1 **Article Type:** Regular Paper

2 **Date of preparation:** 09/10/2015

3 **Number of text pages:** 35

4 **Number of tables:** 2

5 **Number of figures:** 4

6 **Title:** Independent and combined effects of oxytetracycline and antibiotic-resistant *Escherichia*

7 *coli* O157:H7 on soil microbial activity and partial nitrification processes

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ABSTRACT

The common practice of field-spreading animal manure on agricultural land contributes to the dissemination of antibiotics and antibiotic-resistant pathogens, which may disrupt important soil microbial functions. In this study, the potential independent and combined effects of oxytetracycline (OTC) and antibiotic-resistant *Escherichia coli* O157:H7 (*E. coli* O157:H7) on soil microbial activity and partial nitrification processes were investigated by testing the abundance of 16S rRNA and 18S rRNA, ammonia-oxidizing bacteria (AOB) and archaea (AOA). Treatments included manure-amended soil inoculated vs. not inoculated with *E. coli* O157:H7, treated or not treated with OTC at environmentally relevant concentration. Results indicated that OTC did not affect soil bacterial diversity or abundance but increased the abundance of 18S rRNA, the AOB *amoA* gene, and the activity of urease. The invasion of *E. coli* O157:H7 significantly decreased the abundance of 16S rRNA, the AOA *amoA* gene, and soil microbial diversity from 1 to 14 days, while there was no significant impact of *E. coli* O157:H7 on soil microbial activity and function from 14 to 28 days. The dual treatment with OTC and *E. coli* O157:H7 significantly increased the abundance of AOB at day 14 and 28, which resulted in higher concentrations of NO_3^- -N in the soil. The interaction between OTC and *E. coli* O157:H7 on decreasing the abundance of 16S rRNA and microbial diversity was statistically significant after 1 day of incubation. Additionally, OTC and *E. coli* O157:H7 had significant interactive effects on urease activity, which may be also attributed to the impact on the partial nitrification process.

Keywords: oxytetracycline; *Escherichia coli* O157:H7; soil microbial diversity; soil enzyme activity; Q-PCR

1. Introduction

Globally, thousands of tons of veterinary pharmaceuticals are used annually for disease control or growth promotion in intensive animal husbandry (Thiele-Bruhn, 2003; Sarmah et al., 2006; Liu et al., 2009). A large proportion (30-90%) of the antibiotics may be excreted in manure due to incomplete metabolism. Meanwhile, farm animals often harbor a variety of zoonotic pathogens; for example, dairy cattle are the reservoir of antibiotic-resistant bacteria *E. Coli* O157:H7 (Fang et al., 2014). The common practice of field-spreading animal manure on agricultural land is a major pathway for the dissemination of antibiotic residues and antibiotic-resistant pathogens into the agoecosystem and beyond. Indeed, antibiotics and antibiotic-resistant bacteria related to food animal production have been detected in agricultural soils receiving manures (Qiao et al., 2012; Fang et al., 2014), raising concerns over potential soil health as well as food security and ecological sustainability issues (Jechalke et al., 2014).

Oxytetracycline (OTC) is one of the tetracycline class of antibiotics widely used on animal farms with broad-spectrum antimicrobial activities against a variety of bacterial infections (Gao et al., 2013). Residues of OTC have been detected in farm animal manure, water and soils (Qiao et al., 2012; Hou et al., 2009). Several studies have reported adverse effects of OTC on soil enzymes (Yang et al., 2009; Gao et al. 2013; Thiele-Bruhn and Beck 2005), soil microbial community (Liu et al. 2009), microbial function (Liu et al., 2012; Kong et al., 2006) and root and shoot elongation of wheat (Jin et al., 2009). OTC has also been shown to decrease the rate of nitrification through inhibiting the activity of soil nitrifying bacteria (Piotrowska-Seget et al., 2008). However, it is not clear how OTC, when added with manure at environmentally relevant concentration, may affect soil microbial activities or key soil processes such as the N cycling.

Escherichia coli (*E. coli*) is a Gram-negative, rod-shaped bacterium commonly found in the lower intestine of warm-blooded organisms. Most *E. coli* strains are harmless, but some serotypes are pathogenic and can cause serious disease. Among the pathogenic *E. coli* strains, *E. coli* O157:H7 has been shown to pose a significant threat to environmental safety and public health because of its low infective dose (as few as 10 cells), high pathogenicity and ability to survive under harsh environmental conditions (Griffin and Tauxe 1991; Haznedaroglu et al., 2009). Survival of *E. coli* O157:H7 in different substrates, such as soil, manure and soil-manure mixtures, can range from several weeks to more than a year (Kudva et al., 1998; Jiang et al., 2002; Nicholson et al., 2005). Once *E. coli* O157:H7 is introduced into soil through animal manure, they start to interact with the autochthonous microbial community and influence soil microbial activity and diversity. The invasion of *E. coli* O157:H7 changed the soil microbial community to some extent, making the environment stressful and unstable (Yao et al. 2014). However, with regard to pathogenicity, most of the research has focused on the survival and transport of *E. coli* O157:H7 in soil (Van Elsas et al., 2007; Liang et al., 2011; Franz et al., 2008; Semenov et al., 2008). Little information is available on the impact of *E. coli* O157:H7 on soil functional microorganisms, such as N cycle-related microorganisms.

The widespread use of veterinary pharmaceuticals (e.g. OTC) and the high prevalence of *E. coli* O157:H7 in livestock animals (particularly cattle) inevitably lead to manure containing both the chemical and the bacterial agents and their subsequent spreading to agricultural field. Once applied, OTC and resistant *E. coli* O157:H7 may affect the abundance of bacteria and fungi, microbial diversity and activity, and soil microbial function. Therefore, it is important to examine the potential impacts of OTC and resistant *E. coli* O157:H7, when simultaneously released into the

soil environment, on disruption of soil microbial functions. To our knowledge, there has not been research addressing this issue.

