when the inlet COD/S-Sulfate ratio was 8.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$. Incomplete sulfate removal was found at inlet COD/S-Sulfate ratios below 8.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$ despite COD was not completely removed. In fact, complete mineralization of glycerol was never reached. As reported by Hansen et al. (2009) and Hu et al. (2012) crude glycerol can contain more than 50% of organic matter different from glycerol and methanol such as fatty acid methyl esters, free or long chain fatty acids and glycerides, which are complex compounds difficult to be hydrolyzed and even inhibitory (Chen et al., 2008). Thus, the COD consumed probably corresponded to the hydrolysable and readily biodegradable fraction of crude glycerol (Figure 2B), that limited sulfate removal at inlet COD/S-Sulfate ratios below 8.5 g O_2 g^{-1} $S\text{-}SO_4^{2-}$. The low hydraulic residence time set in this study (HRT=2h) also contributed to reduce the capacity of the reactor to hydrolyze the slowly biodegradable fraction of crude glycerol. Based on the COD fed and the remaining COD in the first and second operating periods, the slowly biodegradable fraction of the crude glycerol used herein was estimated to be around 15 to 20%.

When COD was not limiting, SRB activity resulted in a maximum S-EC of 110 mg $S\text{-}SO_4^{2-}$ L^{-1} h^{-1}, which is 2.2-fold that obtained by Bertolino et al. (2014) using glycerol as the carbon source and comparable to that obtained by Celis-García et al. (2007) using lactate and VFAs as carbon sources, which are readily biodegradable organic compounds. However, complete sulfate removal was achieved at expenses of producing an effluent containing an excess COD of around 1100 mg COD L^{-1} (Figure 2B). Results indicate that

335 improvement of the hydrolytic capacity of crude glycerol is warranted to increase the
336 sulfate reduction capacity of the UASB and, concomitantly, to reduce the electron
337 acceptor consumption (generally oxygen) in the downstream processing of the UASB
338 effluent.
339 Biogas was always produced until the pH shock occurred. The high content of methane
340 in the biogas produced before the pH shock was obtained not only due to the high pH set
341 in the UASB but to the presence of both acetoclastic and hydrogenotrophic methanogens
342 capable of converting acetate and hydrogen and CO_2, respectively, into methane. As can
343 be also observed from VFA and methane profiles (Figure 2C), the pH shock certainly
344 caused a severe effect on methanogenic activity since methane production was
345 completely interrupted. Acetate accumulation coincided with methanogenic activity
346 cease, which caused acetate concentration peaks up to 650 mg O_2 L^{-1}. This result indicated
347 that acetate was consumed by acetoclastic methanogens and that heterotrophic sulfate
348 reducers were probably not consuming acetate to reduce sulfate as reported by other
349 authors (Qatibi et al., 1998; Dinkel et al., 2010b; Postgate, 2013; Santos et al., 2017). In
350 addition, and considering that around 15-20% of crude glycerol was slowly
351 biodegradable, an average outlet acetate concentration of 510 mg COD·L^{-1} (Figure 2C)
352 coupled to an outlet total COD concentration of around 700 mg COD·L^{-1} at the end of the
353 UASB operation (Figure 2B) indicated that acidogenesis and acetogenesis were not
354 severely affected by the pH shock.
355 During the UASB performance many operational problems occurred due to the granular
356 sludge rising, before and after the pH shock. It was suspected that crude glycerol
357 diminished the density of the biomass. Viana et al. (2012) also observed severe sludge
358 flotation in anaerobic reactors fed with crude glycerol due to the presence of long chain
359 fatty acids. Moreover, after the pH shock, biogas production disappeared, and so the shear
360 stress that contributes to granulation and, consequently, to the settling capability of the
361 sludge. Overall, the pH shock had detrimental effects on the UASB performance that
362 could not be reversed 80 days after the pH shock. The addition of active carbon or the
363 injection of nitrogen pulses were only temporarily effective. The loss of part of the bed
364 and the rupture of the bed caused a sharp decrease of the contact time of the liquid phase
365 in the sludge bed that led to the incomplete reduction of sulfate. In Figure 4B the effect
366 of the preferential paths formed from the middle of the bed (Figure S2C) are clearly
367 represented and also how the lower part of the bed had a softer effect due to the use of
368 activated carbon in the inlet of the UASB. The complete degranulation of the sludge and

methanogenic activity disappearance also affected the S-RE and COD-RE that decreased down to 60.4% and 25.1%, respectively, at the end of the UASB operation (Table 2). Other authors have also reported the operation difficulties of sulfidogenic reactors with low methane production, which may cause sludge bed clogging due to the lack of shear stress (Tsui et al., 2016). Results confirmed the beneficial effects of using granular biomass in sulfidogenic UASB reactors and the benefits of a minimum production of biogas.

