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AREA 1.1 • BEHAVIOR OF CHEMICALS IN SOILS / INTERACTIONS WITH BIOTA • RESEARCH ARTICLE

Effects of Cd and Pb on soil microbial community structure and activities

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Abstract
Background, aim, and scope Soil contamination with heavy metals occurs as a result of both anthropogenic and natural activities. Heavy metals could have long-term hazardous impacts on the health of soil ecosystems and adverse influences on soil biological processes. Soil enzymatic activities are recognized as sensors towards any natural and anthropogenic disturbance occurring in the soil ecosystem. Similarly, microbial biomass carbon (MBC) is also considered as one of the important soil biological activities frequently influenced by heavy metal contamination. The polymerase chain reaction–denaturing gradient gel electrophoresis (DGGE) has recently been used to investigate changes in soil microbial community composition in response to environmental stresses. Soil microbial community structure and activities are difficult to elucidate using single monitoring approach; therefore, for a better insight and complete depiction of the soil microbial situation, different approaches need to be used. This study was conducted in a greenhouse for a period of 12 weeks to evaluate the changes in indigenous microbial community structure and activities in the soil amended with different application rates of Cd, Pb, and Cd/Pb mix. In a field environment, soil is contaminated with single or mixed heavy metals; so that, in this research, we used the selected metals in both single and mixed forms at different application rates and investigated their toxic effects on microbial community structure and activities, using soil enzyme assays, plate counting, and advanced molecular DGGE technique. Soil microbial activities, including acid phosphatase (ACP), urease (URE), and MBC, and microbial community structure were studied.
Materials and methods A soil sample (0–20 cm) with an unknown history of heavy metal contamination was collected and amended with Cd, Pb, and Cd/Pb mix using the $CdSO_4$ and $Pb(NO_3)_2$ solutions at different application rates. The amended soils were incubated in the greenhouse at 25±4°C and 60% water-holding capacity for 12 weeks. During the incubation period, samples were collected from each pot at 0, 2, 9, and 12 weeks for enzyme assays, MBC, numeration of microbes, and DNA extraction. Fumigation–extraction method was used to measure the MBC, while plate counting techniques were used to numerate viable heterotrophic bacteria, fungi, and actinomycetes. Soil DNAs were extracted from the samples and used for DGGE analysis.
Results ACP, URE, and MBC activities of microbial community were significantly lower ($p<0.05$) in the metal-amended samples than those in the control. The enzyme inhibition extent was obvious between different incubation periods and varied as the incubation proceeded, and the highest rate was detected in the samples after 2 weeks. However, the lowest values of ACP and URE activities (35.6% and 36.6% of the control, respectively) were found in the Cd_3/Pb_3-treated sample after 2 weeks. Similarly, MBC was strongly decreased in both Cd/Pb-amended samples and highest reduction (52.4%) was

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detected for Cd_3/Pb_3 treatment. The number of bacteria and actinomycetes were significantly decreased in the heavy metal-amended samples compared to the control, while fungal cells were not significantly different (from 2.3% to 23.87%). In this study, the DGGE profile indicated that the high dose of metal amendment caused a greater change in the number of bands. DGGE banding patterns confirmed that the addition of metals had a significant impact on microbial community structure.

Discussion In soil ecosystem, heavy metals exhibit toxicological effects on soil microbes which may lead to the decrease of their numbers and activities. This study demonstrated that toxicological effects of heavy metals on soil microbial community structure and activities depend largely on the type and concentration of metal and incubation time. The inhibition extent varied widely among different incubation periods for these enzymes. Furthermore, the rapid inhibition in microbial activities such as ACP, URE, and MBC were observed in the 2 weeks, which should be related to the fact that the microbes were suddenly exposed to heavy metals. The increased inhibition of soil microbial activities is likely to be related to tolerance and adaptation of the microbial community, concentration of pollutants, and mechanisms of heavy metals. The DGGE profile has shown that the structure of the bacterial community changed in amended heavy metal samples. In this research, the microbial community structure was highly affected, consistent with the lower microbial activities in different levels of heavy metals. Furthermore, a great community change in this study, particularly at a high level of contamination, was probably a result of metal toxicity and also unavailability of nutrients because no nutrients were supplied during the whole incubation period.

Conclusions The added concentrations of heavy metals have changed the soil microbial community structure and activities. The highest inhibitory effects on soil microbial activities were observed at 2 weeks of incubation. The bacteria were more sensitive than actinomycetes and fungi. The DGGE profile indicated that bacterial community structure was changed in the Cd/Pb-amended samples, particularly at high concentrations.

