Journal of Environmental Sciences Vol.17, No.5, pp.802—807, 2005

ISSN 1001—0742
CN11—2629/X

Article ID: 1001-0742(2005)05-0802-06 **CLC number**: X172; X53 **Document code**: A

Changes in enzymes activity, substrate utilization pattern and diversity of soil microbial communities under cadmium pollution

Muhammad Akmal, WANG Hai-zhen, WU Jian-jun, XU Jian-ming*, XU De-fu

(Institute of Soil and Water Resources and Environmental Science, Zhejiang University, Hangzhou 310029, China. E-mail: jmxu@zau.edu.cn)

Abstract: Heavy metal pollution has received increasing attention in recent years mainly because of the public awareness of environmental issues. In this study we have evaluated the effect of cadmium (Cd) on enzymes activity, substrate utilization pattern and diversity of microbial communities in soil spiked with 0, 20, 40, 60, 80, and 100 mg/kg Cd, during 60 d of incubation at 25℃. Enzyme activities determined at 0, 15, 30, 45, and 60 d after heavy metal application (DAA) showed marked declines for various Cd treatments, and up to 60 DAA, 100 mg/kg Cd resulted in 50.1%, 47.4%, and 39.8% decreases in soil urease, acid phosphatase and dehydrogenase activities, respectively to control. At 60 DAA, substrate utilization pattern of soil microbial communities determined by inoculating Biolog ECO plates indicated that Cd addition had markedly inhibited the functional activity of soil microbial communities and multivariate analysis of sole carbon source utilization showed significantly different utilization patterns for 80 and 100 mg/kg Cd treatments. The structural diversity of soil microbial communities assessed by PCR-DGGE method at 60 DAA, illustrated that DGGE patterns in soil simplified with increasing Cd concentration, and clustering of DGGE profiles for various Cd treatments revealed that they had more than 50% difference with that of control.

Keywords: enzyme activity; microbial community; soil; cadmium

Introduction

Heavy metals in soil above the ambient levels can adversely affect the surrounding ecology by a variety of ways such as population loss, changes in population structure, physiological activity and shifts or change in the composition of the soil microbial communities (Knight, 1997; Kozdroj, 2000; Anne, 2001; Claudia, 2003). This, in turn can result in adverse effects on various parameters influencing crop quality, yield and possibly human health through food chain. A number of soil microbiological parameters, notably microbial biomass, basal respiration, enzyme activity and physiological profiling (Campbell, 1997; Dick, 1997; Sparling, 1997; Landi, 2000; Trasar-Cepeda, 2000; Yao, 2003) have been suggested as possible indicators of soil environmental quality, and employed in the national and international monitoring programs. However, the advances in molecular biological techniques have also been applied to soil ecosystem; enable the researchers to study microbial diversity at the molecular level (Amann, 1996; Borneman, 1996; Antonio, 1999). The method of polymerase chain reaction (PCR) based on 16S ribosomal DNA and denaturing gradient gel electrophoreses (DGGE) fingerprinting technology has recently been used to assess changes in experimentally altered soil microbial communities (Friedrich, 1997; Jensen, 1998; Claudia, 2003). The 16S ribosomal DNA genes are useful for such studies since these genes are present in all bacteria and have been taken as the base to clarify bacteria. Extraction of total DNA from soil followed by PCR amplification of the 16S ribosomal DNA genes ideally yield a mixture of DNA fragments representing all species present in that soil sample. Denaturing gradient gel electrophoresis (DGGE) is an electrophoretic method to identify single base changes in a segment of DNA. The strength of DGGE as a screening method for diversity is its ability to monitor changes in community structure in response to changes in the environmental parameters (Mette, 2002).

The results of some long term field experiments have indicated negative effects of heavy metals on microbial parameters in soils treated with metal-contaminated sludge's in the past (McGrath, 1995; Baath, 1998; Garcia-Gil, 2000; Yao, 2003; Renella, 2004). However, the effects were observed at modest concentrations of heavy metals (Chander, 1991; McGrath, 1995; Dar, 1996; Knight, 1997; Renella, 2004) and the applied sludge's contained mixtures of several metals, so it is not well known which metal or groups of metals are producing these effects. Also at long-term field sites, soil microbial communities have had time to adapt to the stress presented by the elevated metal concentrations (Kozdroj, 2000). Although comparison of metal-affected soil microbial communities and non-metal affected microbial communities at these sites can provide information on the changes that have occurred in the communities as a result of the metal contamination, such studies do not provide information on the time course of these changes (Giller, 1998; Pennanen, 2001). Therefore, the information regarding the direct application of these heavy metals in organic or inorganic form under better controlled, short-term experiments was required to assess the changes in soil microbial parameters. Cadmium (Cd) concentration in normal agricultural soils is usually less than 1.0 mg/kg (Soon, 1993), and excess of 1 mg/kg Cd in soil is considered to be the evidence of anthropogenic pollution (Uminska, 1993). However, in polluted soils especially near mines or by sewage sludge applications, Cd contents are even higher than 100 mg/kg soil (Elliott, 1989; Peters, 1992; Fuge, 1993; Stefanov, 1995; Pichtel, 2000). It accumulates in soils as result of wet and dry deposition, phosphatic fertilizers, manure inputs and incorporation of sewage sludge, and is considered being the metal having the most adverse effects on microbial biomass and activity in soils (Smith, 1996). Hence, we selected significantly wide levels of Cd in nitrate form to evaluate their effects on enzymes activity, substrate utilization pattern and structure of microbial communities in soil during 60 d of exposure. Here, we combined the traditional assay of enzymes activity and

