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Effects of Typical Antibiotics on Denitrification Processes and Their Degradation Products in Aquatic Environments

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Abstract: The continuous input of antibiotics into groundwater poses a potential threat to nitrogen cycling, but the mechanisms affecting denitrification remain unclear. In this study, ciprofloxacin (CIP) and sulfamethoxazole (SMX) were selected to investigate the effects of single and combined exposure on denitrification performance, enzyme activities, microbial community, functional genes, and degradation product ecotoxicity. Results showed that CIP exhibited the strongest inhibitory effect, followed by CIP+SMX, while SMX showed weak and delayed inhibition. Antibiotics inhibited cell viability more significantly than cell abundance, consistent with changes in nitrogen transformation and enzyme activities. *Pseudomonadaceae* was decreasing under antibiotic stress, particularly in the CIP+SMX group. Functional gene analysis revealed significant reductions in *nirK*, *nirS*, and *nosZ* abundances in CIP-treated groups, while SMX inhibition emerged during recovery. Degradation pathway analysis identified multiple transformation routes, and ecotoxicity predictions indicated that some intermediates were more toxic than parent compounds. This study provides important insights into the ecotoxicological effects of antibiotics on denitrification in groundwater systems.

Keywords: Antibiotics; Denitrification; Microbial Community Structure; Degradation Products; Ecological Toxicity

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1 Introduction

Antibiotics are biologically active compounds synthesized by bacteria, fungi, or other

microorganisms during their metabolic processes, conferring antimicrobial properties. They are categorized by chemical structure, including penicillins, tetracyclines, aminoglycosides, acylamino acids, sulfonamides (SAs), and quinolones, among others. Antibiotics have played a crucial role in preventing bacterial infections and combating infectious diseases across sectors such as agriculture, livestock, and aquaculture (Ma et al., 2025; Mupindu et al., 2025). However, rising antibiotic production and consumption have raised significant public health concerns (Van Boeckel et al., 2014). Global antibiotic consumption surged by approximately 46% from 2000 to 2018, with a further spike in 2019 due to the sudden COVID-19 outbreak. In 2020, total consumption surged by 16.3% compared to 2019 and is projected to increase by 67% by early 2023 (Seethalakshmi et al., 2022; Petromelidou et al., 2024). High-income countries exhibit higher antibiotic consumption rates than middle- and low-income nations (Anh et al., 2021; Harvey et al., 2023).

Antibiotics are frequently released into the environment through three pathways: human use, veterinary use, and pharmaceutical wastewater (Akhter et al., 2024). They permeate soil water bodies, and plants, and have been identified in aquatic environments worldwide (Dong et al., 2026). For instance, in secondary sedimentation tanks of sewage treatment plants in seven European countries, including Portugal, Ireland, and Germany, macrolides and quinolones are the most frequently detected antibiotics (Wang et al., 2020). These antibiotic classes comprise 7-12 distinct compounds, with concentrations ranging from 3 to 598 ng/L (Carvalho et al., 2016). Moreover, antibiotics have been detected in rivers and lakes in specific Asian regions, where concentrations can reach hundreds of ng/L. Quinolones and SAs are prevalent in surface water, with SAs the primary focus of antibiotic surveillance in various regions, showing detection rates up to 84.6% and average concentrations of 466 ng/L (Lyu et al., 2020). SMX was predominantly found in groundwater samples from Germany and other European regions (Jiang et al., 2017). Global environmental assessments identify SAs as the most frequently encountered antibiotic class across five continents, with SMX showing notably elevated environmental persistence; median concentrations range from 3.20 to 122 ng/L.

Antibiotics entering surface water and soil environments can migrate to groundwater through a complex interplay of physical, chemical, and biological processes, including leaching, infiltration, groundwater-surface water interactions, adsorption, hydrolysis, and microbial degradation (Huang et al., 2020). Groundwater typically harbours lower levels of antibiotic pollution than surface water, due to natural attenuation mechanisms such as physical filtration, soil particle adsorption, microbial

biotransformation, and native groundwater dilution (Jurado et al., 2020). Over 80% of monitored groundwater antibiotic concentrations are below 10 ng/L. Despite this, the potential for antibiotics to induce allergies and toxic reactions, and their persistent presence in the environment, may disrupt human resistance to antibiotics. This not only contributes to environmental contamination but also poses significant risks to both ecological systems and human health, underscoring the imperative need for increased research attention (Harvey et al., 2023; Masud et al., 2024).

Statistical results from field sampling and testing by various scholars on antibiotic contamination in Chinese aquatic environments show that ciprofloxacin (CIP) and norfloxacin (NOR) are quinolone antibiotics with relatively high detection rates, with maximum detected concentrations ranging from ng/L to µg/L (Ma et al., 2015; Chen et al., 2018); sulfamethoxazole (SMX) is the predominant sulfonamide antibiotic, detected in all five types of water bodies with generally high concentrations, and SMX has also been identified as the primary contaminant in most cases (Li et al., 2022; Duan et al., 2022).

Groundwater nitrate pollution poses a significant environmental challenge, as the natural denitrification process in water bodies involves a series of reactions: $\text{NO}_3\text{-N} \rightarrow \text{NO}_2\text{-N} \rightarrow \text{NO} \rightarrow \text{N}_2\text{O} \rightarrow \text{N}_2$. These reactions are catalyzed by specific enzymes, namely nitrate reductase, nitrite reductase, nitric oxide reductase, and nitrous oxide reductase (Xiang et al., 2020; Zhu et al., 2026). Denitrification primarily depends on microorganisms as the main agents executing these functions. The escalation of nitrate concentrations in aquatic environments can be attributed to two factors: increased pollution flux and inhibition of denitrification (Rivett et al., 2008).

Currently, research on the effects of antibiotics on the denitrification process in groundwater is relatively limited, and their mechanism of action remains unclear. Therefore, in this study, CIP and SMX were selected as representative antibiotics, and microcosm experiments were designed to investigate their effects on the denitrification process. The objectives of this study were to: (i) elucidate the effects of different types of antibiotics on the anaerobic denitrification process and their underlying mechanisms; and (ii) identify the changes in denitrification-related functional genes and metabolic pathways induced by exposure to the two antibiotics. This systematic research approach provides important insights into the ecotoxicological effects of antibiotic contamination on the nitrogen cycle in groundwater systems.

2 Materials and Methods

2.1 Susceptibility testing methods

Susceptibility testing was performed using the disk diffusion and broth microdilution methods in accordance with Clinical and Laboratory Standards Institute (CLSI) guidelines. The main reagents and materials used in this study were as follows: ciprofloxacin (CIP, >98% purity, Shanghai Anpu Cuishi Standard Technical Service Co., Ltd.), sulfamethoxazole (SMX, >98% purity, Shanghai Anpu Cuishi Standard Technical Service Co., Ltd.), denitrifying bacterial agent (Shanghai Gandu Environmental Engineering Co., Ltd.), Mueller-Hinton (MH) broth medium (Changde Bikeman Biotechnology Co., Ltd.), *Staphylococcus aureus* ATCC-25923 (Shanghai Luwei Technology Co., Ltd.), ciprofloxacin susceptibility test disks (5 µg/disk, Changde Bikeman Biotechnology Co., Ltd.), LB broth medium (Changde Bikeman Biotechnology Co., Ltd.), LB agar plates (Changde Bikeman Biotechnology Co., Ltd.), and blank susceptibility test disks (Changde Bikeman Biotechnology Co., Ltd.).

Disk diffusion method: *Staphylococcus aureus* ATCC-25923 was used as the quality control strain. The diameter of the inhibition zone around a 5 µg/disk ciprofloxacin disk was measured on an LB agar plate inoculated with *S. aureus* ATCC-25923. Clear zone margins with a diameter of ≥ 20 mm indicated reliable. Meanwhile, filter paper disks containing different types and concentrations of antibiotics were placed on the surface of LB agar plates inoculated with the denitrifying bacterial culture. Three parallel replicates were prepared for each treatment. The plates were pre-incubated at 5 °C for 2 h to allow pre-diffusion, and subsequently incubated at 25 °C for 24 h. Inhibition zones formed under different antibiotic conditions were clearly observable. A larger inhibition zone indicated higher susceptibility of the test organism to the corresponding antibiotic.

Broth microdilution method: CIP solutions at mass concentrations of 2.000 00, 0.250 00, and 0.031 25 mg/L, and SMX solutions at 400.00, 50.00, and 6.25 mg/L were added to LB broth medium and then subjected to 2-fold, 4-fold, and 8-fold serial dilutions, respectively. Subsequently, the denitrifying bacterial suspension was mixed with the serially diluted antibiotic solutions and incubated at 30 °C for 24 h in a constant-temperature incubator. Bacterial growth was assessed by measuring the optical density at 600 nm (OD₆₀₀) using a microplate reader. The minimum inhibitory concentration (MIC) was defined as the lowest antibiotic concentration that completely inhibited visible bacterial growth. A lower MIC value indicated higher susceptibility of the denitrifying bacteria to the antibiotic. A growth control group containing only the denitrifying bacterial suspension and medium was also included to confirm that the bacteria could grow normally under the same experimental conditions.

2.2 Microcosm Experiment

Fig.1 shows the schematic diagram and physical device for simulating nitrate degradation in water. The upper part of the reaction device has three openings: the middle opening collecting liquid samples, the left opening is for sample injection, and the right opening is for gas venting. In the 1 L reaction device, the denitrification process of simulated water was carried out to investigate the effects of single and combined antibiotic use on the denitrification process.

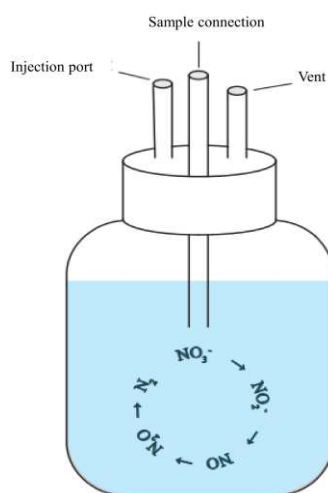


Fig.1. Experimental setup

The background solution was composed of 0.3607 g potassium nitrate (KNO_3), 0.2080 g sodium bicarbonate (NaHCO_3), 0.0739 g sodium sulfate (Na_2SO_4), and 0.0411 g sodium chloride (NaCl), resulting in a $\text{HCO}_3\text{-Na}$ type water with a total dissolved solids (TDS) concentration of 683.7 mg/L, in which the $\text{NO}_3\text{-N}$ was supplied by KNO_3 . After adjusting the pH to 7, the reactor solution was purged with argon for 20 min to remove dissolved oxygen, then incubated under sealed, dark conditions at 25°C. Carbon source plays a crucial role in the growth of denitrifying bacteria and significantly impacts denitrification. Sodium acetate (CH_3COONa) is a commonly utilised carbon source in anaerobic cultivation experiments. To prevent interference from other bacteria during nitrate decrease in the sludge, commercially available denitrifying bacterial agents from Shanghai Gandu Environmental Engineering Co., Ltd. were employed; a 1 L reaction vessel contains 0.7500 g of denitrifying bacterial agent, containing *Bacillus* and *Pseudomonas* denitrifying bacteria. The optimal temperature range is 10-60°C, with a pH range of 6-9.

The experiment consists of two stages. The initial stage is the domestication stage, during which periodic acclimation experiments to decrease nitrate concentration were conducted at an initial

concentration of 50 mg/L of NO₃-N. Samples were collected every 2 days, and the solution was replenished to its initial level after each acclimation cycle to maintain reaction progress. The acclimation phase is deemed complete when nitrite accumulation is no longer apparent, and NH₄-N levels remain consistently low, indicating a stable denitrification environment. At the beginning of each cycle, CH₃COOH and KNO₃ were supplemented to their initial concentrations. The subsequent stage, the formal experiment, begins with the addition of 50 mg/L NO₃-N. This phase comprises two cycles: the first is a blank cycle without adding antibiotic to any reaction vessel, and the second is the experimental cycle, involving one blank group and three experimental groups. Three experimental treatments were designed: 100 µg/L CIP, 100 µg/L SMX, and a combination of 100 µg/L CIP and 100 µg/L SMX. Each treatment was conducted in triplicate. Methanol was used as the co-solvent. Sampling was conducted at regular intervals throughout the experiment.

2.3 Sample Analysis and Identification

The TOC concentration was measured with a total organic carbon analyzer (Model TOC-L, Shimadzu, Japan). As part of the regular evaluation, measurements of pH, EC, TDS, ORP, and DO were conducted using a HACH HQ40D portable water analyzer. The concentrations of NO₃-N, NO₂-N, and NH₄-N were determined according to Standard Methods for the Examination of Water and Wastewater (4th edition) through ultraviolet spectrophotometry (Method B), N-(1-naphthyl)-ethylenediamine spectrophotometry (Method A), and Nessler's reagent spectrophotometry (Method A), respectively.

