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RESEARCH ARTICLE

Toxicity of OTC to *Ipomoea aquatica* Forsk. and to microorganisms in a long-term sewage-irrigated farmland soil

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Abstract Water spinach (*Ipomoea aquatic* Forsk.) was selected to investigate the effects of oxytetracycline (OTC) on the toxicity of soil contaminated by long-term sewage irrigation. After acute toxicity test in petri dish at nine different OTC-spiked levels for 48 h, the germination rate was found to be generally inhibited in all treatments treated with OTC but the root elongation and activities of several antioxidant enzymes, superoxide dismutase (SOD), peroxidase (POD) and catalase (CAT) were either forward or backward stimulated to varying extent. During a 60-day sub-chronic toxicity test by means of a pot experiment, activities of SOD, POD and CAT in both the leaf and root tissue at 25 mg OTC per kg soil (dry weight) and in root tissue at 1 mg OTC per kg soil (dry weight) were significantly different than those in other treatments, which also indicated the higher sensitivity of the root. The foliar photosynthetic rate, stomatal conductance and transpiration rate were all gradually inhibited in spite of elevated water use efficiency under the pressure of the different OTC concentrations, which were highly significant different at 10 mg OTC per kg soil (dry weight). Indices of soil microbial diversity at 4 mg OTC kg^{-1} soil were significantly different from those of the control, indicating the potential adverse effects of OTC to soil microorganisms. The results suggest that the introduction of OTC could damage both plants and soil microorganisms, and during sub-chronic incubation, the sensitivity of different indices generally followed the order of root tissue antioxidant enzyme activities, soil microbial diversity indices, leaf photosynthesis-related index and leaf tissue enzyme antioxidant activities. In addition, the application of livestock and poultry manure containing pollutants like OTC in farmland soil, especially if the soil has been contaminated before, should be taken more seriously in the context of the current pursuit of increased agricultural production.

Keywords Soil contamination · Oxytetracycline · Phytotoxicity · Biolog · Transmission electron microscopy

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Introduction

Antibiotics are widely applied in the therapy of microbial infectious diseases of humans, livestock and poultry or as growth promoters in amounts far exceeding the requisite (Boxall et al. 2004; Zhao et al. 2010). However, antibiotics are so poorly absorbed in the digestive tracts that about 50 to 80 % of the dose is directly excreted through faeces and urine in the form of parent compounds (Sarmah et al. 2006). As a consequence, antibiotic compounds are indirectly released into the environment with the application of manures and sewage sludge as fertilizers or by irrigation with recycled wastewater containing frequently detected antibiotics, usually accompanied by varying levels of organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) or heavy metals

(Wang et al. 2014; Subramanian et al. 2015) due to the development of an agrarian economy (Martínez-Carballo et al. 2007; Radjenović et al. 2009; Leung et al. 2015; Thuy and Nguyen 2013).

Oxytetracycline (OTC) is representative of the tetracycline antibiotics and is one of the most extensively employed in animal nutrition, which occurs at high residual concentrations in agricultural soils from 100 to 1700 μg kg^{-1} soil (Kay et al. 2004). The application of manure containing OTC has resulted in up to 2683- and 5172-μg kg^{-1} OTC concentrations in amended soils of Tianjin city and Zhejiang province, respectively (Hu et al. 2010; Li et al. 2011). Tetracycline antibiotics are the most frequently found in agricultural soils throughout the world, and the residual compounds result in a potential threat to both plants and soil organisms even at concentrations that are commonly detected in agricultural soils (Aga et al. 2005; Thiele-Bruhn and Beck 2005; Sarmah et al. 2006; Karcı and Balcıoğlu 2009; Goetsch et al. 2012). A mass of investigation on the occurrence of antibiotic resistance genes (ARGs) was derived from the selection by antibiotics in recent years, indicating the contamination of natural environments or their antagonistic effects to soil microorganisms and a latent environmental threat besides the promoted genetic toxicity to plants in the soil (Xie et al. 2011; Gao et al. 2013; Zhang et al. 2015).

Phytotoxicity of antibiotics is generally assayed using seed germination and plant growth tests, investigating the effects of different soil types, plant species and pharmaceuticals (Liu et al. 2009; Migliore et al. 2010; Farzaneh et al. 2013). OTC can significantly affect soil microbial activity and high concentrations can produce visual symptoms in plants including light green or yellow colours in the leaves of alfalfa (Boleas et al. 2005; Kong et al. 2007). Application of OTC has been shown to activate existing soil pollutants such as heavy metals in contaminated soils in previous studies in our laboratory, indicating an elevated environmental risk in soils with multiple contaminants (Chen et al. 2014). There may also be effects resulting from long-term exposure to antibiotics and their metabolites on offspring or maturation period of organisms, especially from the usually ignored effects of low toxicity and low concentration, so chronic or sub-chronic toxicity effects on plants and soil microorganisms are issues of concern (Gothwal and Shashidhar 2015; Li et al. 2015).

The objectives of the present study were to elucidate the toxicity of OTC to water spinach and soil microorganisms by means of germination test and whole grow stage incubation test; and to inquire into sensitive parameters and critical concentrations of OTC toxicity effects in soils under the condition of different added concentrations and frequencies, imitating the application of sewage water and sludge in farmland production and providing suggestions for their further use.