Foundation item: The National Basic Research Program of China (2005CB121104); the National Key Project for Science and Technology of China (No. 2001BA804A25) and the Natural Science Foundation of Zhejiang Province (Y504128); * Corresponding author

substrate utilization analyses with modern molecular technique of polymerase chain reaction(PCR) and denaturing gradient gel electrophoresis(DGGE) to evaluate their ability to detect changes in soil microbial parameters as a result of Cd pollution.

1 Materials and methods

1.1 Soil sampling, preparation and heavy metal addition

A bulk soil sample was collected at 0—10 cm depth from the research field of Huajiachi campus of Zhejiang University, Hangzhou, China. The soil was brought to the laboratory, hand-picked to remove stones, discrete plant residues and large soil animals (earthworms etc.), passed through 2 mm sieve and mixed thoroughly. A sub-sample of the soil was taken, air-dried, mixed and analyzed for selected physico-chemical properties (Table 1). The moist soil(equivalent to 100 g oven-dry weight) was transferred to 250 ml capacity glass beaker. The soil samples were first adjusted to 40% of the soil water holding capacity(WHC) by adding distilled water and then pre-incubated at 25℃ for 7 d (conditioning period). After conditioning, cadmium (Cd) was applied as $Cd(NO_3)_2$ solution maintaining the concentrations of 0 (control), 20, 40, 60, 80 and 100 mgCd/kg soil. The moisture contents in the soil were adjusted to 50% of WHC and soil samples were incubated for 60 d at 25℃. The soil moisture was kept at the same level throughout the incubation period.

Table 1 Basic physico-chemical properties of soil used in the study

Properties	Values	Properties	Values
Sand	700 g/kg	CEC	11.3 cmol/kg
Silt	190 g/kg	Total organic carbon	19.3 g/kg
Clay	110 g/kg	Total nitrogen	1.6 g/kg
$pH_{(1:2.5)}$	5.51	Total cadmium	0.045 mg/kg

1.2 Soil enzymes assays

Soil samples in various treatments were taken at 0, 15, 30, 45 and 60 d after heavy metals application(DAA) and analyzed for dehydrogenase, urease, and acid phosphatase activities. Soil dehydrogenase activity was measured by the reduction of triphenyl tetrazolium chloride(TTC) to triphenyl formazan(TPF). Briefly, 5 g soil sample was incubated for 24 h at 37℃ in 5 ml of TTC solution(5 g/L in 0.2 mol/L Tris-HCl buffer, pH 7.4). Two drops of concentration H_2SO_4 were immediately added after the incubation to stop the reaction. The sample was then blended with 5 ml of toluene to extract TPF and shaken for 30 min at 250 r/min (25℃), followed by centrifugation at 5000 × g for 5 min, and the absorbance of color in the extract was measured at 492 nm. For analysis of soil urease activity, 5 g soil was taken into 250 ml conical flask, and 10 ml of 100 g/L urea solution and 20 ml citric acid buffer(pH 6.7) were added into flask. Soil sample was incubated at 37℃ for 24 h. After 24 h, the solution was filtered and 3 ml of filtrate was taken into 50 ml volume flask, and 20 ml distilled water and 4 ml of mixed reagent(phenol + NaOH) were added. Then, 4 ml of sodium hypo chlorite solution was added, mixed and made the volume to 50 ml with distilled water, and absorbance of color was checked at 578 nm. Soil acid phosphatase activity was measured by disodium phenyl phosphate method. Briefly, 5 g of soil sample was carefully transferred into 250 ml flask and 2 ml of toluene was added to inhibit the growth of microorganisms. After standing for 15 min, added 20 ml of 0.5% (w/v) disodium phenyl phosphate prepared in acetic acid buffer(pH 5), and sample was incubated at 37℃ for 24 h. After incubation, 100 ml of 0.3% $Al_2(SO_4)_3$ solution was added to the sample, filtered, and 3 ml of filtrate was taken into 50 ml volume flask. Then 5 ml of borate buffer (pH 9.4) and 4 drops of indicator were added, and made up the volume. The absorbance of color in the solution was measured at 660 nm. Soil dehydrogenase, urease and acid phosphatase activities are expressed as mg TPF g^{-1} dry soil 24 h^{-1}, mg NH_3-N g^{-1} dry soil 24^{-1} and mg phenol produced g^{-1} dry soil 24 h^{-1}, respectively(Li, 1996; Min, 2001). Soil enzymes activity's data for different treatments were analyzed by ANOVA and means($n = 3$) were compared at 5% level of significance using Duncan's multiple test. The effects of different treatments were compared at specific as well as over different incubation periods(Gomez, 1984).

