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Published in:
Bioresource Technology

Link to article, DOI:
[10.1016/j.biortech.2019.121592](https://doi.org/10.1016/j.biortech.2019.121592)

Publication date:
2019

Document Version
Peer reviewed version

[Link back to DTU Orbit](#)

Citation (APA):
Qiu, L-Q., Zhang, L., Tang, K., Chen, G., Kumar Khanal, S., & Lu, H. (2019). Removal of sulfamethoxazole (SMX) in sulfate-reducing flocculent and granular sludge systems. *Bioresource Technology*, 288, [121592]. <https://doi.org/10.1016/j.biortech.2019.121592>

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Removal of sulfamethoxazole (SMX) in sulfate-reducing flocculent and granular sludge systems

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Abstract

This study investigated sulfamethoxazole (SMX) removal and fate in sulfate-reducing up-flow sludge bed (SRUSB) reactors inoculated with sulfate-reducing bacteria (SRB) granules and flocs. The resilience of SRB granules and flocs against varying pHs and hydraulic retention times (HRTs) was also examined. SRB granules and flocs efficiently removed SMX from wastewater, which was significantly higher than the aerobic sludge. SRB granules achieved significantly ($p < 0.05$) higher SMX removal ($\sim 13.3 \mu\text{g/g}$ suspended solids (SS)-d) than the SRB flocs ($\sim 11.2 \mu\text{g/g}$ SS-d) during 150-day of SRUSB reactors operation. The SMX removal by both granules and flocs was mainly attributed to biodegradation. Sorption also contributed to SMX removal, in which aromatic protein-like substances of extracellular polymeric substances played important role in SMX removal. In addition, SRB granules showed higher resilience than SRB flocs against varying pHs and HRTs. Thus, SRB-mediated biological process, especially SRB granules, could be a promising biotechnology to remove SMX from wastewaters.

Keywords: Sulfamethoxazole (SMX), Sulfate reducing bacteria (SRB) granules and flocs, Biodegradation, Adsorption, Extracellular polymeric substances (EPS)

1. Introduction

Sulfamethoxazole (SMX) is one of the extensively used antibiotics by humans, and for animal production and aquaculture (Zuccato *et al.*, 2010, Zhang *et al.*, 2017), and is frequently detected in aquatic environment (Heberer, 2002, Xu *et al.*, 2011). Owing to its slow degradation, SMX is highly persistence in the environment and has received increasing attention in recent years (Chen *et al.*, 2015). Wastewater treatment plants (WWTPs) effluents, aquaculture effluent, digestate / biosolids and animal manures are the main sources of SMX entering into the environment (Joss *et al.*, 2005). Conventional activated sludge (CAS) process is mainly aimed at removal of typical pollutants, such as organics, nitrogen and phosphorus, and is less effective in the removal of antibiotic compounds (Grandclement *et al.*, 2017). Moreover, the process is highly energy intensive and generates large amount of biological sludge. Anaerobic process is cost-effective for treating high strength/high solids waste (water). However, the process is less effective in removing the antibiotics (Falås *et al.*, 2016, Grandclement *et al.*, 2017). The process is also less effective in removing carbon, nitrogen and phosphorus simultaneously. Thus, there is a critical need to develop an economical, effective and energy-efficient biological process for simultaneous removal of organic matters, nitrogen and phosphorus along with antibiotics. One of such innovative biotechnologies is Sulfate-reduction Autotrophic-denitrification and Nitrification Integrated (SANI) process, which has shown to reduce sludge generation by 60–70%, energy consumption by 35% and CO₂ emission by 36% compared to CAS process (Lu *et al.*, 2012, Wu *et al.*, 2016).

