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Removal and biotransformation pathway of antibiotic Sulfamethoxazole from municipal wastewater treatment by anaerobic membrane bioreactor

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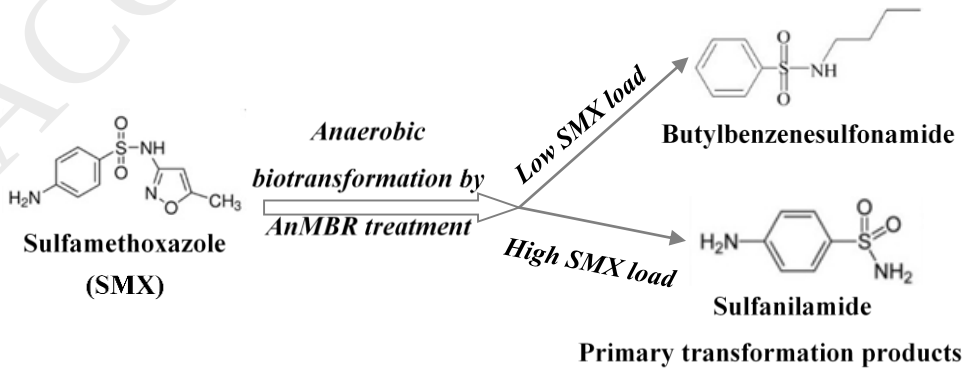
Graphical Abstracts



Highlights

- Good SMX removal was achieved by AnMBR under variable feed SMX of 10-100000 μg/L.
- SMX biotransformation followed pseudo-first order reaction kinetics in AnMBR.
- SMX biotransformation pathway was proposed based on 7 TPs identified by GC-MS.
- Non-antibiotic Butylbenzenesulfonamide was the dominant TP under low SMX load.
- Sulfanilamide with low antibiotic toxicity was the dominant TP under high SMX load.

Abstract: A lab-scale mesophilic anaerobic membrane bioreactor (AnMBR) was used to treat synthetic municipal wastewater with variable concentrations of antibiotic Sulfamethoxazole (SMX) and bulk organics in this study. The removal and biotransformation pathway of SMX in the AnMBR were systematically investigated during a 170 d of operation under hydraulic retention time of 1 d. Average SMX removal was 97.1% under feed SMX of 10-1000 μg/L, decreasing to 91.6 and 88.0% under feed SMX of 10000 and 100000 μg/L due to the inhibition effects of high SMX loading rate on anaerobic microorganisms. SMX biotransformation followed pseudo-first order reaction kinetics based on SMX removal independent of feed SMX of 10-1000 μg/L during continuous operation and also in a batch test under initial SMX of 100000 μg/L. According to the identified 7 transformation products (TPs) by gas chromatography-mass spectrometry, the biotransformation pathway of SMX from municipal wastewater treatment via AnMBR was first proposed to consist of 2 primary

routes: 1) Butylbenzenesulfonamide without antibiotic toxicity dominated under feed SMX of 10-100 μg/L; 2) Sulfanilamide with much lower antibiotic toxicity than SMX dominated under feed SMX of 1000-100000 μg/L, further transforming to secondary TPs (4-Aminothiophenol, Aniline, Acetylsulfanilamide) and tertiary TPs (4-Acetylaminothiophenol, Acetylaniline).

Keywords: Anaerobic membrane bioreactor; Biotransformation pathway; Municipal wastewater; Sulfamethoxazole; Transformation product

1. Introduction

Organic micro-pollutants (OMPs) (e.g., pharmaceutically active compounds, personal care products) have been detected globally in aquatic environments and received increasing attention in recent years due to their potentially harmful effects on aquatic ecosystems and public health [1-4]. Among them, antibiotics have raised more concerns due to their direct toxicity to aquatic organisms as well as the potential development of antibiotic resistance genes and/or species [5]. Wastewater treatment plants (WWTPs) have been identified as a key point source for OMPs release into receiving water bodies [1, 6]. As a widely used antibiotic sulfonamide for both human and veterinary medicines, Sulfamethoxazole (SMX) (chemical abstracts service (CAS) number of 723-46-6) has been frequently detected in raw municipal wastewater at the concentration of ng/L to few tens μg/L [7, 8] and even up to mg/L in single household wastewater [9], in the effluent of WWTPs at the concentration of ng/L to μg/L [6-8], and even in surface water receiving WWTPs effluent at the concentration of ng/L [1-3]. Thus, eliminating SMX in WWTPs is an important solution to control its discharge to the environment.

SMX is mainly removed via biotransformation in WWTPs due to its negatively charged nature and low sorption capacity under neutral pH [10, 11]. The aerobic-based activated sludge processes show a large variation in SMX removal depending on operating conditions (e.g., hydraulic/sludge retention time (HRT/SRT), temperature) with studies reporting from nearly 0% [12], less than 30% [13], 30%-60% [14], 60%-90% [15], more than 90% [16] and even complete removal [12]. In comparison, the anaerobic-based processes like upflow anaerobic sludge blanket [17, 18], anaerobic sequential batch reactor [19, 20] and anaerobic immobilized reactor [21] are reported to show a consistent high removal (over 80%). The anaerobic membrane bioreactor (AnMBR), coupling anaerobic digestion and micro/ultra-filtration (MF/UF) separation, can not only achieve high removal of bulk organics in terms of chemical oxygen demand (COD) and high methane production as a recovered energy source [22], but also achieve a consistently high SMX removal (over 90%) during municipal wastewater treatment [23-26]. Therefore, AnMBR appears to be a promising technology treating municipal wastewater containing SMX.

