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Influence of oxytetracycline on the structure and activity of microbial community in wheat rhizosphere soil

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Abstract

The microbial community composition in wheat rhizosphere was analyzed by detecting colony forming units (CFUs) in agar plates. The total CFUs in rhizosphere were 1.04×10^9/g soil with 9.0×10^8/g bacteria, 1.37×10^8/g actinomyces and 3.6×10^6/g fungi. The 10 dominant bacteria were isolated from wheat rhizosphere and were grouped into genus *Bacillus* according to their full length 16S rRNA gene sequences. Although belonging to the same genus, the isolated strains exhibited different sensitivities to oxytetracycline. When a series of the rhizosphere soil was exposed under various concentrations of oxytetracycline, the microbial community structure was highly affected with significant decline of CFUs of bacteria and actinomyces (22.2% and 31.7% at 10 mg/kg antibiotic, respectively). This inhibition was clearly enhanced with the increase exposure dosage of antibiotic and could not be eliminated during 30 d incubation. There was no obvious influence of this treatment on fungi population. Among the four soil enzymes (alkaline phosphatase, acidic phosphatase, dehydrogenase and urease), only alkaline phosphatase was sensitive to oxytetracycline exposure with 41.3% decline of the enzyme activity at 10 mg/kg antibiotic and further decrease of 64.3%–80.8% when the dosage over 30 mg/kg.

Key words: wheat rhizosphere; microbial community; antibiotic sensitivity; oxytetracycline

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Introduction

With the development of medicine and biological techniques, more and more new antibiotics appear and facilitate the therapy. By statistic, antibiotics consumption in Europe has reached 10000 tons each year. In China, the production and usage of penicillin, tetracycline, doxycycline, cefotaxime sodium has among the front range of the world (Richardson *et al*., 2005).

Antibiotics are not only used in human therapy but also used in farming and in aquaculture for prevention, for therapy and as antimicrobial substances to improve nutrient uptake in the gastrointestinal tract (grow promoters). Approximately 50% of the antibiotics consumption is used in animals and more than 70% of veterinary antibiotics consumption in the USA is as growth promoters. Tetracyclines (TCs) are among the most extensively used growth promoters and therapeutic drugs in animal agriculture. According to previous studies, 4 mg/kg of this drug was detected in liquid manure and was not eliminated during the storage over a period of 120 d (Kummerer, 2001; Hamscher *et al*., 2002). Various veterinary antibiotics could enter into soils through fertilization using animal manure.

Sørensen *et al*. (2005) investigated the fate of chlortetracycline in a field study and could detect the compound for the whole experimental period of 5 months. According to Kunmerer's review (2001), tetracycline could be measured in topsoil treated with manure in high concentration (20 mg/kg soil). Bruhn and Beck (2005) reported that mean concentration of tetracycline in soils approached 300 ng/g. Concentration of tetracycline in the range 0.003–7.35 μg/g could inhibit 10% of soil microbial activity. Due to the persistence and accumulation of such chemicals in soil, pollution of antibiotics may pose a risk to the soil microbial ecosystem even to plants.

At present, antibiotics pollution on aquaculture microbial community has been deeply studied (Eleonor, 2001). Some reports were focused on their operation mechanism in soil except a few about their degradability (Barr and Aust, 1994; Wetzstein *et al*., 1999). As far as their resident in environment after fertilization, the effect on microbial community, activity and diversity were less mentioned (Mohamed *et al*., 2005; Wong *et al*., 2004).

Oxytetracycline is one of tetracyclines and is widely used in chicken and pig farming in China. Therefore, in this study, effects of oxytetracycline on soil microbial community as well as enzyme activities were investigated in detail using dominant microbial isolates and lab cultivated soil systems. Their dosage relation was addressed preliminarily.

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1 Materials and methods

1.1 Sample collection

The experimental cornfield in Henan Normal University, China was used as sampling site due to its antibiotic pollution free. Soil samples in wheat rhizosphere were collected at the growing stage of wheat seedling in the spring of 2006. One sample was the soil mixture from 5 spots in the same cornfield.