The quantity of denitrifying bacteria was characterized by measuring the optical density (OD₆₀₀) of the bacterial suspension using a UV-Vis spectrophotometer at a wavelength of 600 nm, with higher absorbance values indicating greater cell numbers. Cell viability was assessed using the CCK-8 assay. Genomic DNA was extracted from the samples, and adapter primers were designed and synthesized, followed by PCR amplification. Sequencing was performed on a qualified library using the Illumina PE300 sequencing platform to investigate the microbial community structure and relative abundance. Ltd. Shanghai Meiji Biomedical Technology Co., Ltd. constructed the denitrification functional genes. The target denitrification functional genes were *nirK*, *nirS*, and *nosZ*. The primers used for *nirK* were *nirK-876* and *nirK-1040*, with the sequences ATYGGCGVCAYGGCGA and GCCTCGATCAGRTRTGGTT, respectively. For *nirS*, the primers cd3aF and R3cd were employed, with the sequences GTSAACGTSAAGGARACSGG and GASTTCGGRTGSGTCTTGA, respectively.

For *nosZ*, the primers *nosZ-2F* and *nosZ-2R* were used, with the sequences CGCRACGGCAASAAGGTSMSSGT and CAKRTGCAKSGCARTGGCAGAA, respectively.

Antibiotic and intermediate product analysis was performed using an ultra-high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS) system equipped with a ZORBAX Eclipse Plus C18 column (2.1 × 100 mm, 1.8 µm). Antibiotic concentration detection method: Electrospray ionization positive ion mode (ESI+) was employed. The drying gas temperature was set at 325 °C with a flow rate of 6 L/min. The nebulizer temperature was 325 °C, and the nebulizer pressure was 40 psi. The capillary voltage was maintained at 3.5 kV. The mobile phase consisted of an aqueous phase A (0.2% formic acid in water) and an organic phase B (acetonitrile). The sample injection volume was 10 µL, the flow rate was 0.2 mL/min, and gradient elution was performed. For CIP, the declustering potential (DP) was set at 80 V. The ion transitions monitored were m/z 332.1 → 288.1 (with a collision energy of 25 eV) and m/z 332.1 → 245.1 (with a collision energy of 33 eV). For SMX, the DP was set at 70 V, and the ion transitions were m/z 254.1 → 156.0 (collision energy: 23 eV) and m/z 254.1 → 108.0 (collision energy: 32 eV). Antibiotic intermediate product detection method: Electrospray ionization positive ion mode (ESI+) was used. The drying gas temperature was set at 325 °C, and the capillary voltage was 3.5 kV. Full-scan mode was employed in positive ion mode to determine CIP and its degradation products, with the primary mass spectrometry scan range set from 50 to 1000 m/z . For CIP intermediates: the mobile phase consisted of aqueous phase A (0.2% formic acid in water) and organic phase B (acetonitrile), with gradient elution performed at a volume ratio of 9:1 (A:B). The column temperature was set at 35 °C, the flow rate was 0.3 mL/min, and the injection volume was 5 µL, with gradient elution. For SMX intermediates: the mobile phase consisted of aqueous phase A (0.2% formic acid in water) and organic phase B (acetonitrile), with gradient elution performed at a volume ratio of 8:2 (A:B). The column temperature was set at 20 °C, the flow rate was 0.1 mL/min, and the injection volume was 10 µL, with gradient elution. In this research, the ECOSAR Version 2.0 (2000 - 2016, U.S. Environmental Protection Agency) software was utilized to forecast the acute toxicity (LC₅₀ and EC₅₀) and chronic toxicity (ChV) of SMX degradation by-products to aquatic organisms, including fish, water fleas, and algae, during water denitrification. The toxicity levels of antibiotic degradation products and their impacts on ecosystems were assessed through model prediction results, rather than empirical toxicological data, as detailed in Table 1.

Table 1 Toxicity classification according to the Globally Harmonised System for the Classification and

Labeling of Chemicals (Wang et al., 2023)

Toxicity Range (mg/L)	Categorisation
$LC_{50}/EC_{50}/ChV \leq 1$	Extremely Toxic
$1 < LC_{50}/EC_{50}/ChV \leq 10$	Toxic
$10 < LC_{50}/EC_{50}/ChV \leq 100$	Harmful
$LC_{50}/EC_{50}/ChV > 100$	Harmlessness

Note: LC_{50} (for fish)/ EC_{50} (for algae): aquatic acute toxicity, usually expressed using 96 h for fish, 48 h for Daphnia, 72 or 96 h for algae; ChV: aquatic chronic toxicity.

3 Results

3.1 Susceptibility testing result

The disk diffusion method was used to evaluate the susceptibility of denitrifying bacteria to antibiotics by measuring inhibition zone diameters. According to CLSI guidelines, antibiotic susceptibility disks containing 5 μ g/disk of CIP and 300 μ g/disk of SMX were placed on agar plates uniformly spread with a suspension of denitrifying bacteria. All experiments were performed in triplicate, and the results are presented in Fig 2. The mean inhibition zone diameters of the two quality control strains were 27.7 mm and 27.2 mm, respectively, which met the standard criteria for the disk diffusion susceptibility test. As shown in Fig. 2, CIP produced the largest inhibition zone diameters among all tested antibiotics, with mean values of 31.0, 31.3, and 30.8 mm across the three replicates, indicating that the denitrifying bacteria were most sensitive to CIP. In contrast, SMX exhibited no obvious inhibitory effect on the denitrifying bacteria, with mean inhibition zone diameters of 16.3, 17.5, 15.7, and 18.0 mm (note: four values listed), which fell within the range between susceptible and intermediate according to the CLSI interpretive criteria.

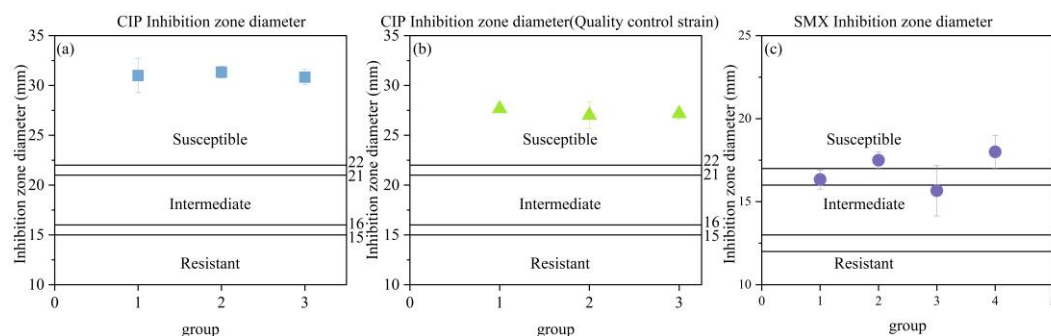


Fig 2. Inhibition zone diameters of the two antibiotics (CIP and SMX) against denitrifying bacteria.

Note: Error bars represent the standard deviation of three inhibition zones on a single plate.

As shown in Fig 3, the intersection angle between the inhibition zones of CIP and SMX was less than 90° , indicating an indifferent effect of the combined action of CIP and SMX.

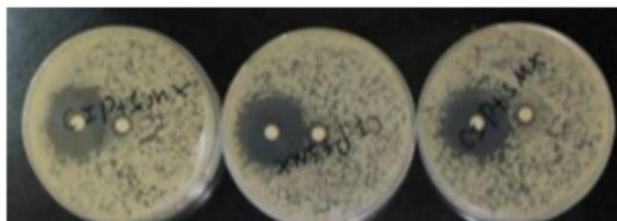


Fig 3. Combined effect of CIP and SMX

The inhibitory effects of antibiotics on denitrifying bacteria were evaluated using the broth microdilution method. The MIC of CIP against the quality control strain *Staphylococcus aureus* ATCC-25923 was $0.25 \mu\text{g/mL}$, which met the quality control criteria for the broth microdilution susceptibility test. According to CLSI guidelines, the MIC of CIP against the denitrifying bacteria was $0.125 \mu\text{g/mL}$, within the susceptible range, while the MIC of SMX was $200 \mu\text{g/mL}$, within the intermediate range. The order of susceptibility of the denitrifying bacteria to the tested antibiotics was therefore $\text{CIP} > \text{SMX}$. Furthermore, the MIC of the CIP + SMX combination was less than or equal to that of each antibiotic alone, indicating an indifferent effect between CIP and SMX.

3.2 Changes in water environmental indicators

Fig 4 illustrates the temporal variations of TOC concentrations in the 12 reactors. The initial TOC concentration was approximately 200 mg/L . After two acclimation periods (Cycles I and II), the TOC concentration decreased to approximately 100 mg/L . To avoid insufficient electron donor supply during denitrification, which could lead to incomplete denitrification and nitrite accumulation, an additional carbon source was supplemented to the initial level at the beginning of each cycle from Cycle III onwards, ensuring rapid denitrification performance.

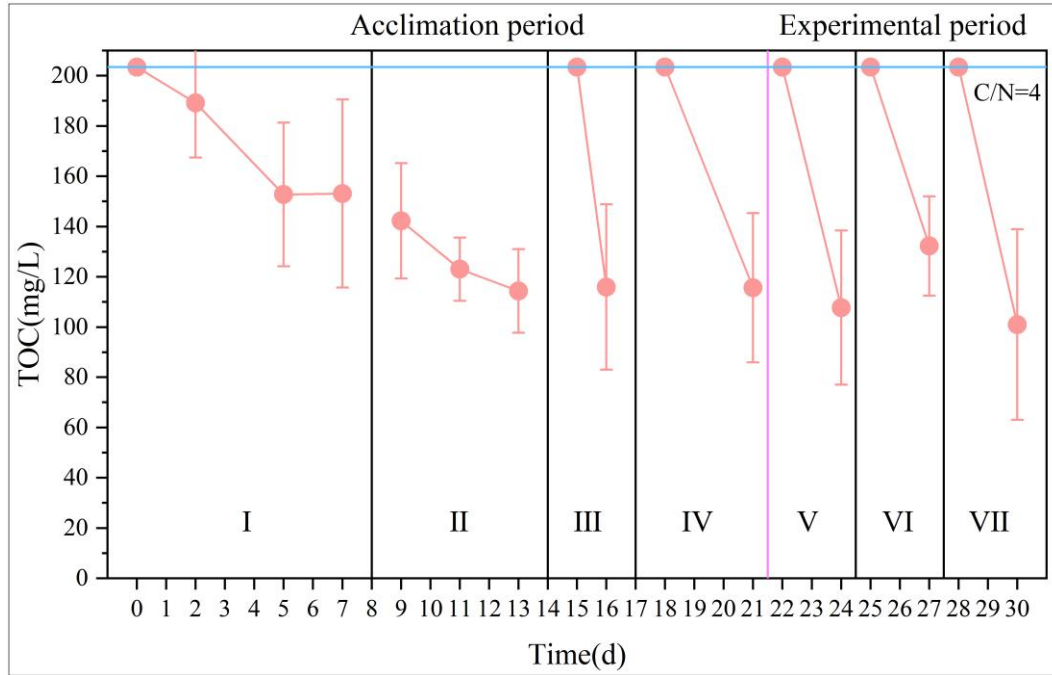


Fig 4. Change of TOC in the device over time

Note: Error bars represent the standard deviation of the measurements from the 12 reactors.

As shown in Fig 5, the pH profile during the experimental process exhibited a convex upward curve. This was attributed to the simultaneous reduction of $\text{NO}_2\text{-N}$ and $\text{NO}_3\text{-N}$, which generated OH^- and consequently increased pH in the system. The initial pH in the reactors was approximately 7.0. As denitrification proceeded, the pH value increased continuously, reaching a mean value of 8.1 at the end of Cycle III. To ensure normal denitrification and maintain optimal growth conditions for denitrifying bacteria (the most favorable pH range for denitrifying bacteria is 7.0–8.5, within which a relatively high denitrification rate can be achieved (Yin et al., 2016)), 1 mol·L⁻¹ HCl was used to adjust the pH to 7.8 at the beginning of each cycle from Cycle IV onward.

