Article Type: Regular Paper

Date of preparation: 07/07/2016

Number of text pages: 34

Number of tables: 1

Number of figures: 7

Title: Improvement of the soil nitrogen content and maize growth by earthworms and arbuscular mycorrhizal fungi in soils polluted by oxytetracycline

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Abstract

Interactions between earthworms (*Eisenia fetida*) and arbuscular mycorrhizal fungi (*Rhizophagus intraradices*, AM fungi) have been suggested to improve the maize nitrogen (N) content and biomass and were studied in soils polluted by oxytetracycline (OTC). Maize was planted and amended with AMF and/or earthworms (E) in the soil with low (1 mg kg^{-1} soil DM) or high (100 mg kg^{-1} soil DM) amounts of OTC pollution in comparison to soil without OTC. The root colonization, shoot and root biomass, shoot and root N contents, soil nitrogen forms, ammonia-oxidizing bacteria (AOB) and archaea (AOA) were measured at harvest. The results indicated that OTC decreased maize shoot and root biomass ($p < 0.05$) by mediating the soil urease activity and AOB and AOA abundance, which resulted in a lower N availability for maize roots and shoots. There was a significant interaction between earthworms and AM fungi on the urease activity in soil polluted by OTC ($p < 0.05$). Adding earthworms or AM fungi could increase the maize biomass and N content ($p < 0.05$) in OTC polluted soil by increasing the urease activity and relieving the stress from OTC on the soil N cycle. AM fungi and earthworms interactively increased maize shoot and root biomass ($p < 0.05$) in the OTC polluted soils through their regulation of the urease activity and the abundance of ammonia oxidizers, resulting in different soil $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ contents, which may contribute to the N content of maize shoots and roots. Earthworms and AM fungi could be used as an efficient method to relieve the OTC stress in agro-ecosystems.

Keywords: OTC polluted soil; Shoot and root biomass; Shoot and root N content; Soil urease; AOA and AOB abundance

1. Introduction

A large amount of pharmacologically active substances are used annually in animal medicines for the treatment and prevention of illness (Liu et al, 2009; Chen et al., 2014). However, up to 90% of these veterinary antibiotics cannot be completely absorbed by animals and are discharged through faeces and urine as the parent compound or as metabolites (ElSayed and Prasher, 2014). In agro-ecosystems, the agricultural application of untreated or even treated animal wastes containing antibiotics might disrupt certain microbial activities in the soil, which can influence plant growth (Chitescu et al., 2013). Because of their biological effects on the normal functions of soil microorganisms, antibiotics are regarded as potential micro-pollutants (De Liguoro et al, 2003). This concern is directed to the potential ecological problems that antibiotics can cause. Several studies have described the inhibitory effects of antibiotics on various indices of microbial activity in the soil, such as respiration, nitrification and soil enzyme activities (Cao et al., 2015b). However, no information is available on whether soil biota, when added into the soil with antibiotics, affect the soil nitrogen content and N-cycling communities.

In the soil ecosystem, earthworms and arbuscular mycorrhiza fungi (AMF) are important macrofauna and microorganisms of the rhizosphere. Earthworms function as ecosystem engineers, i.e., they directly or indirectly modify the chemical, physical and biochemical properties of the soil, thus affecting the fertility and health of the soil at different spatial and temporal scales (Blouin et al., 2013; Jusselme et al., 2013). Earthworms can influence the progress of the nitrogen cycle in the soil (i.e., N-mineralization, nitrification and denitrification) by regulating the soil structure and stability of aggregates, water infiltration and aeration of deep soil layers, microbial biomass and

microbial community (Curry and Schmidt 2007). It was reported that inorganic nitrogen ($\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$) in the soil in the mesocosms inoculated with *P. corethrurus* was higher than in the earthworm-free controls and that earthworms can stimulate the transfer of nutrients from the microbial biomass to plants (Tapia-Coral et al. 2006). Additionally, earthworms have been used recently to promote the bioremediation of contaminants or unrecyclable compounds in soils (Gupta and Garg, 2009). Several studies have reported the positive effects of earthworms on the removal of organic contaminants, such as herbicides (Tejada and Masciandaro, 2011), veterinary antibiotics (Cao, et al. 2015b), PAHs and PCBs (Hernández-Castellanos et al., 2013, Rodriguez-Campos et al., 2014) under laboratory conditions. Clearly, research on the potential impact of earthworm on nitrogen cycling in soil polluted with antibiotics is still in its infancy.