Soil N cycle is one of the fundamental processes critical to soil health, productivity, and food security, driven by soil microorganisms. Of the N transformation processes, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are the drivers for the first and rate-limiting step in the nitrification processes (Zhang et al., 2015). Monitoring AOB and AOA activities has been used as a way for assessing microbial response to soil changes as a result of agricultural management practices, such as oxygen limitation and pH changes (Francis et al., 2007; Schleper and Nicol, 2010; Prosser and Nicol, 2012). It was also suggested that studying AOB and AOA dynamics may help us better understand the impact of climate change on key soil processes, linking microbial community composition with functions (Prosser and Nicol, 2008).

In the present study, we elucidate the potential impact of manure-borne antibiotics OTC (at environmentally relevant rate) and antibiotic-resistant pathogen *E. coli* O157:H7 on soil N processes using AOB and AOA as sentinels, in addition to other soil microbial activity indicators. Our objective was to assess the individual and combined effects of OTC and *E. coli* O157:H7 on the abundance of soil bacteria and fungi, microbial diversity and activity, and the partial nitrification process (by measuring the abundance of AOA and AOB). Based on the rationale above, we hypothesized that OTC and *E. coli* O157:H7 could affect soil N cycling by regulating soil microbial activity and suppressing specific groups of ammonia-oxidizer (Fig. 1). Soil microbial diversity and communities were tested by the terminal restriction fragment length polymorphism (T-RFLP) technique. The abundance of AOB and AOA were assessed using quantitative polymerase chain reaction (qPCR).

2. Materials and Methods

2.1. Soil, antibiotic and *E. coli* O157:H7

The soil, mapped as a silt loam, was obtained from the 0- to 20-cm layer of long-term non-farmed land in the Shangzhuang experimental station, Beijing (116.25°E, 40.13°N), and had received no fertilizer or manure containing veterinary antibiotics for at least 10 years. The bulk soil sample was air-dried and passed through a 2-mm sieve, and stored in a plastic container at ambient temperature. The soil had a total nitrogen concentration of 0.10 %, an organic matter concentration of 1.25 %, a pH of 7.40 (H₂O, soil 2.5:1, v/v), a cation exchange capacity of 10.43 cmolc kg⁻¹, an Olsen-P of 13.65 mg kg⁻¹ and an NH₄Cl-exchangeable K of 143.0 mg kg⁻¹.

The antibiotic used in the study was OTC (Terramycin[®], 200 mg mL⁻¹ base as OTC dihydrate). The international reference strain of enterohemorrhagic *E. coli* O157:H7, EDL933, was purchased from the Institute of Epidemiology and Microbiology, Chinese Academy of Preventive Medicine, and antibiotic-resistant strains were screened out using 1 mg L⁻¹ OTC. The *E. coli* O157:H7 was cultured on eosin methylene blue medium (EMB) containing 1 mg kg⁻¹ OTC, and incubated aerobically at 37 °C for 24-48 h, then the growing colonies were transferred into Luria-Bertani (LB) medium. After 24 h of growth, the *E. coli* O157:H7 was inoculated onto the surface of EMB agar plates. Subsequently, filter paper discs (6 mm in diameter) saturated with 1 mg kg⁻¹ OTC were placed on the surface of each inoculated plate. The plates were incubated at 37 °C for 24 h, and there was no inhibition zone observed. In all the *E. coli* O157:H7 treatments, cells were pre-grown overnight in LB medium, harvested by centrifugation (12,000 g) for 10 min, and washed with phosphate buffer (10 mM, pH 7.2) three times. The cell pellets were resuspended in sterile deionized water and used as inoculum. The cells were added to the soil to a final

concentration of approximately 10^7 CFU gdw⁻¹ according to a method adapted from Franz et al. (2008).

2.2. Experimental design and incubation

Treatments included manure-amended soil inoculated vs. not inoculated with antibiotic-resistant *E. coli* O157:H7 (10^7 CFU gdw⁻¹), treated or not treated with OTC [1 mg kg⁻¹ soil DM, which was selected to encompass environmentally relevant concentrations (Toth et al., 2011)]. Each treatment had three replications. Both the antibiotic and *E. coli* O157:H7 were spiked to cattle manure. The cattle manure, testing negative for presence of *E. coli* O157, was obtained from the floor of a free-stall dairy immediately following excretion from cows that did not receive any antibiotics. After thoroughly mixing with a sterile wooden spatula, the spiked manures were mixed with the prepared soil. The final soil-manure mixture consisted of 35 g cattle manure per kilogram of DM soil, and the manure/soil ratios were calculated to approximate an agronomic nitrogen-based manure application of 180 kg N ha⁻¹. The mixtures were then brought to 80% of field capacity (soil field capacity was 31.3% gravimetric water content), having accounted for the water contained in manure, PSS and antibiotic liquid. After thorough mixing, portions of 500 g were weighed into sterilized 800 mL polypropylene beakers for incubation and the beakers were covered in parafilm to retard moisture loss. During the course of the incubation, beakers were arranged on a laboratory bench in a completely random design, and the ambient temperature was approximately 25 °C. Throughout the incubation, moisture content was monitored weekly and maintained by adding sterile deionized water when necessary. The weekly moisture loss averaged less than 3% of the total weight.

2.3. Sampling and analysis

Soil samples were collected at 1, 7, 14, 28 d and used to determine residual OTC, *E. coli* O157:H7 survival, the abundance of bacteria, fungi, AOA and AOB (Q-PCR), soil microbial diversity (T-RFLP), soil microbial activity (urease and catalase), and NO_3^- -N and NH_4^+ -N concentrations.