Recently, sulfate-reducing bacteria (SRB)-mediated biological process has been employed to treat sulfate-laden pharmaceutical wastewater, and has shown better tolerance of SRB to high concentrations of pharmaceutical compounds compared to aerobic microbes (Mohan *et al.*, 2005, Li *et al.*, 2015). Several studies reported that SRB-enriched sludge flocs could efficiently remove antibiotics such as SMX and ciprofloxacin (CIP) from wastewater (Jia *et al.*, 2017, Jia *et al.*, 2018). Thus, SRB sludge system has the potential of treating wastewater containing antibiotics (e.g., SMX). Anaerobic SRB granular sludge has recently been successfully applied for wastewater treatment (Hao *et al.*, 2013, Hao *et al.*, 2015). Compared to SRB sludge flocs, SRB granular sludge has potential to shorten the start-up time of SRB sludge system and facilitates better removal of pollutants as higher biomass concentration could be maintained in the system (Hao *et al.*, 2013). In addition, granular sludge has a higher extracellular polymeric substances (EPS) content thereby protecting the microbes against inhibitory compounds including antibiotics (Delgado *et al.*, 2010). Despite the above stated inherent merits, there are limited studies on the use of SRB granular sludge system for antibiotics removal. Even for aerobic granular sludge, very few studies examined the use of aerobic granular sludge system for the removal of micro-pollutants, including antibiotics. For example, Balest *et al.* (2008) reported that aerobic granular sludge achieved better removal of endocrine disrupting compounds, bisphenol A, and 4-tert-octylphenol (60-90%) than the CAS process (41-72%). Kang *et al.* (2018b) found that the removal efficiency of SMX was higher in an anoxic/anaerobic/oxic sequencing batch reactors (SBRs) inoculated with aerobic

granular activated sludge (~84%) than that in the similar system inoculated with suspended aerobic activated sludge (~73%). Anaerobic granular sludge has different biological and physical-chemical attributes including redox conditions and microbial community. Thus, there is a need to examine the potential of anaerobic SRB granular sludge systems in treating SMX-laden wastewaters.

Therefore, the objectives of this study were to (a) investigate the effects of SMX on long-term performance of sulfate-reducing upflow sludge bed (SRUSB) reactor inoculated with SRB granules and flocs in terms of organic removal and sulfate reduction; (b) investigate the removal of SMX by the SRUSB reactors during a long-term study; and (c) examine the effects of operational parameters (e.g., pH and hydraulic retention time (HRT)) on SMX removal in the SRUSB reactors with SRB granular and flocculent sludges. In addition, the fate of SMX in the SRUSB reactors was also examined.

2. Materials and Methods

2.1. Chemicals and reagents

SMX (99% purity) was obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany). The physical-chemical properties of SMX are presented in supplementary information. SMX $^{13}\text{C}_6$ was purchased from Cambridge Isotope Labscience Inc. (Tewksbury, MA, USA), which was used as an internal standard to determine SMX concentration in samples. Formic acid, and HPLC gradient grade methanol and ethanol were obtained from Merck (Darmstadt, Germany). Oasis HLB cartridges (200 mg/6 mL) were purchased from Waters (Milford, MA, USA). Filter membrane (0.22-

μm) was obtained from Jinteng (Tianjin, China). All the standards were prepared using Ultra-pure water from Millipore System (Millipore, Billerica, MA, USA). Two individual standard stock solutions of SMX (100 mg/L) and SMX $^{13}\text{C}_6$ (10 mg/L) were prepared in methanol. Both stock solutions were kept in the dark at 4 °C until further use.

2.2. SRUSB reactor setup and operation

Two identical laboratory-scale SRUSB reactors with a working volume of 0.5 L (40 mm diameter $\times$ 500 mm total height) were fabricated using acrylic (Supplementary material). SRB granules taken from a SRUSB reactor in our laboratory, were used as a seed sludge in one SRUSB reactor in this study, which was continuously operated for more than 200 days. The SRB granules taken from the same SRUSB reactor were disintegrated into flocs and then seeded into another SRUSB reactor. The sludge concentration in the two SRUSB reactors was 7.0 ± 0.1 g suspended solids (SS)/L. The properties of the sludge in the two SRUSB reactors are described in Supplementary material. Both SRUSB reactors were fed with synthetic wastewater, and the stock synthetic wastewater was prepared as per Hao *et al.* (2013). Due to well acclimation of SRB sludge, both sludge systems showed COD and sulfate removal efficiency over 85% and 75%, respectively, (data not shown here) during a short acclimation period of 10 days without SMX. After the acclimation period, SMX stock solution was spiked periodically into the influent tank to achieve an influent SMX concentration of about 50 $\mu\text{g/L}$ (the measured influent SMX concentration fluctuated slightly, see Fig. 2). The two SRUSB reactors were then

operated continuously for approximately 150 days during which the influent COD and sulfate concentrations were 498 ± 38 mg/L and 222 ± 12 mg S/L, respectively. The influent pH was maintained at 7.0 ± 0.2 by adding 0.1 mM HCl/NaOH solution using a pH controller. The HRT in both reactors was maintained at 5 h. The effluent from each reactor was recirculated to the SRUSB reactor with an internal flow recycle ratio at 5:1. The liquid up-flow velocity in each reactor was 0.47 m/h. The two SRUSB reactors were placed in a temperature-controlled chamber (25 ± 2 °C) throughout the experiment. During the experiment, influent and effluent samples were periodically collected from both reactors to measure sulfide, total organic carbon (TOC), sulfate, thiosulfate and SMX concentrations, and the effluent pH. Moreover, sludge samples were also periodically collected from the two SRUSB reactors to determine SMX accumulation and EPS content of the sludge.