Materials and methods

Chemicals and instruments

OTC (97.5 %) was obtained from Dr. Ehrenstorfer GmbH, Germany. Nitro blue tetrazolium (NBT), dipotassium phosphate (KH_2PO_4), disodium hydrogen phosphate (Na_2HPO_4), potassium chloride (KCl), potassium hydroxide (KOH), hydrogen peroxide (H_2O_2), o-methoxyphenol, riboflavin, ethylenediaminetetraacetic acid disodium salt (EDTA-Na_2), sodium hydroxide (NaOH), glacial acetic acid (CH_3COOH), sodium chloride (NaCl), ethyl alcohol and acetone were all analytical reagents purchased from the National Pharmaceutical Group Chemical Reagent Co. Ltd., Shanghai or Nanjing Chemical Reagent Co. Ltd.

Assay kits A045-3 for total protein content assay, A001-1 for SOD assay, A084-1 for POD assay and A004 for CAT were purchased from Nanjing Jiancheng Bioengineering Institute, China. Biolog Eco plates™ were purchased from Biolog Company, Hayward, CA. An LI-6400XT Portable Photosynthesis System was used for the determination of photosynthetic rate, transpiration rate and other variables based on the leaf area of water spinach. A Hitachi-7650 transmission electron microscope (TEM) was used for the observation of leaf ultra-structure of water spinach in the College of Life Sciences at Nanjing Agricultural University.

Soil and plant seeds

The soil tested was collected from the top 20 cm of the soil profile layer in an arable area contaminated by wastewater irrigation at Zhangshi wastewater irrigation area, Shenyang city, Liaoning province, northeast China. The history of industrial wastewater irrigation has been over 40 years with a 10-year break, which resulted in the relative high heavy metal contamination background. The soil was an Alfisol according to the US Department of Agriculture classification system (USDA 2011), with 15.6 % clay, 76.4 % silt, 7.98 % sand and 2.92 % organic matter. The soil pH was 6.66, and the available nitrogen, phosphorus and potassium concentrations were 1.29, 2.26 and 16.9 g kg^{-1}, respectively (Chinese Society of Soil Agricultural Chemical Professional Committee 1983). The soil was air dried, passed through a 2-mm nylon sieve before use, and the background concentrations of Cu, Zn, Pb, Cd and ∑PAHs were determined to be 46.0 ± 1.5, 148 ± 2, 48.5 ± 4.7, 2.73 ± 0.24 and 2.31 ± 0.31 mg kg^{-1}, respectively.

Water spinach (*Ipomoea aquatica* Fork.) was selected because of its tolerance and resistance to both inorganic and organic pollutants (Bhaduri and Fulekar 2012; Xin et al. 2013). The seeds were bought from Jiangsu Provincial Academy of Agricultural Sciences (JAAS).

Seed germination tests

Batches of soil (200 g × 3) were adjusted to concentrations of OTC of 0, 0.01, 0.05, 0.1, 0.2, 0.5, 1, 5 and 10 mg kg^{-1} soil by spraying with 5-mL stock solutions in acetone in 15-cm-diameter glass Petri dishes. All the seeds were surface sterilized by immersion in 10 % sodium hypochlorite solution for 10 min (USEPA 1996), rinsed three times with distilled water, soaked in distilled water for 2 h and finally, sown in the prepared Petri dishes. Three replicates of each treatment were set up, and 100 pre-treated seeds of uniform size were sown with equal spacing in each dish. The dishes were placed in the dark in a growth chamber at 25 ± 1 °C for 48 h before the germination test was halted. The germination status of each treatment was checked, seedling root length was measured and the biomass in each dish was determined by fresh weight (FW). Root length was defined as the length from the root tip to root radicle. The seeds were considered to have germinated when the root length was over 5 mm (Wang et al. 2001). Enzyme activities and important indicators related to plant defence mechanisms comprising total protein content, and the activities of peroxidase (POD), superoxide dismutase (SOD) and catalase (CAT) were determined on fresh samples immediately after the germination test or stored at −20 °C for less than 3 days before use.

Plant whole growth stage toxicity tests

The sub-chronic toxicity of OTC to plant growth was assayed using the following method. Pots of soil (1.5 kg × 3) were adjusted to concentrations of OTC of 0, 1, 4, 10 and 25 mg kg^{-1}soil by spraying with 50-mL stock solutions in acetone. The same volumes of pure acetone without OTC were also sprayed onto the control soil. After evaporation of the acetone, the thoroughly mixed soil was adjusted to 60 % of water holding capacity before use. There were three replicates of each treatment, and three germinated seedlings of uniform height were transplanted with equal spacing in each pot and cultivated under the same chamber conditions as used for the germination tests. After 60 days, the photosynthetic rate, transpiration rate, stomatal conductance and intercellular CO_2 concentration of ten randomly chosen leaves were measured and recorded. Leaf samples of both the control and the 25-mg kg^{-1} OTC group were also collected for the observation of ultrastructure. The shoots and roots were harvested for the determination of the parameters biomass, total protein content and activities of SOD, POD and CAT.