Arbuscular mycorrhizal fungi penetrate cortical cells of over 80 % to 90 % of all plant species and are important rhizospheric microorganisms providing a direct physical link between the soil and plant roots (Smith and Read 2008), as their extraradical mycelia increase the nutrient uptake capacity of plant roots by extending the soil volume exploited (Hodge et al. 2010). AM fungi can also influence the progress of the nitrogen cycle in the soil-plant system by regulating some enzyme activities and the microbial community in the soil. Mycorrhizal fungal hyphae may extend the N absorption zones and take up both inorganic and organic N and transport N to the host plants (Hodge et al. 2001, 2010; Leigh et al. 2009). However, ammonia oxidizers are weak competitors for $\text{NH}_4^+\text{-N}$ in the soil compared to AM fungi (Bollmann et al. 2002). So far, mycorrhizal plants consistently support lower potential nitrification rates (PNR) than non-mycorrhizal representatives, indicating a lower abundance of ammonia oxidizers in the mycorrhizosphere (Veresoglou et al. 2011). Additionally, AM fungi can also increase the

decomposition of organic contaminants (i.e., atrazine, PAHs, OTC and DDT) and improve plant growth by alleviating the toxicity of pollutants under laboratory conditions (Hillis et al., 2008; Huang et al., 2009; Cao et al. 2015b). However, very little information is available on the impact of AM fungi on the nitrogen content and on ammonia oxidizers in soil polluted by antibiotics.

In the normal soil-plant system, earthworms and AM fungi can independently or interactively influence nutrient cycles in the soil, nutrient uptake by shoots and roots, microbial and enzymatic activities in the soil, and the physical environment of the soil (Wardle et al., 2004; Zarea et al., 2009; Li et al., 2012a, b). AMF hyphae can assist maize in the uptake of ^{15}N mineralized from wheat straw by earthworms (Li et al., 2013). Additionally, ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing archaea (AOA) are drivers for the first and rate-limiting step in the nitrification process (Zhang et al., 2015). Previous research showed that antibiotics (i.e., sulfadiazine) can impact the abundance of AOB and AOA in greenhouse experiments (Schauss et al., 2009; Ollivier et al., 2010). Therefore, what would happen to the soil nitrogen content and the abundance of AOB and AOA when earthworms and AM fungi were added into soil containing antibiotics?

Earthworms and AM fungi can also interactively increase the degradation of OTC (Cao et al., 2015b), which is an antibiotic in the tetracycline class that acts against a range of Gram-positive and Gram-negative bacteria (Gao et al., 2013). Moreover, several studies have reported adverse effects of OTC on soil enzymes (Yang et al., 2009; Gao et al. 2013), the microbial community in the soil, microbial function (Liu et al., 2012;) and the root and shoot elongation of wheat (Jin et al., 2009). OTC has also been shown to decrease the rate of nitrification by inhibiting the activity of nitrifying bacteria in the soil (Piotrowska-Seget et al., 2008). In the present study, we elucidate the

potential impact of earthworms and AM fungi on N cycle processes in soil polluted with OTC using AOB and AOA as sentinels in addition to other indicators of microbial activity in the soil. Correlations between the N uptake of maize and urease activity in the soil and changes in the content of $\text{NH}_4^+\text{-N}$, $\text{NO}_3^-\text{-N}$ in the soil as well as the abundance of AOA and AOB were monitored. We hypothesized that 1) OTC had a negative impact on maize biomass and N uptake by decreasing the enzyme activity in the soil and the abundance of ammonia oxidizers; 2) earthworms and AM fungi could relieve the negative effects of OTC on maize biomass and N uptake by regulating urease activity in the soil and the abundance of ammonia oxidizers in soil polluted by OTC (Fig. 1). The abundance of AOB and AOA was assessed using quantitative polymerase chain reaction (qPCR).

2. Materials and Methods

2.1 Antibiotics and soil

The antibiotic used in the study was OTC (Terramycin[®], 200 mg ml⁻¹ base as OTC dihydrate, purity>99%), which is the second of the broad-spectrum tetracycline group of antibiotics. The soil, air-dried and sieved through a 2 mm sieve, was taken from the surface layer of a field plot at the Shangzhuang experimental station, Beijing, China. The plot had been cultivated with a winter wheat/summer maize rotation without manure application for the past decade. The soil type was sandy loam, with 67.58% sand, 20.92% silt and 4.89% clay. The soil included an organic matter concentration of 1.25 %, a total nitrogen concentration of 0.10 %, a cation exchange capacity of 96 mg kg⁻¹, an Olsen-P of 13.65 mg kg⁻¹, a NH_4Cl -exchangeable K of 143.0 mg kg⁻¹ and a pH of 7.40 (H_2O , soil 2.5:1, v/v). The sterilized soil was treated with 25 kGy of ⁶⁰Co γ -radiation for 72

h at the Beijing Radiation Application Research Centre.

