Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 2010, 22(7) 1040–1048
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

# Effects of plant species coexistence on soil enzyme activities and soil microbial community structure under Cd and Pb combined pollution

Yang Gao[1,3], Pei Zhou[2,3,*], Liang Mao[2], Yueer Zhi[2], Chunhua Zhang[2], Wanjun Shi[2]

*1. School of Environmental Science and Engineering, Shanghai Jiaotong University, Shanghai 200240, China. E-mail: gaoyang0898@sjtu.edu.cn*
*2. School of Agriculture and Biology, Shanghai Jiaotong University, Shanghai 200240, China*
*3. Key Laboratory of Urban Agriculture (South), Ministry of Agriculture, Shanghai 200240, China*

Received 02 June 2009; revised 13 July 2009; accepted 27 July 2009

**Abstract**

The relationship between plant species coexistence and soil microbial communities under heavy metal pollution has attracted much attention in ecology. However, whether plant species coexistence could offset the impacts of heavy metal combined pollution on soil microbial community structure and soil enzymes activities is not well studied. The modified ecological dose model and PCR-RAPD method were used to assess the effects of two plant species coexistence on soil microbial community and enzymes activities subjected to Cd and Pb combined stress. The results indicated that monoculture and mixed culture would increased microbe populations under Cd and Pb combined stress, and the order of sensitivity of microbial community responding to heavy metal stress was: actinomycetes > bacteria > fungi. The respirations were significantly higher in planted soil than that in unplanted soil. The plant species coexistence could enhance soil enzyme activities under Cd and Pb combined. Furthermore, planted soil would be helpful to enhance soil genetic polymorphisms, but Cd and Pb pollution would cause a decrease on soil genetic polymorphisms. Mixed culture would increase the ecological dose 50% ($ED_{50}$) values, and the $ED_{50}$ values for soil enzyme activities decreased with increasing culture time. The dehydrogenase was most sensitive to metal addition and easily loses activity under low dose of heavy metal. However, it was difficult to fully inhibit the phoshpatase activity, and urease responded similarly with phosphatase.

**Key words**: ecological dose; enzyme activities; DNA; RAPD; heavy metal
**DOI**: 10.1016/S1001-0742(09)60215-1

## Introduction

Soil microbes are important reflection for soil quality and enzyme activity involved in the biogeochemical cycling of carbon, nitrogen, phosphorus, sulphur and other nutrients (Caldwell, 2005). There is strong evidence that soil microbes and enzyme activities are sensitive to heavy metal contamination (Yang et al., 2006; Chen et al., 2005). Therefore, soil enzyme activities are widely used as biological indicators for soil health and estimating the adverse effects of various pollutants on soil quality. Among the different enzymes in soils, dehydrogenase, urease and phosphatases are important in the transformation of different plant nutrients (Muhammad et al., 2005).

Heavy metal is toxic to almost all bacteria, by inhibiting basic cellular functions, which are linked with energy metabolism (Walker et al., 2000; Lorenz et al., 2006). As a result, decreases in microbial populations have been reported in the soils polluted with heavy metal compounds (Renella et al., 2005). Plants can affect the soil biota by influencing the quantity and quality of organic substrates that reach the soil (Viketoft et al., 2005). It is well known that different plant species can associate with microbial communities with unique characteristics (Chen et al., 2002) probably due to differences in amount and quality of root exudates (Nguyen, 2003). Coexistence of multiple plant species may enhance the complexity of soil microorganisms by increasing the heterogeneity of organic substrates during decomposing of litter and living roots (Broughton and Gross, 2000; Stephan et al., 2000).

Random amplified polymorphic DNA (RAPD) has been widely used in species classification and phylogenetic analysis, resistance gene identification, and genetic analysis of populations (Atienzar et al., 2002), as it is quick, simple and inexpensive. In fact, RAPD analysis has become one of the most popular DNA-based methods for assessing genetic diversity in plants (Liu et al., 2005) and has been used in DNA analysis of soil microbial community (Yang et al., 2000; Gao et al., 2010a). Several authors (Hinojosa et al., 2008; Tejada et al., 2008) have quantified the effect of heavy metals on various soil enzyme activities by determining the ecological dose 50% ($ED_{50}$), the concentration of the heavy metal at which the enzyme, or other biological activities, is reduced to 50% of the uninhibited value. Traditionally, only single trace element additions to soils have been used in most studies

* Corresponding author. E-mail: zhoupei@sjtu.edu.cn

for $ED_{50}$ determination, and scarce information is available on the possible synergic effects of multiple trace elements (Yang et al., 2007).

However, the effects of plant species coexistence on soil microbial community structure and soil enzymes activities under Cd and Pb combined pollution are less known. We suggest modifying the dose-response model is used to evaluate the $ED_{50}$ values for enzyme activities subjected to multiple metals coexisting in soil ecological system. In our study, the specific objectives are to: (1) investigate the effects of plant species coexistence on soil microbes, respiration and soil enzymes activities under Cd and Pb combined pollution; (2) and assess the inhibition effect of enzyme activities and the changes of microbial community structure under Cd and Pb combined pollution using modified ecological dose model and PCR-RAPD method.

## 1 Materials and methods

### 1.1 Characterization, sampling and treatment of soils

The pot-culture experiment was conducted in a greenhouse in Shanghai Jiaotong University, with 22–25°C average temperature. The tested soil was paddy soil, with the following soil parameters: soil pH was 8.18; organic matter was 16.17 g/kg; total N and P were 1.14 and 1.36 g/kg, respectively; cation exchange capacity was 15.60 cmol/kg; and the concentrations of Cd, Cu, Zn, Pb and As were 0.18, 22.9, 38.1, 15.1 and 7.4 mg/kg, respectively. Air-dried unpolluted soil was sieved through a 4-mm sieve and thoroughly mixed with basal fertilizers, and then, spiked with Cd/Pb. After two weeks incubation mixed soil was placed into plastic pots (30 cm in height, 40 cm in diameter). The additional levels of Cd were 10 and 25 mg/kg of dry weight soil, respectively, and the levels of Pb were 200 and 500 mg/kg of dry weight soil, respectively. Cd was applied as $CdCl_2 \cdot 2.5H_2O$ and Pb was applied as $Pb(NO_3)_2$.

