# Effects of oxytetracycline on denitrification and anammox processes in riparian buffer zone soil

Dongmei Xue[1†], Jing Li[1,2,3†], Hang Yu[1,2], Ke Jin[3*], Zhongliang Wang[4]

(1. *Tianjin Key Laboratory of Water Resources and Environment, Tianjin Normal University, Tianjin 300387, China*;
2. *School of Geographic and Environmental Sciences, Tianjin Normal University, Tianjin 300387, China*;
3. *Institute of Grassland Research, Chinese Academy of Agricultural Sciences, Huhhot 010010, Inner Mongolia, China*;
4. *Tianjin Key Laboratory of Aquatic Science and Technology, Tianjin Chengjian University, Tianjin 300384, China*)

**Abstract:** Microbial denitrification and anaerobic ammonium oxidation (anammox) are two critical processes involved in nitrate ($NO_3^-$) removal from riparian buffer zones. However, the responses of these processes to antibiotics used in nearby animal farms are not yet fully understood. In this study the effects of oxytetracycline (OTC) short-term exposure on microbial denitrification and anammox in riparian buffer zones were examined via slurry-based $^{15}N$ tracer technique combined with network analysis. Short-term OTC exposure will significantly decrease denitrification and anammox performance by restricting related functional genes, but inhibitory upper will appear at high OTC contents. The dominant bacterial community did not significantly change, while the bacterial network became more complex to keep the network stable. Functional bacteria containing *nos*Z and *hzs*B genes underwent significant changes, manifested as inhibitory effects, promotion effects, and no significant effects. The results indicated that short-term OTC exposure seemed unable to affect all types of functional bacteria, resulting in a considerable part of bacteria maintaining certain nitrogen removal performance. In the bacteria containing *nos*Z gene, it was found that 22.2% are denitrification mode bacteria and 77.8% are non-denitrification mode bacteria, suggesting that the restoration of the denitrification process should rely on the maintenance of a diverse denitrifying community. These results provide scientific knowledge on the effect of antibiotics-containing wastewater on the excessive N loading in aquatic ecosystems, and also indicate that additional efforts are needed to control antibiotic pollution in riparian buffer zones and coastal ecosystems.

**Keywords:** riparian zone, oxytetracycline, denitrification, anammox, co-occurrence network, $^{15}N$ tracer
**DOI:** 10.25165/j.ijabe.20241706.8741

**Citation:** Xue D M, Li J, Yu H, Jin K, Wang Z L. Effects of oxytetracycline on denitrification and anammox processes in riparian buffer zone soil. Int J Agric & Biol Eng, 2024; 17(6): 286–295.

## 1 Introduction

Excessive nitrogen (N) loading exerts serious threats to various ecosystems, especially aquatic ecosystems[1]. Most N enters rivers [mostly in the form of nitrate ($NO_3^-$)] via surface/subsurface runout and atmospheric deposition[1]. Riparian buffer zones create a barrier between terrestrial and aquatic ecosystems and intercept pollutants through a range of hydrological, physical, chemical, and biological processes[2]. Therefore, exploring pathways for reactive N removal from riparian buffer zones to protect rivers is particularly important.

Denitrification and anaerobic ammonium oxidation (anammox) have been identified as the primary processes for removing $NO_3^-$ via emission of nitrogen ($N_2$) and/or nitrous oxide ($N_2O$)[3,4]. Denitrification generally occurs under anaerobic conditions and is driven by heterotrophic microorganisms that convert $NO_3^-$ or nitrite ($NO_2^-$) into $N_2O$ and/or $N_2$[5]. Anammox requires $NO_2^-$ as an electron acceptor to directly oxidize ammonium ($NH_4^+$) to $N_2$ under anaerobic conditions[6,7]. The riparian buffer zone as a complex system possesses widely distributed and intensely active microbial communities, which are considered as hot spots for denitrification and anammox processes. However, the $NO_3^-$ removal capacity of riparian buffer zones may be challenged by extensive use of antibiotics in farming and aquaculture[8]. Therefore, it is necessary to better understand the responses of denitrification and anammox to antibiotics in riparian buffer zones.

About 80%–90% of antibiotics are directly discharged into aquatic and soil environments via urine and feces as parent compounds[9], which can then influence microbial processes including denitrification and anammox[8,10]. Antibiotics can inhibit bacterial growth or survival[11-13], alter functional gene abundances or expression[14,15], and alter community structures of denitrifying and anammox bacteria[10,13]. For example, chloramphenicol exposure significantly inhibited denitrifying enzyme activity in soils[16] and in estuary sediments[17]. Lysozyme, penicillin, and oxytetracycline exposure have been shown to destroy the cell walls and/or impede the synthesis of ribosomes of anammox bacteria, thereby inhibiting their growth and reducing anammox rates[18]. Oxytetracycline inhibits bacteria growth through various pathways, such as preventing ribosome formation, inhibiting peptide chain extension, or blocking protein synthesis[19,20].

In China, up to 162 000 t of antibiotics are used annually, about

**Received date:** 2023-12-15 **Accepted date:** 2024-11-09
**Biographies: Dongmei Xue**, PhD, Professor, research interest: surface processes and environmental evolution, Email: xuedongmei@tjnu.edu.cn; **Jing Li**, PhD, research interest: agricultural ecology, Email: 912592989@qq.com; **Hang Yu**, MS candidate, research interest: surface processes and environmental evolution, Email: 18463758791@163.com; **Zhongliang Wang**, PhD, Professor, research interest: historical changes in regional ecological environment, Email: 82101219406@caas.cn.
†These authors contributed equally
***Corresponding author: Ke Jin**, PhD, Professor, research interest: soil health cultivation and functional improvement. Institute of Grassland Research, Chinese Academy of Agricultural Sciences, Tel: +86-15801362245, Email: jinke@caas.cn.

52% of which are used in livestock and poultry rearing[21]. For example, nearly 20 000 t of veterinary antibiotics are used yearly in the Beijing-Tianjin-Hebei region[22]. Tetracyclines are the most frequently used antibiotics[22], with oxytetracycline (OTC) representing a typical tetracycline that is regularly detected in livestock and poultry rearing areas of the Beijing-Tianjin-Hebei region[23,24]. Indeed, OTC contents have been observed at concentrations of up to 524.4 mg/kg in pig manure in a livestock farm[25]. This high level of antibiotic contamination exposure is a big risk for surrounding soils and waters. Some studies showed that $NO_3^-$ and antibiotic levels in rivers near a livestock or poultry farm are much higher than in other comparison rivers[22,26]. However, few studies have investigated the response of $NO_3^-$ removal process-associated microbial communities to short-term high OTC levels within riparian zones that play critical roles in preventing excess $NO_3^-$ pollution into rivers.

In order to better understand the OTC effects on microbial-driven denitrification and anammox activities in a riparian buffer zone, soil slurry $^{15}N$ tracer incubation experiments combined with microbial network analyses were used to evaluate: 1) variations in denitrification and anammox rates; 2) shifts in the presence of key functional genes; and 3) response and adaptation of soil bacteria communities.

## 2 Material and methods

### 2.1 Study area and soil sampling

The study area is located in a riparian buffer zone (39°29′N, 117°22′E) of the Chaobaixin River in the Beijing-Tianjin-Hebei region, with a livestock farm located upstream of the sampling sites. The riparian buffer zone is comprised of woodlands and weeds. Salinized tidal soil is the dominant soil type of the area with pH 7.1-8.0. The study area has a semi-moist temperate continental monsoon climate. The mean annual temperature is 11.4°C-12.9°C, and the annual precipitation is approximately 520-660 mm, with 75% falling from June to August.

Three plots (5 m×5 m) with 20 m intervals were selected as the experimental sites for the study. In each plot, soil was collected from 0-20 cm depth (i.e., the plow layer) and eight drill samples were mixed as one plot-level soil sample with three replicates, resulting in a total of nine samples (e.g., 3 plots×3 replicates)[27]. After removing roots and stones, the composite soil samples were sieved through a 2-mm mesh and immediately taken to the laboratory on ice bags. To eliminate potential interference, ambient antibiotics in soil samples were removed through a pre-incubation experiment[8]. Then each sample was separated into three parts, one of which was air-dried at room temperature for soil physicochemical properties analyses as described in Table 1; the second part was stored at 4°C for slurry-based $^{15}N$ tracer experiments; and the third part was stored at –80°C for soil molecular analysis.

