

# Oxytetracycline on functions and structure of soil microbial community

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## Abstract

Effects of oxytetracycline (OTC) on soil enzyme activity and structural diversity of soil microbial communities were investigated. The low and moderate OTC inputs of less than 15 mg/kg enhanced microbial growth and increased the total and bacterial and fungal biomasses in the receiving soils. The antibiotic toxicity of OTC although targeted the bacteria in the soils proportionally resulted in greater harmed against the soil fungi than the bacteria and the Gram-negative bacteria fared significantly better than the Gram-positive bacteria. Collectively, the high OTC inputs lowered of soil enzymatic activities and the low and moderate OTC inputs did not have any adverse impact on the soil enzyme activities.

**Keywords:** Veterinary antibiotic, soil enzyme activity, PLFAs, soil microbial mass

## 1. Introduction

Veterinary antibiotics are widely used as growth promoters and for therapies of infectious diseases in livestock farming. Worldwide, about 100,000 to 200,000 tons of antibiotics are used annually (Jeong *et al.*, 2010) with 30 to 90% of the parent compounds find their ways into the environment (Halling-Sorensen *et al.*, 1998; Sarmah *et al.*, 2006). The antibiotics may reach the soil through livestock excreting on pastures or the use of manures as fertilizers. Accumulation of antibiotics in soils poses potential threats to the functions and structure of microbial communities in receiving soils (Demoling *et al.*, 2009; Johnsen *et al.*, 2011; Kong *et al.*, 2006; Liu *et al.*, 2009; Liu *et al.*, 2012).

Oxy-tetracyclines (OTCs), the most common veterinary antibiotics, possess broad-spectrums of antimicrobial properties including block the transfer of RNA and prevent synthesis of proteins. OTCs are poorly adsorbed or metabolized by the targeted livestock animals; resulting in large amounts being inadvertently released to the environment through excretions of feces and urine (Tolls, 2001). They are found in receiving soils at the parts per million concentration levels due to repeated applications of manures, occasionally exceeding the 100 ppm level (Zhou *et al.*, 2011). Consequently, the activities and function of the soil microbial community are adversely

affected (Boleas *et al.*, 2005; Kong *et al.*, 2006; Liu *et al.*, 2009; Thiele-Bruhn and Beck, 2005) and the soil microbes develop pollution-induced tolerant and antibiotic resistant genes (Brandt *et al.*, 2010; Schmitt *et al.*, 2006; Zhu *et al.*, 2010).

The fates of OTCs in soil are related to not only the inherent soil properties of organic matter, pH, solid/solution ratio, and cation speciation (Tolls, 2001; Zhou *et al.*, 2010), but also the nature of aboveground vegetation (Migliore *et al.*, 2010; Yang *et al.*, 2009). Antibiotics, including oxy-tetracyclines, tetracycline, sulfadiazine, sulfamethoxazole, and ciprofloxacin harmed the function and structure of the soil microbial community and reduce the soil enzymatic activities (Demoling *et al.*, 2009; Ding and He, 2010; Kotzerke *et al.*, 2011). Do soil enzyme activities change in proportion to amounts of antibiotics present? What is the role of aboveground vegetation on the soil microbial activity of an OTC-stressed community? We hypothesized that the vegetation would dampen the antibiotics' adverse impacts on activities and functions of soil microbial community.

In this research, greenhouse experiments growing two types of plants in soils receiving three levels of OTC inputs were conducted to assess effects of OTC concentrations and vegetation patterns on changes of soil microbial (1) functions assessing activities of 4 enzymes involved in C, N, P, and S, cyclings, respectively and (2) community structure analyzing the phospholipid fatty acids (PLFAs) profiles.

