Available online at www.sciencedirect.com
ScienceDirect
Journal of Environmental Sciences 19(2007) 834–840
JOURNAL OF ENVIRONMENTAL SCIENCES
ISSN 1001-0742
CN 11-2629/X
www.jesc.ac.cn

Soil enzymatic activities and microbial community structure with different application rates of Cd and Pb

KHAN Sardar[1,2], CAO Qing[1], HESHAM Abd El-Latif[1], XIA Yue[1], HE Ji-zheng[1,*]

1. Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing 100085, China. E-mail: sardar.khan2008@yahoo.com
2. Department of Environmental Sciences, University of Peshawar, 25120 Peshawar, Pakistan

Received 14 August 2006; revised 9 January 2007; accepted 16 March 2007

Abstract

This study focused on the changes of soil microbial diversity and potential inhibitory effects of heavy metals on soil enzymatic activities at different application rates of Cd and/or Pb. The soil used for experiments was collected from Beijing and classified as endoaquepts. Pots containing 500 g of the soil with different Cd and/or Pb application rates were incubated for a period of 0, 2, 9, 12 weeks in a glasshouse and the soil samples were analyzed for individual enzymes, including catalase, alkaline phosphatase and dehydrogenase, and the changes of microbial community structure. Results showed that heavy metals slightly inhibited the enzymatic activities in all the samples spiked with heavy metals. The extent of inhibition increased significantly with increasing level of heavy metals, and varied with the incubation periods. The soil bacterial community structure, as determined by polymerase chain reaction-denaturing gradient gel electrophoresis techniques, was different in the contaminated samples as compared to the control. The highest community change was observed in the samples amended with high level of Cd. Positive correlations were observed among the three enzymatic activities, but negative correlations were found between the amounts of the heavy metals and the enzymatic activities.

Key words: soil contamination; heavy metals; application rate; enzymatic activity; microbial community

Introduction

Soil contamination with heavy metals has become a serious issue throughout the world, as a result of wastewater irrigation, sludge application, solid waste disposal, automobile exhaust and industrial activities (Cui *et al*., 2005). Sewage water contains substantial organic carbon, nutrients and heavy metals, and its long-term irrigation can lead to the build-up of heavy metals in the soil ecosystem (Cui *et al*., 2004; Rattan *et al*., 2005; Khan *et al*., 2006). Heavy metals could have long-term hazardous impacts on soil ecosystem and adverse influence on soil biological processes (Lee *et al*., 2002; Majer *et al*., 2002; Pérez-de-Mora *et al*., 2006). Soil enzymes are synthesized by microorganisms and act as biological catalysts to facilitate different reactions and metabolic processes to decompose organic pollutants and produce essential compounds for both microorganisms and plants (Moreno *et al*., 2003). Soil enzymatic activities are recognized as a more sensitive bio-indicator than plants and animals of any natural and anthropogenic disturbance (Hinojosa *et al*., 2004). Moreover, soil enzyme assays have been considered as one of the cheapest and easiest techniques and used before as sensors for measuring the degree of soil pollution and also to integrate information about microbial community status (Baum *et al*., 2003).

Enzymatic activities are frequently used for determining the influence of various pollutants including heavy metals on soil microbiological quality (Shen *et al*., 2005). Soil enzyme inhibition depends on the nature and concentration of heavy metals, and its extent varies from one enzyme to another and at a certain concentration, some heavy metals can stimulate the activity of an enzyme (Dick and Tabatabai, 1983; Zheng *et al*., 1999). Heavy metals can inhibit enzymatic activities by interacting with the enzyme substrate complexes, denaturing the enzyme protein and interacting with its active sites (Megharaj *et al*., 2003). Heavy metals can also influence microbial community, which ultimately lead to changes in soil enzymatic activities (Kandeler *et al*., 2000; Moreno *et al*., 2002).

In order to investigate the link between soil enzymatic activities and microbial community structure changes with amendments of heavy metals, it is necessary to study microbial community structure using advanced molecular biological techniques because of some limitations of using soil enzyme assays to assess soil quality (Trasar-Cepeda *et al*., 2000). The method of polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) approach has recently been used to assess changes in soil microbial community structure (Jensen *et al*.,

Project supported by the Key Project of Chinese Academy of Sciences (No. KZCX3-SW-431, KZCX2-YW-408) and the National Natural Science Foundation of China (No. 40321101, 40571082). * Corresponding author. E-mail: jzhe@rcees.ac.cn.

1998; Claudia *et al*., 2003, He *et al*., 2005, 2006). The PCR-DGGE technique is used for screening microbial community diversity and also changes in response to environmental stresses (Mette and Neils, 2002).