Determination of physiological and biochemical indices

Analysis of all the indices was performed in triplicate by picking whole seedlings from each Petri dish from the germination tests and the shoots and roots from each pot of the plant growth tests. After cutting into pieces and mixing thoroughly in an ice-cold bath, fresh plant samples of about 1.0 g were homogenised by grinding in ice-cold 50 mmol L^{-1} potassium phosphate buffer (PBS with pH 7.8) and centrifuged at 6000 rev min^{-1} at 4 °C for 20 min. The supernatant was used for activity analysis of antioxidant enzymes. The protocols of protein extraction were determined according to the Bradford method (1976) with bovine serum albumin using A048-3 assay kits. SOD activity was determined by measuring its capacity to inhibit the photochemical reduction of NBT (Giannopolitis and Ries 1977) with A001-1 assay kits. One unit of SOD activity is defined as the amount of enzyme inhibiting 50 % of the initial reduction of NBT under light, expressed as units mg^{-1} protein. POD activity determination was based on the observation of absorbance changes in the reaction solution (60s after the reaction started) at 470 nm (Noreen and Ashraf 2009) with an A084-1 assay kit. POD can catalyse the transformation of guaiacol to tetraguaiacol in the presence of H_2O_2. One unit of POD activity, expressed as unit mg^{-1} protein, was defined as the amount of enzyme required for formation of 1 μmoL of tetraguaiacol in 60s at room temperature. CAT activity was calculated by measuring the decrease due to H_2O_2 decomposition with spectrophotometric assay at 405 nm, using the A004 assay kit. One unit of CAT activity (one unit per mg protein) was defined as the amount that decomposes 1 μmoL of H_2O_2 per second per milligram protein.

Biolog analysis

Biolog Eco plates were used to study the substrate utilization pattern of soil microbial communities. After the plants were harvested, fresh soil (5 g) from each treatment was sieved and added to 100 mL of distilled water in a 250-mL conical flask and shaken at 200 rev min^{-1} for 20 min. Tenfold serial dilutions were made, and 1000-fold soil dilution solution was used for injection into the wells of the Biolog Eco plates. Plates were incubated at 25 °C for 7 days, and colour development was measured daily as difference in absorbance (A) at 590 nm using a μQuant microplate spectrophotometer (BioTek, Winooski, VT) before the data were calculated using Gen5 v1.06 software. Average well colour development (AWCD) values over time for all C sources were calculated as a measure of total microbial activity. Mean values ($n = 3$) of AWCD for the different treatments over time were compared at the 5 % level of significance to evaluate their effects (Yao et al. 2000).

The average well colour development:

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

C_i—the colour absorbance value of reaction well; R—the colour absorbance value of control well; n—the carbon source number, 31.

The Shannon index reflects both the substrate richness and the substrate evenness of test microorganisms. Shannon measures are shown to be the only standard diversity measures that can be decomposed into meaningful independent alpha and beta components when community weights are unequal (Jost, 2007). Shannon diversity index:

$$H = -\sum_{i}^{n} P_i \times lnP_i$$

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.

Evenness index:

$$E = H/lnS$$

H—Shannon index; S—the carbon source number of Biolog Eco plate, 31 for maximum.

The Simpson index, weighted toward the abundances of the most common species, is used to emphasize the dominant population of soil microorganisms (Simpson, 1949). Simpson index:

$$D = 1 - \sum_{i=1}^{n} p_i^2$$

McIntosh index indicates the evenness or homogeneity of soil microorganisms (McIntosh, 1967) and reflects the consistency of bacteria in the multidimensional rhizosphere here. McIntosh index:

$$U = \sqrt{\left(\sum_{i=1}^{n} p_i^2\right)}$$

All the data were processed with Microsoft Excel 2013 and the SPSS v.18.0 software package. The data were analysed for significant differences from the control treatment or between treatments using one-way analysis of variance. To highlight the principal directions of variation of the toxicity results, principal component analysis (PCA) was conducted with the SPSS v.18.0 software package, too.

Results and discussion

Acute phytotoxicity of OTC

The results of the acute phytotoxicity tests indicate that during the seed germination stage, inhibition of water spinach germination occurred in almost all the treatments compared to that in the control (Table 1), with a significant ($p < 0.01$) increase in inhibition rate to 13 % at 0.01 mg OTC kg^{-1} soil and 20 % at 5 mg OTC kg^{-1} soil. Root elongation (Table 1) was generally inhibited from about −23 to −24 % under the doses of 0.2 and 0.5 mg OTC kg^{-1} soil but sharply inhibited to −55 % at 10 mg OTC kg^{-1} soil ($p < 0.01$), which means significant promotion of root elongation appeared at most of the spiked concentrations of OTC. Biomass of water spinach did not present a clear trend at different soil concentrations of OTC (Table 1). No significant change ($p < 0.05$) of the total biomass appeared in the different treatments.

