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Original article

Effects of different concentrations and application frequencies of oxytetracycline on soil enzyme activities and microbial community diversity

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ABSTRACT

Antibiotics in soil can interfere with the structure and function of the soil microbial community and represent a potential genetic pollution risk. The effects of different concentrations and application frequencies of oxytetracycline (OTC) to an agricultural soil on the activities of soil microorganisms and enzymes were investigated during incubation of 120 days. Single once-only high application treatments (1, 3.6, 10, and 30 mg OTC kg^{-1} soil) and one daily low application treatment (0.03 mg OTC kg^{-1} soil every day) were compared to simulate OTC application to the soil in sewage sludges or manures or from waste water irrigation. In the single addition treatments, microbial biomass carbon (C_{mic}) in the soil increased 2.17–3.29 times and 1.37–2.08 times after 7 and 42 d of incubation, respectively, but nitrification potential increased sharply to 3.01–10.9 times after 28 d and dehydrogenase activity was also significantly stimulated after 14 d compared to the zero OTC control and decreased sharply by 120 d. The daily OTC addition treatments promoted C_{mic} (up to 2.64 times) and increased the McIntosh index ($p < 0.05$) between 60 and 90 days as calculated using Biolog data and compared to the zero OTC control. A single high rate of OTC addition showed a generally more pronounced negative effect on soil microbial community metabolism (but not on functional diversity indices of the soil microbial community) than repeated small rates of addition because with equal amounts of added OTC (single 3.6 mg kg^{-1} and daily 0.03 mg kg^{-1} OTC) C_{mic}, nitrification potential and neutral phosphatase activity at 120 d were significantly lower in the single application treatments.

1. Introduction

Each year many tonnes of antibiotics are used in human and veterinary medicine worldwide [1]. Oxytetracycline (OTC) is a tetracycline (TC) antibiotic that has been widely used without strict controls for many years, especially in livestock, poultry, and aquaculture production and in human medicine, with about 30–90% excreted along with the faeces and urine [2,3]. The land application of livestock and poultry manures high in antibiotics and the irrigation of agricultural land with wastewaters high in antibiotics are major sources of contamination in soils, groundwater and sediments [4–6]. Soil contents of OTC in China have often exceeded the trigger value of 100 μg kg^{-1} over the last decade [7–9]. Unlike many other environmental pollutants, antibiotics can have a direct biological action on microbes so that they can interfere with the structure and function of the soil microbial community and induce antibiotic resistance genes (ARGs), bringing about a potential genetic pollution risk in different environmental matrices [10–12]. Microorganisms in the environment are more likely to exhibit resistance to antibiotics in the presence of heavy metals and the effects on soil microbial community function are much greater when multiple pollutants are present [13,14]. It has been reported that OTC can significantly reduce soil microbial biomass and

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alkaline phosphatase activity but increase fungal counts [3,15]. Although OTC is one of the most widely used antibiotics in China, investigations on its residual effects under field conditions have been little investigated at different contents and frequencies of application [16].

It has been suggested that soil microbial biomass and microbial processes such as dehydrogenase activity and nitrification activity can be used as environmentally relevant indicators of soil pollution, nutrient cycling and energy metabolism [16–18]. The Biolog test has often been used to detect pollution-induced soil community tolerance at sites where sewage sludge has been applied [19]. The present study is based on an agricultural soil historically contaminated with long-term wastewater irrigation. The effects of OTC at different application rates and frequencies simulating the application of livestock and poultry manures and wastewater irrigation have been investigated to ascertain the acute toxicity of OTC under high application rates and toxicity under low OTC contents. Effects of both single high rates and one daily low rate of OTC additions on soil microbial biomass carbon and nitrification potential, soil enzyme activity and effects of daily low addition on function of the microbial communities and diversity indices were clarified. It is important to understand the additional effects of OTC under these conditions to enable the safe recycling of plant nutrients in livestock manures.

2. Materials and methods

2.1. Reagents and test soil

OTC (97.5%) was obtained from Dr. Ehrenstorfer GmbH, Augsburg, Germany. All other reagents used were of analytical grade and purchased from the National Pharmaceutical Group Chemical Reagent Co. Ltd., Shanghai or Nanjing Chemical Reagent Co. Ltd.

The soil tested was collected from the arable layer (top 20 cm) of the soil profile of a vegetable field following long-term wastewater irrigation in a suburban district of Shenyang city, Liaoning province, northeast China. The soil is an alfisol according to the US Department of Agriculture classification system (USDA 2011), with 14.8% clay, 77.5% silt, 7.69% sand, 2.38% organic matter and 12.3 cmol (+) kg^{-1} soil cation exchange capacity (CEC). The soil pH is 6.77 and the available nitrogen, phosphorus, and potassium contents were 1.21, 1.68, and 17.5 g kg^{-1}, respectively [20]. The soil collected was air dried and passed through a 2-mm nylon sieve before use. The content of OTC was determined to be 37.3 ± 2.1 μg kg^{-1}. Before the addition of OTC, ultrapure water was used to adjust the soil water content to about 60% of the water holding capacity (WHC) before pre-incubation for 14 days at 25 ± 0.5 °C in the dark.