1.3 Biolog ECO plate analyses

At 60 d after heavy metal application(DAA), Biolog ECO plates were used to study the substrate utilization pattern of soil microbial communities. Briefly, fresh soil(10 g) was added to 100 ml of distilled water in a 250-ml flask and was shaken for 10 min at 200 r/min. Ten-fold serial dilutions were made and 1000-fold dilution was used to inoculate the Biolog ECO plates. Plates were incubated at 25℃ for 7 d and color development was measured as absorbance(A) using an automated plate reader (VMAX, Molecular Devices, Crawley, UK) at 590 nm and data were collected using Microlog 4.01 software(Biolog, Hayward, CA, USA). The data of average well color development(AWCD) over time for all C sources were calculated as a measure of total microbial activity. Means ($n = 3$) of AWCD for different treatments over time were compared at 5% level of significance to evaluate their effects. For multivariate analysis of the Biolog data, absorbance values were first transformed by dividing the AWCD to avoid bias among samples with different inoculum's density and then analyzed by principal component analysis technique(Campbell, 1997; Garland, 1997; Yao, 2000).

1.4 Extraction and purification of total microbial DNA from soil

Total microbial DNA from soil was extracted and PCR-DGGE method was used to study the structure of soil microbial communities in different treatments also at 60 d after heavy metals application(DAA). For that, fresh soil sample of 5 g was added to 13.5 ml extraction buffer(100 mmol/L Tris-HCl(pH 8.0), 100 mmol/L di-sodium ethyl-diamine tetra acetic acid(EDTA)(pH 8.0), 100 mmol/L Na_2HPO_4 (pH 8.0), 1.5 mol/L NaCl, 1% hexadecyl trimethyl ammonium bromide(CTAB)). Then 50 μl of 10 mg/ml proteinase K was added and the mixture was incubated at 37℃ for 30 min while being shaken horizontally at 150 r/min. Sodium dodecyl sulphate(SDS) was then added to a final concentration of 20% and each tube was incubated at 65℃ in water bath for 2 h with gentle end-over-end mixing after every 15 min. The mixture was then centrifuged at 2500 × g for 5 min. The supernatant was collected and the debris was extracted twice more time. Proteins were denatured by the addition of chloroform-isoamyl alcohol and the DNA was

precipitated in isopropanol, overnight at room temperature. DNA was pelleted by centrifugation, washed twice with 5 ml of cold 70% ethanol, and then dissolved in 100 μl sterile water and stored at 4℃ (Zhou, 1996; Bruce, 2003). To reduce the likelihood of chimera formation, the DNA was size fractionated by agarose gel electrophoresis and DNA ≥ 20 kb was recovered using a Geneclean UNIQ-10 Spin kit (Sangon biological company, Shanghai, China) according to the manufacturer's instructions.

1.5 PCR-DGGE analysis

The PCR mixture contained 0.2 μmol of each primer, 1 μl (about 5—15 ng) template DNA, 200 μmol of deoxynucleoside triphosphate, 5 μl 10 × PCR buffer, 37.5 mmol/L magnesium chloride, 5 U Taq polymerase, and sterile water to a final volume of 50 μl. The primers used for PCR were R1401(5'-GCG TGT GTA CAA GAC CC-3') and F968(5'-GC-Clamp [CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG G]-AAC GCG AAG AAC CTT AC-3'), spanning the region roughly between nucleotides 968 and 1401 of the 16S rRNA gene. The GC-rich sequence attached to the 5'-end of primer F968 prevents the PCR products from complete melting during separation via DGGE(Muyzer, 1993). Both primers have been shown to anneal to the majority of bacterial sequences in the ribosomal database, and to amplify plant and soil bacterial communities (Heuer, 1997; Duineveld, 1998; van Elsas, 1998). PCR amplification was performed at 95℃ for 4 min, followed by 40 thermal cycles at 94℃ for 1 min, 54℃ for 1 min, 72℃ for 1 min, and final single extension at 72℃ for 7 min. The size of the PCR product was visualized by electrophoresis in 1% agarose gels after ethidium bromide staining. Strong bands of approximately 430 bp were subjected to DGGE analysis. DGGE analysis was conducted using a DCode system (Bio-Rad Laboratories, Hercules, Calif.). Samples of PCR product(20 μl) were loaded on to 6% (w/v) polyacrylamide gel in 1 × TAE buffer. The polyacrylamide gel was made with a linear denaturing gradient ranging from 40% denaturant at the top of the gel to 65% denaturant at the bottom. The electrophoresis was run at 60℃ and 130 V for 330 min. After electrophoresis, the gel was stained with a silver staining procedure (Bassam, 1991). To get the clear image, the gel was photographed by gel photo system (Gel Doc 2000, BioRad). Photograph was then analysed with BioRad quantity one software package.