Recommendations and perspectives The investigation of soil microbial community structure and activities together could give more reliable and accurate information about the toxic effects of heavy metals on soil health.

Keywords DGGE · Enzymatic activity · Heavy metals · Microbial biomass carbon · Microbial community

1 Background, aim, and scope

Heavy metals are increasing in soil environments as a result of both anthropogenic and natural activities. Wastewater irrigation, sludge applications, solid waste disposal, automobile exhaust, and industrial waste dumping are the major anthropogenic sources of heavy metals (Khan et al. 2008). However, heavy metals could have long-term hazardous impacts on the health of soil ecosystems and adverse influences on soil biological processes (Pérez-de-Mora et al. 2006; Wang et al. 2007; Bhattacharyya et al. 2008). Soil biological characteristics are recognized as bioindicators of soil quality because they are more dynamic and more sensitive than the soil physicochemical properties (Brookes 1995; Hinojosa et al. 2004). Previously, literature has revealed that both short-term and long-term exposures to heavy metals result in the reduction of soil microbial community diversity and activities (Lasat 2002; McGrath et al. 2001; Khan et al. 2007).

Soil enzymes are acting as biological catalysts to facilitate different reactions and metabolic processes occurring in biogeochemical cycles of nutrients, maintenance of soil structure, detoxification of pollutants, and produce essential compounds for both microorganisms and plants (Dick 1997; Moreno et al. 2003). Soil enzymatic activities are known as sensors towards any natural and anthropogenic disturbance occurring in the soil ecosystem (Baum et al. 2003; Hinojosa et al. 2004; Wang et al. 2007). Many researchers have used the soil enzymatic activities as bioindicators to determine toxicological influences of various pollutants on soil microbial quality (Shen et al. 2005). Microbial biomass carbon (MBC) is also considered as one of the important soil biological activities frequently influenced by heavy metal contamination. In the past, MBC has been used as meaningful indexes of soil quality assessment (He et al. 2006; Xu et al. 2008).

In order to investigate the link between soil microbial community structure and activities changes with amendments of heavy metals, it is necessary to study the microbial community structure using traditional approaches like plate counting and/or advance molecular approaches such as polymerase chain reaction–denaturing gradient gel electrophoresis (PCR-DGGE). These techniques have recently been used to investigate changes in soil microbial community composition (Claudia et al. 2003; Oliveira and Pampulha 2006; Wang et al. 2008; Moreels et al. 2008). The DGGE technique is used for screening microbial community composition and also changes in response to environmental stresses (Mette and Neils 2002). Microbial community structure and activity are important indices of soil quality. Furthermore, any alteration in the composition of microbial communities has often been proposed to be an easy and sensitive indicator of anthropogenic effects on soil ecology. Generally, heavy metals have induced influence on microbial community, which ultimately lead to changes in soil microbial activities including enzymes and MBC (Moreno et al. 2002; He et al. 2005).

Soil microbial community structure and activities are difficult to elucidate using single monitoring approach; therefore, for a better insight and complete depiction of the soil microbial situation, different approaches need to be used. This study was conducted in a greenhouse for a period of 12 weeks to evaluate the changes in microbial community structure (using plate counting and advanced molecular DGGE techniques) and activities (using soil enzyme assays and MBC) in the soil amended with different application rates of Cd, Pb, and Cd/Pb mix.

2 Materials and methods

2.1 Soil characteristics

A soil sample (0–20 cm depth) was collected from a cultivated field after harvesting of maize crop and sieved through a 2-mm sieve in the laboratory. Subsample was air-dried and used to measure the basic physicochemical properties of the soil, as shown in Table 1.

2.2 Experimental set up

The experimental set up of this pot experiment was also the same as that used in our previous work (Khan et al. 2007). Briefly, the sieved soil sample was added with Cd and Pb using the $CdSO_4$ and $Pb(NO_3)_2$ solutions at the following application rates (in milligrams per kilogram): (a) Cd as Cd_1=1.5, Cd_2=3, and Cd_3=5; (b) Pb as Pb_1=150, Pb_2=300, and Pb_3=500; and (c) both Cd/Pb as Cd_1/Pb_1=1.5/150, Cd_2/Pb_2=3/300, and Cd_3/Pb_3=5/500g. Plastic pots were filled with 500 g of the treated soil samples and control without Cd, Pb, and Cd/Pb was also included. The soil was incubated in the glasshouse for 12 weeks at 25±4°C.

