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Oxytetracycline, copper, and zinc effects on nitrification processes and microbial activity in two soil types

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Abstract

The distribution, fate, and effects of antibiotics and heavy metal residues in agricultural soil caused by long-term application of organic fertilizers are of increasing concern. However, the ecotoxic effects of the interaction between antibiotics and heavy metals vary with the physicochemical properties of the soil, and it is still unclear how these substances interact with soil microbial functions. A short-term microcosm experiment was conducted to investigate effects of the typical antibiotic oxytetracycline (OTC) with heavy metals (zinc [Zn] and copper [Cu]) alone or in combination on nitrification process and soil microbial activity in two different types of soil (FQ: sandy loam soil and NB: clay loamy soil). Results indicated that soil types influenced the toxic effects of antibiotics and heavy metals. Zn and Cu alone and when combined with OTC inhibited and retarded nitrification processes and reduced nitrous oxide emissions, which were mainly attributed to the inhibitory effects on ammonia-oxidizing microorganisms. Moreover, Zn and Cu alone or combined with OTC increased soil respiration, but decreased the abundances of bacteria and fungi. In contrast, OTC alone had no significant effect on soil respiration but increased the abundance of fungi in both soils. Together, our results suggest that the widespread occurrence of antibiotics and heavy metals in agriculture soils may pose significant eco-environmental risks by altering nitrification process and soil microbial activity.

KEYWORDS

ammonia-oxidizing microorganisms, antibiotics, heavy metals, microbial activity, nitrification, nitrous oxide

1 | INTRODUCTION

Large amounts of antibiotics are extensively used for therapeutic, prophylactic, and growth promotion purposes in intensive animal husbandry (Granados-Chinchilla & Rodríguez, 2017). For example, in the USA the number of antibiotics sold for food animals is approximately fourfold greater than that for human use in the USA (Maron, Smith, & Nachman, 2013). However, a large percentage (25%–75%) of antibiotics are excreted in manure due to incomplete metabolism (Jechalke, Heuer, Siemens, Amelung, & Smalla, 2014). In addition, zinc (Zn) and copper (Cu) are

normally found in animal feeds at concentrations that exceed the nutritional requirements of the animals and for prevention of diarrheal disease, and also as an alternative to in-feed antibiotics for growth promotion (Yazdankhah, Rudi, & Bernhoft, 2014). Consequently, antibiotic and heavy metal compounds are accumulated in agricultural soil when repeated applications of manure occur. With a large number of antibiotics and heavy metals being detected in agricultural soils at concentrations ranging from ng/kg to mg/kg (Guo et al., 2018; Tamtam et al., 2011), questions have been raised concerning possible effects on soil microbial functions and potential implications for the health and balance of the agroecosystem (Abdu, Abdullahi, & Abdulkadir, 2017; Grenni, Ancona, & Barra Caracciolo, 2018).

Nitrification, as an essential component of the global nitrogen cycle, is a microbially regulated process converting ammonia to nitrate via nitrite and leads to changes in plant nitrogen (N) availability and greenhouse gas N_2O emissions (Kuypers, Marchant, & Kartal, 2018). The first step involving conversion of ammonia to nitrite is often assumed to be rate limiting and performed by ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA; van Kessel et al., 2015). Organic fertilization involving manure application affects microbial functional diversity by mediating the availability of soil inorganic N and bioavailable organic matter, thereby affecting the nitrification process in agricultural soils (Kong et al., 2019). In addition to direct effects, organic fertilization could indirectly regulate nitrification processes through the accumulation of antibiotics and heavy metals in manures. Although antibiotics and heavy metals have been detected in many environments, their ecological effects have been poorly investigated, and inconsistent effects of antibiotics and heavy metals on nitrification have been reported (Roose-Amsaleg & Laverman, 2016). For instance, Konopka, Henry, Marti, and Topp (2015) found that bacitracin at 0.1 mg/kg had no effect on nitrification, whereas it increased nitrification when applied at 100 mg/kg in a loamy soil. In contrast, Banerjee and D'Angelo (2013) reported that bacitracin had no effect on nitrification in the silty loam at any concentration. These inconsistencies may be due to differences in the persistence and bioavailability of antibiotics in different environmental matrixes. Research has shown that the fate and effects of such compounds depend not only on the characteristics of the molecules, but also to a large extent on the nature and properties of the main soil components (Albero, Tadeo, Escario, Miguel, & Pérez, 2018). Leal, Alleoni, Tornisielo, and Regitano (2013) reported that the sorption of fluoroquinolones and sulfonamides in soils with different physicochemical and mineralogical properties was most affected by soil texture and cation exchange capacity. However, combined effects of antibiotics and heavy metals on N transformation processes from different soil types remains unclear. Moreover, different plant species have different uptake preferences for inorganic N forms under specific soil conditions (Boudsocq et al., 2012; Britto & Kronzucker, 2013; Zhang, Wang, Müller, & Cai, 2016). Most crops prefer NH_4^+–N over NO_3^-–N, because the conversion of former N species in protein is less energy intensive to the conversation of later N species to protein (Bolan, Saggar, Luo, Bhandral, & Singh, 2004). Crop plant species that adapt to low redox potential, such as rice (*Oryza sativa* L.) prefer NH_4^+–N (Wang, Siddiqi, Ruth, & Glass, 1993). However, NO_3^- is usually more available for uptake, owing to its higher mobility (Wang & Macko, 2011). Some crops that grow in neutral and alkaline soils in arid and semiarid areas (e.g., wheat [*Triticum aestivum* L.]) prefer NO_3^-–N and grow in soils with higher nitrification rates (Hamid, 1972). The interference of antibiotics and heavy metals on N transformation process may lead to a mismatch between preferential N uptake by plants and soil N dynamics, which may affect crop N use efficiency and induce high N loss. Therefore, to determine the influence of soil types and to elucidate how antibiotics and heavy metals, when simultaneously released into the soil environment, alter N-cycle-related processes, are critical to provide more information to understand how to affect the N fertilizer utilization efficiency of crops and the N loss.