2.2. Soil microbial experiments

Sixteen portions of test soil, each 2.5 kg on oven dried basis, were collected after 14 days of pre-incubation and divided into four groups to give four replicates of each treatment. Stock aqueous solutions (5 mL) of OTC were sprayed to give the five high content treatments of 1, 3.6, 10, and 30 mg kg^{-1} soil (dry weight, DW), to mimic the addition of manures high in antibiotics. Replicates were sprayed with water to give the 0 OTC controls (control 1). Soils after adjustment and spiked following the method of Hund-Rinke et al. [21] were placed in plastic pots, covered with perforated PVC film and set up for incubation periods of 0, 3, 7, 14, 28, 42 and 120 days in the dark at 25 ± 0.5 °C. The day of OTC addition is designated 0 day. The soil moisture content was maintained at 60% of water holding capacity (WHC) during incubation.

Eight portions of pre-incubated test soil were prepared for two treatments (control 2 and the daily addition treatment) as described above. Aqueous OTC solution was sprayed to the daily addition 0.03 mg kg^{-1} treatment every day to mimic the addition of wastewater irrigation containing antibiotics and the soil moisture was adjusted to 60% of WHC during the incubation period as described above. Scarification of the soil was carried out daily to minimize the uneven distribution of the OTC applied. Samples were collected after 0, 10, 20, 60, 90 and 120 days for analysis of different indices.

2.3. Sample analysis

2.3.1. Soil pollutants background analysis

About 50 g of soil were sieved through a 60-mesh screen after freeze-drying in a Free Zone 2.5 L Freeze Dry System (Labconco Corp., Kansas City, MO) before the determination of OTC contents following the procedure of Cheng et al. [22]. The target antibiotics were determined using high performance liquid chromatography-tandem mass spectrometry (HPLC-MS/MS). An HPLC system (Shimadzu, Kyoto, Japan) consisting of a LC-20 AD binary pump, a DGU-20A on-line degasser, a SIL-20A auto sampler and a CTO-20A column oven was used for chromatographic separation. The operating conditions are shown in the Supporting Information (Table S1). The tandem mass spectrometric analyses were performed on an API 3200 triple quadrupole mass spectrometer (AB-Sciex, Framingham, MA) equipped with an electrospray ionization (ESI) source that operated in the positive ionization (PI) mode. Sample acquisition was performed in the multiple reaction monitoring (MRM) modes by recording two MRM per compound. The MRM parameters of 16 compounds and four internal standards are listed in the Supporting Information (Table S2) [22].

TC-D6 was used as the internal standards of TCs for quantification and quality control. Calibration standards (0, 1.0, 5.0, 10.0, 20.0, 30.0, 50.0, 70.0, 100.0, 150.0, 200.0 and 300.0 μg L^{-1}) were made by serial dilution of the stock solution. Isotope-labeled internal standards were added to the calibration standards at a concentration of 100.0 μg L^{-1}. Calibration curves were drawn using ten points within the range 5.0–300.0 μg L^{-1} using the internal standard method. Recovery experiments were performed by spiking soil samples with the standard solutions to give contents of 100 and 300 μg kg^{-1} in triplicate. Limit of detection and limit of quantification were 0.44 and 1.48 μg kg^{-1}, determined as the lowest contents resulting in signal/noise ratios of (S/N) ≥3 and ≥ 10. The recovery of OTC at 0.3 μg kg^{-1} was 95.8 ± 1.2 μg kg^{-1} [22]. The residual contents of OTC were determined and are listed in Table 1.

2.3.2. Soil microbial biomass carbon

Soil microbial biomass carbon was determined by the chloroform fumigation extraction method [23] using two 10-g subsamples (DW) of soil from each treatment weighed into Petri dishes. The filtered 0.5 mol L^{-1} K_2SO_4 solutions were analyzed using an organic carbon analyzer (Multi N/C3000, Analytik Jena AG, Jena, Germany).

Table 1
Concentrations of OTC in different treatments tested at the end of the incubation period.

Treatment (mg kg^{-1})	Final concentration (mg kg^{-1})
0	0.028 ± 0.003
1	0.133 ± 0.021
3.6	0.372 ± 0.019
10	0.628 ± 0.028
30	1.373 ± 0.031
Daily 0.03	0.251 ± 0.017

2.3.3. Soil nitrification potential

Soil nitrification potential was determined following the method of Corbel et al. [24]. An aliquot of 15 g of fresh soil was placed in Erlenmeyer flasks with 60 mL of $(NH_4)_2SO_4$ solution (pH 7.2) supplemented with KH_2PO_4 (0.2 M) and K_2HPO_4 (0.2 M). After 3 h of incubation the concentration of nitrate (NO_3^-–N) was measured using a Skalar SanPlus segmented flow analyzer (SA21075320, Skalar Analytical B.V., Breda, The Netherlands).

2.3.4. Functional diversity of soil microbial community

Biolog tests were carried out according to the method of Garland and Mills [25] using 5-g aliquots of fresh soil from each pot sieved through a 250 μm screen and added to 100 mL of distilled water in a 250 mL conical flask and shaken at 200 rpm for 20 min for the Biolog Eco test to study the substrate utilization patterns of the soil microbial communities. Diluted (10^{-2}) inocula (125 μL) were injected into Biolog microplates for incubation at 25 °C in the dark. Colour development was measured at 0, 24, 48, 72, 96, 120, 144 and 168 h as difference in absorbance at 590 nm using a μQuant microplate spectrophotometer (BioTek, Winooski, VT) and the data were collected using Gen5 v1.06 software.

