1 **Exploring the performance limits of a sulfidogenic UASB during the**

2 **long-term use of crude glycerol as electron donor**

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5 **ABSTRACT**

6 SOx contained in flue gases and S-rich liquid effluents can be valorized to recover

7 elemental sulfur in a two-stage bioscrubbing process. The reduction of sulfate to sulfide

8 is the most crucial stage to be optimized. In this study, the long-term performance of an

9 up-flow anaerobic sludge blanket (UASB) reactor using crude glycerol as electron donor

10 was assessed. The UASB was operated for 400 days with different sulfate and organic

11 loading rates (SLR and OLR, respectively) and a COD/S-SO_4^{2-} ratio ranging from 3.8 g

12 O_2 g^{-1} S to 5.4 g O_2 g^{-1} S. After inoculation with methanogenic, granular biomass, the

13 competition between sulfate-reducing and methanogenic microorganisms determined to

14 what extent dissolved sulfide and methane were produced. After the complete washout of

15 methanogens, which was revealed by next-generation sequencing analysis, the highest S-

16 EC was reached in the system. The highest average sulfate elimination capacity (S-

17 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

18 of 24.4 kg O_2 $m^{-3}d^{-1}$ with a sulfate removal efficiency of 94%. The conversion of influent

19 COD to methane decreased from 12% to 2.5% as the SLR increased while a large fraction

20 of acetate (35% of the initial COD) was accumulated. Our data indicate that crude

21 glycerol can promote sulfidogenesis. However, the disappearance of methanogens in the

22 long-term due to the outcompetition by sulfate reducing bacteria, lead to such large

23 accumulation of acetate.

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1. INTRODUCTION

Combustion of sulfur-containing fuels, such as coal, natural gas, peat, wood and oil, results in SO_2 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 SO_2 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 ($\approx$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

102 information regarding S-rich streams valorization but also assesses the use of crude

103 glycerol specifically for sulfate reduction through the analysis of C sinks to the main

104 bioprocess occurring in the system.

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106 **2. MATERIALS AND METHODS**

107 **2.1. Experimental setup**

108 A jacketed glass-made UASB reactor of 2.5 L, with a granular sludge volume of 1L, was

109 used in this study. A detailed scheme of the UASB is presented in Figure 1. During the

110 operation, inlet pH ranged between 8.4 and 8.6 and temperature was controlled at 35ºC

111 by a thermostatic bath connected to the water jacket of the reactor (Figure 1). The

112 composition of the mineral medium was (g L^{-1}): K_2HPO_4 (3), NH_4Cl (0.2) dissolved in

113 tap water to add macro- and micronutrients and adjusted to pH=8.8-9.0 with NaOH (2

114 M). Because of the difficulty to control pH in a plug-flow type bioreactor, the pH in the

115 UASB was not controlled. However, the buffering capacity of the mineral medium

116 allowed maintaining the outlet pH above 7 along the whole operation of the reactor (data

117 not shown). Mineral medium was pumped at a flow rate of 0.5 L h^{-1}, once mixed with the

118 organic influent, from the bottom to the top of the UASB (up-flow velocity of 0.25 m h^{-1}

119 ^{1}). The flow rate of the organic influent was set at 30 mL h^{-1}. Hence, crude glycerol was

120 diluted to adjust the inlet COD concentration. The hydraulic residence time (HRT),

121 calculated as that corresponding to the reaction volume only (sludge blanket), was 2h.

122 Biogas produced in the UASB was collected in a 5 L Tedlar bag (FlexFoil, SKC Inc.) to

123 monitor its composition and flow rate. Inlet and outlet flows were also sampled every

124 two/three days to analyze COD, S compounds (sulfate, thiosulfate and TDS) and VFA.

125 **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-SO_4^{2-} L^{-1} to 859 ± 30 mg S-SO_4^{2-} 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

150 concentration of sulfate, TDS and VFA. In addition, sulfate concentration in the influent

151 was also measured every 12 hours.