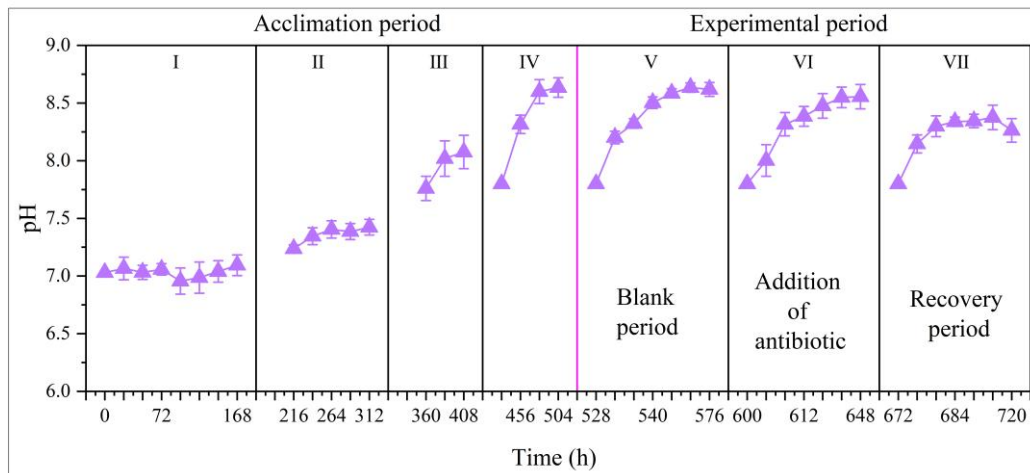


Fig 5. Variation of pH in the device over time

Note: Error bars represent the standard deviation of the measurements from the 12 reactors.

Fig. 6 shows the variations in EC and TDS in the reactors over time. With the supplementation of KNO_3 and CH_3COONa at the beginning of each cycle, both EC and TDS exhibited a continuous upward trend, while remaining relatively stable within each cycle. At the beginning of Cycle I, the EC and TDS values were 1999 $\mu\text{S}/\text{cm}$ and 981 mg/L , respectively. By the end of Cycle VII, they had reached 5735 $\mu\text{S}/\text{cm}$ and 4281 mg/L , respectively. This increase was attributed to continuous supplementation of nitrate and carbon sources, which led to elevated concentrations of dissolved inorganic salts, organic matter, and other soluble substances resulting from bacterial growth and metabolism (Whelinta Sherin et al., 2023).

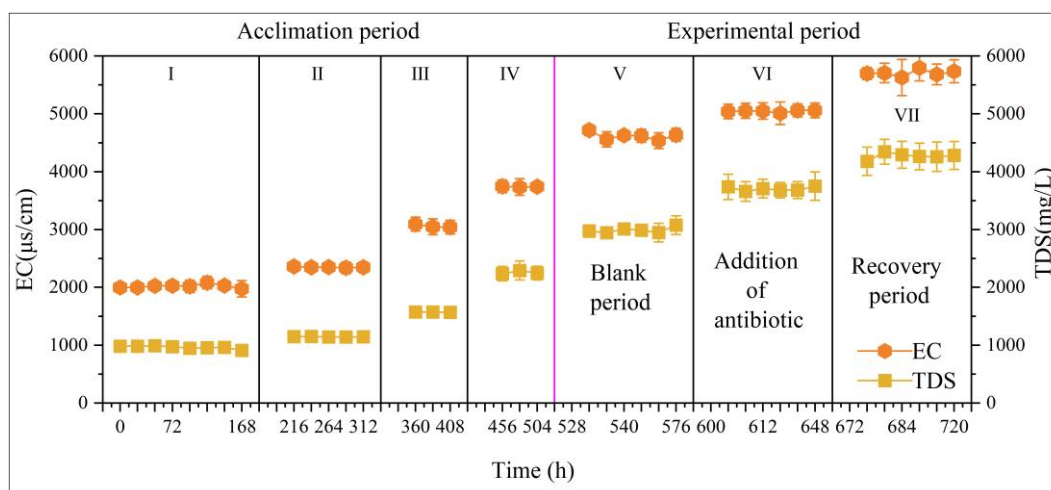


Fig 6. Changes of EC and TDS in the device over time

Note: Error bars represent the standard deviation of the measurements from the 12 reactors.

Dissolved oxygen (DO) can inhibit denitrification; therefore, denitrification is essentially carried out under anoxic conditions. This is because O_2 competes with $\text{NO}_3\text{-N}$ for electron donors, and molecular oxygen also suppresses the synthesis and activity of denitrifying enzymes (Schneider et al., 2014). The oxidation-reduction potential (ORP) reflects the overall redox state of an aqueous solution. Stronger reducing conditions correspond to lower ORP values. The ORP and DO in the reaction system are shown in Fig 7. The initial ORP in the reaction system was 80.9 mV, indicating an oxidative environment. As denitrification proceeded, the residual DO in the water was consumed, and the ORP shifted to negative values, indicating a transition from an oxidative to a reductive environment. Under strictly anoxic conditions, the DO concentration approached zero, and the denitrification process was maintained under strict anoxic conditions throughout.

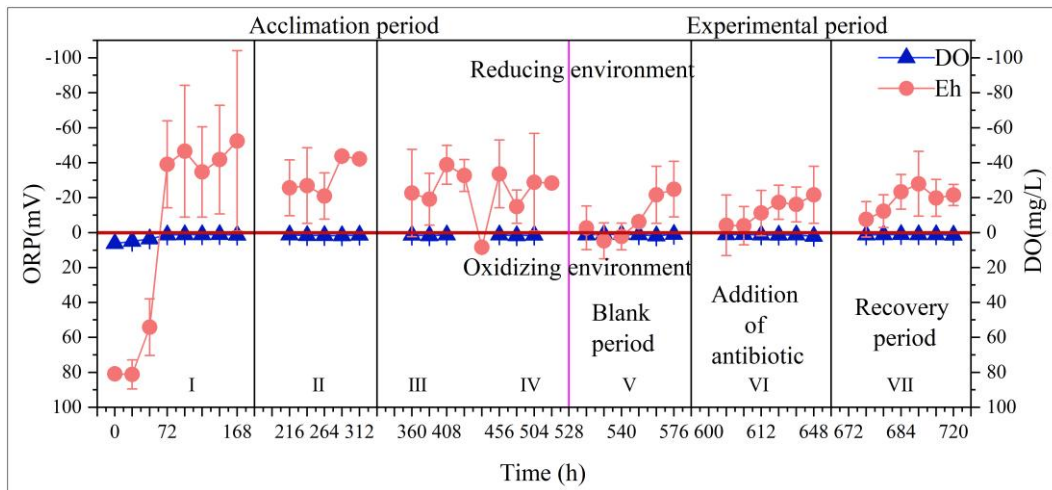


Fig 7. Changes of ORP and DO in the device over time

Note: Error bars represent the standard deviation of the measurements from the 12 reactors.

3.3 Variations in inorganic nitrogen concentrations during denitrification

The variations in $\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, and $\text{NH}_4\text{-N}$ concentrations during the experiment are presented in Fig. 8. In the first cycle, the degradation of 50 mg/L $\text{NO}_3\text{-N}$ required approximately 7 days. However, after five cycles of acclimation, the same amount of $\text{NO}_3\text{-N}$ was degraded within only 12 hours in the fifth cycle. Concurrently, the accumulation of $\text{NO}_2\text{-N}$ progressively decreased with ongoing acclimation. The maximum $\text{NO}_2\text{-N}$ accumulation initially reached 40.75 mg/L, but by the fifth cycle, no detectable $\text{NO}_2\text{-N}$ accumulation was observed. Throughout the process, the $\text{NH}_4\text{-N}$ concentration remained relatively stable, fluctuating around 5 mg/L. These results indicate that a stable denitrifying environment was established in the fifth cycle, at which point the acclimation was considered complete. This cycle was subsequently adopted as the blank control cycle for the formal experiments.

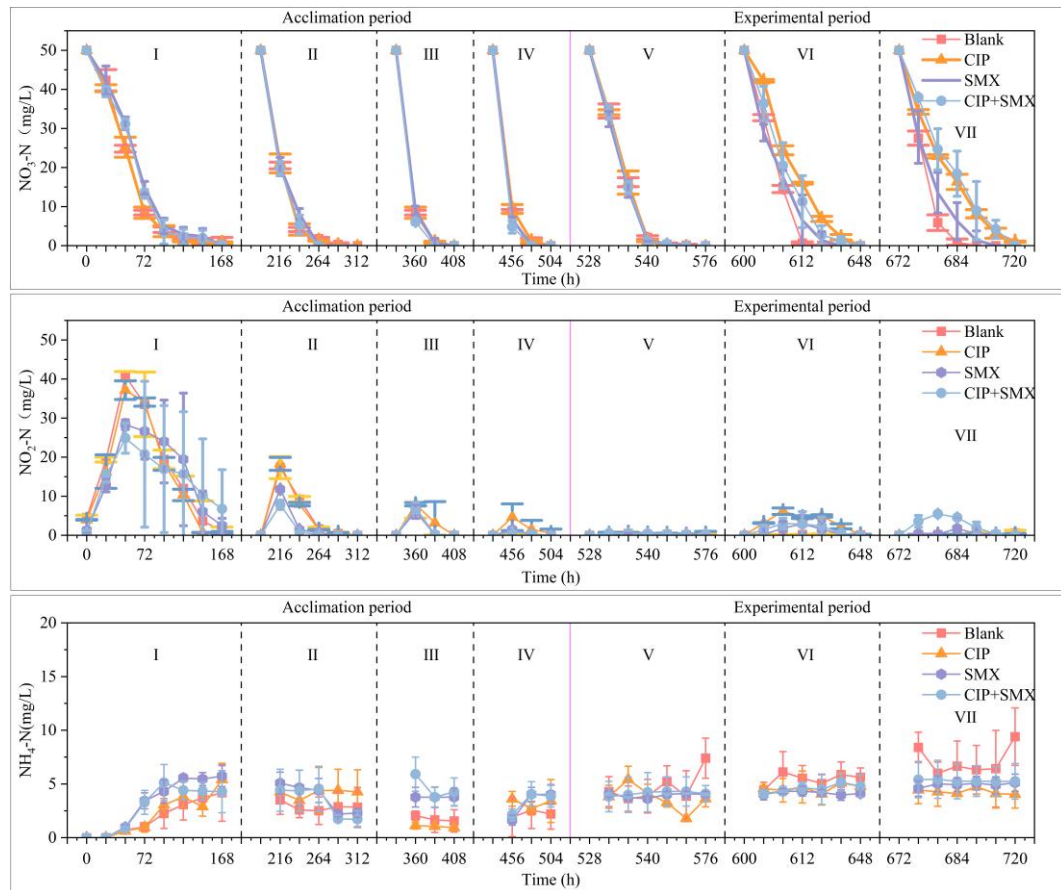


Fig. 8 Changes in nitrate (a), nitrite (b), and ammonium (c) nitrogen in each reaction system

Note: Error bars represent the standard deviation of measurements from three parallel reaction setups.

In the fifth cycle of the formal experiment, $\text{NO}_3\text{-N}$ in all groups was nearly completely degraded within approximately 12 hours. In the sixth cycle, the degradation rates of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ in the blank group remained consistent with those observed in the fifth cycle. However, adding CIP, SMX, and CIP+SMX inhibited denitrification, reducing $\text{NO}_3\text{-N}$ degradation rates and significantly prolonging completion times. In the system spiked with 100 $\mu\text{g/L}$ SMX alone, a slight stimulatory effect was observed during the initial phase, whereas the CIP and CIP+SMX groups primarily exhibited inhibition of denitrification. In the seventh cycle, inhibitory effects on $\text{NO}_3\text{-N}$ degradation persisted in the CIP+SMX and SMX groups, with $\text{NO}_2\text{-N}$ accumulation still observed in the CIP+SMX group. Throughout all cycles, the $\text{NH}_4\text{-N}$ concentrations in each system remained relatively stable.

The inhibitory effect of antibiotic additives on the denitrification process persisted throughout the recovery period. The order of inhibition intensity was $\text{CIP+SMX} > \text{CIP} > \text{SMX}$, with $\text{NO}_3\text{-N}$ degradation inhibition rates of 35.06%, 30.84%, and 10.78%, respectively. Regarding $\text{NO}_2\text{-N}$ reduction, the SMX + CIP group exhibited a sustained inhibitory effect, with the maximum $\text{NO}_2\text{-N}$ accumulation

reaching 5.46 mg/L, corresponding to an inhibition rate of 10.92%. In contrast, SMX alone CIP showed a trend toward alleviation. Similarly, Yin et al. demonstrated that combined antibiotics also inhibited denitrification.