Table 1 Some initial physicochemical characteristics of the soil sample

Properties	Values (mean±SD)
pH (water)	7.9±0.3
Soil particle size (%)	
Sand	40.3±1.6
Silt	50.4±2.
Clay	9.3±0.7
SOM (g kg^{-1})	14.6±2.3
Cd (mg kg^{-1})	0.2±0.08
Pb (mg kg^{-1})	1.79±1.35
Nitrate (mg kg^{-1})	13.47±1.5
Fluoride (mg kg^{-1})	0.55±0.1
Chloride (mg kg^{-1})	3.05±0.9
Sulfate (mg kg^{-1})	8.03±1.2

During the incubation period, the soil moisture contents were monitored by weighing and adjusted to 60% water-holding capacity by adding deionized water. Samples from each pot were collected at 0, 2, 9, and 12 weeks for enzyme assays and DNA extraction and numeration of microbial community.

2.3 Bioavailable fractions of metals

Sequential chemical extraction is a well-established approach for the fractionation of trace metals in soils, but only bioavailable fractions of metals are presented in this study. For bioavailable fractions, 5 g of soil sample was extracted for 2 h with 8 mL of 0.5 M $CaCl_2$ (pH 7.0) and 15 mL of deionized water. After extraction, separation was done by centrifuging at 7,000×*g* for 10 min. The supernatant was filtered through a 0.45-μm filter paper and analyzed for bioavailable metal concentrations using inductively coupled plasma–optical emission spectrometry (ICP-OES; Perkin-Elmer OPTIMA-2000, USA).

2.4 Soil microbial activities

Acid phosphatase (ACP; orthophosphoric monoester phosphohydrolase, EC 3.1.3.2) activities were measured according to Dick et al. (1996). The *p*-nitrophenol phosphatase was used as substrate and the intensity of the 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 milligrams of *p*-nitrophenol per kilogram of dry sample.

Urease (URE; urea amidohydrolase, EC 3.5.1.5) activity was measured by the method described by Kızılkaya and Bayraklı (2005) with minor modification. Briefly, 1 g of soil sample was mixed with 0.25 mL toluene, 0.75 mL citrate buffer (pH 6.7), and 1 mL of 10% urea substrate solution. The soil samples were filtered after 3 h incubation at 37°C and 1 mL of filtrate was diluted to 10 mL with deionized water and 4 mL of sodium phenolate and 3 mL of sodium hypochloride solution was added. The formation of ammonium was determined with a spectrophotometer with wavelength adjusted to 578 nm and results were expressed as milligrams of N per kilogram. Ammonium concentrations were determined using a calibration curve of ammonium chloride standard solutions. 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 and before filtration of the soil suspensions.

Fumigation–extraction method was used to measure the MBC (Vance et al. 1987). Every time, adequate amount of moist soil samples were collected from each pot. Subsample of moist soil (equivalent to 3.0 g dry soil) was

fumigated with ethanol-free chloroform for 24 h at 25°C, while another subsample of the same weight was not fumigated. A 20 mL K_2SO_4 (0.5 M) solution was used to extract MBC from fumigated and nonfumigated samples. The samples were shaken for 30 min, centrifuged at 7,000×*g* for 10 min and the supernatant was filtered and frozen at −20°C. The MBC contents were measured in the extracted samples using a TOC analyzer (Phoenix 8000, Tekmar Dohrmann, USA). The MBC was calculated by the expression: $MBC=2.22\ (C_{fumigated}-C_{nonfumigated})$ where $C_{fumigated}$ and $C_{nonfumigated}$ are C extracted from fumigated and nonfumigated soil samples, respectively.

2.5 Soil microbial community structure

2.5.1 Plate counting

The total number of culturable heterotrophic bacteria, actinomycetes, and fungi were numerated using the plate counting technique. Soil (10 g fresh weight) from each replicate of every treatment was weighed into 300 mL Erlenmeyer flasks containing 90 mL of sterile monopotassium phosphate buffer (K_2HPO_4 (0.65 g), KH_2PO_4 (0.35 g), and $MgSO_4$ (0.10 g) in 1 L water) and shaken for 15 min at 250 rpm. From each soil extract, 100-fold dilutions were prepared in test tubes containing 9 mL buffer. The total number of culturable heterotrophic bacteria was numerated using plate counts made on tryptone soya agar (Oxoid, Basingstone, Hampshire, England) amended with 0.1 g/L cyclohexamide. The colony-forming units (cfu) of fungi and actinomycetes were also numerated using plate counts made on rose Bengal agar (Oxoid) amended with 30 mg/L streptomycin sulfate for fungi, while glycerol casein agar was amended with 0.05 g/L cyclohexamide for actinomycetes. The plates were inoculated with 100 µL soil suspension and cultured in incubator at 25°C for 1–2 weeks (to form visible colonies of heterotrophic bacteria, fungi, and actinomycetes). Control plates of respective media without soil suspension were also included to check any possible contamination.