2.3.1 Determination of OTC concentrations in the soil

Five grams of the treated soil-manure mixtures were weighed and placed into a 50 mL tube with 5 mL of 0.10 M Na_2EDTA -McIlvaine buffer ($\text{pH}=4\pm0.005$). The concentrations of OTC in the soil were extracted as described by Cao et al, 2015, for more details see Supplementary Information Materials and Methods. The eluate was dissolved in 1 mL methanol and filtered through a 0.22- μm syringe filter. The filtrate was injected into autosampler vials to determine the OTC concentrations by HPLC. The mean recoveries of OTC at spiking levels (100, 50, 1 and 0.1 mg L^{-1}) were ranged from 90% to 100%. And the limits of detection (LOD) and the limits of quantification (LOQ) values were 0.017 $\mu\text{g L}^{-1}$ and 0.05 $\mu\text{g L}^{-1}$, respectively.

2.3.2 The survival of *E. coli* O157:H7

To determine the survival of *E. coli* O157:H7 over time, at each sampling point, three samples (approximately 1 g dry weight equivalent) from each triplicate plastic bag were removed from the middle of the soil sample and put into pre-weighed dilution tubes. The tubes containing soil samples were weighed to calculate the mass of the soil sample. A 10 mL aliquot of physiological saline was added to the test tube containing the soil sample, and the soil was thoroughly mixed. The resulting soil paste (cell suspension) was then subjected to 10-fold serial dilutions. Samples were cultured on EMB and incubated aerobically at 37 °C for 24-48 h, the growing colonies were examined by eye to evaluate their shape, size and color, enumerated, and the results were expressed as $\log \text{CFU gdw}^{-1}$.

2.3.3 Enzyme assay

Urease activity was determined by sodium phenolate and sodium hypochlorite spectrophotometry (modified after Kandeler and Gerber, 1988). Briefly, 5 g of fresh soil sample (sieved through 2 mm) was treated with 1 mL methylbenzene in a 250 mL conical flask with stopper, and kept at room temperature for 15 min. Then, 10 mL urea (10 %, w/v) and 20 mL citrate buffer (1 M, pH 6.7) were added and the mixture was incubated at 37 °C for 24 h with shaking. After the solution was filtered, 3.0 mL of the filtrate was mixed with 4 mL sodium phenolate, 3 mL sodium hypochlorite and 40 mL distilled water, which was kept at room temperature for 20 min for the color reaction to develop. Urease activity was determined by a UV-2450 spectrophotometer at 578 nm. The linearity, linear range and sensitivity were obtained from analytical curves using working standard solutions at nine $\text{NH}_4^+\text{-N}$ concentrations (0, 0.2, 0.6, 1.0, 1.4, 1.8, 2.2, 2.6, 3.0 $\mu\text{g mL}^{-1}$) with quintuplicate analyses. Urease activity was expressed as $\text{mg NH}_4^+\text{-N g}^{-1}$ dry soil released in 24 h.

Catalase activity was measured by back-titrating residual H_2O_2 with KMnO_4 (Johnson and Temple, 1964). Briefly, 2 g air-dried soil (sieved through 1 mm) was added to 40 mL distilled water with 5 mL of 0.3 % hydrogen peroxide solution. The control tube contained 40 mL distilled water with 5 mL of 0.3 % hydrogen peroxide solution. The mixture was then shaken for 30 min with 150 r min^{-1} . Five mL of 3 M H_2SO_4 were added to stop the reaction. After the solution was filtered, 25 mL filtrate was titrated by 0.1 M KMnO_4 . The reacted amount of 0.1 M KMnO_4 calculated per gram of air-dried soil was used to express the activity of catalase.

2.3.4 Nitrogen forms in soil

Ten g of soil per replicate were extracted with 40 mL KCl (1 M) for 1 h in an orbital shaker (175 r

min⁻¹; 20 °C). After filtration, the extracts were analyzed for NH₄⁺-N and NO₃⁻-N by continuous flow analysis (Bran+Luebbe, Norderstedt, Germany).

2.3.5 Terminal restriction fragment length polymorphism (T-RFLP) analysis

The total soil DNA was extracted using 500 mg soil (wet weight) with the PowerSoil[®] DNA Isolation Kit (MOBIO laboratories Inc., Carlsbad, CA, USA). Polymerase chain reaction (PCR) amplification for the bacterial community was performed using the bacterial universal primers 27F (5'-AGA GTT TGA TCC TGG CTC AG-3') and 907R (5'-CCG TCA ATT CCT TTG AGT TT-3'). The fungal community amplification was performed with the primer pair EF3RCNL (5'-CAA ACT TGG TCA TTT AGA GGA-3') and ITS4 (5'-TCC TCC GCT TAT TGA TAT GC-3'). In T-RFLP analyses, both the forward primer (27F) of the bacterial community and EF3RCNL of the fungal community were labeled with the fluorescent dye 6-FAM (6-carboxyfluorescein; Sangon Biotech, Shanghai, China) at the 5' end to allow the detection of terminal fragments. PCR conditions for bacterial and fungal community analyses were described in Supplementary Information Materials and Methods. All the PCR products were purified with an OMEGA kit (Beijing, China), bacteria and fungi digested with the restriction enzyme MspI and *Hinf*I (Takara), respectively. The fluorescent labels of the terminal fragments were detected on an ABI 373 DNA sequencer (Applied Biosystems, Inc., USA). The relative abundance of individual terminal restriction fragments (T-RFs) was calculated as the percentage of total peak height in a given T-RFLP profile. Only those T-RFs with a relative abundance > 1% and fragment lengths in the range of 50-500 bp were considered in further analysis.

