Microbial Biomass and Bacterial Community Changes by Pb Contamination in Acidic Soil.

Muhammad Akmal[1*], Xu Jianming[2]

ABSTRACT

Heavy metals contamination of soil above a threshold can adversely affect soil ecology and health. Until recently studies are carried out for modest levels of metals present in the sludges where effect of individual metal could not be segregated. Microbial biomass and bacterial community structure was determined in an acidic soil loaded with 0, 200, 400, 600, 800, and 1000 mg Pb kg^{-1} soil incubated at 25 ^{o}C for 60 days. Decrease in soil microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) and widening C: N ratio was noted with increasing levels of Pb in soil. The diversity of soil bacteria assessed by PCR-DGGE method illustrated the simplification of DGGE patterns in soil with increasing Pb concentration, and less bacterial diversity for Pb > 400 mg kg^{-1} compared with the control. Such decreases in soil microbial biomass and bacterial diversity by Pb contamination may help to evaluate heavy metals contaminated soil ecologies.

Keywords: Heavy Metal; Acidic Soil; Microbial Population; Bacterial Diversity

INTRODUCTION

Elevated levels of heavy metals can adversely affect soil microbial ecology due to population loss, changes in population structure, physiological activity, and shifts or changes in the composition of the microbial communities (Knight *et al.*, 1997; Kozdroj and van Elsas, 2000). The natural concentrations of most heavy metals in soils vary widely and are mainly related to the soil parent materials; however, anthropogenic sources such as smelters, power stations, industries; and the application of metal-containing pesticides, fertilizers, composts, and sludges may contribute to and at times exceed those from natural sources (McGrath *et al*., 1995). Lead (Pb) concentration in normal field soil is in the range of 10 to 100 mg kg^{-1} (Soon and Abboud, 1993), but in contaminated soils especially near mines or by sewage sludge applications, its concentration as high as 1000 mg kg^{-1} has been reported (Peters and Shem, 1992; Pichtel *et al*., 2000).

Soil microbial biomass, basal respiration and enzymes activity (Campbell *et al*., 1997; Trasar-Cepeda *et al.*,2000;Yao *et al*., 2003) have been suggested as possible indicators in monitoring soil environmental quality. However the use of modern biological techniques enables the study of microbial diversity at the molecular level. Polymerase chain reaction (PCR) based on 16S ribosomal DNA and denaturing gradient gel electrophoreses (DGGE) fingerprinting has been used to assess changes in experimentally altered soil microbial communities (Claudia *et al*., 2003). The 16S ribosomal DNA genes have been used for such studies since these genes are present in all bacteria and have been taken as the base to classify the bacteria. Extraction of total microbial DNA from soil followed by PCR amplification of the 16S ribosomal DNA genes ideally yield a mixture of DNA fragments representing all bacterial species present in that soil sample. DGGE is an electrophoretic method to identify single base changes in a segment of DNA. Mette and Niels (2002) used it as a screening method for monitoring changes in community in response to changes in the environmental parameters.

Nevertheless, long term field experiments have already indicated negative effects of metals on microbial parameters in soils treated with metal-contaminated sludge's in the past (Baath *et al*., 1998; Pichtel *et al.*, 2000; and Renella *et al*., 2004). But these studies were carried out at modest concentrations of heavy metals present in the sludges or the applied sludges contained mixtures of several metals, where the effect of individual metal could not be segregated. Secondly, at long-term field sites the soil microbial communities had time gain to adapt to the elevated metal concentrations stress (Kozdroj and van Elsas, 2000). So, the direct application of heavy metal in inorganic form under controlled, short-term experiments was required to assess the

M. Akmal[1*,] X.Jianming[2]. [1]Department of Soil Science and Soil & Water Conservation, PMAS Arid Agriculture University Rawalpindi, 46300 Pakistan. [2]Institute of Soil and Water Resources and Environmental Science, Zhejiang University, 268 Kaixuan Road, Hangzhou, 310029 China. Corresponding author (E-mail: akmal@uaar.edu.pk). Published in J. agric. biol. sci. 1 (1):30-37 (2009). A biannual publication of PMAS Arid Agriculture University Rawalpindi, Pakistan.

changes in soil microbiological parameters as a result of heavy metal contamination. Therefore, in the present incubation study different levels of Pb in nitrate form were selected to evaluate their effects on microbial biomass and bacterial community in an acidic soil.