Based on the relatively complex molecular structure of SMX, the occurrence of some transformation products (TPs) in WWTPs would be expected. Some TPs (e.g., 4-nitro-SMX (CAS 29699-89-6) and 4-hydroxy-SMX (CAS 141233-20-7)) may pose higher toxicity to aquatic organisms than SMX itself [27]. Thus, it is crucial to investigate TPs formation during SMX removal in WWTPs. Only one recent study [28] has reported the detection of two TPs (i.e., 4-nitro-SMX and desamino-SMX (CAS 13053-79-7)) in the effluent of full-scale WWTPs. Other related studies have mostly been based on lab-scale aerobic batch tests using pure cultures [29], activated sludge [30], enriched ammonia oxidizing bacteria [31], or municipal wastewater effluent [32] as inoculum,

and applying SMX as the sole or main substrate with an initial concentration from 100 µg/L to 200 mg/L. Two studies have been found on SMX transformation using anaerobic batch tests. A long-term study [33] using a microbial fuel cell seeded with anaerobic digester sludge and fed with SMX at 20-200 mg/L as the main carbon source in batch mode, reported the detection of 3 TPs: 4-aminobenzenesulfinic acid (CAS 7472-15-3), 3-amino-5-methyl-isoxazole (CAS 1072-67-9) and 4-amino-2-butanol (CAS 39884-48-5). Another anaerobic sludge batch test using skimmed milk as a primary substrate and spiked with an initial ^{14}C- labelled SMX of 271 µg/L also observed the breakdown of the isoxazole ring of SMX [34]. However, there is no information available so far on TPs formation and biotransformation pathway of SMX in AnMBR in the literature.

The objective of this study was to investigate the removal, TPs and biotransformation pathway of SMX from synthetic municipal wastewater treatment by a lab-scale mesophilic AnMBR. A broad range of feed SMX from 10 to 100000 µg/L and variable feed COD from 810 to 3330 mg/L were tested under an HRT of 1 d during a 170 d continuous AnMBR operation. The removal of SMX and COD as well as TPs identification of SMX were intensively investigated during the AnMBR operation. The biotransformation pathway of SMX in AnMBR was first proposed based on the identified TPs. A batch test at initial SMX of 100000 µg/L was also conducted to explore SMX removal kinetics by anaerobic microorganisms.

2. Materials and methods

2.1. AnMBR set-up

The lab-scale AnMBR set-up (shown in Fig. S1) consisted of a completely mixed anaerobic activated sludge bioreactor (effective volume of 2 L, temperature of 35±1 °C, pH of 7±0.1, stirrer speed of 200±2 rpm), and a side-stream biogas-sludge diphase crossflow hollow fiber UF membrane module (polyvinylidene fluoride, nominal pore size of 0.03 µm, filtration area of 310 cm^2). The UF membrane was operated in intermittent permeating mode (on/off: 9/1 min) with a flux of 3 $L/m^2/h$ to maintain an HRT of 1 d for the AnMBR system. Details of the AnMBR set-up can be found in a previous study [22].

2.2. Feed preparation

Synthetic municipal wastewater with a COD of 800 mg/L was based on a recipe (shown in Table 1). A 10 times concentrated stock was prepared weekly using tap water, stored in a refrigerator (4 °C) and diluted for daily use. Through dissolving SMX powder (purity > 99.9 %, Fluka) into methanol (MeOH), two concentrated SMX stocks (1 and 50 g/L) were prepared and stored under 4 °C for daily spiking into the synthetic municipal wastewater at low concentrations (10, 100, 1000 µg/L) to simulate the upper limit of real SMX concentrations detected in raw municipal wastewater, and at high concentrations (10000, 100000 µg/L) to simulate accidental intrusion of SMX production wastewater, respectively.

2.3. Operational phases

The AnMBR operation was classified into 8 Phases depending on feed SMX (shown in Table 3). In Phases 1-3 with low feed SMX of 10-1000 µg/L, the focus was on SMX removal while exploring detection methods of SMX TPs. The experiments were repeated in Phases 4-6, with a focus on SMX TPs. For high feed SMX of 10000-100000 µg/L, both SMX removal and TPs were investigated in Phases 7-8. Between Phases 3-4

and 7-8, a 7 d (Day 55-61) and 10 d (Day 130-139) of operation using normal synthetic municipal wastewater (COD 800 mg/L from 10 times dilution of concentrated wastewater stock) and concentrated synthetic municipal wastewater (COD 2670 mg/L from 3 times dilution of concentrated wastewater stock) without SMX spiking, respectively, was applied to remove/wash out residual SMX and its TPs in the AnMBR. Before Phase 8, a batch test to investigate SMX removal kinetics was conducted (details shown in Section 2.4). No sludge was wasted except twice weekly sampling (10 ml per sampling) for biomass measurement during the whole operation, resulting in a very long SRT of 700 d in the AnMBR. Both SMX and anaerobic sludge were negatively charged at neutral pH in this study, making SMX sorption onto sludge difficult due to the electrostatic repulsion. Combing the long SRT and low sorption capacity, SMX removal via sorption could be neglected in this study. No SMX rejection by UF membrane was verified at the end of batch test, where both filtrate of 0.03 μm UF membrane and 0.45 μm MF membrane contained the same SMX of 5.3 mg/L. Therefore, SMX removal would be mainly from biotransformation in the AnMBR in this study. SMX and its TPs were measured via pre-concentrating using solid-phase extraction (SPE) and evaporation followed by liquid chromatography-mass spectrometry (LC-MS) and gas chromatography-mass spectrometry (GC-MS), respectively.

2.4. Batch test

The batch test was conducted on Day 140, which started with a direct injection of 4 ml concentrated SMX stock of 50 g/L in MeOH into the bioreactor (i.e., the target initial SMX of 100000 μg/L) followed by sludge sampling (50 ml per sampling) at different reaction times (0, 1, 2, 3, 6, 12, 18 and 24 h). The sludge sample was first centrifuged under 15000 g and 20 °C for 15 minutes and then the collected supernatant was filtered

through a 0.45 µm MF glass fiber syringe filter (Whatman) to produce a final supernatant sample around 40 ml, which was stored at 4 °C for both SMX and COD analysis. The 10 d AnMBR operation using concentrated synthetic municipal wastewater before the batch test was also aimed to assimilate sludge to the initial high COD (target 2530 mg/L mainly from MeOH dissolving SMX) during the batch test, which resulted in a high mixed liquor volatile suspended solids of 7.6 g/L at the time of the batch test.