1.2 Soil microbial community analysis

Colony forming units (CFU) of bacterium, actinomyces and fungi were determined by a modified plate dilution technique on meat-peptone agar, Gauses starch agar and Martin agar, respectively (Carter, 1993). The incubation temperatures for microorganisms were 28 and 37°C. The incubation duration was 1–2 d for bacterium, 3–6 d for actinomyces, and 3–5 d for fungi. The number of every group of microbes was determined by the average of three replicate soil samples.

1.3 Isolation and identification of dominant bacteria in wheat rhizosphere soil

Dominant bacteria in the soil of wheat rhizosphere were selected from the above agar plates by their morphologic characteristics of colonies on LB agar and further purified at least 3 times. Genomic DNA was extracted according to the standard method (Stahl, 1988). 16S rRNA genes for the purified dominant bacterial isolates were amplified using an eubacterial primer SSEub27F and a universal primer SS1492R as standard methods (Koblizek, 2003). PCR fragments containing 16S rRNA genes were extracted from agarose gels using TaKaRa purification kit and were cloned into the PMD 18-T vector. The recombinant plasmid DNA was extracted by standard procedures and sequenced by Shanghai Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China).

All the nucleotide sequences were analyzed by performing a BLAST search (http://www.ncbi.nlm.nih.gov/BLAST/). Alignments of 16S rRNA genes of the isolates and their closest relatives were carried out with Clustal X1.8 program. Phylogenetic trees and evolutionary distances were constructed using Mega 4.1 software package.

Sequences of 16S rRNA genes determined in this study were deposited in GenBank under the accession numbers EF636889 to EU636898.

1.4 Antibiotic sensitivity study of the dominant bacterial isolates

One milliliter suspension of dominant bacterial isolate was inoculated into a series of flasks with 100 mL sterile LB liquid medium containing final concentration of tetracycline 10, 50, 100, 500, or 1000 μg/L. The bacteria were cultivated at 150 r/min and 37°C. The growth of bacteria was measured by detecting the OD values every about 6–8 h at 600 nm visible spectrum using type 752 spectrophotometer (Shanghai Precision & Scientific Instrument Co., Ltd.). The sterile LB medium was used as blank and bacteria flask without antibiotic was used as control.

1.5 Influence of antibiotics on soil microbial community and activities

A series of garden pots were prepared with soil samples collected from the wheat rhizosphere. Oxytetracycline was added by mixing together with clean sand at the final antibiotic concentration of 10, 50, 100, and 150 mg/kg, respectively. The soil enzyme activities and structure of microbial community were detected and analyzed at a certain interval. All tests were conducted on three replicate samples.

1.6 Determination of enzyme activities

Four soil enzymes, urease, acidic phosphatase, alkaline phosphatase, and dehydrogenase, were detected under different concentrations of antibiotic exposure. For each of the following enzyme assay, three subsamples replicate per treatment were analyzed using the field-moist soil. Dehydrogenase activity was determined by colorimetric measurement of the reduction of 2,3,5-triphenyltetrazolium chloride (TTC) to triphenylformazan (TPF) according to the method by Casida *et al*. (1964). Acid (or neutral) phosphatase activity at pH 6.5 and also the activity of arylsulfatase were determined by measuring the release of *p*-nitrophenol (PNP) when soil was incubated with *p*-nitrophenyl phosphate or *p*-nitrophenyl sulfate as described by Tabatabai and Bremner (1969, 1970). Urease activity was determined using the nonbuffer method by Zantua and Bremner (1975).

2 Results and discussion

2.1 Structure of microbial community in wheat rhizosphere soil

CFUs of bacteria, fungi, and actinomyces in wheat rhizosphere soil were detected. The results showed that the CFUs for total microbial population in wheat rhizosphere soil was 1.04×10^9/g soil with 9.0×10^8/g bacteria, 1.37×10^8/g actinomyces and 3.6×10^6/g fungi. Bacteria were absolutely the dominant population.

Combining the microscopic observation and colony characteristics, 10 dominant bacterial strains were isolated from the selected medium and further purified at least three times.