**Table 1 Soil physicochemical properties at different OTC addition levels**

| OTC treatment | pH | $NO_3^-$-N | $NH_4^+$-N | TOC | TN |
|---|---|---|---|---|---|
| | | /mg·kg$^{-1}$ | | /g·kg$^{-1}$ | |
| CK | 6.34±0.15$^a$ | 7.49±1.70$^b$ | 1.78±0.19$^b$ | 8.25±0.75$^a$ | 1.08±0.03$^a$ |
| $OTC_1$ | 6.43±0.32$^a$ | 8.93±0.62$^{ab}$ | 1.87±0.13$^{ab}$ | 8.26±0.88$^a$ | 1.08±0.07$^a$ |
| $OTC_2$ | 6.58±0.27$^a$ | 10.09±0.32$^a$ | 1.99±0.09$^{ab}$ | 8.31±0.82$^a$ | 1.10±0.05$^a$ |
| $OTC_3$ | 6.64±0.32$^a$ | 10.56±0.53$^a$ | 2.02±0.09$^a$ | 8.36±0.68$^a$ | 1.11±0.04$^a$ |

Note: TOC: total organic carbon; TN: total nitrogen. Values are means ±standard deviations (SD) for three replicates. Lowercase letters in the same column indicate statistically significant differences at $p<0.05$ level. CK, $OTC_1$, $OTC_2$, and $OTC_3$ indicate OTC contents of 0, 50, 200, and 400 mg/kg, respectively. Same as the tables below.

### 2.2 $^{15}N$ tracer experiments and reaction rate calculations

The potential rates of denitrification (Denitrif-$N_2$) and anammox (Anammox-$N_2$) were determined with a $^{15}N$ tracer soil slurry incubation approach[28,29]. First, fresh soil (3 g) was weighed into 12-mL Exetainer tubes (Labco, High Wycombe, UK) that were injected with helium (He)-purged Milli Q water to completely fill the glass vial, and then sealed using a gas-tight cap. To remove residual $NO_x^-$ and oxygen, samples were pre-incubated for 7 d at 25°C in the dark under anoxic conditions. Then, all vials were divided into three groups that were injected with a stock solution of (a) $^{15}NH_4^+$(99.09% $^{15}N$), (b) $^{15}NH_4^+$ plus $^{14}NO_3^-$, or (c) $^{15}NO_3^-$ (99.14% $^{15}N$) to achieve a final concentration of 100 $\mu$mol/L $^{15}N$ in each vial. $^{15}NH_4^+$ (99.09% $^{15}N$) measurements were used to evaluate whether microorganisms had completely consumed background $^{14}NO_3^-$ in soil samples during pre-incubation. The second treatment was used to demonstrate the presence of anammox bacteria in samples. $^{29}N_2$ was used to calculate the anammox activity, and $^{30}N_2$ was used to calculate the denitrification rates in third treatments with $^{15}NO_3^-$ addition. The $^{15}NO_3^-$ (99.14% $^{15}N$) vials were then injected with 500 $\mu$L of He-purged oxytetracycline (OTC) to achieve contents of 0 (CK), 50 ($OTC_1$), 200 ($OTC_2$), 400 ($OTC_3$), 600 ($OTC_4$), and 800 ($OTC_5$) mg/kg (six replicates per treatment). Consistent with previous results[30], the $N_2$ production increased linearly in the first 8 h of incubation, and then tended to be stable. Therefore, the optimal incubation time was selected as 8 h. After 0 and 8 h of incubation: 1) half of the replicates in each treatment were injected with 200 $\mu$L of a saturated $ZnCl_2$ solution to quench microbial activity for determination of $^{29}N_2$ and $^{30}N_2$, whereas 2) the remaining half of assays were immediately frozen without addition of saturated $ZnCl_2$ solution and then stored at −80°C until subsequent genomic DNA extraction. The isotopic compositions of $N_2$ (i.e., $^{29}N_2$ and $^{30}N_2$) were determined by Membrane Inlet Mass Spectrometry (MIMS) (Bay Instruments, Easton, MD) in soil slurries[31]. The potential rates of Denitrif-$N_2$ and Anammox-$N_2$ were obtained using linear regressions between the $N_2$ production of samples and the corresponding sampling time points. Calculations were conducted as previously described[29], based on equations established by Thamdrup and Dalsgaard[32].

### 2.3 Functional gene and microbial community analyses

#### 2.3.1 Quantitative PCR

Functional genes (*nosZ* and *hzs*B) were quantitatively assessed by real-time fluorescent quantitative PCR (qPCR) (ABI7900, Applied Biosystems Inc., USA). PCR reaction system (25 $\mu$L) contained 5 $\mu$L of Ex Taq buffer, 2 $\mu$L of dNTP Mix (2.5 mM), 0.5 $\mu$L of Ex Taq, 1 $\mu$L of each primer (10 $\mu$mol/L), 1 $\mu$L of template DNA, and 12.5 $\mu$L of $ddH_2O$. Specific primers for *nosZ* and *hzs*B genes are shown in Table 2. The amplification efficiencies were 98% and the $R^2$ of the standard curve was >0.99.

**Table 2 Specific primers and conditions**

| | Primer | Sequence (5′-3′) | Conditions |
|---|---|---|---|
| *nosZ* | *nosZ*F | CGYTGTTCMTCGACAGCCAG | Pre-denaturation: 95°C, 3 min<br>Denaturation: 98°C, 30 sec<br>Annealing: 60°C, 30 sec<br>Extension: 72°C, 30 sec<br>Final-extension: 72°C, 10 min |
| | *nosZ*1622R | CGSACCTTSTTGCCSTYGCG | |
| *hzs*B | Amx809F | GCCGTAAACGATGGGCACT | |
| | Amx1066R | AACGTCTCACGACACGAGCTG | |
| Bacteria | 338F | ACTCCTACGGGAGGCAGCAG | |
| | 806R | GGACTACHVGGGTWTCTAAT | |

2.3.2 DNA extraction and high-throughput sequencing

DNA was extracted from 0.5 g frozen soil samples using the Fast DNA SPIN Kit for soil (MP Biomedical, Solon, OH, USA). DNA concentrations and purity were checked by 1% agarose gel electrophoresis using the spectrophotometer (NanoDrop 2000, Thermo Scientific, Wilmington, USA). PCR amplification was performed using specific primers (Table 2), and the products were purified, quantified, and homogenized to form sequencing libraries. Microbial community compositions were determined based on high-throughput sequencing of amplicons. Sequences of high quality (length>300 bp, without ambiguous bases 'N', and average base quality scores>20) were used for downstream analyses. Operational taxonomic units (OTUs) were constructed by clustering sequences at the 97% nucleotide sequence similarity cutoff using the UPARSE (http://drive5.com/uparse/, version 7.1) program. Chimeric sequences were removed using the UCHIME algorithm[33]. Taxonomic classification was assigned using the Ribosomal Database Project (RDP) classifier[34]. Alpha diversity indices were calculated from the quality-filtered sequence data, including the Chao1 estimator of richness and Shannon's diversity. The raw sequence data were deposited in the National Center for Biotechnology Information database under the accession PRJNA792478 (https://www.ncbi.nlm.nih.gov/sra/PRJNA792478).

2.3.3 Co-occurrence network construction and analyses

Microbial ecological networks were constructed using the "microeco" package[35]. Firstly, the microtable class was created and the basic preprocessing operations were performed. The OTUs table was rarefied to make the sequence number the same across samples (10 000 sequences in each sample). The taxa abundance and alpha diversity were all calculated for the downstream analysis. To further study the species co-occurrence patterns, associations between the microbial communities were examined by calculating all possible Pearson rank correlations using the trans_network class. A valid interaction event was considered to be a robust correlation if the Pearson correlation coefficient was either equal to or greater than 0.8 or less than −0.8 and statistically significant ($p$<0.01). The network structure was explored and visualized with the interactive platform gephi (https://gephi.org) using undirected network and the Fruchterman–Reingold layout.

### 2.4 Statistical analyses

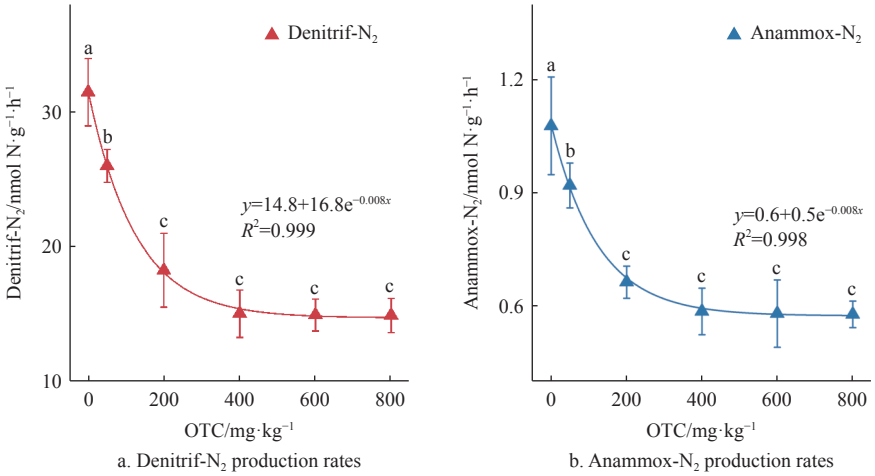
The KEGG PATHWAY database was used to screen the genera containing *nosZ* or *hzs*B genes in Ko00910 nitrogen metabolism.

