Microbial community evolution and fate of antibiotic resistance genes in anammox process under oxytetracycline and sulfamethoxazole stresses

Qian-Qian Zhang\textsuperscript{a,b}, Yu-Hui Bai\textsuperscript{a}, Jing Wu\textsuperscript{a}, Lian-Zeng-Ji Xu\textsuperscript{a}, Wei-Qin Zhu\textsuperscript{a}, Guang-Ming Tian\textsuperscript{b}, Ping Zheng\textsuperscript{b}, Xiang-Yang Xu\textsuperscript{b}, Ren-Cun Jin\textsuperscript{a,*}

\textsuperscript{a} School of Life and Environmental Sciences, Hangzhou Normal University, Hangzhou 311121, China
\textsuperscript{b} Department of Environmental Engineering, Zhejiang University, Hangzhou 310058, China

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ABSTRACT

The microbial community characteristics, functional and antibiotic resistance genes (ARGs), anammox performance under individual and combined oxytetracycline (OTC) and sulfamethoxazole (SMX) were tested under environmentally relevant levels. The results showed that anammox performance was inhibited when the OTC or SMX concentration increased from 0.5 to 1.0 mg L\textsuperscript{−1}. The absolute abundance of \textit{tetX} in OTC (3.03 × 10\textsuperscript{6} copies mg\textsuperscript{−1}), SMX (2.80 × 10\textsuperscript{6} copies mg\textsuperscript{−1}) and OTC + SMX (2.03 × 10\textsuperscript{6} copies mg\textsuperscript{−1}) was the highest and one more order of magnitude higher than that of \textit{tetG}, \textit{tetM}, \textit{intI\textsubscript{1}}, or \textit{sul\textsubscript{2}}. The anammox performance in the presence of OTC or SMX was lower than that sum of their independent effects. The enrichment of sludge resistomes with prolonged exposure time and increasing OTC and SMX doses might be due to succession of bacterial hosts and potential elevation of ARGs by horizontal transfer.

1. Introduction

Due to increasing economic, energy and environmental demands for wastewater treatment, a new treatment technology for wastewater is urgently needed. For nitrogen-rich wastewater treatment processes, an enhancement in nitrogen removal efficiency is urgently needed due to more serious nitrogen pollution issues (Tang et al., 2018; Zhang et al., 2018a). The appearance of anaerobic ammonium oxidation (anammox) results in significant savings in aeration costs and external organic carbon addition (Kartal et al., 2011; Zhang et al., 2018b), and thus, this process has received more attention for use in highly concentrated ammonium wastewater (Yang et al., 2013; Zhang et al., 2014). The anammox process is achieved by an anammox consortium utilizing ammonium and nitrite as electron donors and acceptors, respectively, under anaerobic conditions to produce nitrogen gas (N\textsubscript{2}) (Kartal et al., 2011). However, the presence of antibiotics in highly concentrated...
ammonium wastewater could inhibit the anammox process (Shi et al., 2017; Zhang et al., 2018a) and limit extensive application of the anammox process. Even, the antibiotics give the challenge for the successful application of anammox performance in antibiotic production wastewater (Zhang et al., 2019).

Oxytetracycline (OTC) and sulfamethoxazole (SMX) are broad-spectrum tetracycline and sulfonamide antibiotics, respectively, are used frequently as health safeguards in both humans and animals and growth promoters for animals. Corresponding, they were chosen as the antibiotic selection pressure because they are frequently found in swine wastewater. At the same time, nearly 60–90% of antibiotics are released into the environment due to the low absorption and utilization efficiency of antibiotics (Zhang et al., 2014). Antibiotic residues can negatively affect the environment and human health by producing emerging contaminants, i.e., antibiotic resistance genes (ARGs) (Zhang et al., 2015). The frequent detection of high concentrations of OTC and SMX was observed by a previous investigation (Zhang et al., 2015). The environmental concentration of OTC and SMX in wastewater is very low (ppt–ppb), but non-negligible concentrations are also found in pig slurry (up to 5 ppm) as well as drug manufacture effluents (Sengelov et al., 2003).