Thirteen different treatments were applied to the soil, and each treatment had three replications (Table 1). The plant for monoculture was *Solanum nigrum* L. and the plants for mixed culture were *S. nigrum* and *Zea mays* L. Seedlings were firstly cultured by means of sand culture, and then, the seedlings (about 2-week old and 3–4 cm height with 2–3 leaves) of plant were transplanted into each pot. Total plant density was about 15 for each pot. For each pot, five soil holes (1.5 cm diameter) were taken to a depth of 15 cm and were then mixed (about 100 g) as one sample. Soil microbe population, respiration and soil enzymes activities were assayed at 7, 14, and 35 days after plants transplanted into pot.

**Table 1** Number of 12 treatments responding to factor and level, except number of control treatment 13

| Levels (mg/kg) | Factors | | |
|---|---|---|---|
| | No-planting | Monoculture | Mixed culture |
| Cd10 + Pb200 | 1 | 5 | 9 |
| Cd25 + Pb20 | 2 | 6 | 10 |
| Cd10 + Pb500 | 3 | 7 | 11 |
| Cd25 + Pb500 | 4 | 8 | 12 |

### 1.2 Enumeration of major soil microbial population groups

The enumeration of the soil microflora was done by the dilution plate method (Nair and Subba-Rao, 1977). The total colony forming units (cfu) of bacteria, fungi and actinomycetes were recorded on Ken Knight and Munaier's agar (Allen, 1959), Martin's rose bengal agar (Martin, 1950) and Jensen's agar (Jensen, 1951) media, respectively. The plates were incubated at 28°C and microbial population was calculated and expressed as $10^n$ cfu/g air dried soil, where $10^n$ was dilution factor.

### 1.3 Soil respiration

Respiration was determined as described previously by Bringmark and Bringmark (1993). $CO_2$ evolved from samples was captured with alkali in closed vessels for 24 hr. After precipitation of carbonate in $BaCl_2$, the remaining alkali concentration was determined by titration with HCl. Respiration was expressed as $CO_2$ evolution per gram dry weight and hour (mg $CO_2$/(g·hr)).

### 1.4 Determination of enzymes activity

Soil urease activity was determined by the method of Tabatabai and Bremner (1972), expressed as mg $NH_4^+$-N/(kg·hr). Dehydrogenase activity was tested by reduction of 2,3,5-triphenyltetrazolium chloride (TTC). After 24 hr at 37°C, the triphenyl formazan (TPF) released was extracted with methanol and assayed at 485 nm in an UV spectrophotometer. The unit of dehydrogenase activity was mg TPF/(kg·hr). Acid phosphatase activity was determined by the method of Tabatabai and Bremner (1969). The *p*-nitrophenol (PNP) in the filtrate was determined colorimetrically at 410 nm after 1 hr incubation with *p*-nitrophenyl phosphate, expressed as mg PNP/(kg·hr).

### 1.5 Modified ecological model for soil enzyme activity

The two kinetic models and the sigmoidal dose-response model were used to assess the inhibition of enzymatic activity by heavy metal (Hinojosa et al., 2008; Tejada et al., 2008). Toxicity coefficient was used to improve ecological dose-response model, which can evaluate the inhibition effect for enzyme activity subjected to multiple heavy metal synergic effects (Gao et al., 2010b).

$$E_i = T_i \times C_i \tag{1}$$

$$R_i = \sum_{i=1}^{m} E_i \tag{2}$$

where, $C_i$ (mg/kg soil) is concentration of heavy metal in soil; $T_i$ is the "toxic-response" factor for a given heavy metal, i.e., it is 5 for Cu and Pb, 1 for Zn, 30 for Cd, 2 for Cr and Ni (He et al., 1998); $E_i$ is the potential ecological toxicictiy coefficient for a given heavy metal; $R_i$ is total ecological toxicity coefficient under multiple heavy metal pollution.

The two kinetic models were:

$$\text{Model 1:}\quad v = \frac{c}{1 + bR_i} \tag{3}$$

$$\text{Model 2:}\quad v = \frac{c(1 + aR_i)}{1 + bR_i} \tag{4}$$

where, $v$ is the response variable, and $a$, $b$ and $c$ are fitting parameters with positive values and $b > a$; $R_i$ was total ecological toxicity coefficient under multiple heavy metal pollution in Eq. (2). Model 1 describes the full inhibition of $v$ by $R_i$, and Model 2 describes the partial inhibition. By fitting the equation of Models 1 and 2 to the experimental data, it is possible to calculate the 50% ecological dose ($ED_{50}$) values from the relationship:

$$ED_{50} = \frac{1}{b} \tag{5}$$

where, $ED_{50}$ is total ecological toxicity coefficients which lead to enzyme activity inhibited by 50%.

The mathematical equation for the sigmoidal dose-response model (Model 3) is

$$v = \frac{a}{1 + e^{b(x-c)}} \tag{6}$$

where, $v$ is again the response variable, $x$ is the natural logarithm of $R_i$, $a$ is the uninhibited value of $v$, $b$ is a slope factor, and $c$ is the natural logarithm of $ED_{50}$. The $ED_{50}$ values are calculated using the following expression:

$$ED_{50} = e^{c} \tag{7}$$

This model describes a logistic curve, which represents the relationship between the measured activity and the natural logarithm of toxicity coefficient.

### 1.6 DNA extraction and RAPD

Soil samples of 5 g were mixed with 13.5 mL of DNA extraction buffer (100 mmol/L Tris-HCl (pH 8.0), 1.5 mol/L NaCl, 1% CTAB) and 100 μL of proteinase K (10 mg/mL) in centrifuge tubes by horizontal shaking at 225 r/min for 30 min at 37°C. After shaking treatment, 1.5 mL of 20% SDS was added, and the samples were incubated in a 65°C water bath for 2 hr with gentle end-over-end inversions every 15–20 min. The supernatants were collected after centrifugation at 6000 ×$g$ for 10 min at room temperature and transferred into 50 mL centrifuge tubes. Supernatants from the three cycles of extractions were combined and mixed with an equal volume of chlorformisoamyl alcohol (24:1, *V/V*). The aqueous phase was recovered by centrifugation and precipitated with 0.6 volume of isopropanol at room temperature for 1 hr or overnight. The pellet of crude nucleic acids was obtained by centrifugation at 16,000 ×$g$ for 20 min, resuspended in TE buffer (10 mmol/L Tris-HCl (pH 8.0), 1 mmol/L EDTA (pH 8.0)) to give a final volume of 500 μL. DNA was then purified by the low-melting-point agarose gel recovery method (Zhou et al., 1996).