The analyses were carried out in the SPSS v19.0 software and R v4.2.1 software[36]. All data were evaluated to validate the model assumptions of normality and equality of variance. Means comparisons were conducted by one-way ANOVA, followed by the Least Significant Difference (LSD) test. Spearman's rank correlation was performed to test relations between the variables using the "corr.test" function. Significance for all statistical tests was evaluated at $p$=0.05.

## 3 Results

### 3.1 Denitrification (Denitrif-$N_2$) and anammox (Anammox-$N_2$) rates at different contents of OTC

The $^{29}N_2$ and $^{30}N_2$ production rates were calculated from the slopes of linear regressions of changes in 29 and 30 masses across incubation times with $^{15}NO_3^-$. A negative, exponential relationship was observed between Denitrif-$N_2$ and/or Anammox-$N_2$ rates and OTC contents (Figure 1; $R^2$=0.999 for Denitrif-$N_2$ and $R^2$=0.998 for Anammox-$N_2$). Rates of both processes rapidly decreased at OTC contents < 400 mg/kg, while the decrease rate gradually flattened at OTC contents > 400 mg/kg. As OTC contents increased, the rates reduced from 31.5 (CK: 0 mg/kg) to 14.9 nmol N/g·h ($OTC_5$: 800 mg/kg) for Denitrif-$N_2$ and from 1.1 (CK: 0 mg/kg) to 0.6 nmol N/g·h ($OTC_5$: 800 mg/kg) for Anammox-$N_2$. The rate reduction percentages of Denitrif-$N_2$ and Anammox-$N_2$ substantially increased from 17.0% to 52.5% and from 14.0% to 45.8%, respectively, compared with the CK in an OTC content between 50 ($OTC_1$) and 800 ($OTC_5$) mg/kg (Figure 2). The rate reduction percentages of Denitrif-$N_2$ and Anammox-$N_2$ reached 52.3% and 45.5%, at OTC content of 400 mg/kg. Variations in the rate reduction percentages were small at higher OTC content (i.e., > 400 mg/kg).




a. Denitrif-$N_2$ production rates b. Anammox-$N_2$ production rates

Note: Error bars indicate standard deviations (SD) based on three replicates. [a,b,c] Different lowercase letters indicate significant differences ($p$<0.05) as determined by Fisher LSD test. Same as the figures below.

Figure 1 $N_2$ production rates at different OTC addition levels

### 3.2 Denitrification and anammox functional gene copy numbers at different contents of OTC

Since variation was small at OTC contents > 400 mg/kg (Figure 1), microbial analyses were primarily focused upon groups with OTC contents ranging from 0 to 400 mg/kg. Abundances of denitrification and anammox functional genes (*nosZ* and *hzs*B) that encode nitrous oxide reductase and hydrazine synthetase, respectively, were quantified in the soil slurry incubation

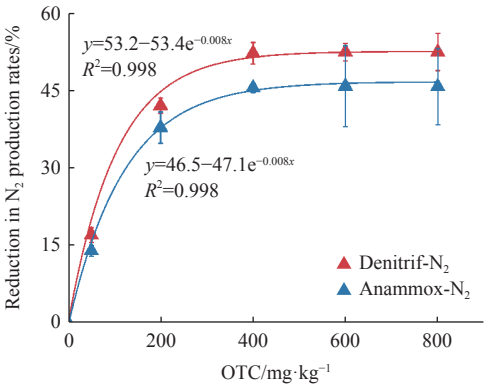



Figure 2 Reduction percentages of $N_2$ production of denitrification (Denitrif-$N_2$) and anammox (Anammox-$N_2$) at different OTC addition levels

experiments. Negative, exponential relationships were observed between the abundances of the functional gene *nosZ* or *hzs*B and OTC contents (Figure 3; $R^2$=0.999 and $R^2$=0.982, respectively). Specifically, the abundances of *nosZ* and *hzs*B genes decreased from $6.3\times10^7$ (CK: 0 mg/kg) to $4.9\times10^7$ ($OTC_3$: 400 mg/kg) copies/g and from $2.9\times10^9$ (CK: 0 mg/kg) to $2.0\times10^9$ ($OTC_3$: 400 mg/kg) copies/g, respectively. Compared with the CK groups, the abundances of *nosZ* and *hzs*B genes were reduced from 21.8% to 10.4% and from 32.1% to 18.4% at OTC contents between 50 ($OTC_1$) and 400 mg/kg ($OTC_3$), respectively. Interestingly, a linear relationship was observed between Denitrif-N2 and Anammox-N2 rates and their corresponding functional genes (*nosZ* and *hzs*B) at different contents of OTC (Figure 4).

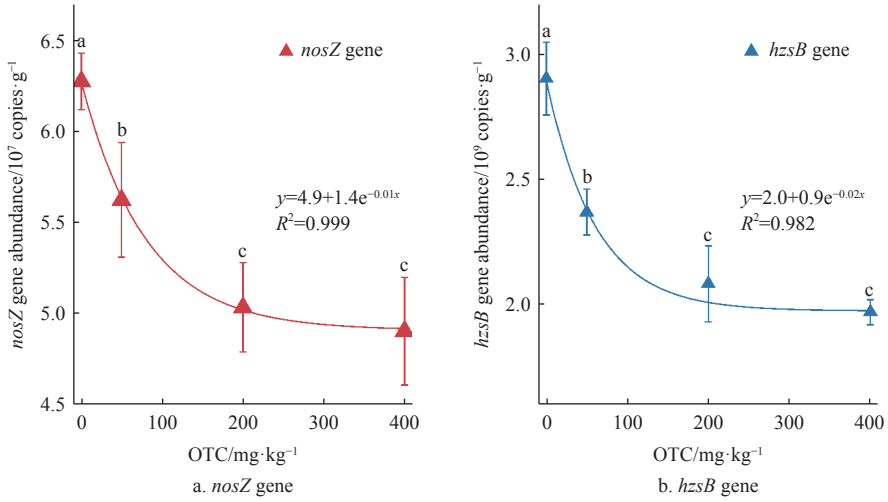



Figure 3 Variations in gene abundances at different OTC addition levels

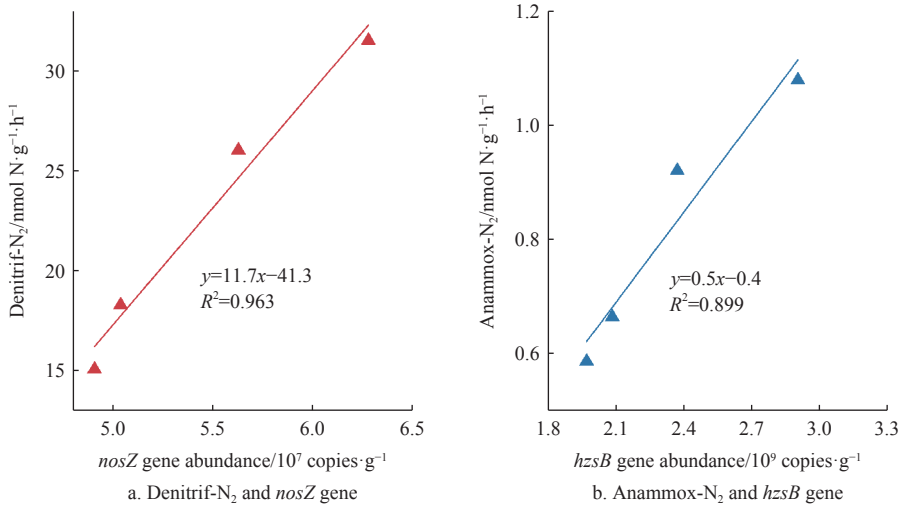


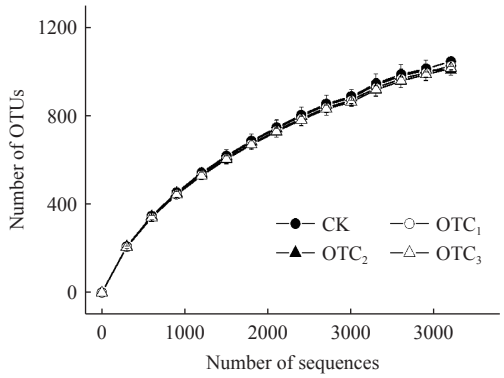

Figure 4 Relationships between $N_2$ production rates and gene abundances

### 3.3 Composition and structure of soil microbial communities at different contents of OTC

A total of 697 053 high-quality sequences (average length of 416 bp) were obtained from 12 samples (3 replicates each for CK, $OTC_1$, $OTC_2$, and $OTC_3$), which were clustered into 4249 OTUs. Rarefaction curves indicated that the bacterial OTUs were fully sampled with increasing sequencing depth (Figure 5). The CK and OTC group communities did not exhibit significantly different Shannon and Chao1 index values (Table 3), but OTC groups tended to reduce the diversity and richness of the communities.

