Effect of veterinary oxytetracycline on functional diversity of soil microbial community

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ABSTRACT

Effects of oxytetracycline (OTC) on the functional diversity of soil microbial community with or without vegetation were analyzed in a 7-week greenhouse pot experiment by use of Biolog-Eco plate. Results showed that along with the increase of OTC, average well color development (AWCD) values increased with the peak at 200 ppm OTC, and the utilization of sugar and its derivatives enhanced. The Shannon diversity and the evenness under different OTC levels followed a general order of red amaranth treatments > white clover treatments > no vegetation. The OTC treatments can be distinguished by two principle components. Our findings suggested that OTC addition stimulated soil microbial activities and vegetation changed carbon source utilization pattern at a certain content.

Keywords: veterinary antibiotic; soil microbe; community-level physiological profiling; metabolic fingerprint

With the rapid development of intensive livestock industry, the usage of veterinary antibiotics is increasing continuously. About 100 000 to 200 000 t of antibiotics is used annually in the world (Jeong et al. 2010), and 60–90% antibiotic is released into environment along with animal feces or other paths (Halling-Sorensen et al. 1998). Oxytetracycline (OTC), a kind of tetracycline, is widely used in pig farming for preventing disease or improving immunity. It could persist at mg level in pig manure that is routinely used for organic fertilizer (Migliore et al. 2010). Some studies disclosed effects of OTC on soil enzyme and soil microbial community (Kong et al. 2006, Liu et al. 2009), uptake by plant (Zhu et al. 2007), and distribution of antibiotic resistance gene related to OTC (Hu et al. 2008). However, effects of OTC pollution under different vegetation patterns are seldom studied.

Soil microorganisms, used as main driving factor for transformation of soil materials, reflect healthy states of soil ecosystem at certain content (Kong et al. 2011). Maintenance of biological activity in soil is generally regarded as a key feature of sustainable production to ensure ecosystem functions, and soil microbial properties are often used as indicators for evaluating soil quality (Gomez et al. 2006, Janvier et al. 2007, Shen et al. 2010). Soil microbes are used in studying the pollution effects of heavy metal, pesticide, PAHs, etc., and were identified as a sensitive factor to soil pollution (Megharaj et al. 2000, Andreoni et al. 2004). Biolog-Eco plate for community-level physiological profiling (CLPP) was widely applied in investigating the functional diversity of soil microbial community in different environments (Gomez et al. 2006, Elfstrand et al. 2007, Andersen et al. 2010, Zhang et al. 2010).

In this study, we evaluated the soil microbial community's function change under four OTC concentrations and three vegetation patterns in a greenhouse plot experiment. By use of the Biolog-Eco plate, community-level physiological profiling (CLPP) was studied through investigation of average well color development (AWCD), Shannon diversity index, evenness index, metabolic fingerprint, and principle component analysis (PCA).

MATERIAL AND METHODS

Soil spiking. Soil for the incubation experiment was from a long-term agricultural experiment station

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in Beijing, China. The ploughed topsoil horizon (0–20 cm) was sampled in April 2010. The soil is loam soil with pH of 8.3, organic matter content of 1.2%, clay content of 9.2%, silt content of 38.6%, and sand content of 52.2%, and is typical in the northern China. Swine manure was sampled from the individual household with no antibiotic feeding. The manure was air-dried, sieved (2 mm), and stored at room temperature for use.

Soil was air-dried and sieved (2 mm). Manure was firstly spiked with OTC (Merck, New Jersey, USA) and then thoroughly mixed with soil (30 mg/g soil) and the final OTC concentration in soil was at 0, 1, 15, and 200 ppm, respectively. Fertilizers (urea, KH_2PO_4 and K_2SO_4) were added to soil to support the growth of plant at concentration of 0.15 g N/kg, 0.12 g P/kg, and 0.15 g K/kg. Distilled water was added to adjust the soil moisture content to 50–60% of maximum field capacity for equilibration of oxytetracycline. OTC-spiked soil was then packed into polyvinylchloride (PVC) pots (15 cm high × 10 cm wide), with 1 kg air-dried soil per pot. The pots were sealed and incubated at dark at room temperature for 6 days.