Microorganisms in soil are sensitive to soil pollution which may lead to changes in ecosystem functioning. Previous researchers have extensively reported the adverse effects of antibiotics and heavy metals on broad functioning, for example, Conkle and White (2012) found ciprofloxacin and sulfamethoxazole exhibited the ability to suppress soil respiration. Tobor-Kapłon, Bloem, Römkens, and Ruiter (2005) demonstrated that under heavy metals stress, the more resistant microorganisms increased soil respiration because oxygen consumption increases during the decontamination process, while vulnerable microorganisms reduced soil respiration due to intoxication. Additionally, bacteria and fungi often share a common substrate, and their ecological interactions lead to synergy or antagonism (Mille-Lindblom, Fischer, & Tranvik, 2006). The misuse and abuse of antibiotics in veterinary and human medicine have accelerated the growing worldwide phenomenon of antimicrobial resistance, which can lead to an increase in the number of antibiotic-resistant genes in bacteria (Martínez, 2008), but the extent to which it influence fungi is unclear. Importantly, there is a growing body of evidence that antibiotics and heavy metals can form complexes, which can hinder (or enhance) antibiotic activity, and heavy metals can also drive the development of antibiotic resistance in metal-exposed bacteria (Poole, 2017). Nonetheless, when antibiotics and heavy metals coexist in soil, it is unclear which takes the dominant role in disturbing microbial activity. Therefore, it is important to examine the potential impacts of antibiotics and heavy metals on disruption of soil microbial function at environment-related concentrations.

In the present study, oxytetracycline (OTC), Cu, and Zn were selected as target contaminants to investigate the individual and combined effects of antibiotics and heavy metals on nitrification processes and soil respiration in two different types of soil. We hypothesized antibiotics and heavy metals have different effects on nitrification and soil respiration in different soil types, Cu and Zn have higher inhibitory effects than OTC when they coexist in soil. The abundance of soil bacteria, fungi, AOB, and AOA *amoA* was studied to elucidate the related microbial mechanisms. This work will provide more realistic data for the understanding of soil functions responding to multiple antibiotics and heavy metals exposure in agricultural soil, which may help to establish an index system for livestock manure agricultural safety assessment.

2 | MATERIALS AND METHODS

2.1 | Soils collection and chemicals purchase

The experiments were performed with two different types of soil: a sandy loam collected from Fengqiu (FQ), Henan Province, China (28°15′N, 116°55′E), where the traditional cropping system is wheat (*T. aestivum* L.) grown in winter and maize (*Zea mays* L.) cultivated in summer. The region has a semiarid, subhumid monsoon climate, with a mean annual temperature of 13.9°C and precipitation of 615 mm; a clay loam collected from Ningbo (NB), Zhejiang Province, China (28°15′N, 116°55′E), where the traditional cropping system is rice (*O. sativa* L.)-wheat (*T. aestivum* L.). The region has a subtropical monsoon climate, with a mean annual temperature of 17.4°C and precipitation of 1,480 mm; Both were taken from the surface layer (0–20 cm) and were sieved (<2 mm), then stored at 4°C prior to the incubation experiment. Soil main physicochemical properties are presented in Table 1. Inorganic N (NH_4^+–N and NO_3^-–N) were measured prior to storage and incubation, and extracted with 2 M of potassium chloride (KCl) solution at 25℃, in a 1:5 soil solution ratio, the contents were determined using a continuous flow analyser (San++ System; Skalar; Henriksen & Selmer-Olsen, 1970). Soil pH was measured by pH meter (Sartorius) at soil/distilled water ratio of 1:5, stirred with a glass rod and allowed to sit for 30 min (Thunjai, Boyd, & Dube, 2001). Then, the contents of soil organic matter (SOC) were determined by wet digestion with H_2SO_4–$K_2Cr_2O_7$. Soil total nitrogen (TN) was determined using a Vario MAX CNS elemental analyser (Elementar). Soil particle size was determined with a laser particle size analyser (LS13320; Beckman Coulter).

The OTC with purity >99% was purchased from Sigma. The Zn and Cu (added as $CuCl_2$ and $ZnCl_2$) and $(NH_4)_2SO_4$ used in this study were of analytical grade and were purchased from Chemical Reagent Factory. The OTC, Zn, and Cu stock solution were prepared as aqueous solutions in sterile deionized water.

2.2 | Experimental design

The experiment design included 11 treatments for each soil type with each treatment three replications, as follows: a control with no contamination (CK); two treatments of individual OTC addition (OTC0.1 and OTC1 at 0.1 and 1 mg/kg, respectively); two treatments of individual Zn (Zn200 and Zn400 at 200 and 400 mg/kg, respectively); two treatments of individual Cu (Cu50 and Cu200 at 50 and 200 mg/kg, respectively); two treatments of combined OTC and Zn (OTC0.1 + Zn200 and OTC1 + Zn400); and two treatments of combined OTC and Cu (OTC0.1 + Cu50 and OTC1 + Cu200).