2.3. SMX and extracellular polymeric substances (EPS) binding experiment

EPS play a crucial role in micropollutants removal. This is because proteins as an important component of EPS, have diverse functional groups, such as carboxyl, amine and hydroxyl groups, and hydrophobic regions, which can provide binding sites for the adsorption of micro-pollutants (Sheng *et al.*, 2010, Xu *et al.*, 2013, Wang *et al.*, 2018). There are limited studies on the role of proteins in EPS derived from SRB sludge on the adsorption of SMX. Thus, following the tests described in Section 2.4, a series of binding batch experiments were conducted using the EPS extracted from both SRB granules and flocs from SRUSB reactors at different SMX concentrations to determine which proteins of EPS play an important role on the adsorption of SMX in

the SRB granular and flocculent sludge systems. Before extracting EPS from the respective sludge samples, the SRUSB reactors were continuously fed with SMX-free synthetic wastewater until SMX concentration was nondetectable in both aqueous and solid phases. The extracted EPS from the SRB granules and flocs, was first purified by polyethersulfone ultrafiltration membrane with 5000 molecular weight cut-off (Millipore, Billerica, MA, USA) to remove ions and small molecules (Xu *et al.*, 2013). The retentate was lyophilized to EPS powder (referred to as pristine EPS) with a freeze dryer (ALPHA Marin Christ, Germany), and stored in a desiccator until further use. In order to obtain a good resolution of excitation-emission matrix (EEM) fluorescence spectra, 50 mg/L of EPS solution was prepared following the procedure given in Zhang *et al.* (2018a). Different volumes of SMX stock solution were spiked into each tube containing the EPS solution to obtain a series of different initial concentrations, ranging from 0, 100, 200, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000 and 4500 µg/L. The initial pH was adjusted to approximately 7.0 by adding 0.1 mM HCl or NaOH solution. All the tubes were mixed for 10 min using oscillator (IKA, Vortex, Germany), and then placed in a dark room for 4 h at 25 ± 0.5 °C to reach adsorption equilibrium before spectral analyses (Zhang *et al.*, 2018a). The details of spectral analysis are described in Chemical analyses section.

2.4. Effects of pH and HRT on SMX removal by SRB granules and flocs

2.4.1. Test I: Effect of pH on SMX removal

The variations in wastewater pH could affect enzyme activity of microorganisms which is essential for biodegradation of complex organic compounds

(Grandclement *et al.*, 2017). As such, the removal of SMX in the SRUSB reactors could be influenced by pH. At the end of the experiment (from days 152 to 205), the effects of different initial pH levels on SMX removal were studied in the two SRUSB reactors. As the sewage pH generally ranges from 6.5 to 8.5 (Qian *et al.*, 2018), a broader pH ranges of 6.0 to 9.0 were examined in this study (Table 1). During each pH condition, the SRUSB reactors were operated for seven days except at pH 7.0 in which the reactors were operated for 5 days. The other operational parameters were kept the same as those described in Section 2.1. During the test, the influent and effluent pH, TOC, sulfate, sulfide and thiosulfate, and SMX concentration in aqueous and sludge samples were measured as discussed in Chemical analyses section.

2.4.2. Test II: Effects of HRT on SMX removal

HRT is a critical operating parameter in bioreactor operation. Long HRT on one hand extends the exposure of high concentrations of intermediates that are more toxic than SMX, to microbes in SRUSB reactor; on the other hand, it provides longer time for microbes to degrade the SMX. Thus, the effects of different HRTs (from 5 to 9 h) on SMX removal and the treatment performance of the SRUSB reactors were investigated at $\text{pH } 7.0 \pm 0.2$ following the completion of studies of pH effect. This experiment lasted for 42 days (from days 210 to 251), in which each HRT condition was tested for at least seven days (Table 1). Before the start of test, the SRUSB reactors were continuously operated at $\text{pH } 7.0 \pm 0.2$ for four days to minimize the interference of effect of varying pH on SMX removal. The other operational parameters were kept the same as those described in Section 2.1. During the test,

the measured parameters were the same as those described in the Test I.