To investigate the specific changes of microbiota in samples, the relative abundance of taxa in OTC and CK groups was assessed. At the phylum level, microbial communities in soils were primarily comprised of *Actinobacteriota*, *Proteobacteria*, *Chloroflexi*, *Acidobacteriota*, and *Gemmatimonadota*, accounting for more than 85% of the total identified phyla (Figure 6a), and their abundance in CK and OTC groups was similar (Figure 7a). At the genus level, the top five genera were *Kaistobacter*, *Balneimonas*, *Streptomyces*, *Rhodoplanes*, and *Mycobacterium* (Figure 6b). There were no significant differences in relative abundance between CK and OTC

groups (Figure 7b).




Note: CK, $OTC_1$, $OTC_2$, and $OTC_3$ indicate OTC contents of 0, 50, 200, and 400 mg/kg, respectively. Same as the figures below.

Figure 5 Rarefaction curves for different OTC addition levels

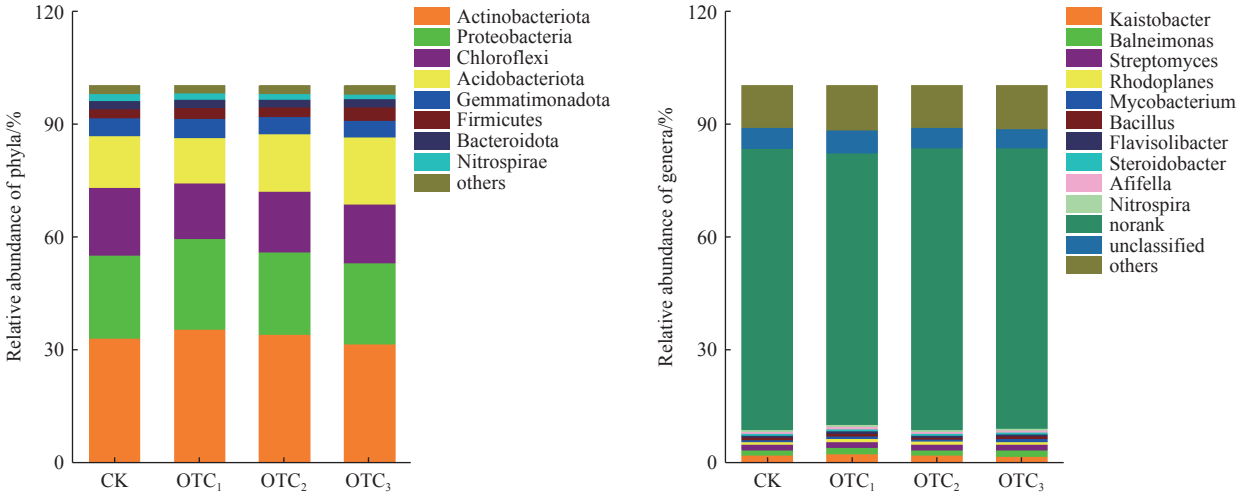
### 3.4 Co-occurrence networks of soil microbial communities at different contents of OTC

To understand the potential interactions of soil bacterial communities at different OTC contents, 16S rRNA gene OTU distributions were used to construct the co-occurrence networks for the CK and OTC groups (Figure 8). Based on the topological features presented in Table 4, the number of nodes ranged from 442 to 446, and the number of links ranged from 21 267 to 21 986. On average, each node had 50.66, 48.99, 48.12, and 48.94 edges, respectively. Moreover, the modularity (MD) demonstrated that OTC groups had a higher value (0.660, 0.641, and 0.654) compared to CK (0.626). As for soil bacterial communities, higher percentages of positive links (from 52.96% to 58.11%) were also observed in the OTC groups compared to in the CK group (51.60%).

**Table 3 Shannon and Chao1 index at different OTC addition levels**

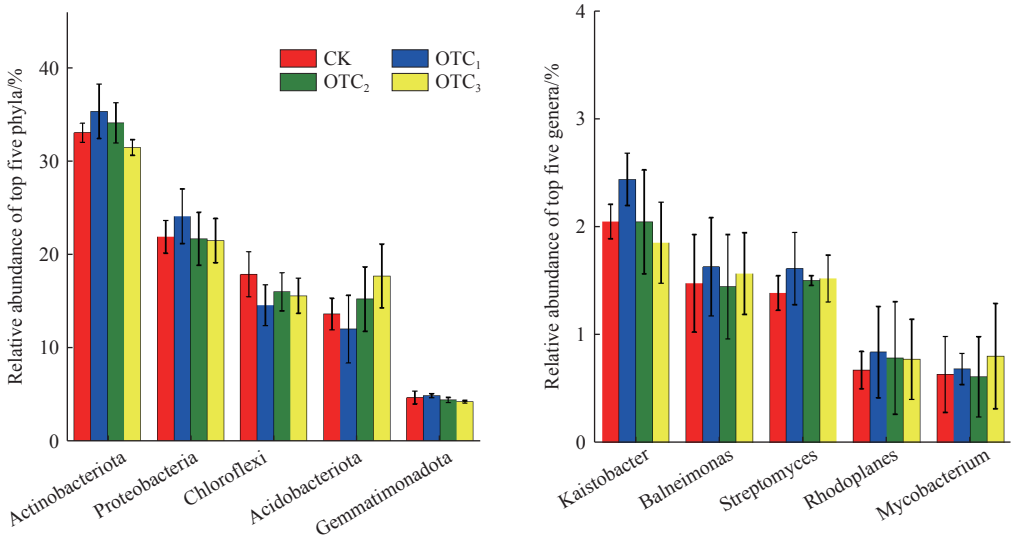
| OTC treatment | Shannon index | Chao1 index |
|---|---|---|
| CK | 6.44±0.06[a] | 2996±233[a] |
| $OTC_1$ | 6.42±0.13[a] | 2992±204[a] |
| $OTC_2$ | 6.38±0.11[a] | 2940±153[a] |
| $OTC_3$ | 6.36±0.09[a] | 2882±80[a] |

Note: Values are means ± standard deviations (SD) for three replicates.



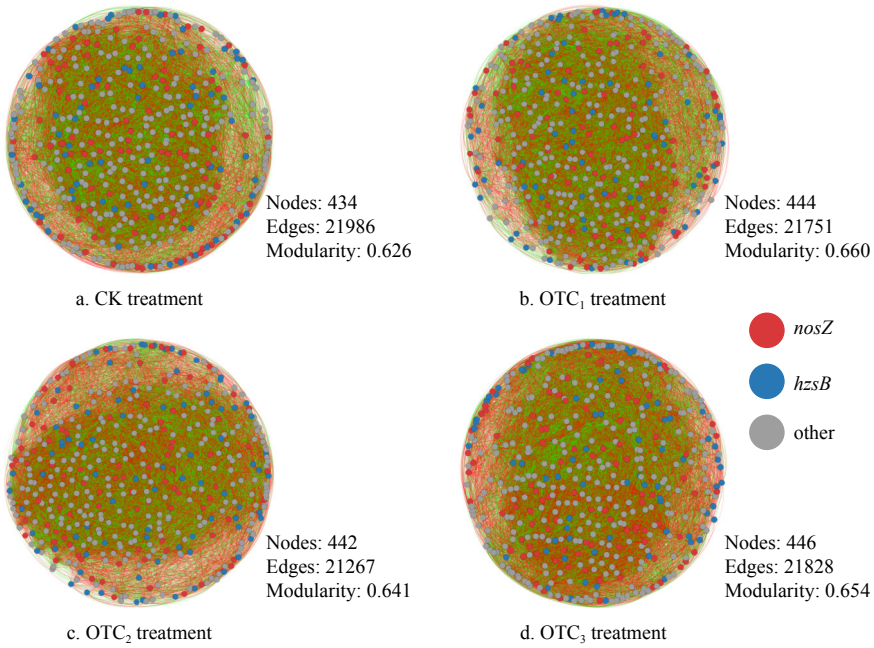

a. phylum level b. genus level

Figure 6 Stacked bar chart showing the relative abundance of bacteria in soils




a. phylum level b. genus level

Figure 7 Relative abundances of the top five phyla and genera as affected by OTC




Note: Only Spearman's correlation coefficient ($r$>0.8 or $r$<–0.8; significant at $p$<0.01) is shown. Each node represents a bacteria genus. Red nodes represent genera containing *nosZ* gene, blue nodes represent genera containing *hzs*B gene, and grey nodes indicate sums for the genera neither containing *nosZ* nor *hzs*B gene. Lines connecting two bacteria genera represent the interactions between them. Red lines represent the positive significant correlations, and green lines represent the negative significant correlations. Same as the figures below.