PCR amplification reaction in a 20 μL total volume, containing 2 μL 10× Taq buffer, 2 mmol/L $MgCl_2$, 1 unit Taq DNA polymerase, 0.25 mmol/L dNTP, 25 pmol primer, and 10 ng soil DNA. DNA amplification was carried out in a MJ research thermocycler (PT-200, China) with the following procedure: an initial denaturing step at 94 μL for 3 min; 40 cycles for 30 sec at 94°C (denature), 45 sec at 36°C (annealing), 90 sec at 72°C (extension), and a final elongation step at 72°C for 5 min. PCR products were separated by electrophoresis on 1.8% agarose gel and after which the gels were stained with ethidium bromide (EB) solution (0.015%) in distilled water and photographed. The standard DNA samples (1 kb DNA ladder marker) were used as molecular size marker.

### 1.7 RAPD fingerprints profiles

The photographic plates were scanned into computer and analyzed using a computer image analysis system (ChampGel$^{3200}$, China). We selected 10 random primers having good repetition from 50 random primers to amplify the microbial community DNA from the treated soils (Table 2). Polymorphism observed in RAPD profiles included disappearance of a normal band and appearance of a new band in comparison to control RAPD profiles. The presence and absence of amplified fragments were scored (Wang et al., 2007).

### 1.8 Data analysis

The values of the constants $a$, $b$ and $c$ of the three models were estimated using the Levenberg-Marquardt method to solve Non-linear Curve Fit by Origin 7.5. Data are expressed by mean ± SD, which was performed on each dependent variable by SPSS 12.0.

## 2 Results

### 2.1 Soil microbe population

The results of quantitative analysis showed that soil microbe populations decreased with elevated heavy metal concentration (Fig. 1). Soil microbe populations in heavy metal contaminated soil showed a significant difference compared with unpolluted soil. Monoculture and mixed culture would enhance microbe populations in heavy metal contaminated soil. The bacteria population size in contaminated soil was bigger under mixed culture than under monoculture. Moreover, mixed culture could increase the bacteria in 15%–20% of population size compared to monoculture. The orders of bacteria population size under

**Table 2** Sequences of ten primers used in this experiment

| No. of primers | Sequences of primers | Percentage of GC (%) |
|---|---|---|
| S1 | GTTTCGCTCC | 60 |
| S2 | CTGCTGGGAC | 70 |
| S3 | TCTCCCTCAG | 60 |
| S4 | TCCGCAACCA | 60 |
| S5 | CACTTCCGCT | 60 |
| S6 | GGTTACTGCC | 60 |
| S7 | CTCCCCAGAC | 70 |
| S8 | TTGCAGGCAG | 60 |
| S9 | GTAAGCCCCT | 60 |
| S10 | GTAAGCCCCT | 60 |

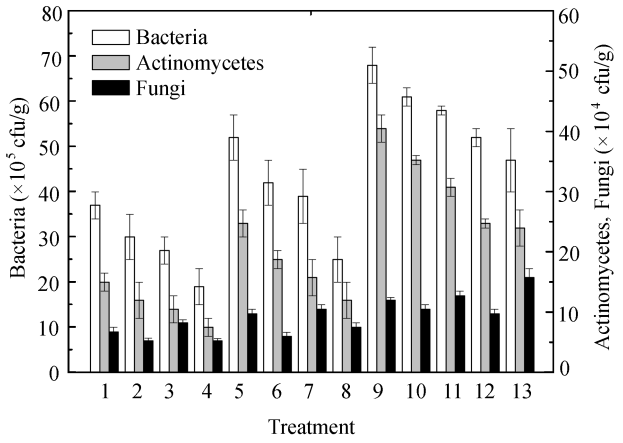


**Fig. 1** Soil microorganism quantities under different treatments. Treatments 1–12 can refer to Table 1, and treatment 13 is blank.

different treatments was: mixed culture > monoculture > unplanted treatment.

The actinomycetes in population size showed a similar behavior to bacteria. The population size for actinomycetes in contaminated soil was bigger under mixed culture than under monoculture. Mixed culture significantly increased actinomycetes population size than bacteria population size, compared to monoculture. The decrease in population size for actinomycetes under unplanted treatment was also obvious as that in bacteria. Although mixed culture could increase fungi population size, the discrepancy between monoculture and mixed culture was smaller for fungi than for bacteria and actinomycetes. The sensitivity of different microbial community responding to Cd and Pb stress was also different. Therefore, the coefficient of variance (CV) for microbial community in heavy metal contaminated soil was used to assess the sensitivity. In heavy metal contaminated soil, the orders of the sensitivity of microbial community responding to heavy metal stress were: actinomycetes > bacteria > fungi.

### 2.2 Soil respiration and enzymatic activity

Respirations were significantly higher in planted soil than that in unplanted soil (Fig. 2a). The behaviors of soil respiration under monoculture and mixed culture showed significant difference with increasing culture time, but there was a slight discrepancy in soil respiration due to monoculture and mixed culture. The soil respiration in planted treatment all increased after 35 day culture time, whereas soil respiration in unplanted treatment showed a decrease or slight fluctuation.

Results from our experiment showed that the coexistence effect of elevated heavy metal concentration and plant species on soil enzyme activities was different (Fig. 2b, c, d). Phosphatase, urease and dehydrogenase

