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PII: S0045-6535(17)30306-5

DOI: 10.1016/j.chemosphere.2017.02.117

Reference: CHEM 18880

To appear in: *ECSN*

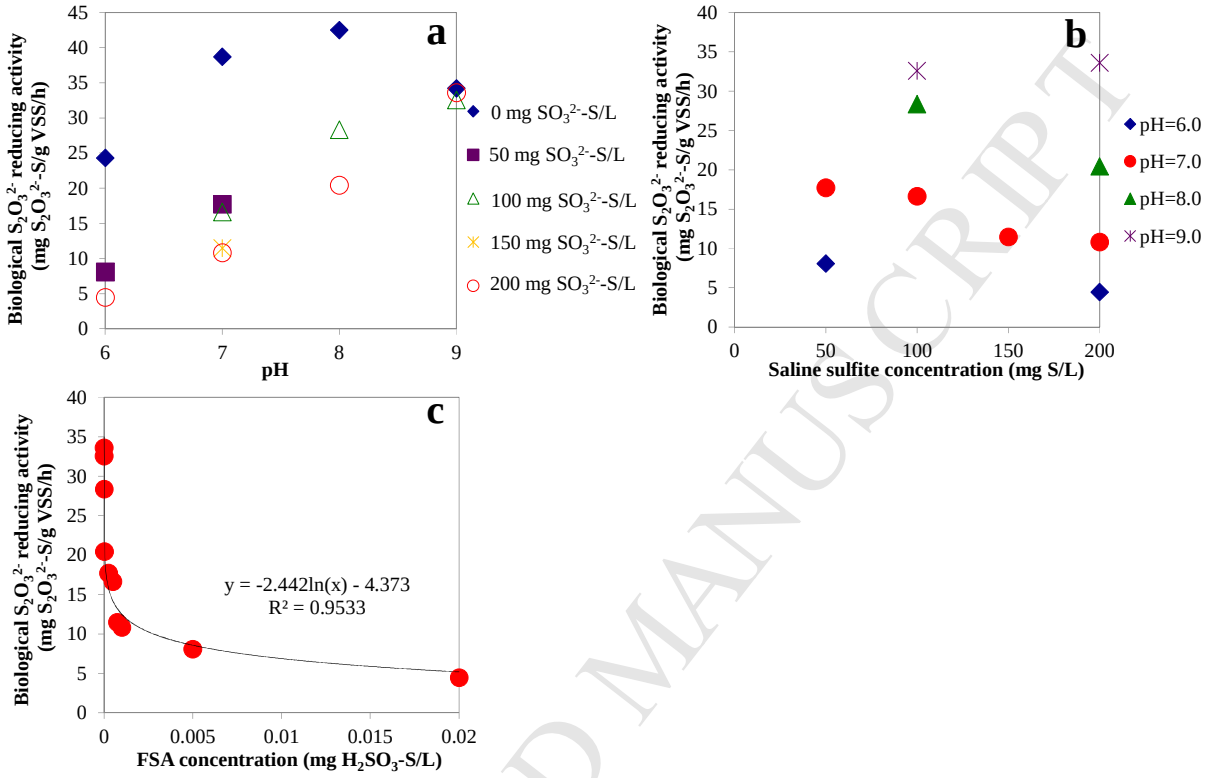
Received Date: 10 November 2016

Revised Date: 20 February 2017

Accepted Date: 23 February 2017

Please cite this article as: Qian, J., Wang, L., Wu, Y., Bond, P.L., Zhang, Y., Chang, X., Deng, B., Wei, L., Li, Q., Wang, Q., Free sulfurous acid (FSA) inhibition of biological thiosulfate reduction (BTR) in the sulfur cycle-driven wastewater treatment process, *Chemosphere* (2017), doi: 10.1016/j.chemosphere.2017.02.117.

Graphical Abstract



Free Sulfurous Acid (FSA) Inhibition of Biological Thiosulfate Reduction (BTR) in the Sulfur Cycle-driven Wastewater Treatment Process

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Abstract: A sulfur cycle-based bioprocess for co-treatment of wet flue gas desulfurization (WFGD) wastes with freshwater sewage has been developed. In this process the removal of organic carbon is mainly associated with biological sulfate or sulfite reduction. Thiosulfate is a major intermediate during biological sulfate/sulfite reduction, and its reduction to sulfide is the rate-limiting step. In this study, the impacts of saline sulfite (the ionized form: HSO_3^- + SO_3^{2-}) and free sulfurous acid (FSA, the unionized form: H_2SO_3) sourced from WGFD wastes on the biological thiosulfate reduction (BTR) activities were thoroughly investigated. The BTR activity and sulfate/sulfite-reducing bacteria (SRB) populations in the thiosulfate-reducing up-flow anaerobic sludge bed (UASB) reactor decreased when the FSA was added to the UASB influent. Batch experiment results confirmed that FSA, instead of saline sulfite, was the true inhibitor of BTR. And BTR activities dropped by 50% as the FSA concentrations were increased from 8.0×10^{-8} to 2.0×10^{-4} mg H_2SO_3-S/L. From an engineering perspective, the findings of this study provide some hints on how to ensure effective thiosulfate accumulation in biological sulfate/sulfite reduction for the subsequent denitrification/denitritation. Such manipulation would result in higher nitrogen removal rates in this co-treatment process of WFGD wastes with municipal sewage.

