Effects of sulfamethoxazole on the denitrifying process in anoxic activated sludge and the responses of denitrifying microorganisms

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ABSTRACT

The presence of antibiotics in municipal wastewater is bound to affect the anoxic denitrifying process in anoxic activated sludge (AAS). This study investigated the effects of sulfamethoxazole (SMZ) on the denitrifying process in AAS and the responses of denitrifying microorganisms. The results showed that SMZ could decrease the speed of nitrate removal significantly when the concentration of SMZ was lower than 10 mg/L, and the removal of nitrate would be completely inhibited when SMZ concentration was higher than 100 mg/L. Weak alkaline condition would enhance the inhibition effect of SMZ on removal of nitrate in the anoxic bioreactor. The results of high-throughput sequencing and qPCR (quantitative polymerase chain reaction) showed that 100 mg/L of SMZ did not decrease the total abundance of denitrifying microorganisms. However, the relative expression levels of key denitrifying genes *NirS* and *NosZ* in AAS treated by 100 mg/L of SMZ versus the raw AAS without SMZ was only 0.030 and 0.036. Therefore, the inhibitory mechanism of SMZ on the denitrifying process in AAS was denoted by an effective inhibition to the expressions of denitrifying genes, rather than a decrease in the total abundance of denitrifying microorganisms.

Key words | anoxic activated sludge, antibiotic, denitrifying gene, denitrifying microorganisms, gene expression

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INTRODUCTION

Because of their resistance capacity against pathogens, a large amount of antibiotics are produced to cure and defend against epidemic diseases originating from humans and animals (Sarmah *et al.* 2006; Arias & Murray 2009). However, about 90% of antibiotics ingested by humans or animals are excreted in the form of archetypes or metabolites (Halling-Sørensen 2001; Kemper 2008). Thus, plenty of antibiotics excreted by humans and animals are transported into the municipal drainage system and then flow into a wastewater treatment plant. To date, various antibiotics, such as sulfamethoxazole (SMZ), tetracycline, azithromycin, and ofloxacin, have been detected in a large amount of municipal wastewater plants all over the world with concentration levels ranging from nanogram to milligram per litre (Campagnolo *et al.* 2002; Haller *et al.* 2002; Hammesfahr *et al.* 2008; Wang *et al.* 2013; Gan *et al.* 2014).

Antibiotics have significant impacts on environmental microbial communities. Antibiotics can change the structure and function of microbial communities at the molecular genetic level, which directly leads to an imbalance in the microbial ecosystem and disorder of ecological function (Kong *et al.* 2006; Yang *et al.* 2009; István *et al.* 2012). Many research achievements related to the effects of antibiotics on the structure and function of environmental microbial communities have been reported, with the most notable findings denoting that antibiotics have conspicuous effects on the microbial denitrifying process. For example, Costanzo *et al.* (2005) found that erythromycin could inhibit denitrifying microorganisms in water (Simon *et al.* 2005). Sun *et al.* (2017) found that antibiotics in soil significantly inhibited the removal of nitrate, the production of nitrous oxide (N_2O), and the abundance of denitrifying genes. Haack *et al.* (2012) found

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that the microbial capacity of nitrate reduction began to be inhibited in groundwater when the concentration of SMZ reached 1.3 μg/L. Hou *et al.* (2015) found that antibiotics could change the microbial community structure and that short-term exposure had a negative influence on denitrification rates. Microbial denitrification is the key process to accomplish nitrogen cycling by transforming nitrate to nitrogen in natural water/soil environments (Granger & Wankel 2016). Also, microbial denitrification has great significance in the treatment of municipal wastewater (Zheng *et al.* 2018).

The upgrade in the quality of wastewater treatment plant effluent in the '12th Five-Year' period of China resulted in the effluent standard of urban wastewater treatment plant improving from first level B to first level A. The concomitant effluent concentration of total nitrogen decreased from 20 to 15 mg/L, and the ammonia concentration decreased from 8 to 5 mg/L (He *et al.* 2017; Li 2017). In order to meet the demand for wastewater denitrification, wastewater treatment plants had to utilize an alternating anoxic–aerobic biochemical process to treat municipal wastewater, for example, the anoxic/oxic (A/O) process. However, the presence of antibiotics in municipal wastewater is bound to affect the denitrifying efficiency of the A/O process. To date, no study has focused on determining the effects of antibiotics on the denitrifying process in anoxic activated sludge (AAS). Moreover, the mechanism related to the effects of antibiotics on microbial denitrification also has not been investigated clearly.

