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Temporal changes in horsebean bioavailability and accumulation after removing extractable oxytetracycline fractions in soils

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Abstract:

Extractable fractions of oxytetracycline in soil affect its bioavailability and accumulation in plant. The objective was to assess the bioavailability of different bound fractions of oxytetracycline remained in soil to horsebean. After freshly spiking with oxytetracycline for 24 h, soils were treated with water, mild (0.1 mol L^{-1} $CaCl_2$) and exhaustive (0.1 mol L^{-1} Na_2EDTA-McIlvaine) extractants, respectively, as treatments of T_1, T_2, T_3 in order to remove different extractable oxytetracycline fractions. The control was nonextracted soil. Horsebean was exposed in above soils. The results showed that oxytetracycline was accumulated in shoot and root at 10d, however, after that less than quantification limits (< 0.001 mg kg^{-1}) of oxytetracycline was in root. The resistant bound fraction of oxytetracycline in T_3 always exhibit the highest shoot concentration factors (SCF) comparing with removing fewer extractable fractions during 14-28d exposure. And the effect of horsebean upon oxytetracycline extractability during exposure was also studied. Adding horsebean did not affect the change trend of three extractable fractions in the control, T_2, T_3 during exposure, but change the trend of $CaCl_2$- and total-extractable fractions in T_1. Horsebean activity can increase total-extractable concentration in the control, but decrease total-extractable concentration in T_1, T_2 and T_3. The correlation analysis showed that there were close relationship between water-, $CaCl_2$-, total-extractable fractions in soil and root accumulation in the horsebean at exposure 10d. The interesting phenomenon that resistant bound OTC had high availability, and its ecological risk in soil has been underestimated in past research.

Key words: Oxytetracycline bioavailability; Accumulation; Extractable fractions; Horsebean; Soil

Introduction

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Tetracycline antibiotics including oxytetracycline (OTC) have been as one of the most widely-used veterinary antibiotics in livestock industry. It has been reported [1,2] that above 75 % of OTC administered to animals is excreted in an antimicrobially active form in urine or feces. Agricultural application of untreated or even treated antibiotics-containing animal wastes has caused serious contamination in soil. [3] At present, their frequent detection and high detected concentrations in soil have occurred. [4-6]

In order to estimate contaminants availability, single chemical extraction techniques are commonly used to extract their different fractions in soil. And the chemical extraction is relatively simple, one-step procedures. [7] Different extractable fractions have different affinities for binding to soil, which determine or govern contaminant mobility and bioavailability. [8] OTC exists predominantly as a zwitterion with a positive charge on the tertiary amine functionality and a negative charge on the deprotonated hydroxyl group, so chemical extraction could also be suitable to estimate the labile fractions of OTC. In soil, water-extractable fraction represents the most available portion and it can be stated with a degree of certainty that this fraction is dissolved and mobile in soil solution. Other OTC fractions will be sorbed on soil as bound residue fractions. In past study, $CaCl_2$ extraction was commonly used as "mild/soft" extraction methods being considered to represent the labile fraction of metals that has the potential to enter terrestrial organisms. [8-12] It was reported that 0.1 mol L^{-1} $CaCl_2$ extraction concentrations were steady for different soil types, and its extraction concentration was between water and exhaustive extraction [13]. 0.1 mol L^{-1} Na_2EDTA-McIlvaine (pH, 4.0 ± 0.05) as exhaustive extraction was applied to extract/remove total extractable fractions containing dissolved, exchangable fraction and loosely bound fraction [14], that is to say, only resistant bound fraction was not extracted. And in our previous paper [13], it was also evident that 0.1 mol L^{-1} $CaCl_2$ as neutralised salt solutions could be suitable to extract loosely bound OTC, and that 0.1 mol L^{-1} Na_2EDTA-McIlvaine (pH 4.0 ± 0.05) as exhaustive extraction can achieve stable and efficient recovery comparing with other buffer solution. However, it can not extract all OTC in soil, and with extraction rate ranging 31.97-80.64 % [13]. Only 43.9 % of total OTC was extracted in contaminated soils in our following study, which showed that resistant bound (or nonextractable) OTC existed in soil.

Over the past decade, a single chemical extraction method have been developed to address this issue by attempting to measure bioavailable OTC concentrations in soil. [15,16] However, there is very little information on the study of biological methods to assess OTC bioavailability in soil and it is unclear whether the chemical method gives the measure that is closest to the biological method. At present, it was found that the antibiotics can accumulated in plant from soil (or aqueous) media. [17-26] It was reported that that soil-bound antibiotic retained bioavailability toward microorganisms [27,28].

However, few researches have been published on the determination of different bound fractions of OTC in soil. Furthermore, little studies have addressed the potential bioavailability of adsorbed (containing nonextracted and labile-extracted) OTC onto soils toward plant. It should be considered whether the bound residues with different binding extent have different bioavailability, and whether resistant bound fraction has low bioavailability with strong adsorbed on soil as we have expected. Hence, the present study aims to investigate OTC accumulation by horsebean in soils after removing different extractable fractions during exposure experiment; and to characterise the effect of horsebean on the extractability of OTC by single chemical extraction in soils during exposure. The objective was to reveal exactly the bioavailability of different OTC bound fractions remained in soil and assess its ecological risk.