Figure 8 Co-occurrence networks for soil bacterial communities in different treatments

**Table 4 Co-occurrence network topological features for soil bacterial communities at different OTC addition levels**

| OTC treatment | Nodes | Links | Modularity (MD) | Positive link/% | Negative link/% |
|---|---|---|---|---|---|
| CK | 434 | 21 986 | 0.626 | 51.60 | 48.40 |
| $OTC_1$ | 444 | 21 751 | 0.660 | 52.96 | 47.04 |
| $OTC_2$ | 442 | 21 267 | 0.641 | 55.38 | 44.62 |
| $OTC_3$ | 446 | 21 828 | 0.654 | 58.11 | 41.89 |

In the co-occurrence network, red nodes represent genera containing *nosZ* gene, blue nodes represent genera containing *hzs*B gene, and grey nodes indicate sums for the genera neither containing the *nosZ* nor *hzs*B gene. The proportion of nodes containing *nosZ* and *hzs*B genes ranged from 20.4% to 22.3% and 16.6% to 19.0%, respectively. To explore the impact of OTC on the genera containing *nosZ* and *hzs*B genes, the sub-networks for *nosZ* and *hzs*B genes without other microbial communities were constructed (Figure 9). The corresponding topological features are summarized in Table 5. Overall, the four sub-networks have similar nodes (31.25±1.26). However, compared with CK (links: 142; modularity: 0.546), OTC groups demonstrated fewer links (137, 133, and 92), but higher modularity (0.562, 0.592, and 0.651). The proportion of positive links ranged from 56.34% to 67.67% in the OTC groups and was 50.36% in the CK group.

There were 36 genera containing the *nosZ* gene in the four sub-networks (Table 6), in which only eight genera (occupying 22.2%) were involved in denitrification (defined as denitrification mode bacteria), and the remaining 28 (occupying 77.8%) were non-denitrification mode bacteria. This result implies the importance of non-denitrification mode bacteria in the denitrification process. The denitrification mode bacteria which appeared in the CK group were all in the OTC groups, and five genera displayed different abundances at different OTC contents ($p$<0.05, Table 6).

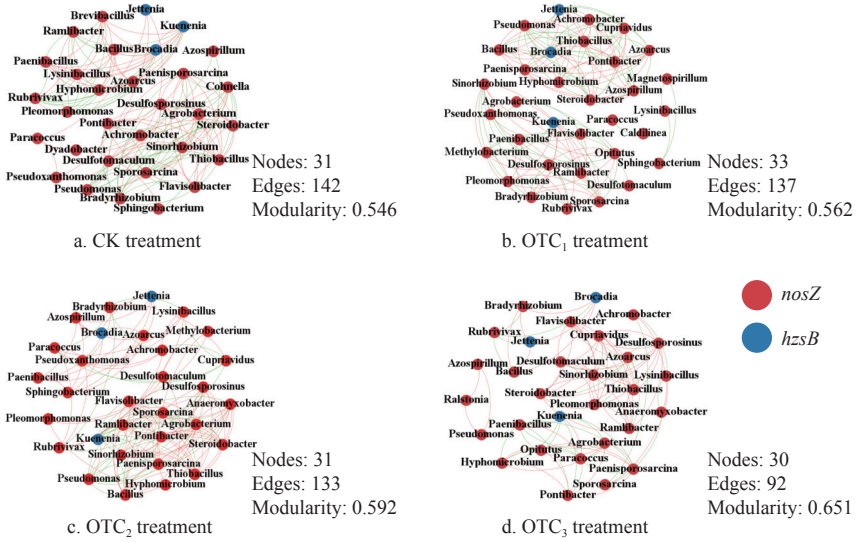



Figure 9 Co-occurrence sub-networks for soil bacterial communities containing *nosZ* and *hzs*B genes in different treatments

**Table 5 Co-occurrence sub-network topological features for soil bacterial communities at different OTC addition levels**

| OTC treatment | Nodes | Links | Modularity(MD) | Positive link/% | Negative link/% |
|---|---|---|---|---|---|
| CK | 31 | 142 | 0.546 | 50.36 | 49.64 |
| $OTC_1$ | 33 | 137 | 0.562 | 56.34 | 43.66 |
| $OTC_2$ | 31 | 133 | 0.592 | 67.67 | 32.33 |
| $OTC_3$ | 30 | 92 | 0.651 | 65.22 | 34.78 |

**Table 6 Genus containing *nosZ* and *hzs*B genes in co-occurrence sub-networks of CK and OTC treatments**

| Functional genes | | Genus | Abundance/% | | | |
|---|---|---|---|---|---|---|
| | | | CK | $OTC_1$ | $OTC_2$ | $OTC_3$ |
| *nosZ* | Denitrification mode bacteria | *Hyphomicrobium* | 0.476$^{a}$ | 0.459$^{a}$ | 0.450$^{a}$ | 0.302$^{b}$ |
| | | *Anaeromyxobacter* | - | - | 0.015 | - |
| | | *Azospirillum* | 0.074$^{a}$ | 0.048$^{b}$ | 0.039$^{bc}$ | 0.026$^{c}$ |
| | | *Bacillus* | 1.926 | 1.973 | 1.676 | 1.860 |
| | | *Paenibacillus* | 0.614$^{a}$ | 0.520$^{b}$ | 0.546$^{b}$ | 0.489$^{b}$ |
| | | *Paracoccus* | 0.165$^{a}$ | 0.055$^{b}$ | 0.091$^{b}$ | 0.067$^{b}$ |
| | | *Pseudomonas* | 0.048$^{a}$ | 0.038$^{ab}$ | 0.043$^{a}$ | 0.031$^{b}$ |
| | | *Thiobacillus* | 0.075 | 0.073 | 0.076 | 0.083 |
| | Non-denitrification mode bacteria | *Achromobacter* | 0.137$^{a}$ | 0.064$^{b}$ | 0.064$^{b}$ | 0.024$^{c}$ |
| | | *Agrobacterium* | 0.356 | 0.320 | 0.368 | 0.322 |
| | | *Azoarcus* | 0.301$^{a}$ | 0.308$^{a}$ | 0.313$^{a}$ | 0.112$^{b}$ |
| | | *Bradyrhizobium* | 0.465 | 0.495 | 0.509 | 0.455 |
| | | *Desulfosporosinus* | 0.094 | 0.101 | 0.109 | 0.107 |
| | | *Desulfotomaculum* | 0.043 | 0.038 | 0.044 | 0.050 |
| | | *Flavisolibacter* | 2.012$^{a}$ | 1.797$^{ab}$ | 1.423$^{b}$ | 1.498$^{b}$ |
| | | *Lysinibacillus* | 0.053$^{a}$ | 0.022$^{b}$ | 0.016$^{b}$ | 0.021$^{b}$ |
| | | *Paenisporosarcina* | 0.544$^{c}$ | 1.052$^{b}$ | 0.949$^{b}$ | 1.770$^{a}$ |
| | | *Pleomorphomonas* | 0.084 | 0.070 | 0.058 | 0.056 |
| | | *Pontibacter* | 1.014$^{a}$ | 0.930$^{ab}$ | 0.786$^{b}$ | 0.854$^{b}$ |
| | | *Ramlibacter* | 0.405$^{c}$ | 0.527$^{b}$ | 0.523$^{b}$ | 0.726$^{a}$ |
| | | *Rubrivivax* | 0.631 | 0.705 | 0.666 | 0.643 |
| | | *Sinorhizobium* | 0.627 | 0.714 | 0.698 | 0.670 |
| | | *Sporosarcina* | 0.119 | 0.145 | 0.109 | 0.053 |
| | | *Steroidobacter* | 1.632 | 1.734 | 1.592 | 1.745 |
| | | *Opitutus* | 0.014 | 0.027 | - | 0.026 |
| | | *Brevibacillus* | 0.041 | - | - | - |
| | | *Cohnella* | 0.011 | - | - | - |
| | | *Dyadobacter* | 0.009 | - | - | - |
| | | *Caldilinea* | - | 0.012 | - | - |
| | | *Magnetospirillum* | - | 0.012 | - | - |
| | | *Pseudoxanthomonas* | 0.085 | 0.037 | 0.071 | - |
| | | *Sphingobacterium* | 0.025 | 0.021 | 0.023 | - |
| | | *Methylobacterium* | - | 0.010 | 0.012 | - |
| | | *Flavobacterium* | - | 0.012 | - | - |
| | | *Cupriavidus* | - | 0.010 | 0.019 | 0.012 |
| | | *Ralstonia* | - | - | - | 0.043 |
| *hzs*B | Anammox mode bacteria | *Candidatus Jettenia* | 0.613$^{b}$ | 0.600$^{b}$ | 0.768$^{ab}$ | 1.088$^{a}$ |
| | | *Candidatus Brocadia* | 0.518 | 0.491 | 0.611 | 0.576 |
| | | *Candidatus Kuenenia* | 0.244 | 0.265 | 0.234 | 0.268 |

Note: "-" indicates that the genus was not detected.