2.5. Chemical analyses

The influent and effluent samples of the SRUSB reactors were collected and filtered through disposable Millipore filter (0.22- μ m pore size). The samples were then analyzed for various important parameters such as dissolved sulfide (H_2S , HS^- and S^{2-}), pH, alkalinity, and total volatile fatty acids (VFAs). TOC was measured instead of COD in this study to eliminate the effect of dissolved sulfide on COD measurement (Zhang *et al.*, 2018b). TOC was analyzed with a TOC analyzer (Shimadzu TOC-5000A, Japan), and the values were converted into equivalent COD based on a theoretical ratio of 2.67 g COD/g TOC (Liang *et al.*, 2016). Dissolved sulfide was determined by the methylene blue method as per Standard Methods (APHA, 2005). Sulfate and thiosulfate concentrations were quantified using an ion chromatograph (DIONEX-900, ICS-900, Fremont, CA, USA) with a conductivity detector and an IC-SA2 analytical column. VFAs and alkalinity were analyzed using the five-point titration method (Moosbrugger *et al.*, 1992). pH was measured with a pH meter (HQ40D). Sludge volume index in 5 min (SVI_5), suspended solids (SS), and volatile suspended solids (VSS) were measured following the Standard Methods (APHA, 2005).

During the long-term operation of the SRUSB reactors, EPS contents of sludge granules and flocs on days 0, 30, 60, 90, 120 and 150 were determined. The EPS were extracted from the sludge granules and flocs using NaOH formaldehyde method described in Bourven *et al.* (2011). EPS mainly contain proteins (PN) and polysaccharides (PS) (Yang *et al.*, 2016). PN was measured using

the modified Lowry method (DC protein assay, BioRad, USA) with bovine serum albumin as a standard (Yang *et al.*, 2016). PS was measured using the phenol-sulfuric acid method with glucose as a standard (Yang *et al.*, 2016). The adsorption of SMX onto EPS was analyzed by three-dimensional excitation-emission matrix (3D-EEM) fluorescence spectroscopy technology, which was further visualized by a three-dimensional fluorescence spectrophotometer (Aqualog- UV-800-C, HORIBA, Japan). The EEM data were analyzed using MATLAB 2016a (Mathworks, Natick, MA, USA) with DOMFluor1.7 tool-box. Parallel factor analysis (PARAFAC) was used to resolve the EEM data. Determination of the correct number of components was mainly based on residual analysis, split-half analysis, and visual inspection (Zhang *et al.*, 2018a). The details of EEM analysis are described in Zhang *et al.* (2018a).

The SMX was measured according to the pre-established method as described in Jia *et al.* (2017). Briefly, the SMX concentration was determined by Ultra Performance Liquid Chromatography (UPLC) equipped with a diode array detection (DAD) detector (Dionex UltiMate 3000, CA, USA) using an Acclaim120 C18 column (2.1 x 150 mm, 3 mm, Dionex, CA, USA) under mobile Phase A (35% ultra-pure water with 0.1% formic acid) and mobile Phase B (65% methanol). The operational parameters of the UPLC, limit of detection (LOD) and limit of quantification (LOQ) of the UPLC are based on the analytical methods described in Jia *et al.* (2017), and the detailed procedures are given in Supplementary material.

2.6. Statistical analysis

The statistical analysis was performed in GraphPad Prism 7.0. A Mann-Whitney

test was used to assess the significant difference of the results between the two SRUSB reactors with granules and flocs at the 95% confidence level ($p < 0.05$). One-way Analysis of Variance (ANOVA) was used to find the significant differences among different parameters in the SRUSB reactors with granules and flocs using a 0.05 significance level ($p < 0.05$).