## 2. Material and Methods

### 2.1. Antibiotics incorporation

Surface horizon (0 to 20 cm) of the soils at an agricultural experiment station farm near Beijing was collected during the autumn, 2011 for the experiments. In the immediate past five years, the land where soils were obtained was in annual corn productions. The soil

was a loam with pH of 8.3, and the organic matter, clay, silt, and sand contents of 1.2, 9.2, 38.6, and 52.2%, respectively. The soil was air-dried and sieved to pass a screen with 2 mm x 2 mm openings. The manure was collected from a local swine farm with no history of feeding antibiotics. It was air-dried, sieved to pass a screen with 2 mm x 2 mm openings and stored at room temperature until use.

Two hundred seventy grams (270 g dry weight) of swine manure were mixed with OTCs to reach three concentration levels and then blended into 9 kg soil, resulting in a typical manure application of 30 mg/g soil. The final OTC concentration in spiked soil was 1, 15, 200 mg OTCs/kg of soil, respectively. The soil OTC concentrations up to 1 mg/kg were typical in ordinary agricultural operations, while those of 119 to 307 mg/kg had been observed in soils irrigated with domestic wastewater (Zhou *et al.*, 2011). The three soil OTC concentrations were selected to bracket levels observed in the fields.

Fertilizers including urea,  $\text{KH}_2\text{PO}_4$  and  $\text{K}_2\text{SO}_4$  were added to soil at 0.15 g N/kg, 0.12 g P/kg, and 0.15g K/kg, respectively. Soil moisture content was adjusted to 50 to 60% of maximum water holding capacity using distilled water. The OTC-spiked soils were then divided evenly and packed into 9 PVC plant growing pots, 15 cm in height and 10 cm in diameter, covered, and incubated in darkness at room temperature for 6 days.

### 2.2. Experimental design

A two-factor ( $P \times A$ ) randomized block design with three replications was used, in which factor "P" denoted plant species including 3 levels of P0: no vegetation, P1: red amaranth (*Amarantus mangestnus* L.), and P2: white clover (*Trifolium repens* L.) and factor "A" denoted OTC concentration of soils including 4 levels A0: 0 mg/kg, A1: 1 mg/kg, A2: 15 mg/kg, and A3: 200 mg/kg). Red amaranth was chosen to represent a plant with short growing period and widespread cultivation in northern China. The

cultivation in northern China. The white clover was selected for its wide applications in pastures and forage productions and its special rhizosphere microorganisms. After the initial incubation, pots for the plant treatment were sown with either 15 red amaranth seeds or 20 white clover seeds each. Seven days after germination, plants were thinned to 6 and 15 plants for red amaranth and white clover, respectively. Soil moisture content was maintained at 50 and 70% of the maximum water holding capacity at early and middle-late stages, respectively. The experiment was carried out in a glasshouse maintained at 20 to 27 and 15 to 20 °C day and night temperature ranges, respectively. The plant growing experiment lasted 7 weeks.