2.5. SMX quantification

The collected sample was first spiked with 100 µl isotope SMX-d4 (Toronto Research Chemicals) of 1 mg/L in MeOH serving as the internal reference for measuring SMX recovery during the following SPE-evaporation concentrating. The sample was then processed via a Dionex Autotrace 280 SPE instrument (Thermo Scientific) using an Oasis hydrophilic/hydrophobic balanced HLB SPE cartridge filter (Waters) in the sequence of conditioning, loading, washing, drying and eluting to produce first elute of 5 ml using MeOH and second elute of 5 ml using 10% MeOH in methyl tert-butyl ether (MTBE). The 10 ml elute was further concentrated to 1 ml final sample by evaporation under 40 °C and nitrogen gas stripping. The final 1 ml sample was quantified through an LC-MS system consisting of an Agilent Technology 1260 Infinity LC unit and an Applied Biosystems Sciex Qtrap 5500 MS unit. The LC unit was equipped with a Phenomenex Luna 5u C18 column, where a positive mobile phase composed of 0.1 % (v/v) formic acid in water and 0.1 % (v/v) formic acid in MeOH was used. The MS detection was done through a multiple reaction monitoring acquisition with positive electrospray ionization (EI) mode. The *m/z* of parent/precursor for SMX and SMX-d4 was 254/156 and 258/160, respectively. Coupling SPE-evaporation and

SMX-d4 recovery control (recovery rate of 80-95%), the SMX detection limit was determined to be 1 ng/L in this study.

2.6. SMX TPs identification

AnMBR permeate (1-12 L) was first processed via the SPE methodology using different eluting solvents – dichloromethane (DCM), ethyl acetate (EA), acetone (ACE), MeOH, MTBE, MeOH followed by 10 % MeOH in MTBE – and the lowest (according to the capacity of the SPE instrument) loading rate of 1 ml/min and eluting rate of 0.6 ml/min for maximizing TPs recovery in the produced 10 ml elute. Then, this 10 ml elute was further concentrated via the evaporation to a final sample of 0.1-0.5 ml, indicating a maximum concentrating factor of up to 120k. The supernatant (30 ml) from the batch test was pre-concentrated via the SPE methodology using ACE as the eluting solvent and the lowest loading and eluting rate to produce 10 ml elute, which was further concentrated via the evaporation to a final 0.1 ml sample. The final concentrated sample was measured via a GC-MS system consisting of an Agilent Technology 7890A GC unit and an Agilent Technology 5975C MS unit. The GC unit was equipped with an Agilent J&W HP-5ms capillary column, where helium was used as carrier gas. The GC oven program started at an initial temperature of 60 °C for 3 min, and ramped up to 320 °C with a heating rate of 20 °C/min and remained at 320 °C for 5 min. The MS detection was conducted via a full scan acquisition (scanning *m/z* of 25-300) under EI mode (standard energy source of 70 eV). The main peaks of total ion chromatogram (TIC) of samples from GC-MS were identified by comparing the mass spectra of samples to standard compounds in the NIST (National Institute of Standards and Technology, USA) mass spectral library. Only compounds with a matching probability over 60 % and repetitively detected under different eluting solvents could be identified

as candidate TPs, which were finally confirmed as SMX TPs via comparing the retention times and mass spectra of candidate TPs with those of pure compounds under the same GC-MS conditions.

2.7. Routine analysis

COD measurement was done by commercial COD kits (Hach) based on the method of rapid digestion (150 °C, 2 h) in a digital heater followed by colorimetric measurement using a spectrometer. Biogas composition and volume (reported at a temperature of 25 °C and pressure of 1 atm) were measured according to the gas bag method using GC (310C, SRI Instruments) [22]. Biomass concentration in terms of mixed liquor (volatile) suspended solids (ML(V)SS) was measured according to the standard method of 0.7 μm glass fiber (Whatman) filtration followed by sequential drying at 105 °C and 550 °C.

3. Results and discussion

3.1. Bulk organics removal

Summarized results of bulk COD removal, methane production and biomass concentration are listed in Table 2 (the detailed operational data with time shown in Figs. S2-S5). The feed COD increased from 810 to 3330 mg/L with feed SMX increase from 10 to 100000 μg/L, which was mainly due to the contribution from MeOH (1 ml MeOH in 1 L feed equivalent to COD of 1200 mg/L) applied to dissolve the concentrated SMX as well as a slight contribution from SMX itself (1 mg SMX equivalent to COD of 1.32 mg). During Phases 1-3 and 4-6, where feed COD increased from 810 to 2000 mg/L while increasing feed SMX from 10 to 1000 μg/L, average permeate COD was below 50 mg/L with average COD removal over 96%. This indicated a good removal of bulk

organics while no inhibition effects of varying feed SMX concentrations on anaerobic degradation was observed. However, during Phases 7 and 8, where feed SMX further increased to 10000 and 100000 µg/L (corresponding feed COD of 1050 and 3330 mg/L), average permeate COD increased to 60.8 and 495.7 mg/L with average COD removal decreasing to 94.2 and 86.2%, respectively, implying the significant drop of bulk organics removal may be caused by inhibition effects at high feed SMX concentrations. Two studies [19, 20] using anaerobic sequential batch reactors for synthetic pharmaceutical wastewater treatment also reported the significant inhibition effects of high SMX concentrations (over 15 mg/L) on bulk COD removal, methane production and SMX removal, and even total collapse of the reactor at feed SMX over 40 mg/L.