As a typical antibiotic widely detected in municipal wastewater, SMZ was chosen for this study to investigate the denitrifying characteristics of AAS affected by the sulfonamide antibiotic. Further, the responses of denitrifying microorganisms to SMZ were studied so as to illuminate the mechanisms in which SMZ affects microbial denitrification. The outcomes of this research provide important information regarding microbiology and basic data for the operation of an A/O process affected by SMZ.

MATERIALS AND METHODS

Experimental AAS

The experimental AAS was sampled from a western wastewater treatment plant in Changchun city. This plant treats mixed wastewater stemming from sanitary wastewater and industrial wastewater discharged from the China Faw Group Corporation.

Experimental wastewater

The wastewater used for the experiment was self-prepared in the laboratory. The experimental wastewater consisted of the following components: 0.50 g/L of glucose, 0.50 g/L of $Na_2HPO_4 \cdot 12H_2O$, 0.32 g/L of KH_2PO_4, 0.10 g/L of $MgSO_4$, 3.00 g/L of NaCl, 0.05 g/L of $NaNO_3$, 0.20 mL/L of mixed trace element solution, and 0.20 mL/L of Ca-Fe solution. The mixed trace element solution was composed as follows: 10.0 mg/L of EDTA, 2.0 mg/L of $ZnSO_4 \cdot 7H_2O$, 1.0 mg/L of $CaCl_2 \cdot 2H_2O$, 5.0 mg/L of $FeSO_4 \cdot 7H_2O$, 0.2 mg/L of $NaMoO_4 \cdot 2H_2O$, 0.2 mg/L of $CuSO_4 \cdot 5H_2O$, 0.4 mg/L of $CoCl_2 \cdot 6H_2O$, and 1.0 mg/L of $MnCl_2 \cdot 2H_2O$. The Ca-Fe solution was made up of 1.00 g/L of $CaCl_2 \cdot 2H_2O$ and 1.00 g/L of $FeSO_4 \cdot 2H_2O$. To expel dissolved oxygen, experimental wastewater was treated by nitrogen-blowing for 10 minutes before use.

Setup of anoxic bioreactor

Seventeen plastic buckets (12 cm in diameter, 25 cm in height, and 5 cm in bucket mouth width) were used as the anoxic bioreactors. Experimental AAS and wastewater were added into the 17 bioreactors simultaneously. A stirrer and a temperature controller were installed in each bioreactor. The rotary speed of the stirrer was 150 rpm, and the water temperature was maintained at 25 °C. The experimental wastewater in each bioreactor was changed every 3 days until the nitrate was removed rapidly and AAS showed good settleability in 30 minutes in all bioreactors.

Operation of anoxic bioreactor with SMZ

Different concentrations of SMZ and fresh experimental wastewater were added into the seven bioreactors resulting in final SMZ concentrations of 0, 1 μg/L, 10 μg/L, 100 μg/L, 1 mg/L, 10 mg/L and 100 mg/L, respectively. Another 10 bioreactors divided into two groups were used to investigate the effect of pH on denitrification in AAS treated by SMZ. The first group included five bioreactors to which 10 mg/L of SMZ was added, and the pH of the experimental wastewater was adjusted to 5, 6, 7, 8 and 9, respectively. The second group had only pH adjustment, to 5, 6, 7, 8 and 9, without adding SMZ.

Sampling and testing

During the whole experimental process, 2 mL of wastewater in each bioreactor was sampled every 5 hours to determine

the concentration of nitrate (NO_3^--N) by external standard method with an ion chromatograph (Metrohm, 861). The concentration of dissolved oxygen in each bioreactor was also monitored online with a dissolved oxygen meter (310D-01A, ORION) as an indicator for microbial denitrification, and the measured results were always lower than 0.20 mg/L. All the above experiments were run in triplicate.

Test of high-throughput sequencing

DNA and RNA extraction

The AASs treated by 0 and 100 mg/L of SMZ were collected at the end of the experiment, to extract total DNA using a Powersoil™ DNA Isolation kit (MoBio, USA) and to extract total RNA using a Powersoil™ RNA Isolation kit (MoBio, USA) according to the procedures provided by kits.