Experimental designs. Red amaranth (*Amarantus mangestanus* L.) was selected due to short growing period and its large cultivation area in farms in the north of China, and white clover (*Trifolium repens* L.) was selected due to its wide application in forage and lawn, in which large amount of organic fertilizer were applied in farms and lawns. OTC concentration is up to 1 ppm in ordinary vegetable soil, and is up to 119–307 ppm in agricultural soil irrigated with domestic wastewater (Zhou et al. 2011). According to reported concentration in the literature, four OTC levels, 0, 1, 15, and 200 ppm, were set in greenhouse experiments, representing the control, normal, high, and extreme high pollution levels of OTC, respectively. A two-factor (P-A) randomized block design was used, in which 'P' was denoted as the vegetation pattern (P0: no vegetation, P1: red amaranth, and P2: white clover) and 'A' was denoted as OTC concentration (A0: 0 ppm, A1: 1 ppm, A2: 15 ppm, and A3: 200 ppm). Each treatment had five replicates and three of them were sampled after 7 weeks for CLPP analysis. Each PVC pot was sown with 15 red amaranth seeds, or 20 white clover seeds. Seven days after their emergence, plants were thinned to 6 and 15 plants. Soil moisture content was maintained at 50% and 70% maximum water holding capacity at early and middle-late stages, respectively. The experiment was carried out in a glass greenhouse (20–27°C during the day and 15–20°C at night).

Community-level physiological profiling. The CLPP of soil microbes was analyzed by the Biolog-Eco plate with 31 carbon source substrates. A pre-experiment of CLPP was carried out to determine reasonable inoculum density. Fresh soil equivalent to 10 g dried soil was dissolved in 95 mL 0.85% NaCl solution, followed by sealing, shaking, standing, transferring 1 mL extraction solution into in a tube containing 9 mL 0.85% NaCl solution, and mixing. And then, 2 mL mixed solution was transferred into another tube containing 18 mL 0.85% NaCl solution and mixed thoroughly. 150 μL of dilution was inoculated into each well of a sterile Biolog-Eco plate. The plates were incubated at 25°C for up to 180 h and measured with a Bio-Rad 1350 automatic Biolog-Eco plate reader at 590 nm, with two measurements per day.

Data analysis. The amount of color development:

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

Where: C_i – the color absorbance value of reaction well; R – the color absorbance value of control well (water only); n – the carbon source number '31'. Shannon diversity index:

$$H = -\Sigma_{i=1}^{n} P_i \times lnP_i$$

Where: P_i – calculated by subtracting the absorbance value of the control well each reaction well and then dividing by the summed color absorbance value of 31 wells. Evenness index:

$$E = H/\ln S$$

Where: H – Shannon index; S – the carbon source number of Biolog-Eco plate, 31.

Metabolic fingerprint was used for evaluating the utilization of sole carbon source. The carbon source substrates were divided into four categories, including sugar and its derivatives (SD), amino acid and its derivatives (AD), fatty acid and its derivatives (FD), and secondary metabolites (SM). AWCD, Shannon diversity index and evenness index were compared by one-way ANOVA. PCA was conducted with SPSS13 to evaluate the influence of OTC and vegetation on soil microbial community.

RESULTS

Average well color development. According to Figure 1, there was a trend to increased AWCD with increased OTC concentrations, which was significantly only for the highest concentration, namely, the soil microbial activity was activated by addition of OTC (Figure 1a). The AWCD values maintained at low levels for 48 h and then increased