3.4 Antibiotic concentration

This indoor incubation experiment was conducted at 25 °C under ambient dark conditions. During the experiment, the concentrations of CIP and SMX were measured over time, and the results are shown in Fig 9. The initial concentration of each antibiotic was 100 µg/L. After the reaction, the mean concentrations of CIP and SMX in the individually spiked groups were 91.55 µg/L and 90.75 µg/L, respectively, while in the combined spiked group (CIP + SMX), the mean concentrations of CIP and SMX were 93.63 µg/L and 88.94 µg/L, respectively. These results indicate that slight degradation of both CIP and SMX occurred during the experiment.

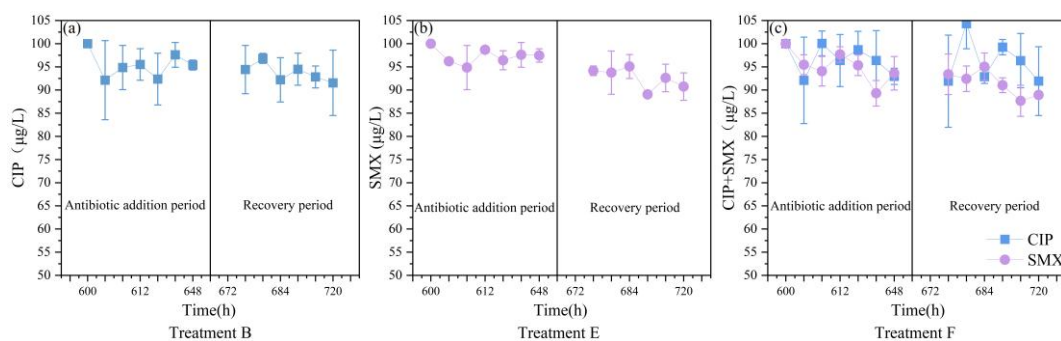


Fig 9. Changes in antibiotic concentrations in each reaction system

Note: Error bars represent the standard deviation of measurements from three parallel reaction setups.

3.5 Denitrifying bacteria count

The microbial growth process generally comprises four phases: the lag phase, the logarithmic (exponential) phase, the stationary phase, and the death (decline) phase. The S-Gompertz model is one of the commonly used growth curve fitting models, which is applicable to scenarios where the death/decline phase is not considered and contains three parameters. For bacterial populations during the acclimation/adaptation phase, the S-Gompertz model can be employed for curve fitting. The fitting equation is as follows:

N_0 : initial OD₆₀₀ value;

N_m : maximum bacterial growth (cell concentration), cm⁻¹;

λ : lag phase duration, d;

t: incubation/growth time, d;

μ_m : maximum specific growth rate of bacteria, cm/d;

N: bacterial population at a given time estimated by curve fitting, cm^{-1} .

Fig. 10 shows the curves fitted with the S-Gompertz model. 10 shows the curves fitted with the S-Gompertz model. The fitted parameters were as follows: $N_0=0.007 \text{ cm}^{-1}$, $N_m=0.475 \text{ cm} \cdot \text{d}^{-1}$, $\mu_m=0.15998 \text{ cm/d}$, $\lambda=2.19976 \text{ d}$. Experimental findings showed that denitrifying bacterial growth stabilized after 8 days, consistent with the measured data.

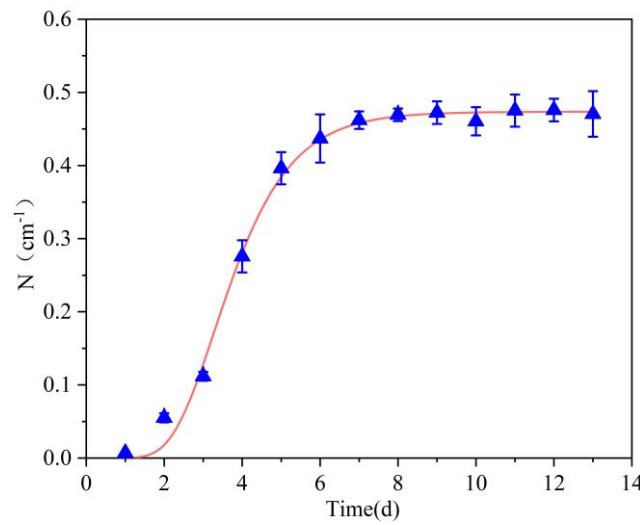


Fig. 10 Growth curve of denitrifying bacteria fitted by S-Gompertz model

Note: Error bars represent the standard deviation of the measurements from the 12 reactors.

4. Discussion

4.1 Denitrifying enzyme activity

As shown in Fig 11(a), during the blank cycle, the nitrate reductase activities (expressed as the degradation capacity of $\text{NO}_3\text{-N}$ per milliliter of reductase per hour) in the blank control, CIP, SMX, and CIP+SMX groups were 65.32, 79.35, 77.03, and 69.96 $\mu\text{mol/h}$, respectively. During the antibiotic-spiked cycle, the corresponding values in the blank control, CIP, SMX, and CIP+SMX groups were 61.94, 49.86, 70.63, and 55.28 $\mu\text{mol/h}$, respectively, with nitrate reductase activities accounting for 94.82%, 62.84%, 91.69%, and 79.02% of those in the respective blank-cycle groups. These results indicate that antibiotic addition inhibited nitrate reductase activity in all treated groups, with the inhibitory effect being more pronounced in the CIP-treated group. The order of inhibition intensity was as follows: $\text{CIP} > \text{CIP+SMX} > \text{SMX}$. In the recovery cycle, the nitrate reductase

activities in the blank control, CIP, SMX, and CIP+SMX groups were 85.84, 62.51, 67.68, and 49.32 $\mu\text{mol/h}$, respectively, corresponding to 138.59%, 125.37%, 95.82%, and 89.22% of those in the respective antibiotic-spiked cycle groups. During the recovery cycle, the inhibitory effect on nitrate reductase activity in the CIP-treated group was alleviated, indicating partial recovery of enzyme activity. In contrast, the SMX-treated group exhibited sustained inhibition, suggesting a delayed inhibitory effect of SMX. Similarly, the combined CIP+SMX group also showed persistent inhibition of nitrate reductase activity.

As shown in Fig 11(b), during the blank cycle, the nitrite reductase activities (expressed as the degradation capacity of $\text{NO}_2\text{-N}$ per milliliter of reductase per hour) in the blank control, CIP, SMX, and CIP+SMX groups were 43.50, 44.24, 42.65, and 46.41 $\mu\text{mol/h}$, respectively. During the antibiotic-spiked cycle, the corresponding values in the blank control, CIP, SMX, and CIP+SMX groups were 35.65, 21.31, 43.11, and 28.77 $\mu\text{mol/h}$, respectively, with nitrite reductase activities accounting for 81.95%, 48.17%, 101.08%, and 61.99% of those in the respective blank-cycle groups. These results indicate that the addition of CIP and CIP+SMX inhibited nitrite reductase activity, whereas the inhibitory effect of SMX alone was not pronounced. The order of inhibition intensity was as follows: CIP > CIP+SMX > SMX. During the recovery cycle, the nitrite reductase activities in the blank control, CIP, SMX, and CIP+SMX groups were 35.80, 32.27, 36.09, and 17.18 $\mu\text{mol/h}$, respectively, corresponding to 100.42%, 151.36%, 83.72%, and 59.71% of those in the respective antibiotic-spiked cycle groups. It can be observed that during the recovery cycle, the inhibitory effect on nitrite reductase activity in the CIP-treated group was weakened, indicating partial recovery of enzyme activity. In contrast, the SMX and CIP+SMX groups exhibited sustained inhibition, which was similar to the trend observed for nitrate reductase activity.

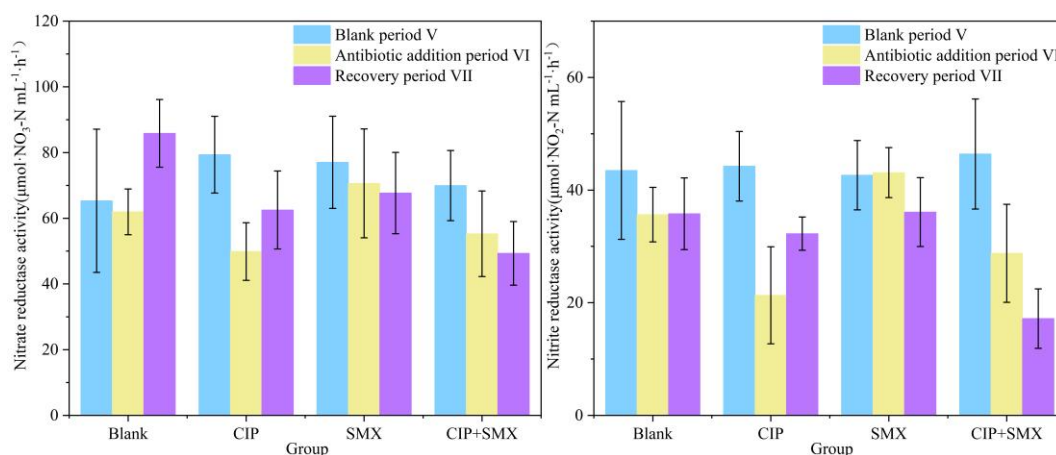


Fig 11. Denitrifying enzyme activities in each reaction system

Note: Error bars represent the standard deviation of measurements from three parallel reaction setups.

4.2 Microbial abundance and activity

The changes in denitrifying bacterial abundance are shown in Fig 12. The OD₆₀₀ values fluctuated within a narrow range, indicating that the bacteria in each system had entered a stationary phase of slow growth after prior acclimation, which is consistent with the growth patterns of bacteria in natural aquatic environments (Zeng et al., 2019; Chen et al., 2015). No significant trend in denitrifying bacterial abundance was observed during the formal experimental period, suggesting that antibiotic addition had little effect on the microbial population within the reaction systems. In complex microbial communities, various microorganisms interact and compete with one another. When antibiotics are introduced into such communities, they may kill certain susceptible microorganisms; however, they may also provide additional resources and space for other microorganisms, promoting their growth and proliferation (Zeng et al., 2019b).

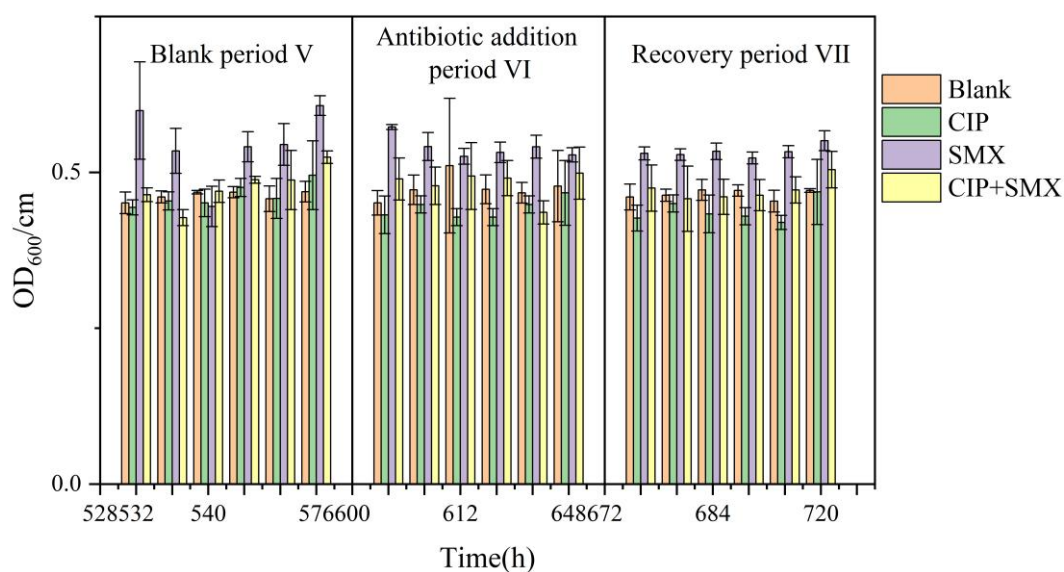


Fig 12. Changes in bacterial abundance in each reaction system

Note: Error bars represent the standard deviation of measurements from three parallel reaction setups.

Fig 13 shows the cell viability results for each reaction system. The mean cell viabilities in the CIP, SMX, and CIP+SMX treatment groups were 68.84%, 98.94%, and 72.21%, respectively, indicating that antibiotic exposure inhibited bacteria. The inhibitory effects were pronounced in the CIP and CIP+SMX groups, while the SMX group exhibited only weak inhibition. These results suggest that antibiotics more strongly affected denitrifying bacterial cell viability than cell abundance, and the

inhibition patterns were consistent with the observed changes in nitrogen species ($\text{NO}_3\text{-N}$, $\text{NO}_2\text{-N}$, $\text{NH}_4\text{-N}$) and denitrifying enzyme activities. Furthermore, the functional groups in the chemical structures of CIP and SMX responsible for inhibiting denitrifying bacterial activity differ (Alhaj-Suliman et al., 2023; Knoll et al., 2021; Kamal El-sagheir et al., 2023).