Key words: biological thiosulfate reduction (BTR); sulfate/sulfite-reducing bacteria (SRB); saline sulfite (HSO_3^- + SO_3^{2-}); free sulfurous acid (FSA, H_2SO_3)

1. Introduction

Sulfur bioconversion-associated sewage treatment processes have been reported extensively in the last two decades, among which biological sulfate reductions (BSR) and biological reduced sulfur (i.e. sulfide, thiosulfate, elemental sulfur, etc) oxidations (BSO) play an essential role in the removal of organics and nitrogen respectively (Lens et al., 1998; Cardoso et al., 2006; Manconi et al., 2007; Mora et al., 2014). By linking BSR with BSO and based on the sulfur sources from wet flue gas desulfurization (WFGD) wastes, an integrated process for co-treatment of fresh sewage and WFGD wastes has been developed (Qian et al., 2013). This co-treatment process mainly depends on the sulfur bioconversions from sulfate/sulfite (alkaline absorption of WFGD wastes) reduction to sulfide/thiosulfate, followed by sulfide/thiosulfate oxidation to sulfate (see Fig. S1 in Supporting Information for schematic diagram of the co-treatment process). Due to the low biomass yields of the bacteria involved in this process, i.e. sulfate/sulfite-reducing bacteria (SRB), sulfur oxidizing-denitrifying bacteria and autotrophic nitrifying bacteria, the sludge production rate is only 0.03 to 0.09 g MLVSS/g COD (Jiang et al., 2013; Qian et al., 2015a) (MLVSS: mixed liquor volatile suspended solids; COD: chemical oxygen demand), and this results in energy savings and reduction in greenhouse gas emission during the sludge treatment.

In this co-treatment process, sulfite produced from the WFGD wastes is one of the major sulfur compounds for biological energy conversions. However, negative effects of sulfite in both ionized form (saline sulfite: SO_3^{2-} + HSO_3^-) and unionized forms (free sulfurous acid, FSA: H_2SO_3) on microorganisms including SRB have been reported. Previous studies have found that

67 saline sulfite inhibition on sulfate/sulfite reduction occurs at concentrations as low as 16 mg S/L
68 (Weijma et al., 2000). Zan et al. (2016) recently demonstrated that FSA from 0.002 to 1 mg
69 H_2SO_3-S/L instead of saline sulfite directly causes the lysis of microorganisms. This implies that
70 FSA, rather than saline sulfite, is the factor exerting the antimicrobial effect on SRB.
71
72 During biological sulfate/sulfite reduction, thiosulfate (i.e. $S_2O_3^{2-}$) is an important intermediate
73 and its reduction to sulfide is the rate-limiting step during the biological SO_4^{2-}/SO_3^{2-} reaction
74 (Brunner and Bernasconi, 2005) (see Fig. S2). Additionally, thiosulfate is important in sulfur
75 dependent denitrification as its oxidation is reported to drive denitrification 4-8 times faster than
76 oxidation by sulfide (Cardoso et al., 2006). Therefore, it is important to understand the potential
77 effects of saline sulfite and FSA on these important thiosulfate transformations, including that of
78 biological thiosulfate reduction (BTR). This insight will determine the role of saline sulfite and
79 FSA in this sulfur cycle-based treatment of WFGD wastes as well as to shed light on how to
80 maintain effective $S_2O_3^{2-}$ accumulation for high nitrogen removal in the subsequent
81 $S_2O_3^{2-}$-driven denitrification/denitritation. To the best of the authors' knowledge, although the
82 effects of saline sulfite and FSA effects on microbial inactivation (Chang et al., 1997), biological
83 SO_4^{2-}/SO_3^{2-} reduction (Weijma et al., 2000) and sludge treatment (Zan et al., 2016) have been
84 examined, no detailed investigations of the effects of saline sulfite and FSA on BTR have been
85 carried out so far.
86
87 This study aims to thoroughly explore the effects of saline sulfite and FSA on BTR. Long-term
88 impacts of saline sulfite and FSA on BTR activities as well as microbial community structures

were investigated during the co-treatment process of WFGD wastes with sewage in an up-flow anaerobic sludge bed (UASB) reactor. Batch experiments were conducted and the quantitative relationship between the BTR activities and FSA concentrations were determined. The findings of this study also identified strategies on how to achieve $S_2O_3^{2-}$ accumulation during biological SO_4^{2-}/SO_3^{2-} reduction, which is then utilized for high nitrogen removal in the co-treatment process.

2. Materials and Methods

2.1 UASB reactor setup and operation

A UASB reactor with an effective reactor volume of 1.0 L (height: 51 cm, diameter: 5 cm) was established (Fig. S3). The seeding sludge for the UASB reactor was from a lab-scale biological sulfate reduction–sequential batch reactor (SBR) (see Table S1 for detailed operating conditions). At which time the SBR was at steady state achieving at least 90% sulfate and organic carbon removal. 500 mL of mixed liquor sludge was taken from this SBR reactor and added to the UASB reactor, resulting in an initial MLVSS concentration of 4300 mg/L. Although the typical COD values of municipal sewage in Mainland China and Hong Kong are between 300 and 400 mg/L (equivalent to about 150 to 200 mg SO_4^{2-}-S/L), 200 mg COD/L was employed for the UASB reactor's influent as the electron accepting capacity of $S_2O_3^{2-}$ is only half of that of SO_4^{2-}. The temperature of UASB reactor was kept at 25±1^{o}C in an air conditioned room and its hydraulic retention time (HRT) was maintained at 4 h during the operation. The internal recirculation flow rate was maintained at three times the influent flow rate, this ensured effective mass transfer between the bulk liquid and the biomass.

The reactor was continuously operated for 181 days consisting of 5 stages during which the same influent organic carbon (200 mg COD/L, sodium acetate was used as the sole organic source) and thiosulfate (220 mg S/L) concentrations were maintained, but varying pH (7.0-8.5), influent saline sulfite (0-150 mg S/L) and influent FSA concentrations (0-6.0×10^{-4} mg S/L) were applied. The detailed experimental conditions for each stage are shown in Table S2. Overall, Stage I (Day 1 to Day 21) was to evaluate the BTR performance of the UASB reactor at pH 7.0 in the absence of added saline sulfite and FSA. Stages II- IV were to examine the effect of saline sulfite and FSA on the BTR in the UASB reactor. The different FSA concentrations in Stages II-IV were obtained by varying the pH and Na_2SO_3 concentrations (see Table S2). The operating conditions of the UASB reactor at Stage V were the same as those at Stage I. This was to determine whether the inhibited BTR, caused by the saline sulfite and FSA, could be recovered after removal of the influent saline sulfite and FSA.