2.5.2 DNA extraction and PCR-DGGE analysis

In this study, Cd/Pb treatments were selected as an example for the study of bacterial community structure because of their high toxicological effects on soil microbial activities. Soil community DNA was extracted from triplicate samples of 0.5 g of fresh soil by a bead-beating procedure using the Fast DNA Spin Kit (for soil) as described by the manufacturer (Bio 101, USA). Yield and quality of the extracted DNA was verified by 1.0% (*w*/*v*) agarose gel electrophoresis and stained with ethidium bromide. Primers 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 and EU500R: 5′-GTA TTA CCG CGG CTG CTG G-3′ were used in this study for amplification of bacterial 16S rDNA genes (Muyzer et al. 1993). PCR amplification was performed on an iCycler Thermal Cycler (Bio-Rad, USA) using 50 µL reaction volumes. The reaction mixture contained 15 pmol of each 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. A touchdown temperature profile was adopted in which the annealing temperature was reduced from 65°C by 0.5°C on successive cycles until the theoretical annealing temperature 55°C was reached after 20 cycles, a further 10 cycles were performed at this temperature. An aliquot of 5 mL PCR products was checked by electrophoresis in 1.0% agarose gels stained with ethidium bromide prior to DGGE.

The DGGE was performed using a Bio-Rad Dcode™ Universal Mutation Detection System (Bio-Rad, USA). The PCR product was loaded on 0.8 mm thick polyacrylamide gel (10% *w*/*v* acrylamide/bisacrylamide 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 and temperature of 60°C for 5 h. After the run, the gels were stained for 20 min in 1× TAE containing 0.5 mg/mL ethidium bromide and rinsed with distilled water, then photographed.

2.6 Data analysis

Means, standard deviation, and analysis of variance (ANOVA) were determined using the SPSS 11.5 computer package. The means were compared using paired-samples *t* test, with a significance level of $p<0.05$. The figures presented are the mean values and standard errors of triplicates.

3 Results

3.1 Bioavailable fractions of metals

The results clearly indicate that the available fractions of Cd and Pb were decreased during the first 2 weeks of incubation and reached nearly steady state at 9 weeks. In the Cd treatments, the available fraction of Cd was 48.2±2.7% of the total concentration at 0 week and decreased to 38.3±2.2% after 2 weeks. At the steady-state period, this fraction concentration was decreased to 35.2±2.1% and

34.7±2.1% at 9 and 12 weeks, respectively. Similarly, in the Pb treatments, the available fraction of Pb was 37.8± 2.7% of the total concentration at 0 week and decreased to 26.9±2.3% after 2 weeks. At the steady-state period, this fraction concentration was decreased to 19.5±2.6% and 17.9±2.2% at 9 and 12 weeks, respectively.

3.2 Soil microbial activities

ACP inhibition rate in the heavy metal-amended samples and control with different incubation times is given in Fig. 1. The ANOVA showed that this enzyme activity in the heavy metal-treated samples was significantly lower ($p<0.05$) than those in the control. The ACP inhibition rate was increased with the increasing metal concentrations. Similarly, the inhibition extent was also obvious between different incubation periods and varied as the incubation proceeded and the highest rate was detected in the samples of 2 weeks. In the Cd-treated soil, the ACP activity had a decrease of 5.1% to 30.6% and the highest inhibition rate was observed in Cd_3 treatments. Similarly, in the Pb treatments, this enzyme activity was decreased with increasing Pb level. In the case of both Cd/Pb treatments, the ACP activity was also significantly ($p<0.05$) decreased as the levels of Cd/Pb increased and ranged from 7.5% to 35.6%. However, the lowest value of ACP activity (35.6% of the control) was found in the Cd_3/Pb_3-treated sample at 2 weeks. For all treatments, the ACP values were more seriously inhibited in the first 2 weeks but later on inhibition was reduced with incubation time.