MATERIALS AND METHODS

Soil Sampling and Preparation

The soil was collected at 0-10 cm depth from the research field of Hua Jia Chi campus, Zhejiang University, Hangzhou, China. Stones, discrete plant residues, and large soil animals (earthworms etc.) were removed and the sample air dried and passed through 2 mm sieve. The soil was analyzed for selected physical, chemical and microbiological properties (Table 1).

Table 1. Some selected properties of the soil used in the study.

Properties	Values
Soil texture	**Sandy loam**
pH $_{(1:2.5)}$	**5.5**
CEC	**11.3 cmol kg^{-1}**
Total organic carbon (TOC)	**19.3 g kg^{-1}**
Total nitrogen (TN)	**1.6 g kg^{-1}**
Total lead (Pb)	**28.5 mg kg^{-1}**
Microbial biomass carbon (C_{mic})	**290 mg kg^{-1}**
Microbial biomass nitrogen (N_{mic})	**50 mg kg^{-1}**
Microbial biomass C: N	**5.8**

Incubation Experiment

Fresh soil equivalent to 250 g oven-dry weight was taken in 500 mL glass beakers. Total eighteen beakers were prepared to accommodate six levels of Pb with three replications, and arranged in CRD manner. The soil in the beakers was first adjusted to 40 % of water holding capacity (WHC) with distilled water, and pre-incubated at 25 ^{0}C for seven days (conditioning period). After conditioning, 0, 200, 400, 600, 800 and 1000 mg Pb kg^{-1} soil was applied as $Pb(NO_3)_2$ solution, and the soil moisture was adjusted to 50 % of WHC and incubated at 25 ^{0}C for 60 days maintaining the soil moisture at 50 % WHC during the incubation period.

Soil Microbial Biomass

Soil microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) was analyzed after 60 days of Pb contamination. The chloroform fumigation-extraction method was applied to measure C_{mic} and N_{mic}. Fresh soil sample of 10 g was exposed to alcohol-free chloroform ($CHCl_3$) vapor in a vacuum desiccator containing soda-lime at 25 ^{0}C for 24 h. The fumigated soil was transferred into empty desiccator and residual $CHCl_3$ was removed from the fumigated soils by repeated evacuations. The fumigated soil was extracted immediately following $CHCl_3$ removal by shaking for 30 minutes with 50 mL 0.5 M K_2SO_4. The un-fumigated 10 g soil (oven dry weight) was extracted at the time of fumigation commencement. Automatic analyzers were used to measure total organic carbon (Shimadzu, TOC-500) and nitrogen (Astoria, Pacific-Inc) in the samples. The total organic carbon and nitrogen data collected were used to get the values of C_{mic} and N_{mic} (Anderson and Ingram, 1993).

Soil Bacterial Community

Total bacterial DNA from soil was extracted and purified for community analysis. For this fresh 5 g soil was added to 13.5 mL extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM di-sodium ethyl-diamine tetra acetic acid (EDTA) (pH 8.0), 100 mM Na_2HPO_4 (pH 8.0), 1.5 M

NaCl, 1 % hexadecyl trimethyl ammonium bromide (CTAB)). Then 50 µL of 10 mg mL^{-1} Proteinase K was added and the mixture was incubated at 37 °C for 30 min while being shaken horizontally at 150 rpm. Sodium dodecyl sulphate (SDS) was added to a final concentration of 20 % and each tube was incubated at 65 °C in water bath for 2 hours with gentle end-over-end mixing after every 15 min. The mixture was then centrifuged at 2500 X 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 in100 µL sterile water and stored at 4 °C (Zhou *et al*., 1996; Bruce *et al*., 2003). To reduce the chimera formation, the DNA was size fractionated by agarose gel electrophoresis (Fig. 1a) and DNA ≥ 20 kb was recovered using a Geneclean UNIQ-10 Spin kit (Sangon Biological Company, Shanghai, China).