*Azospirillum*, *Paenibacillus*, and *Paracoccus* were significantly higher in the CK group than in OTC groups. The differences of *Hyphomicrobium* and *Pseudomonas* were smaller in the CK and OTC groups, but their relative abundances were still significantly lower in $OTC_3$ content. *Azospirillum* only appeared in the OTC groups. The non-denitrification mode bacteria in the CK group (except *Brevibacillus, Cohnella*, and *Dyadobacter*) were mostly in the OTC groups, and seven genera exhibited different abundances at different OTC contents ($p$<0.05, Table 6). *Achromobacter*, *Flavisolibacter*, *Lysinibacillus*, and *Pontibacter* were significantly decreased by OTC, while *Paenisporosarcina* and *Ramlibacter* were increased. *Azoarcus* was significantly lower in $OTC_3$ content than in other groups. There were some genera that only appeared in the OTC groups: *Caldilinea*, *Magnetospirillum*, and *Flavobacterium* were observed in the $OTC_1$ content; *Ralstonia* was only found in $OTC_3$ content; *Methylobacterium* was detected in $OTC_1$ and $OTC_2$ contents; and *Cupriavidus* was observed in all the OTC groups. There were three genera containing the *hzs*B gene, all of which were involved in anammox. The relative abundance of *Candidatus Jettenia* significantly increased ($p$<0.05, Table 6).

## 4 Discussion

### 4.1 Denitrification (Denitrif-$N_2$) and anammox (Anammox-$N_2$) rates and functional genes at different OTC contents

In the denitrification and anammox metabolic pathways, *nosZ*, which catalyzes the conversion of nitrous oxide into nitrogen, and *hzs*B, which induces the transformation of nitric oxide (NO) into hydrazine ($N_2H_4$), are considered as the important functional genes to assess denitrification and anammox processes, respectively[37,38]. This study clearly demonstrated a linear correlation between Denitrif-$N_2$ and Anammox-$N_2$ rates and their corresponding functional genes (Figure 4), which likely suggests a genetic basis for antibiotic inhibition of denitrifying and anammox bacteria activities. When the OTC contents were lower than 400 mg/kg, the rates of Denitrif-$N_2$ and Anammox-$N_2$ (Figure 1), and the abundances of *nosZ* and *hzs*B genes (Figure 3) decreased remarkably. At concentrations higher than environmental concentrations (>1 mg/L), antibiotics are toxic to bacteria and inhibit their growth via preventing ribosome formation, inhibiting peptide chain extension, and blocking protein synthesis[19,20], thereby negatively affecting the removal efficiencies of nitrate. Noophan et al.[39] reported that after 7 h of OTC exposure, at concentrations of 25-100 mg/L, anammox activity was inhibited by 22.15%. Yin et al.[8] observed that even at ultra-low OTC concentrations of 5.13-22.5 ng/L, the denitrification rates were reduced by as much as 44.2%. In this study, at OTC contents of 400 mg/kg, Denitrif-$N_2$ and Anammox-$N_2$ rates were cut down by up to 52.3% and 45.5%, respectively, implying that OTC exposure led to greater inhibition on denitrification and anammox rates in riparian buffer zones. The average OTC concentration in the Chaobaixin River was around 13.1 ng/L (approximately 0.0131 mg/kg)[40], and Denitrif-$N_2$ and Anammox-$N_2$ rates were estimated to be reduced by 10.3% and 8.2% based on the incubation experiments, respectively (Figure 2). This indicates that inorganic nitrogen removal efficiency from denitrification and anammox will rapidly decrease upon addition of even small contents of external antibiotics. These results suggest that the inhibition of OTC on denitrification and anammox performance may be factors causing the retention of excessive inorganic N in the riparian ecosystem, thereby further contributing to hyper-eutrophication in the nearby Bohai Bay. Nitrous oxide is considered a main greenhouse gas with higher radiative forcing potential than carbon dioxide ($CO_2$) and methane ($CH_4$)[41]. The inhibition of OTC on the *nosZ* gene that controls $N_2O$ reduction to $N_2$ (10.4%-21.8% decrease in number) could contribute to an increase in $N_2O$ production in the riparian buffer zones, and the resulting release of $N_2O$ would lead to greenhouse effects and atmosphere ozone depletion[41,42]. When OTC contents were higher than 400 mg/kg, the effects on denitrification and anammox rates were not obvious. This was likely the response of antibiotic

resistance genes (ARGs) in the denitrifying and anammox bacteria. Some denitrifying genera encoded functional genes such as *nir*B, *nir*K, *nir*S, and *nos*Z, which simultaneously contained multidrug resistance genes (e.g., *otr*A, *otr*B, *tet*A, *tet*PB, *tet*W, and *tet*M)[13], which may help the function of denitrifying bacteria exposed to environmental antibiotics. In addition, studies have found that denitrifying and anammox communities may develop ARGs to alleviate the stress caused by antibiotics exposure[43].

### 4.2 Response and adaptation of soil microbial communities at different OTC contents

Co-occurrence networks have been used to identify the key pathways that act in concert to regulate bacteria responses to antibiotics[44]. Modularity features allow different taxa groups to perform independent functions, resulting in less overlap and connectivity between taxa[45]. In this study, compared to the CK group, OTC exposure resulted in bacterial taxa forming high modularity networks (Table 4), which can provide an advantage for bacterial communities to be more resilient to antibiotic stress[46]. Furthermore, antibiotic exposure in the environment could impose severe pressure on the microbial community, so cooperation among microorganisms is necessary for survival[47]. In our study, the percentages of co-presence links in the OTC groups (ranging from 52.96% to 58.11%) were higher than in the CK group (51.60%) (Table 4), suggesting the prevalence of interspecific cooperation of bacteria populations that were promoted by OTC exposure. These results were consistent with the findings of some studies that microbes cooperate for various purposes, including for acquiring nutrients, and providing protection against antibiotics and harsh environments[48-50]. This implies that bacteria adapt to short-term OTC exposure by increasing the cooperation between microbes and reducing their functional overlap.