2. Materials and methods

2.1 Oxytetracycline (OTC) and other chemicals

Oxytetracycline hydrochloride (95 % purity, analytical grade) was bought from the Sigma Co. in St. Louis, USA. The standard reference material of tetracycline hydrochloride was obtained from the Institute of Veterinary Drug Control in Beijing, China. They were stored at 4 °C. Some important physicochemical properties of OTC are involved in molecular weight (MW) (496.89 g mol^{-1}), molecular formula ($C_{22}H_{24}N_2O_9·HCl$), aqueous solubility (500 mg mL^{-1}). And OTC structure was shown in Fig. 1 and has three acid dissociation constants (pK_a = 3.27, 7.32, and 9.11).

All other chemicals were of analytical grade. Deionized water was used for all experiments.

Fig. 1 Molecular structure and molecular formula of oxytetracycline

2.2 Soil properties and treatments

Surface soil (0-20 cm) was manually collected from cinnamon soil of agricultural fields in Weifang, Shandong, China. The soil was air-dried and passed through a 1.00-mm sieve. Adding dairy manure in the ratio of 1:5 increase organic matter content of soil in order to ensure the plant growth with enough nutrient during 28 d exposure. The analysis showed that the properties of mixed soil were as follows: organic matter, 8.64 %; total nitrogen, 0.37 %; total phosphorus, 0.34 %; total potassium, 2.67 %; alkali-hydrolyzable nitrogen, 110 mg kg^{-1}; available phosphorus, 228 mg kg^{-1}; available potassium, 2727 mg kg^{-1}; and pH, 7.4. OTC was not detected in soil and dairy manure.

We wanted to compare the potential bioaccumulation and bioavailability of OTC by horsebeans from different OTC bound fractions remained in soil. Briefly, above mixed soil had been freshly spiked with OTC. After contaminated 24 h, the soils were then extracted with water (T_1 treatment), 0.1 M $CaCl_2$ (T_2 treatment) and 0.1mol/L Na_2EDTA-McIlvaine (pH = 4.0 ± 0.05) (T_3 treatment), respectively, for removing water dissolved fraction, a part of loosely bound fractions, all extractable fractions of OTC. The schematic diagram of OTC removal of three fractions is shown in Fig. 2. T_1 was considered as remaining OTC of all loosely bound and resistant bound fractions. T_2 was considered as remaining a part of loosely bound and resistant bound fractions, because Ca^{2+} can exchange a part of absorbed OTC on soil. T_3 was considered as remaining only resistant bound (nonextractable residues) fractions. In T_3, the treated soils were washed with de-ionized water in order to avoid the effect of extract agent on horsebean growth, because crystalline substance was precipitated on soil surface after EDTA extraction in our preliminary experiment. For non-soluble contaminants, the water extraction is less effective than the extraction with neutralizing solutions or chelating agents. OTC was not determined in all eluate, which showed that washing did not remove OTC. Finally adding clean soil in the ratio of 7:1 increased the volumes of above these extracted soils. [29] And the control was nonextracted treatment soil.

Fig. 2 The schematic diagram of OTC removal of three fractions.

2.3 Exposure experiment

Horsebean (*Pisum sativum Linn*) seed was sourced from Tianjin Academy of Agriculture Science. Seeds were surface sterilized with 10 % H_2O_2 for 10 min, rinsed with distilled water and soaked in deionised water for 2-3 h before planting. Three seeds of uniform size per each replicate were used to reduce the variability between horsebeans. Horsebeans were exposed to a PVC pot (10 cm in diameter, 15 cm in height) containing 500 g soils. Every treatment was twenty four PVC pots which contained twelve pots with horsebean and twelve pots without horsebean. The soils were initially adjusted to approximately 60 % water holding capacity, and then deionised water was added as required to replace losses during exposed experiment and ensure an initial field conditions. Pots were placed in greenhouse to grow at 25 ± 1.0℃ for 28 days. Pots were taken for horsebeans at 10, 14, 21 and 28 d after exposure with triplicate analyses being carried out in order to determine OTC accumulation by root and shoot. We took the horsebean sample from the beginning of exposure 10d because horsebean was still a seed without seedling before 10d. At the same time, soils sample with and without plant was analysed by single-chemical extraction at exposure 10, 14, 21 and 28 d.

2.4 Horsebean-extraction and determination of OTC

The concentrations of OTC in plant samples were determined by adding 25 mL of 0.1 mol L^{-1} Na_2EDTA-McIlvaine and followed the method described in Li *et al* (2011). [14] The extracts were filtered through 0.22 μm polytetrafluoroethylene membraneand then concentrated and cleaned-up by solid phase extraction (SPE) cartridges (Oasis HLB, 6 cc/500 mg). The concentration of OTC in the extraction solution was measured by a reverse-phase HPLC (Waters Corp.) with a 46×255-mm Waters ODS–C18 (5 μm) column followed by UV detection at 360 nm. The mobile phase was a mixture of 0.01 M oxalic acid–acetonitrile (80:20, v/v) in an equilibrium system at a flow rate of 1.0 mL/min. The retention time was 5.24 min.