Mean values of URE activity were also significantly ($p<0.05$) decreased with increasing level of heavy metals in the amended samples (Fig. 2). The values of this enzyme were lower in heavy metal-amended soils compared to the control. In Cd-amended samples, the values of this enzyme dropped from 7.3% to 33.0%. Similarly, the inhibition rate was varied as the incubation proceeded and the highest rate was detected in the samples at 2 weeks. Like ACP, the URE values strongly inhibited up to 2 weeks and then increased with incubation time. In the case of the Pb treatments, the extent of inhibition was lower than the Cd treatments but increased with increasing Pb level, such as from 4.5% to 17.9% in Pb_1, 9.9% to 22.2% in Pb_2, and 11.8% to 26.0% in Pb_3 treatments. In the samples at 2 weeks, the combined effects of both Cd/Pb were also higher on this enzyme activity and values dropped by 27.7%, 33.9%, and 36.6% in the Cd_1/Pb_1, Cd_2/Pb_2, and Cd_3/Pb_3 treatments, respectively. Pearson's correlation coefficients (data not shown) between the heavy metals and two enzyme activities (ACP and URE) indicated that these enzyme activities have significantly positive correlations between each other, but negative correlations were detected between the enzyme activities and heavy metal concentrations.

The mean values of MBC in the heavy metal-amended and control samples with different incubation times are given in Table 2. The MBC values were significantly ($p<0.05$) decreased with increasing level of heavy metals in the amended samples. The effects of heavy metals on MBC were highly varied in the amended samples and also in the different incubation periods. The MBC values were reduced by 19.3%, 22.1%, and 32.4% in Cd_1-, Cd_2-, and Cd_3-amended samples, respectively, after 2 weeks of incubation. In Pb-amended samples, the MBC values were highly decreased in the 2- and 9-week samples, ranging from 16.6% to 25.5% and from 25.9% to 27%, respectively. However, MBC was strongly decreased in both Cd/Pb-amended samples and the highest reduction (52.4%) was detected after Cd_3/Pb_3 treatment at 2 weeks incubation.

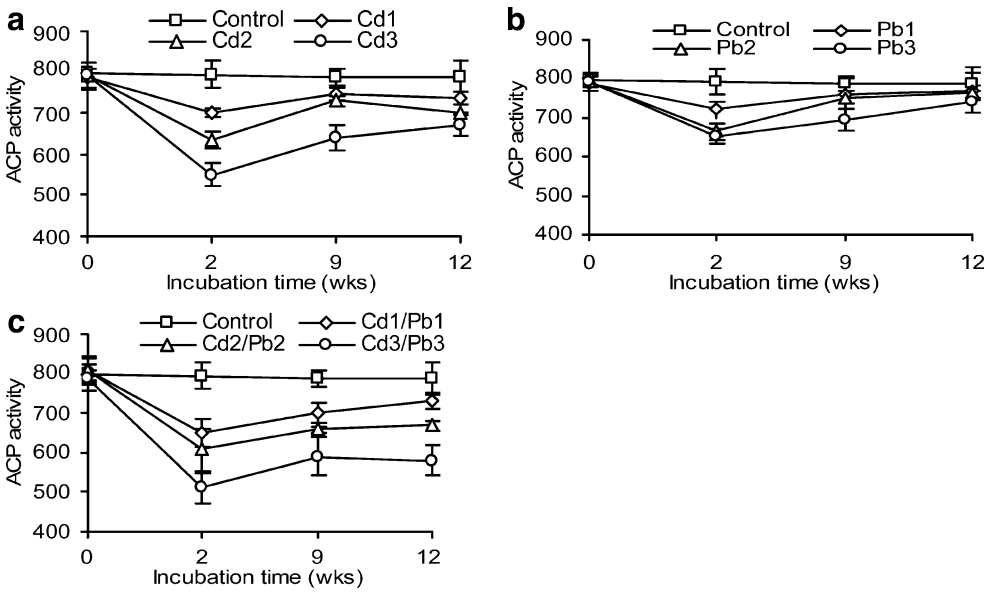

Fig. 1 Inhibitory effects of Cd/Pb mix on ACP activity (milligrams of *p*-nitrophenol per kilogram of soil per hour) in different incubation periods. **a** Cd (Cd_1=1.5 mg/kg, Cd_2=3 mg/kg, and Cd_3=5 mg/kg), **b** Pb (Pb_1=150 mg/kg, Pb_2=300 mg/kg, and Pb_3=500 mg/kg), and **c** Cd/Pb (Cd_1/Pb_1=1.5/150 mg/kg, Cd_2/Pb_2=3/300 mg/kg, and Cd_3/Pb_3=5/500 mg/kg) during different incubation periods. The *error bars* indicate the standard deviation