Barcoded PCR and amplicon sequencing

The V3–V4 regions of the 16S rDNA were amplified from the above total DNAs using the special primers 341F and 805R (Hugerth *et al.* 2014). The polymerase chain reaction (PCR) products were then sequenced on an Illumina MiSeq 2*300 bp Sequencing Platform. The whole process of PCR, sequencing, and the subsequent data analysis was undertaken by the Shanghai Sangon Biological Engineering Technology & Service Co., Ltd. The significance level of sequencing was set at $p < 0.01$.

Real-time qPCR

A relative quantitation method was used to quantify the relative abundances and expressions of the key denitrifying genes *NirS* and *NosZ*. 16S rDNA (V3 region) was used as the internal control gene. For the relative abundances of the genes *NirS* and *NosZ*, diluted total DNA of 10 ng/μL was used as the real-time quantitative PCR (RT-qPCR) template. For the relative expressions of the genes *NirS* and *NosZ*, the diluted cDNA (10 ng/μL) synthesized from the total RNA with an M-MuLV First Strand cDNA Synthesis Kit (Sangon, Shanghai) was used as the RT-qPCR template. The primers for RT-qPCR are shown in Table 1. Amplification was carried out via the Hot Start Fluorescent qPCR Core Reagent Kit for SYBR green I (Sangon, Shanghai). RT-qPCRs were, additionally, performed in the Stratagene Mx 3000 Thermal Cycler with 25 μL of the reaction mixture containing 1 × Hot Start fluo-PCR mix, 15 pmol/L of each primer, and 1 μL of DNA template (10 ng). The RT-qPCR program was the same as described in the literature (An *et al.* 2012). The relative abundances and expressions of the genes *NirS* and *NosZ* were determined using the $2^{-\Delta\Delta Ct}$ method ($\Delta\Delta Ct = \Delta Ct[\text{sample}] - \Delta Ct[\text{calibrator}]$) (Sehringer *et al.* 2005). The control sample (no added SMZ) was considered the calibrator.

RESULTS AND DISCUSSION

The effects of SMZ on the denitrifying process in AAS

Although it has been reported that SMZ could decrease denitrification significantly (Underwood *et al.* 2011), the effects of SMZ on the denitrifying process in AAS have not been investigated in detail, and no estimate has been provided concerning the damage of SMZ on a wastewater treatment plant. In this study, therefore, the effect of different levels of concentration of SMZ on the denitrifying process in AAS was investigated. The results of the real-time concentrations of nitrate (C) compared with the original nitrate concentrations (C_0) are shown in Figure 1.

SMZ had a distinct inhibitory effect on the denitrifying process in AAS (Figure 1). The first significant inhibitory effect on the denitrifying process was noted at SMZ concentrations of 10 μg/L. The higher the concentration of SMZ, the stronger

Table 1 | Primer sequences and length of amplification products

Target DNA fragment	Primer	Oligonucleotide sequence (5′ – 3′)	Length of amplification product	References
16S rDNA region V3 for RT-qPCR	338F 518R	CCTACGGGAGGCAGCAG ATTACCGCGGCTGCTGG	181 bp	An *et al.* (2012); Zhang *et al.* (2016)
Partial *NirS* gene for RT-qPCR	NirS-F NirS-F-R	CACGGYGTBCTGCGCAAGGGCGC CGCCACGCGCGGYTCSGGGTGGTA	702 bp	Scala & Kerkhof (1998); Bai *et al.* (2008)
Partial *NosZ* gene for RT-qPCR	NirS-F-F NirS-F-R	CGYTGTTCMTCGACAGCCAG CATGTGCAGNGCRTGGCAGAA	707 bp	