2.2 Phylogenetic analysis of dominant bacteria isolated from wheat rhizosophere

16S rRNA gene sequences of the 10 dominant bacterial isolates were determined and compared with other sequences contained in public databases (Table 1) and phylogenetic tree (Fig. 1). As shown in Table 1, almost all the isolates had the highest similarity scores with Bacillaceae bacteria, in which only 2 (WRB-2 and WRB-4) had the maximum identity over 97%. The low similarity scores of other isolates with the known bacterial strains suggested a large number of possibly novel microorganisms in the

Table 1 Dominant isolates in wheat rhizosphere soil and their identification by 16S rRNA gene sequences

Strain	Accession number	Similarity strain	Maximum identity (%)
WRB-1	EF636890	Uncultured bacterium clone B15(EF655638)	93
WRB-2	EF636889	*Bacillus* sp. B2(2007) (EU196519.1)	99
WRB-4	EF636891	*Bacillus* sp. DSK25 (D88778.1)	97
WRB-6	EF636892	*Bacillus* sp. 171544 (AF071856)	94
WRB-10	EF636893	Bacillaceae bacterium KVD-1982-07 (DQ490423.1)	95
WRB-11	EF636894	Bacillaceae bacterium KVD-1982-07 (DQ490423.1)	92
WRB-12	EF636895	*Bacillus* sp.171544 (AF071856.1)	96
WRA-1	EF636896	Bacillaceae bacterium KVD-1982-07 (DQ490423.1)	94
WRA-3	EF636897	*Bacillus* sp. BWDY-12 (DQ314537)	96
WRA-4	EF636898	*Bacillus* sp. NAF001 (AB049195)	95