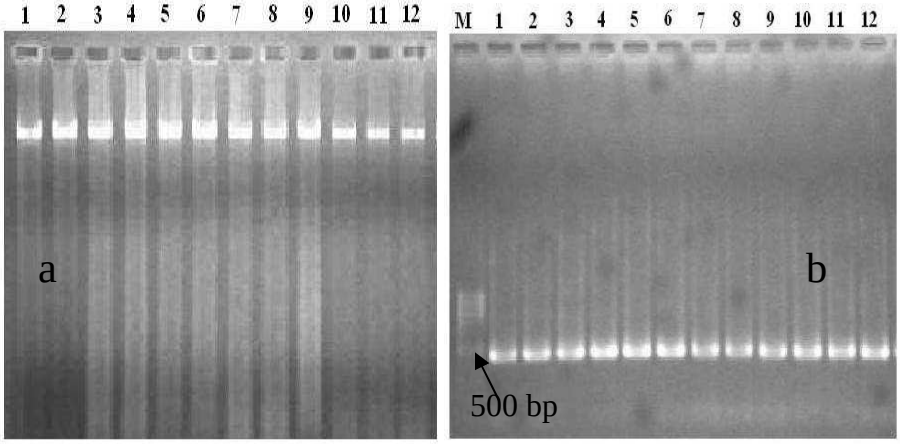

Fig. 1. DNA extraction (a) and PCR amplification results of extracted DNA from soil (b) under different Pb treatments. M; Marker, Lane; 1-2 (Control), 3-4 (200 mg kg^{-1} Pb), 5-6 (400 mg kg^{-1} Pb), 7-8 (600 mg kg^{-1} Pb), 9 10 (800 mg kg^{-1} Pb), and 11-12 (1000 mg kg^{-1} Pb).

The PCR mixture used for DNA amplification contained 0.2 µmol of each primer, 1 µL (about 5-15 ng) template DNA, 200 µmol of deoxynucleoside triphosphate, 5 µL 10 X PCR buffer, 37.5 mmol 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 rDNA gene. PCR amplification was performed at 95 °C for 4 min, followed by 40 thermal cycles of 94 °C for 1 min, 54 °C for 1 min, 72 °C for 1 min, and final single extension at 72 °C for 7 min. The size of the PCR product was visualized by electrophoresis in 1 % agarose gels after ethidium bromide staining (Fig.1b). Strong bands of approximately 430 bp were subjected to DGGE analysis. The 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 X 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 for 330 min at 60 °C and 130 V. After electrophoresis, the gel was stained with a silver staining procedure (Bassam *et al*., 1991). To get the clear image, the gel was photographed by gel photo system (Gel Doc 2000, BioRad).

Analysis of DGGE Pattern

Photographs were then analysed using BioRad Quantity One software package for genetic similarities of soil bacteria among Pb treatments. The structural variations of soil bacteria for various Pb treatments were assessed by Richness (S), Shannon indices (H) and Evenness (E_H) according to the following equations:

$$H = -\sum_{i=1}^{S} p_i \ln p_i = -\sum_{i=1}^{S} (N_i/N)\ln(N_i/N)$$

$$E_H = H / H_{\max} = H / \ln S$$

Where p_i is the ratio between the specific band intensity and the total intensity of all bands in a lane sample, whereas *S* is the total number of bands in each lane sample (Wang *et al*., 2007).

Statistical Analysis

All the data were analyzed statistically by ANOVA and means for different treatments were compared at 5 % level of significance using Duncan's multiple range test (Gomez and Gomez, 1984).

RESULTS

Soil Microbial Biomass and C: N

Soil microbial biomass C and N decreased with the increasing Pb levels in soil. Significantly lower C_{mic} content was found in soils containing 600 mg kg^{-1} or more Pb compared to the control (Table 2). The soil loaded with 1000 mg kg^{-1} Pb had approximately 36 % lower C_{mic} value compared to the control. Similarly, N_{mic} contents were significantly ($P<0.5$) lower in all Pb contaminated soils compared with control. About 62 % lower N_{mic} value was observed in soil contaminated with 1000 mg kg^{-1} Pb compared to control. Microbial C: N increased at enhanced levels of Pb in soil with exception of 600 mg kg^{-1} Pb treatment. However, the soils contaminated with greater than 200 mg kg^{-1} Pb had significantly higher C: N ratio compared to the control.

Table 2. Microbial biomass carbon (C_{mic}), nitrogen (N_{mic}) and C: N ratio as affected by the Pb application level.

