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Soil Biological and Biochemical Response to Cd Exposure

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Though heavy metals can stimulate the activity of soil enzymes in smaller amounts, yet act as inhibitors, if present in high concentrations. Natural and anthropogenic heavy metal contamination and its disturbances on soils can be evaluated by using enzymatic activities as sensors. To study the effects of Cd, soil added with known Cd concentrations (0, 10, 20, 50,100 and 200 mg/kg soil) were incubated for a period of 30 days at 28°C. At intervals of 0, 5, 10, 20 and 30 days samples were withdrawn for enzyme assays like dehydrogenase (DHA), catalase (CAT), phenol oxidase (PHE), and peroxidise (PER). In a separate experiment the effect of Cd on active microbial biomass carbon (AMBC), basal soil respiration (BSR), and metabolic quotient were studied. AMBC showed a reduction trend with increase in Cd concentration, and a maximum reduction of 47% was observed at 30th day for 200 mg/kg treatment. BSR also showed the same trend, with a maximum decrease of 42% at the 30th day. With the rate of Cd amendments and treatment period, DHA showed an inhibition trend; whereas maximum decrease was observed for 200 mg/kg treatment at 30th day. CAT, PER, and PHE were found to be increased with Cd addition and remained at higher levels than in the control soil. These changes can be attributed to the effect of Cd on microbial activities. Based on cluster analysis, AMBC appears to be the sensitive indicators for the soil exposed to Cd contamination.

Keywords: Cadmium, Microbial Biomass, Basal Soil Respiration, Dehydrogenase, Catalase, Peroxidase, Phenol Oxidase, Respiration Quotient

Introduction

Intensified urbanisation and associated anthropogenic activities cause extensive changes on soil and soil-related natural resources. The study of enzymatic activities in soil is a useful tool for assessing the functional diversity of soil microbial communities or soil organic mass turnover (Kandeler et al. 1999). Soil biological activities and biochemical properties get altered with addition of fertilisers, agricultural activities and also with the contamination of chemicals like heavy metals. Soil microbes get influenced by pollutants introduced into the soil, which is manifested by changes in enzyme activities. In this group, heavy metals are of special importance, which can stimulate the activity of soil enzymes in smaller amounts, but can also act as inhibitors if present in high concentrations (Christensen et al., 1982; Frankenbereger et al., 1983; Wyszkowska et al., 2001). In general, an increase of metal concentration influences soil microbial properties (e.g. respiration rate, and enzyme activity), which appear very useful as indicators of soil pollution (Brookes, 1995; Szili-Kovács et al., 1999). In sewage sludge and phosphate fertilisers, Cd is one of the most toxic and has been recognised as an environmental contaminant of considerable interest in various human and animal diseases (Bramley, 1990; Loganathan et al., 1996). Moreover Cd represents a group of heavy metals causing the most severe changes in the biological properties of soils (Milosevic et al., 1997; Landi et al., 2000; Lebedeva et al., 1995; Welp, 1999; Zheng et al., 1999).

The purpose of the study was to determine the influence of cadmium contamination on soil microbial activities. Soil enzyme activities are the driving force behind all the biochemical transformations occurring in soil. Several soil quality monitoring programs employed microbial biomass, basal respiration, and microbial community structure as indicators of soil environmental quality (Doran and Parkin, 1994; Sparling, 1997, Yao et al., 2000). Soil microbial biomass, which plays an important role in nutrient cycling and ecosystem sustainability, has been found to be sensitive to increased heavy metal concentrations in soils (Giller et al., 1998; Huang and Khan, 1998). Basal respiration is also commonly measured and indicates the total carbon turnover. The metabolic quotient, i.e., the ratio of basal respiration to microbial biomass, is inversely related to the efficiency with which the microbial biomass uses the indigenous substrates (Anderson and Domsch, 1990) and can be a sensitive indicator for revealing heavy metal toxicity under natural conditions (Wardle and Ghani, 1995). Though there are few studies on the effect of Cd on soil microbial activities, most of them are carried out in temperate soils, where the microbial activities are quiet higher as compared to tropical soils. Such studies on tropical soils are limited. In view of above, this study was undertaken to determine the effect of cadmium exposure on the soil biological activities in a red soil from tropical region of India.

Materials and Methods

Incubation Experiment

Soil samples collected from Central Institute of Mining and Fuel Research (CIMFR), Digwadih campus were selected for the study, and analysed for their physico-chemical properties by standard methods and data are shown in Table 1.The samples were passed through 2 mm sieve, and added with required quantities of cadmium chloride in solution form to attain different Cd concentrations of 0, 10, 25, 50, 100, 200 mg/kg soil. The samples were then incubated at 28°C, while maintaining

the moisture content of the soil at field capacity level by adding required amounts of water. Samples were taken out at 0, 5, 10, 15, 20, and 30 days of incubation, and analyzed for dehydrogenase, peroxidase, phenol oxidase, and catalase activities. A separate set of soil was incubated for 10 days to measure the soil respiration rate and active microbial biomass.

Methods of Analyses

Dehydrogenase activity (DHA) was determined by adding 0.2 ml of 3% sterile triphenyltetrazolium chloride (TTC) solution and 0.5 ml of 1% sterile glucose into a culture tube containing 1 g of soil sample. After an incubation period of 24 hr at 28℃, 10 ml of methanol was added and re-incubated at 28℃ for 8 hr. The extracted triphenyl formazan (TPF) was measured by absorbance at 485 nm using a spectrophotometer (Klein et al., 1971).