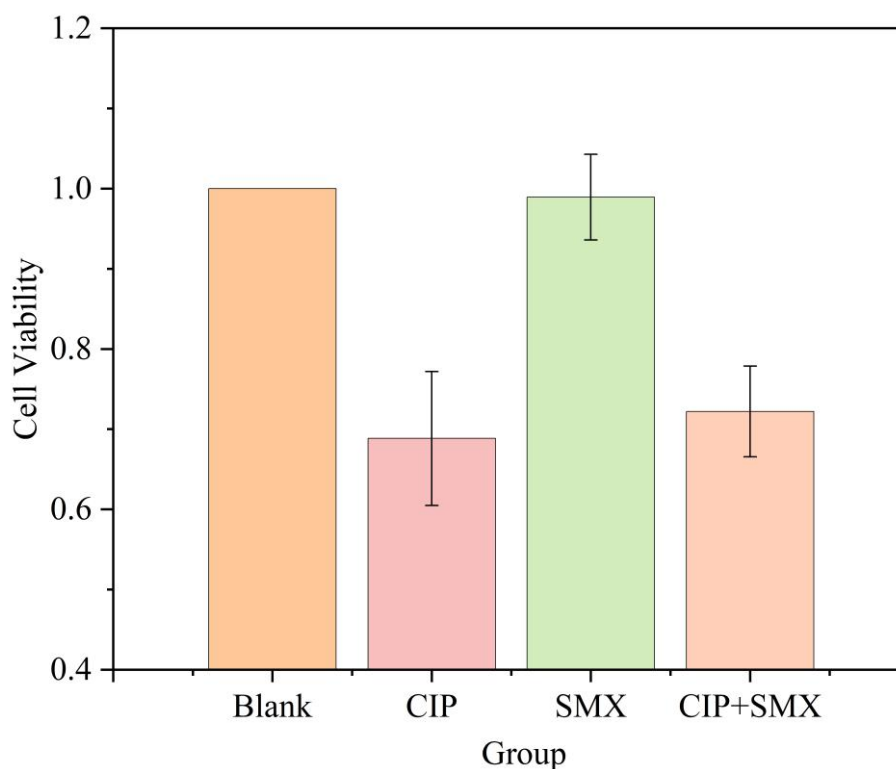


Fig 13. Cell viability in each reaction system

Note: Error bars represent the standard deviation of measurements from three parallel reaction setups.

4.3 Microbial Community Structure

Fig 14 presents the Circos plot of the microbial community composition at the family level in each experimental group, generated using Circos-0.67-7 (taxa with relative abundances below 0.01 were pooled into "others"). The dominant families across all samples included *Enterobacteriaceae*, *Azospirillaceae*, *Pseudomonadaceae*, and *Rhizobiaceae*, all of which are *Gram-negative* bacteria and are widely distributed in aquatic and soil environments (Kuzmanović et al., 2023; Jayasurya et al., 2023; Maestro-Gaitán et al., 2023). Among these, *Pseudomonadaceae* is recognized as a key family involved in denitrification (Eberly et al., 2016). Compared with the blank cycle, *Pseudomonadaceae* abundance in the CIP-treated system decreased from 11% to 4%, then partially recovered to 5% from Cycle VI to Cycle VII. In the SMX-treated group, the abundance increased from 0% to 4% but subsequently decreased to 0% during the recovery cycle. In the CIP+SMX system, the abundance decreased from 3%

to 0% and remained at 0% during the recovery cycle. These results indicate that antibiotic addition generally in the abundance of *Pseudomonadaceae*. Additionally, *Alcaligenaceae* is a major denitrifying family; however, its abundance was relatively low (< others) and thus was not discussed in detail. Overall, the observed changes in *Pseudomonadaceae* abundance appear to be a primary factor contributing to differences in denitrification performance among the treatment systems.

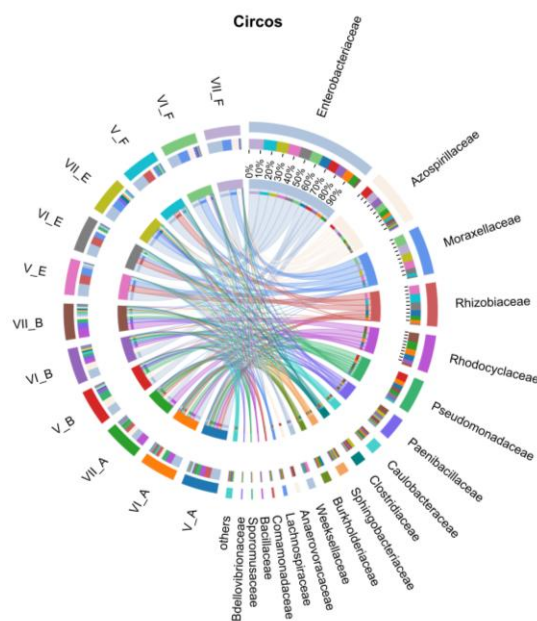


Fig 14. Circos plot of the community composition at the family level in each system during the experimental period

Note: In the Circos plot showing the sample–species relationships, the left semicircle (smaller half) represents the species composition within each sample. The outer ribbons indicate the group to which each sample belongs, while the inner ribbons represent different species, with their lengths corresponding to the relative abundance of each species in the corresponding sample. The right semicircle (larger half) illustrates the distribution proportions of species across different samples at the given taxonomic level. In this section, the outer ribbons denote species, the inner ribbons represent different groups, and their lengths indicate the proportion of each sample's contribution to the total abundance of a given species.

The dominant genera across all groups included *Enterobacter*, *Azospirillum*, *Acinetobacter*, *Allorhizobium*-*Neorhizobium*-*Pararhizobium*-*Rhizobium*, *Dechloromonas*, *unclassified_f_Enterobacteriaceae*, *Pseudomonas*, and *Paenibacillus*. After antibiotic addition, the relative abundance of *Pseudomonas*, a key genus involved in denitrification (Chen A ling et al., 2022), varied across treatment groups. From Cycle V to Cycle VI, the abundance of *Pseudomonas* decreased from 23.67% to 7.79% in the CIP-treated group, increased from 23.06% to 25.21% in the NOR-treated

group, increased from 2.82% to 8.21% in the SMX-treated group, and decreased from 6.55% to 0.45% in the CIP+SMX group. Furthermore, from Cycle VI to Cycle VII, the relative abundance of *Pseudomonas* in the CIP-treated group rebounded to 10.83%, indicating that this genus gradually adapted to CIP. In contrast, the SMX group decreased to 5.35%, and the CIP+SMX group continued to decline to 0.15%. During the recovery cycle, *Pseudomonas* abundance in the CIP group recovered somewhat, while SMX showed a delayed inhibitory effect on this genus, and the CIP+SMX group displayed sustained suppression of its abundance. As shown in Fig. 15, antibiotic addition did not strongly inhibit *Acinetobacter*, as this genus maintained relatively high abundances in Groups E and F, suggesting that *Acinetobacter* and *Pseudomonas* may jointly contribute to denitrification in these groups.

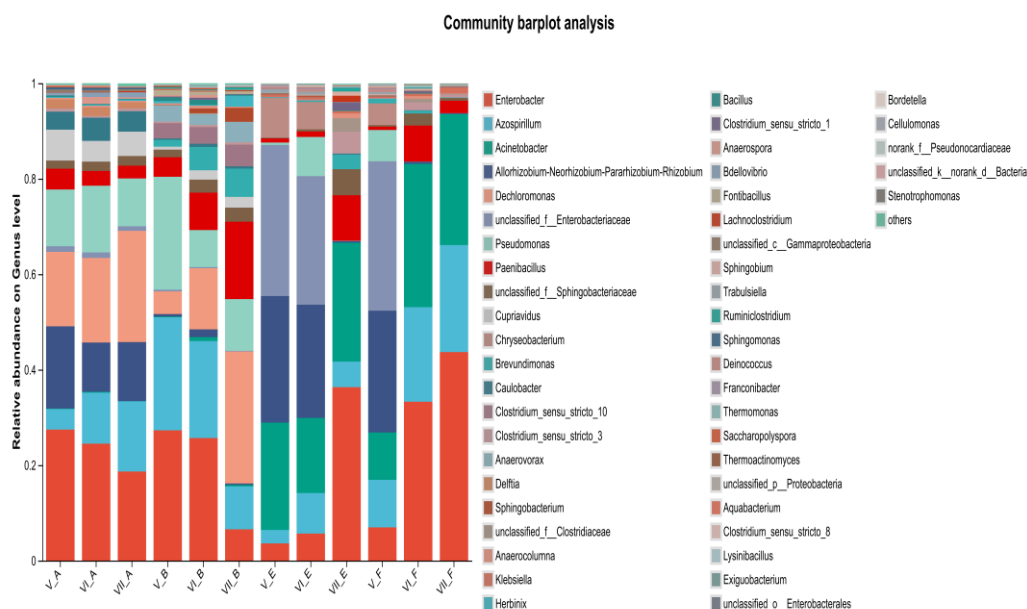


Fig 15. Community structure at the genus level in each sample during the experimental period

Note: The x-axis and y-axis represent sample names, while the y-axis and x-axis indicate the proportion of each species within the corresponding sample.

Different colored bars represent different species, and the length of each bar corresponds to the relative abundance of that species.

4.4 Denitrification functional genes

To compare differences among samples at the functional gene level, we determined the absolute abundances of denitrification functional genes by quantitative PCR using SYBR Green dye. The variations in denitrification functional genes among different samples are presented in Fig. 16. The *nirK*, *nirS*, and *nosZ* genes are key denitrification functional genes, among which *nirS* and *nirK* encode the enzymes responsible for the reduction of $\text{NO}_2\text{-N}$ to NO , while *nosZ* encodes the enzyme catalyzing

the conversion of N_2O to N_2 (Zhang et al., 2022).

During the experiment, the abundances of *nirK*, *nirS*, and *nosZ* genes in the blank group remained relatively stable across different cycles, fluctuating within the ranges of 1.34×10^9 – 1.51×10^9 , 6.82×10^7 – 8.04×10^7 , and 7.54×10^7 – 8.27×10^7 copies/mL, respectively. In the antibiotic-amended cycles, the abundances of *nirK*, *nirS*, and *nosZ* genes decreased markedly in the CIP groups, indicating significant inhibition. In contrast, the SMX group exhibited no obvious inhibitory effect on denitrification functional gene abundances. During the recovery cycle, functional gene abundances in the CIP groups showed signs of recovery. Notably, the SMX group began to exert an inhibitory effect on functional gene abundances during the recovery cycle, suggesting a lagged inhibitory response. These findings are consistent with previous studies by Zhang et al. (Zhang et al., 2022) and Liu et al. (Liu et al., 2020), which also reported that antibiotics can suppress genes.

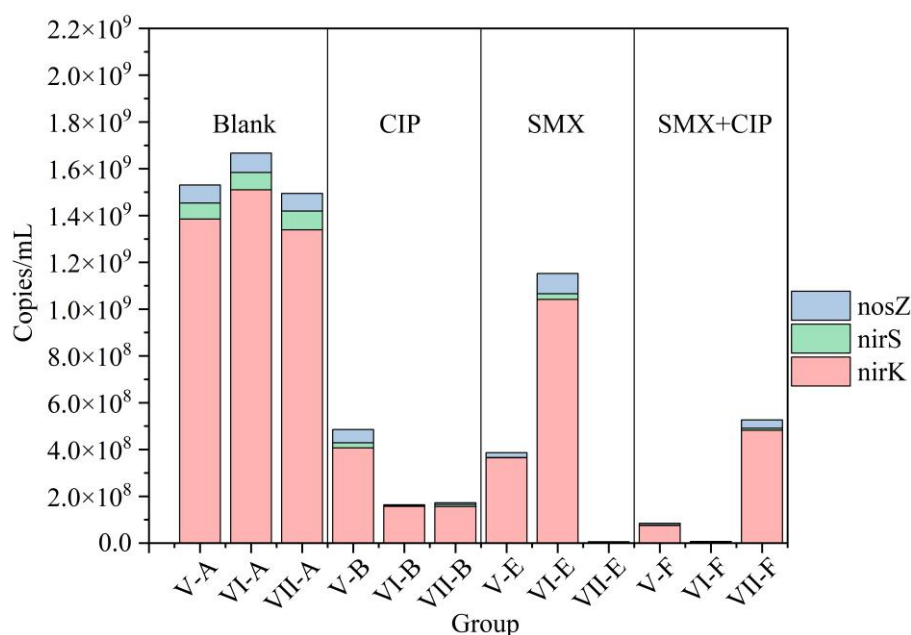


Fig. 16 Functional gene abundances in each reaction system

4.5 Degradation products of CIP and SMX

Based on a literature review, CIP degradation primarily occurs at the piperazine ring, the fluorine atom on the quinolone ring, and the carboxyl group (Hu et al., 2020). By analyzing full-scan LC-MS mass spectra of intermediate products formed during CIP degradation, the possible intermediates generated in the degradation process were identified, and potential degradation pathways were proposed. Based on the mass spectra, seven possible intermediate products were identified. Based on the mass-to-charge ratios (m/z) of the degradation compounds, the structures of the intermediates and

the possible degradation pathways of CIP under the denitrification conditions in aqueous systems were deduced, as shown in Fig 17.