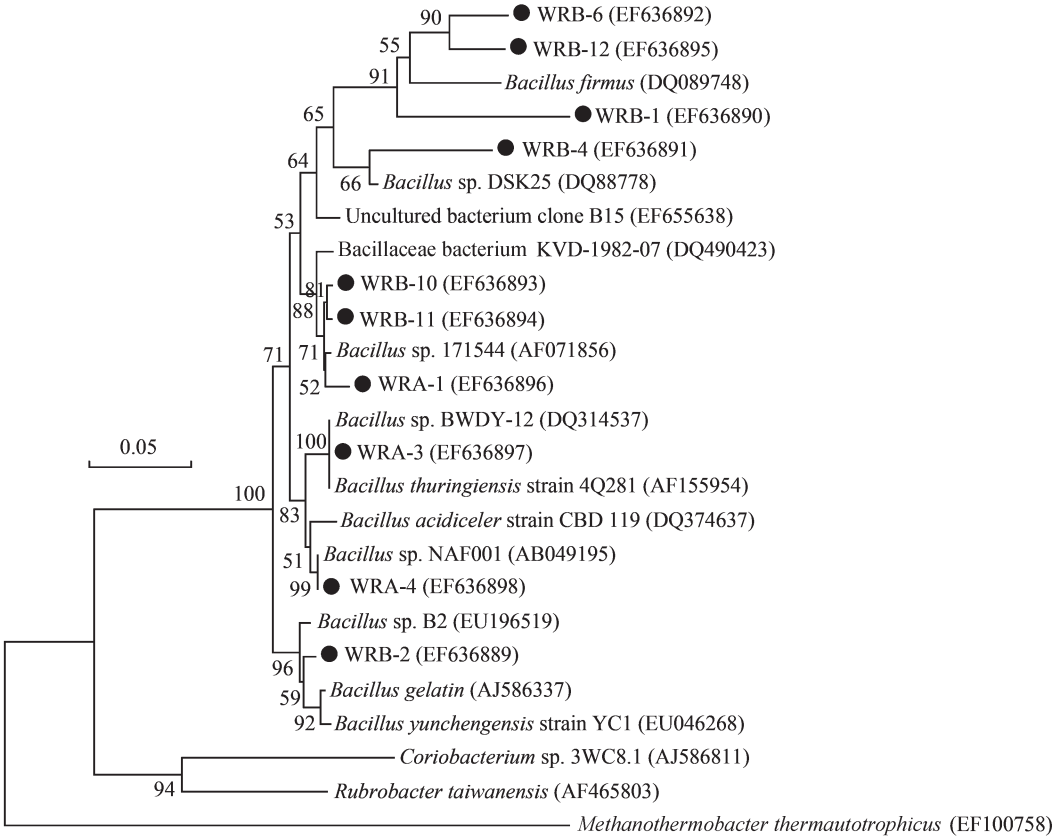

Fig. 1 Neighbor-joining tree established by the alignments of 16S rRNA gene sequences of dominant microbes from the wheat rhizosphere soils and representative references retrieved from Genbank. Clones from this study are indicated in boldface. Bootstrap values above 50 (100 iterations) are shown at each node. Scale bar represents the nucleotide substitution percentage. 16S rDNA sequences from *Actinobacteria* and an archaea, *Corynebacterium glutamicum* were used as outgroups.

wheat rhizosphere.

A phylogenetic tree of the 10 dominant bacterial isolates was constructed by their 16S rRNA gene sequences using the neighbor-joining method (Fig. 1). Phylogenetic analysis clearly indicated that all the isolates fell within genus of *Bacillus*. Strains affiliated to *Bacillus* were further subdivided into three clusters: four strains fell into the first cluster, in which WRB-6 and WRB-12 were grouped tightly with WRB-1 and had a high evolutional relationship with an unpublished strain *Bacillus firmus* (DQ089748) isolated from soils treated with fumigant agents (Mocali *et al*., 2008); WRB-4 had the most comparability with *Bacillus* sp. DSK25 (D88778) isolated from the deep-sea (Li *et al*., 1998). The second cluster happened in WRB-10, WRB-11, and WRA-1, which were closely related with AF071856, endospore-forming bacteria isolated from silage following heat enrichment (Silva *et al*., 1998). WRA-3, WRA-4 and WRB-2 were clustered into other different *Bacillus* species.

Bacillus strains were proved to be a group of plant growth-promoting rhizobacteria (PGPR). Zhang and Lu (1998) reported their isolation results from different plant rhizospheres indicating that high ratio of *Bacillus polymyxa* was existent in wheat rhizosphere. Yang and Sun (1999) screened the entophytic bacteria strains isolated from the roots, stem, leaves and seeds of rice "Yuefu" through measuring the activities of acetylene reducing and $^{15}N_2$-fixing. Strains of bacteria possessing the activity of N_2-fixing were obtained and classified into 9 genera and 14 species including *Agrobacterium*, *Enterobacter* and

Bacillus. The research of Sundarad (1964) gave the conclusion that *Bacillus* was the domain phosphate dissolving rhizobacterium in wheat rhizosphere. Except the nitrogen fixation and phosphate dissolving, *Bacillus* also exerts PGPR function in other aspects. Zhuang *et al*. (2007) found that *Bacillus megaterium* HKP-1 played an important role in avoiding mustard from toxic heavy metal pollution. Prikryl (1980) found that wheat rhizobacterium could transform some organic compounds secreted by plant roots in rhizosphere and could stimulate the roots to secrete more compounds correspondingly.

2.3 Antibiotic sensitivity of the rhizosphere dominant bacteria

As discussed above, all the rhizosphere dominant bacteria fell into the genus *Bacillus* and this genus strains were largely reported to be plant growth-promoting rhizobacteria. Thereby, pollutants which influence this group of bacteria may be disadvantage to microbial community and soil activities.

Oxytetracycline was widely used antibiotic in animal treatment and growth promoter. The mechanism was to inhibit synthesis of protein by working on the 30S ribosome and to prevent the combination of tRNA and ribosome. At the same time, oxytetracycline had a high affinity to soil (Zhang and Dong, 2005), which meant difficult to be decomposed once entering into the soil. Based on those reasons, oxytetracycline was selected as a target pollutant to investigate the potential risk of antibiotics on soil microbial ecology in our study. Figure 2 showed the growth of the isolated rhizosphere dominant bacteria within 24 h under exposure of various oxytetracycline concentrations.