In previous reports on OTC or SMX inhibition of anammox performance, antibiotic concentration was high (nearly more than 10–100 times of the natural level) (Yang et al., 2013; Zhang et al., 2014). For example, Yang et al. (2013) proposed that the OTC concentration was higher than 50 mg L\(^{-1}\) and also Zhang et al. (2014) illustrated that OTC level was higher than 155–1731 mg L\(^{-1}\). The above antibiotic concentration was higher than the environmental level. Therefore, the impact of environmental relevant concentration levels of OTC on the anammox performance should not be overlooked. Less information is available on the relationship among anammox performance, activity, heme c content, extracellular polymeric substances (EPS), microbial community, functional genes, such as nirS, hzsA and hdh, involved in anammox metabolism (Wang et al., 2016; Zhang et al., 2019) and induced ARGs changes in the presence of low levels of individual and combined OTC and SMX selection pressures. Therefore, the present investigation provides basic information regarding the individual and combined effects of OTC and SMX on anammox performance and improves understanding of microbial community variation, functional genes and ARGs succession under different antibiotic selection pressures. Previous investigations on ARGs contribution to environmental sampling and the effect of low dosages of OTC and SMX on the amplification of ARGs in bioreactors are lacking.

The main objective of this study was to assess the possible microbial community succession and formation of resistance in anammox system under environmentally relevant antibiotic concentrations ranging from 0 to 1.0 mg L\(^{-1}\) OTC and SMX. Furthermore, to account for microbial adaption to antibiotic stress, a long-term chronic experiment was conducted for 166 days.

2. Materials and methods

2.1. Reactor configuration and operation

Laboratory-scale up-flow anaerobic sludge blanket (UASB) reactors with an effective volume of 0.5 L and an internal diameter of 50 mm were placed in a thermostatic room at 35 ± 1 °C and covered with black cloth (Zhang et al., 2018a). The inoculated sludge was produced from a laboratory-scale UASB reactor that stably operated for three years with specific anammox activity (SAA) and volatile suspended solid (VSS) respectively for 13.6 ± 5.6 mg TN g\(^{-1}\) VSS h\(^{-1}\) and 2.9 ± 0.2 g L\(^{-1}\). The 0.4 L anammox granules were inoculated into each reactor with an initial sludge concentration of approximately 1.3 g VSS L\(^{-1}\). Synthetic wastewater was pumped into reactors with an equimolar ammonium and nitrite level of 560 mg total nitrogen L\(^{-1}\) and the composition were detailed by Zhang et al. (2018a). The influent pH was approximately 7.8 ± 0.1 and hydraulic retention time (HRT) maintained at 0.6 h. The specific addition level and stages of OTC and SMX are detailed in Table 1.

<table>
<thead>
<tr>
<th>Stage</th>
<th>Time (d)</th>
<th>R(_0)</th>
<th>R(_1)</th>
<th>R(_2)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>OTC (mg L(^{-1}))</td>
<td>SMX (mg L(^{-1}))</td>
<td>OTC (mg L(^{-1}))</td>
</tr>
<tr>
<td>Start-up (P(_3))</td>
<td>1–10</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Sensitivity (P(_1))</td>
<td>11–98</td>
<td>0.1</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>Resistance (P(_2))</td>
<td>99–138</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Tolerance (P(_3))</td>
<td>139–166</td>
<td>1.0</td>
<td>1.0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

2.2. Sample collection, DNA extraction and 16S rRNA gene amplification

Mixed sludge samples were collected on the last day of each stage, i.e., days 10, 98, 138, and 166 from R\(_0\), R\(_1\) and R\(_2\) and then stored at −80 °C for a subsequent analysis of the bacterial community structures and ARGs abundance. The genomic DNA of the sludge samples and the V3-V4 region of the all bacterial 16S rRNA gene was amplified as illustrated by previously investigation (Zhang et al., 2018a).

2.3. RNA extraction, cDNA synthesis and reverse transcription (RT)

RNA extraction, cDNA synthesis, RT, primers and thermal programs information are detailed illustrated by previously investigation (Zhang et al., 2019).

2.4. Detection and representation of ARGs

The nine tetracycline resistance genes (tetA, tetC, tetD, tetG, tetL, tetK, tetQ, tetM, tetT, and tetX), one OTC proper (Otr(A)), one integrase (intI1) gene and three sulfonamide resistance genes (sul1, sul2, sul3) was detected by PCR. The PCR reaction conditions, primers and thermal programs information are provided in the Supplementary Materials. Finally, tetC, tetG, tetX, sulM, sul1, sul2 and intI1 with the high specificity were conducted with three technical replicates.