The present study aimed to evaluate the inhibition of enzymatic activities and changes of microbial community structure in the soil amended with different rates of Cd and Pb. Both traditional method of soil enzyme assays and advanced molecular PCR-DGGE techniques were used to investigate the impacts of Cd and Pb with different application rates on soil microbial community structure. The experiment was conducted in a glasshouse for a period of 12 weeks (wks) and specific soil enzymatic activities including catalase (CAT), alkaline phosphatase (ALP) and dehydrogenase (DEH), and microbial community changes were measured.

1 Materials and methods

1.1 Soil characteristics

A pristine soil sample (0–20 cm, classified as Endoaquepts in USA Soil Taxonomy) was collected from a cultivated land near Beijing, China. After transportation to laboratory, the soil was sieved through a 2-mm sieve. Sub-samples were air dried and some physico-chemical properties were measured according to standard procedures. Soil particle composition included 42.5% sand, 47.1% silt and 10.4% clay. Soil pH was 8.0 measured in H_2O. Total Cd and Pb concentrations of the soil were 0.14 mg/kg and 2.57 mg/kg, respectively. Soil organic matter (SOM) content was 17.9 g/kg.

1.2 Experimental design

The pot experiments were conducted in a glasshouse. The sieved soil samples were added with Cd and Pb using $CdSO_4$ and $Pb(NO_3)_2$ solutions, at the following application rates: (1) Cd as Cd1 = 1.5, Cd2 = 3 and Cd3 = 5 mg/kg; (2) Pb as Pb1 = 150, Pb2 = 300, and Pb3 = 500 mg/kg; and (3) both Cd and Pb as Cd1 + Pb1, Cd2 + Pb2 and Cd3 + Pb3. The samples were then thoroughly homogenized and 500 g of each treated samples were filled into each plastic pots. Thus, all together there were 10 treatments, including control (no Cd and Pb), contaminated with Cd, Pb and both Cd+Pb, respectively. The soil was incubated in the glasshouse for 12 wks at 25±4°C. During the incubation period, the soil moisture contents were monitored by weighing and adjusted to 60% water holding capacity by deionized water. Samples from each pot were collected at 0, 2, 9 and 12 wks for enzyme assays and DNA extraction.

1.3 Soil enzyme assays

ALP (orthophosphoric monoester phosphohydrolase, EC 3.1.3.1) activities were determined according to Dick *et al*. (1996). The *p*-nitrophenol phosphatase was used as substrate and the intensity of yellow color of the filtrate due to *p*-nitrophenol was determined using a UV-Vis spectrophotometer (UV 757 CRT, China) at a wavelength of 410 nm, and the results were expressed as mg *p*-nitrophenol/kg dry sample. DEH (dehydrogenase) activity was determined as described by Casida *et al*. (1964) and the reddish color intensity of the filtrate was measured with a spectrophotometer at a wavelength of 485 nm and methanol was used as a blank. The results of the samples were compared with triphenyl formazan standards. While CAT (EC 1.11.1.6) activity was measured by titration method (Yan, 1988) and the results were expressed as ml (0.02 mol/L $KMnO_4$)/(g·0.5 h). All the enzyme assays were performed using the moist soil samples in triplicates along with control without soil. The substrate was added to blanks after the reaction stopped before filtration of the soil suspensions.

Whole-community DNA was extracted from 0.5 g of triplicate fresh samples using a method adopted from Hesham *et al*. (2006). Yield and quality of the extracted DNA was verified by 1.0% agarose gel electrophoresis and stained with ethidium bromide.

1.4 PCR amplification

The region corresponding to nucleotide positions 341 and 518 in the 16S rDNA of *Escherichia coli* was PCR-amplified using the forward primer EUB341F (5′-CCT ACG GGA GGC AGC AG-3′) with a GC clamp (CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC G) at the 5′ end to stabilize the melting behavior of the DNA fragments and the reverse primer EU500R (5′-GTA TTA CCG CGG CTG CTG G-3′) (Muyzer *et al*., 1993). PCR amplification was performed on an iCycler Thermal Cycler (Bio-Rad, USA) in 50 μl reaction volumes. A touch down temperature profile was adopted in which the annealing temperature was reduced from 65°C by 0.5 degree on successive cycles until the theoretical annealing temperature 55°C was reached after 20 cycles, a further 10 cycles were performed at this temperature. The PCR reaction mixture contained 15 pmol/primer, 200 μmol of each deoxyribonucleoside triphosphate, 5 μl of 10×PCR buffer (free of Mg^{2+}), 3 μl of $MgCl_2$ (25 mmol/L), 1.25 μl unit Taq polymerase, 1–10 ng of the DNA extracts as template, and made a final volume of 50 μl.

1.5 Denaturing gradient gel electrophoresis (DGGE)

The DGGE was performed using a Bio-Rad DcodeTM Universal Mutation Detection System (Bio-Rad, USA). The PCR products were loaded on a 0.8-mm thick polyacrylamide gel (10% w/v acrylamide:bisacrylamide of 37.5:1) using a denaturing gradient from 30% to 50% of urea and formamide (100% corresponds to 7 mol/L urea and 40% w/v formamide) being increased in the direction of the electrophoresis run. The electrophoresis was performed under a constant voltage of 150 V for 5 h and a constant temperature of 60°C. After the run, the gels were stained for 20 min in 1×TAE containing 0.5 mg/ml ethidium bromide, rinsed with distilled water and then photographed.