The water spinach root elongations were more susceptible to OTC at the different concentrations (Table 1). It was not linearly correlated with the increase in OTC concentration, although extremely significant difference occurred at 0.2, 0.5 and 10 mg kg^{-1} ($p < 0.01$); the sensitivity of results to evaluate the acute toxicity of OTC do not so agree with those for lettuce (*Lactuca sativa*), alfalfa (*Medicago sativa*) or carrot (*Daucus carota*) reported by Hillis et al. (2011). Desorption of heavy

Table 1 Effects of OTC on germination, root elongation, biomass and total protein content, SOD, POD and CAT activities of water spinach during germination

OTC concentration (mg kg^{-1})	Germination inhibition (%)	Root elongation inhibition (%)	Total biomass per 100 seeds (g)	Total protein content (mg g FW^{-1})	SOD (U g FW^{-1})	POD (U mg $pr.^{-1}$)	CAT (U ml^{-1})
0	0	0	23.31 ± 0.25	0.030 ± 0.002	113.9 ± 18.2	16.08 ± 3.52	142.6 ± 25.6
0.01	13.0 ± 1.9**	−18.7 ± 8.1*	24.29 ± 0.45	0.009 ± 0.010**	64.9 ± 15.1*	40.23 ± 4.66**	145.6 ± 33.9
0.05	0 ± 1.3	−16.4 ± 11.2*	23.20 ± 0.23	0.021 ± 0.009	130.5 ± 19.8	32.95 ± 4.92**	134.7 ± 28.4
0.1	7.0 ± 1.2*	6.5 ± 6.2	23.20 ± 0.32	0.025 ± 0.007	80.1 ± 18.6	21.58 ± 6.02	182.9 ± 32.2
0.2	3.0 ± 0.2	−24.3 ± 7.7**	23.13 ± 0.35	0.008 ± 0.009**	177.0 ± 17.3*	16.85 ± 6.34	300.8 ± 31.5**
0.5	3.0 ± 0.9	−23.1 ± 7.5**	23.15 ± 0.19	0.016 ± 0.006**	276.5 ± 19.2**	18.57 ± 4.97	137.0 ± 25.6
1	1.0 ± 1.1	5.3 ± 5.8	23.38 ± 0.24	0.019 ± 0.003**	95.6 ± 14.9	41.56 ± 5.28**	315.9 ± 22.2**
5	20.0 ± 1.2**	1.3 ± 6.1	22.84 ± 0.43	0.015 ± 0.005**	129.3 ± 21.3	29.35 ± 1.87**	257.1 ± 30.8**
10	9.0 ± 1.3*	−55.1 ± 9.8**	22.81 ± 0.44	0.053 ± 0.007**	238.4 ± 19.7**	42.12 ± 3.79**	404.4 ± 29.8**

Values are the average of three replicates ± standard deviation (SD)

*It means significantly different from treatment without OTC addition ($p < 0.05$)

**It means highly significant different from treatment without OTC addition ($p < 0.01$)

metal ions (including cadmium) occurs under the competitive adsorption effect of OTC in soils, and this may explain the inhibition of germination observed (Sawidis 2008; Wan et al. 2010). Metal-centric toxicity at low addition rates of OTC occurred, particularly in heavy metal-contaminated soil, so that the existence of Cd in soil might affect the germination rates significantly. The sudden increase in toxicity at high rates OTC might represent a combined effect of the high dose of OTC and the influence of desorbed pollutants.

In other researches, antibiotics, chloramphenicol (CHL), florphenicol (FLO) and OTC inhibited growth of *Tetraselmis suecica* with 96-h IC50 values in the order of OTC > CHL > FLO (Seoane et al., 2014). In accordance to the standard procedures, the acute toxicities (48-h EC50 value, mg/L) in decreasing order were oxolinic acid (OA) (4.6), tiamulin (TI) (40), sulfadiazine (SU) (221), streptomycin (ST) (487), tylosin (TY) (680) and OTC (~1000) to the freshwater crustacean *Daphnia magna* (Wollenberger et al., 2000).

The biochemical index total protein content (Table 1) was inhibited at OTC exposure below 5 mg kg^{-1} soil but was significantly promoted when the OTC concentration increased to 10 mg kg^{-1}. SOD activity (Table 1) decreased slightly at OTC stress below 0.01 mg kg^{-1} and then showed a stimulated trend when the OTC stress exceeded 0.2, 0.5 and 10 mg kg^{-1}. A similar trend in POD activity was observed (Table 1). After elevation at 0.01 mg OTC kg^{-1} soil, POD activity declined with further increase in exposure concentration (0.01 to 0.5 mg OTC kg^{-1} soil) and then a second peak appeared at 1 mg OTC kg^{-1} soil. CAT activity also showed an upward trend except for a low point at 0.5 mg OTC kg^{-1} soil stress (Table 1).

The antioxidant status was of great importance as an indicator of physiological functions against the stress from environmental pollutants. Environmental stress leads to increasing oxygen free radical levels and disturbs the anti-oxidative balance in plants, animals and microorganisms (Bernanke and Köhler 2009). Generally, total protein content has been shown to decrease when expressed in a toxic environment (Santos et al. 1993), perhaps mainly due to the consumption of enzymes as a defence mechanism for plants in the short term or because of the inhibition or blocking of protein synthesis by OTC. However, the clear increase at 10 mg OTC kg^{-1} soil may be related to the accelerated synthesis of antioxidant enzymes under the stimulation of OTC, which might provide enough enzymes in the defence against pollutants in water spinach.