### 2.3. Phospholipid fatty acids and soil enzymatic activities assays

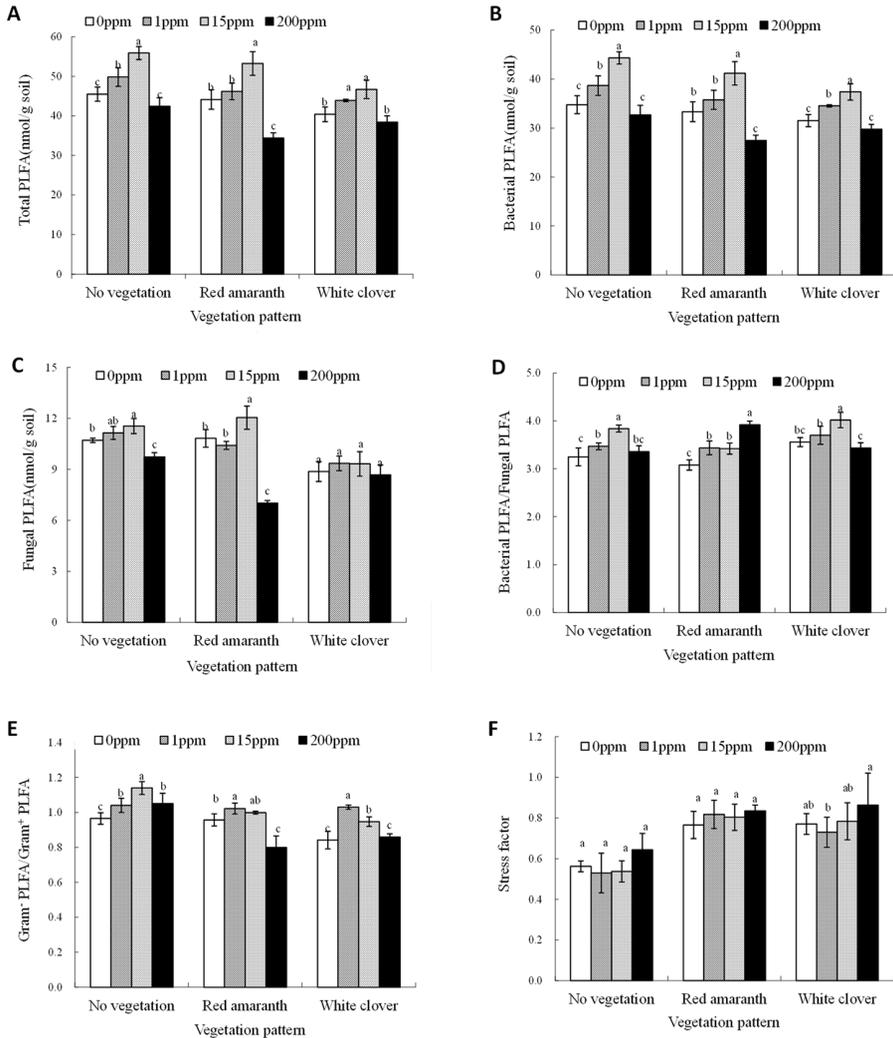
Microbial biomass and community composition were assessed by the PLFAs. After plants were harvested, the soil at field conditions was crushed, mixed, and sieved to remove visible plant roots, freeze-dried at -45 °C, and then passed through a 0.15 mm mesh sieve. All solvents and reagent chemicals for the extractions were either analytical or chromatographic grade. The total phospholipids were extracted from 3 g freeze-dried soil aliquots with a chloroform-methanol-citrate mixture buffer at the 1:2:0.8 volumetric ratios, followed by vortex mixing, shaking and centrifuging and then fractionated into glycolipids, neutral lipids, and polar lipids. The lipid extracts in chloroform phase were transferred to silica-filled solid phase extraction (SPE) columns. Phospholipids were separated from neutral lipids and glycolipids by subsequently eluting with chloroform, acetone and methanol, respectively. Then, the phospholipids in the methanol fraction were hydrolyzed and methylated using a mixed solution of KOH and methanol. Hexane-chloroform mixture was added for repeating extraction process. An internal standard, c19:0 (methylene nonadecanoate) was added to quantify the PLFAs. The PLFAs were separated by an Agilent 6890N gas chromatograph, equipped with a HP-5 column (25.0 m × 0.33 mm

× 200 μm) and identified by a MIDI Sherlock microbial identification system. Nitrogen was used as carrier gas with a flow rate of 0.8 ml/min. The oven temperature started at 170 °C for 1 min, ramped to 260 °C at 5 °C /min, and increased to 310 °C at 40 °C /min, and retained for 1.5 min.