2.5 Single-chemical extraction in soil after exposure experiment

As in section 2.2, three solutions (water, 0.1 mol L^{-1} $CaCl_2$ and 0.1 mol L^{-1} Na_2EDTA-McIlvaine (pH 4.0 ± 0.05)) used in geochemical extraction schemes to determine water dissolved fraction and two bound fractions with different binding extent in order to estimate the effect of horsebean on potential bioavailable of OTC in soil. Briefly, soil samples were firstly frozen dried. 20 mL of extractant was added to 1.000 g dried soil and this mixture was shaken for 30 min at room temperature (25□). The extract was separated from the solid residue by centrifugation (3000 rpm) and then filtered through 0.22 μm polytetrafluoroethylene membrane. OTC concentration was determined as shown in above Section 2.4.

3 Results and Discussion

3.1 The extractability of OTC in soils after removing extractable fractions

According to contamination and extraction treatment in soils before exposure experiment, initial total OTC concentration was 200 ± 0.00, 196 ± 0.23, 153 ± 1.35, 86.1 ± 2.12 mg kg^{-1}, respectively, in the control, T_1, T_2, T_3. It is well known that the all contaminant fractions remained in the soil are not completely available to organism, because a part is progressively sequestrated by soil organic and inorganic constituents. [30,31] Results showed that water-extractable fraction was 4.24 ± 0.23 mg kg^{-1} in the control, and not detected in three treated soils (T_1, T_2, T_3). And $CaCl_2$-extractable fraction was 43.3 ± 1.15 and 47.5 ± 1.35 mg kg^{-1}, respectively, in T_1 and the control, but not detected in T_2 and T_3. Total-extractable fraction was 66.4 ± 0.77, 110 ± 1.97 and 114 ± 2.12 mg kg^{-1}, respectively, in T_2, T_1 and the control, and not detected in T_3. Above extraction results showed that OTC can exist as resistant bound fraction with up to 86.1 ± 2.12 mg kg^{-1} in soils because the fraction was not extracted even exhausted extractant. According to pretreatment, initial various extractable and total concentrations were all in the order the control > T_1 > T_2 > T_3.

3.2 Horsebean growth during exposure experiment

The horsebean growth was observed by measuring the dry weight of the root and shoot. As shown in Fig. 3a, the dry weight of root decreased gradually over time in all soils. At the exposure 10 d, the root weight included root and seed because they can not be divided by hand as a whole, and at exposure 14 d, the seed disappeared and only root existed, which maybe cause the decrease of dry weight of root during 10-14 d. It was worth to consider that above decrease trend was not accurate. During exposure 14 to 21 d, the dry weight of root did not change significantly. From exposure 21 to 28 d, the dry weight of root decreased. Root grew up in the soil, so total-, $CaCl_2$-, water- extractable OTC (as shown in Fig. 5) in soil will affect directly root growth during exposure 10-28 d. Maybe, there are no relationship between root growth influence and extractable OTC concentration according to the results. And the negetive influence is related with exposure time, because dry weight of root decreased significantly during 21-28 d and not 14-21 d. As shown in Fig. 3b, the dry weight of shoot increased from exposure 10 to 21d in all soils with seedling growing up, and decreased from exposure 21 to 28 d. The negative influence may be due to their high concentrations of OTC in shoot at exposure 28 d as seen in the following results of Fig. 4. The low accumulation of OTC in shoot with lower than 1.07 ± 0.09 mg increased the shoot dry weight of plant during 10-21 d. The highest accumulation level of OTC with higher than 1.21 ± 0.06 mg had the worst effects on the shoot growth at exposure 28 d, which is similar to previous research result [32], because antibiotic inhibit the photosynthetic activity [33].

There was insignificantly difference ($p > 0.05$) for root or shoot weight between the control, T_1, T_2, T_3 at same exposure time, which indicated that soil treatment process had no effect on horsebean growth.

Fig. 3 Root (a) and shoot (b) dry weights of horsebean from soils after removing different extractable fractions during 28 d exposure experiment. The bars represent ±SE of the mean.

3.3 OTC accumulation by horsebeans during exposure experiment

The accumulation of OTC in root and shoot over time was shown in Fig. 4. In all soils, OTC accumulation in root (Fig. 4a) was only detected at exposure 10 d, and detected with less than quantification limits (< 0.001 mg kg^{-1}) after that. At exposure 10 d, OTC accumulation in root was in the order T_1 > the control > T_2 > T_3, which was different order with initial various extractable fractions and total concentration of OTC in soils as the control > T_1 > T_2 > T_3. There was insignificant difference ($p > 0.05$) between the control and T_1, and there was significant difference ($p < 0.01$) between T_1, T_2, T_3.

In the control, T_1, T_2 and T_3, OTC accumulation in shoot (Fig. 4b) increased slightly during exposure 10-14 d, and then increased rapidly during 14-28 d. At exposure 10 and 14 d, OTC shoot accumulation in the control and T_1 was significantly ($p < 0.05$) higher than that in T_2 and T_3. And there was insignificant difference ($p > 0.05$) between the control and T_1 or between T_2 and T_3. At exposure 21 d, there was insignificant difference ($p > 0.05$) between four soils. At exposure 28 d, OTC shoot accumulation in the control was significantly ($p < 0.05$) higher than that in T_1, T_2 and T_3. In a word, the highest OTC accumulation concentration was always found in the control or T_1 at same exposure time, which could be caused by higher initial various extractable fractions or total OTC concentration in the control and T_1. But, the order of OTC concentration in shoot in three treatment soils at same exposure time was not consistent with that of intial various extractable and total concentrations in three treatment soils.