Biogas production was observed to have a relatively stable biogas volumetric composition (methane 75-95 %, carbon dioxide < 10 % and nitrogen < 20 %) during the whole operation. During Phases 1-3 and 4-6, average methane production increased from 81.3 to 208.5 and 85.6 to 244.1 ml CH_4/g $COD_{removed}$, respectively, indicating enhanced methane production with increasing bulk organics loading rate, which was in agreement with a previous AnMBR study [22]. Similar to COD removal during Phases 7 and 8, average methane production decreased to 82.6 and 67.5 ml CH_4/g $COD_{removed}$, respectively. This response also confirmed the inhibition effects on methanogenic activity at high SMX concentrations.

Biomass characteristics were found to be relatively stable in terms of sludge composition, expressed as MLVSS/MLSS and resulting in 0.81-0.86 during Phases 1-6 followed by a decrease to 0.79 and 0.72 in Phases 7 and 8, respectively. The decrease in Phases 7 and 8 might reflect the inhibition effects of high SMX concentrations on the

anaerobic microorganisms. MLVSS showed a decreasing trend in Phases 1 and 4 (feed COD of 810 mg/L), a roughly constant value in Phases 2 and 5 (feed COD of 920 mg/L), and an increase in Phases 3 and 6 (feed COD of 2000 mg/L), suggesting that a corresponding sludge organic loading rate of around 0.33 gCOD/gMLVSS/d in Phases 2 and 5 might be regarded as the maintenance energy resulting in zero biomass production. This is also in agreement with the previous AnMBR study [22]. In addition, similar results (e.g., COD removal, methane production, biomass characteristics) were obtained in phases with the same feed (i.e., Phases 1 and 4, Phases 2 and 5, Phases 3 and 6), showing a good reproducibility in this study.

3.2. SMX removal

Results on SMX removal are summarized in Table 3. During Phases 1-3 and 4-6, with a large range of feed SMX (10 to 1000 μg/L) and variable bulk COD (810 to 2000 mg/L), SMX removal was consistently over 96 %, indicating a very good SMX removal capacity by the AnMBR. Considering the dominant co-metabolism mechanism for SMX removal in municipal wastewater treatment systems (mainly due to its very low concentration compared to bulk organics in raw wastewater), it could be deducted that AnMBR can still achieve high SMX removal (no less than the current 96%) for municipal wastewater containing trace SMX concentrations (e.g., ng/L or sub-μg/L). Similar to COD removal, reproducible SMX removal was observed in phases with the same feed (i.e., Phases 1 and 4, Phases 2 and 5, Phases 3 and 6). Average SMX removal for Phases 1-6 was 97.1±0.2 %, operated with the same HRT (1 d) but varying feed SMX concentrations (10, 100, and 1000 μg/L), indicating that SMX removal was independent of feed concentration (for the range applied). Only the first order reaction kinetics (shown below) can result in this pattern:

$$dC/dt = -k_f \cdot C,\ \ln(C/C_0) = -k_f \cdot t,\ C = C_0 \cdot \exp(-k_f \cdot t),\ t_{1/2} = \ln(2)/k_f$$

where C is SMX concentration at reaction time t [µg/L], C_0 is initial SMX concentration at reaction time of 0 d (i.e., feed SMX concentration) [µg/L], k_f is first order rate constant [1/d], t is reaction time [d], and $t_{1/2}$ is half-life [d].

SMX removal was equal to 100(1- C_{1d}/C_0) in this study, where C_{1d} was equivalent to SMX concentration at reaction time of 1 d (i.e., SMX concentration in AnMBR permeate). For Phases 1-6 with the different C_0 (10, 100, and 1000 µg/L), C_{1d}/C_0 was calculated as a constant of 0.029±0.002, indicating SMX removal followed the first order reaction kinetics with an average k_f of 3.53 1/d and $t_{1/2}$ of 0.20 d. This calculation was just derived from only 1 data pair of (C_{1d}/C_0, 1 d) from continuous AnMBR operation and thus did not produce correlation coefficient for linear correlation between $\ln(C/C_0)$ and t or exponential correlation between C/C_0 and t, which was correlated from 7 data pairs of (C/C_0, t) in the batch test (shown in Section 3.5).

Considering that the biomass concentration (i.e., MLVSS of 2.5-7.6 g/L) was far more than SMX concentration (10-100000 µg/L), the mechanism can be described by a pseudo-first order reaction kinetics ($dC/dt = -k_{pf} \cdot MLVSS \cdot C$, $\ln(C/C_0) = -k_{pf} \cdot MLVSS \cdot t$ and $t_{1/2} = \ln(2)/(k_{pf} \cdot MLVSS)$; where k_{pf} is pseudo-first order rate constant (i.e., k_f normalized with respect to MLVSS, also known as biological kinetic constant) [L/gMLVSS/d]). The same approach has been used to describe the biological removal of OMPs, including SMX, in activated sludge systems [9, 34].

The SMX reaction rate constants for both first order and pseudo-first order reaction kinetics, and half-life, are also shown in Table 3. For Phases 1-6, with feed SMX of 10-1000 µg/L, both k_f and $t_{1/2}$ were in the narrow range of 3.53±0.07 1/d and 0.20±0.004 d, respectively, which matched first order reaction kinetics well. For Phases 7 and 8, with

feed SMX of 10000 µg/L and 100000 µg/L, SMX removal showed a significant decrease to 91.6±0.7 % and 88.0±3.0 %, respectively; k_f decreased to 2.48 and 2.12 1/d, respectively; $t_{1/2}$ increased to 0.28 and 0.33 d, respectively. Compared to Phases 1-6 with over 96% SMX removal, this was attributed to the inhibition effects of high SMX concentrations on anaerobic microorganisms as observed in cases of the above-mentioned bulk COD removal and methane production, and in agreement with findings from the studies on anaerobic sequential batch reactors [19, 20]. As for k_{pf}, a decreasing trend with increasing MLVSS can be found from Tables 2 and 3. Compared with Phases 1-3, there was a slightly lower k_f and a higher average MLVSS in Phases 4-6 with the same feed conditions, thus resulting in a lower k_{pf} (1.05 vs 1.22 L/gMLVSS/d, 1.13 vs 1.39 L/gMLVSS/d, 0.94 vs 1.22 L/gMLVSS/d) in Phases 4-6 than Phases 1-3, respectively. This might imply the occurrence of chronic weak inhibition effects of low SMX loading rate (3.1-331.7 µgSMX/gMLVSS/d) on SMX removal capacity by anaerobic microorganisms. Compared with Phases 1-6, the significant inhibition effects of high SMX loading rate (2328.3-13541.0 µgSMX/gMLVSS/d) on anaerobic microorganisms in Phases 7 and 8 resulted in a decreased k_{pf} to 0.58 and 0.29 L/gMLVSS/d, respectively.