2 Results and discussion

2.1 Dynamics of soil enzyme activity

The dynamics of urease activity under cadmium (Cd) pollution in soil (Fig. 1) showed a significant ($P < 0.05$) decline in activity for various Cd treatments from start to 60 d of heavy metal application (DAA). The declining rate of enzyme activity was comparatively higher from start to 30 DAA, then from 30 to 60 DAA. At 30 DAA, 100 mg/kg Cd showed more 50% decrease in activity compared with the initial value, while until 60 DAA, 60, 80 and 100 mg/kg Cd treatments resulted in 55.5%, 58.3% and 61.5 % reduction in activity compared to the initial contents, respectively. However, 100 mg/kg Cd treatment also showed a 50.1% decrease in urease activity compared to that of control at 60 DAA. Soil acid phosphatase activity also showed various degrees of decline for Cd treatments from start to 60 DAA(Fig.2). Significant declines($P < 0.05$) in activity for 60, 80 and 100 mg/kg Cd were observed from 15 to 30 DAA, and at 60 DAA, these treatments decreased up to 50.1%, 50% and 59.7% in phosphatase, respectively, compared to the initial values. However, dehydrogenase activity presented comparatively lesser declines for various Cd levels in soil (Fig. 3), and 20 mg/kg Cd had activity similar to that of control from start up to 45 DAA. The treatments receiving 80 and 100 mg/kg Cd showed significantly lower dehydrogenase activity compared with that of other treatments from 30 to 60 DAA.

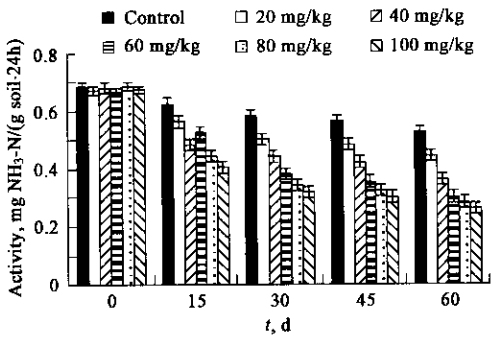

Fig.1 Effect of Cd on the dynamics of urease activity in soil
The error bar is standard error of means($n = 3$)

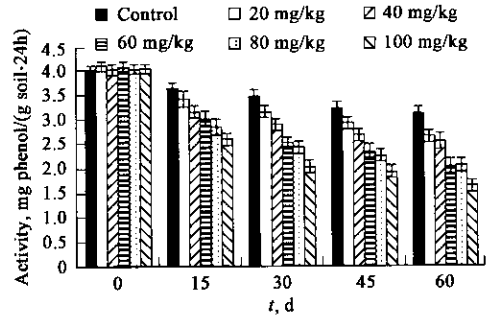

Fig.2 Effect of Cd on the dynamics of acid phosphatase activity in soil
The error bar is standard error of means($n = 3$)

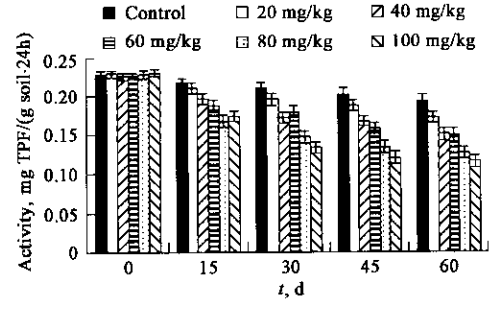

Fig.3 Effect of Cd on the dynamics of dehydrogenase activity in soil
The error bar is standard error of means($n = 3$)

The dynamics of urease, acid phosphatase, and dehydrogenase activities in our experiment at various times during incubation after the heavy metal addition might be related to the dynamics of microbial populations in the soil (Giller, 1998; Renella, 2003). The soil microbial populations decreased upon the depletion of readily utilized carbon substrate resulted from heavy metal toxicity and

starved as the reserves were exhausted, and decreased in size and activity. While another reason could be the binding of Cd with the functional group of enzyme (Wittekind, 1996), denaturizing and thus reducing its activity. Dehydrogenase is an intracellular enzyme that is involved in microbial oxidoreductase metabolism and its activity basically depends on the metabolic state of the soil biota, while urease and phosphatase are hydrolytic enzymes, involved in N and P cycling in the soil. Activities of these enzymes are considered to be sensitive to heavy metals pollution and have been proposed as indicators for measuring the degree of soil fertility sustainability(Brookes, 1995; Dick, 1997; Sparling, 1997; Filip, 2002). Dar(Dar, 1996) found that Cd at 10 mg/kg soil had no significant effect on soil microbial biomass and enzymes activities, whereas significant reductions in biomass and enzyme activities occurred at 25 and 50 mgCd/kg soil (Dar, 1994). However, Belitsyna(Belitsyna, 1989) found a significant suppression of invertase and dehydrogenase activity in soil at 10 mgCd/kg soil. Our results showed that soil spiked with Cd at 20 mg/kg had no significant inhibitory effects on soil acid phosphates and dehydrogenase activities up to 15 and 45 d after heavy metal application(DAA), respectively.

The ecological dose concept represents a promising attempt to quantify the effect of heavy metal on soil microbiological activity. Doelman and Haanstra (Doelman, 1986) defined the ecological dose 50% (ED_{50}) as the concentration of a toxicant that reduces the microbial activity to 50% of its initial value. As soil enzymes are essential factors of soil fertility, and are sensitive to pollutants such as heavy metals, their activity can be used to asses the ecological status in soil monitoring programs(Brooks, 1995; Dick, 1997). In this study we found that the ecological dose 50% (ED_{50}) value of Cd for urease and phosphatase activities was 60 mg/kg, as at this concentration, activities were reduced up to 50% compared to their initial values until 60 DAA. We also observed a higher declining rate for enzymes activities from start to 30 DAA than from 30 to 60 DAA, suggesting a greater toxicity in the first 30 d after which the microbial activities reached at equilibrium. Furthermore, relatively higher declines in various Cd treatments for urease and phosphatase than for dehydrogenase were observed in the present study, which is in line with the findings of Kandeler(Kandeler, 1996) and Filip(Filip, 2002).