Mean values (n = 3) of average well colour development (AWCD), a measure of total microbial activity for different treatments over time, were compared at the 5% level of significance to evaluate effects [26]. Shannon index and McIntosh index were calculated as follows.

$$AWCD = \sum_{i=1}^{n}(C_i - R)/n$$

C_i – the colour absorbance value of reaction well; R – the colour absorbance value of control well; n – the carbon source number.

McIntosh index, Shannon index and Simpson index are used to describe soil microbial diversity,

$$\text{McIntosh index}: \quad U = \sqrt{\left(\sum_{i=1}^{n} p_i^2\right)}$$

$$\text{Shannon diversity index}: \quad H = -\sum_{i}^{n} P_i \times lnP$$

$$\text{Simpson diversity index}: \quad \lambda = \sum S_i^2$$

P_i – each reaction well subtracting the absorbance value of the control well and then dividing by the summed colour absorbance value of 31 wells; S_i – the ratio of the activity on each substrate (OD_i) divided by the sum of activities on all substrates ($\sum OD_i$) [27].

2.3.5. Pollution-induced community tolerance (PICT)

Microbes were extracted from 5-g aliquots of fresh soil of control 1 sieved through a 250 μm screen and added to 100 mL of distilled water in a 250 mL conical flask and shaken at 200 rpm for 20 min before determination of tolerance to OTC of the extracted soil microbial communities. The supernatants containing microbes were diluted with sterilized distilled water solutions of OTC to a gradient of concentrations of 0, 1, 5, 10, 50 and 100 mg L^{-1} and inoculated into Biolog microplates (125 μL per well). The plates were then incubated at 25 °C in the dark and read every 12 h until 168 h for absorbance at 590 nm using a μQuant microplate spectrophotometer (BioTek, Winooski, VT) and a positive well was defined as having 0.5 absorbance units above the blank [28]. Subsequently, for each well the area under the absorbance curve was calculated [29].

2.3.6. Soil enzyme activities

Soil dehydrogenase, neutral phosphatase, and urease activities were determined according to Li et al. [30].

Dehydrogenase was determined by measuring the rate of reduction of 2,3,5-triphenyltetrazolium chloride (TTC) as a substrate. A 2-mL aliquot of TTC-Tris buffer solution (1%) and 2 mL of 1% glucose solution were added to 4 g of soil in 50 mL glass flasks. TTC solution was withheld to give blanks. After incubation at 37 °C for 24 h, reaction product was extracted with methanol to measure absorbance at 485 nm.

Soil neutral phosphatase enzyme activity was determined by adding a 10 mL di-sodium phenyl phosphate solution used as a substrate to 10 g of soil sample and incubating in a pH 7.0 citric-phosphate buffer solution at 37 °C for 24 h. Then, a 5 mL buffer solution was drawn and 1 mL of the solution was filtered into a 100 mL volumetric flask for dilution with deionized water to 25 mL. A 1 mL solution of 2,6-dibromoquinone-chlorimide was then added to the solution for 30 min at room temperature before incubation. Finally, the solution was diluted with deionized water to 100 mL, and the absorbance of the phenol released was measured at 578 nm.

Soil urease activity was determined by mixing soil samples of 5.0 g in 50 mL volumetric flasks with 1 mL toluene for 15 min before 10 mL of 10% urea and 20 mL of citrate buffer (pH 6.7) were added. The samples were placed in an incubator at 37 °C for 24 h, diluted with distilled water and oscillated thoroughly. Immediately after filtration, 3 mL filtrate was transferred into a 50 mL flask to which were added 10 mL distilled water, 4 mL sodium phenate (1.35 M) and 3 mL sodium hypochlorite (active chlorine 0.9%). After 20 min each sample was diluted to volume and the concentration of NH_4^+ ions produced from urea hydrolysis was determined as a blue-coloured complex to represent urease activity.

2.4. Data analysis

All the data were processed using Microsoft Excel 2013 and the SPSS v.18.0 software package. Pairs of mean values were compared for significant differences using the least significant difference (LSD) method at the 5% level.

3. Results

3.1. Effects of single high OTC contents on soil microbial biomass

3.1.1. Effects on carbon and nitrification potential of soil microbial biomass

The effects of high OTC contents on soil microbial biomass carbon (C_{mic}) and nitrification potential can be seen in Fig. 1. After three days of incubation, C_{mic} of control 1 treatment changed significantly with increasing incubation time to over two times the value at day 0, and increased to reach the first maximum value on the 7th day, which is about 2.17–3.29 times the value of day 0. However, under high contents of OTC, on the 28th day C_{mic} returned to a level similar to control 1, although slight promotion continued after 42 d. On the 28th day soil nitrification potential in treatments of 30 mg OTC kg^{-1} soil was greatly stimulated compared to that without OTC but at other contents of OTC after 14 d, soil nitrification potential values were usually lower than that of control 1.