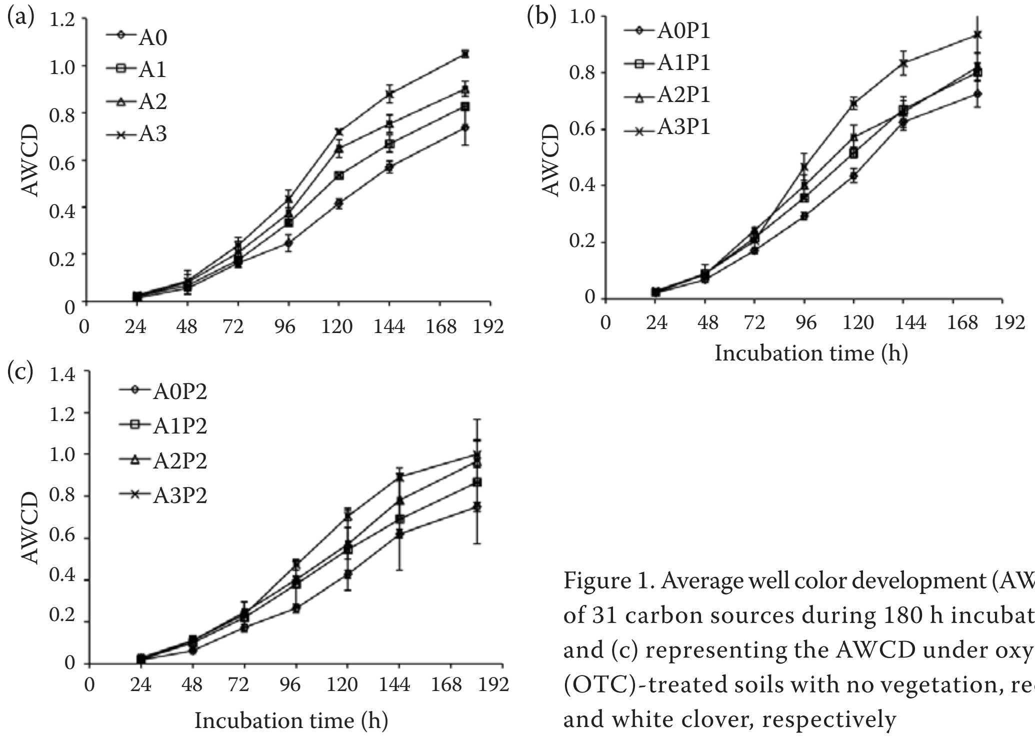

Figure 1. Average well color development (AWCD) change of 31 carbon sources during 180 h incubation, (a), (b), and (c) representing the AWCD under oxytetracycline (OTC)-treated soils with no vegetation, red amaranth, and white clover, respectively

rapidly, with the peak at 200 ppm. The changing trends of AWCD under P1 and P2 treatments were consistent (Figures 1b–c). Figure 2 illustrated the differences of the AWCD values at 180 h incubation. Under P0 treatments, the AWCD value at OTC of 200 ppm was 42.1%, 26.9%, and 16.6% higher than those at OTC of 0, 1, and 15 ppm, respectively. Under P1 treatments, the AWCD values at OTC of 200 ppm was 29.0%, 16.4% and 13.9% higher than those at OTC of 0, 1, and 15 ppm, respectively. Under P2 treatments, the AWCD value at OTC of 200 ppm was 33.2%, 15.0%, and 3.5% higher than those at OTC of 0, 1, and 15 ppm, respectively.

Metabolic fingerprint. As shown in Figures 3a–c, the values of AWCD > 1 were chosen to analyse changes of carbon source utilization. In Figure 3a, the utilization of both SD and AD strongly increased with high OTC concentration. SD was the main carbon source for soil microorganisms. Similarly, under P1 treatments, the utilization of both SD and AD strongly increased under high OTC concentration, and more secondary metabolites were used as carbon sources (Figure 3b). Under P2 treatments (Figure 3c), the carbon source utilization were very similar under the OTC of 1, 15, and 200 ppm; compared to no OTC treatments, much more fatty acid and its derivatives were used with addition of OTC. In general, utilization of SD and AD increased with the increase of OTC concentration. With vegetation present, the utilization of FD and SM increased.