2.3. Analytical methods

153 Sulfate (SO_4^{2-}) and thiosulfate ($S_2O_3^{2-}$) concentrations were analyzed by ion

154 chromatography with conductivity detection using a Dionex ICS-2000 equipment with

155 an Ultimate 3000 Autosampler Column Compartment, and an IonPac AS18 column

156 (ThermoScientific, USA). COD was measured using COD kits and a photometer

157 (Lovibond®).

158 VFA concentration were measured by gas chromatography (7820-A, Agilent

159 Technologies) equipped with a DB-FFA column and using a flame ionization detector

160 (FID) with helium as carrier gas. Prior to VFA analyses, samples were prepared following

161 the procedure described in Baeza et al. (2017) which consisted of pipetting 0.8 mL of

162 filtered samples together with 0.2 mL of a preserving solution (which also contained

163 hexanoic acid as the internal standard) in a glass vial of 1.5 mL. The VFA species

164 analyzed included acetic, propionic, butyric, isobutyric and valeric acids. Only acetic and

165 propionic acids were detected in significant amounts. All samples were filtered at 0.22

166 μm (Millipore, USA).

167 A sulfide selective electrode (VWR International Eurolab, S.L) connected to a benchtop

168 meter (Symphony, VWR) was used for the off-line measurement of TDS concentration.

169 Prior to their measurement, samples were diluted and preserved in sulfide antioxidant

170 buffer (SAOB). The SAOB composition was (g L^{-1}): ascorbic acid (35) and EDTA (67)

171 dissolved in NaOH (2M).

172 CH_4, CO_2 and H_2S in the biogas produced were analyzed by gas chromatography (7820-

173 A, Agilent Technologies, USA). The volume of the gas produced in the UASB reactor

174 was calculated following the Gas Bag Method (GBM) as presented in Ambler and Logan

(2011). 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_2 in this case) in order to produce an appreciable change in the area of the CO_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 ± 17 mg S-SO$_4^{2-}$ L^{-1}

and 12.0 ± 2.1 kg O$_2$ m^{-3}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 g O$_2$ g^{-1} 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 g O$_2$ L^{-1} (640 g C$_3$H$_8$O$_3$ L^{-1}) and a lower BOD$_5$/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

225   SLR was increased in Period V (days 238-288) to verify the maximum treatment capacity

226   of the reactor. In Period V the lowest COD/S ratio was tested despite the system was

227   already overloaded. VFAs accumulated to reaching their maximum concentrations

228   (Figure 3B) as described in section 3.2. During Period VI the UASB operated during 112

229   days under the conditions tested during period III-IV to recover the initial stability of the

230   system.

231   In addition, short-term experiments were performed. For that purpose, different sulfate

232   inlet concentrations were tested at the end of period VI (days 360-370) to verify system

233   robustness to face quickly variable inlet loads during the operation. Figure 4 shows sulfate

234   and sulfide concentration profiles as well as the corresponding S-RE and S-EC obtained

235   during the short-term assays. As can be observed in Figure 4, the sulfate RE was almost

236   doubled for the lowest sulfate concentration tested (120 mg S-SO$_4^{2-}$ L^{-1}) compared to the

237   initial situation before the short-term experiment.

238

239   **3.2 Organic matter sink: sulfate reduction and biogas and VFA production**

240   Even if traces of other VFA were measured from the biodegradation of crude glycerol,

241   only acetate and propionate were predominant and therefore considered for further

242   analysis (Figure 3B). During periods I and II, inlet COD was completely consumed and

243   no VFA were detected in the effluent while some CH$_4$ was produced and recovered as

244   part of the gas phase. From period III onwards, when an average OLR of 25 kg O$_2$ m^{-3}d^{-}

245   ^{1} was fed (Table 1), the effluent contained mainly acetate. As can be observed in Figure

246   3A, this increase in acetate coincided with a decrease in CH$_4$ production, which ceased

247   75 days after the beginning of period III. During period V, the maximum concentration

248   of acetate in the reactor was reached (1000 mg acetate L^{-1}), which progressively decreased

249 until the end of the operation when acetate concentrations below 340 mg L^{-1} were

250 detected.