During the UASB reactor's operation, samples of both the influent and effluent were regularly drawn for analyses of COD, sulfide, saline sulfite and thiosulfate. Sludge samples were taken periodically from the bottom, middle, and top of the reactor to determine the mixed liquor suspended solids (MLSS)/MLVSS concentration. In addition, microbial community structures of the sludge were analyzed at the end of Stages I (Day 21), III (Day 97) and V (Day 181) during the UASB reactor operation.

2.2 Batch Tests

Three sets of batch tests were performed to evaluate the effect of saline sulfite and FSA and reveal the true inhibitor on BTR. These were performed on sludge taken from the abovementioned UASB reactor at the end of its operation (Stage V). For each batch test, the sludge was washed, using a synthetic wastewater (Table S3), three times to remove the background substrate (i.e. acetate, thiosulfate and sulfide, etc). 2 L serum flasks were used as the batch reactors for all the tests. Nitrogen gas was purged into each batch reactor before the assay for half an hour to exclude oxygen and maintain anaerobic conditions. Afterwards, all reactor flasks were sealed tightly with butyl rubber stoppers and aluminum crimp seals. The reactors were well mixed with magnetic stirrers at 150 rpm. The temperature of each reactor was kept at 25±1 ^{o}C in an air-conditioned room. Sodium acetate was used as the sole organic carbon source. In order to exclude the possible influence of generated S^{2-}/H_2S on BTR activity (O'Flaherty et al., 1998), an $FeCl_2$ solution, at 200 mg Fe^{2+}/L was added to each reactor for all the tests (O'Flaherty et al., 1998). During the batch experiments, the mixed liquor was sampled regularly for the analysis of thiosulfate, saline sulfite and FSA.

2.2.1 Batch Test I: BTR under different pH conditions in the absence of saline sulfite and FSA

Batch test I was conducted to evaluate the effect of pH on BTR in the absence of added saline sulfite and FSA. The pH in four reactors (i.e. Batch reactors 1–4) was adjusted to 6.0, 7.0, 8.0 and 9.0, respectively, by addition of Na_2HPO_4/NaH_2PO_4 buffer solution, as shown in Table S4. Initial acetate and $S_2O_3^{2-}$ concentrations were 200 mg COD/L and 200 mg S/L, respectively, by addition of sodium acetate and $Na_2S_2O_3$ stock solutions. The batch tests lasted for 24 h.

155 2.2.2 Batch Test II: Examine the overall effects of saline sulfite and FSA on BTR activities

156 Batch Test II was carried out to investigate the overall effects of both saline sulfite and FSA on
157 the BTR activities. The same amount of thiosulfate (200 mg S/L) was added into the four batch
158 reactors (i.e. Batch Reactors 5–8, see Table S4). The saline sulfite and FSA concentrations were
159 50~200 mg S/L, and 2.0×10^{-4} ~8.0×10^{-4} mg H_2SO_3-S/L, respectively (see Table S4). The pH in
160 each reactor was controlled at 7.0±0.1 using the Na_2HPO_4/NaH_2PO_4 buffer solution. The batch
161 tests lasted for 24 hours.

162

163 2.2.3 Batch Test III: BTR under different FSA levels

164 To explore the correlation between FSA concentration and BTR activities, Batch Test III was
165 also conducted by changing the pH and initial Na_2SO_3 concentrations, according to Eqs. (1) and
166 (2). Different amounts of Na_2SO_3 (i.e. 50~200 mg S/L) and different pH levels (i.e. 6.0~9.0)
167 were applied to the six batch reactors (i.e. Batch Reactor 9–14) in this test, resulting in the
168 different initial FSA concentrations (i.e. 8.0×10^{-8}~0.015 mg S/L) in each batch reactor. Detailed
169 information of each batch reactor test is shown in Table S4. The batch tests lasted for 24 h.

170

171 $H_2SO_3 \rightleftarrows HSO_3^- + H^+$ $pKa_1 = 1.91$ at 25 ^{0}C Eq. (1)

172 $HSO_3^- \rightleftarrows SO_3^{2-} + H^+$ $pKa_2 = 7.0$ at 25 ^{0}C Eq. (2)

173

174 *2.3 Sampling and Chemical/Physical Analysis*

175 Mixed liquor samples from the batch reactors were taken periodically using a 10-mL syringe
176 and these were immediately filtered through disposable Millipore filters (0.22 μm pore size).

177 Saline sulfite concentrations were determined by titration after sample pretreatment as detailed
178 in Qian et al. (2015a). Thiosulfate and acetate were detected with an ion chromatograph
179 (DIONEX-900). Sulfide was measured by the methylene blue method after sample pretreatment
180 with NaOH and ZnAc (APHA, 2005). MLSS/MLVSS were measured according to the Standard
181 Method (APHA, 2005). pH and temperature were monitored using a multi-meter electrode
182 during each test (PHSJ-4F).

183

184 As thiosulfate is an intermediary compound in biological sulfite reduction, BTR activity cannot
185 be directly derived from the profile of $S_2O_3^{2-}$ concentration versus time. In this study, the BTR
186 activity was represented by the rate of thiosulfate utilization (derived from the profile of $S_2O_3^{2-}$
187 concentration versus time) plus the biological sulfite reduction rate (derived from the profile of
188 saline sulfite concentration versus time) and expressed as kg $S_2O_3^{2-}$-S/d/m^3 in the UASB reactor
189 and mg $S_2O_3^{2-}$-S/g MLVSS/h in the batch reactor, respectively.

190

191 *2.4 Microbial analysis*

192 Sludge samples from the UASB reactor were collected at the end period of Stages I (Day 21), III
193 (Day 97) and V (Day 181) to analyze the structure of microbial communities. The samples were
194 collected by centrifugation under 12,000 rpm for 10 minutes. Around 0.5 g of sludge pellet was
195 stored for each sample at −80 °C until the DNA extractions were performed. Genomic DNA was
196 extracted using the PowerSoil DNA Isolation Kit (MoBio Laboratories, Inc., Carlsbad, CA)
197 following the manufacturer's protocols. The quality and quantity of DNA were checked with a
198 NanoDrop device (ND-1000, thermo Fisher, USA).