When OTC induced oxidative stress and disturbed the antioxidant balance, the water spinach initiated anti-oxidation and detoxification responses against OTC exposure by strengthening anti-oxidative enzyme synthesis. As previous assays have shown, SOD performed the function of transforming reactive O_2 species (ROS) into hydrogen peroxide, constituting the first line of defence against excess ROS under stress conditions (Fridovich 1995). Thus, the increase in SOD activity indicates the presence of toxicity. In the tests, the initial decrease in SOD activity was supposed to be the consumption against the rising ROS with the trend in promotion of enzyme activity occurring at OTC addition rates from 0.1 to 0.5 mg kg^{-1} soil, suggesting the response of water spinach to adverse conditions. The minimum dose of OTC to activate SOD defence mechanism was observed, as in previous assays, which showed that the detoxification system was stimulated at a certain exposure concentration (Bigorgne et al. 2011). POD and CAT perform the role of splitting hydrogen peroxide into water and oxygen as found frequently in fishes and plants in some studies (Verma and Dubey 2003; Woo et al. 2009; Wang et al. 2012). Since SOD increased at 0.1–0.5 mg OTC kg^{-1} soil, more hydrogen peroxide was produced from ROS, resulting in high consumption of POD and CAT within any specified OTC dose. There is also a synergism between SOD and CAT in defence mechanisms. The depleting of SOD and CAT will definitely reduce the overall activity of CAT in some way. The SOD results showing specific inhibition at 1 mg OTC kg^{-1} soil indicate that the

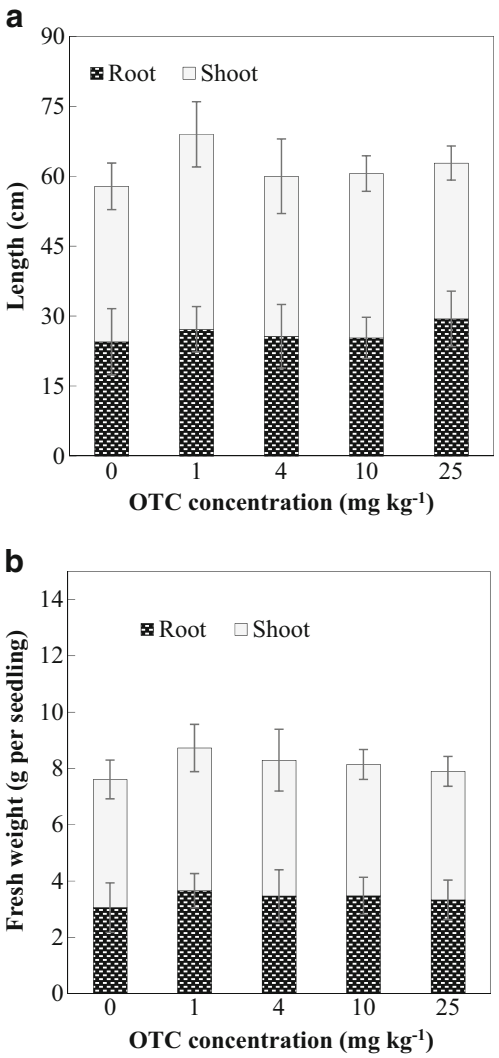

Fig. 1 Effects of OTC on the length and fresh weight of the shoots and roots

Table 2 Effects of OTC on total protein content, SOD, POD and CAT activities of water spinach during the seedling stage

Sample	OTC concentration (mg kg^{-1})	Total protein content (mg g FW^{-1})	SOD (U g FW^{-1})	POD (U mg $pr.^{-1}$)	CAT (U ml^{-1})
Leaf	0	41.75 ± 1.09	4.79 ± 0.52	28.74 ± 0.35	0.59 ± 0.03
	1	35.18 ± 1.84**	7.10 ± 0.99**	34.61 ± 1.66*	0.60 ± 0.02
	4	26.61 ± 3.56**	11.05 ± 1.33**	44.66 ± 4.95**	1.05 ± 0.22
	10	23.92 ± 1.92**	11.35 ± 2.02**	49.63 ± 3.62**	1.15 ± 0.34
	25	33.51 ± 5.18*	5.74 ± 0.81*	27.81 ± 0.38*	0.24 ± 0.13*
Root	0	25.84 ± 2.07	9.25 ± 1.41	42.29 ± 3.95	0.20 ± 0.10
	1	17.44 ± 1.15**	14.68 ± 1.04**	63.05 ± 5.57**	0.53 ± 0.08*
	4	17.60 ± 0.50**	13.54 ± 0.74**	63.47 ± 2.67**	0.45 ± 0.23
	10	19.56 ± 2.99	12.79 ± 2.67	54.20 ± 10.45	0.77 ± 0.31*
	25	18.30 ± 0.76**	13.89 ± 0.95*	59.63 ± 2.48**	1.17 ± 0.11**

Values are the average of three replicates ± SD

*It means significantly different from treatment without OTC addition ($p < 0.05$)

**It means highly significantly different from treatment without OTC addition ($p < 0.01$)

threshold of the defence system was exceeded (Kono and Fridovich 1982). Anti-oxidation and detoxification systems were disturbed and might result in an inhibition of enzymes directly or indirectly because metabolic disorder, tissue damage or other toxicologically relevant responses might be induced by ROS (Zhou et al. 2010). To resist even higher doses of OTC, stronger defence mechanisms might be applied as test results showed that a larger amount of SOD, POD and CAT were synthesized under stimulation of even higher OTC addition rates (1 to 10 mg kg^{-1} soil).