Pb	C_{mic}	N_{mic}	C: N
	(mg kg^{-1})		
Control	**271 a**	**40.0 a**	**6.7 d**
200	**250 ab**	**36.3 b**	**6.8 cd**
400	**243 ab**	**27.6 c**	**8.8 bc**
600	**223 bc**	**27.0 c**	**8.3 bcd**
800	**193 cd**	**21.7 cd**	**8.9 b**
1000	**174 d**	**15.3 d**	**11.5 a**

Note: Means in a column following the same letter (s) are statistically non significant at 95 % confidence level.

Soil Bacterial Community

Lead markedly affected the bacterial community as evidenced by the number of DNA bands detected for various treatments on polyacrylamide gel after denaturing gradient gel electrophoresis (Fig. 2). Profile of DGGE for 800 mg kg^{-1} Pb was the simplest with only 8 visible bands among the profiles for all other treatments. The highest number (21) of visible bands was detected for the control, followed by 17 for 200 mg kg^{-1} Pb treatment (Table 3). The profiles for treatments receiving 400, 600 and 1000 mg kg^{-1} Pb showed less number of visible bands compared with the control. There were several similarities in banding positions among the treatments

receiving different levels of Pb (Fig. 2) indicating many common bacterial community members were still present in each treatment regardless of the metal level. Some of the DGGE bands were unique in each of the different treatments suggesting a change in the community. The diversity index approach was used to estimate changes in the diversity of soil bacterial under Pb contamination. The diversity index consisting of two components; the total numbers of species present or species richness and the distribution of the number of individuals among different species, called species evenness indicated that bacterial diversity for all Pb treated soils was less compared to the control (Table 3).

Table 3. Effect of Pb on soil bacterial community structure.

Pb (mg kg^{-1})	Shannon Indices (H)	Richness (S)	Evenness (E_H)
Control	**2.94 a**	**21 a**	**0.965 ab**
200	**2.83 ab**	**17 b**	**0.998 a**
400	**2.08 c**	**9 c**	**0.945 ab**
600	**2.08 c**	**9 c**	**0.945 ab**
800	**1.94 c**	**8 c**	**0.934 b**
1000	**2.30 bc**	**11 c**	**0.960 ab**

Note: Means in a column following the same letter (s) are statistically non significant at 95 % confidence level.

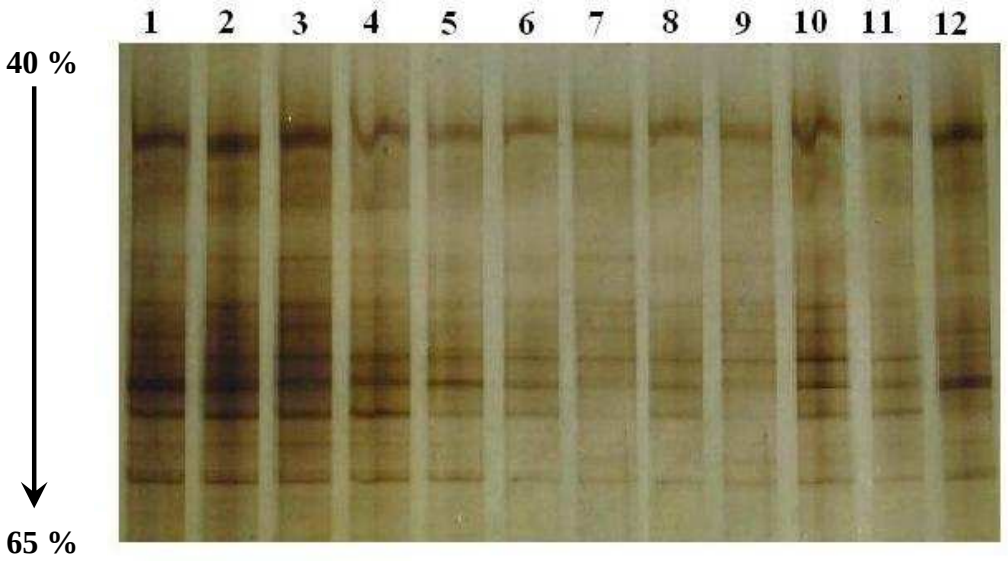

Fig. 2. DGGE profiles of amplified 16S rDNA fragments from soil with different levels of Pb contamination. Lane; 1-2 (Control), 3-4 (200 mg kg^{-1} Pb), 5-6 (400 mg kg^{-1} Pb), 7-8 (600 mg kg^{-1} Pb), 9-10 (800 mg kg^{-1} Pb), and 11-12 (1000 mg kg^{-1} Pb). Increasing denaturant from top (40 %) to the bottom (65 %).