199

200 The primer pair 515 F and 926 R targeting the hypervariable V1 and V3 regions was used to
201 amplify the bacterial 16S rRNA gene (Quince et al., 2011). Barcode sequences were
202 incorporated between the 454 adaptor and the forward primer (Table S5). Each 100 μL PCR
203 reaction mixture contained 5 U of Pfu Turbo DNA polymerase (Stratagene, La Jolla, CA, USA),
204 1× Pfu reaction buffer, 0.2 μM of dNTPs (TaKaRa, Dalian, China), 0.1 μM of each primer and
205 20 ng of genomic DNA template. PCR was performed on a thermal cycler (Bio-Rad, USA) with
206 the cycles including an initial denaturation at 94°C for 5min, followed by 30 cycles of 94°C for
207 30s, 53°C for 30s and 72°C for 45s; and a final extension at 72 °C for 10 min. PCR products
208 were purified using Agarose Gel DNA Purification Kit (TaKaRa, China) and quantified with the
209 NanoDrop device. The purified PCR amplicons were sequenced using the ROCHE 454 FLX
210 Titanium platform (Roche, Basel, Switzerland) at the National Human Genome Centre of China
211 (Shang Hai, China). Analysis of the sequences obtained followed the procedures reported in
212 Qian et al. (2015b).

213

214 **3. Results and Discussion**

215 *3.1 UASB reactor performance under different operating conditions*

216 In Stage I, as the sulfur source was transformed from sulfate (for the sludge cultivation) to
217 thiosulfate in the UASB reactor, thiosulfate reduction efficiency (65%) and organic carbon
218 removal efficiency (60%) were low initially (Fig. 1a and b). However, the sulfide generation
219 became stable at the end of Stage I and reached 185 mg S/L in the UASB effluent (Fig. 1a),
220 indicating approximately 84% (185/220×100%) thiosulfate was converted to sulfide and the

BTR rate was 1.11 kg $S_2O_3^{2-}$-S/d/m^3. Correspondingly, the effluent COD concentrations stabilized at around 18 mg /L after 16 days, corresponding to a COD removal efficiency of 90% through BTR (Fig. 1b).

(Position for Fig. 1)

During the UASB reactor operation Stage II the influent contained 50 mg S/L of Na_2SO_3 (pH 7.0, equivalent to 2.0×10^{-4} mg FSA-S/L). It was seen that the effluent sulfide concentration decreased from 185 mg S/L in Stage I to 120 mg S/L in Stage II (Fig. 1a) and the BTR rate decreased from 1.11 to 0.93 kg $S_2O_3^{2-}$-S/d/m^3. At the same time, the COD removal efficiency dropped immediately and stabilized at about 75%. This implied an inhibitory effect of saline sulfite and FSA on the BTR. As the influent Na_2SO_3 concentration was increased to 150 mg S/L (FSA at 6.0×10^{-4} mg S/L), sulfide generation in UASB reactor's effluent dropped from 185 mg S/L (in Stage I without FSA) to 99 mg S/L in Stage III. In Stage III the BTR rate had also dropped to 0.8 kg $S_2O_3^{2-}$-S/d/m^3, which was only 72% of that in Stage I. As well, the COD removal efficiency continued to drop to 65% (Fig. 1b). In Stage IV, the UASB reactor's influent pH was raised to 8.5 and the influent Na_2SO_3 concentration was kept at 150 mg S/L, corresponding to a lowered FSA concentration of 1.2×10^{-6} mg FSA-S/L. In this stage, sulfide generation, thiosulfate reduction as well as organic carbon removal were restored to some extent. As shown in Fig. 1a and b, there were increases in the BTR rate to 0.96 kg $S_2O_3^{2-}$-S/d/m^3, the effluent sulfide concentration to 157 mg S/L and the organic carbon removal efficiency to about 80%. Therefore, in addition to the Na_2SO_3 concentration, pH may also play a role in the BTR

activity, suggesting that the combined effects of pH and Na_2SO_3 (i.e. FSA) might be the true inhibitor on the BTR activity in the UASB reactor.

At Stage V, the operating conditions of the UASB reactor were fully restored to those of Stage I. After 20 days into Stage V it was seen that the BTR rate (1.06 $S_2O_3^{2-}$-S/d/m^3), sulfide generation (174 mg S/L) and the COD removal efficiency (89%) in UASB reactor were comparable with those in Stage I (Fig. 1). Thus, indicating the biomass activity in the UASB reactor had recovered after eliminating the saline sulfite/FSA addition to the influent.

3.2 Microbial community shift in UASB reactor

9812, 8174 and 9578 quality sequence reads of the 16S rRNA gene (with an average read length of 374 bp) were obtained from the UASB reactor at the end of Stages I, III and V respectively (Fig. 2a). The sequences were clustered into 564, 959 and 670 operational taxonomic units for the three tested sludge samples in Stage I, III and V respectively (Table S6).

(Position for Fig. 2)

Excluding the unclassified Bacteria, altogether, 9 bacterial phyla were recovered from the three sludge samples. The majority of the sequences belong to the Firmicutes, Proteobacteria and Bacteroidetes phyla (Fig. 2b). However, at the phylum level microbial community changes are not obvious between the stages with and without the FSA addition to the reactor's influent. Therefore, the microbial communities were analyzed at the class and genus levels (Fig. 2c and

d). The sequences were further classified into 17 classes (Fig. 2c), and in all three stages, the dominant classes were Bacilli, Deltaproteobacteria and Clostridia. However, variation of abundances of the classes was detected between the different Stages, with the Deltaproteobacteria having the most significant shifts. As most of the functional SRB genera belong to the class Deltaproteobacteria (Castro et al., 2000), it is possible that this relates to variation of the SRB populations corresponding to the absence and presence of FSA.