2.2 Earthworm, mycorrhiza and maize

The species of earthworm was *Eisenia fetida*, whose surfaces or gut contents were pretreated to minimize the impact of naturally occurring mycorrhizal propagules (Li et al. 2012a). The AM fungi inoculum was *Rhizophagus intraradices* (BEG JX04B), propagated on maize and white clover host plants in a growth chamber at 30°C/18°C with a 16 h/8 h light/dark regime and 50–75% relative humidity. Maize seeds were *Zheng Dan 958*, which were surface sterilized with a 10% (v/v) solution of H₂O₂ for 10 min, rinsed thoroughly with deionized water, and then placed on autoclaved filter paper soaked with sterile distilled water and incubated at 25 °C for 24 h (Li et al. 2012a).

2.3 Experimental design and inoculation

The experiment was conducted using plastic pots (inner diameter 18 cm, height 22 cm) as the culture vessels. Each pot was filled with 1.5 kg of sterilized soil that was mixed with 8.75 g of sterilized oven-dry wheat straw (equivalent to 8 t/ha of biomass). There were three groups of pots that received 0, 1 or 100 mg OTC kg⁻¹ soil DM, where 1 mg OTC kg⁻¹ soil DM is the environmentally relevant concentration based on a literature synthesis (Toth et al., 2011) and 100 mg OTC kg⁻¹ soil DM is the simulation scenario in which OTC could accumulate to a level that was dangerous to soil life or health (Gao et al., 2013; Liu et al., 2012). OTC in the soils were detected by HPLC, no OTC detected in the reference soil and the tested concentrations were closely equal to the spiked concentration at all two levels. Each group was a 2 × 2 factorial design: AMF (inoculated or not inoculated) and earthworms (added or not added). For the inoculation treatment, the mycorrhiza inoculum (consisting of spores, mycelium, fine root segments and soil)

was placed approximately 2 cm under the soil surface at 50 g kg^{-1} , whereas the soil that was not inoculated received sterilized inoculum plus 10 ml of soil filtrate (0.45- μm pore size) to provide a similar microbial community in the soil (Calvet et al. 1993). Fifteen earthworms with similar fresh weights ($3.40 \pm 0.03 \text{ g}$) were added to the relevant treatment pots after the third leaf of maize had emerged. All treatments received basal fertilization of $150 \text{ mg kg}^{-1} \text{ N}$ (NH_4NO_3), $50 \text{ mg kg}^{-1} \text{ P}$ (KH_2PO_4), $150 \text{ mg kg}^{-1} \text{ K}$ (K_2SO_4), $100 \text{ mg kg}^{-1} \text{ Mg}$ ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), $5 \text{ mg kg}^{-1} \text{ Mn}$ ($\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$), $5 \text{ mg kg}^{-1} \text{ Zn}$ ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$) and $5 \text{ mg kg}^{-1} \text{ Cu}$ ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$) (Zhang et al. 2008). Each treatment was replicated four times, and the 48 pots were arranged at random, with a spacing of approximately 60 cm between two adjacent maize plants. All pots received 80 ml of deionized water every third day. During the course of the incubation, the ambient temperature was approximately $22^\circ\text{C}/32^\circ\text{C}$ (day/night) with a 16 h/8 h (light/dark) photoperiod and 50 % relative humidity, respectively.

2.4 Sampling and analysis

The experiment was carried out from May 20th to July 15th, 2014. At the end of the experiment, the maize shoots were cut at the soil surface. The roots and soil in the rhizosphere were collected, as described in Li et al. (2012a, b). The biomass of the shoots and roots was determined by drying at 65°C for 48 h. The oven-dried root and shoot samples were analysed for total N concentrations using Kjeldahl oxidation (Axmann et al., 1990). Both the exchangeable NH_4^+ -N and NO_3^- -N in the soil were extracted with 1 M KCl (soil:solution = 1 g:4 ml,) for 1 h in an orbital shaker (175 r min^{-1} ; 20°C) as described by Keeney and Nelson (1982). The filtrates were analysed by a Bran+Luebbe Auto Analyzer 3 (Bran+Luebbe, Norderstedt, Germany). Fresh soil samples were collected and used to determine the urease activity and residual OTC in the soil as quickly as

possible. The soil used for the Q-PCR analysis (the abundance of AOA and AOB) was stored at -70 °C. Earthworms were recovered from the soil by hand sorting, washed, counted, and weighed (fresh).