DISCUSSION

Decrease in microbial biomass carbon (C_{mic}) and nitrogen (N_{mic}) with increasing level of Pb in soil was found in our experiment which was due to decrease in soil microbial populations upon the depletion of readily utilized carbon substrate resulted from Pb toxicity. The differences in C_{mic} and N_{mic} among various Pb treatments were caused by the different concentration of Pb added to the soil, which inhibited the growth of soil microorganisms. Measurement of the microbial

biomass offers a means of assessing the response of total microbial populations to heavy metal pollution in soil (McGrath *et al*., 1995). Previous studies (Dar, 1997) showed that Pb addition of 100 mg kg^{-1} soil as $PbCl_2$ caused no significant change in microbial biomass, but Pb addition at the level of 250 mg kg^{-1} significantly decreased the soil microbial biomass, and at 500 mg kg^{-1} a decrease of 16-26 % in microbial biomass occurred in the soil. A decrease in the growth and yield in response to metal stress was shown in a chemo-stat study using the marine bacterium *Vibrio alginolyticus* and the difference in the cellular energy budget was thought to be directed toward physiological processes required for detoxification mechanism. The suggestion that soil microorganisms under stress divert energy from growth to cell maintenance functions may, therefore, be the possible explanation for the decrease in biomass for metal contaminated soils (Gordon *et al*., 1993).

The cycling and availability of nutrients in the soil is significantly controlled by microbial biomass C: N ratio. Therefore, the microbial biomass C: N ratio is also an indicator of the effects of heavy metals on the functioning of soil ecosystem. Heavy metal stress can induce changes in the microbial biomass C: N ratio (Khan *et al*., 1998). Some studies have also shown an increase in the fungal population proportion compared to bacteria in heavy metal amended soil due to the fact that fungi tend to be more resistant to heavy metals than do the bacteria (Hiroki, 1992; Kelly and Tate, 1998). Generally, the degree of tolerance in microorganism to metal pollutants varies in the order; fungi > bacteria > actinomycetes (Frostegard *et al*., 1993). Hence, this change in bacterial to fungal populations may also be one of the reasons of changes in the C: N ratio for Pb contaminated soils under present investigation.

Bacterial diversity in soil amended with various levels of Pb assessed by PCR-DGGE analyses clearly showed that control had the most complex DGGE pattern (Fig. 2) with 21 visible bands indicating the presence of a high number of different bacterial species. The profile for 800 mg kg^{-1} Pb was the simplest with only 8 bands, among the profiles for all treatments. Shannon indices (H), based on the DGGE bands patterns of the samples, provide a numerical indicator to compare changes in the structure of dominant members in the bacterial community. Since H is based on the summed proportional abundance of individual bacterial types (DGGE bands and their intensity), a change in H either implies a change in the number of bacterial types or a change in the proportional abundance of a given type. A decrease in H for Pb treated soils compared to the control in this study (Table 3) reflected the impact of Pb on the bacterial diversity in soil, which caused a decrease in the number of bacterial types. This can also give partial explanations for above mentioned decreases of microbial biomass C and N observed under Pb contaminated soils. We found several similarities in banding positions among the soils receiving different levels of Pb, which indicated that many common microbial members were still present in each soil regardless of the heavy metal rates. Renella *et al*. (2003) found that high concentration of heavy metal mainly induced physiological adaptations rather than selection for metal-resistant culturable soil microflora. However, in our experiment some of the DGGE bands were unique to each of the different treatments suggesting a change in the structure, as some unique bacteria were forced to adapt to the changed environment, and could exist in the stressed habitat.

We combined two different techniques (traditional and modern) to investigate the effect of Pb on soil microbiological parameters and found that soil contaminated with more than 200 mg Pb kg^{-1} soil adversely affected the soil microbial ecology. The changes noted in soil microbial biomass and bacterial community as a result of Pb contamination would be helpful for future studies on soil microbial ecology under metal stress environments.