2.3.6 Real-time PCR quantification of bacteria, fungi, and amoA genes for AOB and AOA

Real-time quantitative PCR of bacterial 16S rRNA and fungal 18S rRNA genes were performed to

estimate the abundance of the total bacterial and fungal community. Primer pairs used for bacterial 16S rRNA genes were 341F (5-CCTACGGGAGGCAGCAG-3) and 534R (5-ATTACCGCGGCTGCTGGCA-3). For fungal 18S rRNA genes, primer pairs were FR1 (5-AICCATTCATCGGTAIT-3) and FF390 (5-CGATAACGAACGAGACCT) (Nunan et al., 2015). Quantification was based on the fluorescence intensity of the SYBR Green dye and reactions for each sample were carried out in an ABI 7500 thermal cyclor. For more details about the reactions see supplementary information Material and Methods. Standard curves were obtained using serial dilutions of linearized plasmids (pGEM-T, Promega) containing cloned 16S rRNA genes and 18S rRNA amplified from *Pseudomonas aeruginosa* (PAO1) for bacteria and fungi and cloned crenarchaeal 16S rRNA and 18S rRNA genes from soil ($r^2 = 0.99$ for both standard curves).

Real-time quantitative PCR of *amoA* genes was performed to estimate the abundance of the archaeal and bacterial *amoA* genes. The primers *amoA*-1F (5-GGGGTTTCTACTGGTGGT-3) and *amoA*-2R (5-CCCCTCKGSAAAGCCTTCTTC-3) were used for bacteria generating a 491 bp fragment (Rotthauwe et al., 1997); and Arch-*amoA*F (5-STAATGGTCTGGCTTAGACG-3) and Arch-*amoA*R (5-GCGGCCATCCATCTGTATGT-3) were used for archaea generating a 628 bp fragment (Francis et al., 2005; Zhang et al., 2015). Quantification was based on the fluorescence intensity of the SYBR Green dye and reactions for each sample were carried out in an ABI 7500 thermal cyclor. For more reactions details see supplementary information Material and Methods. The PCR reaction runs had an efficiency of 94 and 99% for the AOB and AOA, respectively. Standard curves for the AOB and AOA were obtained using serial dilutions of linearized plasmids (pGEM-T, Promega) containing cloned *amoA* genes amplified from environmental clones ($r^2 =$

0.99 for both standard curves).

2.4. Data analysis

Data were subjected to analysis of variance of all parameters/response variables, which was carried out using SPSS software, version 17.0 (SPSS Institute, Inc., Cary, NC, USA). Fisher's LSD (Least Significant Difference) test was used to test for significant differences between treatment means at the 5% level. The redundancy analysis (RDA) procedure utilized the Canoco version 4.5 software package, which related the main environmental factors to bacterial communities. The Shannon diversity index H was used to calculate the band pattern diversity of bacteria based on the following equation (Lang et al., 2012):

$$H = -\sum_{i=1}^S p_i \ln p_i = -\sum_{i=1}^S (n_i/n) \ln (N_i/N)$$

Where N_i was the abundance of the i th ribotype, N was the total abundance of all ribotypes in the sample and S was the number of ribotypes.

3. Results

3.1 OTC concentration and *E. coli* O157:H7 survival

OTC concentration decreased over time in soil (Fig. 2); the extracted OTC content was 0.83 mg kg⁻¹ at the beginning and 0.28 mg kg⁻¹ at the end of the experiment. After inoculation, *E. coli* O157:H7 in soil declined notably by day 7 but its concentration remained steady thereafter (Fig. 2).

3.2 Impacts on soil enzyme activity

Throughout the experimental period, the OTC treatment significantly increased the soil urease and catalase activity compared to the control ($p < 0.05$, Table 1), while the *E. coli* O157:H7 treatment significantly decreased soil urease activity. Additionally, *E. coli* O157:H7 significantly increased

soil catalase activity during the experimental period, but the effect was statistically significant only on days 14 and 28 ($p < 0.05$). The interactions between OTC and *E. coli* O157:H7 on soil urease and catalase activity were significant at day 7 through day 28 ($p < 0.05$, Table 1).

3.3 Impacts on the abundance and diversity of bacteria and fungi

The bacterial 16S rRNA gene abundance was in the range of 6.61×10^7 to 1.17×10^9 gene copies per gram of dry soil. Neither OTC nor *E. coli* O157:H7 affected the abundance of bacteria. Additionally, the fungal 18S rRNA gene abundance ranged from 5.37×10^6 to 2.04×10^8 gene copies per gram of dry soil. OTC significantly increased fungi abundance ($p < 0.05$, Fig. 3), but *E. coli* O157:H7 decreased it at day 1 and increased it at day 28 ($p < 0.05$, Fig. 3). As for combined pollution, the abundance of 18S rRNA gene was significantly increased at day 28 ($p < 0.05$), and there was a significant interaction at day 7, 14 and 28. Repeated-measures ANOVA showed that the four treatments (CK, OTC, O157, OTC+O157) did significantly differ in 18S rRNA ($r = 45.221$, $p < 0.001$, Table 2), and OTC increased the abundance of 18S rRNA in the whole period.

OTC treatment significantly increased the diversity of bacteria and fungi at day 14 and day 28 (as shown by the T-RFLP results, see Supplementary Information Fig. S1), although there was a decrease in fungal diversity during the first part of the experiment (day 1 and 7 ($p < 0.05$, Table 3)). *E. coli* O157:H7 treatment significantly decreased soil bacterial and fungal diversity at day 1, but increased soil bacterial and fungal diversity at day 14 and day 28, respectively. OTC and *E. coli* O157:H7 had significant negative interactive effects on soil bacterial and fungal diversity at day 1, but significant positive interaction at day 14 and day 28 respectively (Table 3). The interaction between OTC and *E. coli* O157:H7 on soil fungal diversity at day 7 was also significant ($p < 0.05$, Table 3).