Within the microbial community analysis at the genus level, four to five types of recognized SRB were detected at different levels in each stage, with *Desulfomicrobium* and *Desulfobulbus* as the most two abundant genera (see Fig. 2d and Table S7). Species of these two genera can reduce $S_2O_3^{2-}$ to HS^-/S^{2-} coupled with the oxidation of organic substrates that include lactate, pyruvate, glycerol and acetate (Barton and Hamilton, 2007; Widdel, 1998; Brenner et al., 2005). These are also previously reported to be the dominant SRB groups in both sulfate and/or sulfite-reducing UASB reactors (Jiang et al., 2013; Qian et al, 2015b). In Stage I, with thiosulfate as the sole sulfur source, the total SRB population accounts for 42.6% at the genus level, of which *Desulfomicrobium* and *Desulfobulbus* make up 21.5 and 20.6%, respectively (see Fig. 2d). The enrichment of SRB in Stage I supports the high BTR and COD removal rate in UASB reactor without FSA and saline sulfite (Fig. 1a and b). When the reactor influent was supplemented with FSA at 6.0×10^{-4} mg S/L in Stage III, the total SRB abundance sharply decreased to 7.1% at the genus level. The levels of two major SRB genera, i.e. *Desulfobulbus* and *Desulfomicrobium* dropped to 3.4 and 0.8%, respectively. The small SRB population in Stage III corresponds to the low BTR activity (Fig. 1a and b). In Stage V, when FSA addition

was excluded, there was a recovery period according to performance that lasted for around 60 days (from Day 122 to Day 181). During that stage the SRB population rebounded to 33.1%, and this corresponded to the recovered BTR activity (see Fig. 1a and b). After this recovery, the *Desulfomicrobium* and *Desulfobulbus* levels rose to 21.1% and 11.6%, respectively. Based on the changes of microbial community and reactor performance at the different Stages, these strongly implicate a negative affect of FSA and saline sulfite on the SRB population.

Typically, in a sulfur reducing reactor operating for municipal sewage treatment, fermentation of organic compounds is an essential microbial process (Jiang et al., 2013). Although the single and simple organic compound (acetate, which is not fermentable) was utilized as electron donor and energy source in this study, certain levels of typical fermenting genera were still detected in the reactor communities (Fig. 2d). The most possible reason should be the sludge lysis to some extent as no sludge was purposely taken during the whole operation period. The organic products from cell lysis (the reactor had a long sludge retention time) and extracellular polymeric substances could contribute the fermentable substrates in the reactor (Wang et al., 2013, 2014). High abundance of *Trichoccocus*, a well-known fermenting bacteria (Liu et al., 2002), was detected at 16.9% in Stage III (see Table S7), compared with 0.67 and 6.62% in Stages I and V. Thus, suggesting higher levels of fermentation occurred when FSA was added in Stage III, and possibly this was due to increased cell lysis caused by FSA, as has been reported for sludge treatment previously (Zan et al., 2016).

3.3 Effects of pH on the BTR

309 In Batch Test I the BTR activities were determined at different pH in the absence of saline
310 sulfite and FSA (Fig. 3 and Fig. S4a). The BTR activity peaked at between 40 to 43 mg
311 $S_2O_3^{2-}$-S/g MLVSS/h at pH 7.0 and 8.0 (see Fig. 3a). This activity was 1.7 and 1.3 times that at
312 pH 6.0 and 9.0 respectively. Also, this pH related trend is the same as that detected for biological
313 co-sulfate/sulfite reduction where the reducing activity is also highest between pH 7.0 and 8.0
314 (Qian et al., 2015c). Consequently, this finding supports the notion that thiosulfate reduction is
315 the rate-limiting step in biological sulfate/sulfite reduction.

316

317 (Position for Fig. 3)

318

319 *3.4 The effects of saline sulfite and FSA concentrations on BTR*

320 The effects of different Na_2SO_3 (that includes both saline sulfite and FSA) concentrations (50 to
321 200 mg S/L) on the BTR activity were examined in Batch Test II when the pH was controlled at
322 7.0 (Fig. 3b). It was seen that the highest biomass-specific thiosulfate reduction rate of 17.7 mg
323 $S_2O_3^{2-}$-S/g MLVSS/h was achieved with the lowest initial addition of Na_2SO_3 (50 mg S/L) and
324 the activity continually lower when the higher initial Na_2SO_3 concentrations were added. Such
325 that the biomass-specific $S_2O_3^{2-}$ reducing rate dropped by 39% when the Na_2SO_3 concentration
326 increased from 50 mg S/L to 200 mg S/L (Fig. 3b). Therefore, the results of this test confirm that
327 Na_2SO_3 consists of saline sulfite and FSA played a role in the inhibition of the BTR activity.

328

329 *3.5 Correlation between FSA (H_2SO_3) and BTR activity*

The BTR rates under different FSA levels were examined in Batch Test III. Both the pH and Na_2SO_3 concentrations were varied in each reactor in this test (Table S4). Based on the results from Batch Tests I, II and III, we examined for the correlations between BTR activity versus pH (Fig. 4a), BTR activity versus saline sulfite concentration (Fig. 4b) and BTR activity versus FSA concentration (Fig. 4c). As confirmed in Batch Test I, the pH really impacts the BTR activity, but the correlation between pH and BTR activity is not strong in the presence of Na_2SO_3 (Fig. 4a). At the same pH level, lower activity was observed at higher Na_2SO_3 concentrations. Generally, as the saline sulfite concentration increased, the BTR activity was reduced. However, this relationship also depends on pH (Fig. 4b). For example, with initial concentration of 200 mg S/L saline sulfite, the BTR activity varied from 4.4 to 33.6 mg $S_2O_3^{2-}$-S/g MLVSS/h as the pH changed from 6.0 to 9.0. Therefore, the correlation between BTR and saline sulfite concentrations is also not strong. These observations imply that saline sulfite and pH jointly cause the inhibitory effect on thiosulfate reduction.