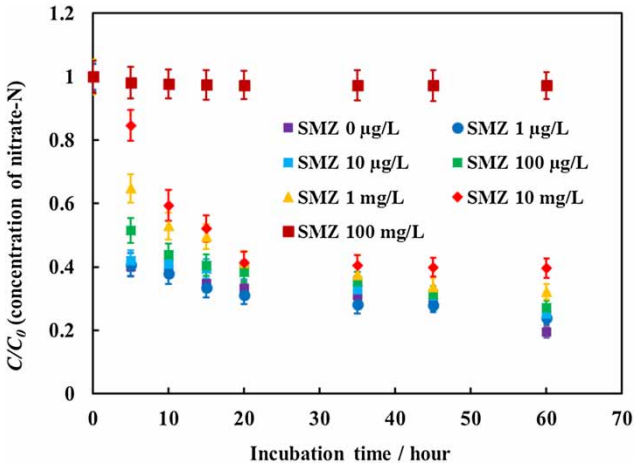

Figure 1 | The effects of SMZ on the denitrifying process in AAS (error bars represent standard deviation of triplicate runs).

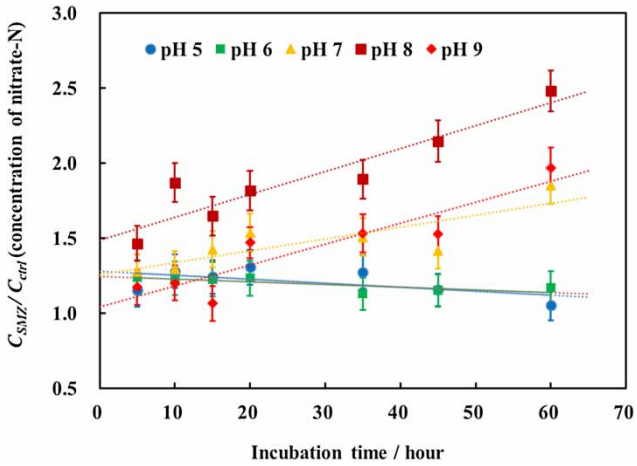

Figure 2 | The effects of pH on the denitrifying process in AAS containing SMZ (error bars represent standard deviation of triplicate runs).

the inhibitory effect. When the concentration of SMZ was less than 10 mg/L, the terminal removal rates of NO_3^- were all similar to those where no SMZ had been added, as long as the experimental time was long enough (Figure 1). However, when the concentration of SMZ was 100 mg/L, the denitrifying process in the AAS was completely inhibited.

Although the concentrations of SMZ in the actual wastewater treatment plant were not high (concentration levels ranging from nanogram to milligram per litre), a long denitrifying time would be required because SMZ could impact the denitrifying speed. However, the hydraulic retention time (HRT) of wastewater was limited in the A/O process. If the HRT were excessively prolonged to ensure denitrification efficiency, it would have a major impact on both the treatment flux and the treatment cost for municipal wastewater treatment plants.

The effects of pH on the denitrifying process in AAS containing SMZ

The SMZ molecule contains both acid and alkaline functional groups. Different pH values are likely to affect microbial denitrifying activities. Research to date, however, has only focused on the concentration of SMZ, but not pH. Therefore, the effect of pH on the denitrifying process in AAS containing SMZ was studied herein. The results of the real-time nitrate concentrations of the sample group (C_{SMZ}, adding 10 mg/L of SMZ) compared with the control group (C_{ctrl}, not adding SMZ) are shown in Figure 2.

When the pH value was 8, the ratio of C_{SMZ}/C_{ctrl} was the highest, followed by pH 7 and pH 9, and the lowest were pH 6 and pH 5. These results demonstrate that alkalinity can enhance the inhibitory effects of SMZ on the denitrifying process, and weak acidity just the opposite. The optimal pH for anoxic denitrification in the A/O process was noted to be 6.5–7.5. The degree of inhibition imparted on microbial denitrification caused by SMZ in anoxic tanks may change significantly as a function of the variation in pH of the wastewater.

The effects of SMZ on changes in the microbial community in AAS

Ecological indexes, such as the Shannon index (H) and the Simpson index (D), were used to assess the ecological diversity of microorganisms in AAS. The numbers of DNA sequences analyzed by high-throughput sequencing were 64,981 and 52,621 for the control and the SMZ-treated AAS, respectively, and the coverage indices were 0.99 and 0.98, respectively, which indicated that the outcomes of high-throughput sequencing were credible. The actual operational taxonomic unit (OTU) numbers determined by the clustering analysis of sequences were 2,681 and 2,618, and the total OTU numbers estimated by the ACE (abundance-based coverage estimator) index were 3,569 and 3,485, and estimated by Chao1 index were 3,505 and 3,398, repectively, which demonstrated that AAS control expressed more microbial species than in AAS treated with SMZ (100 mg/L). The H of microorganisms in AAS treated with 100 mg/L of SMZ was a little higher than that not treated with SMZ (control). The D distinctly decreased in the SMZ-treated AAS; which demonstrated that SMZ did not decrease the microecological diversity of AAS; if anything, it slightly increased it (Table 2).