1.6 Data analysis

Means, standard deviation and variance (ANOVA) were

determined using SPSS 11.5 computer program. The means were compared using Duncan test with a significance level of $P<0.05$.

2 Results

2.1 Enzymatic activities

The mean values of CAT (catalase) activity were lower in all heavy metals amended samples than those in the control (Fig.1). The CAT inhibition rate increased with increasing metal concentrations. The inhibition extent was also obvious between different incubation periods. The lowest CAT activity (39.9% of the control) was found in the treatment of Cd3+Pb3 at 2 wks. In the Cd treated soil, the CAT activity had a reduction ranging from 5.96% to 39.3%, and the highest inhibition rate was observed in Cd3 treatments. Similarly, in the Pb treatments, this enzyme activity decreased with increasing Pb level. In the case of both Cd+Pb treatments, the CAT activity was also significantly decreased with increasing levels of Cd+Pb, and ranged from 8.8%–39.9%. For all treatments, the CAT values were more seriously inhibited in the first two wks, but the inhibition eased off with incubation time.

ALP (alkaline phosphatase) activity was significantly affected by the heavy metals. The values of this enzyme were lower in heavy metals amended soils as compared to the control (Fig.2). In Cd treated samples, the activity of this enzyme dropped by 7.8%–41.5%, and the highest inhibition rate was detected in the samples incubated for 2 wks. Like CAT, the ALP activity was strongly inhibited up to 2 wks and then increased with incubation time. In the case of Pb treatments, the extent of inhibition was lower than Cd treatments, and increased with increasing Pb level, such as 7.8%–19.3% in Pb1, 11.9%–20.9% in Pb2 and 13.1%–24.3% in Pb3 treatments. The combined effects of both Cd+Pb were also higher on this enzyme activity and values dropped by 25.5%, 40.5% and 43.5% in Cd1+Pb1, Cd2+Pb2 and Cd3+Pb3 treatments respectively, in samples of 2 wks.

Mean values of DEH activity also decreased significantly with increasing levels of heavy metals in the amended samples (Fig.3). The inhibition rate of DEH varied in samples with different incubation periods. Lower DEH activity was detected in all metal treated samples than in control. In the case of Cd treatments, DEH activity was significantly inhibited, by 19.3%, 25.9% and 32.4% in Cd1, Cd2 and Cd3 treatments, respectively, after 2 wks incubation. This highest inhibitory effect of heavy metals on soil enzyme activities may be due to the sudden exposure of the microbes to heavy metals in the first two wks, and thus resulted in a significant decrease in the enzyme activity. Later on the microbes may have adapted to the polluted environment, and the enzyme activity tended to recover. In Pb treated samples, this inhibition was 2.9%–15.8% in Pb1, 7.2%–23.7% in Pb2 and 12.1%–18.2% in Pb3 treatments. This enzyme activity was also strongly inhibited in both Cd+Pb treatments, by 8.9%–24.1% in Cd1+Pb1, 11.9%–32.5% in Cd2+Pb2 and 15.5%–41.6% in Cd3+Pb3 treatments.

Results showed that CAT, ALP and DEH activities

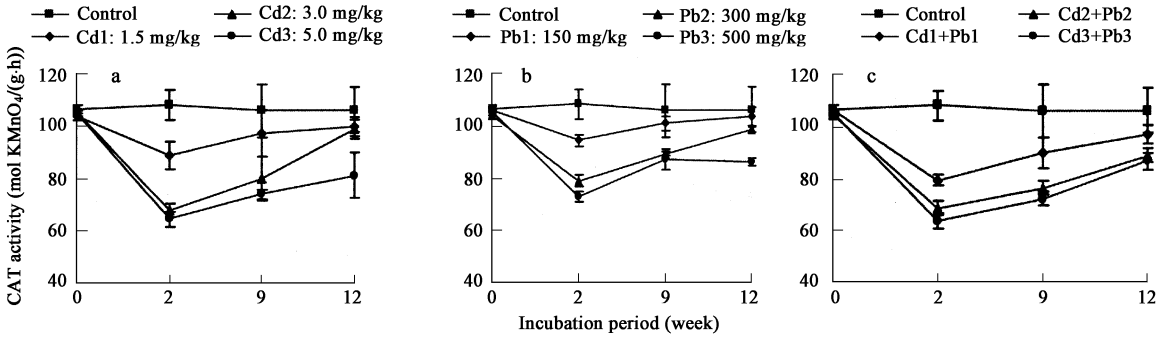

Fig. 1 Inhibitory effects of heavy metals on CAT activity (ml (0.1 mol/L $KMnO_4$)/(g·0.5 h)) during different incubation periods. (a) Cd treatment; (b) Pb treatment; (c) Cd+Pb treatment. The error bars indicate standard deviation.