4.2 Sulfur mass balance

The recovery of sulfide from sulfate in the sulfidogenic UASB was of high importance since the aim of this study was the valorization of sulfate rich effluents to obtain elemental sulfur in a microaerobic bioreactor located downstream of the UASB. If sulfate is converted into other compounds different from sulfide, the productivity decreases compromising the technical and economic feasibility of the process.

After avoiding a slight air diffusion in the first operating period on top of the UASB headspace, still the sulfur imbalance was over 20% for the second and third operating periods, when the OLR was higher compared to that of the first period. In the third operating period, crude glycerol and biomass samples at three different heights of the UASB (lower, medium and upper part of the riser) were analyzed for metals in order to check if there were metallic sulfides precipitating in the reactor. Results did not reveal any compound in crude glycerol that could precipitate with sulfide to result in such a high percentage of imbalance. Also, biomass samples had less than 4 mg S g^{-1} solid. Moreover, crude glycerol used in this study had large quantities of S, P, K, Na and Mg. These findings all together indicated that organic sulfur compounds were probably produced as intermediate compounds of the biodegradation process leading to higher sulfur imbalances at higher COD loads. As reported by Andersson et al. (2004) volatile organic sulfur compounds are typically produced from the anaerobic digestion of sulfur containing organic wastes, which is the case of crude glycerol and the bioprocess studied in this work. It would also be in concordance with VFAs concentration in the effluent, which represented around 75-80% of its COD content (Figure 2B and 2C). Then, other organic compounds (with or without sulfur) could represent the remaining 20-25% of the COD in the effluent. Further analyses are warranted to assess the organosulfur compounds formed and their potential impact on biogas composition as well as on the emissions in downstream reactors.

4.3 Assessment of the sludge bed stratification

Results obtained from the monitoring of liquid and gas phases presented above allowed generating some hypothesis about the processes that could be ongoing in the sludge bed. However, the analysis of the microbial diversity was essential to determine which of the hypothesis were unlikely. Assessment of the sludge bed stratification also helped linking sludge granulation, microbial diversity and the presence of different compounds (such as sulfide, sulfate, VFA and pH) to identify the main mechanisms occurring along the sludge bed in each operation stage analyzed.

In terms of granulation, the performance of the sulfidogenic UASB was complex since the up-flow velocity set was lower (0.25 m h^{-1}) than the minimal velocity of 0.5 to 1 m h^{-1} reported to keep the sludge granulated (Pol et al., 1983). The rationale behind starting up the UASB with methanogenic granular sludge in this study was the low availability of granular sulfidogenic sludge in full-scale systems. Thus, the closer type of granular sludge for sulfidogenic UASB inoculation in practice is that from granular methanogenic UASBs. Figure 3 shows the fast degranulation of part of the bed that occurred during the first 10 days of the UASB operation indicating that the development of sulfate reducing bacteria and the presence of sulfide besides the low up-flow velocity set in the UASB interfered in granules preservation. After 110 days of operation, the high OLR set allowed reaching maximum D(0.5) in each bed height, which indicated that methanogenic activity was present along the whole bed even at sulfide concentrations above 100 mg S L^{-1}, contributing to sulfidogenic sludge granulation. As reported by Liu and Tay (2004), high OLR sustains fast microbial growth and drives to a rapid granulation and stable treatment process. Microbial diversity analysis on day 110 showed that the most abundant genera in the sludge bed (*Anaerosinus, Citrobacter, Eubacterium, Klebsiella, Dysgonomonas* and *Trichococcus*) were able to convert glycerol, or its fermentation products, into metabolites such as 3-hydroxypropionaldehyde, 1,3-propanediol, ethanol, lactate, hydrogen and VFAs (mainly acetate and propionate) among others (Schauder and Schink, 1989; Homann et al., 1990; Yazdani and Gonzalez, 2007; van Gelder et al., 2012). These compounds could be easily consumed by SRB to produce sulfide. A larger abundance of *Desulfovibrio* and *Desulfobulbus* were identified in the upper part of the UASB rather than in the lower part. This result was in concordance with the analysis of sulfate and sulfide along the bed (Figure 4A), which revealed that the higher sulfate reducing activities were obtained in the medium (H=25cm) and upper (H=44cm) parts of the bed.

On the other hand, the main ongoing process in the lower part of the bed (H=5cm) was the hydrolysis and acidogenesis of glycerol instead of the reduction of sulfate, which is in agreement with the main genera found at this bed height: *Citrobacter* (9.47%), *Klebsiella* (11.1%) and *Trichococcus* (31.0%). Acetic and propionic acids were produced in large amounts as result of their activity. Then, acetic acid was progressively consumed along the bed while propionic acid was still increasing, even if could not be ensured that sulfate reduction was occurring using whether VFA (mainly propionic), hydrogen (an intermediate product obtained from glycerol fermentation) or both. *Eubacterium*, a hydrogenotrophic methanogenic bacterium, was also found in high abundance (11.6%). The presence of *Eubacterium*, together with the presence of the methanogenic archaea *Methanosaeta* (5.55%), explained the high percentage of methane in the biogas produced (above 85%). Despite reduced HRTs of 2h-4h enhanced hydrogen production (Si et al., 2015), hydrogen was not always detected, indicating that H_2 was probably produced and consumed along the granular sludge bed.