2.3 | Measurements

2.3.1 | Nitrification

Nitrification processes were determined by monitoring the dynamics concentrations of ammonium-N (NH_4^+–N), nitrite-N (NO_2^-–N), and nitrate-N (NO_3^-–N) in soils amended with substrate ($(NH_4)_2SO_4$) over a 72-hr incubation period (Keeney & Bremner, 1966). Twenty grams of fresh soil (on a dry weight basis) was weighed into a series of 250-ml Erlenmeyer flasks. Each treatment had three replications. After a 7 days preincubation, $(NH_4)_2SO_4$ solution was applied evenly to the flasks at a rate of 50 mg NH_4^+–N per kg to stimulate nitrification activity. The OTC, Zn, and Cu stock solutions were subsequently applied uniformly according to the required treatment levels, and the samples were finally moistened to 60% WHC with distilled water. The flasks were covered with parafilm with needle holes to maintain aeration and then incubated at 25°C. At 6, 24, 48, and 72 hr after the addition of the substrate, the soil

TABLE 1 Soil basic properties of FQ and NB soils before incubation study

Sampling site	pH	C/N	Total *N* (%)	SOC (%)	NH_4^+–N (mg/kg)	NO_3^-–N (mg/kg)	Clay (%)	Silt (%)	Sand (%)	Cu (mg/kg)	Zn (mg/kg)
FQ	8.32	13.18	0.16	2.05	3.63	22.16	23.2	21.7	55.1	17.1	82.5
NB	5.83	10.45	0.27	2.76	8.64	15.76	30.5	33.4	36.1	20.0	96.5

from three replicates per treatment (selected randomly) was sampled destructively and extracted with salt solution (2 M KCl) to monitor dynamics concentrations of NH_4^+–N, NO_2^-–N, and NO_3^-–N.

2.3.2 | N_2O and carbon dioxide emissions

Another group of flasks was used to measure N_2O and carbon dioxide (CO_2) emissions (soil respiration) at 6, 24, 48, and 72 hr after substrate addition. Before gas sampling, the flasks were sealed with rubber stoppers for 6 hr at each time point, and then 20 ml of gas samples were transferred with a syringe into pre-evacuated 20 ml vials to analyze N_2O and CO_2 concentrations with a gas chromatograph (Agilent 7890). Gas fluxes were calculated assuming concentrations followed a linear change during the 6 hr period and the cumulative emissions for the 72 hr incubation were calculated by linear interpolation between two consecutive measurement times (Chen et al., 2015).

2.3.3 | Molecular and bioinformatics analysis

At the end of incubation, the soil samples in each flask were stored at −20°C prior to analysis of the abundances of nitrification functional genes, bacteria, and fungi. Briefly, the total DNA was extracted with a Fast DNA SPIN Kit for soil (MP Biomedical), according to the manufacturer instructions. The quantity and purity of DNA solutions were determined by a spectrophotometer (NanoDrop 2000; Thermo Scientific). Quantitative PCR amplification (qPCR) for the *amoA*- AOA and AOB genes was used to assess the nitrification functional abundances, the primers and thermal cycle conditions for the qPCR were described by Xia et al. (2011). The copies of 16S rRNA genes and 18S rRNA genes were measured to estimate the bacterial and fungal abundances, the primers and thermal cycle conditions were according to Rousk et al. (2010). The copy numbers of genes were quantified using SYBR Green qPCR methods with Applied Biosystems QuantStudio™ 3.

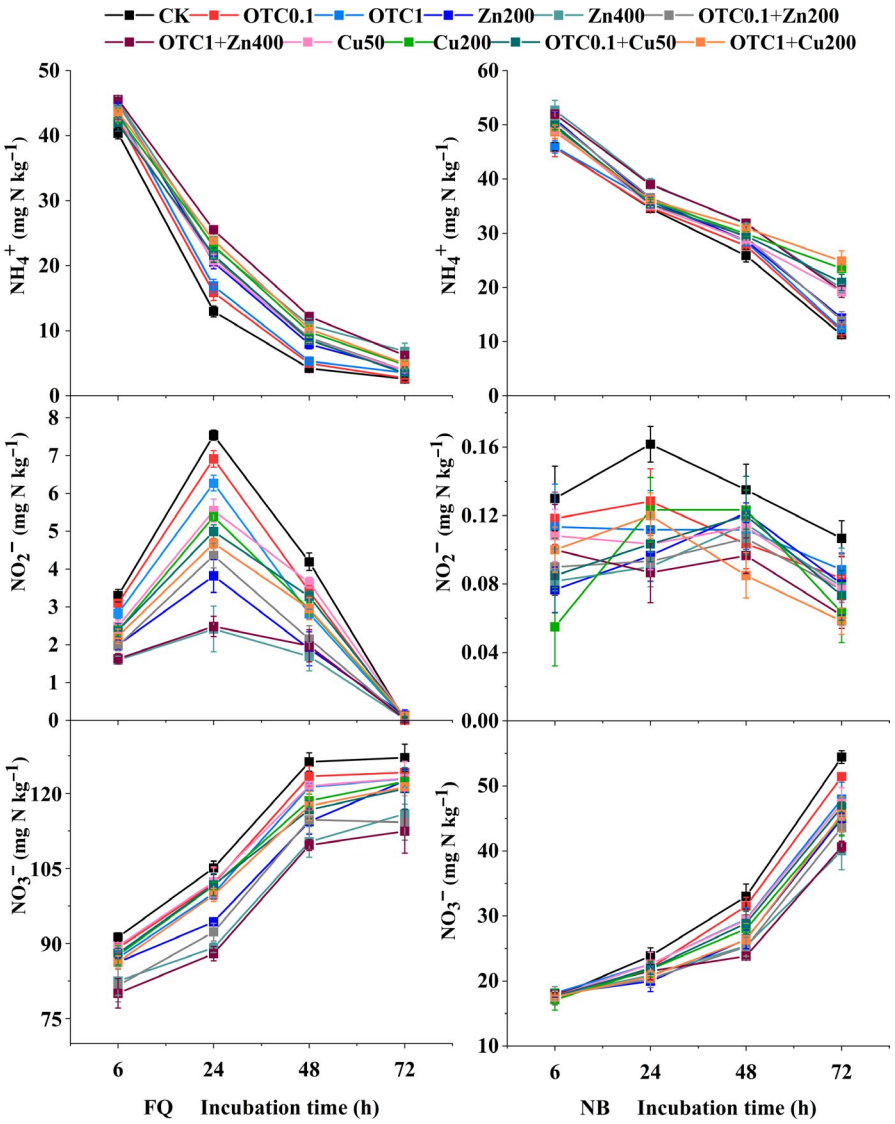

FIGURE 1 Dynamics of NH_4^+–N, NO_2^-–N, and NO_3^-–N over the 72-hr incubation for the NB (a) and FQ (b) soils; error bars indicate the standard deviations ($n = 3$)