Shannon diversity index and evenness index. According to Figure 4a, the Shannon diversity followed a general order of P1 > P2 > P0, and

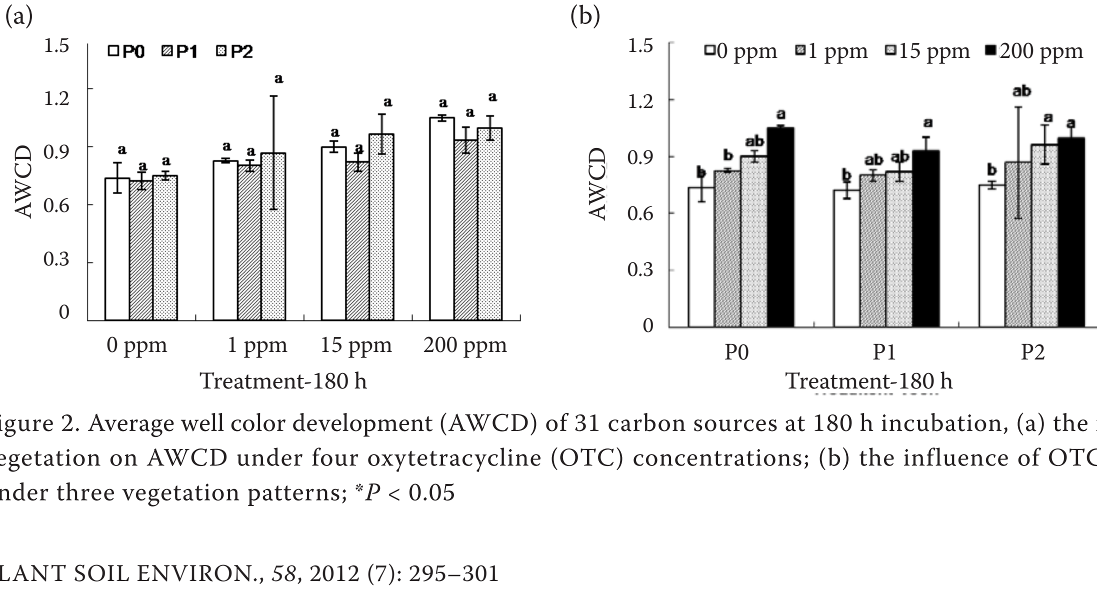

Figure 2. Average well color development (AWCD) of 31 carbon sources at 180 h incubation, (a) the influence of vegetation on AWCD under four oxytetracycline (OTC) concentrations; (b) the influence of OTC on AWCD under three vegetation patterns; *$P < 0.05$