A completely different performance was found after the reduction of the organic load and the pH shock (day 120 on). The UASB operation became completely unstable. Granulation was lost (Figure 3) and operational problems such as the sharp decrease of the HRT in the blanket section of the bed, caused by the sludge bed breakage and the formation of preferential paths (Figure S2, Supplementary Material), emerged. However, it was observed that, even with such a malfunctioning of the reactor, the S-RE was 60.4% with a low COD/S-Sulfate consumed (around 2.5 g O_2 g^{-1} S). Then, bacteria overcoming the pH shock were capable of hydrolyzing glycerol and to produce VFA and reduce sulfate to obtain acetic acid and CO_2 (such as *Desulfobacterium* sp. or some *Desulfovibrio* species) as reported by other authors (Qatibi et al., 1991a; Qatibi et al., 1991b). However, the analysis of microbial diversity revealed that the main genera that remained in the UASB after the pH shock were not only *Desulfobulbus* (19.8%) and *Desulfovibrio* (8.5%) but also Unclassified *Clostridiales* (45.2%). The theoretical COD/S-Sulfate required to completely reduce sulfate to sulfide with the complete mineralization of glycerol is 2 g O_2 g^{-1} S (Eqs. 1 and 2) which is 25% lower than that obtained at the end of the UASB operation. However, it was observed that the remaining COD in the effluent was mostly acetate. The acetogenesis from glycerol without the reduction of sulfate implies the consumption of 0.43 g O_2 g^{-1} O_2-glycerol (Eq. 3). This means that the conversion of 1100 mg O_2-glycerol L^{-1} (COD concentration in the influent at the end of the UASB operation) into acetate requires a consumption of 470 mg O_2 L^{-1}, which is similar to the COD

consumption obtained at the end of the UASB operation (Figure 2). A production of 58.9 mg H_2 L^{-1} could be also produced from the acetogenesis (Eq. 3), which is high enough to reduce 140 mg S L^{-1} (sulfate reduced at the end of the UASB operation) since the theoretical ratio is 0.25 g H_2 g^{-1} S-Sulfate (Eq. 4). The high abundance of Unclassified *Clostridiales*, together with the COD/S-Sulfate consumed and the VFAs composition in the effluent are in agreement with the hypothesis that acetogenesis and hydrogenotrophic sulfate reduction were the main processes occurring in the sludge bed at the end of the UASB operation. Complementary experiments such as microcosms are warranted to define clearly the bioprocesses occurring in each height of the sulfidogenic glycerol-degrading sludge bed.

$$C_3H_8O_3 + 0.75\ SO_4^{2-} \rightarrow CH_3COOH + 0.75HS^- + 0.75\ OH^- + H_2CO_3 + 0.25\ H_2O \quad (1)$$

$$CH_3COOH + SO_4^{2-} \rightarrow HS^- + HCO_3^- + H_2CO_3 \quad (2)$$

$$C_3H_8O_3 + 2H_2O \rightarrow CH_3COOH + 3H_2 + H_2CO_3 \quad (3)$$

$$4H_2 + SO_4^{2-} \rightarrow HS^- + 3H_2O + OH^- \quad (4)$$

5. CONCLUSIONS

This work demonstrates that sulfate can be successfully treated in UASB reactors using crude glycerol as carbon source at low up-flow velocities and short HRTs. An excess of crude glycerol was needed since 15-20% of the crude glycerol COD was not hydrolyzed and, consequently, not utilized. The study of the sludge bed stratification performed before the system failure revealed that crude glycerol hydrolysis and acidogenesis happened in the first half part of the reactor while methanogenic activity was present along the whole bed even at sulfide concentrations above 100 mg S L^{-1}. Sulfate was reduced once the crude glycerol was converted into VFAs or hydrogen to become the main process concomitantly with hydrogenotrophic and acetoclastic methanogenesis. The lack of a minimum methane production leads to poor sludge granulation, thus driving the UASB performance to an unstable operation. Also, a short, temporary pH shock up to pH=12 dramatically affected the UASB performance by ceasing methanogenic activity and leading to biomass degranulation. SRB revealed as more resistant and resilient to pH shocks compared to methanogenic biomass. Further research is needed to enhance the identification of the biodegradation mechanisms and intermediate compounds such

organosulfur compounds and VFA produced from the biodegradation of crude glycerol in sulfidogenic UASBs.

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