3. Results and Discussion

3.1. Performance of SRUSB reactors

The two SRUSB reactors inoculated with granules and flocs reached steady state within ten days of operation without SMX addition. Both reactors achieved average COD and sulfate removal efficacy of 85% and 75%, respectively (data not shown here). The two SRUSB reactors were then continuously operated for nearly 150 days at an initial SMX concentration of 50 $\mu\text{g/L}$ (Fig. 1). In the SRUSB reactor with granules, the settleability ($\text{SVI}_5 = 28 \text{ mL/g}$) and size ($\sim 620 \mu\text{m}$) of SRB granules did not change substantially during the entire operational period, and the sludge concentration gradually increased from $7.06 \pm 0.01 \text{ g SS/L}$ on day 0 to $10.08 \pm 0.02 \text{ g SS/L}$ on day 150 (Supplementary material). The COD and sulfate removal efficiency remained stable, and were similar to those in the acclimation period without SMX. The results apparently showed that the presence of SMX did not affect the performance of SRUSB reactor with granules. In the SRUSB reactor with flocs, flocs size ($\sim 81 \mu\text{m}$) and sludge concentration ($6.94 \pm 0.02 \text{ g SS/L}$) did not change significantly during the first 30 days of operation. During the same time period, COD

and sulfate removal efficiency decreased to $75 \pm 3 \%$ and $62 \pm 4\%$, respectively (Fig. 1b). The results suggest that SMX had an inhibitory effect on SRB flocs at the beginning of the reactor operation. The COD and sulfate removal efficiency, however, gradually recovered to the levels similar to that in the SRUSB reactor with granules. Thus, the SRB were able to gradually adapt to SMX with time. The flocs size and sludge concentration also gradually increased to $\sim 147 \mu\text{m}$ and $9.41 \pm 0.17 \text{ g SS/L}$, respectively (Supplementary material).

Furthermore, fluorescence in situ hybridization (FISH) analysis results showed that SRB abundance in the granules and flocs did not decrease during the long-term operation with SMX addition of $50 \mu\text{g/L}$ (see Supplementary material). The performance of the two SRUSB reactors was similar to that of a full-scale SANI system (Wu *et al.*, 2016). Unlike the SRUSB reactor with flocs, the performance of SRUSB reactor with granules did not decrease from the starting of the reactor operation, indicating that the SRB granules showed a higher tolerance to SMX than the SRB flocs. This could be due to the fact that the SRB granules had a higher content of EPS than the SRB flocs (see Section 3.4), thereby protecting the SRB community against the inhibitory effect of SMX (Schmidt *et al.*, 2012).

3.2. Removal of SMX in SRUSB reactors

The measured SMX concentration in the influent was $48.0 \pm 4.9 \mu\text{g/L}$ during 150 days of SRUSB reactors operation (Fig. 2). During the first 15 days of operation, the SMX removal efficiencies in the SRUSB with granules and flocs were $30 \pm 3\%$ and $22 \pm 5\%$, respectively. Starting from day 15, the SMX removal efficiency in SRUSB

reactors with granules and flocs gradually increased to $51 \pm 4\%$ and $40 \pm 3\%$, respectively, and then remained fairly stable during the rest of the operation. Overall, SRUSB reactor with granules achieved significantly higher SMX removal ($p < 0.05$) than the SRUSB reactor with flocs. The specific SMX removal rate in the SRUSB reactor with granules was also significantly greater ($13.3 \mu\text{g/g SS-d}$) than that in the SRUSB reactor with flocs ($11.2 \mu\text{g/g SS-d}$) during the steady-state operation ($p < 0.001$). The higher SMX removal by the SRB granules could be attributed to the longer solids retention time (SRT) of the granular system ($92 \pm 1 \text{ d}$) than that of suspended sludge (flocs) system ($76 \pm 2 \text{ d}$). Long SRT could improve the removal of pharmaceutical compounds by facilitating the growth of diverse microbial community in the granules and retaining the slow-growing bacteria which may have the ability to degrade organic micro-pollutants (Grandclement *et al.*, 2017).

Several studies examined the removal of SMX in activated sludge system (aerobic, anoxic and anaerobic compartments), and the removal was primarily via biodegradation and sorption processes (Rosal *et al.*, 2010, Alvarino *et al.*, 2014, Alvarino *et al.*, 2016, Kang *et al.*, 2018b). However, studies on SMX removal in SRB sludge system, especially in SRB granule system are very limited. Jia *et al.* (2017) reported SMX removal of around 34% by SRB flocs at an initial concentration of $100 \mu\text{g/L}$, and the specific SMX removal rate was $15.2 \mu\text{g/g SS-d}$, which is comparable to that found in this study. The specific SMX removal rates obtained in the anaerobic SRB granule and floc systems in this study were significantly higher than those reported in the CAS processes ($1.4\text{-}2.2 \mu\text{g/g SS-d}$) (Alvarino *et al.*, 2014, Kang *et al.*,

2018b), and upflow anaerobic sludge blanket (UASB) reactor (methanogenic process, 0.22 µg/g SS-d) (Alvarino *et al.*, 2014), and were comparable to that obtained in aerobic or anoxic membrane bioreactors (MBR) (0.019-48.8 µg/g SS-d) (Clara *et al.*, 2005, Hai *et al.*, 2011, Prasertkulsak *et al.*, 2016).