At exposure 10 d, OTC was detected both in root and shoot, and the accumulation in root from 0.707 ± 0.104 to 1.491 ± 0.129 mg was higher than in root from 0.088 ± 0.012 to 0.164 ± 0.014 mg. After that, OTC concentration in root was low than quantification limit, and high OTC concentrations from 0.176 ± 0.016 to 1.602 ± 0.107 mg were detected in shoot, which showed that OTC translocation from root to shoot was more stronger after 10d than at 10d.

It has been known that initial total OTC concentrations were different in three treated soils and the control in our study. For better presentation and to be able to directly compare treated soils, root concentration factors (RCF) and shoot concentration factors (SCF) for OTC were calculated in Table 1. Accumulation factors were given in mg of OTC over mg of initial measured OTC in soil. RCF values were from 0.012 ± 0.002 to 0.016 ± 0.003 at exposure 10 d, and there was insignificant difference ($p > 0.05$) between four soils. In four soils, SCF increased slightly from exposure 10 to 14 d, then increased rapidly during exposure 14-28 d. At exposure 10 d, there was insignificantly difference ($p > 0.05$) between the control, T_1, T_2 and T_3. At exposure 14-28 d, the highest SCF was always in T_3, and there were insignificantly difference ($p > 0.05$) between the control, T_1 and T_2. Especially in T_3 at exposure 28 d, SCF was high up to 0.031. Results showed that resistant bound OTC remained in T_3 was more readily accumulated in horsebean shoot.

Fig. 4 OTC accumulation by horsebean from soils after removing different extractable fractions during 28 d exposure experiment. Data are expressed as root accumulation concentration (a) and shoot accumulation concentration (b). The bars represent ±SE of the mean.

In the present study, the pretreatment caused only different OTC fractions remained in soils, however, other soil properties in the control and three treated soils were not affectted by pretreatmnet. Consequently, we speculated that the variability in OTC accumulation by horsebean might be mainly explained by the differences in remained OTC fractions in soils. Heise *et al* [34] have pointed that research activities on the phenomenon of non-extractability under different laboratory and field conditions were necessary to complete the data pool for a respective environmental risk assessment because the nature of non-extractable antibiotic residues in soil is unknown now. In the study, it is worth noting nonextractable (resistant bound) OTC residue in soil has still higher bioaccumulation factors (SCF values) by horsebean although it was not extracted by 0.1 mol L^{-1} Na_2EDTA-McIlvaine as exhaustive extractant than other extractant and the control. It was reported [35] that easily extractable antibiotic fraction was accelerated near roots throughout the plant growth period because of the function the root exudates and microbial communities in the rhizosphere. However, as shown in Fig. 5, water-, $CaCl_2$-, total-extractable concentrations in soils with horsebean were not higher than that without horsebean in T_3. Maybe is that non-extractable OTC residues firstly is transformed easily into extractable OTC in the rhizosphere and then is absorbed quickly by plant root, which cause that three extractable OTC concentrations were not high in soils with horsebean in our study. Thus, high OTC accumulation concentration in shoot was also found in T_3 as same as other treated soils (Fig. 4b). And, initial total OTC concentration in T_3 soil was lower than other trated soils (as shown in 3.1), which will cause that SCF values as the ratio (OTC mg in shoot)/(OTC mg in soil) in T_3 were always higher than other trated soils. Consequently, above tests confirmed the low affinity of the non-extractable OTC residues to the soil matrix for horsebean bioavaibility. In the future, it's worth noting that resistant bound fraction of OTC in soil could still be related with its high ecological risk for plant, which indicated OTC was transfered through food chain to crop or other food of human being, and finally caused human health risk.