Sub-chronic phytotoxicity of OTC

Plant growth tests were conducted for 60 days to investigate the effects in whole growth stage of water spinach seedlings under different rates of OTC. At the end of the plant growth test, the residual OTC concentrations were 0.22 ± 0.03, 0.50 ± 0.04, 2.16 ± 0.20 and 5.19 ± 0.32 mg kg^{-1} in soil, decreasing by 78.0, 87.5, 78.4 and 79.2 %, respectively, compared to the initial OTC concentrations of 1, 4, 10 and 25 mg kg^{-1}. Among all the indices determined, the length and biomass (FW) of the shoots and roots of seedlings showed no significant change compared to that in the control (Fig. 1) in general.

The biochemical indices of total protein content, SOD, POD and CAT were measured in the leaves and roots after harvest. As shown in Table 2, total protein content was inhibited without obvious differences among the treatments in the roots and with a gradual decrease in the leaves. The leaves had higher total protein contents, which increased at an OTC rate of 25 mg kg^{-1} soil compared with the roots. The changes in both SOD and POD activities in the leaves were similar to those of the roots (Table 2). Activities of SOD and POD were promoted by OTC, exhibiting a decrease in the leaves and remaining stable in the roots at OTC rates less than 10 mg kg^{-1} soil. However, at an OTC level of 25 mg kg^{-1}, the activities of both SOD and POD in the leaves were inhibited and similar to those in the controls. Unlike SOD and POD, CAT activity was higher in the leaves in all the treatment except 25 mg OTC kg^{-1} soil. CAT activity increased continuously in both the leaves and roots with increasing OTC level but at 25 mg OTC kg^{-1} soil, the foliar CAT activity declined markedly to levels even lower than that in the controls.

Table 3 Effects of OTC on photosynthetic rate, transpiration rate, stomatal conductance and water use efficiency of water spinach

Parameter	Initial OTC concentration (mg kg^{-1})				
	0	1	4	10	25
Photosynthetic rate/μmoL CO_2 ($m^2 \cdot s$)	14.159 ± 1.054	11.814 ± 2.713	11.139 ± 1.974	6.900 ± 1.904**	6.409 ± 0.244**
Transpiration rate/mmoL H_2O ($m^2 \cdot s$)	3.035 ± 0.637	2.292 ± 0.732	2.017 ± 0.230*	1.192 ± 0.286**	1.175 ± 0.088**
Stomatal conductance/mmoL ($m^2 \cdot s$)	0.133 ± 0.038	0.112 ± 0.034	0.101 ± 0.006	0.057 ± 0.0115**	0.059 ± 0.007**
Water use efficiency/mmoL CO_2 moL H_2O	4.733 ± 0.647	5.125 ± 0.472	5.494 ± 0.401	5.783 ± 0.213**	5.339 ± 0.137

Values are the average of three replicates ± SD

*It means significantly different from treatment without OTC addition ($p < 0.05$)