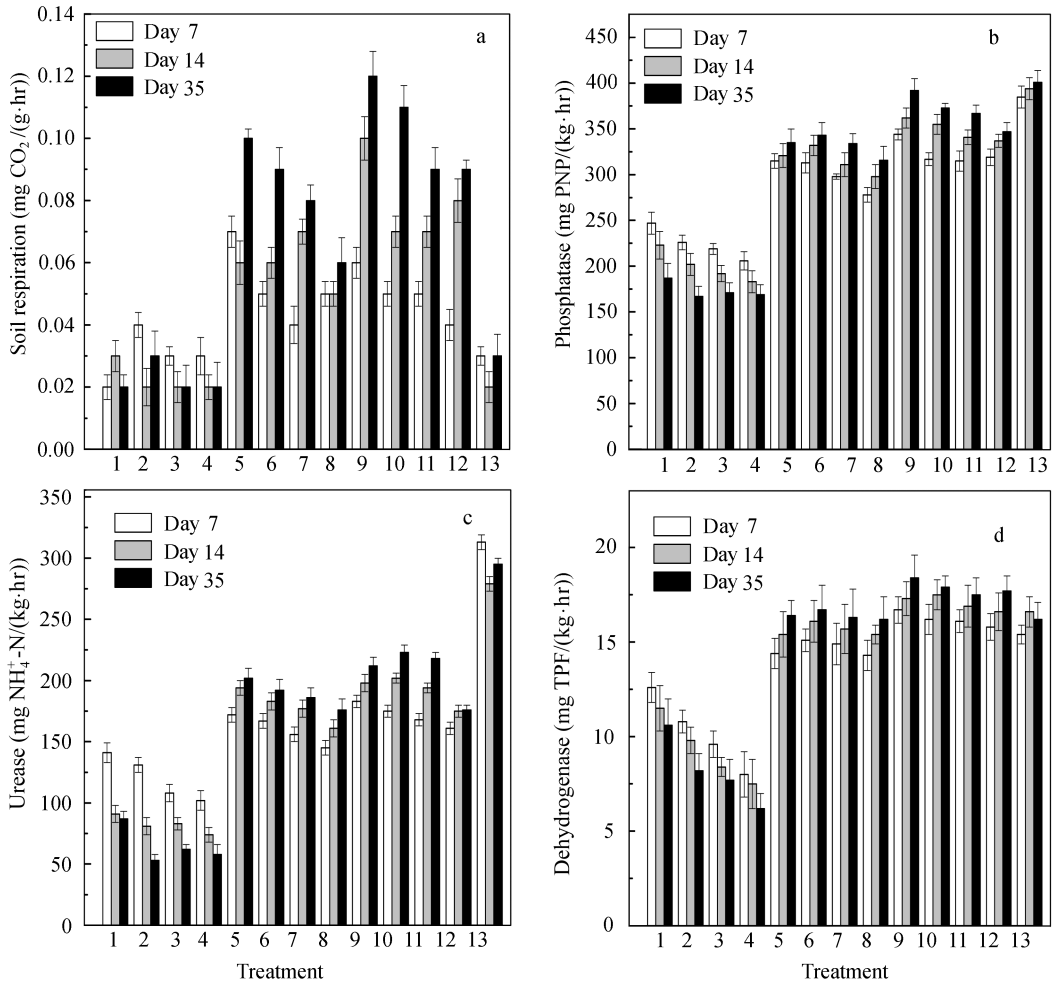


**Fig. 2** Changes of soil respiration (a), phosphatase (b), urease (c), and dehydrogenase (d) at day 7, 14 and 35 under different treatments.

activities were significantly higher in planted treatment than in unplanted treatment. Plant species coexistence increasing soil enzyme activities under heavy metal stress was observed. In planted treatment phosphatase activity significantly increased with increasing culture time. The change of phosphatase activity with increasing culture time showed a significant difference between monoculture and mixed culture (Fig. 2b). Urease activity was also affected by heavy metal stress and plant species coexistence, and increased with increasing culture time in planted treatment (Fig. 2c). The order of urease activity responding to heavy metal stress was: mixed culture > monoculture > unplanted treatment. There was significant difference on dehydrogenase activity due to Cd and Pb combined pollution treatment compared to unpolluted treatment. Dehydrogenase activity in planted treatment increased with increasing culture time, whereas dehydrogenase activity in unplanted treatment showed opposite behavior (Fig. 2d).

Table 3 shows the evolution of the three enzyme activities measured during the experimental period and $ED_{50}$ values calculated from the best fit model, and $R^2$ values from the regression analysis for the three enzyme activity. Although all three models were adequately fitted to experimental data, Model 2 was the best fitted in most of the cases. The $ED_{50}$ values for urease activity were predicted with Model 2, whereas the $ED_{50}$ values for unplanted soil at day 7 of incubation were well predicted with Model 1. Nevertheless, for dehydrogenase activity under monoculture at day 45 of incubation the best fit was achieved by the sigmoidal dose-response Model 3. For phosphatase activity, the $ED_{50}$ values for unplanted soil and monoculture at day 7 of incubation and unplanted soil at day 35 of incubation were all well predicted with Model 1.

The $ED_{50}$ was higher under mixed culture than under other treatments, and $ED_{50}$ values for three enzyme activity significantly decreased with increasing culture time. The dehydrogenase was most sensitive to metal addition and easily loss activity under low heavy metal concentration. The effect of heavy metal on phosphatase was smaller and urease responded similarly with phosphatase. Except for high dose respond, it is difficult to fully inhibit the phosphatase activity,

### 2.3 RAPD profiles

The electrophoresis indicated that the size of DNA obtained from the soil samples was about 23.130 kb and the DNA yields was about (13.8 ± 1.2) μg/g soil (Fig. 3). Concentration and purity of DNA extracted were usually measured at $OD_{260}$ and by 260 nm/280 nm absorbance ratio. The RAPD fingerprints showed substantial differences between different treatments, with apparent changes in the number and size of amplified DNA fragments, for example, using primer S3 (Fig. 4).

The greater numbers of disappearing bands were 20 and 19 and the greater number of new bands were 20 and 18 (Table 4). The number of denoting polymorphic bands was greater in planted treatment and the greatest value of polymorphisms was 51.9%. These results indicated that heavy metal pollutant might decrease the richness of DNA sequence for soil microbial community, while monoculture and mixed culture would maintain at DNA sequence diversity staying at high level. The DNA polymorphisms under mixed culture treatment were similar in heavy metal contaminated soil to that in unpolluted treatment, indicating that plant species coexistence would alleviate the effect of heavy metal stress on soil genetic polymorphisms.

**Table 3** Values of $R^2$ ($p < 0.05$) obtained for Gauss-Newton analysis, which best describe the inhibition of urease, phosphatase and dehydrogenase of different treatment and $ED_{50}$ expressed by toxicant coefficient predicted

| Treatment | Urease | | | Dehydrogenase | | | Phosphatase | | |
|---|---|---|---|---|---|---|---|---|---|
| | Model | $ED_{50}$ | $R^2$ | Model | $ED_{50}$ | $R^2$ | Model | $ED_{50}$ | $R^2$ |
| Culture for 7 days | | | | | | | | | |
| No-planting | 3 | 2504 | 0.77 | 1 | 655 | 0.87 | 1 | 2604 | 0.92 |
| Monoculture | 2 | 3805 | 0.73 | 2 | 956 | 0.92 | 2 | 4903 | 0.85 |
| Mixed culture | 2 | 4885 | 0.88 | 2 | 1174 | 0.85 | 1 | 3875 | 0.82 |
| Culture for 14 days | | | | | | | | | |
| No-planting | 2 | 2248 | 0.78 | 2 | 545 | 0.75 | 2 | 3256 | 0.91 |
| Monoculture | 2 | 3575 | 0.91 | 2 | 826 | 0.84 | 2 | 4545 | 0.83 |
| Mixed culture | 2 | 4984 | 0.79 | 2 | 1143 | 0.93 | 2 | 4954 | 0.77 |
| Culture for 35 days | | | | | | | | | |
| No-planting | 2 | 2072 | 0.88 | 2 | 333 | 0.75 | 1 | 2378 | 0.84 |
| Monoculture | 2 | 3026 | 0.86 | 3 | 773 | 0.84 | 2 | 4094 | 0.76 |
| Mixed culture | 2 | 4411 | 0.82 | 2 | 1021 | 0.89 | 2 | 3517 | 0.72 |