3.4 Effects of horsebean on OTC extractability from soil during exposure experiment

In order to investigate the effect of horsebean on bioavailable OTC concentrations in soil after removing various extractable fractions over exposure experiment, three fractions were extracted by water, 0.1 mol L^{-1} $CaCl_2$, 0.1 mol L^{-1} Na_2EDTA-McIlvaine, respectively, as water-extractable fraction, $CaCl_2$-extractable fraction, total-extractable fraction. It is well known that water-extractable fraction has the highest bioavailable in all extractable fractions. The second is $CaCl_2$-extractable fraction as the total concentration of Ca^{2+} readily exchangeable and water soluble fractions. Following is total-extractable fraction as the sum of all extractable fractions. As shown in Fig. 5, three extractable concentrations increased during 0-10 d and then decreased after that in the control whether horsebean was present or not. Adding horsebean decreased three extractable concentrations during 0-10 d and 14-21 d, and increased their concentrations during 10-14 d and during 21-28 d exposure. There were significant effect of horsebean on $CaCl_2$-, total-extractable concentrations ($p <$ 0.05), but not water-extractable fraction. In T_1, water-extractable concentration was 0 mg kg^{-1} at 0 d, and the concentration increased during 0-10 d and then decreased after that whether horsebean was present or not. For soils without horsebean, $CaCl_2$-extractable concentration decreased during 0-10 d, and increased during 10-14 d, and again decreased after then. Total-extractable concentration increased firstly during 0-10 d and then decreased. For soils with horsebean, $CaCl_2$- and total-extractable concentrations decreased during whole exposure. Adding horsebean decreased water- and total-extractable concentrations during whole exposure. The effect on total-extractable fraction was significant level ($p < 0.05$) during exposure, and the effect on water-extractable fraction was significant level ($p < 0.05$) at exposure 10 and 21 d. Adding horsebean increased ($p < 0.05$) $CaCl_2$-extractable concentrations at exposure 10, 21, 28 d, but decreased ($p < 0.01$) $CaCl_2$-extractable concentrations at exposure 14 d. In T_2, initial water- and $CaCl_2$-extractable concentration was 0 mg kg^{-1}. Whether horsebeans are present or not, their concentrations increased quickly during 0-10 d, and then decreased during 10-28 d. Total-extractable concentration decreased gradually during whole exposure experiment whether horsebeans were present or not. Horsebean activity decreased water-, $CaCl_2$- and total-extractable concentractions during whole exposure with significant difference ($p <$ 0.05), except for water-extractable fraction at 10, 14 d with insignificant difference ($p > 0.05$). In T_3, initial water- and $CaCl_2$- and total-extractable concentrations were 0 mg kg^{-1}. Water-extractable concentrations were still 0 mg kg^{-1} at exposure 10 d, and increased quickly during 10-14 d, and then decreased during 14-28 d whether horsebeanwas present or not. $CaCl_2$- and total-extractable concentrations increased quickly during 0-10 d and then decreased during 10-28 d exposure whether horsebean was present or not. Horsebean activity decreased ($p < 0.05$) significantly $CaCl_2$- (during 14-28 d) and total-extractable (during 10-28 d) concentrations, but not affect water-extractable concentrations.

Fig.5 Effects of horsebean on OTC extractability from soils after removing different extractable fractions during 28 d exposure experiment.

In the end of exposure, adding horsebean increased significantly ($p < 0.05$) total-extractable concentrations of 40.6 mg kg^{-1} in the control, but decreased significantly ($p < 0.05$) in T_1, T_2 and T_3. The reason was as following. In all soils without plant, water- and $CaCl_2$- extractable fractions were released from resistant bound fraction under the experiment condition. Then horsebean activity further promoted the release of resistant bound OTC in soils. In the control, initial three extractable OTC concentrations were high, and then experiment condition and horsebean increased their concentrations by root exudate and so on during exposure, which would cause three extractable concentrations increasing in the end of exposure although horsebean can absorb a part of extractable fractions comparing with three treated soils. In T_1, T_2 and T_3, there was few initial extractable OTC concentrations due to the pretreatment. Although experiment condition and horsebean can increase extractable OTC concentration according to results of the control, horsebean itself will absorb and utilize the released extractable OTC as high accumulation ratio as shown in Section 3.3, which caused that total or various extractable concentrations decreased in treated soils with horsebean in the end of exposure comparing with not adding horsebean.

Regardless of the presence or absence of horsebean, the concentrations of three extractable concentrations at same exposure time always were in the order: the control or $T_1 > T_2 > T_3$, which the order was approximately consistent with initial three extractable and total concentrations. During whole exposure, total-extractable concentration range was 51.6 (± 0.91) – 143 (± 2.23), 43.4 (± 5.85) – 129 (± 6.65), 9.66 (± 0.98) - 58.5 (± 0.19), 1.15 (± 0.00) - 14.2 (± 0.38) mg kg^{-1}, respectively, in the control, T_1, T_2, T_3. And $CaCl_2$-extractable concentration range was 17.6 (± 0.37) - 67.3 (± 3.79), 13.9 (± 2.32) - 50.3 (± 2.74), 3.99 (± 0.13) - 16.3 (± 2.30), 1.15 (± 0.00) - 4.77 (± 0.66) mg kg^{-1}, respectively, in the control, T_1, T_2, T_3. And water-extractable concentration range was 4.33 (± 1.69) - 15.7 (± 4.03), 3.67 (± 2.12) - 11.1 (± 0.64), 1.13 (± 0.01) - 5.07 (± 0.93), 1.15 (± 0.00) - 1.29 (± 0.01) mg kg^{-1}, respectively, in the control, T_1, T_2, T_3.

In the study, adding horsebean did not affect the change trend of three extractable fractions in the control, T_2, T_3 during exposure. Regardless of horsebean was present or absence, water-extractable concentration was firstly increased and then decreased in the control, T_2, and T_3. $CaCl_2$- and total-extractable concentrations were firstly increased and then decreased in the control. $CaCl_2$- and total-extractable concentrations were decreased gradually during exposure in T_2 and T_3. However, adding horsebean affectted the change trend of $CaCl_2$- and total-extractable fractions in T_1, and did not affect the change trend of water- extractable fraction. In T_1, $CaCl_2$-extractable concentration decreased during 0-10 d, and increased during 10-14 d, and then decreased during 14-28 d without horsebean. $CaCl_2$-extractable concentration decreased gradually during whole exposure with horsebean.