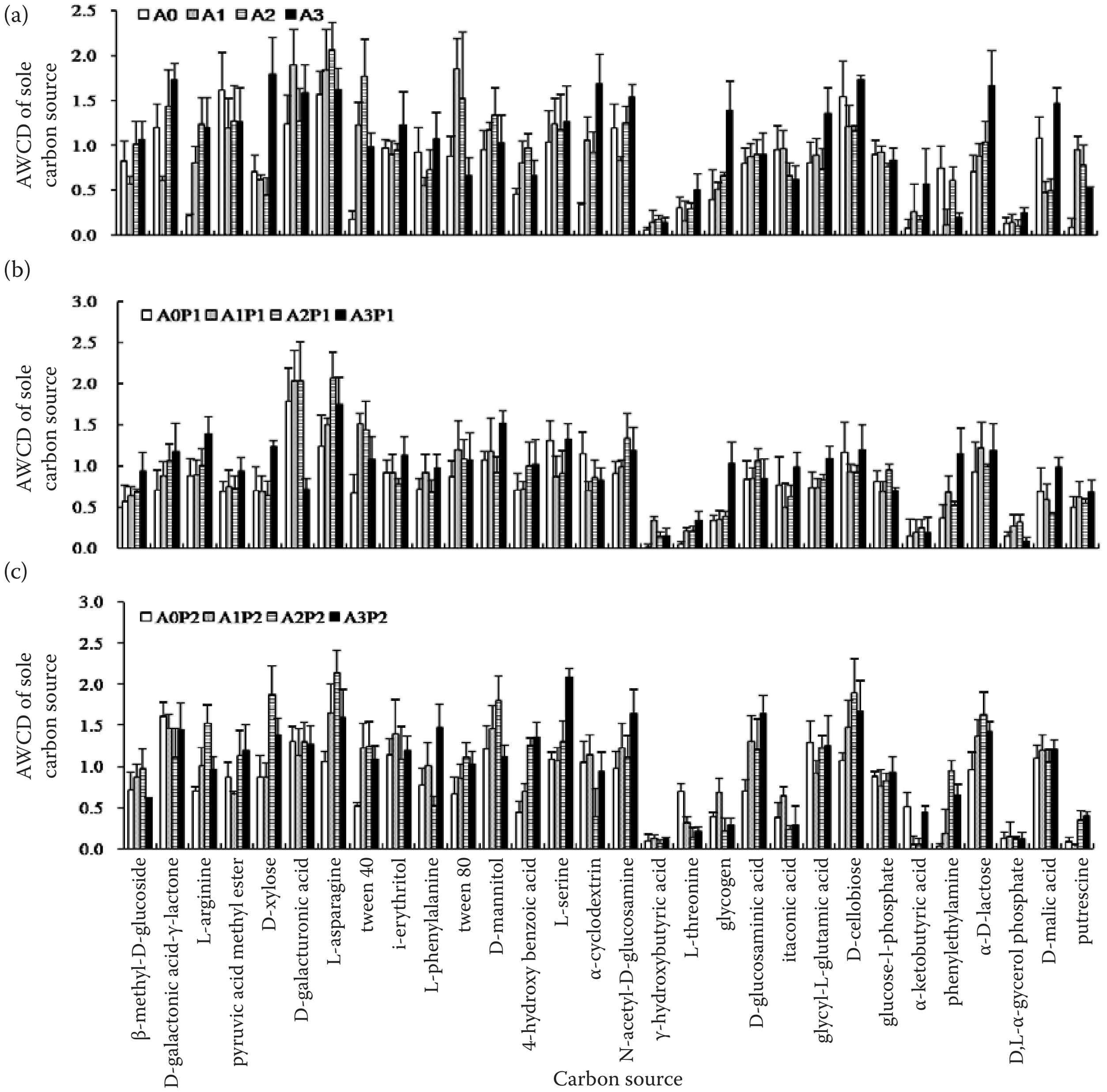

Figure 3. The metabolic fingerprint of sole carbon source at 180 h incubation, each carbon source substrate belonging to sugar and its derivates (SD), amino acid and its derivates (AD), fatty acid and its derivates (FD), or secondary metabolites (SM), and average well color development (AWCD) indicating the average value of three replicated results of one Biolog-Eco plate

there was no significant difference among them by ANOVA analysis. As shown in Figure 4b, under P0 treatments, the Shannon diversity with OTC treatments under 200 ppm was significantly different from that without OTC. Under P1 treatments, the Shannon diversity at 15 and 200 ppm were significantly different from that under no-OTC treatments. Under P2 treatments, there were no significant differences among the Shannon diversity index at four OTC levels. Similarly, the evenness index followed a general order of P1 > P2 > P0 (Figure 4c), but there were no significant differences among different treatments at *P* level of 0.05. Figure 4d further showed that while the evenness index with OTC treatments were clearly higher than those with no OTC treatments, effects of OTC on soil microbal community were not significant for three types of vegetation treatments. In general, additon of OTC tended to increase Shannon diversity and evenness of soil microbial community, which was significant for the effect of the highest OTC treatment on Shannon diversity, while the impacts of vegetation were quite limited.