3.4 Impacts on the abundance of AOB and AOA

AOA and AOB abundance, as measured by *amoA* gene abundance, responded differently to OTC and *E. coli* O157:H7 (Fig. 3). The AOB *amoA* gene copy numbers differed across treatments, ranging from 2.51×10^7 to 1.86×10^8 per gram of dry soil. OTC significantly increased the abundance of AOB *amoA* gene ($p < 0.05$), and the highest AOB *amoA* gene copy numbers (1.86×10^8 copies per gram of dry soil) were found in the OTC treatment at day 14. As for *E. coli* O157:H7 treatment, the abundance of AOB *amoA* gene was not significantly affected at day 7 and 28, while it was significantly increased at day 1 and 14. As for combined pollution (OTC+*E. coli* O157:H7), the AOB *amoA* gene copy numbers were relatively unaffected at day 1 and 7, but they were significantly increased at day 14 and 28. The abundance of the AOA *amoA* gene ranged from 5.13×10^8 to 2.09×10^9 per gram of dry soil. OTC and *E. coli* O157:H7 had no effect on AOA *amoA* gene abundance at day 14 and 28. The combination of OTC and *E. coli* O157:H7 showed no effect on AOA *amoA* gene abundance except for day 14, and there was a significant interaction at day 1 and 7. Repeated-measures ANOVA showed that the four treatments (CK, OTC, O157, OTC+O157) did significantly differ in AOA gene abundance ($r=11.319$, $p=0.003$) and AOB gene abundance ($r=14.842$, $p=0.001$) (Table 2). OTC increased the abundance of AOB, while *E. coli* O157 decreased the abundance of AOB and increased the abundance of AOA gene abundance throughout the whole period.

3.5 Impacts on nitrogen forms in soil

OTC significantly increased soil NH_4^+ -N concentration throughout the experiment, compared to control ($p < 0.05$, Table 4), while the impact on NO_3^- -N concentration was inconsistent (1 and 7 d) or insignificant (14 and 28 d). The *E. coli* O157:H7 treatment significantly increased NH_4^+ -N

contents at day 1 and day 7, while significantly decreasing them at day 14 and day 28 ($p < 0.05$, Table 4). *E. coli* O157:H7 significantly decreased NO_3^- -N content at day 1 and day 14. The interaction between OTC and *E. coli* O157:H7 on soil NH_4^+ -N contents was significant at day 1 and day 7, as the interaction on NO_3^- -N contents was significant at day 14 and day 28 ($p < 0.05$, Table 4). Repeated-measures ANOVA showed that OTC increased the NH_4^+ -N content in the whole period.

4. Discussion

4.1 Impact of OTC on soil N cycle.

OTC was proposed as a bacterial inhibitor (Bailey et al., 2003), which may affect soil microbial processes by directly changing soil microbial structure and the abundance of some functional bacteria. Previous research showed that OTC at environmentally relevant concentrations ($0.003\text{--}7.35 \text{ mg kg}^{-1}$) exerted a temporary selective pressure on soil microbial activity and microbial biomass (Thiele-Bruhn and Beck, 2005). Low concentrations of OTC may stimulate bacteria growth by supplying energy and carbon at early exposure, which resulted in the large increase in the number of bacteria at day 1 in the present study. The OTC treatment significantly increased the abundance of the 18S rRNA gene ($p < 0.05$), and the abundance of fungi showed a significant positive correlation with NH_4^+ -N content throughout the experiment ($p < 0.05$, Fig. 4). OTC significantly increased NH_4^+ -N content which may suggest that the presence of OTC enhances gross N mineralization by increasing the abundance of fungi.

OTC significantly increased the activity of urease. OTC contains one amide (CONH_2) group which may stimulate urease activity by providing substrate. Additionally, urease activity showed significant positive correlations with the abundance of fungi over the entire experiment ($p < 0.05$,

Fig. 4). The correlation between fungal abundance and urease supports the conclusion that the growth and reproduction of soil microbes can promote secretion of enzymes (Gao et al., 2013). This is in line with previous study that the antibiotic sulfadiazine and manure showed interaction effect on soil microorganism, which result in the changes in N turnover (Hammesfahr et al. 2011a b). Urease had a significant positive correlation with $\text{NH}_4^+\text{-N}$ in the present study ($p<0.05$, Fig. 4), which was consistent with the hypothesis that OTC affects soil N cycling by stimulating soil microbial activity. The higher concentrations of $\text{NH}_4^+\text{-N}$ in the soil treated with OTC may also imply that OTC decreased rates of nitrification. Similarly, it has also been shown that OTC at a concentration of 3.3 mg g^{-1} resulted in higher $\text{NH}_4^+\text{-N}$ (Piotrowska-Seget et al., 2008) and inhibited the nitrification process in surface water (Klaver and Matthews, 1994).

Both AOB and AOA are major controllers of gross nitrification rate, and the abundance of these functional groups may be influenced by soil pollutants such as antimicrobial compounds. Previous research showed that sulfadiazine (SDZ) decreased AOB abundance; AOA were impacted to a lower extent by SDZ than AOB in greenhouse experiments (Schauss et al., 2009; Ollivier et al., 2010). In the present study, OTC significantly increased the abundance of AOB, which may indicate that lower concentrations of OTC did not depress the growth of AOB but stimulated it by supplying energy and carbon sources. There was a significant decrease in AOA *amoA* gene copies at day 1 but increase at day 7 ($p<0.05$), followed by no effect thereafter. The different responses to OTC stress by AOB and AOA might be attributed to their differences in cell structure, metabolism and physiology (Hu et al., 2014). Additionally, the significantly higher content of $\text{NO}_3^-\text{-N}$ in soil at day 7 could be indirectly explained by the increased AOA abundance at day 7. Possibly, the reduced susceptibility of AOA to OTC can be explained by a shift in the

AOA diversity toward more OTC-tolerant phylotypes over time. Furthermore, AOA showed a significant negative correlation with NH_4^+ -N ($r=-0.632$, $r=-0.585$, $r=-0.621$ at day 1, 14, 28, respectively, Fig. 4) and AOB showed a significant positive correlation with NO_3^- -N ($r=0.777$, $r=0.654$ at day 7 and 14, respectively, Fig. 4) which may implied that AOA and AOB play an important role in the nitrification process. Overall, OTC may have enhanced the nitrification process by increasing the abundance of AOB, which may make NO_3^- -N vulnerable to leaching and/or denitrification in the ecosystem.