2.5. Analytical methods

The influent and effluent NH\(_4^+\)-N, NO\(_2^−\)-N, NO\(_3^−\)-N were determined according to standard methods. The determination of SAA, heme c content and EPS was illustrated by previous investigation (Zhang et al., 2014). The relative abundance of ARGs were defined as the ARGs with the bacterial 16S rRNA in samples. The relative abundance of nirS, hzsA, and hdh during the testing phase was defined as functional genes in the test phase to the abundance of the anammox bacterial 16S rRNA gene and then was normalized with respect to levels in the initial test, as OTC was not added into the influent. The absolute abundance of functional genes and ARGs was termed as the mRNA gene copies and ARGs within the mass of sludge samples. The copy numbers of functional genes and ARGs in the anammox metabolic process were normalized by log-transformation prior to additional analysis. The relationship between ARGs and the anammox in the samples collected at different periods was assessed by Pearson’s correlation analysis. One-way ANOVA was used to assess statistically significant differences (p < 0.05). A redundancy analysis (RDA) was performed using CANOCO 4.5 software based on the community composition and environmental variable.
3. Results and discussion

3.1. Nitrogen removal performance

The anammox operation over 166 days had four distinct stages: start-up (P0), sensitivity (P1), resistance (P2) and tolerance phases (P3). In P0 phase, the nitrogen removal rate (NRR) and total nitrogen removal efficiency (TNRE) of each reactor were higher than 20.0 kg N m⁻³ d⁻¹ and 88.0%, respectively. In P1 phase, the effluent NH₄⁺-N and NO₂⁻-N concentrations in R₀, R₁ and R₂ first increased and then decreased; thus, the anammox performance fluctuated. But, OTC, SMX and OTC + SMX at low dosage had nearly the same impact on the anammox performance (p < 0.05). The time for increase of effluent NH₄⁺-N and NO₂⁻-N concentrations in R₀, R₁ and R₂ was the same; thus, the sensitivity of the anammox performance response to OTC, SMX and OTC + SMX was similar.

As anammox consortium gradually adapts to low level of OTC and SMX, the resistance of the anammox performance to above antibiotics was poor. At the same time, the anammox performance gradually deteriorates over time under stress from 0.5 and 1.0 mg L⁻¹ OTC, SMX and OTC + SMX. The TNRE was ultimately reduced to 62.4 ± 12.5%, 68.6 ± 10.7% after exposure to 1.0 mg L⁻¹ and OTC + SMX. The TNRE was signally decreased by 54.4% of the one in P0 phase as the antibiotic concentration improved in three reactors. The stronger groups (Hou et al., 2016). Furthermore, the PN/PS ratio increased as the antibiotic concentration improved in three reactors. The stronger hydrophobic and functional groups (Sheng et al., 2010). Thus, EPS could adsorb sulfamethazine, sulfamerazine, sulfadiazine, and tetrahydroxyl groups and hydrophobic regions (Sheng et al., 2010). Thus, EPS concentration increased, but the PS content responded differently. PN was the main component in all EPS samples (in the Supplementary Materials), suggesting that the anammox performance could tolerate low OTC and SMX stress. The performance was significantly inhibited at OTC and SMX doses of 0.1 mg L⁻¹, and adaptation was indicated by the observed gradually remitted performance in the Supplementary Materials, suggesting that the anammox performance could tolerate low OTC and SMX stress. The performance was significantly inhibited at OTC and SMX doses of 0.1 mg L⁻¹ after the reactor was operated for 98 days, and later, a gradual resistance to high antibiotic treatment concentrations (e.g., 1.0 mg L⁻¹) was found. The resistance capacity of the anammox performance to OTC + SMX was higher than that of OTC and SMX. Kang et al. (2018) reported that performance of sequencing batch reactors inoculated with conventional and granular biomass slightly changed in the presence of 0.002 mg L⁻¹ SMX. Collado et al. (2013) revealed that addition of 0.05 mg L⁻¹ SMX for 60 days in an activated sludge reactor has no substantial impact on organic matter content and nitrogen removal. Ghosh et al. (2009) also indicated that 0.5 mg L⁻¹ SMX did not influence the nitrification process.