Pathway1:Piperazine Epoxidation. OH oxidation of CIP yields product P1-A ($m/z = 346$). Defluorination of P1-A yields product P1-B ($m/z = 344$). Oxidative cleavage of the piperazinyl ring in P1-B yields P1-C ($m/z = 316$), P1-D ($m/z = 288$), and P1-E ($m/z = 245$). Based on the molecular structure of P1-E, the final product of this pathway is the result of complete elimination of the piperazine ring.

Pathway 2: Defluorination reaction. The fluorine atom in the quinolone ring is substituted under the action of $\cdot\text{OH}$, yielding product P2-A ($m/z = 330$). Subsequently, upon further reaction with $\cdot\text{OH}$, P2-A undergoes an addition reaction in the piperazine ring, forming product P2-B ($m/z = 346$), which contains a hydroxyl group.

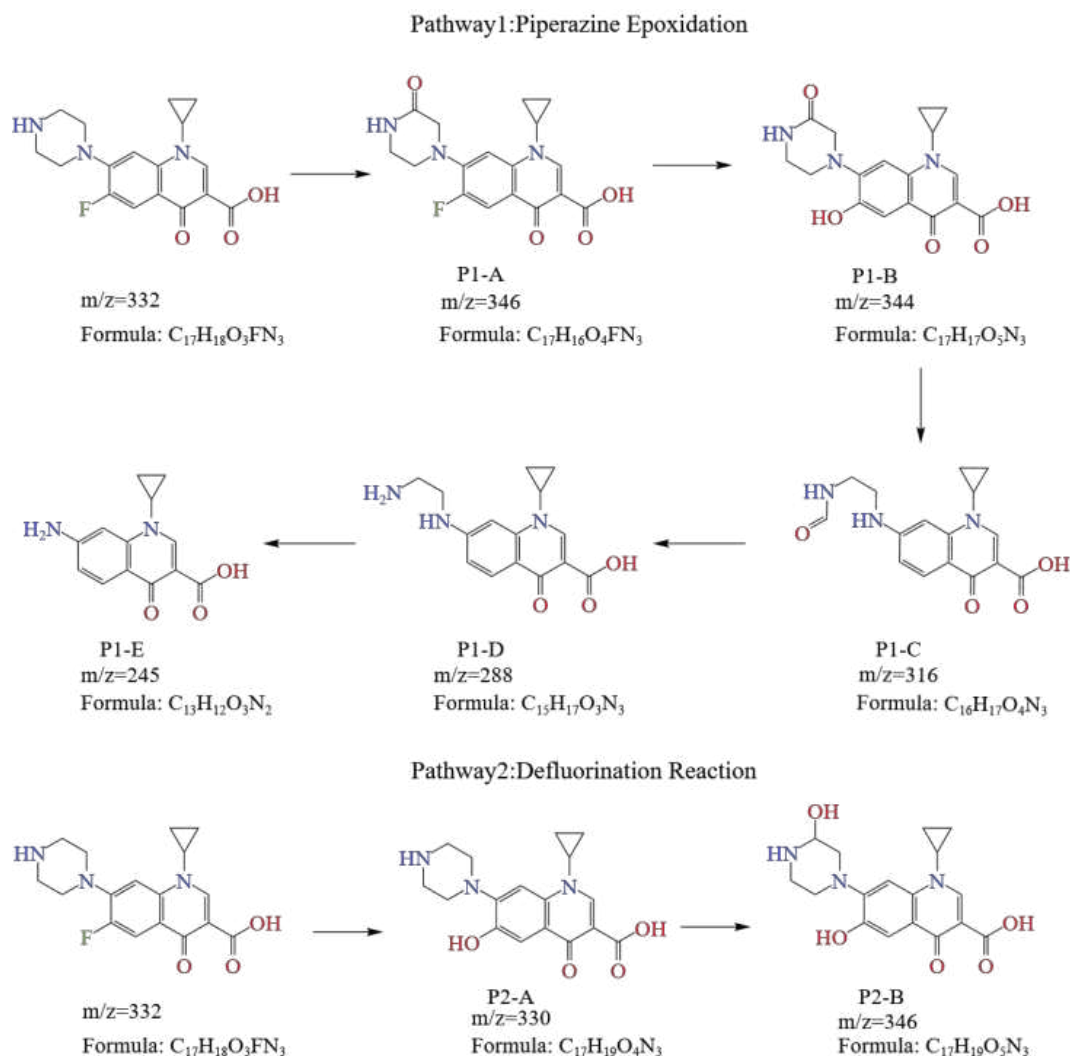


Fig. 17 Possible degradation pathways of CIP

SMX follows distinct degradation pathways, yielding various intermediate products in different degradation systems. To examine the intermediate products formed during SMX involvement in denitrification, liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis was conducted. The study revealed twelve intermediate products in the degradation solution, characterized by mass-to-charge ratios (m/z) of 156, 99, 193, 190, 270, 284, 300, 245, 284, 216, and 202. By consulting pertinent literature (Du et al., 2018; Sang et al., 2020; Chen et al., 2022), we can infer degradation of SMX at the S-N bond, the NH_2 group linked to the aromatic ring, and the benzene ring. Fig 18 illustrates three conceivable degradation pathways of SMX.

Pathway 1: S-N Bond Cleavage: The sulfonamide group in SMX carries a negative charge, making it susceptible to electrophilic attack by species such as $\cdot\text{OH}$ or SO_4^- . Consequently, radicals preferentially target the S-N bond in SMX, leading to the generation of P1-B (4-sulfophthalimide/hypothiocyanite, $m/z=156$) and P1-A (3-amino-5-methylisoxazole, $m/z=99$) through the S-N bond cleavage (Bai et al., 2024). Additionally, LC-MS/MS identified two other compounds, P1-C ($m/z=193$) and P1-D ($m/z=190$). It is hypothesised that P1-C4 forms through a coupling reaction between two P1-B molecules via the $-\text{NH}_2$ groups (Chen et al., 2022), while P1-D likely arises from $\cdot\text{OH}$ attacking P1-A.

Pathway 2: Amino Group Oxidation: The amino group ($-\text{NH}_2$) attached to the benzene ring in SMX is susceptible to radical attacks, leading to the generation of P2-A ($m/z=270$), a hydroxylamine derivative. P2-A then undergoes further oxidation to produce nitro-SMX derivatives, such as P2-B ($m/z=268$) and P2-C ($m/z=284$) (Ji et al., 2015). Subsequently, P2-C participates in a substitution reaction with a hydroxyl radical ($\cdot\text{OH}$), resulting in the creation of P2-D ($m/z=300$), which is then subjected to additional oxidation and ring-opening processes to form P2-E ($m/z=245$) (Bai et al., 2024).

Pathway 3: Methyl Group Oxidation: The methyl group on the isoxazole ring in SMX undergoes oxidation, resulting in the formation of a carboxyl group ($-\text{COOH}$) and leading to the production of P3-A ($m/z = 284$). Next, the isoxazole ring in P3-A opens, producing P3-B ($m/z = 216$), which then undergoes further oxidation to yield P3-C ($m/z = 202$).

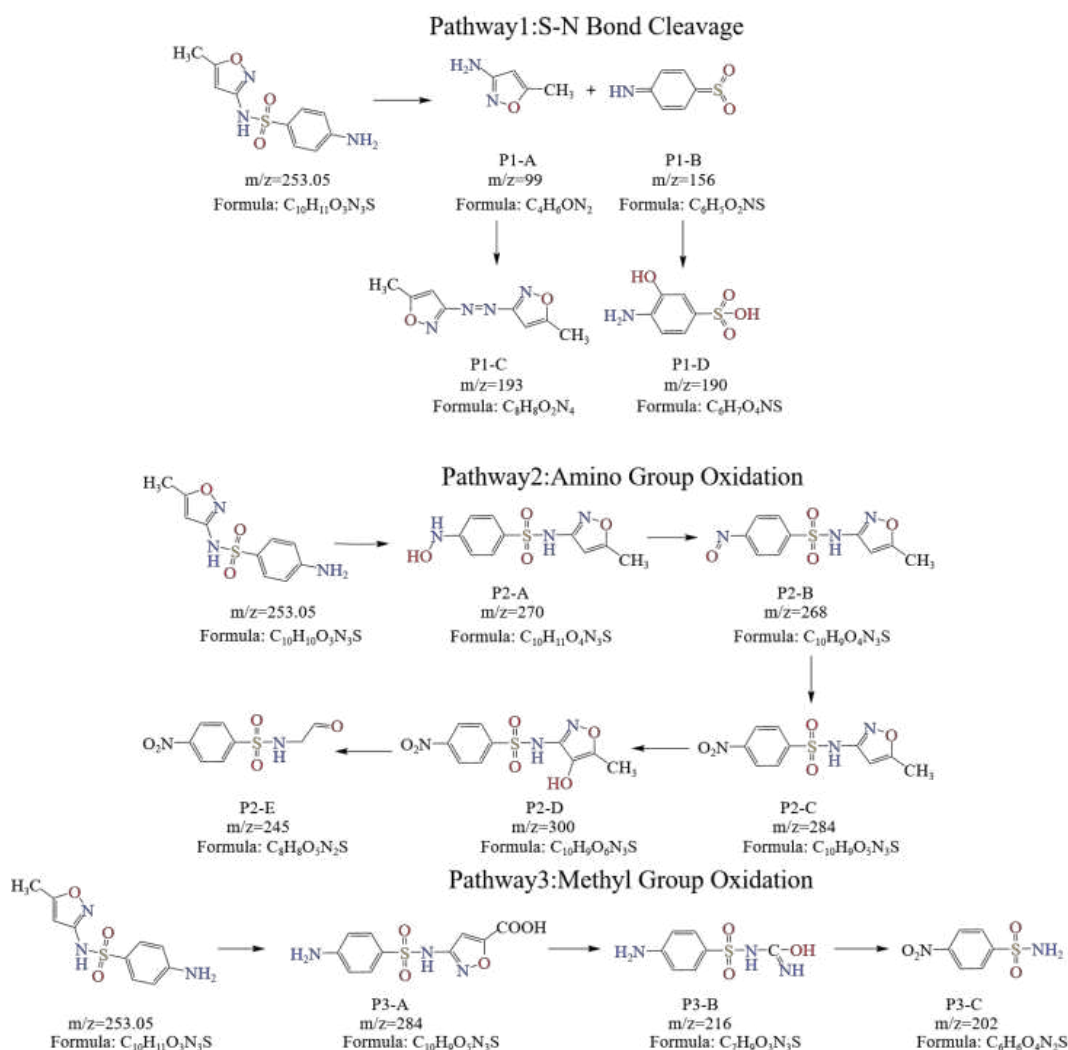


Fig.18. Possible degradation paths of SMX

4.6. Prediction of Toxicity for Degradation Products

The ECOSAR software was used to predict and analyze the toxicity of CIP and its intermediates. According to the results shown in Fig 19, during the degradation process of CIP, the toxicity predictions for P1-A, P1-B, P1-C, P2-A, and P2-B all indicated that they are non-toxic substances, and their ecotoxicity is lower than that of the parent compound. This is mainly attributed to the cleavage of the piperazinyl ring and the defluorination reaction in the CIP structure. As the reaction time progresses, the intermediates generated from CIP degradation undergo continuous cleavage, resulting in a gradual decrease in molecular weight, which facilitates their entry into biological cells and thus exhibits higher toxicity. The toxicity of P1-D increases, but it remains non-toxic to all three aquatic organisms. However, P1-E exhibits an acute toxicity of 44.9 mg/L to *Daphnia magna* (LC_{50}), indicating a harmful level, while its acute toxicity to algae (EC_{50}) and fish (LC_{50}) are 139 and 1130 mg/L, respectively,

which are at non-toxic levels, mainly due to the different sensitivities of different organisms. The chronic toxicity of P1-E to fish, *Daphnia magna*, and algae are 16.9, 0.502, and 56.4 mg/L, respectively, corresponding to harmful, very toxic, and harmful levels. Among these, the intermediate exhibits the strongest chronic toxicity to *Daphnia magna*, suggesting that the health of organisms exposed to such aquatic environments over the long term is susceptible to adverse effects.