4.2 Impact of *E. coli* O157:H7 on soil N cycle

In the present study, *E. coli* O157:H7 significantly increased the NH_4^+ -N content at day 1 and 7, which may be linked to the significantly-decreased AOA abundance and urease activity in the early stages of incubation. That is, *E. coli* O157:H7 may affect the soil N cycle by inhibiting AOA and urease activities. But such inhibition effect faded away in the later stage of the experiment as *E. coli* O157:H7 abundance decreased gradually. The microbial activity and function recovered, resulting in a lower NH_4^+ -N content at day 14 and 28 ($p<0.05$, Table 4) and a higher NO_3^- -N content at day 28 in the soil.

Previous studies reported that the diversity and community composition of soil microbial communities influence the survival of *E. coli* O157:H7 in soil (Yao et al., 2014; Franz et al., 2008; Semenov et al., 2008), meanwhile the introduced microorganisms can change the structure of the soil microbial community and even disrupt ecological balance (De Carcer et al., 2007; Chao and Cheng 2007). In our study, the addition of *E. coli* O157:H7 significantly decreased the abundance of bacteria and microbial diversity at day 1. The suppressive effect caused by *E. coli* O157:H7 on indigenous microorganisms may be explained by competition for nutrients and habitats (Anyango

et al., 1998). *E. coli* O157:H7 significantly decreased soil urease activity at all exposure times, which probably implies that the nutrient turnover ability in soils had declined after *E. coli* O157:H7 inoculation (Yao et al., 2014). Additionally, *E. coli* O157:H7 increased the abundance of the AOB *amoA* gene except for day 7, which is inconsistent with our hypothesis that *E. coli* O157:H7 suppressed the growth of some members of the *Proteobacteria* phylum (e.g. AOB) by competing for nutrients and habitats. *E. coli* O157:H7 decreased AOA *amoA* gene abundance in the early incubation stage which resulted in a higher NH_4^+ -N content.

4.3 Combined effects of OTC and *E. coli* O157:H7 on the soil N cycle

When added together, OTC and O157:H7 significantly decreased the abundance of 16S rRNA and microbial diversity in the beginning of incubation ($p < 0.05$, Fig.3, Table 3), which resulted in less nitrification with higher NH_4^+ -N content in the soil. There was a significant interaction effect between OTC and *E. coli* O157:H7 on the NH_4^+ -N content at day 1 and 7. Meanwhile there was a significant interaction effect on the inhibition of urease activity. Also, NH_4^+ -N concentration correlated significantly with 18S rRNA and urease activity. This suggests that the interaction between OTC and *E. coli* O157:H7 could inhibit the soil nitrogen cycle by decreasing soil urease activity, bacterial abundance and microbial diversity at the beginning of incubation. The dual treatment with OTC and *E. coli* O157:H7 significantly increased the abundance of AOB at day 14 and 28, and AOB showed a positive correlation with soil NO_3^- -N content, which resulted in higher NO_3^- -N in the soil, respectively. That is, with the degradation of OTC and gradual die-off of *E. coli* O157:H7, the combined inhibitory effect on soil microbial and N cycle disappeared. Similarly, the effects on microbial processes tended to be attenuated and soil parameters rapidly rebounded to normal levels during the antibiotic incubation (Westergaard et al., 2001; Zielesny et al., 2006).

The phenomenon reflects the soil resilience and its ability to recover from antibiotic disturbance, and is important since the microbial diversity is highly correlated with the ecological functions of soil (Müller et al., 2002).

Conclusions

OTC and *E. coli* O157:H7 could affect soil N cycling by regulating soil microbial activity and suppressing specific groups of ammonia-oxidizer. OTC and *E. coli* O157:H7 interaction increased soil NH_4^+ -N content at the beginning of incubation, while increasing soil NO_3^- -N content in the later stage. OTC increased the abundance of soil fungi, the enzyme activity of urease, and the ammonium oxidizing bacteria (AOB), with an end result of greater NH_4^+ -N but lower NO_3^- -N concentrations in the soil. In contrast, *E. coli* O157:H7 inhibited the soil nitrification at the early stages of incubation with decreased AOA *amoA* gene abundance. Findings from this research help improve our understanding of the potential implications of antibiotics and antibiotic-resistant bacteria in the soil environment.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Project 31570514), The Da Bei Nong's Youth Researchers Program (2413002), Chinese Universities Scientific Fund (Project 2015TC028), Innovative Group Grant of the National Science Foundation of China (31421092), and the National Basic Research Program of China (973 Program, 2015CB150405). We also acknowledge Dr. Zhenjun Sun and Dr. Xiaolin Li at China Agricultural University for laboratory assistance.

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555 chlorotetracycline on soil bacterial community structure and respiratory activity. Soil Biology

556 and Biochemistry 38, 2372-2380.