According to the fatty acid nomenclature described by Frostegard et al. (1993), the branched phospholipids i15:0, a15:0, i16:0, i17:0, and a17:0 were used as indicators for Gram-positive bacteria (Gram<sup>+</sup>), while the PLFAs 16:1ω5c, 16:1ω7c, 17:1ω8c, cy17:0, and cy19:0 were considered as Gram-negative bacteria (Gram<sup>-</sup>). The unsaturated PLFAs 18:1ω9c and 18:2ω6c were used as fungal biomass indicators, while 15:0 and 17:0 PLFAs were considered as general bacteria markers. The ratios of Gram<sup>-</sup>/Gram<sup>+</sup> PLFAs and bacterial/fungal PLFAs were used for elucidating the changes in microbial community. The stress level (cyc/precursor) for the microbial community was calculated according to the ratio of (cyc17:0+cyc19:0)/(16:1ω7c+18:1ω7c) (Frostegard *et al.*, 1993; Hammesfahr *et al.*, 2008). The activities of urease, dehydrogenase (DH), alkaline phosphatase (ALP), and arylsulphatase (ARS) were assayed according to the methods described by Guan (1983). The urease activity was measured in citrate buffer solution (pH 6.7) using sodium phenolate substrate. The dehydrogenase was measured in Tris-HCl buffer solution (pH 7.6) using 2,3,5-triphenyltetrazolium chloride substrate. The alkaline phosphatase was measured in citrate buffer solution (pH 7.0) with 2,6-dibromo-N-chloro-p-benzoquinoneimide substrate. The aryl sulphatase was measured in sodium acetate buffer solution (pH 5.8) with 4-nitrophenyl sulfate substrate.

### 2.4. Data analysis

Data were subject to analysis of variance and significant difference between means were separated by using Duncan's new multiple range tests at the 5% level using SPSS 18.0.



**Figure 1.** Phospholipid fatty acids (PLFAs) of soils seven weeks after receiving 0, 1, 15 and 200 mg/kg of oxytetracycline under the types of vegetative covers. Each bar and its range bracket denote the arithmetic means  $\pm$  standard deviation ( $n=3$ ) of the indicated parameter and experimental condition. Different letters within each group of bars indicate values are significantly different at  $p<0.05$  according to ANOVA.

### 3. Results

#### 3.1. Microbial biomass and community structure

The total PLFAs of planted soils increased under the low and medium OTC inputs of 1 and 15 mg/kg and significantly decreased at 200 mg/kg OTC treatment comparing to those of the no antibiotic experimental control (Figure 1). The total PLFAs of the soils followed the descending order of 15 mg/kg > 1 mg/kg > no antibiotics experimental control > 200 mg/kg. The trend was consistent across the board for all three plant treatments. For the no plant experimental control, those at 15 mg/kg of OTC treatment were 22.8, 12.1, and 31.6% higher than those at 0, 1 and 200 mg/kg OTC treatment, respectively. For soils planted with red amaranth, the total PLFAs at 15 mg/kg OTC treatment were 20.6, 15.3, and 54.9% higher than those at 0, 1 and 200 mg/kg OTC treatments, respectively. For soils treated with white clover, the total PLFAs at 15 mg/kg of OTC treatment were 15.6, 6.4, and 21.5% higher than those at 0, 1 and 200 mg/kg OTC treatments, respectively. The total PLFAs of soils planted with red amaranth sustained the greatest extent of harms when OTC treatment was at the 200 mg/kg.

Similarly, the bacterial PLFAs of the planted soils responded to the antibiotic treatments in descending order of 15 mg/kg > 1 mg/kg > no antibiotics experimental control > 200 mg/kg (Figure 1B). At the 15 mg/kg OTC treatment, the bacterial PLFAs of soils were 18.6 to 27.5% higher, and at the 200 mg/kg OTC treatment, the bacterial PLFAs were significantly reduced regardless of aboveground covers. Cross the board, the bacterial PLFAs were approximately 3.5 times more than those of the fungal PFLAs. However, the fungal PLFAs of plant treated soils responded to the soil borne antibiotics in different manners (Figure 1C). For soils of the no plant experimental control and red amaranth treatment, the amounts of fungal PLFAs in soils containing 200 mg/kg were significantly lower than those containing 0, 1 and 15 mg/kg OTC, while the fungal PLFAs of soils treated with white clover were not significantly different across the 0 to 200 mg/kg OTC treatments. The fungal PLFAs of