**It means highly significantly different from treatment without OTC addition ($p < 0.01$)

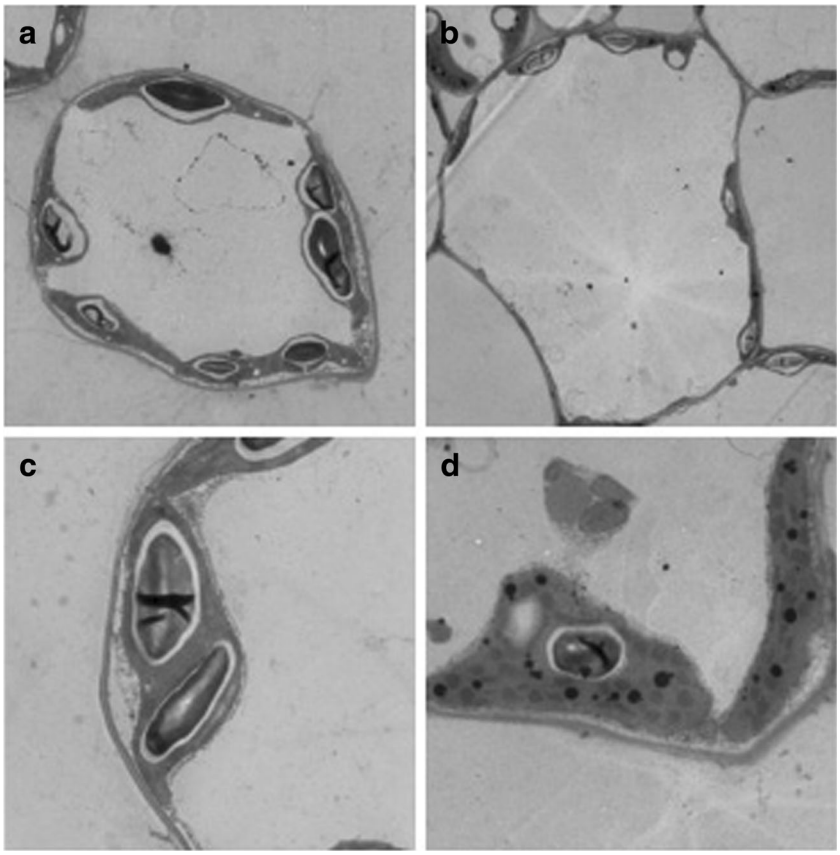

Fig. 2 TEM images of water spinach leaves with and without OTC treatment (**a** and **c**, control; **b** and **d**, 25 mg OTC kg^{-1} soil; **a** and **b** at 2000×; **c** and **d** at 5000×)

Spinach has been reported to be highly tolerant to many abiotic stresses (Shimazaki et al. 1992; Fan et al. 2012). Tolerant plant species protect themselves by using strong defence mechanisms, mostly as intensified antioxidant enzyme activity, and specialization of plant tolerance may be related to the influence of tissue and subcellular localization of enzymes and upstream sequences in the genomic sequence (Wilhelm 2006; Sánchez-Rodríguez et al. 2011; Mittal et al. 2012). In the plant growth tests, the leaves contained more total protein for their enhanced metabolic activities. Higher levels of SOD and POD activities in the roots resulted from the direct stimulation of toxic compounds in the rhizosphere, which in turn contributed to the tolerance of the roots against the toxic environment. At an OTC level of 25 mg kg^{-1} soil, CAT activity in the roots also exceeded that in the leaves. Compared to the leaves, the roots showed higher tolerance to OTC stress as shown by increasing the amount of synthetic anti-oxidative enzymes as the increasing OTC level. Anti-oxidative enzymes in the leaves decreased sharply at 25 mg OTC kg^{-1}, indicating a collapse of defence systems and interference with plant

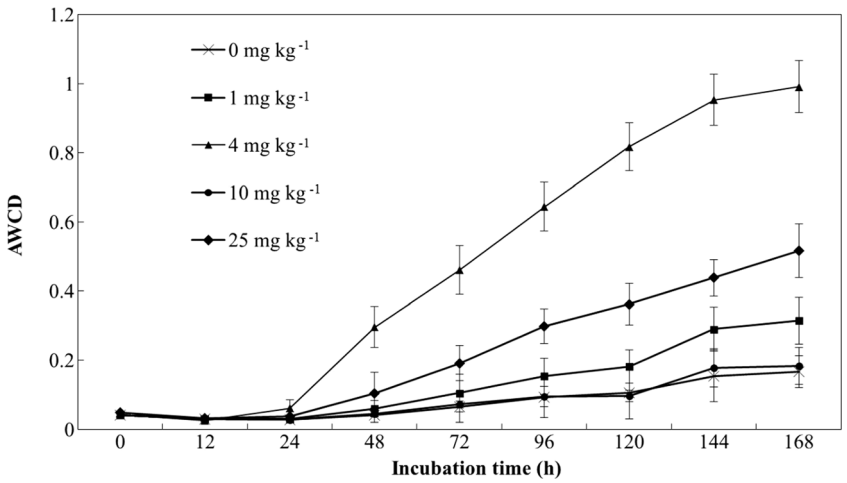

Fig. 3 Changes in AWCD values under the different treatments

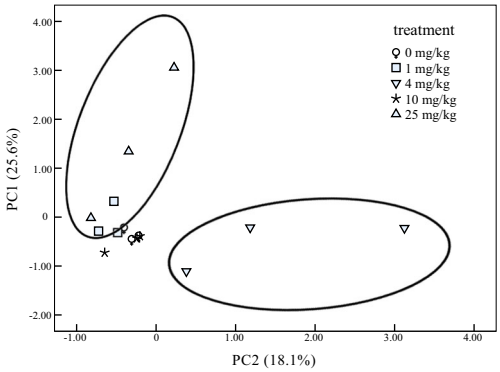

Fig. 4 Principal component analysis (PCA) results of substrate utilization potential of the soil microbial community

physiological activities. Total protein content in the leaves was also seriously shut down at the highest OTC level, demonstrating possible damage to functional tissues related to metabolism.