2.2 Substrate utilization pattern of soil microbial communities

The Biolog data were analyzed in two ways. First, the rate of color intensity on the Biolog plates over time was determined by calculating the average well color development (AWCD) on each plate at each reading time (Garland, 1997) and then Biolog profiles for different treatments were compared by principal component analysis(PCA). The effect of cadmium(Cd) on the activity of soil microbial communities in soil (Fig. 4) showed decrease in AWCD with increasing level of heavy metal, but the differences in AWCD for various Cd treatments were not significant up to 96 h of incubation. After 96 h of incubation, all Cd amended soils showed significantly ($P < 0.5$) lower in AWCD compared with control. Cd is considered being the metal having the most adverse effects on microbial biomass and activity in soils because it is more mobile than other heavy metals due to its low affinity for soil colloids(Alloway, 1995; Smith, 1996). In general the solubility of heavy metals follows the order: Cd > Pb > Ni > Cu(Soon, 1993). As Cd is more soluble than other heavy metals, so its mobility and consequently toxicity to soil microorganism and microbial processes(Smith, 1996) is also higher than by other heavy metals(Pb, Ni, Cu).

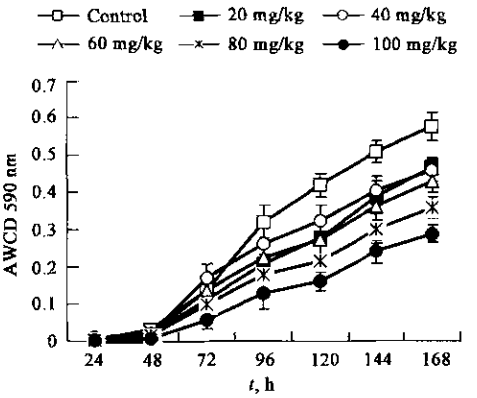

Fig.4 Effect of Cd on the functional activity of soil microbial communities as indicated by average well color development(AWCD) at 590 nm
The error bar is standard error of means($n = 3$)

A functional approach in community analysis can provide an ecologically meaningful measure of heavy metal toxicity to soil microbial communities, since the functional abilities of the microbial community are related to the essential processes in the ecosystem (Pennanen, 2001). In present study, principal component analysis using all 31 carbon sources revealed a separation of soil samples, indicating the different patterns of potential C utilization and different microbial communities. The variability in the metabolic profiles under Cd addition showed significantly different pattern for 80 and 100 mg/kg Cd treatments (Fig. 5). Kandeler(Kandeler, 2000) found that elevated metal loadings resulted in changes in the structure of soil microbial communities, as indicated by changes in their metabolic profiles, while Knight(Knight, 1997) has reported that both the metal concentration and reduced pH values had significant effects on the Biolog pattern of soil microbial communities. In our experiment, correlation and analysis of the loadings of the most influential C sources on PC1 indicated that microbial communities of Cd-amended soils had more utilization of L-asparagine, L-phenylalanine, pyruvic acid methyl ester acid and L-threonine while less utilization of N-acetyl-D-glucosamine, D-galacturonic acid, α-cyclodextrin and D-xylose.

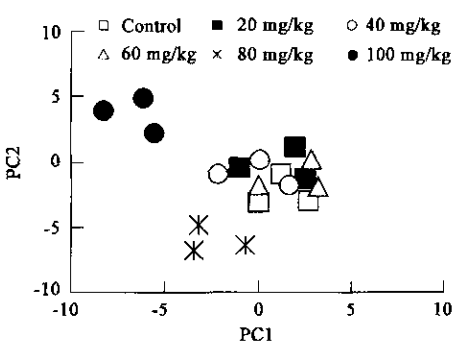

Fig.5 Plot of ordination of principal components(PC); PC1 against PC2 generated by principal component analysis showing the effect of Cd on the pattern of substrate utilization by soil microbial communities at 96 h of incubation

2.3 Diversity of soil microbial communities

Method of PCR-DGGE was used to investigate the structural diversity of soil microbial communities for different treatments at 60 d after heavy metals application (DAA). DGGE profiles generated from the universal bacterial primers (F968 and R1401) revealed the structural composition of communities for various Cd treatments (Fig.6). Each of the distinguishable bands in the separation pattern is represented as an individual bacterial species (Luca, 2002). The Fig.6 showed that heavy metal has markedly affected the pattern of community as evidenced by the number of DNA bands detected for various treatments on polyacrylamide gel after denaturing gradient gel electrophoresis (DGGE). Control had the most complex DGGE pattern indicating the presence of a high number of different bacterial taxa, while profiles for Cd treated soils were relatively simple. The profiles for treatments receiving 60, 80 and 100 mg/kg Cd showed significantly less number of visible bands compared with that of control, which can also explain the above decreases in enzymes activity observed under Cd pollution (Fig.1, 2 and 3) in the present experiment; however, reduction of soil enzymes activities without changes in the soil microbial community structure has also been reported by Kandeler (Kandeler, 2000).