3.5 Relationship between extractability concentration in soil and the accumulation by horsebean

Correlation analysis showed that there was insignificant correlation ($p > 0.05$) between OTC concentration in horsebean shoot (or whole plant) and various extractable OTC in soil during exposure in the control, T_1, T_2 and T_3. It is well known that root is the passage of OTC from soil to plant, so the close relationship between the accumulation of horsebean root and different extractable OTC at exposure 10 d in soils suggests the bioavailability of different extractable OTC fractions in soils. As shown in Fig. 6, the significant positive correlation was observed between concentration of OTC in root and water-extractable fraction (r = 0.914*; $p < 0.05$), $CaCl_2$-extractable fraction (r = 0.963**; $p < 0.01$) and total-extractable fraction (r = 0.937*; $p < 0.05$), which showed that there were close relationship between water-, $CaCl_2$-, total-extractable fractions in soil and root accumulation in the horsebean.

Fig. 6 The correlation analysis between different extractable OTC concentrations and OTC accumulation by root at exposure 10d

Conclusion

This study provides, for the first time, evidence for OTC accumulation by horsebean from soils after removing various labile extractable fractions by pretreatment. Findings of this study clearly demonstrate that resistant bound fraction of OTC in soil after removing all extractable fractions with exhaustive extraction of 0.1 mol L^{-1} Na_2EDTA-McIlvaine always exhibit the highest bioaccumulation factors in horsebean comparing with removing fewer extractable fractions during 14-28 d exposure. However, most of these previous studies have been focused on assessing total OTC contamination concentration in soil through 0.1 mol L^{-1} Na_2EDTA-McIlvaine extraction and little attention was paid on the biological availability of OTC remained in soil as resistant bound fraction. Findings of this study indicated that it was not accurate that resistant bound OTC had low availability as we expected, and the ecological risk of resistant bound OTC in soil has been underestimated in past research. OTC was accumulated in shoot and root during exposure, however, after exposure 10d with less than quantification limit of OTC in root. Adding horsebean did not affect the change trend of various extractable fractions in the control, T_2, T_3 during exposure. However, adding horsebean affectted the change trend of $CaCl_2$- and total-extractable fractions in T_1, and did not affect the change trend of water extractable fraction. Comparing with not adding horsebean, horsebean activity can increase total-extractable concentration in the control, but decrease total-extractable concentration in T_1, T_2 and T_3. The correlation analysis showed that there were close relationship between water-, $CaCl_2$-, total-extractable fractions in soil and root accumulation in the horsebean. An important future research direction might be to derive the release and bioavailability of resistant bound OTC in soil. Such study would greatly facilitate risk assessment and ecological benchmark calculations for soil OTC contamination.

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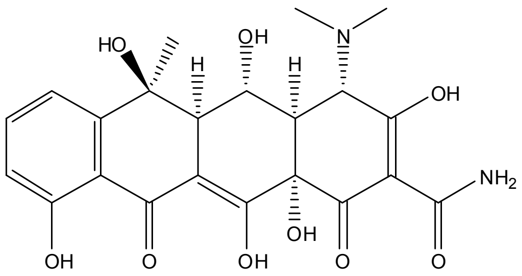


Oxytetracycline

$pK_{a1,2,3}$=3.27,7.32,9.11

Fig.1 Molccular structure and molecular formula of oxytetracycline

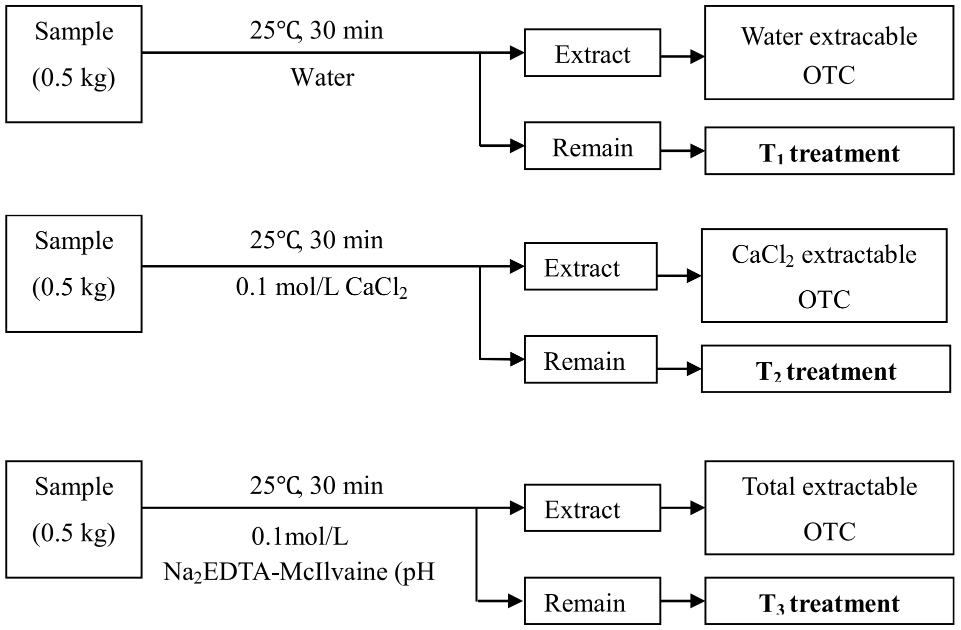


Fig. 2 The schematic diagram of OTC removal of three fractions.