Table 2 | Ecological indices of microorganisms in AAS treated with SMZ

Sample ID	Sequence number	OTU number	Shannon Index	ACE index	Chao1 index	Coverage	Simpson Index
Control	64,981	2,681	5.20	3,569	3,505	0.99	0.05
100 mg/L of SMZ	52,621	2,618	5.74	3,485	3,398	0.98	0.01

Cluster analysis based on the neighbor-joining method was used to construct phylogenetic trees of the microbial groups in AAS according to the results of high-throughput sequencing. When the sample group (adding 100 mg/L of SMZ, Figure 3) is compared with the control (not adding SMZ, Figure 4), significant changes were noted, including a 11.10% decrease in the abundance of *β-proteobacteria*, a 6.77% increase in *γ-proteobacteria*, a 0.24% decrease in *α-proteobacteria*, a 0.58% decrease in *Chlamydiae*, a 1.33% decrease in *Anaerolineae*, a 0.48% increase in *Gemmatimonadetes*, a 1.18% increase in *Nitrospira*, and a 0.22% decrease in *Sphingobacteria*. *Flavobacteria* was stimulated by SMZ and the abundance increased from not detected to 0.80%.

The variation in microbial abundance in AAS treated by 100 mg/L of SMZ is shown in Table 3. The abundances of denitrifying microorganisms, including *Hydrogenophaga*, *Dechloromonas*, *Thauera*, and *Ferribacterium*, in SMZ-treated AAS were all different from those in raw AAS. As a whole, the total abundance of denitrifying microorganisms not only remained stable but also increased under the stress of the presence of 100 mg/L of SMZ. However, Underwood *et al.* (2011) found that the growth of denitrifying bacteria exposed to SMZ (1.5 μg/L) was inhibited significantly in groundwater. In addition, SMZ could inhibit the growth and function of denitrifying bacteria in groundwater (Ahmad *et al.* 2014). These were actually opposite to our research outcomes. This may be a result of the species of denitrifying microorganisms and environmental conditions in AAS being significantly different from those in groundwater. Therefore, inhibition of the growth of denitrifying bacteria was not the reason why SMZ decreased the denitrifying rate in AAS.