The 20 μl of reaction system contained 10 μl SYBR Premix Ex Taq (Takara), 1 μl of each primer and 7.02 μl distilled water, 0.08 μl of ROX reference solution and 2 μl of template DNA. Standard curves were generated using serial dilutions linearized plasmids (pGEM-T; Promega) containing gene fragments of interest from model organisms, plasmids were diluted to produce standards of known copy number to allow the generation of curves containing 10^{10}–10^{3} copies per reaction in a 10-fold dilution series. The amplification efficiencies of all functional gene copies were 90%–110% and standard curve R^2 values were >.99.

2.4 | Statistical analysis

Data analyses were performed with SPSS (version 19.0). The concentration of NH_4^+–N, NO_2^-–N, and NO_3^-–N at each sampling time point, cumulative of N_2O and CO_2 emissions, and abundances of all studied genes were assessed for variation among treatments by one-way ANOVA, and the average value of each treatment was compared using Tukey-HSD test to determine significant differences ($p < .05$).

3 | RESULTS

3.1 | Effects of individual and combined OTC, Cu and Zn on inorganic N dynamics

During incubation, NH_4^+–N concentration in all treatments gradually declined in both soils (Figure 1). In the FQ soil, the NH_4^+–N concentration sharply dropped down to less than 2 mg N per kg within 72 hr. However, the NB soil responded more slowly, and NH_4^+–N concentrations remained higher than 10 mg N per kg at the end of the incubation. Compared with the CK treatment, the interaction between OTC and Cu or Zn significantly increased NH_4^+–N concentration in both soils. The increment level of NH_4^+–N content indicating the rate of nitrification followed: soils amended with antibiotic and heavy metals (27.2%–75.6%) > soils amended individual heavy metal alone (25.4%–71.3%) > soils amended with the antibiotic alone (4.5%–18.8%). On average, the toxic effect was relatively higher in FQ soil (62.5%) than in NB soil (21.9%).

As intermediate product of nitrification, NO_2^-–N levels increased before they went down, the peak of NO_2^- concentration was observed in the CK treatment after 24 hr of incubation with values of was 7.5 mg/kg in the FQ soil and 0.16 mg/kg in the NB soils. The NO_2^- concentrations in FQ soil were one order of magnitude higher than those from NB soil until at the end of incubation, at the last time point in FQ soil, the NO_2^- concentration dropped sharply to 0.04–0.27 mg N per kg, the same level as that in NB soil (Figure 1). Compared to the CK treatment, NO_2^- concentrations decreased by 35.2% in the FQ soil and 27.3% in the NB soils on average when OTC, Zn, or Cu were added alone or in combination.

In correspondence with the decrease in NH_4^+–N concentrations, the NO_3^-–N concentrations in all treatments increased during the incubation (Figure 1). Compared with the CK treatment, Zn and Cu alone or combined with OTC contamination led to decrease in NO_3^-–N production (by 6.7% on average) in FQ soils at each sampling time. OTC combined with Zn contamination (11.8%) further inhibited NO_3^-–N producing rate than that combined with Cu (8.5%). However, no significant change was observed across all treatments at 6 hr in NB soil, and a significant decrease in NO_3^-–N was observed at 48 hr incubation when compared to the control.

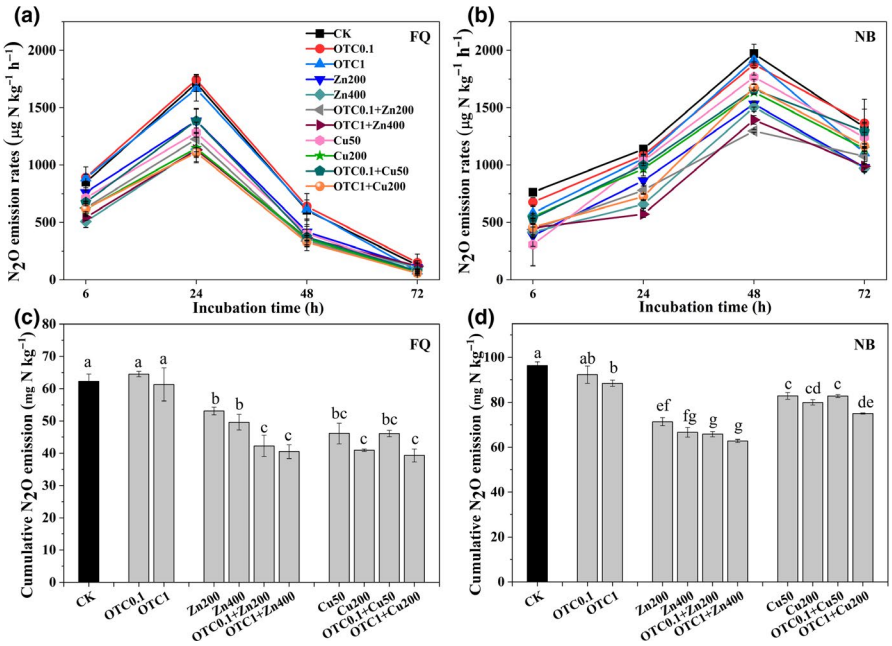

FIGURE 2 N_2O emission rate and cumulative N_2O emissions over the 72-hr incubation for the FQ (a,c) and NB (b,d) soils. Error bars indicate the standard deviations ($n = 3$). Different lowercase letters above the error bars indicate significant differences among treatments ($p < .05$)

In general, compared with the CK treatment, soil under OTC, Zn, and Cu alone or combined contamination led to more NH_4^+–N but less NO_2^-–N, and NO_3^-–N during the 72 hr incubation in both soils.