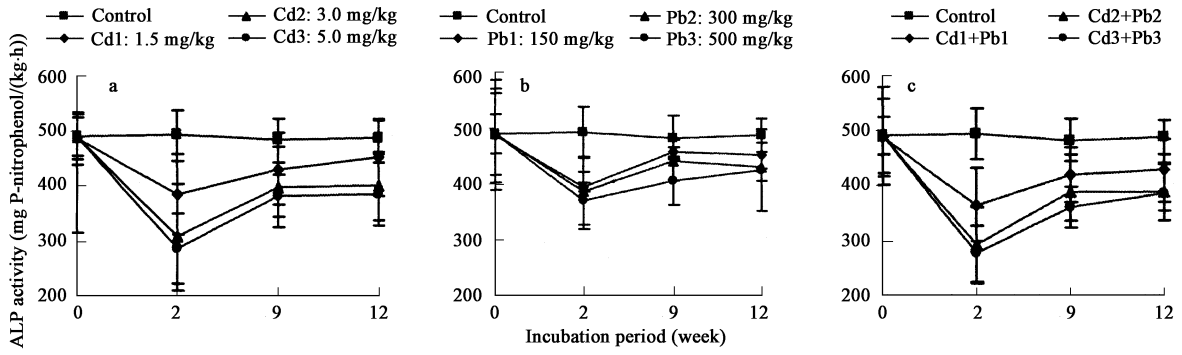

Fig. 2 Inhibitory effects of heavy metals on ALP activity (mg *p*-nitrophenol/(kg·h) during different incubation periods. (a) Cd treatment; (b) Pb treatment; (c) Cd+Pb treatment. The error bars indicate standard deviation.

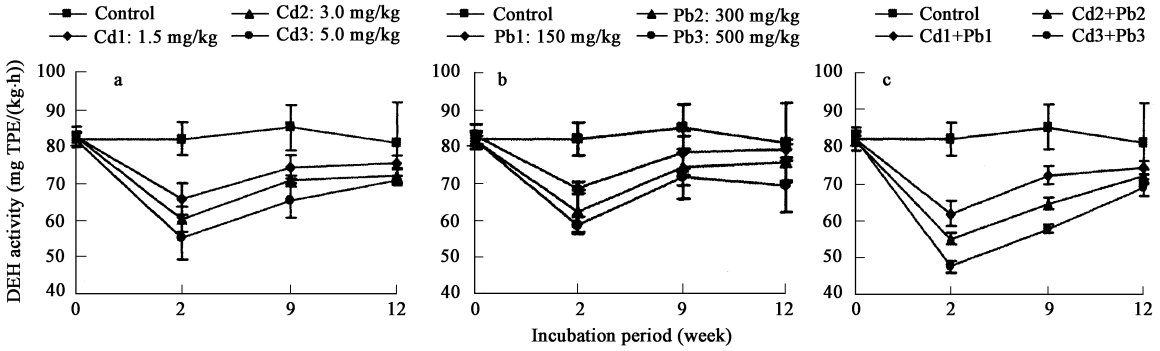

Fig. 3 Inhibitory effects of heavy metals on DEH activity (mg TPF/(kg·h) during different incubation periods. (a) Cd treatment; (b) Pb treatment; (c) Cd+Pb treatment. The error bars indicate standard deviation.

varied widely among different treatments and incubation periods. The enzymatic activities decreased significantly with increasing levels of heavy metals. The data also showed that the combined inhibitory effects of Cd and Pb were more significant than their individual effects on the enzymatic activities. Table 1 shows the Pearson's correlation coefficients between the heavy metals and all the three enzyme activities. All enzyme activities showed significant positive correlations among each other, but negative correlations were detected between the enzyme activities and the heavy metal concentrations.

2.2 PCR-DGGE patterns for bacterial community structure

DNA was extracted from the Cd treated samples and the DGGE patterns of PCR-amplified 16S rDNA is shown in Fig.4. Here Cd treatments were selected as an example for the study of community structure because of its high toxicity on soil microorganisms. In these samples, the PCR-DGGE patterns revealed a shift in soil bacterial community structure with a greater complexity of banding pattern in Cd1 samples than in Cd2 and Cd3 samples. Similarly, the PCR-DGGE patterns also indicated a change in microbial community structure with the incubation period, with a greater impact in Cd3 samples than Cd1 and Cd2 samples. The PCR-DGGE patterns provided the evidence that high dose of Cd caused a greater change

Table 1 Correlation coefficients among soil enzymes activities and between the soil enzyme activity and heavy metals (Cd and Pb)

	Cd	CAT	ALP	DEH
Cd	1*	–0.704	–0.572	–0.618
CAT	–	1	0.760	0.861
ALP	–	–	1	0.616
DEH	–	–	–	1
	Pb	CAT	ALP	DEH
Pb	1	–0.703	–0.479	–0.574
CAT	–	1	0.591	0.811
ALP	–	–	1	0.436
DEH	–	–	–	1
	Cd+Pb	CAT	ALP	DEH
Cd+Pb	1	–0.696	–0.596	–0.656
CAT	–	1	0.768	0.925
ALP	–	–	1	0.682
DEH	–	–	–	1

CAT: catalase; ALP: alkaline phosphatase; DEH: dehydrogenase; *all the coefficients are significant at the 0.01 significant level (2-tailed).