2.5 Determination of AM fungi colonization and the OTC concentration

AM fungi colonization was determined by the method modified after McGonigle et al. (1990). Briefly, 1g of root was cut into 1cm pieces, and thirty random pieces were then cleared in 10% KOH, acidified in 1% HCl, and stained in 0.05% Trypan blue in lactoglycerol. The colonization of each root sample was determined at 250×magnification using the line intersect method. Determination of OTC in the soils was accomplished using a high performance liquid chromatographic (HPLC) method with UV detection. The mean recoveries of OTC at spiking levels (100, 50, 1 and 0.1mg L⁻¹) were ranged from 90% to 100%. And the limits of detection (LOD) and the limits of quantification (LOQ) values were 0.017 µg L⁻¹ and 0.05 µg L⁻¹, respectively.

2.6 Soil urease activity

The urease activity was determined by sodium phenolate and sodium hypochlorite spectrophotometry (modified after Kandeler and Gerber, 1988). Briefly, 5 g of a fresh soil sample (sieved through 2 mm) was treated with 1 mL of methylbenzene in a 250-mL conical flask with a stopper and kept at room temperature for 15 min. Then, 10 mL of urea (10 %, w/v) and 20 mL of citrate buffer (1 M, pH 6.7) were added, and the mixture 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 of sodium phenolate, 3 mL of sodium hypochlorite and 40 mL of distilled water and was kept at room temperature for 20 min for the colour reaction to develop. The 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. The urease activity was expressed as $\text{mg NH}_4^+\text{-N g}^{-1}$ dry soil released in 24 h.

2.7 Real-time PCR quantification of *amoA* genes for AOB and AOA

Soil DNA was extracted from 0.25 g of soil using the PowerSoil DNA Isolation Kit (Mebio Laboratories Inc., Carlsbad, CA, USA) following the manufacturer's instructions. A real-time quantitative PCR of *amoA* genes was performed to estimate the abundance of 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); Arch-*amoA*F (5-STAATGGTCTGGCTTAGACG-3) and Arch-*amoA*R (5-GCGGCCATCCATCTGTATGT-3) were used for archaea, generating a 628-bp fragment (Zhang et al., 2015). Quantification was based on the intensity of the fluorescence of the SYBR Green dye, and reactions for each sample were carried out in an ABI 7500 thermal cycler. The quantification of the *amoA* genes was performed in a total volume of 20 μL using a DyNAmo Flash SYBR Green qPCR Kit (Finnzymes), 0.5 μM of each primer, and 10 ng of soil DNA. Bovine serum albumin (BSA) was added to reach a final concentration of 800 ng mL^{-1} . The fragments for the AOB and AOA were amplified using an initial denaturation step at 95 °C for 15 min, followed by 35 cycles of 15 s at 95 °C, 30 s at 55 °C and 30 s at 72 °C and 30 s at 80 °C. Data were retrieved at 80 °C, and all reactions were finished with a melting curve starting at 60 °C with an increase of 0.5 °C up to 95 °C to verify amplicon specificity. The PCR reaction runs had efficiencies of 94 and 99% for AOB and AOA, respectively. Standard curves for 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).

2.8 Data analysis

The data were subjected to an analysis of variance of all of the 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 principal component analysis (PCA) procedure utilized the Canoco version 4.5 software package.

3. Results

3.1 Mycorrhizal colonization and residual OTC in the soil

Compared to the treatment without OTC, the treatments with low and high OTC concentrations significantly decreased root colonization in both the groups with and without earthworms (Fig. 2). Root colonization was 65.10%, 61.98 and 43.08%, respectively, in the 0, 1 mg and 100 mg OTC treatments in the no earthworm group and 85.31%, 81.60% and 56.88% in the earthworm added group, which indicated that the concentration of OTC might influence the root colonization of AM fungi. Adding earthworms to the AM fungi treatment (i.e., the earthworm $\times$ AMF treatment) improved the maize root colonization rate at all OTC concentrations (Fig. 2).

3.2 Maize biomass and N uptake

Compared to the treatment without OTC, the treatments with low and high levels of OTC significantly decreased the maize (shoot and root) biomass and N contents ($p < 0.05$), while the OTC concentration had no significant impact on the maize biomass and N contents between the low and high OTC levels (Fig. 3). Among all of the OTC levels, the maize biomass and N contents

significantly increased by adding AMF and earthworms ($p<0.01$, Fig. 3, Table 1). In addition, the maize biomass and N contents in the earthworm $\times$ AMF treatment were significantly greater than those in other treatments ($p<0.05$, Fig. 3), which suggested a significant interaction between earthworms and AM fungi regarding the maize biomass and N contents in soil polluted by OTC ($p<0.01$, Table1).