It should be noted that the $t_{1/2}$ of 0.19-0.33 d for SMX removal obtained in this study is much lower than the 1-3 d reported in a microbial full cell fed with SMX of 20-200 mg/L [33], while the k_{pf} of 0.29-1.39 L/gMLVSS/d obtained in this study is significantly higher than the 0.2 L/gMLVSS/d from an aerobic MBR treating single-house wastewater [9], and the 0.01-0.1 L/gMLVSS/d from batch tests using different biomass [34]. Results from this study indicate a superior SMX removal capacity in the AnMBR.

3.3. SMX TPs identification

A total of 7 TPs (shown in Table 4) were identified via GC-MS analysis (shown in Figs. S6-S19) of permeate samples during AnMBR operation. In Phases 1 and 4 with a feed SMX of 10 μg/L (i.e., the normal maximum level detected in centralized municipal WWTPs), only Butylbenzenesulfonamide (CAS 3622-84-2, marked as TP 1) was detected under all tested SPE eluting solvents and a large range of the SPE-evaporation concentrating factor from 20k to 100k using EA elution and from 1k to 120k using MeOH followed by 10% MeOH in MTBE elution. Compared to the parent SMX, Butylbenzenesulfonamide as a biotransformation product does not contain the antibiotic sulfonamide group, indicating that even through the AnMBR treatment did not achieve complete mineralization of SMX it still produced a permeate free of antibiotic functions for a feed SMX of 10 μg/L. In Phases 2 and 5 with a feed SMX of 100 μg/L, Sulfanilamide (CAS 63-74-1, marked as TP 2) being an antibiotic sulfonamide, but showing much lower antibiotic toxicity than SMX [27], was detected besides TP 1. In Phases 3 and 6 with a feed SMX of 1000 μg/L, 4-Aminothiophenol (CAS 1193-02-8, marked as TP 3) and Aniline (CAS 62-53-3, marked as TP 4), without antibiotic functions, were detected besides TPs 1-2. In Phases 7 and 8 with feed SMX of 10000 and 100000 μg/L, Acetylsulfanilamide (CAS 121-61-9, marked as TP 5) being an antibiotic sulfonamide, 4-Acetylaminothiophenol (CAS 1126-81-4, marked as TP 6) and Acetylaniline (CAS number 103-84-4, marked as TP 7) without antibiotic functions, were detected besides TPs 1-4. The sequential detection of TP 1, TP 2, TPs 3-4 and TPs 5-7 under feed SMX of 10, 100, 1000 and 10000 μg/L might depend on the corresponding average SMX loading rate to biomass of 3.3, 35, 288 and 2328 μgSMX/gMLVSS/d, resulting in different metabolic responses of anaerobic microorganisms. In addition, the mass spectrum of all TPs was well matched (more than

95% similarity) with that of pure compounds, while the GC elution time of most TPs was kept constant and very close to that of pure compounds among different samples except TPs 2 and 5, which were delayed mainly due to the increased peak intensity of TP 2 with increased feed SMX concentration.

Unlike the aerobic-based studies [28-32, 35], where several SMX derivatives have been commonly detected as TPs, most TPs detected in this study are the break-down products of SMX. Among the detected TPs of SMX in this study, Sulfanilamide, 4-Aminothiophenol and Aniline have been detected in batch tests using an aerobic cold-adapted bacterium *Pseudomonas psychrophila* HA-4 for SMX removal [36]. Another 4 compounds have been first reported as SMX TPs from this study. Interestingly, 3-Amino-5-methylisoxazole as a stable hydrolyzed product of SMX has been reported in a few aerobic-based batch studies for SMX biotransformation using a bacterium *Microbacterium sp.* strain BR1 [37], an activated sludge inoculum [30], a cold-adapted bacterium *Pseudomonas psychrophila* HA-4 [36] and a bacterium *Achromobacter denitrificans* PR1 [38]. However, the 5-methylisoxazole group of SMX would either directly or indirectly (after hydrolysis) accept protons to form butane (shown in Fig. 1 below) in this study. This could be due to that the anaerobic microorganisms used in this study were totally different from the aerobic microorganisms in these aerobic-based studies. The break-down of 5-methylisoxazole group of SMX has been observed in a microbial fuel cell [33] seeded anaerobic digester sludge and in a batch test [34] using anaerobic sludge.

3.4. SMX biotransformation pathway

Based on the molecular structure and detection sequence of TPs in this study, the biotransformation pathway of SMX during AnMBR treatment is proposed in Fig. 1, and

identified by 2 primary routes. The first route involves the amine and 5-methylisoxazole groups of SMX accepting protons to form Butylbenzenesulfonamide (i.e., the first primary TP), which could be further removed based on the observation in Phase 2 where its peak area (i.e., concentration) in 1k concentrated permeate samples using MeOH followed by 10% MeOH in MTBE as SPE eluting solvents decreased significantly with operation time (shown in Fig. S20). The first route was activated under the lowest feed SMX of 10 μg/L and was consistently detected under all conditions tested in this study. The second route involves the sulfonamide group of SMX accepting a proton to form Sulfanilamide (i.e., the second primary TP), which is further biotransformed to 4-Aminothiophenol (i.e., the first secondary TP) via deamination and/or to Aniline (i.e., the second secondary TP) via simultaneous deamination and desulfurization. There is also a possible biotransformation path from 4-Aminothiophenol to Aniline via desulfurization. Sulfanilamide could be further biotransformed to Acetylsulfanilamide (i.e., the third secondary TP) via acetylation. In addition, 4-Aminothiophenol and Aniline could be further biotransformed to 4-Acetylaminothiophenol (i.e., the first tertiary TP) and Acetylaniline (i.e., the second tertiary TP) via acetylation, respectively. Aniline and Acetylaniline might be finally mineralized based on their simple molecular structures [36]. The second route was activated under a feed SMX of 100 μg/L in this study. In addition, based on the biotransformation pathway of the main TPs in the AnMBR, it could be deducted that the amine group of SMX transformed into ammonia; 5-methylisoxazole group of SMX transformed into butane, ammonia and water; sulfonamide group of SMX transformed into hydrogen sulfide, ammonia and water.