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REFERENCES

Anderson, J.M., and J.S.I. Ingram. 1993. Tropical soil biology and fertility: A hand book of methods. CAB. International, London, UK. 2nd edition: 62-87.

Baath, E., M. Diaz-Ravina, A. Frostegard, and C.D. Campbell. 1998. Effect of metal-rich sludge amendments on the soil microbial community. Appl. Environ. Microbiol. 64:238-245.

Bassam, B.J., G. Caetano-Anolles, and P.M. Gresshoff. 1991. Fast and sensitive silver staining of DNA in polyacrylamide gels. Ann. Biochem. 80:81-84.

Bruce, F., F.A. Moffett, N.C. Nicholson, B.J. Uwakwe, J.A.H. Chambers, and C.J.H. Tom. 2003. Zinc contamination decreases the bacterial diversity of agricultural soil. FEMS. Microbiol. Ecol. 43:13-19.

Campbell, C.D., S.J. Grayston, and D.J. Hirst. 1997. Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. J. Microbiol. Method 30:33-41.

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

Dar, G.H. 1997. Effect of heavy metals (Cd, Cr, Ni and Pb) on soil microbial biomass, C mineralization and enzyme activities. Int. J. Ecol. Environ. Sci. 21:87-95.

Frostegard, A., A. Tunlid, and E. Baath. 1993. Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. Appl. Environ. Microbiol. 59:3605-3617.

Gomez, A.K. and A.A. Gomez. 1984. Statistical Procedures for Agricultural Research. John Wiley and Sons, New York, USA.

Gordon, A.S., V.J. Harwood, and S. Sayyar. 1993. Growth, copper-tolerant cells, and extracellular protein production in copper stressed cultures of *Vibrio alginolyticus*. Appl. Environ. Microbiol. 59:60-66.

Hiroki, M. 1992. Effects of heavy metal contamination on soil microbial population. Soil Sci. Plant Nutr. 38:141-147.

Kelly, J.J., and R.L. Tate. 1998. Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. J. Environ. Qual. 27:609-617.

Knight, B., S.P. McGrath, and A.M. Chaudri. 1997. Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol. 63:39-43.

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

Khan, K.S., Z.M. Xie, C.Y. Huang. 1998. Relative toxicity of lead chloride and lead acetate to microbial biomass in red soil. Chin. J. Appl. Environ. Biol. 4:179-184.

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

McGrath, S.P., A.M. Chaudri, and K.E. Giller. 1995. Long-term effects of metals in sewage sludges on soils, microorganisms and plants. J. Indus. Microbiol. 14:94-104.

Peters, R.W., and L. Shem. 1992. Adsorption/ desorption characteristics of lead on various types of soil. Environ. Progress. 11:234-240.

Pichtel, J., K. Kuroiwa, and K Sawyerr. 2000. Distribution of Pb, Cd and Ba in soils and plants of two contaminated sites. Environ. Pollut. 110:171-178.

Renella, G., A. Reyes, L. Ortigoza, L. Landi, P. Nannipieri. 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}). Soil Biol. Biochem. 35:1203–1210.

Renella, G., M. Mench, and D. vander-Lelie. 2004. Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils. Soil Biol. Biochem. 36:443-451

Soon, Y.K., and S. Abboud. 1993. Cadmium, Chromium, Lead, and Nickel. p. 101-108. *In* M.R.Carter (ed.) Soil Sampling and Methods of Analysis. Canadian Society of Soil Science, Lewis, Canada.

Trasar-Cepeda, C., M.C. Leirós, S. Seoane, and F. Gil-Sotres. 2000. Limitations of soil enzymes as indicators of soil pollution. Soil Biol. Biochem. 32:1867-1875.

Wang, Y., J. Shi, H. Wang, Q. Lin, X. Chen, and Y. Chen. 2007. The influence of soil heavy metals pollution on soil microbial biomass, enzyme activity, and community composition near a copper smelter. Ecotoxicol. Environ. Saf. 67: 75-81.

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

Zhou, J., M.A. Bruns, and J.M. Tiedje. 1996. DNA recovery from soils of diverse composition. Appl. Environ. Microbiol. 62:316-322.