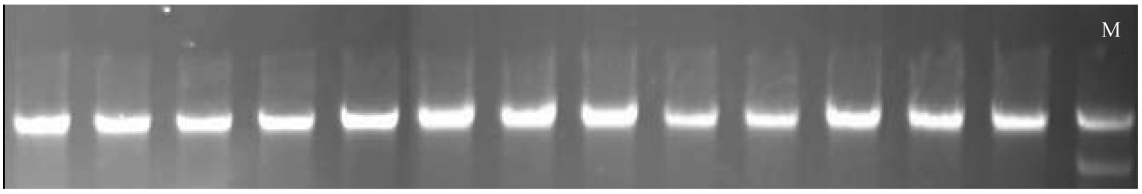


**Fig. 3** Genomic DNA extracted by CTAB in the treated samples (1–13 from left to right). M: DNA marker and the bands bottom was 23.130 bp.

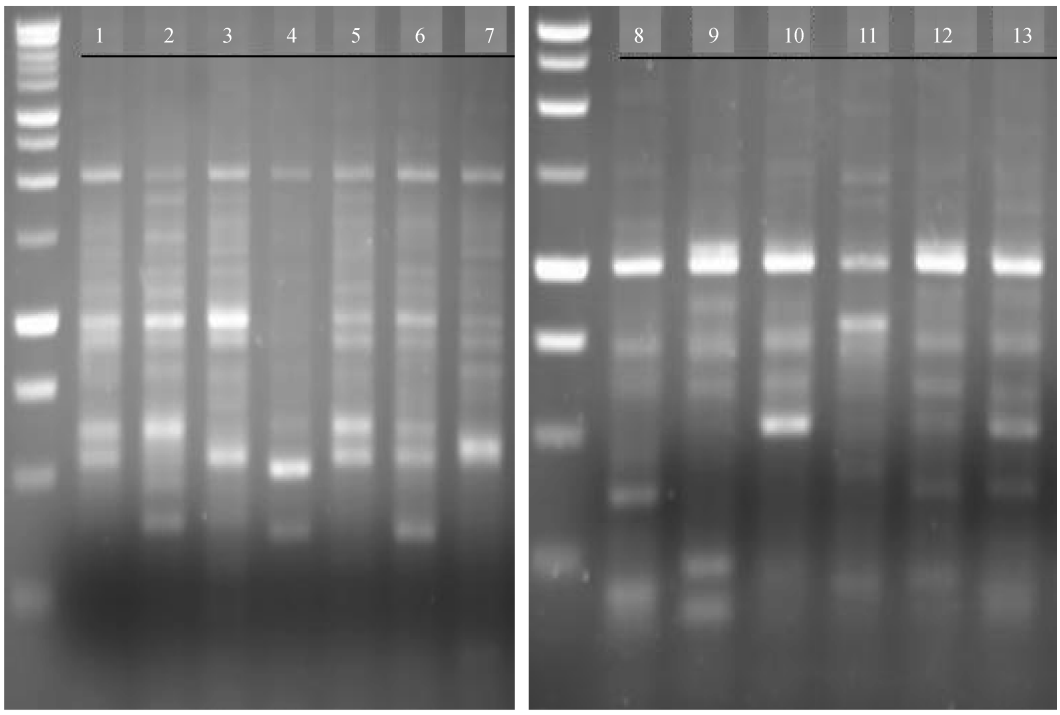


**Fig. 4** RAPD fingerprints of primer S3 from sample 1 to 13.

**Table 4** Changes of total bands, polymorphic bands in treatments (1–13)

| No. | 1 | | 2 | | 3 | | 4 | | 5 | | 6 | | 7 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b | a | b | a | b |
| S1 | 0 | 2 | 2 | 1 | 1 | 0 | 0 | 2 | 1 | 1 | 2 | 1 | 2 | 3 |
| S2 | 1 | 1 | 2 | 3 | 3 | 2 | 2 | 2 | 2 | 1 | 2 | 2 | 2 | 3 |
| S3 | 1 | 2 | 0 | 3 | 3 | 2 | 2 | 1 | 3 | 3 | 2 | 2 | 1 | 1 |
| S4 | 1 | 2 | 2 | 1 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 1 | 0 | 0 |
| S5 | 2 | 1 | 2 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 1 | 3 | 2 | 1 |
| S6 | 0 | 3 | 1 | 2 | 2 | 0 | 1 | 1 | 2 | 3 | 2 | 1 | 1 | 2 |
| S7 | 1 | 2 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 2 | 2 |
| S8 | 2 | 1 | 1 | 1 | 1 | 2 | 1 | 1 | 3 | 1 | 0 | 0 | 3 | 1 |
| S9 | 1 | 1 | 1 | 2 | 2 | 0 | 2 | 1 | 2 | 2 | 2 | 1 | 2 | 1 |
| S10 | 0 | 2 | 2 | 0 | 0 | 1 | 1 | 2 | 1 | 3 | 1 | 3 | 2 | 1 |
| Total | 9 | 17 | 14 | 15 | 12 | 15 | 14 | 15 | 20 | 20 | 17 | 20 | 17 | 17 |
| a + b | 26 | | 29 | | 27 | | 29 | | 40 | | 37 | | 34 | |
| *P* (%) | 33.7 | | 37.6 | | 35.1 | | 37.7 | | 51.9 | | 48.1 | | 44.1 | |