3.1.2. Effects on soil enzyme activities

Effects of high levels of OTC at different rates of addition and incubation time on the activities of soil dehydrogenase, neutral phosphatase and urease are shown in Fig. 2. In this study no marked effect on soil neutral phosphatase activity was observed

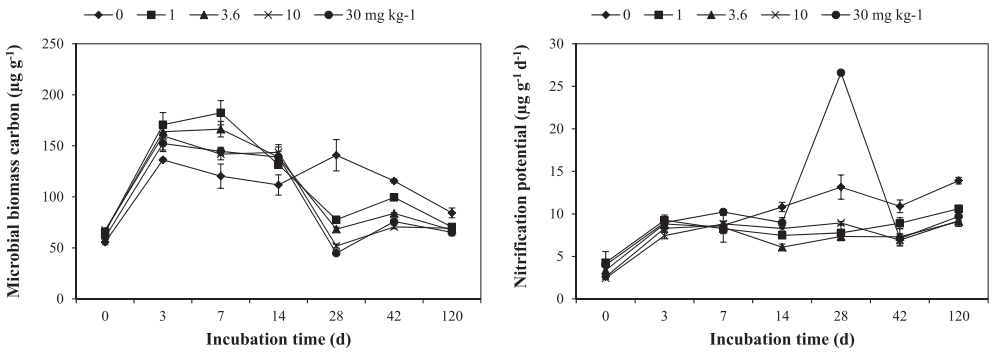

Fig. 1. Effects of high OTC concentrations on soil microbial biomass carbon and nitrification potential at 0, 3, 7, 14, 28, 42 and 120 d. 0 mg kg^{-1} (control 1), no OTC addition; 1, 3.6 10 and 30 mg kg^{-1} denote OTC addition of 1, 3.6 10 and 30 mg kg^{-1} only once on the first day of the experiment. Each value is the mean of four replicates ± standard error of the mean value (SEM).

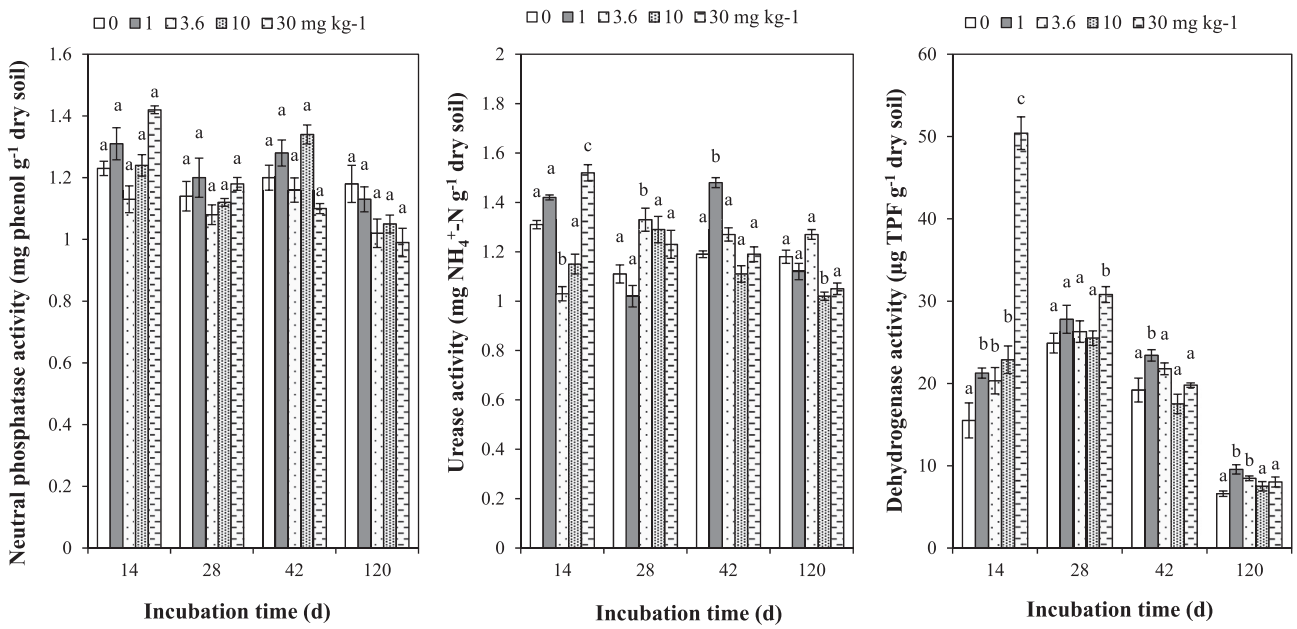

Fig. 2. Effects of high OTC concentrations on soil enzyme activities at 14, 28, 42 and 120 d. Each value is the mean of four replicates ±SEM. Different letters in columns denote significant difference at $p < 0.05$ level compared with control 1. For other abbreviations, see Fig. 1.

over the 120-day incubation period, and even high contents of OTC (up to 30 mg kg^{-1}) in soil had no discernible effect on soil neutral phosphatase activity which remained in the different treatments between 0.99 and 1.42 mg phenol g^{-1} soil (DW). However, significant promotion of soil dehydrogenase activity occurred compared with control 1. As shown in Fig. 2, prior to incubation (before 14 d) soil dehydrogenase activity was stimulated with increasing OTC content, rising to over 325% of the control in the 30 mg kg^{-1} treatment, 50.41 μg TPF g^{-1} soil (DW). However, on days 42 and 120 of incubation the soil dehydrogenase activities in treatments with high OTC contents were generally restricted to the same level as control 1, especially at OTC contents > 10 mg kg^{-1}. After long incubation periods, dehydrogenase activity values in different treatments declined to less than half the values of the 14th day. Dehydrogenase activity on the 120 th day of single additions of 3.6 mg kg^{-1} was higher than the daily 0.03 mg kg^{-1} OTC addition treatment, at 8.47 ± 0.29 and 6.8 ± 1.1 μg TPF g^{-1} dry soil, respectively. However, there were no significant differences in the other two enzyme values between the two treatments.