According to the changes of peak area of individual TPs in the AnMBR permeate samples eluted by the same solvent during Phases 4-8 (feed SMX of 10-100000 μg/L), it is possible to show the relative contribution of the different biotransformation routes in the AnMBR (shown in Fig. 2). TP 1 is the only TP under a feed SMX of 10 μg/L, and is also the dominant TP under a feed SMX of 100 μg/L. The peak area first increases with increasing feed SMX from 10 to 1000 μg/L, then remains stable as increasing feed SMX further from 1000 to 10000 μg/L, and finally decreases with increasing feed SMX from 10000 to 100000 μg/L. This indicates that the contribution of SMX removed via the primary TP 1 route significantly decreases with increasing feed SMX, and plays a minor role under a high feed SMX of 1000-100000 μg/L. The peak area of TP 2 increases 162 times from a feed SMX of 100 to 1000 μg/L (much higher than 2 times increase of TP 1), implying a dominant SMX biotransformation via the primary TP 2 route under a feed SMX of 1000 μg/L. The changes of SMX biotransformation routes with increasing feed SMX could be related to the metabolic response of the anaerobic microorganisms with increasing SMX loading rates. For the secondary and tertiary TPs, the individual peak area increases with the peak area of the corresponding parent TPs (i.e., TP 2 as parent of TPs 3-5, TP 3 as parent of TP 6, TP 4 as parent of TP 7) as well as feed SMX.

3.5. SMX removal and TPs formation in batch test

Both SMX and COD concentration as well as their removals with time during the batch test are shown in Fig. 3. The initial SMX was 105.2 mg/L, close to the target 100 mg/L. The plot of SMX vs reaction time was well fitted (correlation coefficient R^2 of 0.9845) for first order reaction kinetics with a half-life of 0.24 d. The reaction rate constants for first order and pseudo-first order kinetics were 2.90 1/d and 0.38

L/gMLVSS/d, respectively, which were higher than the corresponding values in Phase 8 (continuous AnMBR operation with a feed SMX of 100 mg/L, shown in Table 2). SMX removal after 24 h reaction was 95.0%, also higher than in Phase 8. The difference in results obtained from the batch test and the long-term continuous operation in Phase 8, was attributed to no chronic inhibition effects on the anaerobic microorganisms during the 24 h batch test. The plot of COD vs reaction time was also well fitted (correlation coefficient R^2 of 0.9811) for first order reaction kinetics with a half-life of 0.59 d and reaction rate constants for first order and pseudo-first order kinetics of 1.18 1/d and 0.15 L/gMLVSS/d respectively. COD removal after 24 h reaction was 71.5%, lower than in Phase 8, which could be attributed to a transient COD shock from previous feed COD of 2670 mg/L versus the initial COD of 3018 mg/L in the batch test.

Due to the supernatant sample volume limitation for TPs detection (30 ml sample concentrated to the minimum 0.1 ml via SPE-evaporation, thus only 300 times concentrated), only TPs 1-4 were detected by GC-MS analysis during the batch test (shown in Fig. S21). The peak area changes of TPs with reaction time could provide some insights into TPs formation during the batch test (shown in Fig. 4). In general, the primary TP 2 route (including secondary TPs 3-4) was the dominant SMX biotransformation pathway and the primary TP 1 route played a minor role. This was in agreement with results from continuous operation with feed SMX of 100 mg/L in Phase 8. TPs 1-4 were clearly detected in the first supernatant sample of time 0 h (shown in Fig. S21), which was just taken after 2-3 minutes of mixing the injected SMX stock into the bioreactor. This could imply that SMX biotransformation reaction occurred very quickly once SMX reached to anaerobic microorganisms and the primary TP 1 and TP 2 routes occurred simultaneously. This was probably due to the well-assimilated

anaerobic microorganisms to SMX prior to the batch test and high SMX loading rate (13842 μgSMX/gMLVSS/d) in the batch test, respectively. The peak area of TPs 1-4 first increased with reaction time, up to maximum value around 9 h, then decreased sharply until 12 h followed by a roughly stable level to the end of 24 h (i.e., reaching steady state after 12 h of reaction).

4. Conclusions

Good removal (97%) for both SMX and COD was achieved by AnMBR under feed SMX of 10-1000 μg/L (covering normal municipal wastewater), indicating AnMBR as a promising way to treat municipal wastewater containing SMX effectively. SMX biotransformation followed pseudo-first order reaction kinetics with reaction rate constant of 0.29-1.39 L/gMLVSS/d based on both continuous operation and batch test. Under low SMX loading rate (< 35 μgSMX/gMLVSS/d), Butylbenzenesulfonamide without antibiotic functions was the dominant TP. Sulfanilamide with much lower antibiotic toxicity than SMX was the dominant TP under high SMX loading rate (up to 13541 μgSMX/gMLVSS/d).