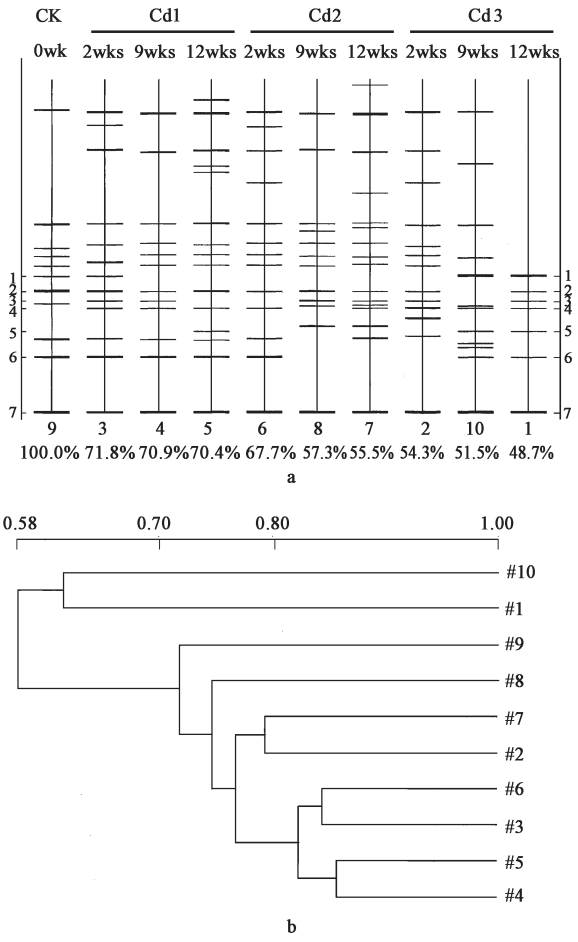

Fig. 4 Similarity dendrograms and cluster analysis of banding patterns generated by PCR-DGGE of 16S rDNA fragments from Cd treated sampls. (a) similarity dendrograms; (b) cluster analysis.

in the soil microbial diversity. In the treatments of Cd1 and Cd2, the majority of bands were more or less evenly localized particularly in the 12 wks treatment, while in the Cd3 treated samples the bands were localized in the middle part of the gel, whereas in 12 wks samples, more bands appeared in the lower part of the gel under higher denaturing conditions. In Cd-treated samples, different levels caused different patterns of decrease in soil bacterial diversity. Similarity dendrograms and phylogenetic analysis were generated by the image analysis of DGGE coupled with the Dice similarity coefficient, showed that PCR-DGGE

patterns of Cd treated samples could be well discriminated from the control (Fig.4). Dendrogram analyses of PCR-DGGE banding patterns confirmed that the addition of metals had a significant impact on the microbial community structure. Dendrogram analysis of a replicate DGGE gel created from separate amplification products indicated that the observed clustering was reproducible and stable.

3 Discussion

In the soil environment, almost all reactions are catalysed by enzymes that are largely of microbial origin and associated with viable cells (Dick, *et al*., 1996). CAT is an intracellular enzyme and involved in microbial oxidoreductase metabolism (García-Gil *et al*., 2000). The CAT inhibition rate increased with increasing heavy metals, particularly, in Cd+Pb treated samples. Moreover, the inhibiting extent of CAT activity by heavy metals was also strong in Cd+Pb amended samples. This finding is in contrast with the results of Belyaeva *et al*. (2005) who found that CAT activity was not markedly inhibited by heavy metals.

Soil phosphatases are important in soil P cycling, involving in mineralization of organic P and releasing phosphate for plants (Dick and Tabatabai, 1983; Pant and Warman, 2000; Gil-Sotres *et al*., 2005). In this study, ALP activities were significantly inhibited with increasing concentration of heavy metals. Similarly, Moreno *et al*. (2003) also concluded that Cd and Ni had clear inhibition effects on phosphatase activities.

DEH plays an important role in the oxidation of organic matters (Dick *et al*., 1996). In this study DEH activity was significantly inhibited with heavy metals and confirming the results from pervious studies by Chander and Brookes (1991), Marzadori *et al*. (1996) and García-Gil *et al*. (2000). These studies found that DEH activity was inhibited by the toxic effect of heavy metals added to the soil system. DEH activity appeared to be one of the most sensitive parameters among the selected enzyme activities. It could be used as a good indicator of soil ecotoxicological test caused by heavy metals (Maliszewsk-Kordybach and Smeeczak, 2003; Hinojosa *et al*., 2004).