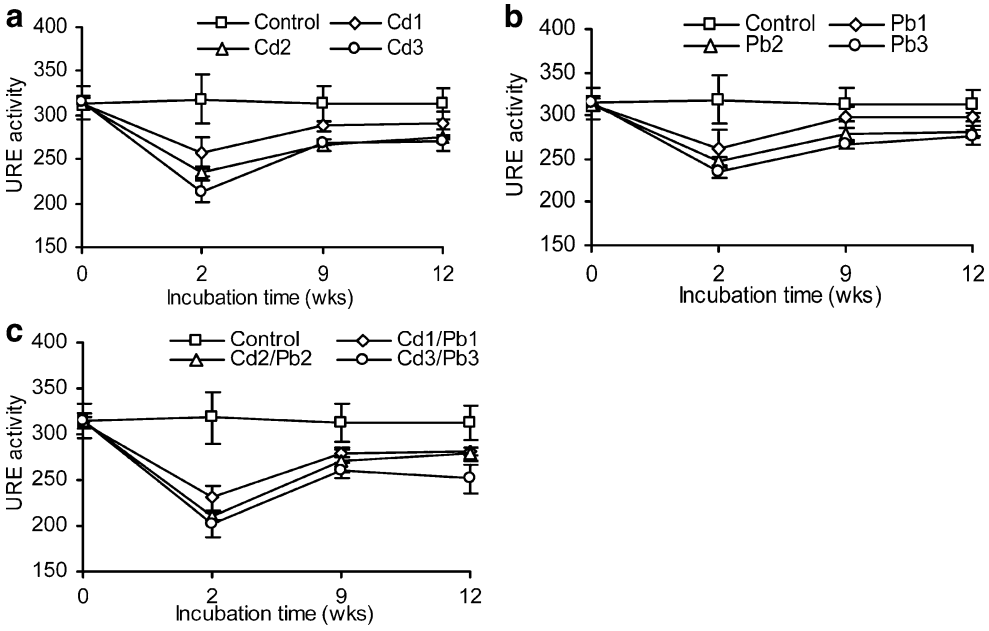

Fig. 2 Inhibitory effects of heavy metals on URE activity (milligrams of N per kilogram of soil every 3 h): **a** Cd, **b** Pb, and **c** Cd/Pb during the different incubation periods. The *error bars* indicate the standard deviation

Like enzymatic activities, MBC had significant negative correlation (from $r=-49$ to $r=-0.71$) with heavy metals (data not shown).

3.3 Soil microbial community structure

3.3.1 Plate counting

Table 3 shows the results of the quantitative analysis of soil microbial community. The number of bacteria was significantly decreased ($p<0.05$) in the heavy metal-amended samples compared to the control. This decrease in the number of bacteria was markedly high in Cd_3-amended (from 34.5% to 63.5%) and Cd/Pb-amended (from 63.6% to 76.2%) samples. The viable count of actinomycetes has shown a significant decrease (from 4.2% to 35.0%) in the metal-amended sample compared to the control, while fungal cells were not significantly different (from 2.3% to 23.87%).

Table 2 MBC (in milligrams per kilogram) in different treatments and incubation time

Treatments	0week	2weeks	9weeks	12weeks
Control	123±9.2	145±10.5	162±12.6	128±10.2
Cd_1	124±8.6	117±6.2	119±11.2	121±9.3
Cd_2	120±4.7	113±8.3	114±10.8	110±8.7
Cd_3	118±5.3	98±9.7	107±6.3	109±4.3
Pb_1	123±6.4	120±3.8	118±7.4	122±4.5
Pb_2	122±5.6	121±3.6	120±8.3	121±5.6
Pb_3	120±9.5	108±8.4	118±6.5	119±6.8
Cd_1/Pb_1	123±7.3	97±4.2	108±5.7	107±4.2
Cd_2/Pb_2	121±4.9	78±5.8	100±3.2	99±5.8
Cd_3/Pb_3	125±10.4	69±7.1	80±6.1	81±7.1

3.3.2 PCR-DGGE for bacterial community structure

Soil DNA was extracted from the Cd/Pb mix-treated samples and the DGGE fingerprinting of PCR-amplified 16S rDNA is shown in Fig. 3. DGGE running conditions were optimized and it was observed that DGGE profiles of triplicate samples were highly reproducible. Differences in the bacterial community composition between heavy metal-amended samples and control could be readily distinguished after 2 weeks of incubation (see Fig. 3). Furthermore, the number of bands in heavy metal-amended samples was apparently and consistently decreased with the increasing level of metals. Similarly, there was a significant decrease in the number of bands with the incubation period in all selected samples. The DGGE profiles provided evidence that a high dose of metal amendment caused a greater change in the number of bands. DGGE banding patterns confirmed that the addition of metals had a significant impact on microbial community structure.