Principle component analysis. As shown in Figure 5, the four OTC treatments formed distinct groups under P0 treatments and P1 treatments,

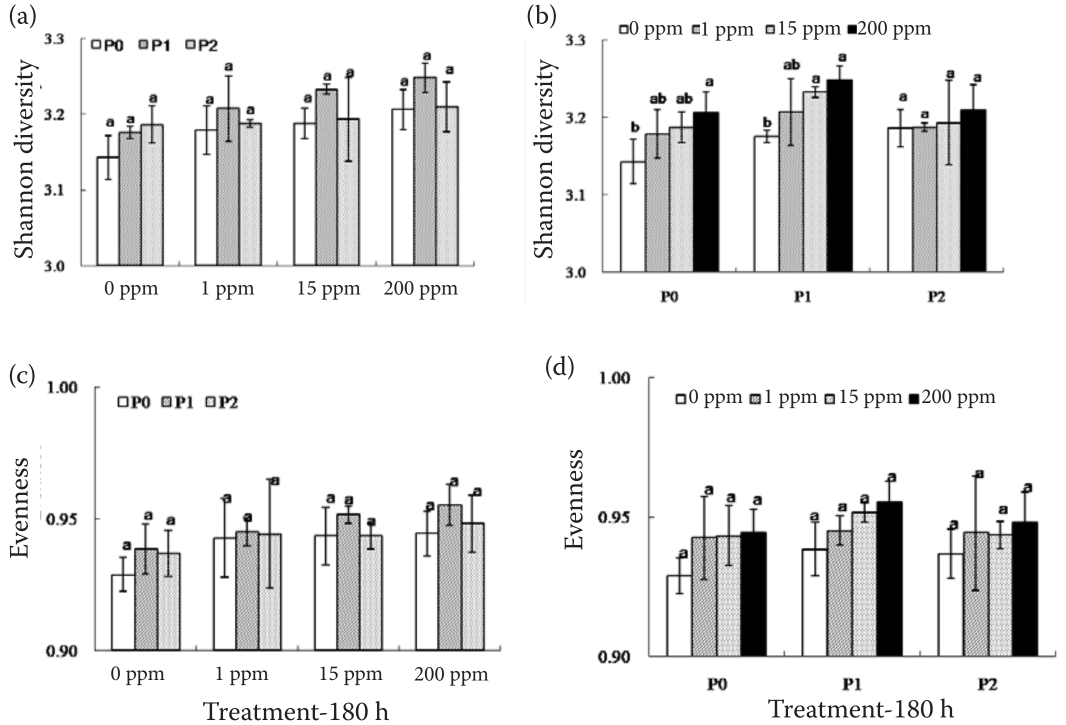

Figure 4. Shannon diversity and evenness of soil microbial community following addition of oxytetracycline (OTC); $^*P < 0.05$

while under P2 treatments, no obvious groups formed. In Figure 5a, under the OTC levels of 1 and 15 ppm, OTC treatment shifted communities along the 2nd axis while it shifted microbial communities along both axes at 200 ppm OTC level. In Figure 5b, shifts of communities were smaller and restricted to the highest OTC treatment. In Figure 5c, addition of 1 ppm OTC seemed to be without an effect on the structure of community, while both higher OCT concentrations (15 ppm and 200 ppm) shifted the communities along the first axis. In general, the treatments at four OTC levels could be seperated by two principle components.

DISCUSSION

In our experiment, the AWCDs at OTC-treated soils were higher than those without OTC treatments, and along with increase of OTC concentration, the AWCDs increased (Figure 1). The AWCD values of 15 and 200 ppm OTC were significantly higher than those with no OTC (Figure 2). And the Shannon diversity at soils treated with highest or higher OTC concentrations were significantly different from that without OTC, further indicating OTC effects on the function of soil microbial community (Figure 4b). Higher AWCD at high OTC