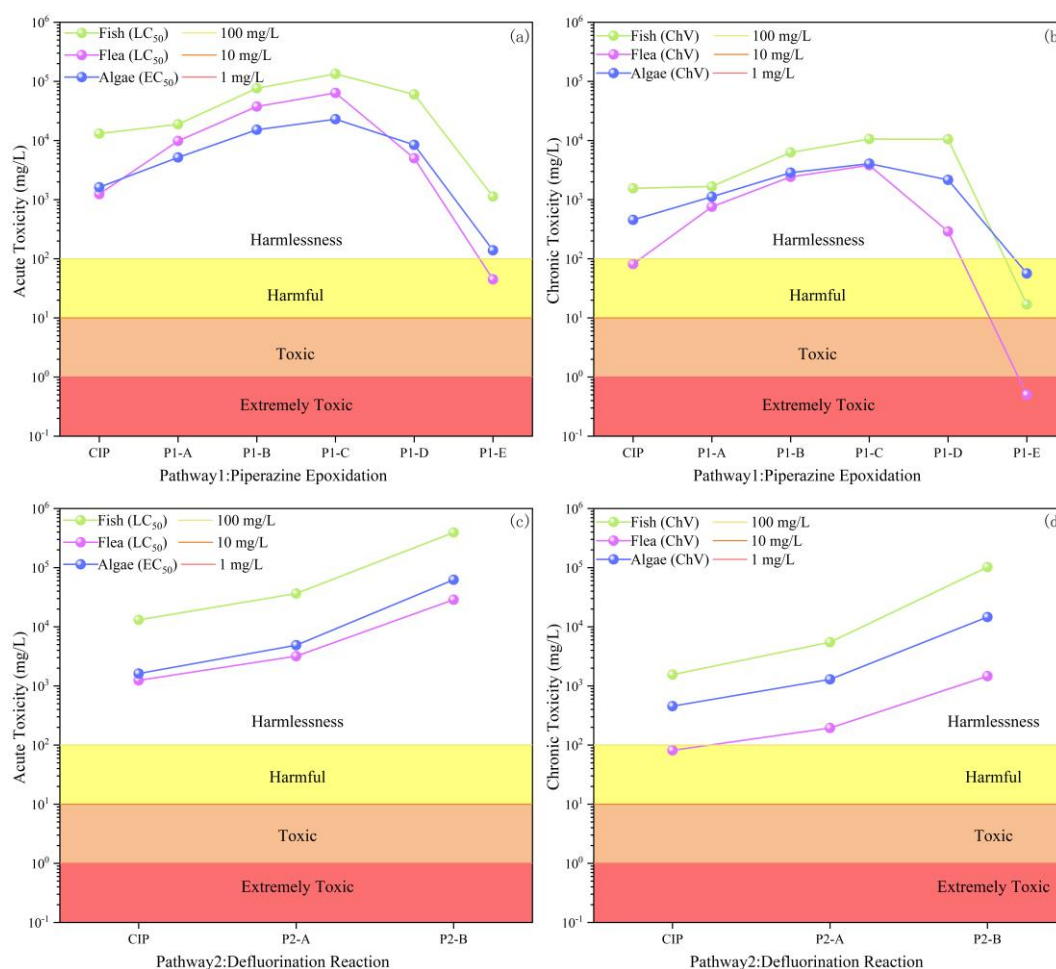


Fig.19 Toxicity of CIP and its intermediates to aquatic plants and animals

Fig. 20 depicts the toxicity prediction and analysis results for SMX and its intermediate products. In Pathway 0, the predicted toxicity levels of S-N bond cleavage are detailed. Intermediate product P1-A showed no toxicity to fish (LC₅₀) but acute toxicity to water flea (LC₅₀) and algae (EC₅₀) at 3.63 and 13.8 mg/L, respectively, indicating harmful effects. Chronic toxicity levels for fish, water flea, and algae were observed at concentrations of 6.59, 0.0363, and 9.16 mg/L, respectively, suggesting toxic effects. Intermediate product P1-C was predicted to be non-toxic. Intermediate product P1-D exhibited non-toxic acute toxicity to fish and algae, as well as non-toxic chronic toxicity to both. However, it showed toxic chronic effects on the water flea at 2.31 mg/L. Intermediate product P-A2

displayed non-toxic acute effects but harmful chronic toxicity.

In Pathway 2, during pathway, intermediate product P2-A is considered non-toxic. Intermediate product P2-B shows acute effects on fish, water fleas, and algae, with chronic exposure indicating toxic levels at 89.5 mg/L, 42.5 mg/L, and 66.9 mg/L, respectively. Intermediate product P2-C demonstrates acute toxicity to water fleas (LC_{50}) and algae (EC_{50}) at concentrations of 6.43 mg/L and 21.8 mg/L, respectively, while exhibiting non-toxic effects on fish (LC_{50}). Chronic toxicity is observed at concentrations of 5 mg/L (fish), 0.0682 mg/L (water fleas), and 11.1 mg/L (algae), indicating toxic, extremely toxic, and harmful levels, respectively. Intermediate product P2-D shows acute toxicity to water fleas (LC_{50}) and algae (EC_{50}) at 9.08 mg/L and 33.5 mg/L, respectively, but is non-toxic to fish (LC_{50}). Chronic toxicity is observed at concentrations of 13.3 mg/L (fish), 0.092 mg/L (water fleas), and 20.9 mg/L (algae), indicating harmful, extremely toxic, and dangerous levels, respectively. Intermediate product P2-E demonstrates acute toxicity to water fleas (LC_{50}) and algae (EC_{50}) at concentrations of 12.6 mg/L and 55.8 mg/L, respectively, while showing non-toxic effects on fish (LC_{50}). Chronic toxicity is observed at concentrations of 75 mg/L (fish), 0.116 mg/L (water fleas), and 53.9 mg/L (algae), indicating harmful, extremely toxic, and unhealthy levels, respectively.

In Pathway 3, the intermediate products show lower toxicity than the parent compound. Intermediate product P3-A displays no toxicity to fish and algae but demonstrates acute toxicity to water fleas (LC_{50}) at a concentration of 32.9 mg/L, indicating a harmful and highly toxic level. Intermediate product P3-B is non-toxic to fish and algae but exhibits acute toxicity to water fleas (LC_{50}) at a level of 18.6 mg/L, indicating a harmful level for water fleas. Intermediate product P3-C is not toxic to fish but shows acute toxicity to water fleas (LC_{50}) and algae (EC_{50}) at 8.04 mg/L and 32.6 mg/L, respectively, suggesting toxic levels for water fleas and harmful levels for algae. This indicates that SMX degradation in natural water bodies poses a higher risk.

SMX degradation via different pathways produces intermediate products that are more toxic than the parent compound. Specifically, these intermediates toxicity and are more toxic to water fleas, underscoring the potential hazards posed by SMX degradation in natural aquatic environments.

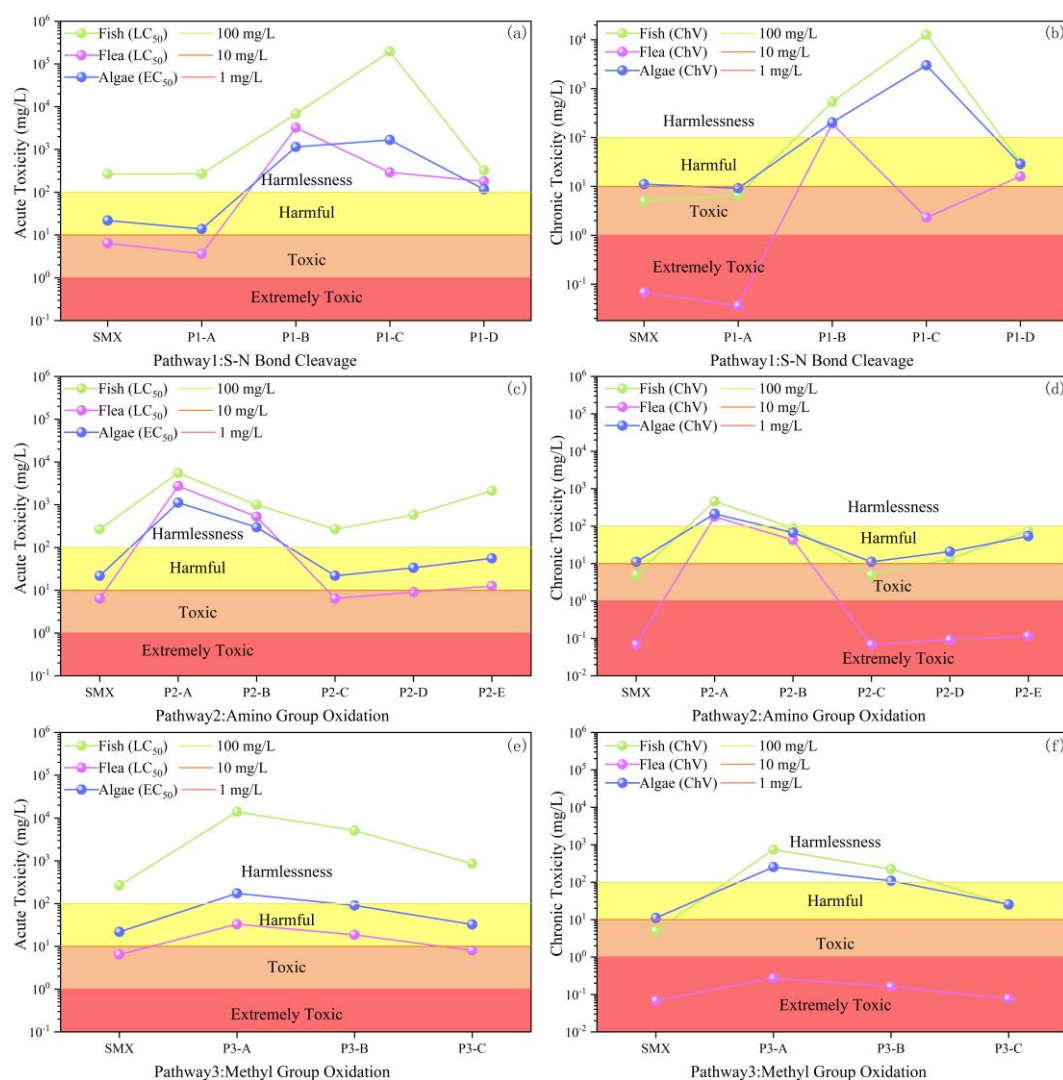


Fig.20 Toxicity of SMX and its intermediates to aquatic plants and animals

5. Conclusions

This study systematically investigated the effects of CIP and SMX on denitrification processes. The results showed that antibiotic exposure inhibited denitrification to varying degrees, with inhibition intensity following the order CIP > CIP+SMX > SMX. Among the tested antibiotics, CIP exhibited the strongest antibacterial activity and the most pronounced suppression of nitrate reductase and nitrite reductase activities, while SMX showed relatively weak inhibition with a delayed effect. Antibiotics inhibited cell viability much more strongly than cell abundance, suggesting they primarily act by disrupting bacterial metabolism rather than directly reducing cell numbers. Community structure analysis revealed that *Pseudomonadaceae* was the key denitrifying functional family, and its abundance generally decreased under antibiotic stress, with particularly notable reductions in the CIP and CIP+SMX treatment groups. Quantitative analysis of functional genes indicated that CIP

significantly reduced the abundances of *nirK*, *nirS*, and *nosZ* genes, while SMX's inhibitory effect became evident during the recovery cycle, consistent with the trends observed in enzyme activities. Furthermore, degradation pathway analysis identified multiple transformation routes for CIP and SMX, and ecotoxicity predictions suggested that some intermediates—especially those exhibiting chronic toxicity to *Daphnia magna*—were more toxic than their parent compounds, indicating that antibiotic degradation processes may pose increased ecological risks to aquatic ecosystems. Overall, this study provides important scientific evidence on the ecotoxicological effects of typical antibiotics on denitrification and highlights the need to pay close attention to the environmental risks associated with their degradation products.