| No. | 8 | | 9 | | 10 | | 11 | | 12 | | 13 | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | a | b | a | b | a | b | a | b | a | b | a | b |
| S1 | 1 | 1 | 0 | 1 | 1 | 1 | 2 | 1 | 1 | 1 | 9 | |
| S2 | 3 | 1 | 2 | 2 | 2 | 1 | 4 | 2 | 2 | 2 | 6 | |
| S3 | 3 | 1 | 2 | 2 | 1 | 4 | 2 | 1 | 2 | 1 | 8 | |
| S4 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 0 | 1 | 3 | 9 | |
| S5 | 1 | 2 | 2 | 1 | 2 | 3 | 2 | 1 | 1 | 2 | 8 | |
| S6 | 3 | 2 | 2 | 4 | 1 | 3 | 2 | 2 | 1 | 2 | 8 | |
| S7 | 1 | 1 | 3 | 2 | 2 | 0 | 1 | 2 | 3 | 1 | 7 | |
| S8 | 0 | 2 | 1 | 3 | 3 | 1 | 2 | 1 | 2 | 1 | 9 | |
| S9 | 1 | 3 | 1 | 1 | 2 | 1 | 1 | 3 | 1 | 2 | 6 | |
| S10 | 2 | 2 | 3 | 0 | 0 | 2 | 2 | 2 | 2 | 2 | 7 | |
| Total | 18 | 16 | 18 | 19 | 15 | 19 | 21 | 15 | 16 | 17 | 77 | |
| a + b | 34 | | 37 | | 34 | | 36 | | 33 | | | |
| *P* (%) | 44.2 | | 48.1 | | 44.2 | | 46.8 | | 42.9 | | | |

a: appearance of new bands; b: disappearance of normal bands; a + b: polymorphic bands; *P*: value of polymorphism compared to the control.

## 3 Discussion

### 3.1 Changes of microbe population and respiration

Activation of microbial activities, and inhibitions at selected scales of time and space are found in almost all structures produced by the rhizosphere (Viketoft et al., 2005). In this study, micro-organism quantity in planted treatment significantly increased compared with unplanted treatment and micro-organism quantity was bigger under mixed culture than under monoculture. Plants can affect the soil biota by influencing the quantity and quality of organic substrates that reach the soil (Chen et al., 2004). Plant species coexistence can enhance interactions between micro-organisms and macro-organisms in soils and rhizospheric activity and complex substrates.

Heavy metals are toxic to living organisms primarily due to their protein-binding capacity and hence inhibition of enzymes. Hiroki (1992) reported that the sensitivity of different microbial community responding to heavy metal stress was different. Various studies have found that fungi are more resistant than bacteria to long-term heavy metal contamination (Fliessbach et al., 1994; Frostegård et al., 1996). The results in our study indicated that the order of sensitivity was: actinomycetes > bacteria > fungi. This may be that the roots of plants associate with mycorrhizal fungi or stimulate bacterial activity in their rhizosphere. The relationship between microbial diversity and soil respiration is a function of both the microorganism presence and activities of functional groups and their interactions. Our results for soil respirations could be used to estimate the potential of heavy metals in contaminated field soils to negatively affect soil microbial community responses, and the behavior of soil respiration were similar with that of microbe population under Cd and Pb combined pollution.

### 3.2 Changes of enzyme activity

The inhibition of urease and dehydrogenase activities by heavy metal pollution was reported by Zheng et al. (1999). As a result, the decreases in microbial populations have been reported in the soils polluted with heavy metal compounds (Renella et al., 2005). As compared to unplanted soil, planted soils have frequently been reported to have higher rates of microbial activity due to the presence of additional surfaces for microbial colonization and organic compounds released by the plant roots (Delorme et al., 2001). The planted treatment after 35-day culture led to significantly higher values for many the biological indicators and root growth, so soil enzyme activities increased with increasing culture time.

Zak et al. (2003) reported that plant diversity influenced microbial biomass mainly through plant products (i.e., litter inputs and root exudates) rather than through diversity per se. Chen et al. (2004) showed that higher plant diversity did not induce higher soil microbial biomass, as the dominant plant species might exert the most influence over the microbial community, regardless of plant diversity. Results from our experiment indicated that plant species coexistence would affect microbe population and urease activity compared with monoculture, and the results of which were similar with Yang et al. (2007). This is because the composition of soil microbial communities may vary markedly in relation to roots and plant species, which mainly through development of beneficial rhizobacteria release specific sugars and amino acids into the rhizosphere (Kowalchuk et al., 2002). Hence, higher plant diversity may produce a higher diversity of root exudates and therefore select for complex microbial communities and more diverse microbial functional groups.

Dehydrogenase activity reflects the total oxidative activity of the microbial biomass being involved in central aspect of metabolism and does not function extracellularly. Urease catalyzes the hydrolysis of urea into ammonia or ammonium ion depending on soil pH, and carbon dioxide. Among the enzymes that are involved in soil N cycling, urease is the most prominent. Phosphatases play an important role in transforming organic phosphorus into inorganic forms, suitable for plants. The enzyme activity investigated in our study all related with plant nutrients transformation, so it is not difficult to explain why monoculture or mixed culture would be helpful to enhance those soil enzyme activities under heavy metal pollution. It is suggested that coexistence of more plant species may alleviate Pb and Cd impacting on the activity of enzymes.

Model 1 is a full inhibition model, whereas in fact, the inhibition of these enzyme activities was always less than 100% of the control value and it is not possible to be determined if the decrease in soil enzyme activities may be due to a direct metal inhibition to enzymes, to a lower synthesis and/or release of enzymes, or to a combination of both (Renella et al., 2005; Mench et al., 2006; Hinojosa et al., 2008). Model 2 is a partial inhibition model, suggesting that a fraction of the enzymatic activities is not inhibited by Cd and Pb addition to soil, and Tejada et al. (2008) also showed similar results. Model 3 indicates that the relationship between enzyme activity and toxicity coefficient is sigmoidal dose. Indeed, enzymes in soils can be physically and chemically protected by soil constituents (organic and inorganic ligands), which interacted with trace elements (Renella et al., 2003), whereas enzyme was influenced by many factor no only heavy metal. Therefore, Model 2 was the best fitted in most of the cases. The $ED_{50}$ values for different enzyme activity at same culture time could be predicted with same model or the $ED_{50}$ values for the same enzyme activity at different culture time could be predicted with different model.

The results of our study showed that it is difficult to fully inhibit the urease activity. Similarly, Karaca et al. (2002) described a significant reduction on many hydrolytic enzymes after residual waste Cd enriched without any inhibition in urease activity. Maliszewska-Kordybach and Smreczak (2003) obtained the similar results that dehydrogenase was the most sensitive to metal addition. This may be that the dehydrogenase activity reflects the total oxidative activity of the microbial biomass. Plant need assimilate inorganic phosphorus to maintain normal metabolize, so plant species coexistence means that they need more inorganic phosphorus transformed. This may

be why phosphatases activity was higher under mixed culture than under monoculture, but the $ED_{50}$ values was lower for mixed culture than for monoculture. These $ED_{50}$ values may be more suitable indicators of the sensitivity of an ecosystem to stress, because a 50% reduction of a basic ecological process may be too extreme for its continued functioning. Our results emphasize the need for the use of soil functioning indicators and toxicant coefficient calculated by ecological risk factor method, in addition for the current analytical chemical measurements, for the risk assessments and evaluations of contaminated sites.