(Position for Fig. 4)

It was seen that the level of inhibition of the BTR had a strong correlation with the FSA concentration, indicating that FSA may be directly causing the inhibition (Fig. 4c). The inhibitory effect of FSA on the BTR was well described by an exponential function (Fig. 4c). The BTR activity decreased significantly with the increased FSA concentration even in the very low range of $0\sim1.5\times10^{-5}$ mg H_2SO_3-S/L. The BTR activity decreased by 50% as FSA concentrations increased from 8.0×10^{-8} (Na_2SO_3 concentration of 100 mg S/L at pH 9.0) to

352 2.0×10^{-4} mg H_2SO_3-S/L (equivalent to a Na_2SO_3 concentration of 50 mg S/L at pH 7.0). When
353 the FSA concentration increased from 8.0×10^{-8} (100 mg Na_2SO_3-S/L at pH 9.0) to 0.015 mg S/L
354 (200 mg Na_2SO_3-S/L at pH 6.0), the BTR activity was inhibited by 90%. Consequently, these
355 results suggest that FSA alone rather than saline sulfite or pH, is the true inhibitor of the BTR.
356
357 *3.6 Toxicity and inhibition of FSA to microorganisms*
358 Sulfite, either in the ionized form (saline sulfite: SO_3^{2-} + HSO_3^-) or unionized form (FSA:
359 H_2SO_3), is characterized as having potential toxicity to microbial metabolism. Its negative
360 effects are suggested to be through damaging the biomacromolecules such as proteins, lipids and
361 DNA (Armentia-Alvarez et al., 1993; Shi and Mao, 1994; Trotter and Grant, 2002; Pena-Egido
362 et al., 2005), thus leading to the inhibition of microbial activity and growth. Other studies show
363 that after exposure of microorganisms to sulfite, the cellular ATP levels are lowered and cell
364 destruction is observed (Schimz and Holzer, 1979; Hinze and Holzer, 1986; Maier et al., 1986;
365 Prakash et al., 1986). In addition, Park and Hwang (2008) provided the evidence that the
366 addition of saline sulfite/FSA represses the expression of genes involved in transcription, protein
367 biosynthesis and cell growth.
368
369 Other studies also show that the antimicrobial action of saline sulfite/FSA is found to be the
370 greatest at low pH (Ough, 1993; Wedzichab, 1984), further adding support that H_2SO_3 (FSA) is
371 the true antimicrobial agent rather than saline sulfite. The precise mechanisms of how FSA
372 causes its antimicrobial effect is yet to be determined. The presence of FSA could change the
373 structure of the cell membrane (Jiang et al., 2015), enter the cell and damage intracellular

components (Stratford and Morgan, 1987), and/or possibly directly cause cell lysis (Zan et al., 2016).

3.7 Implications of this study

Thiosulfate, as an intermediate of biological sulfate/sulfite reduction, is an effective electron donor for chemolithoautotrophic denitrification. Unlike the end product of biological sulfate/sulfite reduction, i.e. sulfide, thiosulfate is not reported to be toxic to microorganisms including the denitrifying bacteria (Cardoso et al., 2006). So use of thiosulfate as the electron donor in a wastewater treatment system could induce a high nitrogen removal rate as well as lead to a low sludge yield. Recently, we developed a "nitritation coupled with thiosulfate-driven denitritation (Nitritation-TDD)" process, that achieved a high biological ammonia-nitrogen removal rate of 0.43 kg NH_3-N/d/m^3 (Qian et al., 2016). To facilitate the application of the Nitritation-TDD process, a key point is to ensure adequate thiosulfate is generated as thiosulfate is generally not directly available from the wastewater. This study on FSA inhibition of BTR provides some hints on how to obtain thiosulfate accumulation in a biological sulfate/sulfite reducing reactor's effluent. By utilizing the inhibitory potential of FSA on BTR, an optimized sulfur cycle-driven biological process with three short-cut bioreactions is proposed here: 1) biological sulfate/sulfite reduction to thiosulfate (SO_4^{2-}/SO_3^{2-} → $S_2O_3^{2-}$,) 2) denitritation with thiosulfate as the electron donor ($S_2O_3^{2-}$ + NO_2^- → SO_4^{2-} + N_2↑) and 3) nitritation (NH_3 → NO_2^-). Consequently, this will result in higher nitrogen removal rates and lower sludge yields. The study to achieve this optimized process for co-treatment of wet flue gas desulfurization wastes with freshwater sewage is required and will be carried out in the near future.

4. Conclusions

The effects of FSA on biological thiosulfate reduction in a sulfur cycle-driven wastewater treatment process were examined in this study. It was concluded that FSA, instead of saline sulfite, is the true inhibitor of biological thiosulfate reduction. Based on the microbial community analysis, the abundance of the SRB population in the thiosulfate-reducing UASB reactor was sharply decreased from 46.2 to 7.1% at genus level when FSA was added to the reactor's influent at 6.0×10^{-4} mg H_2SO_3-S/L. The biological thiosulfate reducing activity decreased markedly with the addition of FSA, this was inhibited by 50% when initial FSA concentrations were altered from 8.0×10^{-8} to 2.0×10^{-4} mg H_2SO_3-S/L in the batch reactor. The inhibition of FSA on biological thiosulfate reduction was found to recover after the elimination of FSA.

Acknowledgements

This work was financially supported by Natural Science Foundation of China (No. 51608444) and the Open Fund of State Key Laboratory of Geohazard Prevention and Geoenvironment Protection (Grant No. SKLGP2017K026). The authors also gratefully acknowledge the support from National Training Programs of Innovation and Entrepreneurship for undergraduate of China (No. 201610699132 and No. 201610699369). Dr. Qilin Wang acknowledges the supports of Australian Research Council Discovery Early Career Researcher Award (DE160100667), Australian Research Council Discovery Project (DP170102812) and the Philanthropic Grant for Early Career Engineering Researcher (GE12015).