The difference in SMX removal rates between the SRB sludge system and other biological systems could be attributed to the biodegradation rather than the sorption process. SRB (e. g., *Desulfobulbus*, *Desulfobacter* and *Desulfomicrobium*) are the predominant microbial community in the anaerobic SRUSB reactors (Hao *et al.*, 2013, Jiang *et al.*, 2013, Hao *et al.*, 2015, Zhang *et al.*, 2016), and are significantly different from those in aerobic (e. g., *Nitrosomonas*, *Pseudomonas*, and *Acinetobacter*). (Kang *et al.*, 2018a) and anaerobic (e.g., *Clostridium*) systems treating SMX-laden wastewater. Moreover, SMX has a low octanol-water distribution coefficient ($\log K_{ow} = 0.86$), and is therefore more hydrophilic and is difficult to be sorbed (Chen *et al.*, 2011, Hyland *et al.*, 2012). Jia *et al.* (2017) also reported that only a small fraction of SMX (~9.1%) was sorbed by inactivated anaerobic SRB sludge (flocs) at an initial concentration of 50 µg/L. In another study by Yang *et al.* (2011), 7.2% of SMX was adsorbed by sterilized aerobic activated sludge at an initial concentration of 100 µg/L.

3.3. Fate of SMX in the SRUSB reactors with granules and flocs

Microbial degradation, hydrolysis, volatilization, photodegradation, and sorption are the different possible pathways of organic micro-pollutants removal in activated sludge systems (Grandclement *et al.*, 2017). Hydrolysis and volatilization

can be neglected based on the physical-chemical properties of SMX.

Photodegradation can also be ruled out as the SRUSB reactors were always covered with aluminum foil. Therefore, the main removal pathways in the SRUSB reactors could be via biodegradation and sorption processes.

As shown in Fig. 3, the accumulated SMX in the SRB granules and flocs were 24.2 ± 0.4 mg/kg SS and 19.5 ± 0.4 mg/kg SS, respectively, during the first ten days of SRUSB reactors operation, and both sludges showed decreasing trend and reached nearly stable values after 50 days of operation. At the end of operation (150 days), the SMX concentrations accumulated in the SRUSB reactors with granules and flocs were 12.5 ± 0.3 mg/kg SS and 10.9 ± 0.2 mg/kg SS, respectively. The results apparently showed that SMX biodegradation improved with time as apparent from higher contribution of biodegradation over adsorption to SMX removal during long-term operation of SRUSB reactors. Based on mass balance, 23.8% and 24.1% of the removed SMX were attributed to sorption process in the SRUSB reactors with SRB granules and flocs, respectively, during the first ten days. The contribution of sorption on SMX removal decreased with time, and the contribution of sorption on SMX removal during entire operational period (150 days) was only 0.8% and 0.9% of the removed SMX in the SRUSB reactors with SRB granules and flocs, respectively. The results apparently indicate that biodegradation was the main pathway of SMX removal during the long-term operation. Jia *et al.* (2017) observed that 93% of SMX was biodegraded by SRB flocs at an initial concentration of 50 $\mu\text{g/L}$. In aerobic activated sludge systems, Kang *et al.* (2018b) found that SMX removal by aerobic

granules and CAS was mainly attributed to biodegradation. It should be noted that, although SMX removal was mainly attributed to biodegradation in the SRUSB reactors, sorption also played an important role on SMX removal process (Jia *et al.*, 2017). The authors reported that SMX removal by SRB flocs was characterized by a rapid sorption followed by desorption until reaching equilibrium, and then followed by slow biodegradation.

3.4. Roles of EPS on SMX removal in the SRB sludge system

EPS were extracted periodically from the SRB granules and flocs during long-term operation of SRUSB reactors at different SMX concentrations (Fig. 4). In the SRUSB reactor with granules, the EPS content increased slightly from 106.0 ± 0.7 mg/g VSS on day 0 to 112.8 ± 1.1 mg/g VSS on day 30, and then remained fairly stable during the rest of operation. In the SRUSB reactor with flocs, the EPS content increased significantly from 49.7 ± 0.7 mg/g VSS on day 0 to 68.8 ± 0.3 mg/g VSS on day 30 and reached a plateau. The increase in EPS content could be due to the fact that microbes secrete more EPS to protect against the toxic compounds such as antibiotics (Schmidt *et al.*, 2012, Zhang *et al.*, 2018a). A less increase in EPS content in SRB granules over SRB flocs shows that SRB granules with higher EPS content had a higher tolerance to SMX than SRB flocs. Zhang *et al.* (2018a) also observed higher EPS content in SRB sludge system with increasing ciprofloxacin (CIP) concentration.