### 3.3 Effects on soil microbial community of DNA sequence

Our results indicated that heavy metal pollution might decrease the richness of DNA sequence for soil microbial community, while plant species coexistence increased the diversity of DNA sequence. This is because that the composition and diversity of the soil microbial community may be closely associated with the plant community and diversity. Furthermore, plant diversity can increase interactions among micro-organisms and macro-organisms in soils and rhizospheric activity and complex substrates, which lead to the changes in the balance of microbial populations and genetic recombination contributed to the increased diversity.

This study indicated that genomic template stability in soil was significantly affected by the addition of Cd and Pb. Changes in oligonucleotide priming sites due mainly to genomic rearrangements and less likely to point mutations and DNA damage in the primer binding sites (because the binding site is only ten base long whereas genomic rearrangements occur in much longer fragments), which could act to block or reduce polymerization of DNA in the PCR reaction (Wang et al., 2007). Pollutants could induce DNA damage such as single- and double-strand breaks, modified bases, abasic sites and so on in organisms (Waisberg et al., 2003; Atesiet et al., 2004), which may also induce important structural changes that can significantly affect the kinetics of PCR events.

The inadequacy of tools to assess microbial diversity has been a long-standing limitation to our understanding of soil microbial communities. Using the DGGE technique, it is possible to separate PCR amplified fragments by the base composition of the fragments. Originally, application of DGGE with environmental samples was used to analyze the genetic diversity of bacterial populations, but recently the use of DGGE has expanded to include the study of fungal communities (Vainio and Hantula, 2000; Anderson et al., 2003). The analysis of fungal communities in compost by DGGE has only been used a few times, and the compost types used in previous studies differs from the two types used in our investigation. RAPD is more sensitive than classic genotoxic tests, and recently, RAPD technique has been successfully utilized to detect various types of DNA damage and mutation in animals, bacteria and plants induced by pollutants (Atienzar et al., 2002; Rong and Yin, 2004). Detection of genotoxic effect using RAPD involves the comparison of profiles generated from control (unexposed) and treated (exposed) DNA. Using multiple primers also helps ensure that a sufficiently large region of the target DNA is scanned when an estimate of overall variance between samples is desired.

The development of molecular techniques more sensitive to shifts in composition may reveal natural shifts from one closely related microbe to another along a gradient, just as there are shifts among closely related plant species along gradients. RAPD analysis in conjunction with other biomarkers such as soil microbe population, respiration, soil enzyme parameter etc. would prove a powerful ecotoxicological method to evaluate soil quality. Furthermore, more experiments should to be considered to better understand the relationships of plant, soil and heavy metal pollution, and more plant species coexistence improving soil heath and microbial diversity.

**Acknowledgments**

This work was supported by the Science Foundation of Shanghai (No. 07DZ12055, 07DZ19604, 08d21900404), and the National Hi-Tech Research and Development Program (863) of China (No. 2007AA10Z441).

## References

Allen O N, 1959. Experiments in Soil Bacteriology (3rd ed.). Burgess Publication, Minneapolis. 117.

Anderson I C, Campbell C D, Prosser J I, 2003. Diversity of fungi in organic soils under a moorland-Scots pine (*Pinus sylvestris* L.) gradient. *Environmental Microbiology*, 5: 1121–1132.

Atesiet I, Suzen H S, Ayin A, 2004. The oxidative DNA base damage in tests of rats after intraperitoneal cadmium injection. *Biometals*, 17: 371–377.

Atienzar F A, Venier P, Jha A N, 2002. Evaluation of the random amplified polymorphic DNA (RAPD) assay for the detection of DNA damage and mutations. *Mutation Research*, 521: 151–163.

Bringmark E, Bringmark L, 1993. Standard respiration, a method to test the influence of pollution and environmental factors on a large number of samples. *Swedish Environmental Protection Agency, Stockholm*. 42–62.

Broughton L C, Gross K L, 2000. Patterns of diversity in plant and soil microbial communities along a productivity gradient in a Michigan old field. *Oecologia*, 125: 420–427.

Caldwell B A, 2005. Enzyme activities as a component of soil biodiversity: A review. *Pedobiologia*, 49: 637–644.

Chen C L, Liao M, Huang C Y, 2005. Effect of combined pollution by heavy metals on soil enzymatic activities in areas polluted by tailings from Pb-Zn-Ag mine. *Journal of Environmental Sciences*, 17(4): 637–640.

Chen X, Tang J J, Fang Z G, Hu S, 2002. Phosphate-solubilizing microbes in rhizosphere soils of 19 weeds in southeastern China. *Journal of Zhejiang University Science*, 3: 355–361.

Chen X, Tang J J, Fang Z G, Shimizu K, 2004. Effects of weed communities with various species numbers on soil features in a subtropical orchard ecosystem. *Agriculture Ecosystems and Environment*, 102: 377–388.

Delorme T A, Gagllardi J V, Angle J S, Chaney R L, 2001. Influence of the zinc hyperaccumulator *Thlaspi caerulescens* J. & C. *Presl*. and the nonmetal accumulator *Trifolium pratense* L. on soil microbial populations. *Canadian Journal of Microbiology*,

47: 773–776.

Fliessbach A, Martens R, Reber H H, 1994. Soil microbial biomass and activity in soils treated with heavy metal contaminated sewage sludge. *Soil Biololgy and Biochemistry*, 26: 1201–1205.

Frostegård Å, Tunlid A, Bååh E, 1996. Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biololgy and Biochemistry*, 28: 55–63.

Gao Y, Mao L, Miao C Y, Zhou P, Cao J J, Zhi Y E et al., 2010a. Spatial characteristics of soil enzyme activities and microbial community structure under different land uses in Chongming Island, China: Geostatistical modelling and PCR-RAPD method. *Science of the Total Environment*. DOI:10.1016/j.scitotenv.2010.04.007.

Gao Y, Zhou P, Mao L, Zhi Y E, Shi W J, 2010b. Assessment of effects of heavy metals combined pollution on soil enzyme activities and microbial community structure: modified ecological dose-response model and PCR-RAPD. *Environmental Earth Science*, 60: 603–612.

He M C, Wang Z J, Tang H X, 1998. The chemical, toxicological and ecological studies in assessing the heavy metal pollution in Le An River, China. *Water Research*, 32(2): 510–518.