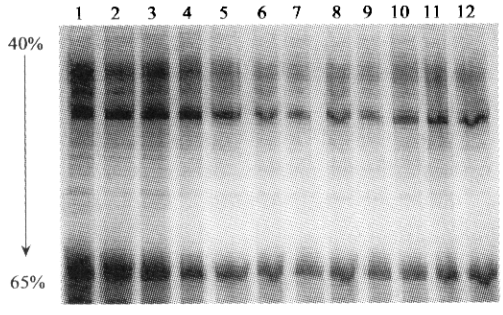

Fig. 6 DGGE profiles of amplified 16S rDNA fragments from soil with different levels of Cd pollution
Lane: 1,2(control); 3,4(20 mg/kg Cd); 5,6(40 mg/kg Cd); 7,8(60 mg/kg Cd); 9,10(80 mg/kg Cd); and 11,12(100 mg/kg Cd); increasing denaturant from top(40%) to the bottom(65%)

Several similarities in banding positions were also found among the treatments receiving different levels of Cd(Fig.6), indicating that many common microbial members were still present in each treatment regardless of the heavy metal rates. Renella (Renella, 2004) found that heavy metal contamination mainly induced physiological adaptations rather than selection for metal-resistant culturable soil microflora. However, some of the DGGE bands in our experiment were unique to each of the different treatments suggesting a change in the structural diversity. Clustering of DGGE profiles further explained the differences existed among different Cd treatments(Fig.7). The biggest difference was found between the profiles for control, 20 and 40 mg/kg Cd, and for other treatments. Control, 20 and 40 mg/kg Cd treatments belonged to a common cluster having 47% similarity among them but 63% difference with the profiles for other treatments (60, 80 and 100 mg/kg Cd). A decrease in the structural diversity of microbial communities in soil amended with higher levels of Cd in the present study is supposed to be the result of the direct toxicity of Cd to soil microbial communities and changes in the microflora, as Kozdroj and van Elsas (Kozdroj, 2000) reported that elevated concentrations of heavy metals can change the composition of soil microflora as detected by DGGE analysis and also can select for metal-resistant microorganisms(Anne, 2001).

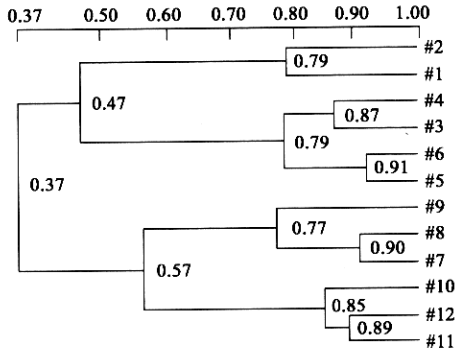

Fig.7 Cluster analysis showing the differences among the profiles for different Cd treatments in soil
Lane: 1,2(control); 3,4(20 mg/kg Cd); 5,6(40 mg/kg Cd); 7,8(60 mg/kg Cd); 9,10(80 mg/kg Cd); and 11,12(100 mg/kg Cd)

These heavy metal-induced changes in microbial community structure under present investigation may in turn cause alteration of some microbial functions in soil, which could be important information while evaluating soil quality and ecosystems sustainability. However, our results showed a difference in inhibitory response of different soil microbiological parameters to heavy metal pollution. As soil microbiological and biochemical properties are indicator of soil quality(Visser, 1992) and these properties can be used individually, as simple indices, or in combination using complex equations derived from mathematical combinations or the application of statistical programs (Smith, 1993; Trasar-Cepeda, 1998). The results described in the literature are contradictory, and the greatest problem posed by the use of these properties as soil quality indicator include the lack of reference values, and regional variations in expression levels (Filip, 2002). Complex expressions, in which different properties are combined, are thought to be highly suitable for estimating soil quality indices (Bentham, 1992; Trasar-Cepeda, 2000). Here, in this study we combined different techniques, both traditional and modern, for better evaluation of soil quality indicators under Cd pollution. However, our experimental results have limitation to compare with the studies which have used surprisingly modest levels of Cd and also their extrapolation to soils at low levels of Cd pollution.

3 Conclusions

The results showed that Cd pollution has detrimental effects on enzymes activity, substrate utilization and microbial diversity in soil. It appeared that the thresh hold level of Cd toxicity to soil enzymes activity is between 20 to 40 mgCd/kg soil, however higher levels (80 and 100 mg/kg Cd) caused significant changes in the structural diversity of soil microbial community as assessed by Biolog ECO Plates and PCR-DGGE analysis. It is suggested that the integration of traditional assays of enzymes activity and substrate utilization analyses with modern molecular technique of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE) can give better assessment of negative effects of heavy metal on the structure and functions of soil microbial communities

and ecosystem processes.

References:

Alloway B J, 1995. Cadmium[M]. In: Heavy metals in soils(Alloway B. J. ed.). Glasgow: Blackie Academic and Professional. 122—151.

Amann R, Anaidr J, Wagner M *et al.*, 1996. *In situ* visualisation of high genetic diversity in a natural microbial community [J]. Journal of Bacteriology, 178: 3496—3500.