251 The conversion of COD resulting from each operating period was also assessed in terms

252 of CH_4 composition in biogas, VFA concentrations in the effluent (acetate and

253 propionate), and COD used for sulfate reduction (Table 3). The COD balance was

254 calculated based on measurements of inlet and outlet COD and VFAs and corresponding

255 methane production. According to the methane composition in biogas, the biogas flowrate

256 and the TDS and residual COD in the effluent, COD conversion proportions along the

257 different periods were obtained according to processes stoichiometry (see Supplementary

258 Information for calculation details). Table 3 shows that along Periods I and II around 11%

259 of the inlet COD was directed to methane production while almost no VFA accumulated

260 in the reactor. However, between 33 and 41% of the influent COD ended up in acetic acid

261 from Period III until the end of the operation while between 3 and 6% of the inlet COD

262 was converted to propionic acid from Period III onwards. Concomitantly, the COD

263 fraction converted to methane had the opposite behavior and was around 0% from Period

264 III onwards, which was taken into account as a way of reporting the percentage of

265 electrons utilized by methanogens. The potential use of COD for sulfate reduction was

266 more stable along the operation even if a progressive deterioration could be detected that

267 accounted for a 26.5% less of organic matter calculated for this purpose comparing the

268 last and the first periods. The rest of COD was assumed to be used for growth and CO_2

269 formation, although it could not be accurately quantified.

270 **3.3** . **Illumina sequencing analysis and bacterial community assessment**

271 The scope of the microbial analysis was not to describe the evolution of the microbial

272 diversity along the UASB operation but to provide further data to explain the switch from

273 methane production to non-methane production conditions from a microbial perspective.

274   Thus, the bacterial community through Illumina analysis of the 16S rRNA gene was

275   applied to compare the methanogenic granular sludge used as inoculum with the biomass

276   developed after 190 days of the UASB operation when no methane production was

277   observed. Results obtained from the microbial analysis are presented in Figure 5.

278   *Deltaproteobacteria* and *Methanomicrobia* were the main classes detected in the

279   inoculum with a relative abundance of 20% and 16% respectively (Figure 5). *Clostridia*

280   was the third class in order of abundance (13.5%). After operating the UASB for 190

281   days, *Deltaproteobacteria* increased their relative abundance to 49% in the sludge bed

282   sample, clearly the most abundant class of the total reads; while *Methanomicrobia*

283   decreased to 0% without detecting any other methanogenic microorganism. *Clostridia*

284   was the second class in order of abundance (12.15%), followed by *Gammaproteobacteria*

285   and *Bacteroidia* (11 and 7.3% of total reads, respectively).

286   As the operation proceeded, in the biomass community of day 190 (Tables S1 and S2;

287   Supplementary Information), *Desulfovibrio* was the most abundant OTU at genus level,

288   with a 35.3% of total retrieved sequences. Oude Elferink et al. (1994) reported that

289   *Desulfovibrio* had higher affinity for sulfate and higher growth rate than other SRB genera

290   such as *Desulfobulbus* and *Desulfobacter*. Interestingly, *Desulfatirhabdium* sp. accounted

291   for 2% of total operational taxonomic units at genus level which has been described as

292   butyrate-oxidizing bacteria (Balk et al., 2008). In the case of *Proteobacteria* that were not

293   SRB,   the   highest   relative   proportion   of   microorganisms   belonged   to   the

294   *Enterobacteriaceae* family (11%).