3.2. Effects of daily low OTC contents on soil microbial biomass

3.2.1. Effects on AWCD

Changes in AWCD values under low OTC contents are shown in Fig. 3. The AWCD values on each specific day of sample collection show marked elevation in both treatments with increasing time but the AWCD values of control 2 remained at low levels (between 0.28 and 0.61) until the end of the incubation period. At the later stages of the incubation experiment (60, 90 and 120 d) the AWCD values increased to 50.5, 46.2 and 28.0% of control 2 and large advances were observed in substrate utilization on the Biolog plates.

3.2.2. Effects on index of diversity of soil microbial community function

Table 2 shows the observed changes in functional diversity indices of microbial communities in the toxicity test with daily addition of OTC. During incubation up to 120 days the Simpson index of control 2 was low but stable between 0.77 and 0.86, and no significant difference in Simpson index between the two treatments was observed. However, between 60 and 90 days of

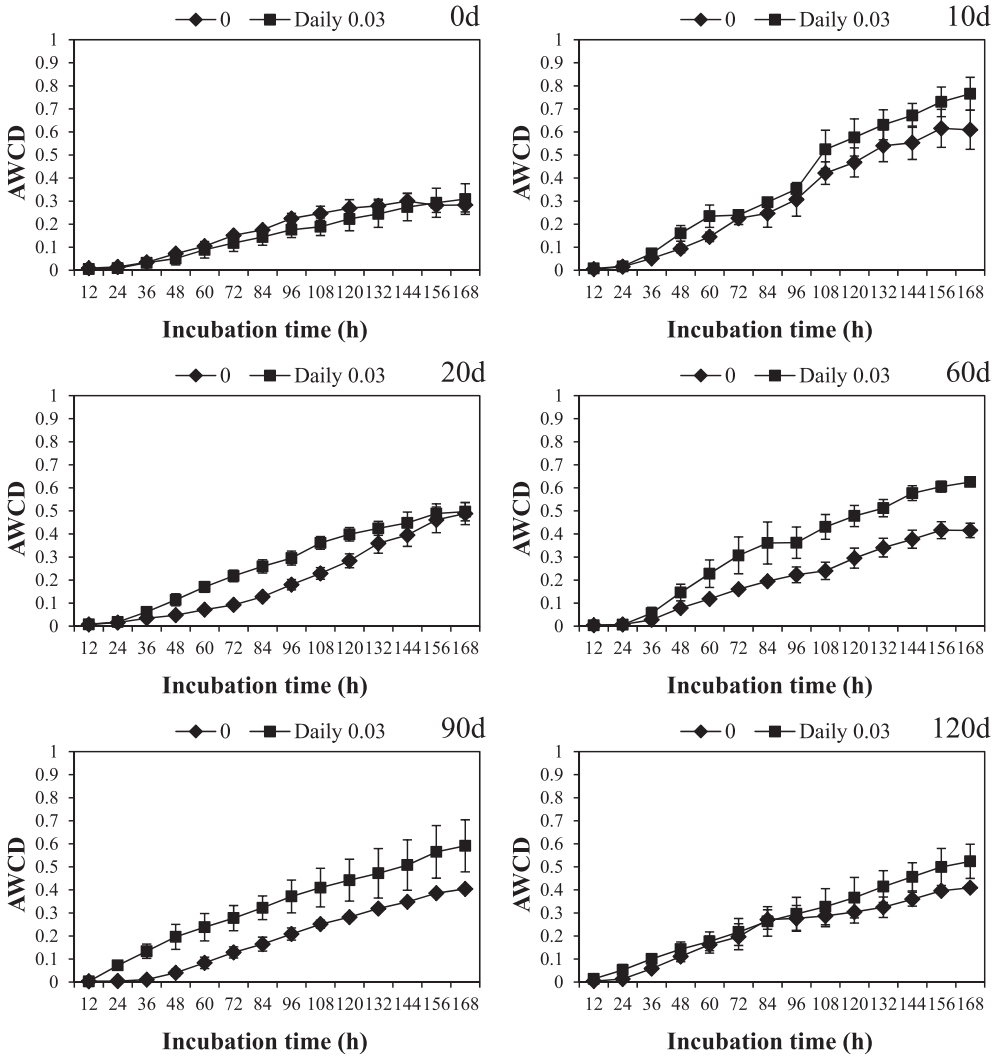

Fig. 3. Changes in AWCD at low OTC concentrations at 0, 10, 20, 60, 90 and 120 d. 0 mg kg^{-1} treatment (control 2), soil without OTC addition; and daily 0.03 mg kg^{-1} treatment, addition of 0.03 mg OTC kg^{-1} soil per day. Each value is the mean of four replicates ± SEM.

Table 2
Changes in functional diversity indices of microbial communities in the toxicity test.