3.3 Urease activity, soil NH_4^+ -N and NO_3^- -N concentration

Compared to the treatment without OTC, OTC significantly decreased the urease activity in the soil, and the high OTC treatment had the lowest urease activity ($p<0.05$, Fig. 4 E). The addition of earthworms or AM fungi alone significantly increased the urease activity in the soil ($p<0.05$, Fig. 5 E), and there was a significant interaction between earthworms and AM fungi on the urease activity in soil polluted by OTC ($p<0.01$, Fig. 5 E, Table 1). Both the low and high OTC treatments significantly decreased the soil NH_4^+ -N concentrations compared to the no OTC group ($p<0.05$, Fig. 4 C). However, the soil NO_3^- -N concentrations increased at the low OTC concentration, while it decreased at the high OTC concentration ($p<0.05$, Fig. 4 D). Earthworms had no effect on the soil NO_3^- -N and NH_4^+ -N concentrations in soil polluted by OTC (Fig. 5C, D). In addition, AM fungi alone significantly decreased the NH_4^+ -N concentration, while it had no impact on the soil NO_3^- -N concentration in soil polluted by OTC (Fig. 5C, D) Additionally, the E+AM fungi treatment significantly decreased the soil NH_4^+ -N and NO_3^- -N contents ($p<0.05$, Fig. 5 C, D), and the interaction between earthworms and AM fungi on the NO_3^- -N content was particularly marked in soil polluted by OTC ($p<0.01$, Table 1).

3.4 Abundance of AOB and AOA

The responses of the gene abundances of AOA and AOB *amoA* were different in the soils with

different OTC concentrations. In the high OTC treatment, both the AOB and AOA *amoA* gene decreased significantly compared to the no OTC group ($p < 0.05$), but there was no significant difference in the low OTC treatment (Fig. 4 A, B). Earthworms or AM Fungi alone had no effect on the abundance of the AOA *amoA* gene, and there was no significant interaction between earthworms and AM Fungi on the AOA *amoA* gene in the soil polluted by OTC (Fig. 5A). Earthworms alone also had no effect on the abundance of the AOB *amoA* gene (Fig. 5 B). However, the AOB *amoA* gene copy numbers decreased significantly in the AM fungi and E+AM fungi treatments ($p < 0.05$, Fig. 5 B), and there was a significant interaction between earthworms and AM fungi on the abundance of the AOB *amoA* gene in soils polluted by OTC ($p < 0.05$, Table1).

4. Discussion

4.1 Impact of OTC on the abundance of ammonia oxidizers, soil and maize N content

The conversion of ammonia to nitrate is performed primarily by bacteria that live in the soil and by other nitrifying bacteria. In the primary stage of nitrification, both AOB and AOA are the drivers for the first and rate-limiting step in the nitrification processes (Zhang et al., 2015). Previous research showed that antibiotics (SDZ) could decrease the abundance of AOB and AOA (Schauss et al., 2009; Ollivier et al., 2010). In the present study, the results showed that the AOB and AOA *amoA* genes decreased significantly in the soil with a high OTC concentration, while there was no significant impact from the low OTC treatment. OTC was proposed to be a bacterial inhibitor (Bailey et al., 2003), which might directly change the microbial structure and abundance of some of the functional bacteria in the soil. Therefore, we believe that OTC could directly

decrease the abundances of AOB when the residual concentration reaches a certain range. The relatively low AOA and Arch-amoA gene abundances in the oxytetracycline system had been showed in the previous study (Zhang et al., 2015), which might be related to the antibiotic type, which requires further study.

Soil bacteria or fungi convert organic nitrogen back into $\text{NH}_4^+\text{-N}$ in the ammonification process. Urease is an enzyme that transforms organic N and urea N in the soil to available inorganic N in the soil (Don et al., 2008), which catalyses the hydrolysis of urea into carbon dioxide and ammonia (Liang et al., 2014). Previous study showed that some soil microorganisms, such as *Mycobacterium* sp., *Streptomyces* sp., *Mesorhizobium* sp., *Bacillus* sp., *Brucella* sp. and *Pseudomonas* sp., can produce ureases (Liang et al., 2011). OTC was proposed as a bacterial inhibitor, which may explain why OTC can affect the urease levels in soil. In the present study, OTC significantly decreased the urease activity and $\text{NH}_4^+\text{-N}$ content in the soil. It is possible that the decrease in the urease activity in the soil reduced the $\text{NH}_4^+\text{-N}$ content in soil polluted by OTC. The different responses of $\text{NO}_3^-\text{-N}$ in the soil to low and high OTC concentration stress might be attributed to their difference impacts on nitrification. AOB and AOA usually use soil $\text{NH}_4^+\text{-N}$ as an energy source in the nitrification process. In the low OTC treatment, the abundance of AOA and AOB had no significant change and $\text{NH}_4^+\text{-N}$ in the soil could subsequently be oxidized by ammonia oxidizers. Therefore, the soil $\text{NO}_3^-\text{-N}$ concentration increased at the low OTC concentration. It is possible that the decrease in the $\text{NO}_3^-\text{-N}$ content in the soil at the high OTC concentration was attributed to the reduction in the abundance of AOB and AOA, which reduces the transformation from $\text{NH}_4^+\text{-N}$ to $\text{NO}_3^-\text{-N}$ in the soil.