Bacterial communities containing *nos*Z and *hzs*B genes as the crucial participants in denitrification and anammox processes should play a crucial role in regulating the Dentrif-$N_2$ and Anammox-$N_2$ rates[37,38]. Thus, the sub-networks were constructed to further explore the impact of OTC on the genera containing *nos*Z and *hzs*B genes. Higher modularity and positive links were observed in the co-occurrence sub-networks containing *nos*Z and *hzs*B genes as well (Table 5). This suggests that altered interspecies interaction and increased community complexity are important strategies for bacteria to cope with antibiotic stress. In the sub-networks, functional bacteria containing *nos*Z and *hzs*B genes underwent significant changes in short-term OTC exposure (Table 6), which would seriously affect the ecosystem function of denitrification and anammox processes. More specifically, both the denitrification and non-denitrification mode bacteria containing *nos*Z gene which appeared in the CK group were mostly in the OTC groups (23 genera), indicating that they are an important component of maintaining network stability and complexity when exposed to antibiotics. Due to different sensitivity of bacteria to antibiotics, some researchers stated distinct conclusions that included inhibition[51] as well as no significant effects[13]. In addition, bacteria with antibiotic degrading enzymes were more resistant to antibiotics than those without them. Moreover, the abundance may not be affected or may slightly increase compared to those that were inhibited[13]. The duration of antibiotic exposure can also influence their effectiveness[52,53], and at short time scales (8h), antibiotics may only affect a subset of sensitive microorganisms. In this study, the responses of the denitrification and non-denitrification mode bacteria on antibiotics exposure can be attributed to a combination of factors including antibiotic sensitivity, microbial resistance, and duration of antibiotic exposure. Thus, some genera demonstrated inhibitory effects with abundance decrease (e.g., denitrification mode bacteria: *Hyphomicrobium*, *Azospirillum*, *Paenibacillus*, *Paracoccus*, and *Pseudomonas*; and non-denitrification mode bacteria: *Achromobacter*, *Azoarcus*, *Flavisolibacter*, *Lysinibacillus*, and *Pontibacter*); some demonstrated promoting effects with abundance increase (e.g., non-denitrification mode bacteria: *Paenisporosarcina* and *Ramlibacter*); while some had no significant effects (the remaining 11). Moreover, some genera (non-denitrification mode bacteria: *Brevibacillus*, *Cohnella*, and *Dyadobacter*) containing *nos*Z gene only appeared in the CK group, and they were quite sensitive to OTC. The *Brevibacillus*, *Cohnella*, and *Dyadobacter* disappear under low OTC exposure, which can explain the rapid decline of Denitrif-$N_2$ at the low OTC content (Figure 1a). Simultaneously, some genera containing *nos*Z gene only appeared in the OTC group. The non-denitrification mode bacteria of *Caldilinea*, *Magnetospirillum*, *Flavobacterium*, and *Methylobacterium* were observed in the $OTC_1$ and/or $OTC_2$ content, which played a substantial role in antibiotic resistance but whose proliferation was inhibited by high OTC ($OTC_3$) content. It is quite interesting to find the strong adaptability of non-denitrification mode bacteria of *Cupriavidus* and *Ralstonia*, which tend to be enriched in $OTC_3$ group (Table 6). Ryan et al.[54] and Ruiz et al.[55] reported that *Cupriavidus* and *Ralstonia* were the emerging opportunistic pathogens and naturally resistant to many classes of antibiotics, as well as potentially contributing to the denitrification process. The three anammox mode bacteria which appeared in the CK group were all in the OTC groups. OTC increased the abundance of *Candidatus Jettenia* but did not significantly impact the *Candidatus Brocadia* and *Candidatus Kuenenia* (Table 6). *Candidatus Jettenia* released extracellular polymeric substances in excess and enveloped cells with them, and could thus protect themselves from the toxic effects of other substances[56]. The results indicate that the denitrification performance recovery under antibiotics exposure should depend on the maintenance of a diverse denitrifying community, as well as highlight the importance of non-denitrification mode bacteria in maintaining the denitrification process under short-term antibiotics exposure. Moreover, short-term OTC exposure seemed unable to affect all types of bacteria containing *nos*Z and *hzs*B genes, which allowed a considerable portion of the bacteria to continue to participate in denitrification and anammox processes to maintain a certain nitrogen removal performance.

## 5 Conclusions

Denitrification and Anammox performance intensely decreased in the presence of OTC, but inhibitory effects appeared at high OTC contents. The dominant bacterial community under short-term OTC exposure did not significantly change, while the bacterial network became more complex to keep the network stable. Moreover, the functional bacteria containing *nos*Z and *hzs*B genes demonstrated major changes in short-term OTC exposure, but there was still a considerable portion of unaffected bacteria which continued to participate in denitrification and anammox processes in order to accomplish the N removal process. The results imply that the denitrification performance recovery after short-term OTC exposure should depend on the maintenance of a diverse denitrifying community and highlight the importance of non-denitrification mode bacteria in maintaining the denitrification process under short-term OTC exposure, as they account for a larger proportion than denitrification mode bacteria. These results enhance our

understanding of the role of OTC in altering denitrification and anammox rates in riparian buffer zones and will help guide future efforts to control antibiotics pollution in riparian buffer zones and coastal ecosystems, which suffer from an increasing input of antibiotics and reactive nitrogen.

## Acknowledgements

This work was financially supported by the National Natural Science Foundation of China (Grant No. 41973017, 32071861, 41930862) and the Natural Science Foundation of Tianjin (Grant No. 19JCZDJC40700). We thank LetPub (www.letpub.com) for its linguistic assistance during the preparation of this manuscript.

## [References]

[1] Hou L J, Yin G Y, Liu M, Zhou J L, Zheng Y L, Gao J, et al. Effects of sulfamethazine on denitrification and the associated $N_2O$ release in estuarine and coastal sediments. Environmental Science & Technology, 2015; 49(1): 326–333.

[2] Hill A R. Groundwater nitrate removal in riparian buffer zones: A review of research progress in the past 20 years. Biogeochemistry, 2019; 143: 347–369.

[3] McPhillips L E, Groffman P M, Goodale C L, Todd Walter M. Hydrologic and biogeochemical drivers of riparian denitrification in an agricultural watershed. Water, Air, & Soil Pollution, 2015; 226: 169.

[4] Naeher S, Huguet A, Roose-Amsaleg C L, Laverman A M, Fosse C, Lehmann M F, et al. Molecular and geochemical constraints on anaerobic ammonium oxidation (anammox) in a riparian zone of the Seine Estuary (France). Biogeochemistry, 2015; 123: 237–250.

[5] Ribera-Guardia A, Kassotaki E, Gutierrez O, Pijuan M. Effect of carbon source and competition for electrons on nitrous oxide reduction in a mixed denitrifying microbial community. Process Biochem, 2014; 49(12): 2228–2234.

[6] Davidson E A. Fluxes of nitrous oxide and nitric oxide from terrestrial ecosystems. In: Rogers J E, Whitman W B (Eds.). Microbial Production and Consumption of Greenhouse Gases: Methane, Nitrogen Oxides and Halomethanes. Washington DC: American Society for Microbiology, 1991; pp.21–28.

[7] Jahangir M M R, Fenton O, Müller C, Harrington R, Johnston P, Richards K G. In situ denitrification and DNRA rates in groundwater beneath an integrated constructed wetland. Water Research, 2017; 111: 254–264.

[8] Yin G Y, Hou L J, Liu M, Zheng Y L, Li X F, Lin X B, et al. Effects of multiple antibiotics exposure on denitrification process in the Yangtze Estuary sediments. Chemosphere, 2017; 171: 118–125.

[9] Yan C X, Yang Y, Zhou J L, Liu M, Nie M H, Shi H, et al. Antibiotics in the surface water of the Yangtze Estuary: Occurrence, distribution and risk assessment. Environmental Pollution, 2013; 175: 22–29.

[10] Zhang Q Q, Bai Y H, Wu J, Xu L Z J, Zhu W Q, Tian G M, et al. Microbial community evolution and fate of antibiotic resistance gene in anammox process under oxytetracycline and sulfamethoxazole stresses. Bioresource Technology, 2019; 293: 122096.

[11] Zou Y, Lin M X, Xiong W G, Wang M, Zhang J X, Wang M Z, et al. Metagenomic insights into the effect of oxytetracycline on microbial structures, functions and functional genes in sediment denitrification. Ecotoxicology and Environmental Safety, 2018; 161: 85–91.

[12] Fan N S, Zhu X L, Wu J, Tian Z, Bai Y H, Huang B C, et al. Deciphering the microbial and genetic responses of anammox biogranules to the single and joint stress of zinc and tetracycline. Environment International, 2019; 132: 105097.

[13] Feng L J, Yang J Y, Yu H, Lan Z Y, Ye X, Yang G F, et al. Response of denitrifying community, denitrification gene and antibiotic resistance gene to oxytetracycline stress in polycaprolactone supported solid-phase denitrification reactor. Bioresource Technology, 2020; 308: 123274.

[14] Zhang K Y, Gu J, Wang X J, Zhang X, Hu T, Zhao W Y. Analysis for microbial denitrification and antibiotic resistance during anaerobic digestion of cattle manure containing antibiotic. Bioresource Technology, 2019; 291: 121803.

[15] Zhang Q Q, Zhao Y H, Wang C J, Bai Y H, Wu D, Wu J, et al. Expression of the nirS, hzsA, and hdh gene and antibiotic resistance gene in response to recovery of anammox process inhibited by oxytetracycline. Science of The Total Environment, 2019; 681: 56–65.

[16] Yan C, Dinh Q T, Chevreuil M, Gamier J, Roose-Amsaleg C, Labadie P, et al. The effect of environmental and therapeutic concentrations of antibiotics on nitrate reduction rates in river sediment. Water Research, 2013; 47(11): 3654–3662.

[17] Yin G Y, Hou L J, Liu M, Zheng Y L, Li X F, Lin X B, et al. Effects of thiamphenicol on nitrate reduction and $N_2O$ release in estuarine and coastal sediments. Environmental Pollution, 2016; 214: 265–272.

[18] Hu Z Y, van Alen T, Jetten M S M, Kartal B. Lysozyme and penicillin inhibit the growth of anaerobic ammonium-oxidizing planctomycetes. Applied and Environmental Microbiology, 2013; 79(24): 7763–7769.