The toxic effects of added heavy metals on enzymatic activities were considerably variable for different levels of Cd and Pb and also during different incubation period. The extent of inhibition increased with increasing levels of heavy metals. According to the studies conducted by Tyler (1974) and Kızılkaya *et al*. (2004), soil enzymatic activities diminished with increasing available concentrations of heavy metals. In this study, the rate of inhibition varied widely with incubation period and a positive correlation was found between all enzymatic activities but negative correlation was observed between heavy metals and enzymatic activities. The rapid inhibition in enzymatic activities was found in the 2-wks incubation which should be related to the fact that the microorganisms were suddenly exposed to heavy metals.

It is well documented that the heavy metals react with sulphydral groups of enzymes and inhibit and/or inactivate the enzymatic activities. Heavy metals could also indirectly affect soil enzymatic activities by altering the microbial community which synthesizes enzymes (Nannipieri, 1994; Kandeler *et al*., 2000). Previous studies such as Doelman and Haanstra (1984), Kuperman and Carreiro (1997), Bandick and Dick (1999) and Kunito *et al*. (2001) have reported that heavy metals are inactivating extracellular enzymes due to their binding with some amino acids in enzymes and reducing the number of microorganisms that produce enzymes. Enzymes toxicity of Cd was found higher than Pb because of its greater mobility and lower affinity for soil colloids. Soil enzymatic activities are good indicators to assess both beneficial and harmful effects on soil health.

The PCR-DGGE patterns showed that while numerous bands were common to all treatments, there were also changes in band presence and relative intensity due to different treatments and temporal effects of metal toxicity. In the Cd treated samples, the bacterial diversity was affected by high rates of Cd, in agreement with the lower enzymatic activity in different level of Cd treatments but not consistent with incubation period within the same treatment. Significant lower CAT, ALP and DEH activities detected in all treated soils could be due to both a lower synthesis and/or release of extracellular enzymes by microbes or to the inhibition of extracellular enzymes. Renella *et al*. (2005) found that it is not easy to interpret the effect of heavy metals on soil enzymatic activities because the meaning of these measurements was not clear, due to the different locations of enzymes in soils. According to Nannipieri *et al*. (2003), these enzyme activities were not discriminative between extra- and intra-cellular enzyme activities. The PCR-DGGE profile showed that the structure of bacterial community changed in heavy metal treated samples. Similarly, Bååth *et al*. (1998) has also reported that the microbial community structure changed in soils amended with Zn (359 mg/kg) and Ni (89 mg/kg) rich sludge. Akmal *et al*. (2005) has also observed the changes of microbial community structure in metal amended soils. In this study, the control and low level of Cd treatments had the most complex PCR-DGGE patterns, indicating the presence of a higher number of different bacterial texa (Luca *et al*., 2002). A great community change in this study, particularly at high level of contamination, was probably resulted from metal toxicity and also unavailability of nutrients. In the previous work it has also been reported that elevated concentrations of heavy metals can change the microbial community structure as detected by PCR-DGGE analysis, which was also used for metals resistant microorganisms (Kozdroj and Van Elsas, 2000; Anne *et al*., 2001; Kong *et al*., 2006). In this study, different soil properties such as enzymatic activities and microbial community structure were used together for estimating soil quality indices which is a better approach for evaluation of soil quality (Trasar-Cepeda *et al*., 2000). Akmal *et al*. (2005) has also reported that soil enzymatic activities and PCR-DGGE analysis can be combined for soil quality assessment. In the present work, the enzymatic activities were inhibited by the application of heavy metals, par-

ticularly, at 2 wks incubation, and microbial community was changed. According to the findings of this study, the applied heavy metals severely affected the enzymatic activities and microbial community structure.

4 Conclusions

It was concluded that the heavy metals have strong inhibitory effects on soil enzymatic activities and microbial community structure. The highest inhibitory effect of heavy metals to soil enzymatic activities were observed at initial 2 wks of incubation and later on they tended to replenish. Microbial community structure examined by PCR-DGGE technique was also changed in the Cd treated samples, particularly at high Cd concentrations. It was noted that soil enzymatic activities and PCR-DGGE technique together could give more information about function and structure of soil microbial community in heavy metal contaminated samples. A negative correlation was found between heavy metals and all enzymatic activities, but positive correlations were observed among all enzymatic activities. The combined effects of both Cd+Pb on enzymatic activities were higher than Cd or Pb alone.

Acknowledgements

Mr. Sardar Khan would like to thank Higher Education Commission, Islamabad, Pakistan for supporting his Ph.D study.

References

Akmal M, Wang H Z, Wu J J *et al*., 2005. Changes in enzymes activity, substrate utilization pattern and diversity of soil microbial communities under cadmium pollution[J]. J Environ Sci, 17: 802–807.