Figure 2 shows that the sensitivity of the 10 isolates to various concentrations of oxytetracycline was quite different. The most obvious inhibition happened on WRB-4 and WRB-6, in which a very low concentration of oxytetracycline as 0.05 mg/L could completely hinder their growth. Growth of WRB-1 and WRB-2 was completely inhibited under the concentration of 0.10 and 0.50 mg/L oxytetracycline, respectively. WRA-3 and WRA-4 had stronger resistance to oxytetracycline and could grow well even under the concentration of 1.00 mg/L. Other isolates exhibited different degree of inhibition by oxytetracycline. Similar study was conducted by Ni (1999), in which the sensitivity of beneficial *Bacillus* to 10 mg/L tetracycline was detected and all of the experimental strains exhibited high sensitivity. However, from our results, we can detect that although the dominant bacterial isolates of wheat rhizosphere were clustered in the same genus, their sensitivity to oxytetracycline were completely diverse. It is easily speculated that once the soil was polluted by antibiotic, the microbial community structure would be highly affected.

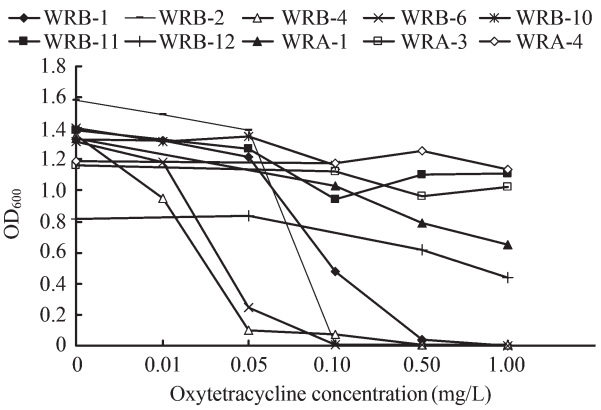

Fig. 2 Growth of the dominant bacterial isolates from wheat rhizosphere soil under various concentrations of oxytetracycline (24 h cultivation). WRA-1 to WRA-4 and WRB-1 to WRB-12, dominant bacterial isolates; OD_{600}, spectrophotometric values detected at the wavelength of 600 nm; Values are means ± standard deviations for three replicates.

2.4 Influence of oxytetracycline on soil microbial community

Influence of oxytetracycline on soil microbial community was investigated in a series of incubated soil pots. CFUs of bacteria, actinomyces, and fungi in the soils were counted in a certain period as described above. The dynamic changes of CFUs against incubation time under different concentrations of oxytetracycline were shown in Fig. 3.

As shown in Fig. 3, the CFUs of bacteria, actinomyces, and fungi fluctuated in 6.3×10^8–9.0×10^8/g, 7.7×10^7–1.4×10^8/g, and 2.7×10^6–3.6×10^6/g, respectively, during the whole incubation period. Adding oxytetracycline even only 10 mg/kg in the soil would affect the microbial community structure positively, especially the populations of bacteria and actinomyces. The most obvious response was observed at day 5 of incubation, in which