The mass ratio of proteins-to-polysaccharides (PN/PS) in EPS is an important factor influencing the adsorption of micro-pollutants as PN/PS ratio can be used as an indicator of hydrophobicity (Xu *et al.*, 2013). Thus, EPS with higher PN/PS

ratio have stronger hydrophobicity and provide more adsorption sites (Xu *et al.*, 2013). As shown in Fig. 4, the PN/PS ratios in the SRB granules were significantly higher than that in the SRB flocs ($p < 0.05$), indicating that the EPS in the SRB granules have a greater capacity to adsorb SMX than that of SRB flocs. This finding was in consistent with the sorption results in which the accumulated SMX concentration in the SRB granules was significantly higher than that in the SRB flocs throughout the operation of SRUSB reactors (Fig. 4).

The 3D-EEM fluorescence spectra of EPS extracted from SRB granules at the end of experiment was analyzed as shown in Fig. 5. Three main components of EPS were identified at excitation/emission: 230/296-305 nm (Peak a), 215-225/355-367 nm (Peak b) and 220/342-349 nm (Peak c). The peaks a, b and c were assigned to the peaks of tryptophan, tyrosine, and aromatic protein-like substances, respectively. The compositions of EPS extracted from SRB flocs were similar to that extracted from SRB granules. Binding experiments between EPS and SMX were also performed to determine which proteins significantly influenced the adsorption of SMX onto EPS (see Supplementary material). The results showed that only the fluorescence intensity of aromatic protein-like substances in both granules and flocs significantly changed with increased SMX concentration indicating that aromatic protein-like substances played an important role on SMX adsorption by EPS. Gu *et al.* (2017) also reported that the fluorescence intensities of protein-like substances of EPS extracted from aerobic biofilms in wastewater treatment systems including aromatic protein-like substances increased with increasing CIP concentration. This study for the first

time demonstrated the role of aromatic protein-like substances of EPS on SMX adsorption by SRB granules.

3.5. Operational parameters influencing SMX removal

3.5.1. pH

The results apparently showed that COD and sulfate removals were stable under different influent pH levels (see Supplementary material), which were also similar to those observed in the long-term operation of SRUSB reactors (Fig. 1). Thus, the SRB activity was not affected at the pH range of 6.0-9.0, which is in consistent with studies by Widdel *et al.* (1983) and Hao *et al.* (2016), who also reported that SRB activity did not change at pH range of 6.0-9.0. However, with decreasing influent pH from 7.0 to 6.0 in the SRUSB reactors, SMX removal gradually declined (Fig. 5a). In detail, SMX removal in the SRUSB reactor with granules significantly decreased from $51\% \pm 2$ at pH 7.0 to $42 \pm 3\%$ at pH 6.0 ($p=0.001$). In the SRUSB reactor with flocs, the SMX removal reduced significantly from $41 \pm 1\%$ at pH 7.0 to $22 \pm 5\%$ at pH 6.0 ($p=0.0003$). When the influent pH was increased to 7.0, the SMX removal in both reactors recovered to the original levels (Fig. 6). When the influent pH was further increased to 9.0 in both SRUSB reactors, the SMX removal remained fairly stable. The results suggest that SRB granules possess higher resilience against varying pH than the SRB flocs.

Although the log D (D: distribution-coefficient) of SMX could be influenced by the varying pH, even with increasing pH from 6.0 to 9.0, the log D of SMX only declined slightly from 0.60 at pH 6.0 to -0.15 at pH 9.0 (see Supplementary material). It apparently shows that the effect of varying pH on SMX adsorption capacity of SRB

sludge was negligible, which is consistent with the SMX adsorbed onto the sludge at different pH levels (see Supplementary material). Therefore, the decrease in SMX removal at low pH levels (6.0 and 6.5) could be mainly attributed to the low biodegradation of SMX by the SRB at pH range of 6.0-6.5. However, the SRB activity in terms of COD removal and sulfate reduction was not affected at the pH range of 6.0-6.5. Thus, some functional genes and enzymes responsible for SMX biodegradation in the SRB were likely inhibited at a low pH, which merits further investigation.