Anne K M, Kamma W, Soren C *et al*., 2001. The effect of long-term mercury pollution on the soil microbial community[J]. FEMS Microbiol Ecol, 36: 11–19.

Bååth E, Diaz-Ravina M, Frostegård A *et al*., 1998. Effect of metal-rich sludge amendments on the soil microbial community[J]. Appl Environ Microbiol, 64: 238–245.

Bandick A K, Dick R P, 1999. Field management effects on soil enzyme activities[J]. Soil Biol Biochem, 31: 1471–1479.

Baum C, Linweber P, Schlichting A, 2003. Effects of chemical conditions in re-wetted peats temporal variation in microbial biomass and acid phosphatase activity within the growing season[J]. Appl Soil Ecol, 22: 167–174.

Belyaeva O N, Haynes R J, Birukova O A, 2005. Barely yield and soil microbial and enzyme activities as affected by contamination of two soils with lead, zinc or copper[J]. Biol Fert Soils, 41(2): 85–94.

Casida L E, Klein D A, Santoro T, 1964. Soil dehydrogenase activity[J]. Soil Sci, 98: 371–376.

Chander K, Brookes P C, 1991. Is the dehydrogenase assay invalid as a method to estimate microbial activity in copper-contaminated soils?[J]. Soil Biol Biochem, 23: 909–915.

Claudia S G, Werner L, Sabine R, 2003. Application of broad-range 16S rRNA PCR amplification and DGGE fingerprinting for detection of tick-infecting bacteria[J]. J Microbiol Methods, 52: 251–260.

Cui Y, Zhu Y G, Zhai R H *et al*., 2004. Transfer of metals from soil to vegetables in an area near a smelter in Nanning, China[J]. Environ Int, 30: 785–791.

Cui Y, Zhu Y G, Zhai R H *et al*., 2005. Exposure to metal mixtures and human health impacts in a contaminated area in Nanning, China[J]. Environ Int, 31: 784–790.

Dick W A, Tabatabai M A, 1983. Activation of soil pyrophosphatase by metal ions[J]. Soil Biol Biochem, 15: 359–363.

Dick R P, Breakwell D P, Turco R F, 1996. Soil enzyme activities and biodiversity measurements and integrative microbial indicators[M]. In: Methods of assessing soil quality. Madison, WI: Soil Science Society of America Publication 49. 247–271.

Doelman P, Haanstra L, 1984. Short-term and long-term effects of cadmium, chromium, copper, nickel, lead and zinc on soil microbial respiration in relation to abiotic soil factors[J]. Plant Soil, 79: 317–327.

García-Gil J C, Plaza C, Soler-Rovira P *et al*., 2000. Long-term effects of municipal solid waste compost application on soil enzyme activities and microbial biomass[J]. Soil Biol Biochem, 32: 1907–1913.

Gil-Sotres F, Trasar-Cepeda C, Leirós, M C *et al*., 2005. Different approaches to evaluate soil quality using biochemical properties[J]. Soil Biol Biochem, 37: 877–887.

He J Z, Xu Z H, Hughes J, 2005. Analyses of soil fungal communities in adjacent natural forest and hoop pine plantation ecosystems of subtropical Australia using molecular approaches based on 18S rRNA genes[J]. FEMS Microbiol Lett, 247: 91–100.

He J Z, Xu Z H, Hughes J, 2006. Molecular bacterial diversity of a forest soil under residue management regimes in subtropical Australia[J]. FEMS Microbiol Ecol, 55: 38–47.

Hesham A, Khan S, Liu X *et al*., 2006. Application of PCR-DGGE to analyse the yeast population dynamics in slurry reactors during degradation of polycyclic aromatic hydrocarbons in weathered oil[J]. Yeast, 23: 879–887.

Hinojosa M B, Carreira J A, Garcła-Rułz R *et al*., 2004. Soil moisture pre-treatment effects on enzyme activities as indicators of heavy metal-contaminated and reclaimed soils[J]. Soil Biol Biochem, 36: 1559–1568.

Jensen S, Ovreas L, Daae F L *et al*., 1998. Diversity in methane enrichments from agricultural soil revealed by DGGE separation of PCR amplified 16S rDNA fragments[J]. FEMS Microbiol Ecol, 26: 17–26.

Kandeler E, Tscherko D, Bruce K D *et al*., 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil[J]. Biol Fert Soils, 32: 390–400.

Khan S, Cao Q, Chen B *et al*., 2006. Humic acids increase the phytoavailability of Cd and Pb to wheat plants cultivated in freshly spiked, contaminated soil[J]. J Soils Sediments, 6: 236–242.

Kızılkaya R, Aşkın T, Bayraklı B *et al*., 2004. Microbiological characteristics of soils contaminated with heavy metals[J]. Eur J Soil Biol, 40: 95–102.