3.2. Sludge characteristics

3.2.1. Specific anammox activity and heme c content

The SAA of the anammox granules after P₃ phase was greater than 35.0 mgNO₂⁻-N g⁻¹ VSS d⁻¹ and slightly decreased by 43.5%, 71.5% and 67.7% respectively of the one in the P₀ phase as the OTC level increased from 0.1 to 1.0 mg L⁻¹ in R₀. Obviously, the inhibition of the SAA for 0.5 and 1.0 mg L⁻¹ OTC has no obvious differences (p < 0.05), indicating that anammox granules possessed tolerance to OTC. For R₁, the SAA was significantly decreased by 54.4% of the one in P₀ phase as stressed by 0.1 mg L⁻¹ SMX. While, the value of SAA in P₀ to P₃ phase have no obviously difference (p < 0.05), suggesting that anammox bacteria could tolerate 0.1 to 1.0 mg L⁻¹ SMX. For R₂, the SAA was deep by 54.4%, 81.4% and 57.1% of the one in the P₀ phase as the OTC + SMX level increased from 0.1 to 1.0 mg L⁻¹ respectively. The most inhibition of the SAA was found at 0.5 mg L⁻¹ OTC + SMX in the Supplementary Materials and later the SAA remitted to the value of one in P₀ phase, suggesting that the resistance of the anammox aggregate to OTC + SMX was gradually increased after further domestication of the lower antibiotics exposure. Thus, the sensitivity of anammox consortium to SMX is higher than OTC and OTC + SMX. Meanwhile, the resistance capacity of the anammox bacteria was elevated most under stress from OTC + SMX followed by OTC and SMX.

The variation in heme c was similar to that observed for SAA. The heme c content of anammox granules in R₀ and R₁ was reduced by one as the OTC and SMX levels increased from 0.1 to 1.0 mg L⁻¹. Notably, the response of heme c content to OTC + SMX was fluctuant. The addition of 0.5 mg L⁻¹ OTC + SMX increased the heme c content by 8.76% relative to the value obtained with 0.1 mg L⁻¹ in P₀ phase, whereas 1.0 mg L⁻¹ OTC + SMX greatly decreased the heme c content by 70.1% relative to the value in P₀ phase. These results may be related to the higher resistance of OTC + SMX to anammox consortium compared to OTC and SMX.

3.2.2. EPS

EPS are produced by microorganisms in response to stress situations (Zhang et al., 2014; Zhang et al., 2018c) and can affect activated sludge characteristics (Sheng et al., 2010). In P₀ phase, the total content of EPS in three reactors was over 29.7 ± 1.98 mg g⁻¹ VSS. EPS content greatly declined to 11.9 ± 1.5, 9.2 ± 1.4 and 12.6 ± 1.2 mg g⁻¹ VSS at treatments of 0.1 mg L⁻¹ OTC, SMX, and OTC + SMX (p > 0.05). Conversely, the EPS content increased to 317.3 ± 29.5, 70.2 ± 7.3 and 36.8 ± 4.7 mg g⁻¹ VSS in the presence of 0.5 mg L⁻¹ of above antibiotics. The impact force of OTC to EPS is higher than SMX and OTC + SMX, the EPS in R₀ treated by 0.5 mg L⁻¹ OTC was highest one than that SMX (R₁) and OTC + SMX (R₂) in any phases. While, the EPS content decreased further with 1.0 mg L⁻¹ above antibiotics but was higher than that in P₀ phase. Thus, the response of EPS to antibiotics disturbance is limited.

Notably, PN was the main component in all EPS samples (in the Supplementary Materials), with the reason may be that the same influent wastewater can be reduced to EPS with the same physicochemical characteristics (Le et al., 2016). The PN content first decreased and then increased to a level higher than the initial value as the antibiotic concentration increased, but the PS content responded differently. PN accounts for the majority of EPS and can provide adsorption binding sites via multiple functional groups, such as carboxyl, amine, and hydroxyl groups and hydrophobic regions (Sheng et al., 2010). Thus, EPS could adsorb sulfamethazine, sulfamerazine, sulfadiazine, and tetracycline antibiotics by the above hydrophobic regions and functional groups (Hou et al., 2016). Furthermore, the PN/PS ratio increased as the antibiotic concentration improved in three reactors. The stronger hydrophobicity and greater availability of adsorption sites contributed to the higher PN/PS ratio (Sheng et al., 2010). Thus, more adsorption sites were available for OTC, SMX or OTC + SMX, especially for OTC because the ratio was highest in R₀ for 0.5 mg L⁻¹ OTC inhibition.