Acknowledgments

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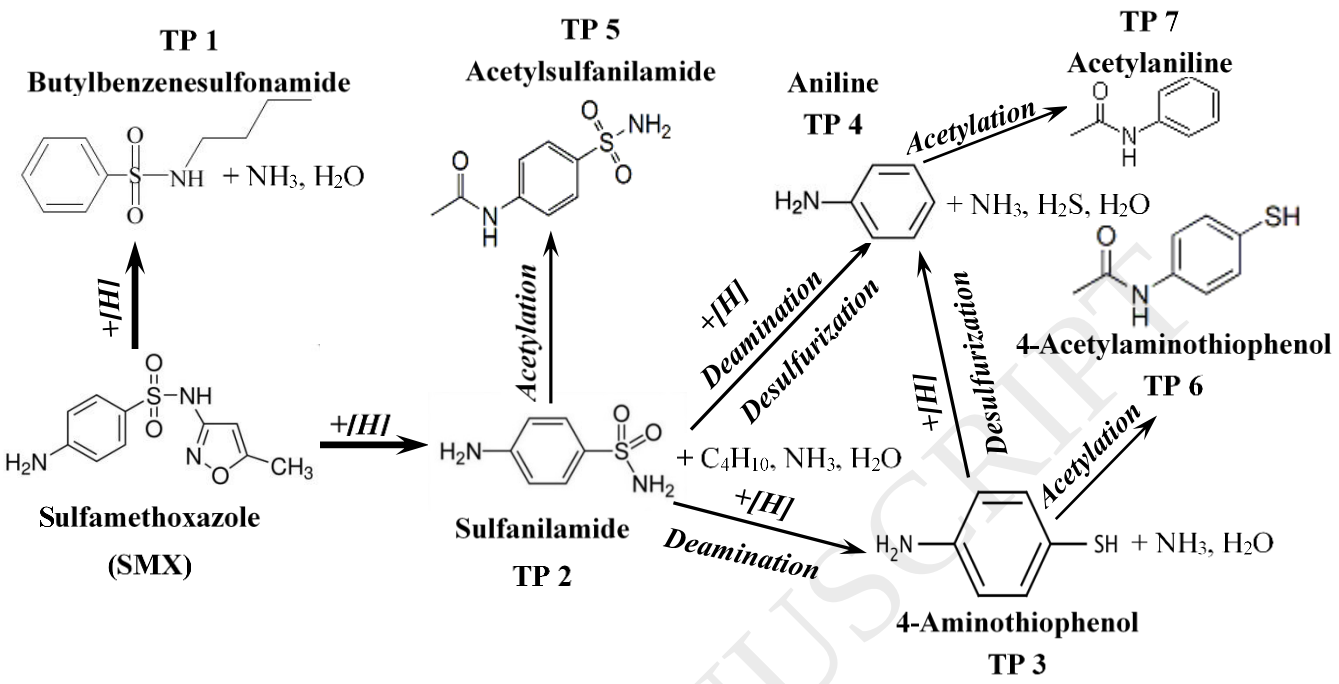
Figure captions

Fig. 1. Proposed biotransformation pathway of SMX in the AnMBR.

Fig. 2. TPs detection and their peak intensity from GC-MS in the AnMBR permeate (20000 times concentrated via ACE elution) under different feed SMX.

Fig. 3. SMX and COD removal kinetics in the batch test.

Fig. 4. TPs detection in the supernatant (300 times concentrated via ACE elution) from GC-MS and their peak intensity changes with reaction time in the batch test.



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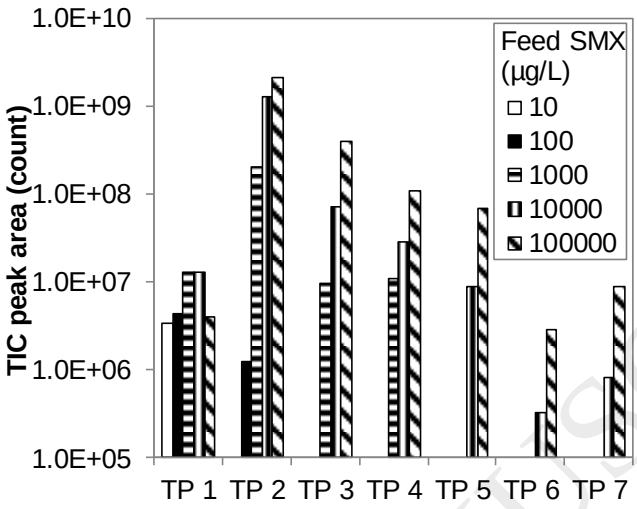

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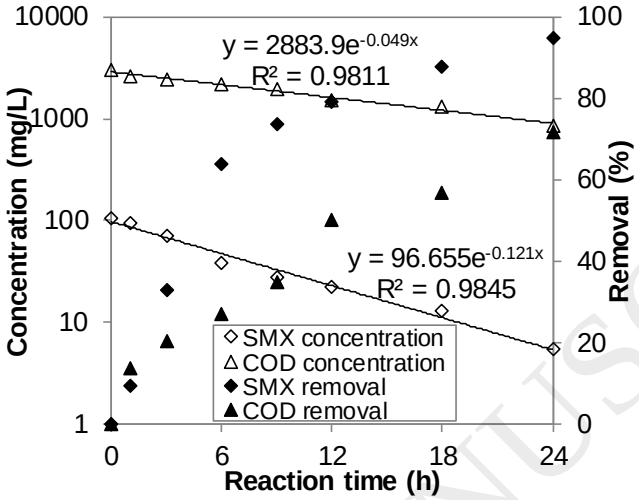


Fig. 3. SMX and COD removal kinetics in the batch test.