Previous studies have shown that OTC decreased the number of roots, total length, total

surface area, root volume and root activity at the seedling stage of wheat, which would further reduce the ability to take up nutrients from the soil, such as nitrogen (Li et al., 2011). Our results showed that OTC significantly decreased the maize (shoot and root) biomass and N content. Maize absorbs most of its N in NH_4^+ -N or NO_3^- -N forms (Bloom, 2015). In the present study, the treatments yielded distinctively separated results, as is presented in the PCA plot (Fig. 6). Both the N contents of the shoots and roots were strongly correlated with the urease activity (Fig. 7), while OTC significantly decreased the concentration of NH_4^+ -N in the soil. Therefore, we believed that OTC reduced the urease activity and NH_4^+ -N content in the soil, which may decrease the maize (shoot and root) biomass and N content in the present study.

4.2 Regulation of earthworms on the soil N content and maize growth in soil polluted by OTC

Earthworms play an important role in soil and plant health (Rodriguez-Campos et al., 2014) and have recently been used as an efficient method to accelerate the bioremediation of soil contaminated by hydrocarbons (Singer et al., 2001, Contreras-Ramos et al., 2006). In the present study, the results indicated that earthworms significantly increased the maize shoot and root biomass by enhancing the N contents of shoots and roots (Fig. 3, 6). Our previous study suggested that earthworms increased the degradation of OTC in soils by regulating the microbial biomass, function and communities in the soil (Cao et al., 2015b). Earthworms facilitate and increase the contact between a contaminant and soil microorganisms (Hickman and Reid, 2008), which can accelerate the removal organic contaminants. It is possible that the presence of earthworms increased the degradation of OTC in soils, which may consequently minimize their damage on the growth of roots in maize and on N cycling in the soil. Additionally, earthworms could increase the

bacterial diversity and change the abundance of bacterial diversity by gut-associated processes (Dempsey et al., 2011, Cao et al., 2015a, c), which enhanced urease activities in the soil through their surface and underground casts (Tao et al., 2009). Therefore, in the present study, earthworms significantly increased the urease activity in soil polluted by OTC, which could consequently relieve the stress from OTC on soil urease and support the balance of the contents of NH_4^+ -N and NO_3^- -N in the soil (Fig. 5).

4.3 Regulation of AM fungi on the soil N content and maize growth in soil polluted by OTC

Most previous studies have found that AM fungi have variable and wide ranging beneficial effects on host plants, including improved nutrient uptake, stress tolerance, and disease and herbivore resistance (Solís-Domínguez et al., 2011; Trouve et al., 2014). Recently, AM fungi have been used as an efficient method to remediate soil and sediment contaminated by oils, petroleum products, pesticides, alkaloids and other toxins (Huang et al., 2009). AM fungi have positive effects on plant growth by alleviating the toxicity of pollutants (Gao et al., 2010). In the present study, AM fungi significantly increased the biomass and N content of maize in soil polluted by OTC. It is possible that AM fungi increases the degradation of OTC in soils (Cao et al., 2015b), which consequently alleviated the toxicity of OTC to maize root growth and N cycling in the soil. On the other hand, AM fungi can modify the bacterial community in the soil (Nuccio et al., 2013) and increase soil enzymes by altering the pattern of root and fungal exudation (Zhang et al. 2011). In our experiment, AM fungi significantly increased the urease activity in soil polluted by OTC (Fig. 5), which may consequently increase the ammonification in soil polluted by OTC.

Mycorrhizal fungal hyphae could help the host plant acquire both NH_4^+ -N and NO_3^- -N, and AMF hyphae may be better able to access N in the less mobile NH_4^+ -N form and acquire N before

it is converted to NO_3^- -N (Hodge and Storer, 2015). In the present study, the NH_4^+ -N content in the soil decreased significantly after AM fungi inoculation, which indicated that AM fungi helped the host maize take up more NH_4^+ -N than NO_3^- -N in soil polluted by OTC. Additionally, ammonia oxidizing bacteria and archaea were essential for the conversion of NH_4^+ -N to NO_3^- -N during nitrification. However, AM fungi would have to compete for NH_4^+ -N with ammonia oxidizers. Therefore, AM fungi inoculation could decrease the abundance of AOB (Fig. 5) and reduce the conversion of NH_4^+ -N to NO_3^- -N. This also helped AM fungi acquire NH_4^+ -N from the soil for maize roots and shoots. Together, these factors might have contributed to the overall positive impact of AM fungi on the maize biomass and N content in soil polluted by OTC.