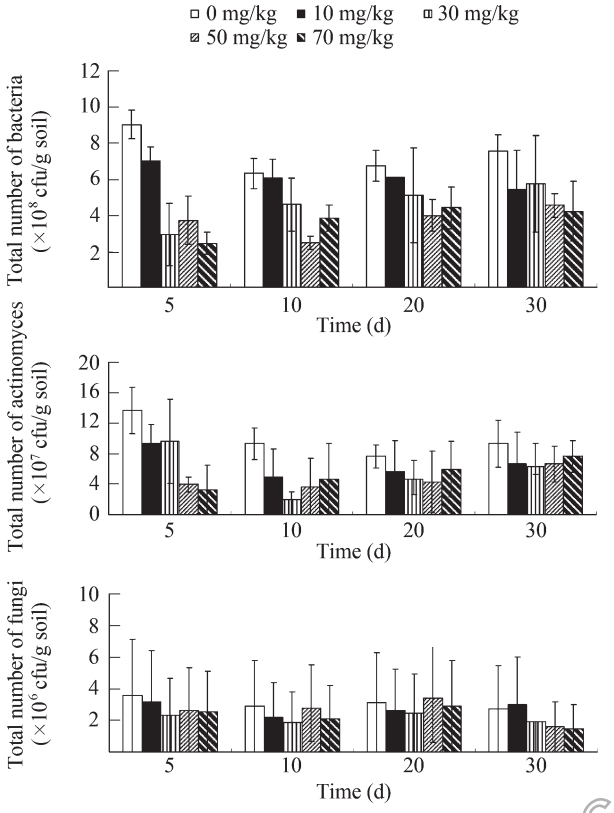

Fig. 3 Dynamic change of microbial community compositions under different concentrations of oxytetracycline exposure. Values are means ± standard deviations for three replicates.

the CFUs of bacteria had a 22.2% decline at 10 mg/kg oxytetracycline exposure and a dramatic drop of 58.7%–72.7% at the concentration over 30 mg/kg. The effect of oxytetracycline lasted till the end of the experiment (30 d cultivation and detection) although a slight recovery of the CFUs was observed comparing with the control. Combining the results of Fig. 2, the reduced bacterial CFUs should be mainly from the dominant bacteria, species of genus *Bacillus*.

Changing trend of CFUs of actinomyces was similar with bacteria. Oxytetracycline obviously reduced the CFUs of actinomyces under all the tested concentrations. The inhibition rate of actinomyces reached 31.7% at the concentration of 10–30 mg/kg and 70.7%–75.6% at 50–70 mg/kg antibiotic exposure, respectively, during 5 d cultivation. After 30 d this inhibition could not be relieved completely.

Compared with bacteria and actinomyces, fungi had no significant response to the treatment of oxytetracycline. The CFUs of fungi fluctuated in the same order of magnitude in all the series of treatment and in the whole cultivation process. This result was identical to our expectation because oxytetracycline mainly worked on prokaryotic microorganisms.

2.5 Influence of oxytetracycline on soil enzymes

Four soil enzymes, urease, acid phosphatase, alkaline phosphatase and dehydrogenase were detected in the above treated soil systems during the incubation process. The dynamic changes of the enzyme activity against incubation time were plotted in Fig. 4.

Phosphatase catalyzes organic phospholipid into inorganic phosphate that is available for plants. This enzyme is a mostly found soil enzyme originating from bacteria or fungi and used as an indicator for soil activity (Liu *et al*., 2008). As shown in Fig. 4, the activity of alkaline phosphatase was significantly higher than acid phosphatase, which was possibly related with the slight alkaline of soil (pH 7.80). Contrary to the unchanged acid phosphatase, alkaline phosphatase was very sensitive to oxytetracycline exposure under all tested concentrations and exhibited obvious dosage effects. The highest inhibition was observed at day 5, in which the activity of alkaline phosphatase decreased 41.3% under the concentration of 10 mg/kg oxytetracycline and had a further drop 64.3%–80.8% under over 30 mg/kg antibiotic exposure. This inhibition lasted till day 20 and had a slight recovery by the end of the experiment. Although the soil system was very complicated, the changing trend of alkaline phosphatase was completely in accordance with the CFUs of bacteria and actinomyces suggesting that bacteria and actinomyces had a great contribution to this enzyme. Our results were consistent with the results about the relation between microbial community structure and soil enzyme activity reported by Chen *et al*. (2008). They found that the amount of bacteria in the canopy soil of *Tamarix* spp. strongly affected the activities of alkaline phosphatase, hydroperoxidase, invertase and dehydrogenase, but had no effect on urease and polyphenol oxidase. However, the population of fungi had a significant correlation with the activities of urease and polyphenol oxidase. In this study, genus *Bacillus* was the dominant bacteria in wheat rhizosphere soil. Therefore, exposure of oxytetracycline mainly inhibited this group of bacteria and resulted in the decline of soil enzyme activity. This conclusion was also supported by the research of Sundarad *et al*. (1963), in which genus *Bacillus* were proved to be the domain phosphate dissolving rhizobacterium in wheat rhizosphere.