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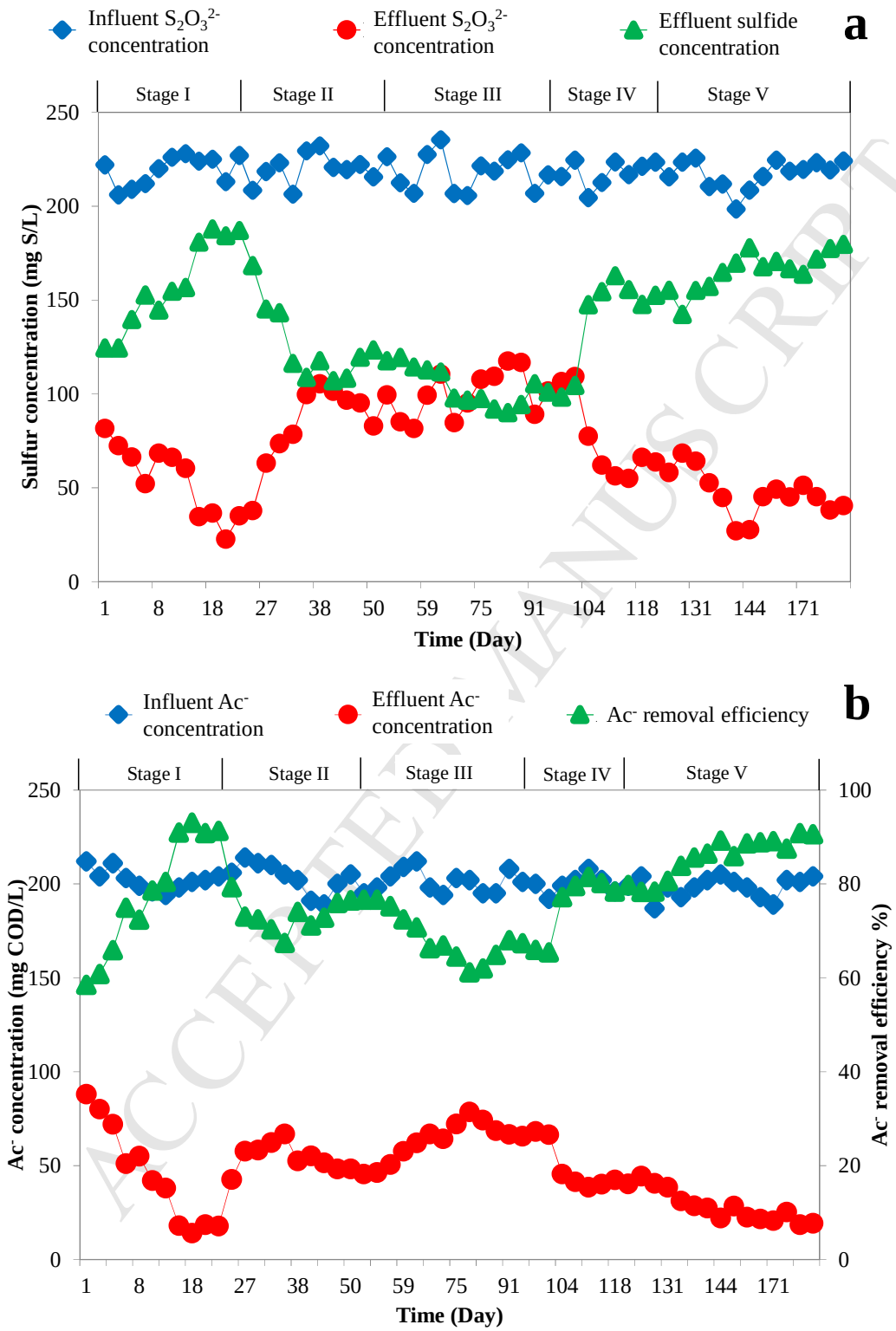

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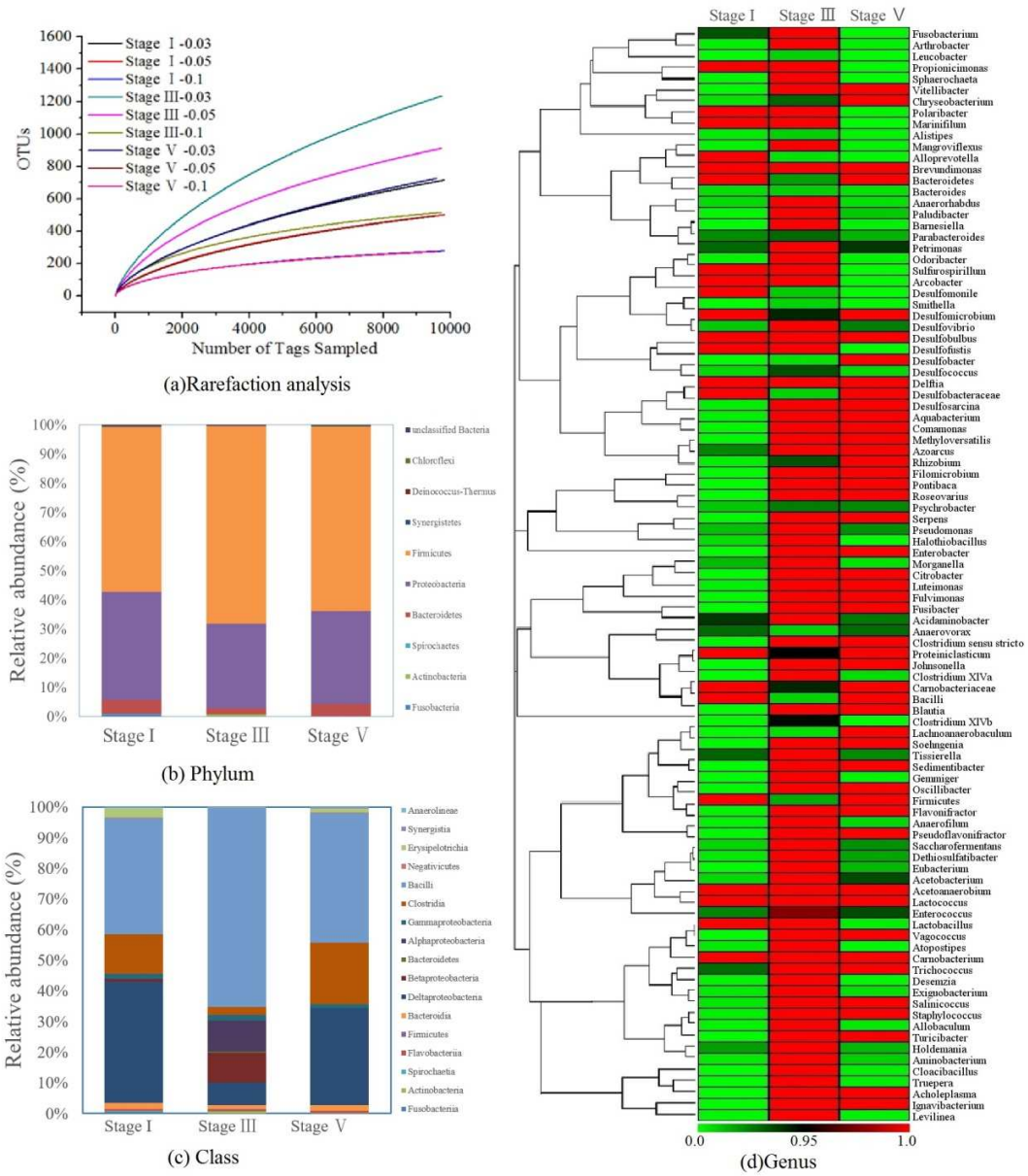