4.4 Interaction between earthworms and AM fungi on the soil N content and maize growth in soil polluted by OTC

Both earthworms and AM fungi are belowground ecosystem engineers that play fundamental roles in shaping ecosystem properties and processes (Mammitzsch, 2012). Previous studies have suggested that there are neutral, positive or negative interactions between earthworms and AM fungi on plant biomass and nutrient uptake (Li et al., 2012a). In the present study, the results showed a significant interaction between earthworms and AM fungi on the maize biomass and N content in soil polluted by OTC. It is probable that earthworms and AM fungi interactively enhance the decomposition of OTC (Cao et al., 2015a, c), which can reduce the negative effects of OTC on the N cycle in the soil and maize biomass. Additionally, earthworms can increase the mineralization of N from organic matter through direct and indirect effects on the microbial community, which directly enhance the N content of roots and shoots (Li et al., 2012 a, b). Our group also found that the AM fungi hyphae assisted maize in the uptake of N mineralized from

wheat straw by earthworms (Li et al., 2013). This might also be the reason for the positive interaction between earthworms and AM fungi on the maize biomass and N content in soil polluted by OTC. In the present study, there was a significant interaction between earthworms and AM fungi on the urease activity in soil polluted by OTC. There were also positive correlations between plant N uptake and urease activity in the soil. Therefore, the interaction between earthworms and AMF could also improve the N uptake of maize shoots and roots by regulating the urease activity in soil polluted by OTC.

5. Conclusions

OTC could affect the root colonization of AM fungi, soil urease activity, the contents of $\text{NH}_4^+\text{-N}$ and $\text{NO}_3^-\text{-N}$ in the soil, and the abundance of AOB and AOA, which resulted in a reduction of the (shoot and root) biomass and N content in maize. Adding earthworms to the AM fungi treatment could improve the root colonization rate of maize at all OTC concentrations. Both earthworms and AM fungi could increase the biomass and N content of maize in soil polluted by OTC by increasing the urease activity in the soil and relieving the stress from OTC on the soil N cycle. AM fungi could also decrease the abundance of AOB, which might consequently affect the N availability in soil polluted by OTC. There was a significant interaction between earthworms and AM fungi on the shoot biomass and shoot N in soil polluted by OTC. All of these factors indicated that earthworms, AMF and their interaction could be used as an efficient method to remediate soil polluted by OTC in agro-ecosystems.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (Project 31570514),

The Da Bei Nong's Youth Researchers Program (2413002), Innovative Group Grant of the National Science Foundation of China (31421092). We also acknowledge Dr. Zhenjun Sun and Dr. Xiaolin Li at China Agricultural University for laboratory assistance.

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Figure Captions

Fig. 1 Schematic representation of the effect of OTC on N cycling processes in the soil and the regulation of earthworms and arbuscular mycorrhizal fungi in OTC polluted soils. We hypothesized that OTC decreased the maize biomass and N uptake (a) by decreasing the soil enzyme activity and the abundance of ammonia oxidizers (b). Earthworms increased the maize biomass and N uptake by increasing the soil urease activity (c); AM increased the soil urease activity (d) and transported N to the host plant directly (f).

Fig. 2 Root colonization rate of mycorrhizal plants inoculated with or without earthworms in soil polluted by OTC.

Fig. 3 Biomass and N uptake of maize in mesocosms with or without earthworms and with and without AMF inoculation.

Fig. 4 The effect of OTC on the abundance of AOA and AOB; the NH_4^+ -N and NO_3^- -N contents in the soil; and the urease activity.

Fig. 5 The effect of earthworms and AM fungi on the abundance of AOA and AOB; the NH_4^+ -N and NO_3^- -N contents in the soil; and the urease activity.

Fig. 6 Two-dimensional similarities among the selected samples revealed by the PCA analysis. The PCA illustrated that PC1 explained 87.3% of the total variations and PC2 explained 6.8% of the total variations; 1, 5, and 9 are the soils without earthworms and mycorrhizae; 2, 6, and 10 are the soils with earthworms; 3, 7, and 11 are the soils with mycorrhizae; and 4, 8, and 12 are the soils with both earthworms and mycorrhizae.

Fig. 7 Correlations between shoot N, root N and urease activity in the soil. The urease in the soil showed positive correlations with shoot and root N.