3.3. Dynamics of microbial communities

The dynamic variation of microbial communities under stress from OTC and SMX was assessed by MiSeq sequencing (Fig. 1). In P₀ phase, the dominated phyla Planctomycetes and Proteobacteria in R₀, R₁ and R₂ were 73.5%, 67.7% and 62.3%, respectively. While, the dominated phyla were reduced to 47.3%, 51.8% and 41.6%, respectively as addition of OTC, SMX and OTC + SMX in the P₀ phase. The abundance of Planctomycetes first decreased and then increased to a level lower than the initial one (Fig. 1A), which suggested that Planctomycetes may...
tolerate antibiotics to some extent. Or the antibiotic-resistant bacteria are included in the Proteobacteria phylum (Shi et al., 2013). Firmicutes was detected in all samples as another common antibiotic-resistant phylum that has numerous tetracycline resistance genes (Ghosh and LaPara, 2007), thus, suggested that anammox process has some resistance to tetracycline and sulfonamide. In the present study, the abundance of Betaproteobacteria first increased and then was stable with an average level of 10.3%, 8.2% and 9.2%. Meanwhile, Betaproteobacteria possess a high antibiotic resistance and are the dominant bacterial community in OTC-containing wastewater treatments (Liu et al., 2012) or high and low concentrations of antibiotics wastewater (Deng et al., 2012). Zhu et al. (2017) proposed that Gammaproteobacteria could obtain resistance to antibiotics, especially in the presence of high concentrations of antibiotics. In the current investigation, Gammaproteobacteria was found in the three reactors with an average abundance of 0.88–1.79%. Thus, the tolerance of anammox sludge to above antibiotics is partially due to increased antibiotic resistance from resistant microorganisms.

The primary family was Candidatus Brocadiaceae with an average relative abundance of over 40.7% (Fig. 1B) in R0, R1 and R2 with the range of 30.9–46.9%. Long-term exposure to low concentrations of antibiotics could result in presence of resistant microorganisms. Ca. Kuenenia was the dominant anammox genus found in all samples (Fig. 1C), which gradually adapted to OTC and SMX stress and maintained a relative abundance over 31.0%. In addition to the resistance of Ca. Kuenenia, as a potential antibiotic-resistant bacterium (Zhang et al., 2018a), another resistance mechanism may exist.

The long-term adaptation of the anammox consortium to OTC and SMX might improve the tolerance of these bacteria to antibiotics (Tian et al., 2018). The suppression of anammox performance might be due to OTC and SMX inhibition of the dominant microbial consortium. The decreasing tendency of the Ca. Kuenenia was lower in R2 than R1 and the lowest in R0 (Fig. 1C). Thus, the OTC + SMX inhibition of anammox microorganisms is more severe than that of a single antibiotic based on the fact that the abundance of Ca. Kuenenia decreased as the antibiotic exposure time increased (Fig. 1C). At the same time, the abundance of Ca. Kuenenia slightly increased in P3 phase of R0 and R1 rather than R2, suggesting that Ca. Kuenenia is a potential resistant bacterium (Zhang et al., 2018a) and further demonstrating that the resistance capacity possessed by the anammox consortium is the reason for the tolerance of the performance.

3.4. Variation in functional genes

Substrate-utilizing activities can be monitored by the abundance of mRNA acts as a biomarker in nature and engineering systems containing biochemical reactions mediated by diverse bacterial consortia. As illustrated in Fig. 2, the responses of nirS, hzsA and hdh in three reactors were different from each other (p < 0.05) in P0 phase. In P1 phase, the absolute abundances of nirS, hzsA and hdh in R1 were up-regulated to 12019.7, 180.6 and 176.2 times of one in P0 stage respectively. While, the change of nirS, hzsA and hdh in R0 and R2 have no

![Fig. 1. System microbial community taxonomic compositions A) at the phylum level, B) at the family level. C) at the genus level in R0 (OTC-T), R1 (SMX-S) and R2 (OTC + SMX-TS). Relative abundance was defined as the number of OTUs assigned to that taxon divided by the total number of OTUs per sample.](image-url)
obviously variation compared to that in R1. In P2 stage, the changes of nirS, hzsA and hdh in the three reactors have the same tendency as those in P1 phase but a gentler response (p < 0.05). Furthermore, the absolute abundances of nirS, hzsA and hdh in R1 increased by 85.0, 9.8 and 21.1 times, respectively, relative to level in P0 phase. However, the responses of nirS and hzsA in R0 during P2 period were higher than those in R2 but with the same change, which was different from that observed for hdh. In P3 phase, the resistances of nirS and hdh to OTC and OTC + SMX were nearly the same tendency but greater than those in P1 phase (p < 0.05). Meanwhile, the absolute abundance of hzsA in the three reactors decreased to 2.9, 0.3 and 1.3 times of one in P0 period, respectively. Meanwhile, the relative abundance of the above functional genes has the same tendency with that of absolute abundances.