3.5.2. HRT

The SRUSB reactors with granules and flocs removed $49 \pm 2\%$ and $39 \pm 2\%$ of the influent SMX, respectively at an initial concentration of $50 \mu\text{g/L}$ at HRT of 5 h (Fig. 6). Again, SRB granules achieved significantly higher SMX removal than SRB flocs regardless of varying HRTs from 5 to 9 h ($p < 0.001$) (Fig. 6). As for the SRB granules, SMX removal gradually increased to $61 \pm 1\%$ when the HRT was increased to 7 h and then remained nearly stable with further increase in HRT to 9 h. The accumulated SMX in the sludge was similar among the different HRTs ($12.4 \pm 0.2 \text{ mg/kg SS}$) (see Supplementary material), indicating that longer HRT facilitated SMX biodegradation. In addition, COD and sulfate removals remained stable when the HRT was increased from 5 to 9 h. In the SRUSB reactor with flocs, removal of SMX ($41 \pm 2\%$) and the accumulated SMX ($10.2 \pm 0.2 \text{ mg/kg SS}$) in the flocs did not change significantly when the HRT was increased from 5 to 9 h. However, the treatment performance of the SRUSB reactor with flocs showed a decreasing trend in terms of COD removal and

sulfate reduction when the HRT was higher than 7 h (see Supplementary material), indicating that SRB activity decreased when HRT was longer than 7 h. The decrease in SRB activity could be attributed to the increasing toxicity caused by the formation of intermediates from SMX biodegradation (Protocols and results are described in Supplementary material). Notably, SMX removal was expected to decrease when HRT was higher than 7 h owing to the decreased SRB activity. In contrast, the SMX removal during these conditions remained fairly stable. It may be due to the fact that a longer HRT allowed a longer reaction time, which has likely overcome the negative effect of decreased SRB activity on SMX removal to some extent. The result also demonstrates that SRB granules had a higher resilience against the variations of HRT than the SRB flocs.

3.6. Practical implications

Previous studies reported that CAS and anaerobic (methanogenic) processes as well as MBR could remove SMX from wastewater (Clara *et al.*, 2005, Hai *et al.*, 2011, Alvarino *et al.*, 2014, Alvarino *et al.*, 2016, Prasertkulsak *et al.*, 2016, Kang *et al.*, 2018b). According to the discussion in Section 3.2, the SMX removal rates in these processes were significantly lower than that obtained in SRB-mediated process especially in the SRB granular sludge system except for MBR. Although MBR can achieve similar or higher SMX removal rate than the SRB-mediated process, the capital and operational costs of MBR systems are generally higher due to costs associated with membrane fouling control and frequent replacement of membrane (Grandclement *et al.*, 2017). SRB-mediated process can also effectively remove COD

from wastewater with lower energy consumption and less sludge production compared to CAS process (Lu *et al.*, 2012, Wu *et al.*, 2016). Further studies on assessing the extended application of SRB-mediated process for removing other antibiotics, such as isoxazole-containing compounds should also be conducted. Additionally, SRB-mediated process could produce high amount of sulfide, an odorous gas, which can cause corrosion of metals and concrete. Additional processes may be needed to remove or convert the formed sulfide into benign form such as elemental sulfur or sulfate (van den Bosch *et al.*, 2007, Zhang *et al.*, 2018c).

4. Conclusions

This study investigated and compared SMX removal by SRB granules and flocs in SRUSB reactors under the same operational conditions for 150 days. SMX removal by SRB granules (13.3 µg/g SS-d) was significantly higher than that by SRB flocs (11.2 µg/g SS-d) ($p < 0.05$). SMX had no significant effect on COD and sulfate removals in the two reactors. Biodegradation was the main pathway for SMX removal in the SRB sludge system. SRB granules had a better resilience against varying pHs and HRTs than SRB flocs. Collectively, SRB granular sludge system could be employed to efficiently remove SMX from various SMX-laden wastewaters.

Appendix A: Supplementary Information

Supplementary information can be found in the online version.

Acknowledgment

We gratefully acknowledge the financial supports from the Natural Science Foundation of China (No. 51638005 and 51778643) and the Tip-top Scientific and Technical Innovative Youth Talents of Guangdong Special Support Program (No. 2016TQ03Z336).

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