Acknowledgement

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References

- AKHTER S, BHAT M A, AHMED S, et al., 2024. Antibiotic residue contamination in the aquatic environment, sources and associated potential health risks[J/OL]. *Environmental Geochemistry and Health*, 46(10): 387. DOI:10.1007/s10653-024-02146-5.
- ALHAJ-SULIMAN S O, NAGUIB Y W, WAFA E I, et al., 2023. A ciprofloxacin derivative with four mechanisms of action overcomes paclitaxel resistance in p53-mutant and MDR1 gene-expressing type II human endometrial cancer[J/OL]. *Biomaterials*, 296: 122093. DOI:10.1016/j.biomaterials.2023.122093.
- ANH H Q, LE T P Q, DA LE N, et al., 2021. Antibiotics in surface water of east and southeast Asian countries: a focused review on contamination status, pollution sources, potential risks, and future perspectives[J/OL]. *Science of The Total Environment*, 764: 142865. DOI:10.1016/j.scitotenv.2020.142865.
- BAI J, LIU G, ZHANG Y, et al., 2024. Autotrophic degradation of sulfamethoxazole using sulfate-reducing biocathode in microbial photo-electrolysis system[J/OL]. *Science of The Total Environment*, 916: 170332. DOI:10.1016/j.scitotenv.2024.170332.
- CARVALHO I T, SANTOS L, 2016. Antibiotics in the aquatic environments: a review of the European scenario[J/OL]. *Environment International*, 94: 736-757. DOI:10.1016/j.envint.2016.06.025.
- CHEN A, CHEN Y, DING C, et al., 2015. Effects of tetracycline on simultaneous biological wastewater nitrogen and phosphorus removal[J/OL]. *RSC Advances*, 5(73): 59326-59334. DOI:10.1039/C5RA08434B.
- CHEN K, LIANG J, XU X, et al., 2022. Roles of soil active constituents in the degradation of sulfamethoxazole by biochar/persulfate: contrasting effects of iron minerals and organic matter[J/OL]. *Science of The Total Environment*, 853: 158532. DOI:10.1016/j.scitotenv.2022.158532.
- CHEN L, LANG H, LIU F, et al., 2018. Presence of antibiotics in shallow groundwater in the northern and southwestern regions of China[J/OL]. *Groundwater*, 56(3): 451-457. DOI:10.1111/gwat.12596.
- DONG Y H, SHAO Q, LI J L, et al., 2026. Transformation patterns and response mechanisms of typical antibiotics in the phosphorus-accumulating bacteria coexisting system[J/OL]. *Journal of East China University of Technology (Natural Science)*, 1-11[2026-09-09]. <https://link.cnki.net/urlid/36.1300.N.20260603.0931.002>.
- DU J, GUO W, WANG H, et al., 2018. Hydroxyl radical dominated degradation of aquatic sulfamethoxazole by Fe⁰/bisulfite/O₂: kinetics, mechanisms, and pathways[J/OL]. *Water Research*, 138: 323-332. DOI:10.1016/j.watres.2017.12.046.
- DUAN W, CUI H, JIA X, et al., 2022. Occurrence and ecotoxicity of sulfonamides in the aquatic environment: a review[J/OL]. *Science of The Total Environment*, 820: 153178. DOI:10.1016/j.scitotenv.2022.153178.
- EBERLY J O, INDEST K J, HANCOCK D E, et al., 2016. Metagenomic analysis of denitrifying wastewater enrichment cultures able to transform the explosive, 3-nitro-1,2,4-triazol-5-one (NTO)[J/OL]. *Journal of Industrial Microbiology and Biotechnology*, 43(6): 795-805. DOI:10.1007/s10295-016-1755-5.
- HARVEY E J, DE BRÚN C, CASALE E, et al., 2023. Influence of factors commonly known to be associated with health inequalities on antibiotic use in high-income countries: a systematic

scoping review[J/OL]. *Journal of Antimicrobial Chemotherapy*, 78(4): 861-870. DOI:10.1093/jac/dkad034.

HU Xi, HU Xinjiang, PENG Q, et al., 2020. Mechanisms underlying the photocatalytic degradation pathway of ciprofloxacin with heterogeneous TiO₂[J/OL]. *Chemical Engineering Journal*, 380: 122366. DOI:10.1016/j.cej.2019.122366.

HUANG F, AN Z, MORAN M J, et al., 2020. Recognition of typical antibiotic residues in environmental media related to groundwater in China (2009-2019)[J/OL]. *Journal of Hazardous Materials*, 399: 122813. DOI:10.1016/j.jhazmat.2020.122813.

JAYASURYA A, GROVER M, 2023. Studies on endurance of azospirillum formosense strains to osmotic stress and modulation of early stage pearl millet growth[J/OL]. *Applied Biochemistry and Microbiology*, 59(2): 206-215. DOI:10.1134/S0003683823020023.

JI Y, FAN Y, LIU K, et al., 2015. Thermo activated persulfate oxidation of antibiotic sulfamethoxazole and structurally related compounds[J/OL]. *Water Research*, 87: 1-9. DOI:10.1016/j.watres.2015.09.005.

JIANG J, WANG G, 2017. Hazard of sulfonamides and detection technology research progress[J/OL]. *IOP Conference Series: Earth and Environmental Science*, 100: 12040. DOI:10.1088/1755-1315/100/1/012040.

JURADO A, MARGARETO A, PUJADES E, et al., 2020. Fate and risk assessment of sulfonamides and metabolites in urban groundwater[J/OL]. *Environmental Pollution*, 267: 115480. DOI:10.1016/j.envpol.2020.115480.

KAMAL EL-SAGHEIR A M, ABDELMESEH NEKHALA I, ABD EL-GABER M K, et al., 2023. Rational design, synthesis, molecular modeling, biological activity, and mechanism of action of polypharmacological norfloxacin hydroxamic acid derivatives[J/OL]. *RSC Medicinal Chemistry*, 14(12): 2593-2610. DOI:10.1039/D3MD00309D.

KNOLL K E, LINDEQUE Z, ADENIJI A A, et al., 2021. Elucidating the antimycobacterial mechanism of action of ciprofloxacin using metabolomics[J/OL]. *Microorganisms*, 9(6): 1158. DOI:10.3390/microorganisms9061158.

KUZMANOVIĆ N, DICENZO G C, BUNK B, et al., 2023. Genomics of the “tumorigenes” clade of the family Rhizobiaceae and description of *Rhizobium rhododendri* sp. nov[J/OL]. *MicrobiologyOpen*, 12(2): e1352. DOI:10.1002/mbo3.1352.

LI S, LIU Y, WU Y, et al., 2022. Antibiotics in global rivers[J/OL]. *National Science Open*, 1(2): 20220029. DOI:10.1360/nso/20220029.

LIU S, WANG C, HOU J, et al., 2020. Effects of Ag NPs on denitrification in suspended sediments via inhibiting microbial electron behaviors[J/OL]. *Water Research*, 171: 115436. DOI:10.1016/j.watres.2019.115436.

LYU J, YANG L, ZHANG L, et al., 2020. Antibiotics in soil and water in China—a systematic review and source analysis[J/OL]. *Environmental Pollution*, 266: 115147. DOI:10.1016/j.envpol.2020.115147.

MA T F, YU X Y, XING C Y, et al., 2025. Impacts of sulfamethoxazole on heterotrophic nitrification-aerobic denitrification bacteria and its response strategies: insights from physiology to proteomics[J/OL]. *Journal of Environmental Management*, 379: 124890. DOI:10.1016/j.jenvman.2025.124890.

MA Y, LI M, WU M, et al., 2015. Occurrences and regional distributions of 20 antibiotics in water bodies during groundwater recharge[J/OL]. *Science of The Total Environment*, 518-519:

498-506. DOI:10.1016/j.scitotenv.2015.02.100.

MAESTRO-GAITÁN I, GRANADO-RODRÍGUEZ S, REDONDO-NIETO M, et al., 2023. Unveiling changes in rhizosphere-associated bacteria linked to the genotype and water stress in quinoa[J/OL]. *Microbial Biotechnology*, 16(12): 2326-2344. DOI:10.1111/1751-7915.14337.

MASUD M A A, SHIN W S, SEPTIAN A, et al., 2024. Exploring the environmental pathways and challenges of fluoroquinolone antibiotics: a state-of-the-art review[J/OL]. *Science of The Total Environment*, 926: 171944. DOI:10.1016/j.scitotenv.2024.171944.

MUPINDU P, ZHAO Y G, PAN C, et al., 2025. Enhancement of aerobic denitrification process on antibiotics removal: mechanism and efficiency: a review[J/OL]. *Water Environment Research*, 97(3): 1-24. DOI:10.1002/wer.70050.

PETROMELIDOU S, ANAGNOSTOPOULOU K, KORONAIIOU L A, et al., 2024. Exploring patterns of antibiotics during and after COVID-19 pandemic in wastewaters of northern Greece: potential adverse effects on aquatic environment[J/OL]. *Science of The Total Environment*, 914: 169832. DOI:10.1016/j.scitotenv.2023.169832.

RIVETT M O, BUSS S R, MORGAN P, et al., 2008. Nitrate attenuation in groundwater: a review of biogeochemical controlling processes[J/OL]. *Water Research*, 42(16): 4215-4232. DOI:10.1016/j.watres.2008.07.020.

SANG W, LI Z, HUANG M, et al., 2020. Enhanced transition metal oxide based peroxymonosulfate activation by hydroxylamine for the degradation of sulfamethoxazole[J/OL]. *Chemical Engineering Journal*, 383: 123057. DOI:10.1016/j.cej.2019.123057.

SCHAUER L A, RUDEL R A, ACKERMAN J M, et al., 2014. Pharmaceuticals, perfluorosurfactants, and other organic wastewater compounds in public drinking water wells in a shallow sand and gravel aquifer[J/OL]. *Science of The Total Environment*, 468-469: 384-393. DOI:10.1016/j.scitotenv.2013.08.067.

SEETHALAKSHMI P S, CHARITY O J, GIAKOUMIS T, et al., 2022. Delineating the impact of COVID-19 on antimicrobial resistance: an indian perspective[J/OL]. *Science of The Total Environment*, 818: 151702. DOI:10.1016/j.scitotenv.2021.151702.

VAN BOECKEL T P, GANDRA S, ASHOK A, et al., 2014. Global antibiotic consumption 2000 to 2010: an analysis of national pharmaceutical sales data[J/OL]. *The Lancet Infectious Diseases*, 14(8): 742-750. DOI:10.1016/S1473-3099(14)70780-7.

WANG J, CHU L, WOJNÁROVITS L, et al., 2020. Occurrence and fate of antibiotics, antibiotic resistant genes (ARGs) and antibiotic resistant bacteria (ARB) in municipal wastewater treatment plant: an overview[J/OL]. *Science of The Total Environment*, 744: 140997. DOI:10.1016/j.scitotenv.2020.140997.

WHELINTA SHERIN A, PALANIVELU K, SRI SHALINI S, et al., 2023. Impacts of climate change on groundwater quality in coimbatore smart city areas of tamil nadu, india[J/OL]. *Environmental Earth Sciences*, 82(21): 488. DOI:10.1007/s12665-023-11179-5.

XIANG Y, SHAO Z, CHAI H, et al., 2020. Functional microorganisms and enzymes related nitrogen cycle in the biofilm performing simultaneous nitrification and denitrification[J/OL]. *Bioresource Technology*, 314: 123697. DOI:10.1016/j.biortech.2020.123697.

YIN G, HOU L, LIU M, et al., 2016. Effects of thiamphenicol on nitrate reduction and N₂O release in estuarine and coastal sediments[J/OL]. *Environmental Pollution*, 214: 265-272. DOI:10.1016/j.envpol.2016.04.041.

ZENG S, HOU D, LIU J, et al., 2019. Antibiotic supplement in feed can perturb the intestinal

microbial composition and function in pacific white shrimp[J/OL]. *Applied Microbiology and Biotechnology*, 103(7): 3111-3122. DOI:10.1007/s00253-019-09671-9.

ZHANG R, XU X, LYU Y, et al., 2022. Impacts of engineered nanoparticles and antibiotics on denitrification: element cycling functional genes and antibiotic resistance genes[J/OL]. *Ecotoxicology and Environmental Safety*, 241: 113787. DOI:10.1016/j.ecoenv.2022.113787.

ZHU T, MA Z, SU Z, et al., 2026. Sulfamethoxazole disrupts denitrification pathway and enhances nitrous oxide (N₂O) accumulation through enzymes and microbial community shifts[J/OL]. *Bioresource Technology*, 451: 134497. DOI:10.1016/j.biortech.2026.134497.