As the antibiotic stress increased from 0.1 to 0.5 mg L\(^{-1}\), the sensitivity order of mRNA levels of nirS, hzsA and hdh to antibiotic was SMX and OTC followed by OTC + SMX. Low doses of SMX (e.g., 0.1 mg L\(^{-1}\)) stimulated the up-regulation of the mRNA levels of nirS, hzsA and hdh to remit SMX inhibition, but the self-compensation of hzsA is limited. Thus, when a higher value of SMX was used (e.g., 1.0 mg L\(^{-1}\)), the up-regulation of hzsA was minimal. In contrast, the response of the mRNA level of nirS under 0.1 mg L\(^{-1}\) or higher concentrations (e.g., 0.5 and 1.0 mg L\(^{-1}\)) of OTC and SMX stress showed no significant differences (p < 0.05). The response of hzsA to 1.0 mg L\(^{-1}\) OTC was higher than that to OTC + SMX, and SMX resulted in the lowest response. The mRNA level of hdh was sensitive to low doses of OTC rather than medium and high levels (e.g., 0.5 and 1.0 mg L\(^{-1}\)) and was not obvious to OTC + SMX.

Although anammox aggregations can resist low OTC and SMX concentrations, the self-regulation of the mRNA levels of functional genes and the EPS defense mechanisms based on the increase of OTC and SMX dose should not be ignored. The variation of nirS, hzsA, and hdh at the transcript level was not unequivocally linked with the response of SAA in the present study (Fig. 2) that was consistent with previous investigations (Wang et al., 2016). The up-regulation of functional genes occurred in the P1 period and then decreased, while the SAA and heme c content decreased during the experiment. Self-regulation of functional genes as the self-regulation response to OTC and SMX inhibition has been suggested to be limited.

However, functional genes have no obvious correlation with SAA or heme c (p > 0.05). The sensitivity of nirS, hzsA and hdh to SMX is obvious, followed by OTC and OTC + SMX. Furthermore, EPS as a defense layer could protect anammox bacteria from OTC and SMX toxicity (Zhang et al., 2014) and even provide more adsorption sites for OTC, SMX or OTC + SMX (Sheng et al., 2010). However, this self-defense capacity is limited. Zhang et al. (2016) proposed that significant enhancement of EPS content with increasing tetracycline or SMX concentration (p < 0.05). The EPS acts as protective mechanism for cells due to secrete and form a network structure for protection against external disturbances (Kunacheva et al., 2014). Huang et al. (2014) illustrated that total average EPS content in activated sludge increased with the enhancement of tetracycline concentration. Zhu et al. (2018) proposed that PN content was greater than PS suppressed by combination of SMX and tetracycline hydrochloride, which may be because sensitivity of PN to antibiotics is greater than that of PS.

### 3.5. Changes in ARGs abundances

The detected genes could be divided into three classes according to different resistance mechanisms, such as efflux pumps, ribosome protection and enzymatic inactivation genes, induced by tetracycline and sulfonamides. The absolute abundance of tetC in R0, R1 and R2 was the highest among all the assessed ARGs and was more than one order of magnitude greater than that of tetG, tetM, intI1, sul1, sul2 and the lowest one was tetC. The overall abundances of tet and sul subtypes are designated as tetR and sulR.

The absolute abundance of tetC in R0 gradually increased from 1.53 × 10^5 to 6.22 × 10^5 copies mg\(^{-1}\) with the increase of OTC from 0 to 1.0 mg L\(^{-1}\) but waved in R1 and R2. The homologous tetracycline antibiotic was absent in R1, the corresponding tetracycline resistance genes tetC first decreased and then increased as the SMX dosage increased. The one in R2 had the same tendency as those in R1 but reached higher levels at higher OTC and SMX dosages (e.g., 0.5 and 1.0 mg L\(^{-1}\)) due to the double selection pressure. Thus, the ability of OTC + SMX to induce amplification of tetC was higher than that of single homology antibiotic or non-homologous antibiotic. The variation in tetG had the same tendency as that of tetC in the three reactors, which is possibly due to the same resistance mechanism. The contribution of the single homology antibiotic to the amplification of tetG was more that of OTC + SMX combination and individual non-homologous antibiotic.

At the same time, the absolute abundance of tetM gradually increased as the OTC dose in R0 increased. The variation of tetM in R1 and R2 first increased and then decreased as the OTC and SMX dosages increased. The reason for this result may be the same as that for tetC. The absolute abundance of tetX in R0, R1 and R2 first increased and then decreased. The reason may be that individual selection pressure induced by OTC or SMX on the anammox bacteria for tetX resistance was higher than that of OTC + SMX. The average absolute abundances of tetR in R0, R1 and R2 were 3.39 × 10^6, 3.10 × 10^6 and 2.47 × 10^5 copies mg\(^{-1}\) respectively. The changes of the tetR in the three reactors had the same variation tendency and first increased and then decreased as the antibiotic dose gradually increased.