3.2 | Effects of individual and combined OTC, Cu, and Zn on N_2O emissions

The N_2O emissions increased rapidly, reaching a maximum value of 1,744 μg kg^{-1} hr^{-1} after incubation in the FQ soil for 24 hr, and then rapidly decreased to 126 μg kg^{-1} hr^{-1} in the CK treatment after 72 hr incubation (Figure 2a). In contrast, the NB soil responded more slowly and emissions reached their highest peak 1,972 μg kg^{-1} hr^{-1} after adding substrate for 48 hr, and then tended to decrease (Figure 2b). When OTC was applied alone, no obvious inhibitory effect was observed at each sampling time in FQ soil, while Zn and Cu alone or that combined with OTC contamination inhibited N_2O emissions at 24 and 48 hr, with the highest inhibition at 24 hr; however, these toxic effects were reduced at the end of the incubation. The toxic effects of Zn and Cu alone or that combined with OTC contamination on N_2O emission lasted for the entire incubation cycle in NB soil. Cumulative N_2O emissions were in the range of 39.3–64.5 and 62.8–96.4 mg/kg in the FQ and NB soils, respectively (Figure 2c,d). In the FQ soil, OTC addition alone had no significant influence on cumulative N_2O emissions, whereas in NB soil, N_2O emissions showed a trend toward decreasing when OTC was added alone and significantly decreased at 1 mg/kg. Compared with the CK treatment, the cumulative N_2O emissions were significantly decreased in Zn and Cu alone or those combined with OTC contamination by 14.7%–36.8% and 14.1%–34.8% in FQ and NB soil. Furthermore, compared with the soils applied with OTC or Zn alone, N_2O emissions were inhibited greatly when the mixture of OTC and Zn was added.

3.3 | Effects of individual and combined OTC, Cu, and Zn on ammonia oxidation microorganisms

The abundances of AOA *amoA* ranged from 1.80×10^9 to 3.16×10^9 and 1.95×10^9 to 3.58×10^9 copies/g dry weight soil in the FQ and NB soils, respectively (Figure 3a,b). Compared with the CK treatment, OTC added alone at the concentration of 0.1 mg/kg had no significant inhibition effect on the abundance of AOA and AOB *amoA* gene, but at 1 mg/kg significantly reduced the abundance of AOB *amoA* gene in NB soil. Except for the Cu addition alone at 50 mg/kg, Zn and Cu added alone or combination with OTC significantly decreased the abundances of AOA *amoA* genes by 7.9%–40.1% and 15.6%–38.9% in the FQ and NB soils, respectively. The toxicity of the combined contamination was higher than that of individual contamination, and the inhibition rate was enhanced with the increase of concentration, indicating antagonistic reactions. The abundances of AOB *amoA* ranged from 4.98×10^6 to 8.87×10^6 and from 9.57×10^8 to 1.72×10^9 copies/g dry weight soil in the FQ and NB soils, respectively (Figure 3c,d). The numbers of AOB *amoA* gene copies in the NB soil were approximately two orders of magnitude higher than those in the FQ soil. The copy number of AOB *amoA* gene in Zn and Cu alone or combined with OTC contamination treatments was significantly different from that in CK and was reduced by 15.8%–44.0% and 14.2%–42.0% in FQ and NB soils, respectively.

3.4 | Effects of individual and combined OTC, Cu, and Zn on soil microbial activity

At the beginning of the incubation (6 hr), lower respiration rates were detected following individual OTC addition, while higher respiration rates were detected following Zn and Cu alone or combined with OTC addition in both soils. This effect appeared to be temporary and the soil respiration trended to recover to CK level at the end of incubation (72 hr; Figure 4a,b). Soil cumulative respiration was in the range of 46.6–79.5 and 58.6–84.1 g CO_2–C per kg in the FQ and NB soils, respectively (Figure 4c,d). Compared with the CK treatment, soil respiration was stimulated in all Zn and Cu alone or combined with OTC contamination treatments by 12.1%–59.6% and 16.4%–36.7% in FQ and NB soil, respectively. However, OTC added alone had no significant influence on both soils.

The abundance of the bacterial 16S rRNA gene ranged from 2.94×10^{11} to 4.43×10^{11} and 1.79×10^{10} to 3.03×10^{10} copies/g dry weight soil in the FQ and NB soils, respectively (Figure 5a,b). Compared with the CK treatment, except for the OTC addition alone at 0.1 mg/kg, all contaminated treatments significantly decreased 16S rRNA gene abundance by 7.6%–33.7% and 7.90%–41.03% in the FQ and NB soils, respectively. The fungal 18S rRNA gene abundance ranged from 2.22×10^8 to 4.09×10^8 and 1.41×10^8 to 2.11×10^8 copies/g dry weight soil in the FQ and NB soils, respectively (Figure 5c,d). The abundance of 18S rRNA gene was increased by OTC alone but not significantly, and reduced by 6.4%–33.7% and 6.8%–28.7% in the all Zn and Cu alone or combined with OTC contamination treatments in the FQ and NB soils. In addition, the combined OTC and heavy metal (Zn or Cu) further decreased the abundance of the 18S rRNA gene than that of individual contamination, and the inhibition rate was enhanced with the increase of concentration.