Incubation time (d)	Treatment (mg kg^{-1})	McIntosh index	Shannon index	Simpson index
0	0	3.11 ± 0.08 a	1.95 ± 0.03 a	0.80 ± 0.01 a
	Daily 0.03	2.62 ± 0.18 a	1.90 ± 0.04 a	0.75 ± 0.02 a
10	0	4.50 ± 0.07 b	2.02 ± 0.08 a	0.84 ± 0.02 a
	Daily 0.03	3.32 ± 0.28 a	2.15 ± 0.09 a	0.85 ± 0.02 a
20	0	2.10 ± 0.13 c	2.19 ± 0.02 a	0.86 ± 0.01 a
	Daily 0.03	3.15 ± 0.26 a	2.07 ± 0.06 a	0.83 ± 0.01 a
60	0	2.36 ± 0.09 c	1.84 ± 0.01 b	0.77 ± 0.01 a
	Daily 0.03	4.66 ± 0.37 b	2.14 ± 0.03 a	0.85 ± 0.01 a
90	0	2.39 ± 0.01 c	1.71 ± 0.02 b	0.78 ± 0.01 a
	Daily 0.03	4.88 ± 0.23 b	2.17 ± 0.07 a	0.83 ± 0.02 a
120	0	3.06 ± 0.29 a	1.99 ± 0.03 a	0.83 ± 0.01 a
	Daily 0.03	3.49 ± 0.20 a	2.05 ± 0.03 a	0.79 ± 0.02 a
	Single 3.6	3.27 ± 0.11 a	1.91 ± 0.04 a	0.85 ± 0.02 a

Each value is the mean of four replicates ± SEM. Different letters denote significant difference at $p < 0.05$ level compared with samples collected at day 0.

incubation there was a substantial rise in Shannon index in control 2 although it returned to the same level as the control treatment at the end of the incubation period. Significant promotion of the McIntosh index in the daily addition treatment at 60 and 90 d and significant variation in control 2 between 10 and 90 d were found. The additional determination of diversity indices in the single 3.6 mg kg^{-1} treatment have been added and compared with that of daily additions of 0.03 mg kg^{-1}, in which no significant difference occurred in the three indices between the two treatments.

3.2.3. Effects on soil microbial biomass carbon and nitrification potential

The effects of low OTC contents on C_{mic} and nitrification potential shown in Fig. 4 indicate different regulation from that of treatments with high addition rates of OTC. C_{mic} increased under daily addition of 0.03 mg kg^{-1}, especially at 60 d, to a maximum of 134 μg g^{-1} (142% of control 2) and the increasing trend subsequently slowed down but remained significantly higher than the control. On day 60 of incubation the most significant inhibition was observed in the low OTC content treatments at 40.4% of control 2. As the incubation time increased, the inhibitory effect of low OTC contents on soil nitrification potential declined but was still significantly lower than the control. Between single additions of 3.6 mg kg^{-1} and the daily 0.03 mg kg^{-1} OTC addition treatment, soil microbial biomass carbon was higher for about twice the value in the latter but at a similar level for nitrification potential values at 120 d, indicating that the addition frequency and content of OTC had greater effects on the metabolism of soil carbon.

3.2.4. Effects on soil enzyme activities

Effects of low OTC contents on the activities of soil dehydrogenase, neutral phosphatase and urease are presented in Fig. 5. As in the high OTC content treatments, soil neutral phosphatase activity was unaffected in the daily addition treatment (0.03 mg kg^{-1} OTC) but urease activity was unaffected and soil dehydrogenase activity declined, in contrast to the trends under the high OTC contents. During the incubation period of 120 days the neutral phosphatase and urease activities were between 1.15 and 1.33 mg g^{-1} and 1.39 and 1.65 mg g^{-1}, respectively. On the 60th day the soil dehydrogenase activity was markedly reduced in the daily addition treatment (0.03 mg kg^{-1} OTC) at about 45.2% of the control (6.59 μg g^{-1}). However, at the end of the incubation (120 d) soil dehydrogenase activity returned to the same level as control 2.

4. Discussion

4.1. Effects of OTC on soil microbial biomass carbon and nitrification potential

C_{mic} can act more rapidly than soil total carbon as an indicator of response to pollution stress and this is why C_{mic} is often used to monitor soil quality under different pollution stresses [31]. In the present study high contents of OTC affected the soil microbial biomass greatly. On the third day the C_{mic} in different treatments changed significantly with increasing incubation time to over two times the value at 0 d and up to 2.17–3.29 times the 0 d value on the 7th day. Under low OTC contents, C_{mic} activity increased in the daily 0.03 mg kg^{-1} treatment but slowed down after 60 days, consistent with the OTC effects on soil microbial activity (AWCD). It has been demonstrated that dynamic moisture changes can accelerate the susceptibility of the soil microbial community to antibiotics [32]. Promotion of C_{mic} with increasing OTC content is consistent with a study on TC by Cui et al. [33] because addition of TC clearly activated the soil metabolic quotient. Between single additions of 3.6 mg kg^{-1} and the daily 0.03 mg kg^{-1} OTC addition treatment, the much higher soil microbial biomass carbon values in the daily addition treatment also indicate activated soil basal metabolism. Under the pressure of high contents of OTC, soil microbes consume more energy to maintain growth and metabolism, leading to a reduction in microbial numbers and then in C_{mic} with the extension of the incubation time (after 28 d in this experiment) which is consistent with the result of Thiele-Bruhn and Beck [3] on OTC and this may become a negative factor affecting plant growth. The slight recovery of Cmic after 42 d of incubation indicates that inhibitory effects of OTC on Cmic vanished at later stages of the experiment. It is possible that when OTC first entered the soil environment it disturbed the balance of the soil microbial ecological system but with increasing time more tolerant micro-organisms may have become dominant and promoted the recovery of the microbial ecological system. The decline over time in the bioavailable fraction of OTC is another possible explanation from the results of Table 1 because 89.7–99.4% could not be recovered. This may be due to degradation or formation of non-extractable residues [34]. PICT has been determined to justify the level of basal tolerance to OTC of microorganisms in the test soil, which is EC_{10}: 0.215 ± 0.011–0.490 ± 0.19 mg kg^{-1}.