295

296   **4. DISCUSSION**

297   **4.1 Startup of a sulfate reducing UASB reactor and influence of inoculum**

298   In practice, start-up of full-scale UASB reactors for sulfate reduction is handicapped

299   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 at., 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

325 period I (OLR of 12.0±2.1 kg O_2 $m^{-3}d^{-1}$) and II (OLR of 15.8±4.6 kg O_2 $m^{-3}d^{-1}$), the

326 average organic matter consumption was 86% and 89% respectively. COD concentrations

327 were below 100 mg O_2 L^{-1} in the effluent, which probably corresponded to the less

328 biodegradable matter contained in crude glycerol considering that the anaerobic

329 biodegradability of crude glycerol due to presence of such inhibitory impurities has been

330 reported to be between 65-85% (Viana et al., 2012). Furthermore, 10% of the oxidized

331 organic matter was used for methane production during period I (Table 3). Similarly,

332 during period II, 11% of the transferred electrons were utilized for methane production.

333 Despite such methanogenic activity, Figure 2 shows that a stable operation in terms of

334 sulfate reduction was reached by the end of period II with almost complete removal of

335 sulfate and COD (S-RE of 96.5% and COD-RE of 89.3%). Despite methane production,

336 results confirmed that organic substrates were available for sulfate reduction and that

337 microbial communities underwent a fast and gradual acclimation to their environment.

338 Our work demonstrates that using methanogenic granular sludge from a paper and pulp

339 industry leads to a fast start-up of sulfidogenic UASBs when moderate inlet sulfate

340 concentrations of 235±17 mg S-SO_4^{2-} L^{-1} are treated using crude glycerol as electron

341 donor at C/S ratio of 3.8 g O_2 g^{-1} S.

4.2 Shifts in the organic matter sink

343 The sink of the organic matter can shift drastically due to the evolution of the microbial

344 population, which influences the performance of the UASB reactor. During Period III, a

345 high S-RE was reached after few days of operation which allowed obtaining a maximum

346 S-EC of 6.6 kg S-SO_4^{2-} m^{-3} d^{-1} (273 g S-SO_4^{2-} m^{-3} h^{-1}) from this period (Period III).

347 Compared to previous periods, the COD-RE dropped drastically (Figure 3A) and an

348 accumulation of VFA was observed (Figure 3B), which indicated that a steady, almost

349 complete sulfate reduction could be reached at a SLR of 4.6 kg S-SO_4^{2-} m^{-3} d^{-1} and COD/S

350 ratios lower than 5 g C g^{-1} S. As previously described by Pol et al. (1998), when a sulfate-

351 rich wastewater is fed into an anaerobic reactor, organic matter will be removed both via

352 methanogenesis and sulfate reduction and when methanogenesis becomes suppressed

353 then a gradual decrease in the organic matter conversion (COD removal) is observed,

354 which was corroborated herein with crude glycerol instead. During this third period (OLR

355 of 24.4 kg COD m^{-3}d^{-1}), the average organic mater consumption was 38 % while only 2.3

356 % of the COD removed ended in methane production when the COD/S was increased to

357 5.4g O$_2$ g S^{-1} (Tables 2 and 3). Taking into account that the reported cellular yield for

358 acidogenic bacteria (0.14-0.17 gVSS/gCOD) is five times higher than that of acetogenic

359 bacteria (0.025-0.051 gVSS/gCOD) or methanogenic archaea (0.01-0.054 gVSS/gCOD)

360 (Pavlostathis and Giraldo-Gomez, 1991) glycerol will be readily available for acidogenic

361 bacteria, and the limiting step will be the methanogenesis.

362 During the anaerobic digestion of glycerol, some organic acids (acetic, propionic, butyric,

363 valeric and others), produced by fermentative acidogenic bacteria, cannot be consumed

364 by methanogenic archaea at the same rate at which they are produced (Viana et al., 2012).