Fig. 1 Schematic representation of the effect of OTC on N cycling processes in the soil and the regulation of earthworms and arbuscular mycorrhizal fungi in OTC polluted soils. We hypothesized that OTC decreased the maize biomass and N uptake (a) by decreasing the soil enzyme activity and the abundance of ammonia oxidizers (b). Earthworms increased the maize biomass and N uptake by increasing the soil urease activity (c); AM increased the soil urease activity (d) and transported N to the host plant directly (f). Earthworm and AM fungi showed an interaction in increasing the soil urease activity and AOB abundance in soil polluted by OTC.

Fig. 2 Root colonization rate of mycorrhizal plants inoculated with or without earthworms in soil polluted by OTC. Values are mean $\pm$ standard error ($n = 4$). Different letters indicate significant differences between inoculation treatments. No E is without earthworms; E is the soil with the addition of earthworms; OTC0+AM is the OTC free soil with the addition of AM fungi; OTC1+AM is the 1 mg kg^{-1} OTC polluted soil with the addition of AM fungi; OTC100+AM is the 100 mg kg^{-1} OTC polluted soil with the addition of AM fungi

Fig. 3 Biomass and N uptake of maize in mesocosms with or without earthworms and with and without AMF inoculation. Values are mean $\pm$ standard error ($n = 4$). Upper case letters denote differences between different groups. OTC0 is the OTC free soil; OTC1 is the 1 mg kg^{-1} OTC polluted soil; OTC100 is the 100 mg kg^{-1} OTC polluted soil; OTC is the different OTC level treatments without earthworms and AM fungi; OTC+E is the soil with the addition of earthworms; OTC+AM is soil with the addition of AM fungi; OTC+E+AM is the soil with the addition of both earthworms and AM fungi.

Fig. 4 The effect of OTC on the abundance of AOA and AOB; the NH_4^+ -N and NO_3^- -N contents in the soil; and the urease activity. The middle lines of the box plots represent median values ($n = 4$), with bars showing value ranges (minimum to maximum). Different letters indicate significant differences among treatments. OTC0 is the OTC free soil; OTC1 is the 1 mg kg^{-1} OTC polluted soil; OTC100 is the 100 mg kg^{-1} OTC polluted soil.

Fig. 5 The effect of earthworms and AM fungi on the abundance of AOA and AOB; the NH_4^+ -N and NO_3^- -N contents in the soil; and the urease activity. The middle lines of the box plots represent median values ($n = 4$), with bars showing value ranges (minimum to maximum). Different letters indicate significant differences among treatments. OTC is the different OTC level treatments without earthworms and AM fungi; OTC+E is the soil with the addition of earthworms; OTC+AM is soil with the addition of AM fungi; OTC+E+AM is the soil with the addition of both earthworms and AM fungi.

Fig. 6 Two-dimensional similarities among the selected samples revealed by the PCA analysis.

The PCA illustrated that PC1 explained 87.3% of the total variations and PC2 explained 6.8% of the total variations; 1, 5, and 9 are the soils without earthworms and mycorrhizae; 2, 6, and 10 are the soils with earthworms; 3, 7, and 11 are the soils with mycorrhizae; and 4, 8, and 12 are the soils with both earthworms and mycorrhizae.

Fig. 7 Correlations between shoot N, root N and urease activity in the soil. The urease in the soil showed positive correlations with shoot and root N.

Table 1 Results of two-way ANOVA for AOB and AOA amoA copy numbers, soil urease activity, NH₄⁺—N concentrations, NO₃[—]—N concentrations, maize biomass and N content in different treatments.

	OTC1	OTC100	E	AM	OTC1*E	OTC1*AM	OTC100*E	OTC100*AME*AM	OTC1*E*AM	OTC100*E*AM	
AOB	0.489	0.000	0.000	0.000	0.708	0.551	0.075	0.905	0.025	0.843	0.001
AOA	0.002	0.000	0.496	0.018	0.035	0.219	0.013	0.000	0.112	0.039	0.244
Urease	0.037	0.008	0.000	0.000	0.016	0.002	0.859	0.011	0.008	0.021	0.028
NH4+-N	0.189	0.141	0.473	0.000	0.506	0.001	0.016	0.002	0.376	0.773	0.214
NO3--N	0.000	0.773	0.682	0.000	0.354	0.000	0.063	0.096	0.000	0.068	0.000
Shoot N	0.507	0.183	0.000	0.000	0.15	0.554	0.155	0.874	0.001	0.000	0.000
Root N	0.103	0.073	0.000	0.000	0.002	0.051	0.009	0.282	0.655	0.468	0.772
Shoot biomass	0.002	0.006	0.000	0.000	0.223	0.002	0.454	0.004	0.004	0.001	0.763
Root biomass	0.001	0.000	0.000	0.000	0.248	0.179	0.468	0.057	0.723	0.205	0.068