Kızılkaya R, Bayraklı B, 2005. Effects of N-enriched sewage sludge on soil enzyme activities[J]. Appl Soil Ecol, 30: 192–202.

Kong W D, Zhu Y G, Fu B J *et al*., 2006. The veterinary antibiotic oxytetracycline and Cu influence functional diversity of the soil microbial community[J]. Environ Pollu, 143: 129–137.

Kozdroj J, Van Elsas J D, 2000. Response of the microbial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches[J]. Soil Biol Biochem, 32: 1405–1417.

Kunito T, Saeki K, Goto S *et al*., 2001. Copper and zinc fractions affecting microorganisms in long-term sludge-amended soils[J]. Bioresource Technol, 79: 135–146.

Kuperman M M, Carreiro R G, 1997. Soil heavy metal concentrations, microbial biomass and enzyme activities in a contaminated grassland ecosystem[J]. Soil Biol Biochem, 29: 179–190.

Lee I S, Kim O K, Chang Y Y *et al*., 2002. Heavy metals concentrations and enzymes activities in soil from a contaminated Korean shooting range[J]. J Biosci Bioeng, 94(5): 406–411.

Luca C, Daniele A, Marisa M *et al*., 2002. An application of PCR-DGGE analysis to profile the yeast population in raw milk[J]. Int Dairy J, 12: 407–411.

Majer B J, Tscherko D, Paschke A *et al*., 2002. Effects of heavy metals contamination on micronucleus induction in *Traescandia* and on microbial enzymes activities: a comparison investigation[J]. Mutation Res, 515: 111–124.

Marzadori C, Ciavatta C, Montecchio D *et al*., 1996. Effects of lead pollution on different soil enzyme activities[J]. Biol Fert Soils, 22: 53–58.

Maliszewsk-Kordybach B, Smeeczak B, 2003. Habitat function of agricultural soils as affected by heavy metals and polycyclic aromatic hydrocarbons contamination[J]. Environ Int, 28: 719–728.

Megharaj K V M, Sethunathan N, Naidu R, 2003. Bioavailability and toxicity of cadmium to microorganisms and their activities in soil: a review[J]. Adv Environ Res, 8: 121–135.

Mette H N, Neils B R, 2002. Denaturing gradient gel electrophoresis (DGGE) approaches to study the diversity of ammonia-oxidizing bacteria[J]. J Micriobiol Methods, 50: 189–203.

Moreno J L, Hernández T, Pérez A *et al*., 2002. Toxicity of cadmium to soil microbial activity: effects of sewage sludge addition to soil on the ecological dose[J]. Appl Soil Ecol, 21: 149–158.

Moreno J L, Garcła C, Hernández T, 2003. Toxic effect of cadmium and nickel on soil enzymes and the influence of adding sewage sludge[J]. Euro J Soil Sci, 54: 377–386.

Muyzer G, Waal de E C, Uitterlinden A G, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA[J]. Appl Environ Microbiol, 59: 695–700.

Nannipieri P, Ascher J, Ceccherini M T *et al*., 2003. Microbial diversity and soil functions[J]. European J Soil Sci, 54: 655–670.

Nannipieri P, 1994. The potential use of soil enzymes as indicators of productivity, sustainability and pollution[M]. In: Soil biota management in sustainable farming systems (Pankhurst C. E., Double B. M., Gupta V. V. S. R., Grace P. R. ed.). Victoria, Australia: CSIRO, East Melbourne. 238–244.

Pant H K, Warman P R, 2000. Enzymatic hydrolysis of soil organic phosphorus by immobilized phosphatases[J]. Biol Fert Soils, 30: 306–311.

Pérez-de-Mora A, Burgos P, Madejón E *et al*., 2006. Microbial community structure and function in a soil contaminated by heavy metals: effects of plant growth and different amendments[J]. Soil Biol Biochem, 38: 327–341.

Rattan R K, Datta S P, Chhonkar P K *et al*., 2005. Long-term impact of irrigation with sewage effluents on heavy metal content in soils, crops and groundwater–a case study[J]. Agr Ecosyst Environ, 109: 310–322.

Renella G, Mench M, Gelsomino A *et al*., 2005. Functional activity and microbial community structure in soils amended with bimetallic sludges[J]. Soil Biol Biochem, 37: 1498–1506.

Shen G, Lu Y, Zhou Q *et al*., 2005. Interaction of polycyclic aromatic hydrocarbons and heavy metals on soil enzymes[J]. Chemosphere, 61: 1175–1182.

Trasar-Cepeda C, Leirós M C, Seoane S *et al*., 2000. Limitation of soil enzymes as indicators of soil pollution[J]. Soil Biol Biochem, 32: 1867–1875.

Tyler G, 1974. Heavy metal pollution and soil enzymatic activity[J]. Plant Soil, 41: 303–311.

Yan C S, 1988. Research method of soil fertility[M]. Beijing: Chinese Agriculture Press.

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[J]. Pedosphere, 3: 251–258.