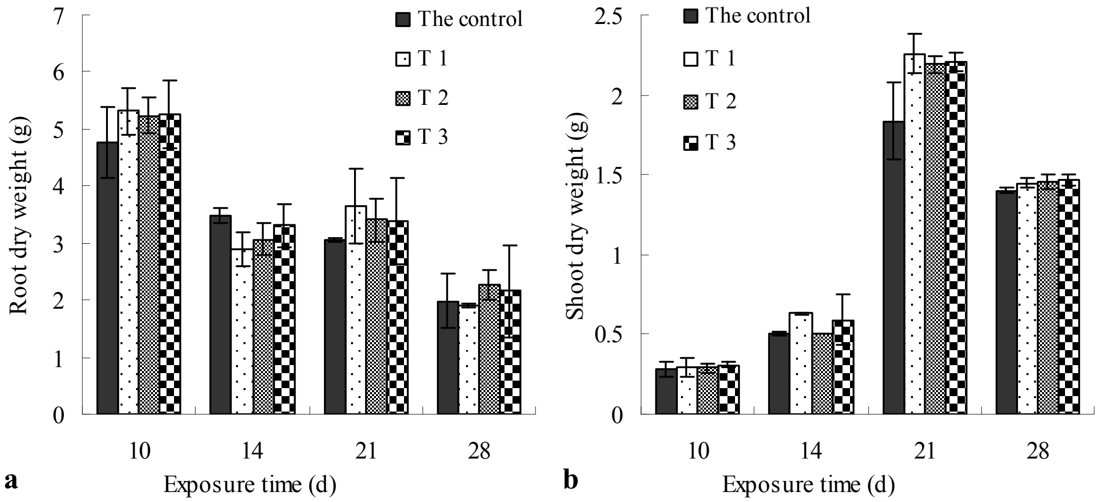


Fig. 3 Root (a) and shoot (b) dry weights of horsebean from soils after removing different extractable fractions during 28 d exposure experiment. The bars represent ±SE of the mean.

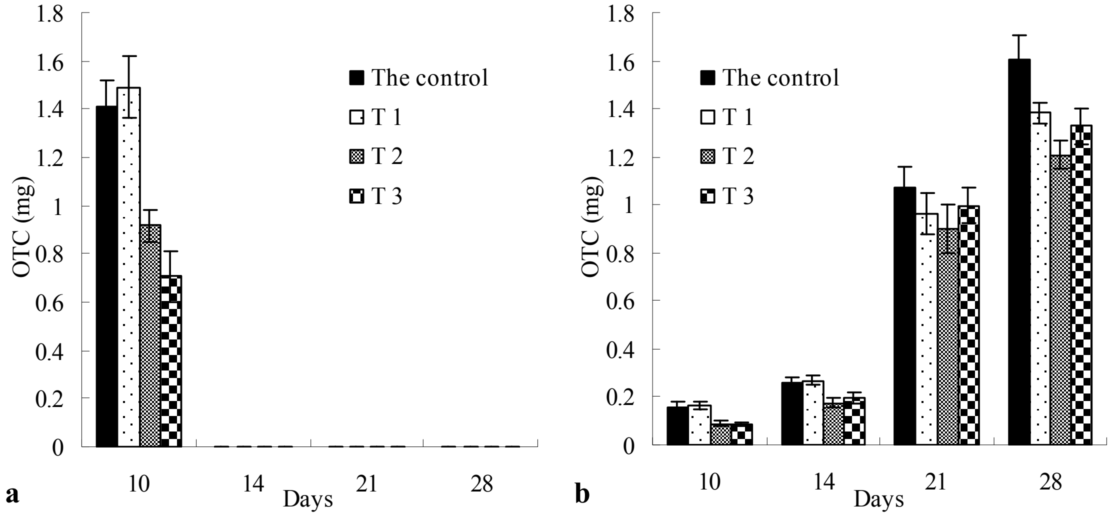
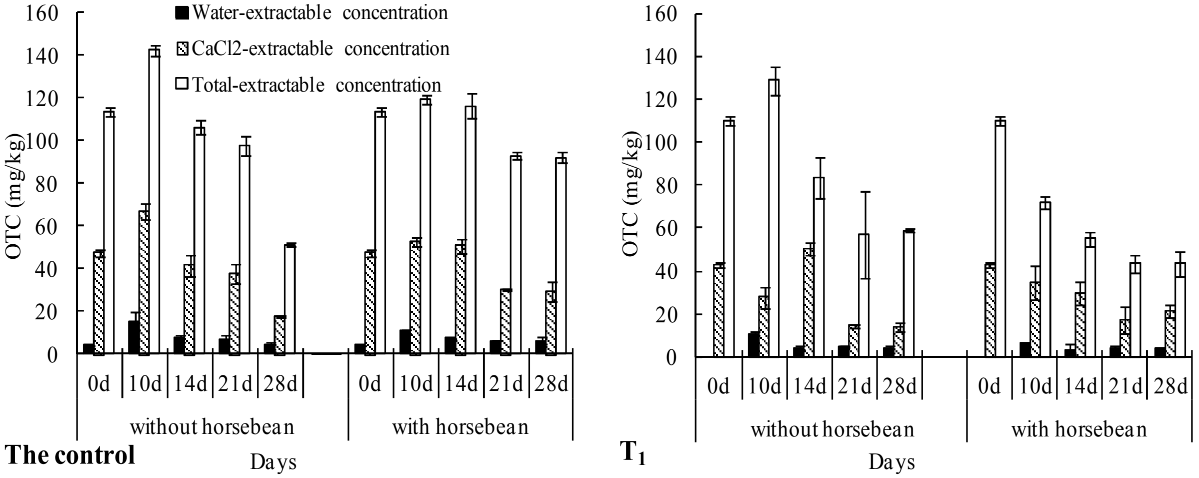


Fig. 4 OTC accumulation by horsebean from soils after removing different extractable fractions during 28 d exposure experiment. Data are expressed as root accumulation concentration (a) and shoot accumulation concentration (b). The bars represent ±SE of the mean.