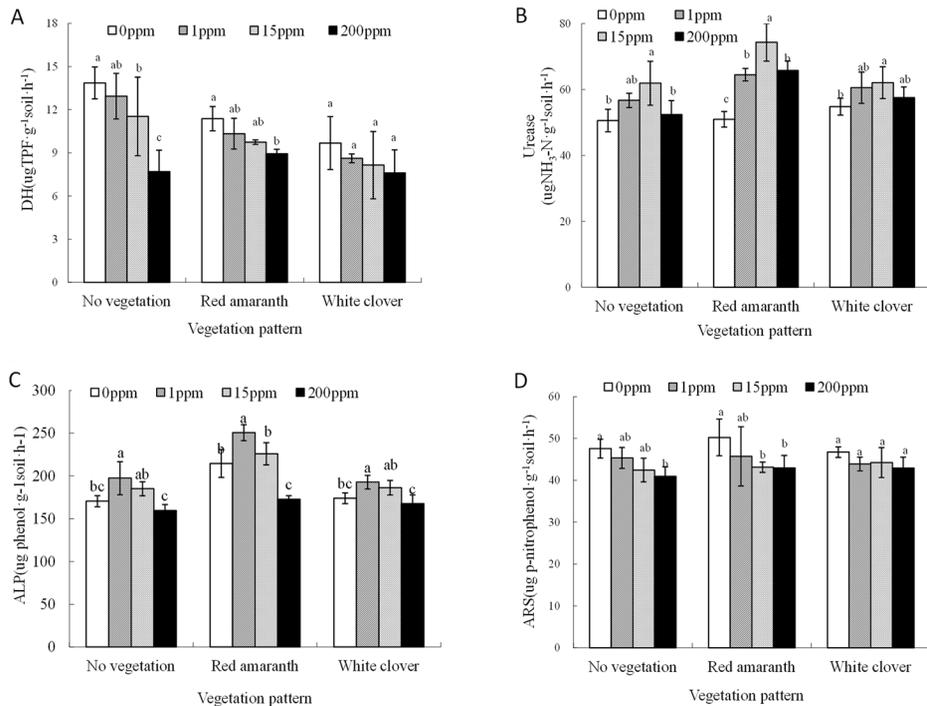
soils planted with red amaranth decreased by 41.8% from 12.0 nmol PLFAs g<sup>-1</sup> soil at the 15 mg/kg OTC treatment to 7.0 nmol PLFAs g<sup>-1</sup> soil at the 200 mg/kg OTC treatment.

The aboveground plant species had significant influences on the bacteria vs. fungi and bacteria vs. bacteria distributions in the OTC antibiotic treated soils and the outcomes appeared to be soil OTC concentration-dependent. Compared to the no OTC treatment, adding OTC to the soils, with the exception of 200 mg.kg OTC treated soils of white clover, increased the soil's bacterial/fungal PLFAs ratios (Figure 1D). The ratios of Gram<sup>-</sup>/Gram<sup>+</sup> bacterial PLFAs in soils of 1 and 15 mg/kg OTC treatments were significantly higher while the ratio of Gram<sup>-</sup>/Gram<sup>+</sup> bacterial PLFAs of the 200 mg/kg OTC treatment was significantly lower than that of the 0 mg/kg OTC level experimental control (Figure 1E).

The stress factors for the soils remained unchanging across the OTC treatments (Figure 1F). The stress factors of soils receiving antibiotic treatments were not significantly different if the soils were not planted and were treated with 1 mg/kg of OTC. The exception was the stress factor of soils planted with white clover when the soil OTC was at the 200 mg/kg.