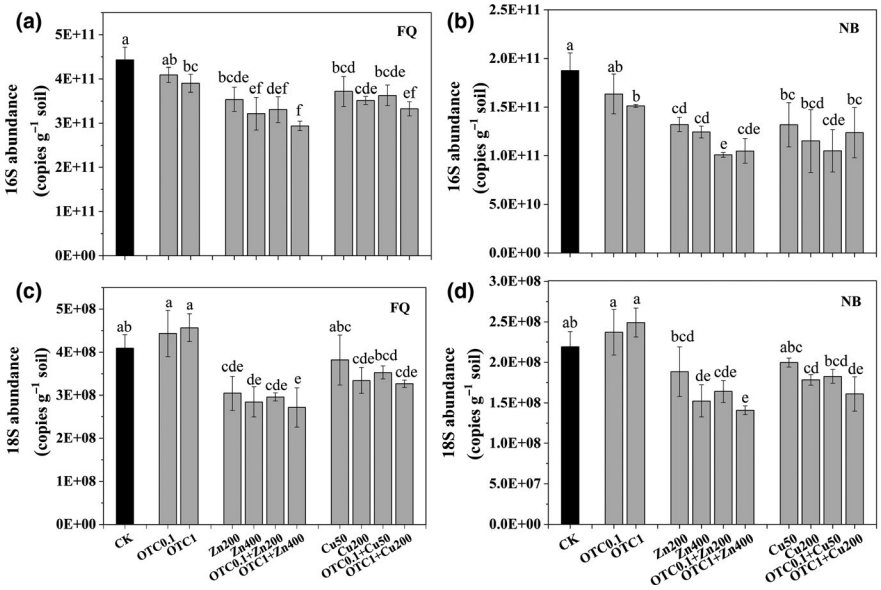

FIGURE 3 Abundance of AOA and AOB *amoA* gene copy numbers over the 72-hr incubation for the FQ (a,c) and NB soils (b,d), respectively. Error bars indicate the standard deviations ($n = 3$). Different lowercase letters above the error bars indicate significant differences among treatments ($p < .05$)

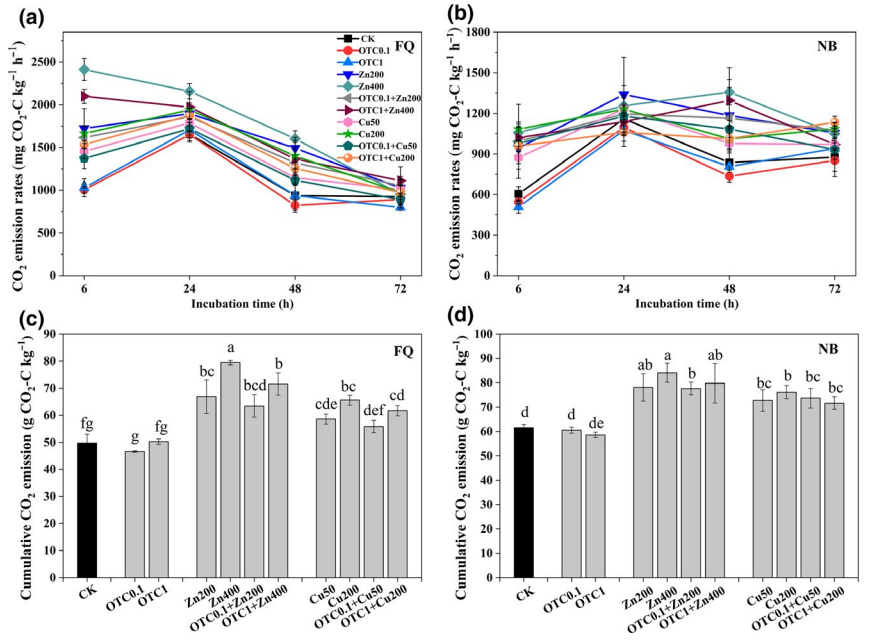

FIGURE 4 CO_2 emission rate and cumulative CO_2 emission over the 72-hr incubation for the FQ (a,c) and NB (b,d) soils. Error bars indicate the standard deviations ($n = 3$). Different lowercase letters above the error bars indicate significant differences among treatments ($p < .05$)

4 | DISCUSSION

4.1 | Influence of individual or combined OTC and Cu/Zn on nitrification process

Conducting a long-term experiments is difficult because of degradable antibiotics and rapidly consumed nutrients, unless continuously added (Alighardashi, Pandolfi, Potier, & Pons, 2009). Therefore, a short-term laboratory simulation experiment was conducted to investigate the acute effect of a typical antibiotic (OTC) with heavy metals (Zn and Cu) alone or in combination on nitrification processes and soil microbial respiration in two different types of soil. As a bacterial inhibitor, OTC may affect soil N cycling by directly changing the abundance of nitrifying functional genes (Konopka et al., 2015). However, the relative distribution and potential contribution of AOA and AOB under antibiotics and heavy metals contaminate conditions are unclear. In this study, OTC alone at 1 mg/kg decreased the abundance of AOB *amoA* gene, while showed no obvious effect on abundance of AOA *amoA* gene in both types of soil, indicating that the inhibitory effects of OTC on AOB were stronger than on AOA. The observed stronger resistance of AOA than AOB toward OTC might be due to the differences in archaeal and bacterial cell walls and membranes, archaea peptidoglycan-free cell walls, and ether lipids membranes may be more resistant to Cu and Zn (Conkle & White, 2012). Schauss et al. (2009) showed that contribution of AOAs activity to ammonia oxidation increased from 50% to 70% under antibiotic sulfadiazine stress, indicating the