557 Table 1 The soil urease and catalase activities under different treatments

Treatments	Urease (mg NH ₃ -N g ⁻¹ soil day ⁻¹)				Catalase (mL/(g·min))			
	1 d	7 d	14 d	28 d	1 d	7 d	14 d	28 d
CK	3.58±0.03a	3.50±0.12b	3.18±0.01b	2.75±0.08b	22.32±0.53c	22.87±0.21b	17.30±0.72b	16.12±0.46c
OTC	3.81±0.05a	5.62±0.04a	4.34±0.04a	4.04±0.02a	23.95±0.02a	25.35±0.26a	18.94±0.55a	20.31±0.12a
O157	2.97±0.19b	2.52±0.30c	1.45±0.32d	0.67±0.09d	23.09±0.91bc	22.73±0.32b	21.38±2.90a	18.74±0.96b
O157+OTC	3.09±0.24b	2.24±0.55c	1.75±0.20c	0.81±0.06	23.72±0.34ab	22.77±0.76b	21.25±0.82a	20.48±0.89a
Significance due to								
OTC	*	***	***	***	**	***	NS	***
O157	***	***	***	***	NS	***	**	**
OTC*O157	NS	***	**	***	NS	***	*	*

558

559 NS = Not significant; ***p<0.001; **p<0.01; *p<0.05

560 Data are the means of three replicates ±SD and were compared by Duncan's multiple range tests. Within each column (3 values) the values with the same lower case

561 letter are not significantly different at p<0.05. CK is the soil without OTC and *E. coli* O157:H7 addition; OTC is the soil with 1mg kg⁻¹ OTC addition; O157 is soil

562 with *E. coli* O157:H7 addition; O157+OTC is the soil with both OTC and *E. coli* O157:H7 addition.

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566 Table 2 The repeated measures ANOVA results under different treatments through the whole incubation period

Treatments	NH ₄ ⁺ -N (mg kg ⁻¹)		NO ₃ ⁻ -N (mg kg ⁻¹)		Urease (mg NH ₃ -N g ⁻¹ soil day ⁻¹)		Catalase (mL/(g·min))		Shannon-Wiener index (H) bacteria	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CK	4.93	1.10	18.84	3.37	3.23	0.34	19.81	3.02	2.67	0.12
OTC	9.40	4.70	19.80	5.32	4.47	0.73	22.12	2.82	2.76	0.16
O157	5.33	3.14	18.10	5.67	1.94	0.93	21.90	2.03	2.68	0.18
O157+OTC	5.27	1.57	26.92	9.36	1.99	0.89	22.30	1.23	2.63	0.11
Analysis	F=80.845, p<0.001		F=20.758, p<0.001		F=1582.592, p<0.001		F=58.518, p<0.001		F=6.154, p=0.018	

Treatments	Shannon-Wiener index (H) fungi		Log 16S rRNA gene copies g ⁻¹ dry soil		Log 18S rRNA gene copies g ⁻¹ dry soil		Log AOB gene copies g ⁻¹ dry soil		Log AOA gene copies g ⁻¹ dry soil	
	Mean	SD	Mean	SD	Mean	SD	Mean	SD	Mean	SD
CK	2.61	0.48	8.46	0.45	7.25	0.35	5.54	3.52	4.99	1.72
OTC	2.52	0.54	8.61	0.44	7.85	0.43	5.64	1.65	4.39	1.79
O157	2.69	0.38	8.42	0.21	7.36	0.33	3.94	1.41	5.81	2.61
O157+OTC	2.43	0.43	8.57	0.33	7.33	0.32	7.66	4.97	4.08	0.88
Analysis	F=41.941, p<0.001		F=3.002, p=0.095		F=41.941, p<0.001		F=11.319, p=0.003		F=14.842, p=0.001	

Table 3 Bacterial and fungal diversity under different treatments

Treatments	Shannon-Wiener index (H) bacteria				Shannon-Wiener index (H) fungi			
	1d	7d	14d	28d	1d	7d	14d	28d
CK	2.80±0.06a	2.65±0.16a	2.64±0.01b	2.58±0.06ab	3.00±0.14a	2.07±0.15a	3.11±0.03a	2.24±0.13c
OTC	2.71±0.06ab	2.66±0.11a	2.98±0.13a	2.67±0.11a	2.47±0.17c	1.71±0.07b	2.93±0.08ab	2.97±0.03a
O157	2.55±0.09c	2.50±0.09a	2.92±0.05a	2.73±0.17a	2.81±0.007b	2.10±0.09a	3.04±0.14ab	2.82±0.02ab
O157+OTC	2.64±0.03bc	2.66±0.09a	2.74±0.04b	2.49±0.04a	1.88±0.07d	2.23±0.12a	2.89±0.10b	2.73±0.06b
Significance due to								
OTC	NS	NS	NS	NS	***	NS	*	***
O157	**	NS	NS	NS	**	**	NS	*
OTC*O157	*	NS	**	NS	*	**	NS	***

569

570 NS = Not significant; ***p<0.001; **p<0.01; *p<0.05

571 Data are the means of three replicates ±SD and were compared by Duncan's multiple range tests. Within each column (3 values) the values with the same lower case

572 letter are not significantly different at p<0.05. CK is the soil without OTC and *E. coli* O157:H7 addition; OTC is the soil with 1mg kg⁻¹ OTC addition; O157 is soil

573 with *E. coli* O157:H7 addition; O157+OTC is the soil with both OTC and *E. coli* O157:H7 addition.

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575

576 Table 4 The soil NH_4^+ -N and NO_3^- -N content under different treatments

Treatments	NH_4^+ -N (mg kg ⁻¹)				NO_3^- -N (mg kg ⁻¹)			
	1 d	7 d	14 d	28 d	1 d	7 d	14 d	28 d
CK	6.27±0.76c	3.76±1.13b	4.93±0.91b	4.97±0.28b	21.11±1.35a	18.15±0.78b	20.85±4.45a	15.75±3.85b
OTC	16.62±1.26a	7.35±1.08a	7.08±0.32a	6.11±0.06a	18.42±0.24bc	26.15±4.51a	20.90±3.11a	13.67±2.08b
O157	8.95±0.48b	7.54±0.94a	2.05±0.13c	2.78±0.39d	16.36±1.63c	20.81±0.99ab	11.06±0.84b	23.67±6.21b
O157+OTC	7.48±1.07bc	5.40±0.33ab	4.6±0.27b	3.61±0.13c	20.45±2.05ab	25.59±2.14a	21.49±2.57a	40.97±9.58a
Significance due to								
OTC	***	NS	***	***	NS	**	*	NS
O157	***	NS	***	***	NS	NS	*	**
OTC*O157	***	**	NS	NS	**	NS	*	*

577

578 NS = Not significant; ***p<0.001; **p<0.01; *p<0.05

579 Data are the means of three replicates ±SD and were compared by Duncan's multiple range tests. Within each column (3 values) the values with the same lower case
580 letter are not significantly different at p<0.05. CK is the soil without OTC and *E. coli* O157:H7 addition; OTC is the soil with 1 mg kg⁻¹ OTC addition; O157 is soil
581 with *E. coli* O157:H7 addition; O157+OTC is the soil with both OTC and *E. coli* O157:H7 addition.