The increase in nitrification potential at single high OTC contents at 28 d reached a maximum especially in treatments with 30 mg OTC kg^{-1} soil. The inhibitory effects of different OTC contents on soil nitrification potential were similar at different time points with the daily low treatment. However, the stimulation at 28 d at 30 mg kg^{-1} indicates that OTC addition stimulated soil microbial activities at a certain content [33]. Except for the special value on 28 d at 30 mg kg^{-1}, inhibitory effects on soil nitrification potential occurred at all other OTC addition treatments conducted, and this is in agreement with findings from other studies on effects of pharmaceutical antibiotics on nitrification potential [35,36]. One possible explanation might be the inhibitory effects of OTC on nitrobacteria which are typical Gram-negative bacteria [37].

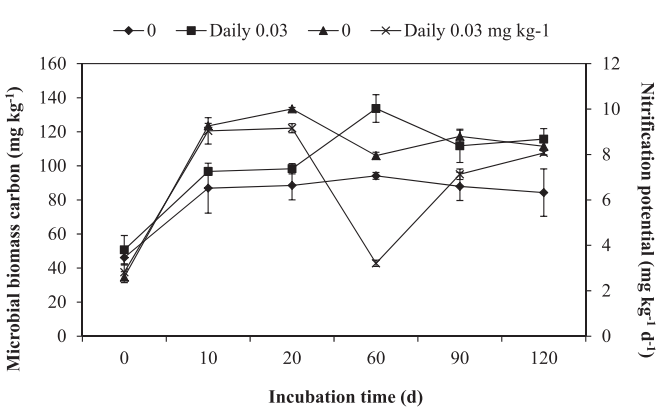

Fig. 4. Effects of low OTC concentrations (0 mg kg^{-1} treatment/control 2, soil without OTC addition; and daily 0.03 mg kg^{-1} treatment, addition of 0.03 mg OTC kg^{-1} soil per day) on soil microbial biomass carbon and nitrification potential at 0, 10, 20, 60, 90 and 120 d. Each value is the mean of four replicates ± SEM.

4.2. Effects of OTC on soil enzyme activities

Different contents of OTC showed quite regular effects on soil dehydrogenase activity, i.e. more promotion at higher contents at early stages of incubation and less promotion as the incubation proceeded, due to several factors such as the OTC residual contents in soil, residual duration and microbial resistance.

In the present study, OTC up to 30 mg kg^{-1} in soil had no discernible effect on the activity of soil neutral phosphatase, indicating that phosphorus cycling was not greatly affected by the addition of high contents of OTC. It has been reported that OTC at 10 mg kg^{-1} can lower soil alkaline phosphatase activity by 41.3% [38] and at about 30 mg kg^{-1} the decrease can be 64.3–80.8%. However, no evident effects of OTC addition on soil neutral phosphatase activity were observed in our study, and this agrees with the results of Wei et al. [39] who carried out a similar investigation on tetracycline.

Metabolic characteristics such as N mineralization potential and enzyme activities are known to be sensitive to management [40–43] and can also provide information on the status and activity