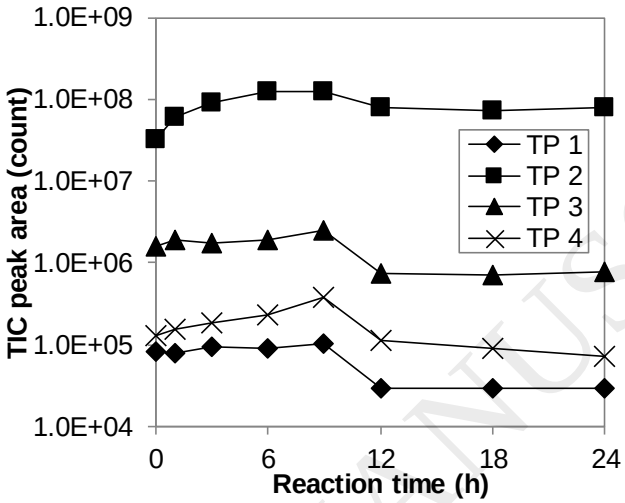


Fig. 4. TPs detection in the supernatant (300 times concentrated via ACE elution) from GC-MS and their peak intensity changes with reaction time in the batch test.

Table 1 Recipe of synthetic municipal wastewater.

Chemical compounds	Concentration (mg/L)	Food ingredients	Concentration (mg/L)	Trace metals	Concentration (mg/L)
Urea	183.4	Starch	244.0	$Cr(NO_3)_3 \cdot 9H_2O$	1.540
NH_4Cl	25.6	Milk powder	232.0	$CuCl_2 \cdot 2H_2O$	1.072
Na-acetate	158.8	Yeast	104.4	$MnSO_4 \cdot H_2O$	0.216
Peptone	34.8			$NiSO_4 \cdot 6H_2O$	0.672
$MgHPO_4 \cdot 3H_2O$	58.0			$PbCl_2$	0.200
KH_2PO_4	46.8			$ZnCl_2$	0.416
$FeSO_4 \cdot 7H_2O$	11.6				

Table 2

Bulk COD removal, methane production and biomass concentration in the AnMBR.

Phase	COD			Methane production*	MLVSS[#]
	Feed (mg/L)	Permeate* (mg/L)	Removal* (%)	(ml CH_4/g $COD_{removed}$)	(g/L)
1 (Day 1-20)	810	25.2±5.0	96.9±0.6	81.3±23.3	3.05-2.57 (2.81)
2 (Day 21-33)	920	27.3±2.9	97.0±0.3	111.8±21.2	2.57-2.51 (2.54)
3 (Day 34-54)	2000	39.3±9.8	98.0±0.5	208.5±46.6	2.51-3.52 (3.02)
4 (Day 62-88)	810	30.0±5.5	96.3±0.7	85.6±21.3	3.45-3.03 (3.24)
5 (Day 89-98)	920	31.9±3.8	96.5±0.4	120.4±19.5	3.03-3.15 (3.09)
6 (Day 99-113)	2000	48.6±6.3	97.6±0.3	244.1±49.1	3.15-4.42 (3.79)
7 (Day 114-129)	1050	60.8±9.9	94.2±0.9	82.6±20.2	4.42-4.17 (4.30)
8 (Day 141-170)	3330	495.7±152.8	86.2±4.8	67.5±15.2	7.62-7.15 (7.39)

*Average value ± standard deviation of at least 5 samples.
[#]Value in chronological order and average value in bracket.

Table 3

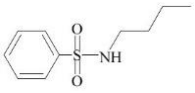
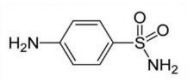
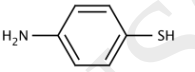
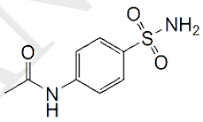
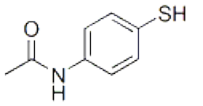
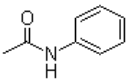
SMX removal and reaction rate constant in the AnMBR.

Phase	Feed	Permeate*	Removal*	Average loading rate	Reaction rate constant		Half-life
	(μg/L)	(μg/L)	(%)	(μg/gMLVSS/d)	First order (1/d)	Pseudo-first order (L/gMLVSS/d)	(d)
1 (Day 1-20)	10	0.32±0.09	96.8±0.9	3.6	3.44	1.22	0.20
2 (Day 21-33)	100	2.9±0.8	97.1±0.8	39.4	3.54	1.39	0.20
3 (Day 34-54)	1000	25.4±3.3	97.5±0.3	331.7	3.69	1.22	0.19
4 (Day 62-88)	10	0.33±0.16	96.7±1.6	3.1	3.41	1.05	0.20
5 (Day 89-98)	100	3.0±0.2	97.0±0.2	32.4	3.51	1.13	0.20
6 (Day 99-113)	1000	27.7±3.5	97.2±0.4	264.2	3.58	0.94	0.19
7 (Day 114-129)	10000	839.6±65.9	91.6±0.7	2328.3	2.48	0.58	0.28
8 (Day 141-170)	100000	11960.2±1483.1	88.0±3.0	13541.0	2.12	0.29	0.33

*Average value ± standard deviation of at least 5 samples.

Table 4

Biotransformation products of SMX in the AnMBR permeate identified by GC-MS.

No.	Compound name	CAS number	Molecular formula	Molecular weight (Dalton)	Molecular structure	GC elution time (min)	Detection under feed SMX (μg/L)
1	Butylbenzenesulfonamide	3622-84-2	$C_{10}H_{15}NO_2S$	213		11.1-11.2 (11.1)*	10, 100, 1000, 10000, 100000
2	Sulfanilamide	63-74-1	$C_6H_8N_2O_2S$	172		12.2 -15.6 (12.3)	100, 1000, 10000, 100000
3	4-Aminothiophenol	1193-02-8	C_6H_7NS	125		8.1-8.5 (8.3)	1000, 10000, 100000
4	Aniline	62-53-3	C_6H_7N	93		5.5-5.7 (5.5)	1000, 10000, 100000
5	Acetylsulfanilamide	121-61-9	$C_8H_{10}N_2O_3S$	214		14.7-15.8 (15.0)	10000, 100000
6	4-Acetylaminothiophenol	1126-81-4	C_8H_9NOS	167		10.7-10.8 (10.8)	10000, 100000
7	Acetylaniline	103-84-4	C_8H_9NO	135		8.6-8.7 (8.6)	10000, 100000

*: the value in the parentheses was the GC elution time of the pure compound dissolved in ACE (1 mg/L).