Physiological indices related to metabolism were determined before the plants were harvested (Table 3). Results show that photosynthetic rates, transpiration rates and stomatal conductance exhibited a downward trend as the OTC level increased and were significantly reduced at 10 mg OTC kg^{-1}, illustrating the sub-chronic negative effect of OTC on water spinach. Water use efficiency (WUE) is an important agronomic character that also decreased slightly at the highest OTC stress (Table 3). Combined with related assays showing that damage to plant tissues may reduce WUE (Shan et al. 1996; Reich et al. 1985) and the marked reduction in enzyme activities in the leaves (Table 2), it can be inferred that OTC might injure the functional tissues in the leaves. Leaf ultrastructure was observed using transmission electron microscopy (TEM) to demonstrate this assumption. Comparing Fig. 2a and b, starch grains decreased sharply in number and in size. Starch grains play an important role in the deposition of photosynthetic products. When the photosynthetic process was inhibited or slowed down, the accumulation rate of the plants could not match the hydrolysis rate. Comparing Fig. 2c and d, a large number of osmiophilic bodies were found in the tissues of the OTC-treated plants. Osmiophilic bodies are often observed in damaged tissues within multiple assays and are considered necessary in the rebuilding of the integrity of the plasma membrane (Pareek et al. 1997), and this confirms that the foliar tissues were damaged by high rates of OTC in sub-chronic toxicity experiments.

In former investigations, it has been found that OTC concentrations range from 0 to 25 mg L^{-1} in concentration compete for adsorption sites on the surface of soils with heavy metal contamination, and as a result, the HM desorption rate increases with increasing OTC concentration (Chen et al. 2014). However, when the OTC concentration ranges from 25 to 100 mg L^{-1}, some free HM ions form complexes with OTC and become fixed again on the surfaces of the soil matrix (Chen et al. 2014). The changes in HM availability in contaminated soils are mostly attributed to competition for adsorption sites, and complexation reactions between OTC and HMs, together with changes in pH, upset the dynamic equilibrium (adsorption and desorption) of the soil pollutants.

Biolog Eco tests

In these tests, soil microbial diversity in soil samples from the plant growth tests was evaluated using Biolog Eco tests. According to Fig. 3, average well colour development (AWCD) values at treatment with 4 mg OTC kg^{-1} were extremely high, followed by 25 and 1 mg OTC kg^{-1}, and 10 mg OTC kg^{-1}, and the controls showed the lowest values. AWCD values did not change initially but then increased rapidly after 48 h. The AWCD values at 1, 4, 10 and 25 mg OTC kg^{-1} were 88.9, 495, 10.5 and 211 % higher than those at the controls after incubation for 168 h.

The Shannon index showed a visible decline at 4 mg OTC kg^{-1} ($p < 0.05$) but no significant differences among the treatments in line with the conclusion of Zielezny et al. (2006) were shown, except that the values of the OTC-treated soils were all lower than those of the control. Evenness showed a similar trend with the values of each treatment following the order: 0 > 10 > 1, 4 and 25 mg kg^{-1} and no significant differences

Table 4 Indices of microbial diversity

Index	Initial OTC concentration (mg kg^{-1})				
	0	1	4	10	25
Shannon	1.487 ± 0.258	0.889 ± 0.385*	0.832 ± 0.088**	1.112 ± 0.236	1.026 ± 0.226
Simpson	0.575 ± 0.043	0.718 ± 0.126	0.726 ± 0.029**	0.646 ± 0.046	0.555 ± 0.229
McIntosh	1.336 ± 0.551	1.709 ± 0.633	3.998 ± 1.368**	1.334 ± 0.171	3.904 ± 0.647**
Evenness	0.608 ± 0.022	0.339 ± 0.191*	0.313 ± 0.05**	0.448 ± 0.064**	0.327 ± 0.072**

Values are the average of three replicates ± SD

*It means significantly different from treatment without OTC addition ($p < 0.05$)

**It means highly significantly different from treatment without OTC addition ($p < 0.01$)

among the OTC concentrations. The Simpson index at 4 mg OTC kg^{-1} and the McIntosh index at 4 and 25 mg kg^{-1} were markedly higher than those at the control, in accordance with the AWCD results.

Principal component analysis of the Eco plate data shows the effects of OTC on substrate utilization in rhizosphere soil (Fig. 4). Statistical analysis was conducted using the SPSS software package with two principal components (25.6 and 18.1 % of the total variance). Treatments with 4 and 25 mg OTC kg^{-1} were divided from the other treatments and the control.

In Biolog Eco plate analysis, the microbial activities were generally promoted by addition of OTC, and the degree of promotion was not linearly correlated with the addition rate of OTC (Fig. 3 and Table 4). Microbial diversity and evenness were also influenced by OTC according to changes in the indices. Principal component analysis (PCA) indicates that the contributions of rhizosphere microorganisms in the utilization of substrate were greatly influenced by the addition of OTC at particular levels. At 4 mg OTC kg^{-1} soil, L-arginine, ß-methyl-D-glucoside, N-acetyl-D-glucosamine, L-asparagine and glycyl-L-glutamic acid were utilized more efficiently and comprised the main contributor of PC2. At 25 mg OTC kg^{-1}, utilization of Tween 80, glycogen, D-cellobiose, a-D-lactose, D,L-a-glycerol and L-arginine was promoted, and these were the main components of PC1.

Conclusions

In the experimental soil, OTC inhibited the seed germination, disturbed the anti-oxidative system and damaged the functional tissues of water spinach. Microbial activity and diversity in rhizosphere soil were simultaneously affected in the variety of carbon sources utilized at different rates of addition of OTC. Considering the risk of soil OTC introduction to agricultural plants, food safety and human health, antibiotics themselves and stuff, like water and fertilizer, containing them should be strictly considered before application in the future.

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