#### 3.2. Soil enzyme activities

The antibiotics OTC adversely affected the enzyme activities in the receiving soils including those of dehydrogenase, urease, alkaline phosphatase, and arylsulfatase (Figure 2). The dehydrogenase arylsulfatase activities of the soils with no vegetation and red amaranth decreased significantly along the OTC concentration gradient of 0, 1, 15, and 200 mg/kg (Figure 2A and 2D) and the dehydrogenase and arylsulfatase activities of white clover were not significantly different along the same OTC concentration gradient. The aboveground plants affected the outcomes.



**Figure 2.** The activities of dehydrogenase (DH), urease, alkaline phosphatase (ALP), and arylsulphatase (ARS) of soils seven weeks after receiving 0, 1, 15 and 200 mg/kg of oxy-tetracycline under three types of vegetative covers. Each bar and its range bracket denote the arithmetic means  $\pm$  standard deviation ( $n=3$ ) of the indicated parameter and experimental condition. Different letters within each group of bars indicate values are significantly different at  $p < 0.05$  according to ANOVA.

Small and moderate inputs of OTC stimulated the enzyme activities, 15 mg/kg in the case of urease activity (Figure 2B) and 1 mg/kg OTC in the case of arylsulphatase, of soils regardless of the aboveground vegetative covers (Figure 2C). The high OTC input of 200 mg/kg treatment always resulted in significantly lower soil enzyme activities.

The enzymatic activities were normalized with respect to their respective activity maxima (Figure 3) and summed up by categories. The standardized mean soil enzyme activities of soils receiving 0, 1, and 15 mg/kg OTC were significantly greater than that of the 200 mg/kg OTC treatment for soils with no vegetation and

the red amaranth aboveground cover but not the white clover aboveground cover. Again, the aboveground cover plants red amaranth and white clover yielded significantly different extent of harms on the soil enzymatic activities. (Figure 3).

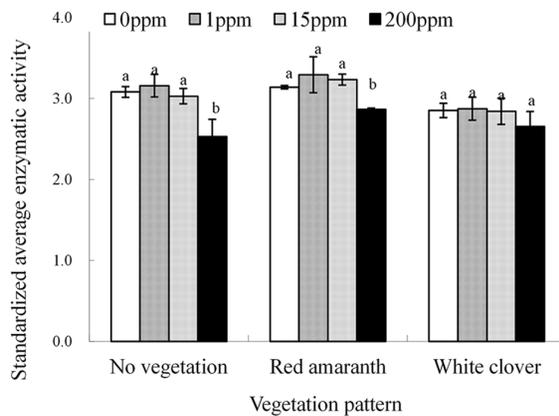
#### 4. Discussion and Conclusions

##### 4.1. Oxy-tetracycline on soil microbial biomass

We used the phospholipids fatty acid profiles to indicate the structural changes of the microbial communities in the OTC affected soils and showed that the total bacterial and fungal biomass of soils receiving the low

and moderate OTC inputs of less than 15 mg/kg were enhanced while those in soils receiving the extremely high OTC input of 200 mg/kg were significantly depressed with respect to outcomes of the no antibiotic experimental control. The aboveground vegetative covers, red amaranth and white clover, enhanced the total microbial biomass in soils receiving low and

moderate OTC treatments and dampened the toxicity of antibiotics in soils receiving the high OTC treatment. It appeared that the manure in which the OTC was incorporated attenuated the potencies of antibiotics in the receiving soils. Only when the attenuation capacity was exceeded, did we observe the harms of antibiotics on the soil microbial biomass.



**Figure 3.** Aggregated enzymatic activity factor of dehydrogenase, urease, alkaline phosphatase, and arylsulfatase of soils seven weeks after receiving 0, 1, 15 and 200 mg/kg of oxy-tetracycline under three types of vegetative covers. The soil enzyme activities were normalized by dividing the them with their respective maxima and then the aggregation was achieved by summing up the normalized values of each category.