365 The accumulation of VFA indicated that the slowly growing methanogens could not

366 sufficiently and rapidly metabolize the intermediate products from VFA producers

367 (acidogenic and acetogenic populations). Since acetate is mainly converted by

368 methanogens and no methanogens were found in the sludge sample from day 190 (Figure

369 5), increasing concentrations of acetic acid were found in the reactor between period III

370 and V. This is in agreement with the production of methane measured from the gas phase

371 (Figure 3A) and with some authors statements (Harada et al., 1994; Omil et al., 1998),

372 who pointed out that the predominance of SRB over methanogens in sulfate-rich streams

373 is only achieved after long-term operation (more than 100 days) in UASB reactors. As

374 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_2 L^{-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_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, Desulfobacter* 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/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

425 undesirable since the reducing power supplied with glycerol is only partly used. In

426 addition, further resources must be used to treat the excess COD from the anaerobic

427 reactor. In the sulfur recovery process depicted in Figure S1, a reduction-oxidation

428 bioprocess was proposed. Then, the COD in the effluent could be treated in the CSTR

429 reactor for the partial oxidation of sulfide to elemental sulfur although an extra

430 consumption of oxygen to treat COD would be required. Consequently, optimization in

431 the use of the electron donor is warranted.

432 **4.4 Potential process limitations**

433 Inhibitory substances are often found to be the main cause of anaerobic reactor

434 disturbance and failure as they cause an adverse shift in the microbial population or

435 inhibition of bacterial growth (Chen et al., 2008). Inhibition of anaerobic digestion is

436 usually diagnosed by a decrease of the steady-state rate of methane gas production and

437 accumulation of organic acids (Kroeker et al., 1979), which was found in the long-term

438 operation of the UASB. The organic acid and methane forming microorganisms differ

439 widely in terms of physiology, growth kinetics, and sensitivity to environmental

440 conditions (Pohland and Ghosh, 1971). At pHs below 7.0, most carboxyl groups are

441 undissociated, thus they pass freely through the membrane and can inhibit the growth of

442 many bacteria. Uncharged molecules such as acetic acid may be inhibitory because they

443 diffuse across the cell membrane and act as an uncoupler (Ghose and Wiken, 1955),

444 whereas acetate ion is not permeant. Inhibition concentrations of $4.68 \cdot 10^{-3}$ mg free acetic

445 acid L^{-1} (pH=7.5) have been reported to block acetoclastic methanogenesis (Fukuzaki et

446 al., 1990). Despite the buffer used, the pH in the reactor varied from 8.4-8.7 at the inlet

447 to 6.7-7.5 at the outlet due to VFAs accumulation, and particularly acetic acid.

448 Considering the pk_a of acetic acid (4.76) and the concentrations of acetate found in the

449 early stages of period III (around 375 mg acetate L^{-1}), concentrations above 0.23 mg free

450   acetic acid L^{-1} found in the UASB could have led to significant methanogens inhibition.

451   In addition, free H_2S concentrations leading to 50% inhibition of methanogenesis of 250

452   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

453   (Koster et al., 1986), indicating that methanogens were also inhibited by sulfide

454   accumulation in the early stages of Period III. Overall, TDS and acetate accumulation

455   lead to a fast decrease of the methanogenic activity in Period III. No methane production

456   from period IV until the end of the operation indicated that methanogenic communities

457   were more susceptible to dissolved sulfide concentration than SRB as was also observed

458   in Jing et al. (2013).

459   Despite the high concentrations of acetate found in the reactor during period III, IV and

460   V (400-1100 mg O_2 L^{-1}), sulfate reduction proceeded at high S-RE during period III,

461   indicating that SRB were not affected by acetic acid. Similarly to methanogens, free H_2S

462   may inhibit SRB. However, inhibitory free H_2S concentration in literature are often

463   contradictory and confusing probably due to the difference in anaerobic inocula used, the

464   susceptibility of anaerobes and the experimental methods and conditions tested in each

465   study, and particularly the pH in the bioreactor. Anaerobic treatment of sulfate-rich

466   wastewater proceeds successfully at COD/sulfate ratios lower than 10 g COD g^{-1} SO_4^{2-}