Hinojosa M B, Carreira J A, Rodríguez-Maroto J M, García-Ruíz R, 2008. Effects of pyrite sludge pollution on soil enzyme activities: Ecological dose-response model. *Science of the Total Environment*, 396: 89–99.

Hiroki M, 1992. Effect of arsenic pollution on soil microbial-population. *Soil Science and Plant Nutrition*, 39: 227–235.

Jensen H I, 1951. Notes on the biology of Azotobacter. *Journal of Applied Microbiology*, 14: 89–94.

Karaca A, Tcherko D, Bruce K D, Stemmer M, Hobbs P J, Bardgett R D, 2002. Effect of cadmium contamination with sewage sludge and phosphate fertiliser amendments on soil enzyme activities, microbial structure and available cadmium. *Biololgy and Fertility of Soils*, 35: 428–434.

Kowalchuk G A, Buma D S, De-Boer W, Klinkhamer P G L, Van-Veen J A, 2002. Effects of above-ground plant species composition and diversity on the diversity of soilborne microorganisms. *Antonie Van Leeuwenhoek International Journal of General and Molecular Microbiology*, 81: 509–520.

Liu W, Li P J, Qi X M, Zhou Q X, 2005. DNA changes in barley (*Hordeum vulgare*) seedlings induced by cadmium pollution using RAPD analysis. *Chemosphere*, 61: 158–167.

Lorenz N, Hintemann T, Kramarewa T, Katayama A, Yasuta T, Marschner P et al., 2006. Response of microbial activity and microbial community composition in soils to long-term arsenic and cadmium exposure. *Soil Biology and Biochemistry*, 38: 1430–1437.

Maliszewska-Kordybach B, Smreczak B, 2003. Habitat function of agricultural soils as affected by heavy metals and polycyclic aromatic hydrocarbons contamination. *Environment International*, 28: 719–728.

Martin J P, 1950. Use of acid, rose bengal and streptomycin in the plate method for estimating soil fungi. *Soil Science*, 69: 215–232.

Mench M, Renella G, Gelsomino A, Landi L, Nannipieri P, 2006. Biochemical parameters and bacterial species richness in soils contaminated by sludge-borne metals and remediated with inorganic soil amendments. *Environmental Pollution*, 144: 24–31.

Muhammad A, Wang H Z, Wu J J, Xu J M, Xu D F, 2005. Changes in enzymes activity, substrate utilization pattern and diversity of soil microbial communities under cadmium pollution. *Journal of Environmental Sciences*, 17(5): 802–807.

Nair S K, Subba-Rao N S, 1977. Microbiology of the root region of coconut and cacao under mixed cropping. *Plant Soil*, 46: 511–519.

Nguyen C, 2003. Rhizodeposition of organic C by plants: mechanisms and controls. *Agronomie*, 23: 375–396.

Renella G, Mench M, Gelsomin A, Landi L, Nannipieri P, 2005. Functional activity and microbial community structure in soils amended with bimetallic sludges. *Soil Biology and Biochemistry*, 37: 1498–1506.

Renella G, Ortigoza A L R, Landi L, Nannipieri P, 2003. Additive effects of copper and zinc on cadmium toxicity onphosphatase activities and ATP content of soil as estimated by the ecological does ($ED_{50}$). *Soil Biology and Biochemistry*, 35: 1203–1210.

Rong Z Y, Yin H W, 2004. A method for genotoxicity detection using random amplified polymorphism DNA with *Danio rerio*. *Ecotoxicology Environmental Safety*, 58:96–103.

Stephan A, Meyer A H, Schmid B, 2000. Plant diversity affects culturable soil bacteria in experimental grassland communities. *Journal of Ecology*, 88: 988–998.

Tabatabai M A, Bremner J M, 1969. Use of *p*-nitrophenyl phosphatefor assay of soil phosphatase activity. *Soil Biology and Biochemistry*, 4: 301–307.

Tabatabai M A, Bremner J M, 1972. Assay of urease activity in soils. *Soil Biology and Biochemistry*, 4: 479–487.

Tejada M, Morenob J L, Hernándezb M T, Garcíab C, 2008. Soil amendments with organic wastes reduce the toxicity of nickel to soil enzyme activities. *European Journal of Soil Biology*, 44: 129–140.

Vainio E J, Hantula J, 2000. Direct analysis of wood-inhabiting fungi using denaturing gradient gel electrophoresis of amplified ribosomal DNA. *Mycological Research*, 104: 927–936.

Viketoft M, Palmborg C, Sohlenius B, Huss-Danell K, Bengtsson J, 2005. Plant species effects on soil nematode communities in experimental grasslands. *Applied Soil Ecology*, 30: 91–103.

Waisberg M, Joseph P, Hale B, 2003. Molecular and cellular mechanisms of cadmium carcinogenesis. *Toxicology*, 192: 95–117.

Walker C, Goodyear C, Anderson D, Titball R W, 2000. Identification of arsenic resistant bacteria in the soil of a former munitions factory at Locknitz, Germany. *Land Contamination and Reclamation*, 8: 13–18.

Wang J H, Lu Y T, Shen G Q, 2007. Combined effects of cadmium and butachlor on soil enzyme activities and microbial community structure. *Environmental Geology*, 51: 1221–1228.

Yang R Y, Tang J J, Chen X, Hu S J, 2007. Effects of coexisting plant species on soil microbes and soil enzymes in metal lead contaminated soils. *Applied Soil Ecology*, 37: 240–246.

Yang Y H, Yao J, Hu S, 2000. Effects of agricultural chemicals on DNA sequence diversity of soil microbial community: A study with RAPD marker. *Microbial Ecology*, 39: 72–79.

Yang Z X, Liu S Q, Zheng D W, Feng S D, 2006. Effects of cadium, zinc and lead on soil enzyme activities. *Journal of Environmental Sciences*, 18(6): 1135–1141.

Zak D R, Holmes W E, White D C, Peacock A D, Tilman D, 2003. Plant diversity, soil microbial communities, and ecosystem function: are there any links? *Ecology*, 84: 2042–2050.

Zheng C R, Tu C, Chen H M, 1999. Effect of combined heavy metal pollution on nitrogen mineralization potential, urease and phosphatase activities in a Typic Udic Ferrisol. *Pedosphere*, 9(3): 251–258.

Zhou J Z, Bruns M A, Tiedje J M, 1996. DNA recovery from soils of diverse composition. *Applied and Environmental Microbiology*, 62: 316–322.