Figure Captions

Fig. 1 Schematic representation of the effect of OTC and *E. coli* O157:H7 on soil N cycling processes. a. OTC increases the abundance of 18S rRNA, AOB *amoA* gene and soil urease activity; b. *E. coli* O157:H7 effect on soil microbial diversity and activity and the abundance of AOA *amoA* gene; d. *E. coli* O157:H7 and OTC combined effect on microbial diversity and activity and the abundance of AOB *amoA* gene.

Fig. 2 OTC degradation and the survival of *E. coli* O157:H7 for up to 28 days. The line graph represents the OTC degradation, and the histogram represents the *E. coli* O157:H7 survival. The concentration of OTC and the log number of *E. coli* O157:H7 ranged from 0.28-0.83 mg kg⁻¹ and 5.48-7.00 g dry soil⁻¹ respectively over the entire experimental period.

Fig. 3 Bacterial 16S rRNA gene (A), fungal 18S rRNA gene (B), bacterial *amoA* gene (C) and archaeal *amoA* gene (D) copy numbers in different treatments.

Fig. 4 Two-dimensional similarities among the selected samples revealed by RDA ordination plots for the first two dimensions of the relationship between environmental parameters and soil NH₄⁺-N and NO₃⁻-N content. For day 1 (A), NH₄⁺-N showed a positive correlation with fungal 18S rRNA gene and urease, the NO₃⁻-N contents were significantly correlated with bacterial 16S rRNA gene, bacterial diversity and AOA *amoA* gene. For day 7 (B), NH₄⁺-N showed a positive correlation with fungal 18S rRNA gene, the NO₃⁻-N contents were significantly correlated with 18S rRNA gene and AOB *amoA* gene. For day 14 (C), NH₄⁺-N showed significantly correlation with AOA *amoA* gene and urease. The NO₃⁻-N contents had significant correlation with AOA *amoA* gene and AOB *amoA* gene. For day 28 (D), NH₄⁺-N showed significant correlation with 18S rRNA gene, AOA *amoA* gene and urease; NO₃⁻-N showed a negative correlation with urease.

Figures

Fig. 1 Schematic representation of the effect of OTC and *E. coli* O157:H7 on soil N cycling processes. a. OTC effect the abundance of fungi and ammonia-oxidizer, and soil urease activity; b. *E. coli* O157:H7 effect on soil microbial diversity and activity and the abundance of ammonia-oxidizer; d. *E. coli* O157:H7 and OTC combined effect on microbial diversity and activity and the abundance of AOB *amoA* gene.

612

613 Fig. 2 OTC degradation and the survival of *E. coli* O157:H7 for up to 28 days. The line graph

614 represents the OTC degradation, and the histogram represents the *E. coli* O157:H7 survival. The

615 concentration of OTC and the log number of *E. coli* O157:H7 ranged from 0.28-0.83 mg kg⁻¹ and

616 5.48-7.00 g dry soil⁻¹ respectively over the entire experimental period.

Fig. 3 Bacterial 16S rRNA gene (A), fungal 18S rRNA gene (B), bacterial *amoA* gene (C) and archaeal *amoA* gene (D) copy numbers in different treatments. Data are the means of three replicates $\pm$ STD, the values with the same lower case letter are not significantly different at $p < 0.05$. CK is the soil without OTC and *E. coli* O157:H7 addition; OTC is the soil with 1 mg kg^{-1} OTC addition; O157 is soil with *E. coli* O157:H7 addition; O157+OTC is the soil with both OTC and *E. coli* O157:H7 addition.

Fig. 4 Two-dimensional similarities among the selected samples revealed by RDA ordination plots for the first two dimensions of the relationship between environmental parameters and soil NH_4^+ -N and NO_3^- -N content. For day 1 (A), NH_4^+ -N showed a positive correlation with fungal 18S rRNA gene ($r=0.767$, $p<0.01$) and urease ($r=0.582$, $p<0.05$), the NO_3^- -N contents were significantly correlated with bacterial 16S rRNA gene ($r=0.586$, $p<0.05$), bacterial diversity ($r=0.712$, $p<0.01$) and AOA *amoA* gene ($r=-0.632$, $p<0.05$). For day 7 (B), NH_4^+ -N showed a positive correlation with fungal 18S rRNA gene ($r=0.617$, $p<0.05$), the NO_3^- -N contents were significantly correlated with 18S rRNA gene ($r=0.619$, $p<0.05$) and AOB *amoA* gene ($r=0.777$, $p<0.01$). For day 14 (C), NH_4^+ -N showed significant correlation with AOA *amoA* gene ($r=-0.585$, $p<0.05$) and urease ($r=0.843$, $p<0.01$). The NO_3^- -N contents had significant correlation

637 with AOA *amoA* gene ($r=-0.577$, $p<0.05$) and AOB *amoA* gene ($r=0.654$, $p<0.05$). For day 28 (D),
638 NH_4^+ -N showed significant correlation with 18S rRNA gene ($r=0.579$, $p<0.05$), AOA *amoA* gene
639 ($r=-0.621$, $p<0.05$) and urease ($r=0.970$, $p<0.01$); NO_3^- -N showed a negative correlation with
640 urease ($r=-0.721$, $p<0.01$).