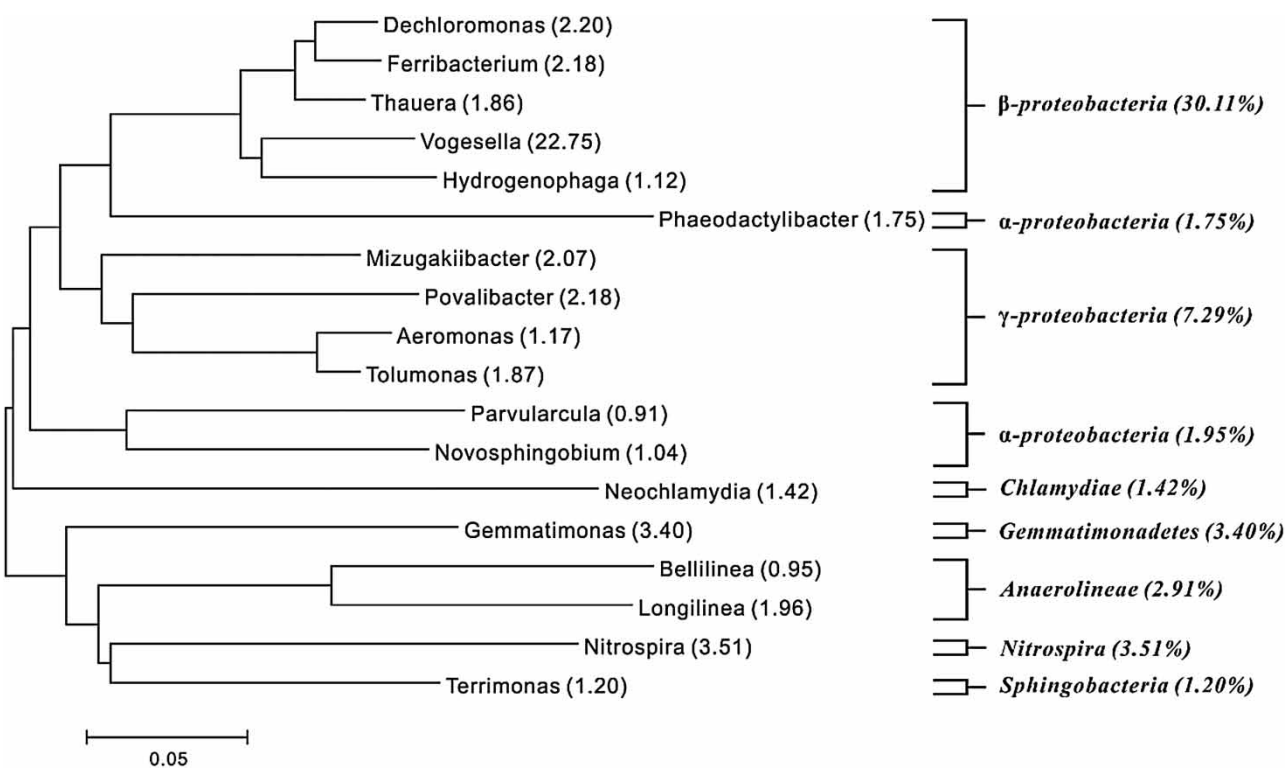

Figure 3 | Neighbor-joining tree of microbial groups in raw AAS (no added SMZ).