467   when precautions are taken to prevent sulfide toxicity (Pol et al., 1998). The TDS during

468   the operation reached 460 mg S L^{-1} by day 240 (Period V). Reis et al. (1992) found that

469   more than 547 mg H_2S L^{-1} can completely inhibit SRB activity at pH 6.2, whereas at pH

470   9.0, dissolved H_2S is mainly in the form of HS^-, which does not penetrate into cells easily

471   (Mora-Naranjo et al., 2003) and therefore would not have a strong inhibitory effect over

472   SRB. As Reis et al. (1992) observed, sulfate uptake decreased when sulfide concentration

473   in the medium increased, and increased again when it was removed from the medium,

474   which pointed out that sulfide is a reversible inhibitor of SRB. A pH range of 6.7-6.8 at

475 the outlet of the reactor points out at a reduction of the potential maximum SRB rates due

476 to inhibition of SRB by hydrogen sulfide.

477 **4.5 Overall performance of the sulfidogenic UASB**

478 Sulfidogenesis was achieved through adaptation of granular sludge with important

479 methanogenic activity, using electrons derived from substrate towards sulfate reduction.

480 While adapting methanogenic granular sludge to sulfate reduction is one of the most

481 common and widespread procedures to engineer microbial sulfate reduction (García-

482 Solares et al., 2014), the stability of the system during long-term operations is still a cause

483 of concern. During period V, SLR was maintained during 50 days but the high sulfate

484 inlet resulted in a sulfate-reduction failure. The system was overloaded and its maximum

485 capacity, 6.5 kg S $m^{-3}d^{-1}$, was reached after 165 days of operation at a SLR of 6.7 kg S-

486 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

487 comparable to that obtained by Bijmans et al. (2008) (9.7 kg S m^{-3} d^{-1}) using formate,

488 which is more biodegradable than crude glycerol. Higher S-ECs were found compared to

489 Boshoff et al. (2004) (600 mg SO_4 L^{-1} d^{-1}) who used tannery effluent as carbon source.

490 After a long-term operation of 360 days, short-term SLR assays (Figure 4) were

491 performed to study the capability of the UASB to reduce sulfate at such a low HRT (2h)

492 and under dynamic conditions with non limiting COD availability. The UASB adapted

493 well to transient load changes and, more interestingly, recovered to the initial load

494 exhibiting a 25% higher S-RE compared to that before the short-term experiment.

495 However, it remains to be investigated why such temporary load decrease resulted

496 apparently beneficial for the UASB performance considering that the same sulfide

497 concentration was found before and after the stepwise decrease of the inlet sulfate

498 concentration. Based on a sulfur balance, a larger C/S ratio during the short-term

499 experiment (up to 19.1 g COD g S^{-1}) could have led to an increase in the production of

500 organosulfur compounds. Overall, a sulfur balance of 85-95% along the UASB operation

501 was obtained based on inlet and outlet sulfate (S-SO$_4^{2-}$) concentrations and produced TDS

502 (Figure 1). Such imbalance was attributed to other organosulfur compounds such as

503 dimethyl sulfide (DMS) or dimethyl disulfide (DMDS) amongst others that were

504 qualitatively detected in the effluent of the UASB (see Table SI-4 in Supplementary

505 Information).

506

4. CONCLUSIONS

508 Long-term operation of a sulfidogenic UASB reactor can be successfully achieved using

509 crude glycerol as carbon source at low up-flow velocities. It was demonstrated that at

510 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.

511 The TDS concentration increase together with VFA accumulation were potential

512 inhibitors of methanogenic activity and when methane production decreased, glycerol

513 was converted mainly to acetic acid and propionic acid. It was not only the COD/S-SO$_4^{2-}$

514 ratio, but a sum and combination of factors along the operation that determined the

515 competition between SRB and methanogenic archaea. However, further batch activity

516 tests are warranted to properly validate the results obtained herein.

517

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