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

Antonio G, Anneke C, Giovanni C *et al.*, 1999. Assessment of bacterial community structure in soil by polymerase chain reaction and denaturing gradient gel electrophoresis[J]. Journal of Microbiological Methods, 38: 1—15.

Baath E, Diaz-Ravina M, Frostegard A *et al.*, 1998. Effect of metal-rich sludge amendments on the soil microbial community[J]. Applied Environmental Microbiology, 64: 238—245.

Bassam B J, Caetano-Anolles G, Gresshoff P M, 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels[J]. Annals of Biochemistry, 80: 81—84.

Belitsyna G D, Dronova N Y, Skovortsova I N *et al.*, 1989. Alteration of certain indices of soil biological activity under anthropogenic stress[J]. Soviet Soil Science, 21: 40—44.

Bentham H, Harris J A, Birch P *et al.*, 1992. Habitat classification and soil restoration assessment using analysis of soil microbiological and physico-chemical characteristics[J]. Journal of Applied Ecology, 29: 711—718.

Borneman J, Skroch P W, O'Sullivan K M *et al.*, 1996. Molecular microbial diversity of an agricultural soil in Wisconsin[J]. Applied Environmental Microbiology, 62: 1935—1943.

Brookes P C, 1995. The use of microbial parameters in monitoring soil pollution by heavy metals[J]. Biology and Fertility of Soils, 19: 269—275.

Bruce F, Moffett F A, Nicholson N C *et al.*, 2003. Zinc contamination decreases the bacterial diversity of agricultural soil[J]. FEMS Microbiology Ecology, 43: 13—19.

Campbell C D, Grayston S J, Hirst D J, 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities[J]. Journal of Microbiological Methods, 30: 33—41.

Chander K, Brookes P C, 1991. Effects of heavy metals from past applications of sewage sludge on microbial biomass and organic matter accumulation in a sandy loam and silty clay loam UK soil[J]. Soil Biology and Biochemistry, 23: 927—932.

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]. Journal of Microbiological Methods, 52: 251—260.

Dar G H, 1996. Effects of cadmium and sewage sludge on soil microbial biomass and enzyme activities[J]. Bioresource Technology, 56: 141—145.

Dar G H, Mishra M M, 1994. Influence of cadmium on carbon and nitrogen mineralization in sewage sludge amended soils[J]. Environmental Pollution, 84: 285—290.

Dick R P, 1997. Soil enzyme activities as integrative indicators of soil health[M]. In: Biological indicators of soil health(Pankhurst C. E., Doube B. M., Gupta V. V. S. R., ed.). CAB International, Wallingford. 121—156.

Doelman P, Haanstra L, 1986. Short- and long-term effects of heavy metals on urease activity in soils[J]. Biology and Fertility of Soils, 2: 213—218.

Duineveld B M, Rosado A S, van Elsas J D *et al.*, 1998. Analysis of the dynamics of bacterial communities in the rhizosphere of Chrysanthemum via denaturing gradient gel electrophoresis (DGGE) and substrate utilization patterns[J]. Applied Environmental Microbiology, 64: 4950—4957.

Elliott H A, Brown G A, 1989. Comparative evaluation of NTA and EDTA for extractive decontamination of Pb-polluted soils[J]. Water Air and Soil Pollution, 45: 361—369.

Filip Z, 2002. International approach to assessing soil quality by ecologically-related biological parameters[J]. Agriculture Ecosystems and Environment, 88: 169—174.

Friedrich V, Wintzingerode U B, Erko S, 1997. Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis[J]. FEMS Microbiology Reviews, 21: 213—229.

Fuge R, Pearce F M, Pearce N J G *et al.*, 1993. Geochemistry of Cd in the secondary environment near abandoned metalliferous mines, Wales[J]. Applied Geochemistry, 2: 29—35.

Garcia-Gil J C, Plaza C, Soler-Rovira P *et al.*, 2000. Long-term effects of municiple solid waste compost application on soil enzyme activities and microbial biomass[J]. Soil Biology and Biochemistry, 32: 1907—1913.

Garland J L, 1997. Analysis and interpretation of community-level physiological profiles in microbial ecology[J]. FEMS Microbiology Ecology, 24: 289—300.

Giller K E, Witter E, McGrath S P, 1998. Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review[J]. Soil Biology and Biochemistry, 30: 1389-1414.

Gomez A K, Gomez A A, 1984. Statistical procedures for agricultural research [M]. New York, USA: John Wiley and Sons.

Heuer H, Smalla K, 1997. Application of denaturing gradient gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities[M]. In: Modern soil microbiology(van Elsas J. D., Trevors J. T., Wellington E. M. H., ed.). New York: Marcel Decker. 353—373.

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 Microbiology Ecology, 26: 17—26.

Kandeler E, Kampichler C, Horak O, 1996. Influence of heavy metals on the functional diversity of soil microbial communities[J]. Biology and Fertility of Soils, 23: 299—306.

Kandeler E, Tcherko D, Bruce K D *et al.*, 2000. Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil[J]. Biology and Fertility of Soils, 30: 390—400.

Knight B, McGrath S P, Chaudri A M, 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc[J]. Applied Environmental Microbiology, 63: 39—43.