#### 4.2. Oxy-tetracycline shifted soil microbial community structure

When the antibiotic effect of OTC was suppressed, the manures stimulated the growth of both bacterial and fungal biomass in the soils with the bacteria outpaced the fungi and the Gram negative bacteria outpaced the Gram positive bacteria. When the toxicity of OTC was expressed, both the bacterial and fungal biomasses were reduced with respect to those of the no antibiotic experimental control. However, the ratios of bacterial/fungal biomass shifted in favor of the bacteria especially

the Gram-negative bacteria in the soils. The antibiotic toxicity of OTC although targeted the bacteria in the soils proportionally resulted in greater harmed against the soil fungi than the bacteria and the Gram-negative bacteria fared significantly better than the Gram-positive bacteria.

The stress factor reflected the overall conditions associated with soils' ability to support plant growth. The low stress factor represented healthy conditions for plant growth while high stress factor indicated the opposite.

Apparently, the antibiotics did not present any significant harmful consequences for plant growth. The stress factors of the vegetated soils were consistently higher than those of the non-vegetated soils, reflected that red amaranth and white clover extracted nutrients to sustain their growth. The soil stress factors shifted higher as nutrients were withdrawn.

#### 4.3. Oxytetracycline lowered soil enzymatic activities

The activities of dehydrogenase, urease, alkaline phosphatase, and arylsulfatase reflected the microbial well beings of the OTC treated soils. We showed that, when exposed to OTCs, not all soil enzymes behaved in the same manner. The activities of urease and alkaline phosphatase in OTC treated soils followed the same pattern as microbial biomass. The activities of dehydrogenase and arylsulfatase in the OTC affected soils decreased with the increases of OTC inputs. Collectively, the high OTC inputs lowered of soil enzymatic activities and the low and moderate OTC inputs did not have any adverse impact on the soil enzyme activities.

#### 4.4. Role of aboveground vegetative cover

The aboveground vegetative covers played a minor role in dampened toxic effects of antibiotics.

### Acknowledgements

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### References

- Boleas, S., Alonso, C., Pro, J., Fernández, C., Carbonell, G., Tarazona, J.V. 2005. Toxicity of the antimicrobial oxytetracycline to soil organisms in a multi-species-soil system (MS.3) and influence of manure co-addition. *J. Hazard. Mater.* 122, 233-241.
- Brandt, K.K., Berg, J., Thorsen, M.K., Holm, P.E., Jensen, J., Nybroe, O. 2010. Cu exposure under field conditions coselects for antibiotic resistance as determined by a novel cultivation-independent bacterial community tolerance assay. *Environ. Sci. Technol.* 44, 8724-8728.
- Demoling, L.A., Baath, E., Greve, G., Wouterse, M., Schmitt, H. 2009. Effects of sulfamethoxazole on soil microbial communities after adding substrate. *Soil Biol. Biochem.* 41, 840-848.
- Ding, C., He, J.Z. 2010. Effect of antibiotics in the environment on microbial populations. *Appl. Microbiol. Biot.* 87, 925-941.
- Frostgard, A., Tunlid, A., Baath, E. 1993. Phospholipid fatty-acid composition, biomass, and activity of microbial communities from 2 soil types experimentally exposed to different heavy-metals. *Appl. Environ. Microb.* 59, 3605-3617.
- Guan, S.Y. 1983. Soil enzymes and their determination methods. Chinese Agriculture Press, Beijing, China, 294-318p.
- Halling-Sorensen, B., Nielsen, S.N., Lanzky, P. F., Ingerslev, F., Lutzhoft, H.C.H., Jorgensen, S.E. 1998. Occurrence, fate and effects of pharmaceutical substances in the environment - A review. *Chemosphere.* 36, 357-394.
- Hammesfahr, U., Heuer, H., Manzke, B., Smalla, K., Thiele-Bruhn, S. 2008. Impact of the antibiotic sulfadiazine and pig manure on the microbial community structure in agricultural soils. *Soil Biol. Biochem.* 40, 1583-1591.
- Jeong, J., Song, W., Cooper, W.J., Jung, J., Greaves, J. 2010. Degradation of tetracycline antibiotics: Mechanisms and kinetic studies for advanced oxidation/reduction processes. *Chemosphere.* 78, 533-540.