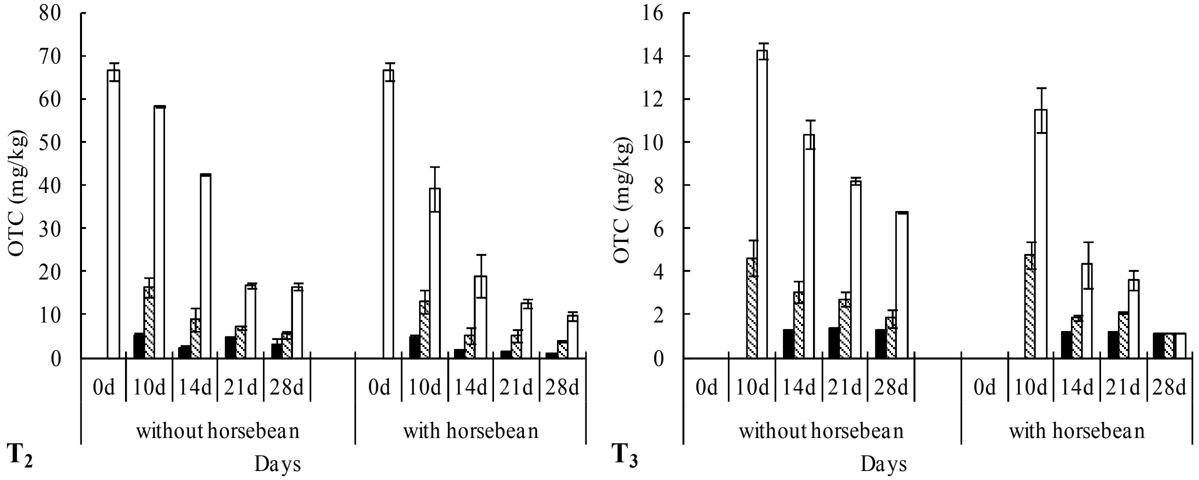


Fig.5 Effects of horsebean on OTC extractability from soils after removing different extractable fractions during 28 d exposure experiment.

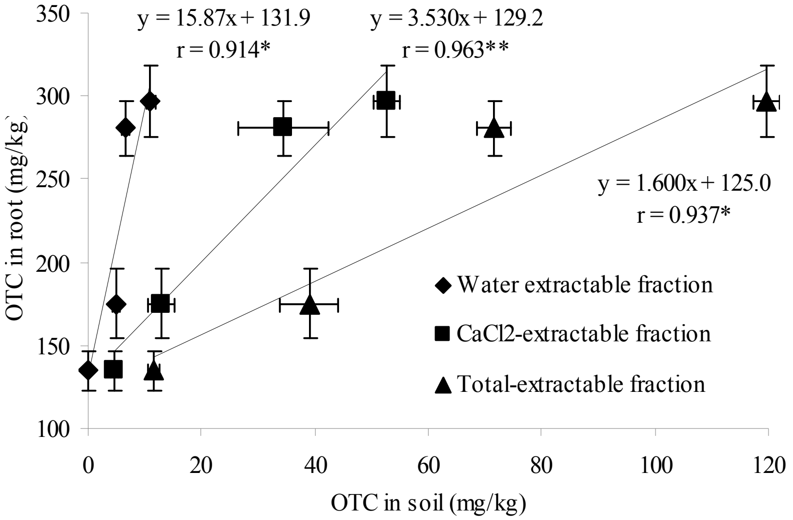


Fig. 6 The correlation analysis between different extractable OTC concentrations and OTC accumulation by root at exposure 10d

Table 1 Root concentration factors (RCF) and shoot concentration factors (SCF) for OTC in soil.

Treatment soils	**Root/Shoot**	**Days**			
		10	**14**	**21**	**28**
The control	Root	0.014 ± 0.005	0	0	0
	Shoot	0.002 ± 0.001	0.003 ± 0.001	0.011 ± 0.004	0.016 ± 0.002
T_1	Root	0.015 ± 0.003	0	0	0
	Shoot	0.002 ± 0.0004	0.003 ± 0.001	0.010 ± 0.003	0.014 ± 0.001
T_2	Root	0.012 ± 0.002	0	0	0
	Shoot	0.001 ± 0.0007	0.002 ± 0.001	0.012 ± 0.003	0.016 ± 0.004
T_3	Root	0.016 ± 0.003	0	0	0
	Shoot	0.002 ± 0.001	0.005 ± 0.002	0.023 ± 0.006	0.031 ± 0.008

Accumulation factors are given in mg of OTC over mg of initial OTC measured in soil.