Urease is involved in the terminal N-cycle in which organic N is transformed to plant-available ammonia and protease–BAA, which is sensitive to N fertilization (Liu *et al*., 2008). Dehydrogenase is an intracellular enzyme

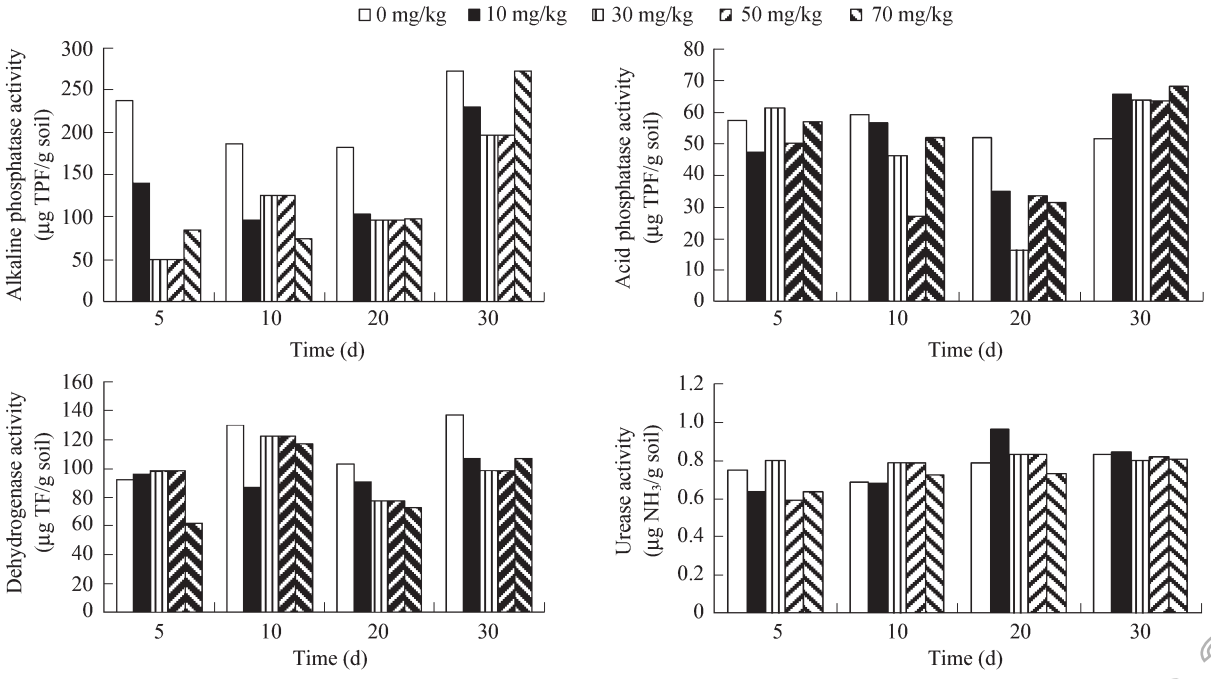

Fig. 4 Dynamic change of soil enzyme activity under different concentrations of oxytetracycline.

involved in the energy transfer in respiration chain and is considered as an index of viable microbial activity in soils. Figure 4 indicated that there was no clear effect of oxytetracycline on the activities of urease and dehydrogenase. For urease activity, it was coincident with the conclusion by Chen *et al*. (2008) as described above. However, the lack of clear correlation between dehydrogenase activity and variation of microbial structure may be interpreted by the possible confounding chemical reactions affecting the measured activity (Udawatta *et al*., 2008) or by soil abiotic conditions such as pH, metal ions, etc., influencing the enzyme activity (Liu *et al*., 2008). Another possible interpretation was existence of large amount of uncultivable microorganisms in the soil. As well known, only about 1% microorganisms was cultivable in the soil. When some sensitive bacteria or actinomyces were inhibited by antibiotics, some other insensitive microorganisms might grow better and contribute more activity in a certain ecosystem. However, in our similar experiments using tetracycline, dehydrogenase was more sensitive to the treated conditions and other three enzymes had no response although the changing trends of CFUs were similar in the two cases. Considering the complex of microbial community in the soil, further study is required on molecular biological analysis and the relation between microbial population and enzymes.

Acknowledgments

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