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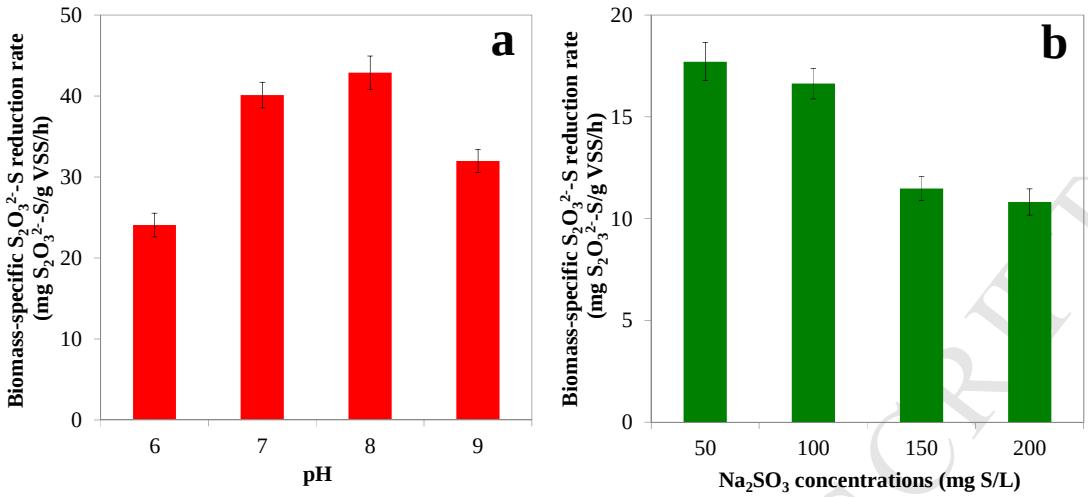

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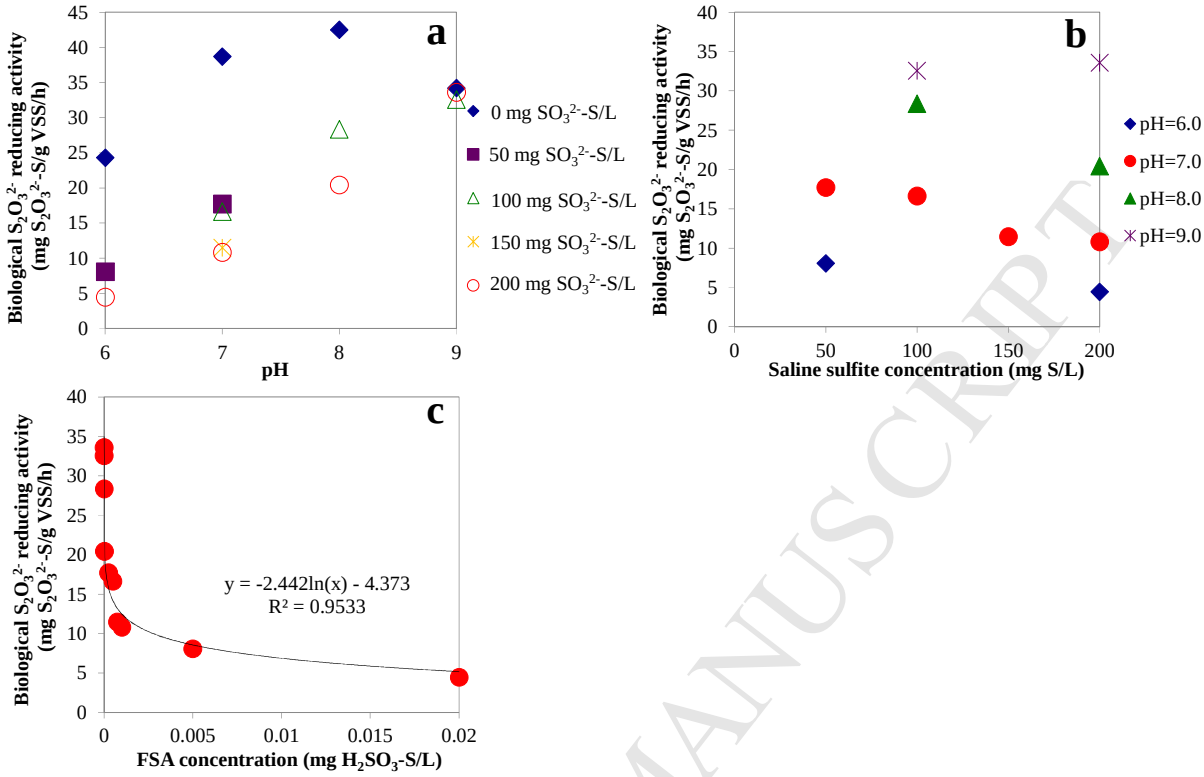

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Highlights

- Free sulfurous acid was the true inhibitor of biological $S_2O_3^{2-}$ reduction
- $S_2O_3^{2-}$ reducing activity was depressed at an FSA concentration of 1.5×10^{-5} mg S/L.
- SO_4^{2-}/SO_3^{2-}-reducing bacteria population decreased in the presence of FSA
- FSA inhibition on biological $S_2O_3^{2-}$ reduction is reversible