4 Discussion

In soil ecosystem, heavy metals exhibit toxicological effects on soil microbes which may lead to the decrease of their numbers and activities. This study demonstrated that toxicological effects of heavy metals on soil microbial community structure and activities depend largely on the type and concentration of metal and incubation time. Soil

Table 3 Microbial population groups in different treatments and incubation time

Treatments	0week	2weeks	9weeks	12weeks
Culturable heterotrophic bacteria (10^5 cfu/g)				
Control	5.3±0.9	6.3±1.1	4.9±0.5	5.5±1.2
Cd_1	6.0±1.0	3.8±0.6	3.9±0.4	4.0±1.0
Cd_2	5.4±0.7	3.0±0.5	2.8±1.1	3.9±0.7
Cd_3	4.9±1.8	2.3±0.8	3.0±0.7	3.6±0.3
Pb_1	5.2±1.0	4.9±0.5	4.5±0.3	5.0±0.4
Pb_2	5.5±0.7	4.2±0.7	4.0±0.5	4.9±0.5
Pb_3	6.2±1.8	3.8±0.7	3.2±0.7	4.5±1.0
Cd_1/Pb_1	5.3±0.4	2.3±0.8	2.9±0.4	3.4±0.8
Cd_2/Pb_2	5.6±0.6	1.9±0.6	2.0±0.3	2.7±0.6
Cd_3/Pb_3	4.9±1.5	1.5±0.2	1.7±0.5	2.0±0.5
Actinomycetes (10^3 cfu/g)				
Control	12.6±1.2	13.7±1.0	12.5±1.2	11.8±1.4
Cd_1	11.3±1.5	10.2±1.1	9.8±1.3	10.5±0.6
Cd_2	10.9±0.8	9.6±0.9	9.6±0.8	11.3±1.0
Cd_3	12.8±1.2	9.2±0.8	10.2±1.1	11.0±0.7
Pb_1	11.5±1.0	11.2±0.4	10.8±1.6	12.5±1.2
Pb_2	10.3±1.3	10.9±1.3	9.1±0.9	10.4±0.9
Pb_3	10.6±0.8	9.8±0.7	8.9±1.5	10.2±0.8
Cd_1/Pb_1	12.5±1.0	9.7±0.6	10.0±1.0	9.9±1.6
Cd_2/Pb_2	11.4±0.7	9.2±0.7	10.2±0.6	10.6±1.0
Cd_3/Pb_3	11.6±0.5	8.9±0.6	11.1±0.8	11.3±0.7
Fungi (10^4 cfu/g)				
Control	3.2±0.5	4.2±0.7	4.3±0.6	4.5±0.4
Cd_1	3.7±0.3	3.5±0.5	4.2±0.5	3.9±0.7
Cd_2	4.0±0.7	3.9±0.4	3.7±0.8	3.9±1.0
Cd_3	4.1±0.6	4.0±0.8	3.9±0.4	4.2±0.6
Pb_1	4.3±0.2	4.0±0.4	4.1±0.5	4.2±0.6
Pb_2	3.9±0.5	3.5±0.5	3.9±0.3	4.0±0.3
Pb_3	4.2±0.8	3.9±0.7	4.1±0.6	4.2±0.3
Cd_1/Pb_1	4.1±0.2	3.6±0.4	3.9±0.7	4.0±0.5
Cd_2/Pb_2	4.2±0.5	3.9±0.6	4.0±0.6	3.9±0.4
Cd_3/Pb_3	3.9±0.4	3.2±0.5	3.7±0.5	4.1±0.5

enzymes and MBC are used to estimate the adverse effects of various pollutants on soil health (Dick 1997; Masto et al. 2008; Xu et al. 2008). In this study, ACP and URE inhibition rates in the heavy metal-amended samples were significantly higher than the control and increased with increasing concentrations of heavy metals. ACP and URE activities were slightly replenished as the bioavailable concentrations of Cd and Pb decreased with the progression of incubation. Previous research showed that soil enzymes diminished with increasingly available concentrations of heavy metals (Kızılkaya et al. 2004). Similarly, Chen et al. (2005) observed that ACP were decreased with increasing heavy metal concentrations in mine-polluted areas, while Moreno et al. (2003) also concluded that Cd and Ni had clear inhibition effects on URE and phosphatase activities. The inhibition extent was varied widely among different incubation periods for these enzymes. These results are in agreement with the findings of previous studies (Shen et al. 2005). Like previous published work, a significant negative correlation was observed between heavy metals and soil enzymatic activities.