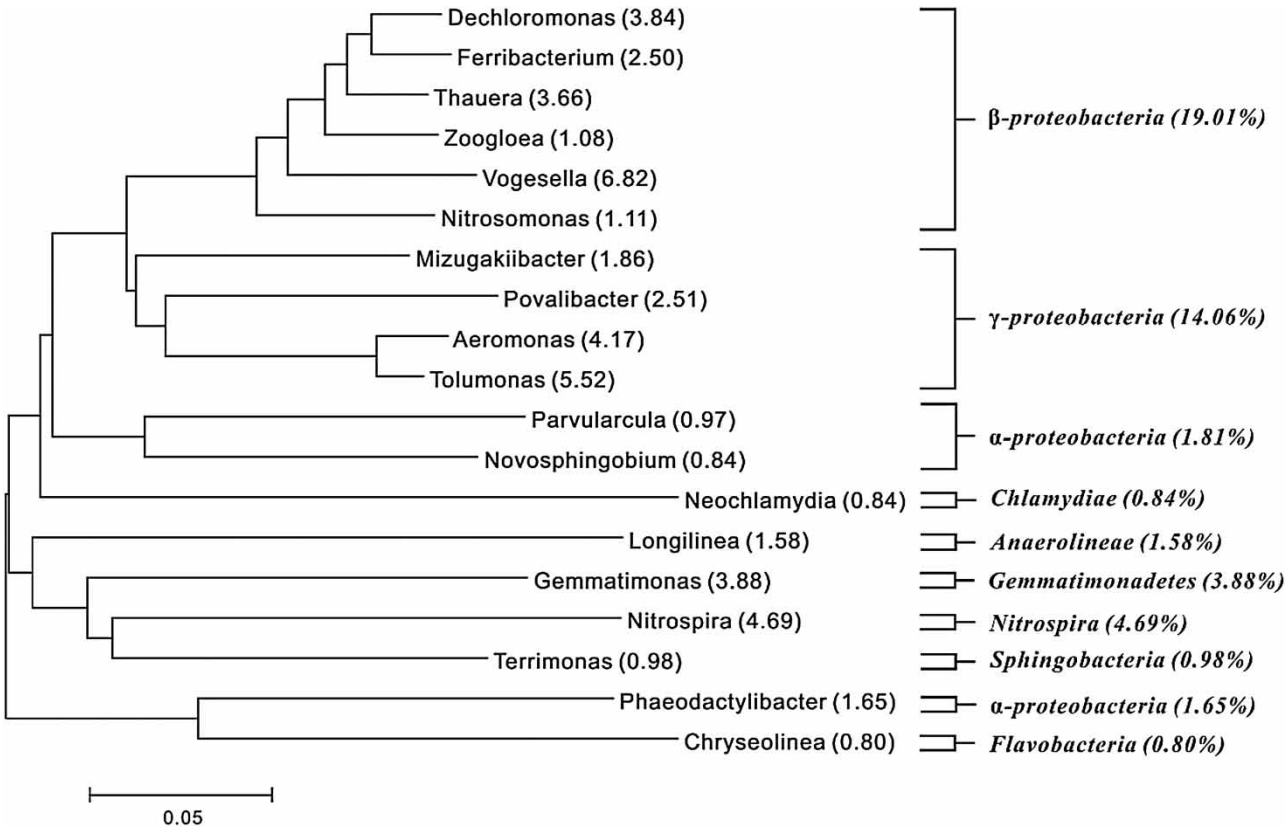

Figure 4 | Neighbor-joining tree of microbial groups in AAS treated with 100 mg/L of SMZ.

Table 3 | The effects of SMZ on changes in the microbial community in AAS

No.	Microbial genera	Microbial function	Abundance without SMZ	Abundance with SMZ	Increase (↑) or Decrease (↓)	Functional category
1	*Hydrogenophaga*	Aerobic denitrification	1.12%	Not detected	−1.12% (↓)	Denitrification
2	*Dechloromonas*	Denitrification, degrade perchlorate	2.20%	3.84%	+1.64% (↑)	
3	*Thauera*	Denitrification	1.86%	3.66%	+1.80% (↑)	
4	*Ferribacterium*	Anoxic reduction of nitrate and Fe(II, III)	2.18%	2.50%	+0.32% (↑)	
5	*Nitrospira*	Nitrification	3.51%	4.69%	+1.18% (↑)	Nitrification
6	*Vogesella*	Aerobic degradation of organics	22.75%	6.82%	−15.93% (↓)	Degrade organics
7	*Novosphingobium*	Aerobic degradation of organics (aromatic hydrocarbon)	1.04%	0.84%	−0.20% (↓)	
8	*Longilinea*	Degrade carbohydrate	1.96%	1.58%	−0.38% (↓)	
9	*Parvularcula*	Degrade organics (polycyclic aromatic hydrocarbons)	0.91%	0.97%	+0.06% (↑)	
10	*Tolumonas*	Aerobic degradation of organics	1.87%	5.52%	+3.65% (↑)	
11	*Neochlamydia*	Restrained by tetracycline, pathogenic bacterium	1.42%	0.84%	−0.58% (↓)	Pathogenic bacterium
12	*Aeromonas*	Aerobic or facultative anaerobic pathogenic bacterium	1.17%	4.17%	+3.00% (↑)	

The effects of SMZ on the relative abundance of the genes *NirS* and *NosZ* in AAS

It is well known that four fundamental denitrifying enzymes, respiratory nitrate reductase (encoded by gene *Nar*), nitrite reductase (encoded by gene *Nir*), nitric oxide reductase (encoded by gene *Nor*), and N_2O reductase (encoded by gene *Nos*), have been employed together to catalyze denitrification reactions (Zumft 1997; Cui *et al.* 2016). Further, nitrite (NO_2^-) reduction and N_2O reduction to N_2 are key steps involved in the denitrification process (Pan *et al.* 2013). Several studies have demonstrated that the reason for the inhibition of antibiotics on denitrification was that the abundance of denitrifying genes was suppressed (Hou *et al.* 2015; Yin *et al.* 2016). As a result, the relative abundances of the key denitrifying genes *NirS* and *NosZ* affected by SMZ in AAS were investigated in this study.

The results show that the relative abundances of key denitrifying genes *NirS* and *NosZ* did not decrease (Figure 5). It is interesting that these results were different from other research findings. For example, Yin *et al.* (2017) found that oxytetracycline affected the abundance of the gene *NirS* and decreased the denitrification rate significantly, as a result of the high tendency to adsorb with sediment (Xu *et al.* 2009; Chen *et al.* 2015). During the treatment process with antibiotics, the abundances of both *NirS* and *NosZ* genes were inhibited significantly (Conkle & White 2012; Yan *et al.* 2013). These results also indirectly infer that the inhibitory effects of SMZ on the denitrifying process in AAS had no correlation with the biomass of denitrifying bacteria.