[19] Lotti T, Cordola M, Kleerebezem R, Caffaz S, Lubello C, van Loosdrecht M C M. Inhibition effect of swine wastewater heavy metals and antibiotics on anammox activity. Water Science & Technology, 2012; 66(7): 1519–1526.

[20] Uddin T M, Chakraborty A J, Khusro A, Zidan B M R M, Mitra S, Emran T B, et al. Antibiotic resistance in microbes: History, mechanisms, therapeutic strategies and future prospects. Journal of Infection and Public Health, 2021; 14(12): 1750–1766.

[21] Zhang Q Q, Ying G G, Pan C G, Liu Y S, Zhao J L. Comprehensive evaluation of antibiotics emission and fate in the river basins of China: source analysis, multimedia modeling, and linkage to bacterial resistance. Environmental Science & Technology, 2015; 49(11): 6772–6782.

[22] Ling W C, Fan Y M, Fang Y Y, Sun C H, Liu G Z. Antibiotics pollution of livestock and poultry breeding in Beijing-Tianjin-Hebei region. Journal of Environmental Engineering Technology, 2018; 8(4): 390–397. (in Chinese)

[23] Zhang W, Huang M H, Qi F F, Sun P Z, Van Ginkel S W. Effect of trace tetracycline concentrations on the structure of a microbial community and the development of tetracycline resistance gene in sequencing batch reactors. Bioresource Technology, 2013; 150: 9–14.

[24] Chen C Q, Li J, Chen P P, Ding R, Zhang P F, Li X Q. Occurrence of antibiotics and antibiotic resistances in soils from wastewater irrigation areas in Beijing and Tianjin, China. Environmental Pollution, 2014; 193: 94–101.

[25] Zhang S Q, Zhang F D, Liu X M, Wang Y J, Zhang J F. Degradation of antibiotics and passivation of heavy metals during thermophilic composting process. Scientia Agricultura Sinica, 2006; 39(2): 337–343. (in Chinese)

[26] Xue D M, Boeckx P, Wang Z L. Nitrate sources and dynamics in a salinized river and estuary - a $\delta^{15}N–NO_3^-$ and $\delta^{18}O–NO_3^-$ isotope approach. Biogeosciences, 2014; 11(20): 5957–5967.

[27] Wang A, Fang Y T, Chen D X, Phillips O, Koba K, Zhu W X, et al. High nitrogen isotope fractionation of nitrate during denitrification in four forest soils and its implications for denitrification rate estimates. Science of The Total Environment, 2018; 633(15): 1078–1088.

[28] Risgaard-Petersen N, Meyer R L, Schmid M, Jetten M S M, Enrich-Prase A, Rysgaard S, et al. Anaerobic ammonium oxidation in an estuarine sediment. Aquatic Microbial Ecology, 2004; 36(3): 293–304.

[29] Xue D M, Yu H, Fang Y T, Shan J, Xi D, Wang Y D, et al. $^{15}N$-tracer approach to assess nitrogen cycling processes: Nitrate reduction, anammox and denitrification in different pH cropland soils. Catena, 2020; 193: 104611.

[30] Li S J, Xie X F, Li H, Xue D M. Relationship between denitrification and anammox rates and $N_2$ production with substrate consumption and pH in a riparian zone. Environmental Technology, 2023; 45(13): 2531–2540.

[31] Shan J, Zhao X, Sheng R, Xia Y Q, Ti C P, Quan X F, et al. Dissimilatory nitrate reduction processes in typical Chinese paddy soils: Rates, relative contributions and influencing factors. Environmental Science & Technology, 2016; 50(18): 9972–9980.

[32] Thamdrup B, Dalsgaard T. Production of $N_2$ through anaerobic ammonium oxidation coupled to nitrate reduction in marine sediments. Applied and Environmental Microbiology, 2002; 68(3): 1312–1318.

[33] Edgar R C. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods, 2013; 10: 996–998.

[34] Wang Q, Garrity G M, Tiedje J M, Cole J R. Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. Applied and Environmental Microbiology, 2007; 73(16): 5261–5267.

[35] Liu C, Cui Y M, Li X Z, Yao M J. Microeco: an R package for data mining in microbial community ecology. FEMS Microbiology Ecology, 2021; 97(2). doi: 10.1093/femsec/fiaa255.

[36] R Core Team. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. 2013; Available: http://www.R-project.org/. Accessed on [2023-11-20].

[37] Zhou Z C, Chen J, Meng H, Dvornyk V, Gu J D. New PCR primers targeting hydrazine synthase and cytochrome c biogeneis proteins in anammox bacteria. Applied Microbiology and Biotechnology, 2017; 101: 1267–1287.

[38] Zhao S Y, Wang X M, Pan H W, Wang Y T, Zhu G B. High $N_2O$ reduction potential by denitrification in the nearshore site of a riparian zone. Science of The Total Environment, 2022; 813: 152458.

[39] Noophan P L, Narinhongtong P, Wantawin C, Munakata-Marr J. Effects of oxytetracycline on anammox activity. Journal of Environmental Science and Health, Part A, 2012; 47: 873–877.

[40] Huang F Y, An Z Y, Moran M J, Liu F. Recognition of typical antibiotic residues in environmental media related to groundwater in china (2009-2019). Journal of Hazardous Materials, 2020; 399: 122813.

[41] Ravishankara A R, Daniel J S, Portmann R W. Nitrous oxide ($N_2O$): The dominant ozone-depleting substance emitted in the 21st century. Science, 2009; 326(5949): 123–125.

[42] Revell L E, Bodeker G E, Huck P E, Williamson B E, Rozanov E. The sensitivity of stratospheric ozone changes through the 21st century to $N_2O$ and $CH_4$. Atmospheric Chemistry and Physics, 2012; 12(23): 11309–11317.

[43] Roose-Amsaleg C, Yan C, Hoang A M, Laverman A M. Chronic exposure of river sediments to environmentally relevant levels of tetracycline affects bacterial communities but not denitrification rates. Ecotoxicology, 2013; 22: 1467–1478.

[44] Kohanski M A, Dwyer D J, Wierzbowski J, Cottarel G, Collins J J. Mistranslation of membrane proteins and two-component system activation trigger antibiotic-mediated cell death. Cell, 2008; 135(4): 679–690.

[45] Faust K, Raes J. Microbial interactions: from networks to models. Nature Reviews Microbiology, 2012; 10: 538–550.

[46] Newman M E J. Modularity and community structure in networks. Proceedings of the National Academy of Sciences of the United States of America, 2006; 103(23): 8577–8582.

[47] Hui C, Richardson D M, Pyšek P, Le Roux J J, Kučera T, Jarošík V. Increasing functional modularity with residence time in the co-distribution of native and introduced vascular plants. Nature Communications, 2013; 4: 2454.

[48] Gore J, Youk H, van Oudenaarden A. Snowdrift game dynamics and facultative cheating in yeast. Nature, 2009; 459: 253–256.

[49] Nadell C D, Xavier J B, Foster K R. The sociobiology of biofilms. FEMS Microbiology Reviews, 2009; 33: 206–224.

[50] Frost I, Smith W P J, Mitri S, Millan A S, Davit Y, Osborne J M, et al. Cooperation, competition and antibiotic resistance in bacterial colonies. The ISME Journal, 2018; 12: 1582–1593.

[51] Ahmad M, Vithanage M, Kim K, Cho J-S, Lee Y H, Joo Y K, et al. Inhibitory effect of veterinary antibiotics on denitrification in groundwater: A microcosm approach. The Scientific World Journal, 2014; 2014(1): 879831.

[52] Chen A, Chen Y, Ding C, Liang H, Yang B. Effects of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal. RSC Advances, 2015; 5(73): 59326.

[53] Pulicharla R, Zolfaghari M, Brar S K, Drogui P, Auger S, Verma M, et al. Acute impact of chlortetracycline on nitrifying and denitrifying processes. Water Environment Research, 2018; 90(7): 604–614.

[54] Ryan M P, Adley C C. *Ralstonia* spp.: emerging global opportunistic pathogens. European Journal of Clinical Microbiology & Infectious Diseases, 2014; 33: 291–304.

[55] Ruiz C, McCarley A, Espejo M L, Cooper K K, Harmon D E. Comparative genomics reveals a well-conserved intrinsic resistome in the emerging multidrug-resistant pathogen Cupriavidus gilardii. MSphere, 2019; 4(5): e00631–19.

[56] Kallistova A Y, Nikolaev Y A, Mardanov A V, Berestovskaya Y Y, Grachev V A, Kostrikina N A, et al. Investigation of formation and development of anammox biofilms by light, epifluorescence, and electron microscopy. Microbiology, 2020; 89: 708–719.