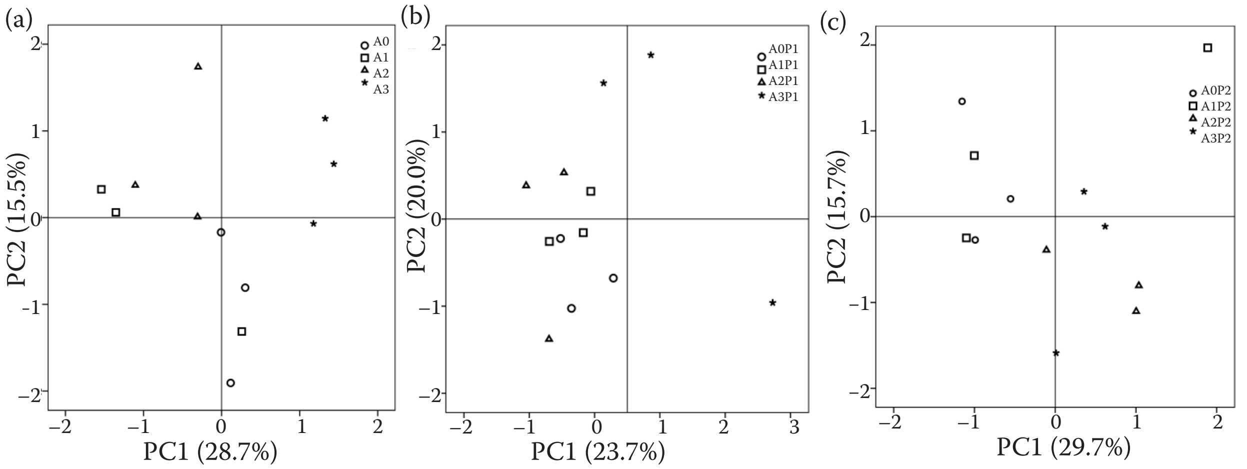

Figure 5. Principle component analysis (PCA) results of carbon utilization potential of soil microbial community

level may be related to changes in community composition at these OTC concentrations, which were shown to occur for tetracyclines in soil (Schmitt et al. 2004, Demoling et al. 2009). Thiele-Bruhn and Beck (2005) indicated that basal respiration and dehydrogenase activity were uninfluenced by 1000 ppm tetracycline, which may be contributed to biostasis and dormant state of most soil microbes, and OTC can exert a temporary selective pressure on soil microbes. Moreover, manure can greatly change ecological toxicity effects in soil (Boleas et al. 2005), and in our study addition of manure used as fertilizer may be one factor for high AWCD at high OTC concentration.

Vegetation can influence composition and biomass of microbial community in natural soil or treated soil (Yang et al. 2007, Ladygina and Hedlund 2010). Root exudates related to plant species are key factors for reflecting soil microbial activites. Under stress of pollutants, the activities of soil microbes are inhibited, and some soil enzymes and amino acid, sugars, and other secondary metabolites are released by roots for relieving pollutant toxicity (Muratova et al. 2009). In this study, compared with non-vegetated treatments, the vegetation treatments were different in the Shannon diversity and evenness index (Figure 4). The AWCDs under P1 treatments at 15 and 200 ppm were lower than those of the other two treatments (Figure 2a). The activities of soil microbes may be inhibited by the exudates from red amaranth root, while the activities of soil microbes of P2 treatments had no obvious change due to slow growth of white clover. Therefore, root exudates may contribute to the function change of soil microbial community. Also, utilization of SD and AD increased with increase of OTC concentration. With the presence of vegetation, the utilization of FD and SM increased, suggesting that root exudates of vegetation accelerated the utilizaiton of FD and SM (Figure 3). Previous studies also indicated that utilization of carbon sources by soil microbes was related to root exduation (Gao et al. 2010, Toyama et al. 2011). The PCA results also showed the compositions and community structures of antibiotics exposed soil microbial communities differred according to the vegetations (Figure 5).

It is difficult to clearly disclose effects of OTC on soil microbes due to multiple properties of soil and OTC itself. Here, high AWCD of soil micrbial compostion indicated high microbial activities, which could be partly due to stress effect of OTC on soil microorganisms, or due to higher energy demand for their survival, eventually for the change of microbial community structure. However, considering the limitation of Biolog-Eco plate, further investigation is needed to understand effect of OTC on the structure of soil microbial community, interactions of soil microbe and root exudates induced by OTC, and OTC-induced PICT.

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