Soil microbial biomass can be investigated using several approaches. In this research, the MBC method was used to study the toxic effects of metals on soil microbial activities. Like enzymatic activities, the values of MBC in the heavy metal-amended samples were significantly decreased with increasing metal concentrations and highly varied in different incubation periods. According to a study conducted by Zhang et al. (2008), MBC was decreased with increasing Cd concentration in soil. The data indicated that the strong inhibitive effects of MBC were observed in the samples amended with the Cd/Pb mix. The values of MBC had a significantly negative correlation with the metal concentrations. These results are in agreement with the findings of previous studies (Bhattacharyya et al. 2008). Furthermore, the rapid inhibition in microbial activities such as ACP, URE, and MBC were observed after 2 weeks, which should be related to the fact that the microbes were suddenly exposed to high concentrations of available fractions of heavy metals. These microbial activities were steadily replenished as the available fraction concentrations decreased with incubation because of the sequestration in the soil.

The increased inhibition of soil microbial activities is likely to be related to tolerance and adaptation of the microbial community, concentration of pollutants, and mechanisms of heavy metals. On exposure to a high concentration of heavy metals, nontolerant (metal-sensitive)

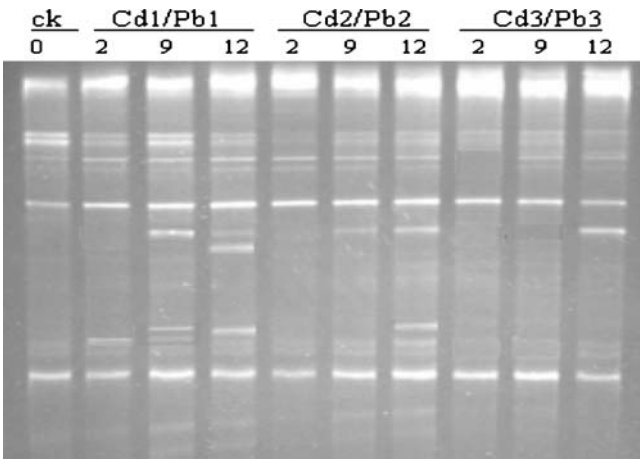

Fig. 3 DGGE profiles of the PCR products obtained from the 16S rDNA extracted from bacterial population. *Lane 0* 0 time control, *lanes 2*, *9*, and *12* represent the incubation time in weeks for different treatments shown *above* them

species can be diminished, while tolerant species survived and increased in abundance. Typically, this enhancement in abundance is due to physiological adaptation and genetic modifications in tolerant species, which may lead to replacement of more sensitive species (Briuns et al. 2000).

In this study, heavy metals (Cd and Cd/Pb mix) have significantly decreased the number of culturable heterotrophic bacteria. It means that the culturable heterotrophic bacteria were more sensitive than other microbes. These findings confirm the results obtained by Oliveira and Pampulha (2006). In the studied microbes, the sensitivity was observed in order of fungi<actinomycetes<bacteria. These results are in agreement with the findings of a previous study (Frostegård et al. 1996).

As an advanced molecular and widespread approach, the DGGE was used to monitor the changes in bacterial community structure. In this research, the DGGE profiles showed that numerous bands were common to all treatments, but there were also changes in bands due to different application rates of heavy metals and their toxic effects on the bacterial community. In the Cd- and Cd/Pb mix-treated samples, the microbial community structure was highly affected, consisting of lower microbial activities with the different levels of heavy metals. The DGGE profile has shown that the structure of the bacterial community changed in heavy metal-amended samples. Similarly, Wang et al. (2008) has also reported that the bacterial community structure was changed in the soils amended with Cu and cultivated with different plants. In our previous work, microbial community structure was also changed in heavy metal-amended soils (Khan et al. 2007). Furthermore, a great community change of indigenous microbes in this study, particularly at a high level of contamination of Cd, Pb, and Cd/Pb mix, was probably a result of metal toxicity and also unavailability of nutrients because no nutrients were supplied during the whole incubation period. In this study, different monitoring approaches were used to investigate soil microbial community structure and activities to estimate soil quality indices which are a better approach for the evaluation of soil health. Soil microbial community structure and activities can be studied together to investigate the soil quality completely and precisely.

5 Conclusions

It was concluded that the added concentrations of heavy metals have changed the indigenous microbial community structure and activities. The highest inhibitory effects of heavy metals on soil microbial activities, including ACP, URE, and MBC, were observed at 2 weeks of incubation. The number of culturable heterotrophic bacteria was significantly decreased in the amended samples, while actinomycetes and fungi were less sensitive towards the toxicity of tested metals. The DGGE profile indicated that the bacterial community structure was changed in the Cd/Pb-amended samples, particularly at high concentrations.

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