In R0, the absolute abundance of sul1 increased as the OTC stress increased even without SMX. The inoculated sludge of the laboratory-scale reactor was from secondary sedimentation tank of the domestic wastewater treatment plant. However, the sewage treatment plants are a collection of ARGs (Zhu et al., 2013). So, the sul1 and other ARGs such as tetC, tetG, tetX, tetM and sul2 were detected in R0 and other no corresponding antibiotic selective pressure added reactor. In R1, there was no obvious variation of sul1 abundance. However, the variation of sul1...
in R2 had the same tendency as that in R0 under the OTC + SMX stress. The amplification of sul1 induced by SMX and OTC + SMX was much higher than that induced by OTC. The sul2 abundance in R1 and R2 fluctuated during the experiment phase with the range from 2.83 × 10^{5} to 4.98 × 10^{5} copies mg^{-1} and 3.37 × 10^{5} to 5.82 × 10^{5} copies mg^{-1}, respectively. The reason for this result may be that the induction ability of a homologous antibiotic (SMX) combined with a non-homologous antibiotic (OTC) was better for sul2 than SMX and OTC individually. Because sul2 is dominant in sulR, the variation of sulR is similar to that of sul2. Furthermore, the absolute and relative abundances of sul2 were usually slightly higher than those of sul1, which was in agreement with previous reports (Wu et al., 2015). The sul2 can obtain non-conjugative plasmids by horizontal and vertical transfer and even normally locate on large transmissible multi-resistant plasmids (Gao et al., 2012). Thus, sul2 could amplify widespread abundance. Without SMX or OTC treatment, the corresponding ARGs subtypes were produced and increased as the exposure time increased above one (Fig. 3) possibly because multidrug resistance in bacteria can be induced by the presence of OTC or SMX (Liu et al., 2012).

In addition to ARGs, intI1 was successfully detected. The average absolute abundances of intI1 in R0, R1 and R2 were 1.46 × 10^{6}, 1.37 × 10^{6} and 1.06 × 10^{6} copies mg^{-1}, respectively. The resistance obtained by the anammox consortium through production of ARGs could act as a defense mechanism against external stress. Finally, the impact of OTC + SMX on the anammox performance (R2) was less than the sum of their independent effects (R0 or R1). A comparison of the ARGs abundances (the sum of the tetracycline and sulfonamides ARGs) in the three reactors revealed that the R0 reactor had the highest ARGs abundance, followed by R1 and R2.

### 3.6. Relevancy of environmental factors and microbial community

The RDA was performed to describe the complicated correlations between the nitrogen removal performance, functional genes, ARGs and species in samples of days 10, 98 and 166 in three reactors (Fig. 3). The addition of 0.1 mg L^{-1} OTC, SMX and OTC + SMX slightly changed the microbial community structure (sample 1), while addition of 0.5 mg L^{-1} above antibiotic obviously increased the degree of difference between microbial community of sample 2 compared with that of sample 0. After long-term exposure of microbial community to OTC and SMX, the microbial community structure was similar to that of the initial state (P_{0}), which was consistent with the performance of the reactor (P_{S}). This result suggested that the microbial community gradually increased its tolerance to the antibiotic as the influent OTC and SMX concentration increased. Obviously, the RDA1 divided the four samples in R1 suppressed by SMX into two separate groups consisting of sample S0 and the other three samples (Fig. 3b), respectively. This separation can be explained by the significantly decreased abundance of genus Kuenenia from sample S0 compared with the other samples (this genus had the minimum score observed in the negative direction of the RDA1 score).

The OTC, SMX and OTC + SMX concentrations appeared a positive correlation with the effluent NH_{4}^{+}-N and NO_{3}^{-}-N concentrations and a strongly negative correlation with NRR and effluent NO_{2}^{-}-N concentration (Fig. 3). These results indicated that OTC and SMX were the primary factors responsible for the performance deterioration of the reactor. NRR, TNRE and effluent NO_{2}^{-}-N concentration was strongly related to the abundance of the genus Kuenenia (Fig. 3), indicating that these predominant anammox bacteria were responsible for nitrogen removal during the OTC, SMX and OTC + SMX suppressed anammox process. Obviously, the abundance of Caldilinea, Syntrophobacter, Nitrosomonas, Ignavibacterium was positively related to the effluent NH_{4}^{+}-N and NO_{3}^{-}-N concentrations and a strongly negative correlation was observed with effluent NO_{3}^{-}-N concentration (Fig. 3a). Meanwhile, the abundance of Nitrosomonas was positively related to the effluent NH_{4}^{+}-N and NO_{3}^{-}-N concentrations and a strongly negative correlation was observed with effluent NO_{3}^{-}-N concentration with Ignavibacterium abundance (Fig. 3b). Specifically, the abundance of Caldilinea, Syntrophobacter, Nitrosomonas, Ignavibacterium was positively related to the effluent NH_{4}^{+}-N, NO_{2}^{-}-N and NO_{3}^{-}-N concentrations (Fig. 3c). While, the Thauera gathered around the origin coordinates (Fig. 3c), indicating less impacts on the distribution of the four samples compared with Kuenenia, Caldilinea, Syntrophobacter, Nitrosomonas, Ignavibacterium.