Kozdroj J, van Elsas J D, 2000. Response of the bacterial community to root exudates in soil polluted with heavy metals assessed by molecular and cultural approaches[J]. Soil Biology and Biochemistry, 32: 1405—1417.

Landi L, Renella G, Moreno J L *et al.*, 2000. Influence of cadmium on the metabolic quotient, L-D-glutamic acid, respiration ratio and enzyme activity: microbial biomass ratio under laboratory conditions[J]. Biology and Fertility of Soils, 32: 8—16.

Li F D, 1996. Analysis of soil enzyme activity[M]. In: Experimental techniques in agricultural microbiology(Li F. D., Yu Z. N., He S. J., ed.). Beijing: Chinese Agricultural Press. 137—139.

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

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

McGrath S P, Chaudri A M, Giller K E, 1995. Long-term effects of metals in sewage sludges on soils, microorganisms and plants[J]. Journal of Industrial Microbiology, 14: 94—104.

Min H, Ye Y F, Chen Z Y *et al.*, 2001. Effects of butachlor on microbial populations and enzyme activities in paddy soil[J]. Journal of Environmental Science and Health, 36: 581—595.

Muyzer G, de Waak E C, Uitterlinden A G, 1993. Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding 16S rDNA[J]. Applied Environmental Microbiology, 59: 695—700.

Pennanen T, 2001. Microbial communities in boreal coniferous forest humus exposed to heavy metals and changes in soil pH; a summary of the use of phospholipids fatty acids, Biolog and H-thymidine incorporation methods in field studies [J]. Geoderma, 100: 91—126.

Peters R W, Shem L, 1992. Adsorption/desorption characteristics of lead on various types of soil[J]. Environmental Progress, 11: 234—240.

Pichtel J, Kuroiwa K, *et al.*, 2000. Distribution of Pb, Cd and Ba in soils and plants of two contaminated sites[J]. Environmental Pollution, 110: 171—178.

Renella G, Reyes A, Ortigoza L *et al.*, 2003. Additive effects of copper and zinc on cadmium toxicity to phosphatase activities and ATP content of soil as estimated by the ecological dose(ED_{50})[J]. Soil Biology and Biochemistry, 35: 1203—1210.

Renella G, Mench M, vander-Lelic D *et al.*, 2004. Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils [J]. Soil Biology and Biochemistry, 36: 443—451.

Smith S R, 1996. Agricultural recycling of sewage sludge and the environment [M]. Wallingford: CAB International.

Smith J L, Halvorson J J, Papendick R I, 1993. Using multiple-variable indicator kriging for evaluating soil quality[J]. Soil Science Society of America Journal, 57: 743—749.

Soon Y K, Abboud S, 1993. Cadmium, chromium, lead, and nickel[M]. In: Soil sampling and methods of analysis(Carter M.R., ed.). Lewis, Canada: Canadian Society of Soil Science. 101—108.

Sparling G P, 1997. Soil microbial biomass, activity and nutrient cycling as indicators of soil health [M]. In: Biological indicators of soil health (Pankhurst C. E., Doube B. M., Gupta V.V.S.R., ed.). Wallingford, UK: CAB International. 97—119.

Stefanov K, Seizova K, Yanishlieva N *et al.*, 1995. Accumulation of lead, zinc and cadmium in plant seeds growing in metalliferous habitats in Bulgaria[J]. Food Chemistry, 54: 311—313.

Trasar-Cepeda C, Leirós M C, Gil-Sotres F *et al.*, 1998. Towards a biochemical quality index for soils; an expression relating several biological and biochemical properties[J]. Biology and Fertility of Soils, 26: 100—106.

Trasar-Cepeda C, Leirós M C, Seoane S *et al.*, 2000. Limitations of soil enzymes as indicators of soil pollution[J]. Soil Biology and Biochemistry, 2: 1867—1875.

Uminska R, 1993. Cadmium contents of cultivated soils exposed to contamination in Poland[J]. Environmental Geochemistry and Health, 15: 15—19.

van Elsas J D, Duarte G F, Rosado A S *et al.*, 1998. Microbiological and molecular biology methods for monitoring microbial inoculants and their effects in the environment[J]. Journal of Microbiological Methods, 32: 133—154.

Visser S, Parkinson D, 1992. Soil biological criteria as indicators of soil quality: soil microorganisms[J]. American Journal of Alternative Agriculture, 7: 33—37.

Wittekind F, Werner M, Reinicke A *et al.*, 1996. A microtiter-plate urease inhibition assay-sensitive rapid and cost-effective screening for heavy metals in water[J]. Environmental Technology, 17: 597—603.

Yao H, He Z, Huang C *et al.*, 2000. Microbial biomass and community structure in a sequence of soils with increasing fertility and changing land use [J]. Microbial Ecology, 40: 223—237.

Yao H, Xu J, Huang C, 2003. Substrate utilization pattern, biomass and activity of microbial communities in a sequence of heavy metal-polluted paddy soils [J]. Geoderma, 115: 139—148.

Zhou J, Bruns M A, Tiedje J M, 1996. DNA recovery from soils of diverse composition[J]. Applied Environmental Microbiology, 62: 316—322.

(Received for review January 3, 2005. Accepted March 7, 2005)