The effects of SMZ on the relative expressions of the *NirS* and *NosZ* genes in AAS

Although many scholars have investigated the inhibitory mechanism of antibiotics on anoxic denitrification in terms of the abundance of denitrifying genes and denitrifying enzyme activity (Gui *et al.* 2017), studies on the expressions of denitrifying genes in AAS have been, to date, rather scarce. In this study, using raw AAS (no addition of SMZ) as the control (i.e., the relative expressions were all 1), the relative expressions of the key denitrifying gene *NirS* and *NosZ* were about 0.030 and 0.036, respectively (Figure 6), which demonstrates that SMZ had intense inhibitory effects on the relative expressions of key denitrifying genes in the AAS. This effect was consistent with the removal of nitrate (Figure 1). Of course, the presence of substrate is necessary for the continued expression of denitrifying genes (Philippot *et al.* 2001; Zheng *et al.* 2014). Therefore, an important conclusion was inferred that the reason why SMZ had negative effects on the denitrifying process in AAS was that the expressions of denitrifying genes were inhibited by SMZ.

CONCLUSIONS

SMZ presented significant inhibition effects on the denitrifying process of AAS, especially under weak alkaline condition. Low concentration of SMZ (<10 mg/L) mainly inhibited the speed of nitrate removal, whereas high concentration of SMZ (>100 mg/L) would completely prevent AAS from removing nitrate in municipal wastewater. The

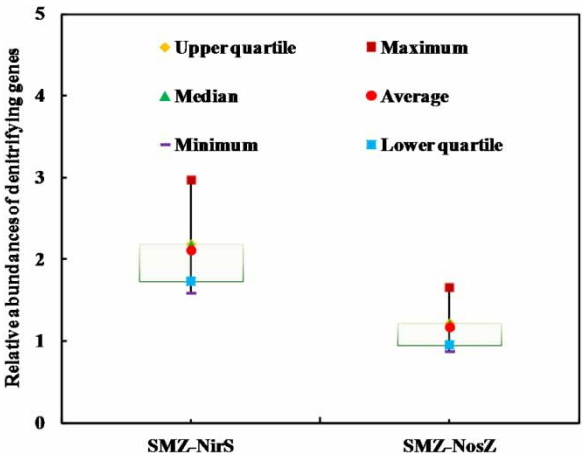

Figure 5 | The effects of SMZ on the relative abundances of the *NirS* and *NosZ* genes in AAS (error bars represent standard deviation of six replicate runs).

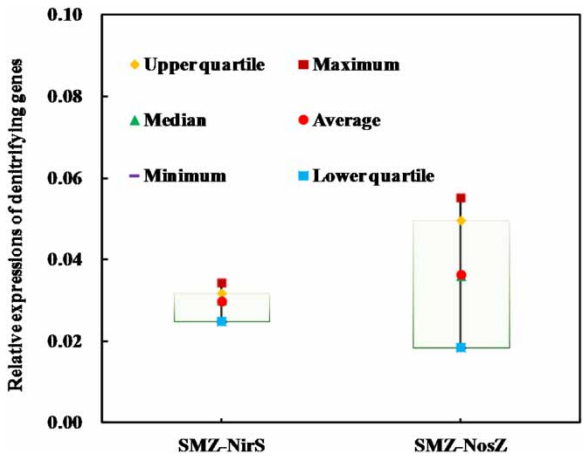

Figure 6 | The effects of SMZ on the relative expressions of the *NirS* and *NosZ* genes in AAS (error bars represent the standard deviation of six replicate runs).

microbial diversity of AAS was a little enhanced, and the total abundance of denitrifying microorganisms increased 2.64% after the treatment of 100 mg/L of SMZ. However, the relative expression levels of key denitrifying genes *NirS* and *NosZ* in AAS treated by 100 mg/L of SMZ versus the raw AAS without SMZ was only 0.030 and 0.036. Therefore, an important conclusion inferred from this study was the reason why SMZ inhibits the denitrifying process in AAS is that SMZ actually can inhibit the expressions of key denitrifying genes, rather than decrease the biomass of denitrifying microorganisms.

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