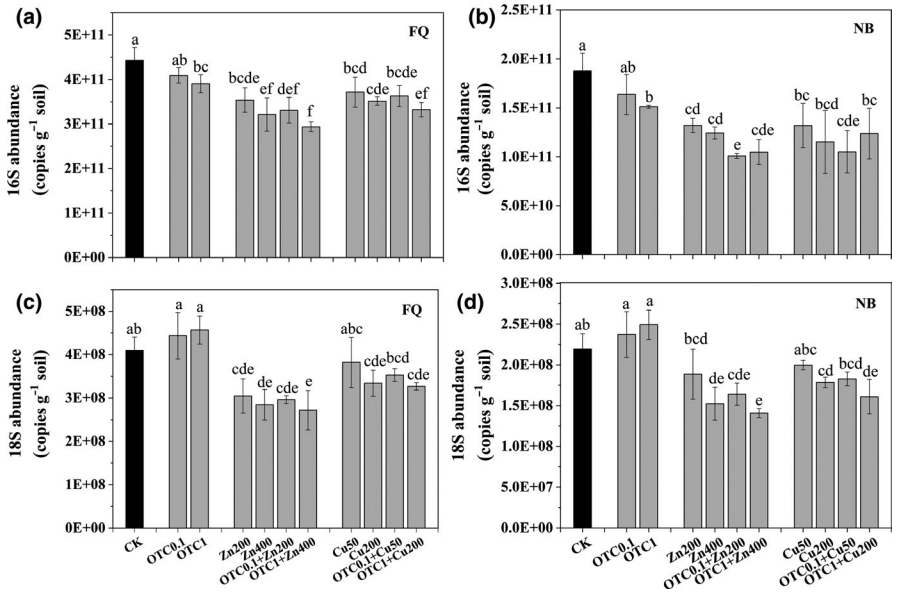

FIGURE 5 Abundance of bacteria (16S) and fungi (18S) gene copy numbers over the 72-hr incubation for the FQ (a,c) and NB (b,d) soils, respectively. Error bars indicate the standard deviations ($n = 3$). Different lowercase letters above the error bars indicate significant differences among treatments ($p < .05$)

functional redundancy between AOA and AOB. Therefore, these might explain that OTC alone at low dose showed no obvious inhibition effect on the oxidation of NH_4^+ to NO_3^- and N_2O emission. The soil ecosystem appears to have a buffering mechanism to antibiotic stress and recover from the inhibition of certain sensitive microorganisms by substituting their functions with less sensitive but functionally redundant groups. However, the combination of OTC with Zn or Cu significantly decreased the abundance of AOA and AOB *amoA* genes and inhibited the nitrification process, resulting in higher NH_4^+–N, lower NO_2^-–N, NO_3^-–N concentration and reduced N_2O emissions in both of soils, indicating that the effect of compounded contaminations was higher than that of individual contaminations. These findings are in agreement with other studies where antibiotics and heavy metals have been applied together (Wang, Wang, Zhu, & Wang, 2018). This might be organic antibiotics and inorganic heavy metals coexisting in the soil and resulting in more complex forms of pollution that affect its functional microorganisms. Turel (2002) demonstrated that nonpolar core and multiple polar functional groups in antibiotics can be complexed with metal ions and interact with each other. OTC-metal chelation causes a spectral redshift of OTC, indicating that an OTC + metal complex is indeed formed in the culture system (Wu & He, 2019). Importantly, there is a growing evidence that Cu/Zn can drive the development of antibiotic resistance in metal-exposed bacteria due to metal selection of genetic elements that harbor both metal and antibiotic resistance genes, and metal recruitment of antibiotic resistance mechanisms (Becerra-Castro, Machado, Vaz-Moreira, & Manaia, 2015). Cu and Zn can also enhance antibiotic activity through metal–drug synergy (Poole, 2017). For example, Zn-ciprofloxacin complexes were shown to have greater biological activity against Gram-positive and Gram-negative bacteria compared with uncomplexed ciprofloxacin (Chohan, Supuran, & Scozzafava, 2005). Similarly, the Cu complex of the OTC was markedly more active than OTC alone influence functional diversity of the soil microbial community (Kong, Zhu, Fu, Marschner, & He, 2006). However, whether the synergy reflects metal influences on antibiotic activity directly or their combined stresses enhance the activity of one or the other of these agents is unclear. In addition, a higher nitrification rate was observed in FQ soil than that in NB soil. This could be explained by the higher pH in FQ soil than that of NB soil (Table 1), it has been found that rates of nitrification are relatively low in acidic soil (Lan, Han, Roelcke, Nieder, & Car, 2014). Furthermore, we found that the toxic effect of heavy metals was relatively higher in FQ soil than in NB soil. Previous studies indicated that the higher pH value decreased the availability of heavy metal concentrations in soils (Kazlauskaitė-Jadzevičė, Volungevičius, Gregorauskienė, & Marcinkonis, 2014). However, the mobility and availability of antibiotics and heavy metals also depends on organic carbon content, clay minerals, inorganic anions, and cations, etc. (Park & Huwe, 2016; Zeng et al., 2011). A large proportion of heavy metals present in the soil solid phase is immobile because of association with organic C. Borggaard, Holm, and Strobel (2019) suggest that the organic C present has also a role in the bioavailability of Zn and Cu, leading to its reduced bioavailability. In the current study, higher soil organic carbon was found in NB soil than in FQ soil (Table 1), which could immobilize OTC, Zn, and Cu through absorption and complexation reactions in the NB soil (Beesley et al., 2011). The adsorption behavior reduces the bioavailability and mobility of the OTCs but, on the other hand, extends their persistence in soil environments (Thiele-Bruhn, 2003). Compared with Table 1, there is a big difference of initial values of NH_4^+ to

NO_3^- in Figure 1. This may be due to the soil basic properties were measured immediately after the collection of the soil samples, and storied in 4℃ until further use (2 weeks), which may result in change NH_4^+ and NO_3^- content during this period (nitrification can also occur in 4℃). Sakai (1959) reported that soils which have high nitrifying activities at ordinary temperature show fairly good nitrification even at lower temperatures (1, 2, 5℃). Minimum temperatures for nitrification in soil have also been reported as low as 2°C (Malhi & Nyborg, 1979).