- Johnsen, A.R., Svenningsen, H., Henriksen, T., Prieme, A. 2011. Triclosan affects the microbial community in simulated sewage-drain-field soil and slows down xenobiotic degradation. *Environ. Pollut.* 159, 1599-1605.
- Kong, W.D., Zhu, Y.G., Fu, B.J., Marschner, P., He, J.Z. 2006. The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community. *Environ. Pollut.* 143, 129-137.
- Kotzerke, A., Hammesfahr, U., Kleineidam, K., Lamshoft, M., Thiele-Bruhn, S., Schloter, M., Wilke, B.M. 2011. Influence of difloxacin-contaminated manure on microbial community structure and function in soils. *Biol. Fert. Soils.* 47, 177-186.
- Liu, F., Ying, G.G., Tao, R., Zhao, J.L., Yang, J.F., Zhao, L.F. 2009. Effects of six selected antibiotics on plant growth and soil microbial and enzymatic activities. *Environ. Pollut.* 157, 1636-1642.
- Liu, W.N., Pan, N., Chen, W.P., Jiao, W.T., Wang, M.E. 2012. Effect of veterinary oxytetracycline on functional diversity of soil microbial community. *Plant Soil Environ.* 58, 295-301.
- Migliore, L., Godeas, F., De Filippis, S.P., Mantovi, P., Barchi, D., Testa, C., Rubattu, N., Brambilla, G. 2010. Hormetic effect(s) of tetracyclines as environmental contaminant on *Zea mays*. *Environ. Pollut.* 158, 129-134.
- Sarmah, A.K., Meyer, M.T., Boxall, A.B.A. 2006. A global perspective on the use, sales, exposure pathways, occurrence, fate and effects of veterinary antibiotics (VAs) in the environment. *Chemosphere.* 65, 725-759.
- Schmitt, H., Martinali, B., Van Beelen, P., Seinen, W. 2006. On the limits of toxicant-induced tolerance testing: Cotolerance and response variation of antibiotic effects. *Environ. Toxicol. Chem.* 25, 1961-1968.
- Thiele-Bruhn, S., Beck, I.C. 2005. Effects of sulfonamide and tetracycline antibiotics on soil microbial activity and microbial biomass. *Chemosphere.* 59, 457-465.
- Tolls, J. 2001. Sorption of veterinary pharmaceuticals in soils: A review. *Environ. Sci. Technol.* 35, 3397-3406.
- Yang, Q., Zhang, J., Zhu, K., Zhang, H. 2009. Influence of oxytetracycline on the structure and activity of microbial community in wheat rhizosphere soil. *J. Environ. Sci.* 21, 954-959.
- Zhou, Q.X., Bao, Y.Y., Wan, Y., Yu, Q.A., Xie, X.J. 2010. Effects of soil/solution ratios and cation types on adsorption and desorption of tetracycline in soils. *Soil Sci. Soc. Am. J.* 74, 1553-1561.
- Zhou, Q.X., Xie, X.J., Lin, D.S., Guo, J.M., Bao, Y. Y. 2011. Toxic effect of tetracycline exposure on growth, antioxidative and genetic indices of wheat (*Triticum aestivum* L.). *Environ. Sci. Pollut. R.* 18, 566-575.
- Zhu, Y.G., Wu, N., Qiao, M., Zhang, B., Cheng, W. D. 2010. Abundance and diversity of tetracycline resistance genes in soils adjacent to representative swine feedlots in China. *Environ. Sci. Technol.* 44, 6933-6939.