The Caldilinea, Syntrophobacter, Ignavibacterium were identified as fermentative bacteria, and Nitrosomonas were classified as the dominant ammonia oxidizing bacteria. It is well-known that the anammox process is the co-metabolism process with fermentation bacteria and denitrification (Wang et al., 2016). Thauera was recently reported to prefer to use nitrate over nitrite as electron acceptor in the partial denitrification process (Ma et al., 2017). Due to the presence of the above co-metabolism substance, the effluent NO_{3}^{-}-N concentrations have no significant variation over whole experiment in the three reactors.

The transfer of ARGs is usually achieved by horizontal and vertical gene transfer (Tian et al., 2018). Thus, the variation of ARGs could be based on contributions from mobile gene elements and the succession of bacterial communities. The contribution of Ca. Kuenenia, as potential antibiotic-resistant bacteria, was verified as vertical gene transfer (Fig. 3). The tetC had significant correlations with intI1 (p < 0.01, R^{2} = 0.997**) in R_{0}. Similarly, strongly significant correlations were observed between tetC and intI1 and between sul2 and intI1 and sulR and intI1 (p < 0.05, R^{2} = 0.971∗; R^{2} = −0.964∗; R^{2} = −0.962∗, respectively) in R_{1}. Additionally, there were significant correlations for tetG with intI1 (p < 0.05, R^{2} = 0.974∗), tetG with sul2 (p < 0.05,
R² = 0.959*) and tetG with sulR (p < 0.05, R² = 0.960*), and strongly significant correlations were observed between sul2 and intI1 (p < 0.01, R² = 0.994***) and sulR and intI1 (p < 0.01, R² = 0.995***) in R. Thus, horizontal transfer of ARGs between diverse bacteria was expedited by antibiotic exposure, and OTC + SMX accelerated the horizontal transfer of ARGs. Meanwhile, the abundance of the tetX gene first increased and then decreased over time as the OTC and SMX addition increased (Fig. 3c). A possible reason for this result is that horizontal transfer of this gene is difficult (Ghosh et al., 2009); thus, the amplification of tetX might be mediated via vertical gene transfer mediated by unknown resistant hosts induced by OTC and SMX stress.

Anammox performance can tolerate individual and combined OTC and SMX stress, and the tolerance mechanism may be divided into four parts (Fig. 4). First, a protective layer is formed by EPS. Second, the dominant microbial aggregate becomes a potentially resistant species. Third, the functional genes regulated by the dominant microbes adapt to external disturbances. Fourth, the sludge resistome is enriched may occur due to elevated potential of ARGs mediated by horizontal transfer and succession of bacterial hosts under persistent OTC and SMX stress. Furthermore, slow-growing bacteria easily develop antibiotic resistance (Levin-Reisman et al., 2017). Liu et al. (2018) illustrated slow-growing microbes involved in the nitritification process easily develop tetracycline resistance in the presence of trace tetracycline. In the present study, anammox bacteria are typical slow-growth microorganisms due to external disturbances. Fourth, the sludge resistome is enriched may occur due to elevated potential of ARGs mediated by horizontal transfer and succession of bacterial hosts under persistent OTC and SMX stress.

4. Conclusions

The research indicated that anammox processes result in dissemination and even decrease of ARGs during the last period in low concentrations of OTC and SMX. The resistance capacity of anammox performance to OTC + SMX was higher than that to OTC and SMX. The tolerance of the anammox consortium to 1.0 mg L⁻¹ OTC and SMX may be linked to the protective layer formed by EPS, the dominant anammox bacteria became potentially resistant species. Furthermore, enrichment of sludge resistomes may occur due to elevated potential of ARGs mediated by horizontal transfer and succession of bacterial hosts under persistent OTC and SMX stress.

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Appendix A. Supplementary data

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