4.2 | Influence of individual or combined OTC, Zn, and Cu on microbial activity

In this study, OTC at high concentration was found to have clear inhibiting effects on bacteria, while stimulating fungal abundance (Figure 5). Similar results were found in previous research (Ding & He, 2010) and could be due to competition displacement, since the modes of action of antibiotics are generally highly specific for prokaryotes (especially bacteria). Soil respiration was not significantly affected by OTC regardless of the dosages of antibiotics, which also suggested that shifts in the microbial community structure that compensated for effects on individual species, fungi are not inhibited by the addition of antibiotics and may overtake the function of bacterial in soil. Furthermore, OTC is only selectively inhibiting bacteria growth and does not kill all of them, and the tolerant bacteria can even profit from dead biomass. Kotzerke et al. (2008) demonstrated that during incubation of soils with antibiotics, resistance mechanisms can be spread easily by the transfer of the corresponding plasmids over the bacterial community. This result indicates that antibiotics have induced impact on soil microbial structure, which will ultimately result in changes to soil ecosystem functions. Given that agricultural plants are often susceptible to pathogenic fungi (Rossman, 2009), the increase in fungi abundance in soil may threaten the yield and quality of agriculture products.

However, the abundance of both bacteria and fungi decreased upon OTC addition alongside Zn or Cu contamination, mainly because of the toxic effect of heavy metals on most microorganisms in soil (Sharaff & Archana, 2015). Soil respiration was significantly stimulated by Zn and Cu alone or combined contamination with OTC. The stimulated soil respiration may have occurred because soil microorganisms under heavy metal stress need to consume more energy to survive, resulting in enhanced CO_2 production (Dai et al., 2004; Zhang, Nie, et al., 2016). Similar findings were reported by Barajas-Aceves (2005), who suggested that microorganisms in heavily polluted soils are under stress and have lower efficiency of C assimilation, resulting in more evolved CO_2 per unit of substrate.

4.3 | Perspective and mitigation strategies

Although only through a 72 hr incubation experiment, our results provide direct evidence that Zn and Cu alone or combined with OTC have acute impacts on both broad (microbial activity) and specialized (nitrification) functions. Long-term exposure also needs to be studied to gain a greater understanding of tolerance changes. Antibiotics and heavy metals also pose a potential risk to ecosystem and human health because their toxicity mode is conserved in environmental microorganisms, and some of them are persistent in the environment, and can be absorbed through the food chain (Gall, Boyd, & Rajakaruna, 2015) and drinking water (Kavcar, Sofuoglu, & Sofuoglu, 2009). Furthermore, resistance to trace elements such as Zn and Cu and concomitant cross-resistance to antimicrobial agents has been found in enteral bacteria of farmed animals, which may be transferred to environment and pose a threat to human health (Manaia, 2017; Poole, 2017). In addition, the interference of combined contamination of antibiotics and heavy metals on soil N dynamics may create a mismatch between preferential N uptake by plants and soil N dynamics, resulting in high N loss. Different plants species have different preferences for taking up certain N forms, with many crops occurring in drylands exhibiting NO_3^- preference (e.g., wheat and maize). The nitrogen use efficiency of crops is dependent on N uptake efficiency and N utilization efficiency. Thus, inhibition of nitrification by combined contamination with antibiotics and heavy metals, especially in soils in alkaline regions, may therefore pose additional risks for enhanced losses via ammonia volatilization. Therefore, the first and most important measure would be reducing unnecessary use of antibiotics and heavy metals in livestock production to tackle this problem at the source. An international regulatory and economic survey suggested that restrictions on antimicrobial use in food animal production in Denmark and Sweden may not be detrimental to production in the long run (Maron et al., 2013). According to the World Health Organization, Denmark's ban on antimicrobial growth promoters reduced human health risk without significantly sacrificing animal health or overall economic impacts (World Health Organization, 2003). Secondly, treatment technologies need to be developed to capture nutrients, reduce contaminants, kill harmful pathogens, and minimize impact of livestock manure on the environment. For example, Hou et al. (2019) synthesized thermo-responsive flocculants CS-g-PNNPAM with different grafted branch lengths for the removal of different types of combined contaminants of antibiotics and heavy metals from water.

5 | CONCLUSION

Our findings provide clear indications that antibiotic and heavy metals residues in agricultural fields could exert a

temporary selective pressure on both broad and specialized functioning, which is influenced by soil type. Overall, our results suggest that individual and combined contamination of antibiotics and heavy metals have varying degrees of interference with soil microbial activity. Levels generally decreased as follows: combined heavy metals and antibiotic > individual heavy metals > individual antibiotic. Combined contamination stimulated soil respiration and reduced bacterial and fungal abundance, while OTC alone had no significant effect on soil respiration and increased fungal abundance. In addition, specialized functioning was inhibited by suppressing the functional genes of ammonia-oxidizing microorganisms, where AOB appeared to be more sensitive to the contaminants than AOA. Application of heavy metals alone or combined with OTC inhibited NH_4^+ oxidation to NO_2^- and then decreased NO_3^-–N concentration in both soils, also having an inhibitory effect on N_2O emissions, due to reduced nitrification which produces N_2O as a by-product, and reduced production of NO_3^- consequently, which is the substrate of denitrification. Future research to predict ecosystem multifunctionality based on microbial community structure under antibiotic-heavy metals disturbed environments both in laboratory and field in the long term is needed to comprehensively evaluate the ecological risk of organic fertilizer application.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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