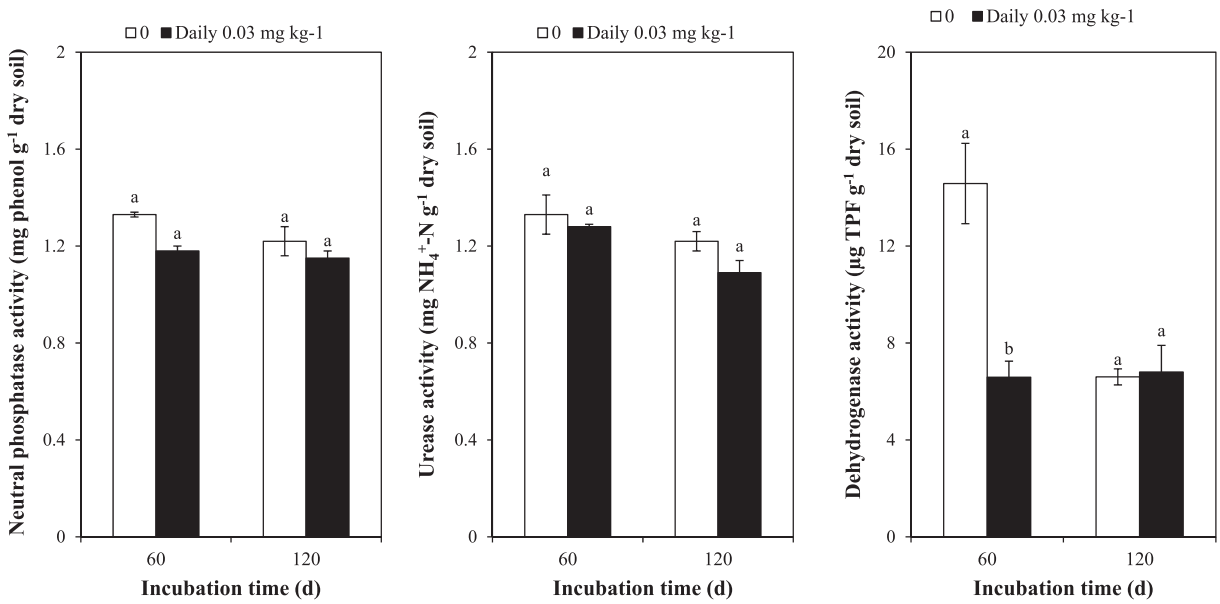

Fig. 5. Effects of low OTC concentrations (0 mg kg^{-1} treatment/control 2, soil without OTC addition; and daily 0.03 mg kg^{-1} treatment, addition of 0.03 mg OTC kg^{-1} soil per day) on soil enzyme activities at 60 d and 120 d. Each value is the mean of four replicates ±SEM. Different letters in columns denote significant difference at $p < 0.05$ level compared with control 2.

of the microbial community as well as the resilience of the community to stress. Agricultural fields may be considered to be ecological systems in which living organisms and their physical and biological environments constitute a single interacting unit which occupies an arbitrarily defined volume of the biosphere at the earth-atmosphere interface [44]. The uncontrolled application of fertilizers derived from livestock and poultry manures and irrigation with wastewaters rich in antibiotics will disturb the transformation of materials and energy in agricultural soils.

4.3. Effects of OTC on soil microbial activity and functional diversity

The changes in absorbance in different wells of the Biolog plates reflect the different carbon source metabolism and utilization processes of soil microbes and the variation in AWCD values can be used as an effective indicator to evaluate the overall level of soil microbial activity. According to Garland and Mills [24], the AWCD values are definitely related to the numbers and types of the specific microorganisms that can live with a single carbon source in the soil. Effects of OTC alone on the functional diversity of soil microbial community with or without vegetation were studied in a seven-week greenhouse pot experiment using Biolog Eco-plates by Liu et al. [44]. The results indicate that together with an increase in OTC alone, AWCD values increased with a peak at 200 mg kg^{-1} OTC and the utilization of sugar and its derivatives was enhanced [45]. However, it must be emphasized that the Biolog approach is limited to cultivable organisms and especially r-strategists that make up a very small minority of the soil microbial community.

The stable appearance of the Simpson index in control 2 throughout the incubation indicates the relative stability of the microbial community. Low contents of OTC, also suspected to be an environmental hormone, have a stimulatory effect on the growth and reproduction of different soil microorganisms. OTC may also have inhibited soil bacteria but not actinomycetes or fungi which may have increased and contributed to the final increase in the number and function of microbial diversity. However, after 60 days the substantial rise in the McIntosh index in the daily addition treatments suggests that the exogenous application of OTC is conducive to the augmentation of microbial community function. Throughout the incubation experiment there was no significant difference in Simpson index between the two treatments, indicating no significant impact on the common dominant species in the soil as a result OTC addition. The significant promotion of the Shannon index in control 2 between 60 and 90 days indicates an increasing effect in soil microbial community richness but the promotion effect was not persistent judging from the return to the control level at 120 d. The different contents and frequencies of OTC addition did not result in clear effects on soil microbial community functional diversity indices.

At present there is a particular interest in the relationship between the number of species present in the system and their soil function with respect to the conservation of biodiversity and its role in maintaining a functional biosphere [46]. Focusing on the current status of soil pollution, soil contamination with antibiotics requires further research on pollution status, toxicity effects and possible mechanisms for implementation of appropriate control strategies and methods in the future.

It is important to note that even a soil OTC content of 37.3 μg kg^{-1} (the background content of the test soil) may cause long-term contamination effects such as adaptation of the microbial community. However, the data in Table 1 suggest that the initial spiked content was substantially higher than the content of that remaining until 120 d. Also, the method used detected almost 20 antibiotic fractions among which only OTC reached the limit of detection, indicating that the unamended soil subsamples represented suitable controls.

5. Conclusions

The effects of OTC on soil microbes are bound up with numerous factors such as basic soil properties and the types and activities of soil microorganisms, OTC residual contents, and also time. High OTC contents generally increased C_{mic}, nitrification potential and soil dehydrogenase activity. However, in the daily addition (0.03 mg kg^{-1}) treatment C_{mic} and McIntosh index were elevated in the polluted soil studied, indicating a slight recovery in the soil

microbial community and function. C_{mic} and dehydrogenase activity can be recommended for the characterization and sensitive detection of microbial toxicity of OTC in soil. An added content of 1 mg kg^{-1} OTC is also proposed as the threshold content of OTC contamination in combined contaminated soil. More significant negative effects were observed in single high rates of OTC addition rather than daily low treatment especially on soil microbial community metabolism. Conservation of agricultural soils calls for further research to improve management of animal manures and wastewaters for agricultural recycling of plant nutrients with minimum soil pollution in the future because of the potential risks of both antibiotics and their resistance genes.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejsobi.2016.07.004.

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