Catalase activity (CAT) was determined as the amount of H_2O_2 consumed by the soil as described by Xu and Zheng (1986). Twenty five ml of 3% H_2O_2 was added to 5 g soil sample. After incubation at 4℃ for 30 minutes, 25 ml of 1M H_2SO_4 was added to it. The contents were filtered; 20 ml of 0.5M H_2SO_4 was then added to the 5 ml filtrate. The resulting solution was titrated against 0.005 M $KMnO_4$ to measure the un-reacted H_2O_2.

Phenol oxidase (PHE) and peroxidise (PER) activities were measured with L-DOPA (L-3, 4-dihydroxyphenylalanine) as substrate in acetate buffer (Robertson et al., 1999). Phenol oxidase activity was determined by adding 5 ml of 50 mM sodium acetate buffer and 5ml of 5mM L-DOPA to 0.5 g soil sample. After incubation, the solution was centrifuged and the supernatant was measured by absorbance at 460 nm. Control was kept for each sample by adding 5 ml of acetate buffer instead of L-DOPA. For determination of peroxidase activity, H_2O_2 was added in addition to L-DOPA, the increment in the absorbance at 460 nm due to H_2O_2 was expressed as the peroxidase activity.

For determination of active microbial biomass carbon (AMBC), 20 g soil at 60% WHC was placed in each of two conical flasks. The soil in one flask was amended with nutrients (120 mg glucose, 30 mg yeast extracts, 45 mg NH_4Cl, 12 mg $MgSO_4.7H_2O$, and 10 mg KH_2PO_4), the other flask was kept as control, without nutrient amendment. A vial containing 5.0 ml of 0.5 M NaOH was placed in each of the flasks to trap evolved CO_2. The flaks were sealed and incubated in the dark for 24 h at 20°C. The trapped CO_2 was measured by back titration with 0.5 M H_2SO_4.The AMBC was measured as follows:

$$AMBC = (CO_2\text{-}C24_{amend} - CO_2\text{-}C24_{unamend}) \times AC \quad (1)$$

where CO_2-$C24_{amend}$ − CO_2-$C24_{unamend}$ are the amount of CO_2 evolved from the glucose-nutrient-amended and unamended soils during 24 h incubation, respectively, and AC is the coefficient (0.283) to convert CO_2-C into AMBC (Islam and Weil, 2000).

Basal soil respiration (BSR) was measured as the CO_2 evolution from the un-amended moist soil adjusted to 60% WHC for an incubation period of 10 days at 25 ± 1°C in the dark (Islam and Weil, 2000). The BSR was calculated as follows:

$$BSR = (CO_2\text{-}C_{soil} - CO_2\text{-}C_{air})/10 \quad (2)$$

where CO_2-C_{soil} - is the amount of CO_2 evolved from soil and CO_2-C_{air} is the atmospheric CO_2 absorbed by 0.5 M NaOH in a blank flask.

Metabolic quotient (qCO_2) was calculated as BSR per unit of AMBC.

Statistical Analysis

The data were analyzed using a statistical software SYSTAT-12. One-way analysis of variance was carried out to compare the means of different treatments and least significant differences at $P < 0.05$ were obtained using Duncan's multiple range test (DMRT). The data were also subjected to Pearson correlation analysis, and cluster analysis, to identify the relationship between the variables and to find out the key soil parameters that are sensitive to Cd exposure.

Results and Discussion

Soil Enzymes

DHA activity showed a significant decline ($P < 0.05$) with increase in Cd concentration (Figure 1). And the mean activity decrease was found to be 15, 18, 35, 40, and 56% in case of 10, 20, 50, 100 and 200 mg/kg Cd treatments, respectively. The reduction in AMBC (Figure 5) must have contributed to the decrease in DHA as this is a group of intracellular enzyme present in active microorganism in the soil (Nannipieri et al., 1990). The increased sensitivity of DHA to metal contamination can be explained by the fact that the dehydrogenase is active only within living cells, intact, unlike other enzymes that act outside the cell. DHA was found most sensitive to pollution with Cd (Violeta, 2011). Other reason may be the interaction of heavy metals with the enzyme substrate complex, denaturation of the enzyme protein or interaction of Cd with protein-active group (Nannipieri et al., 1994). Karaca et al (2002) and others confirm the same pattern in their studies also. Similarly, the inhibition extent was also obvious between different incubation periods, and varied as the incubation proceeded, the highest inhibition rate was detected in at 30th day. Sardar et al (2007) showed that in the case of Cd treatments, DHA activity was significantly inhibited, after 2 weeks of incubation. This highest inhibitory effect of heavy metals on soil enzyme activities in the first two weeks may be due to the sudden exposure of the microbes to heavy metals. Later on the microbes may have adapted to the polluted environment, and the enzyme activity tended to recover. Similar results were obtained by Maliszewska-Kordybach and Smreczak, (2003) and Zhang et al. (2007).

Table 1.
Some initial physicochemical characteristics of the soil sample.

	Parameters	value
1	Clay (%)	22.0
2	Silt (%)	9.5
3	Sand (%)	68.5
4	Bulk density (Mg/m^3)	1.44
5	Maximum water holding capacity (%)	32.54
6	pH	4.65
7	EC (dS/m)	0.054
8	LOI (%)	4.62
9	Organic carbon (%)	1.69
10	CEC	21.3
11	Nitrogen (mg/kg)	19.1

Contrary to DHA, other enzymes like catalase, phenol oxidase, and perioxidase increased significantly ($P < 0.05$) with increase in Cd concentration (Figure 2). The mean CAT activity has increased by 6, 11, 22, 29, and 41% in case of 10, 20, 50, 100 and 200 mg/kg Cd treatments respectively. Similarly, the corresponding increase in PHE activity was 12, 25, 31, 38, 51%; and increase in PER was 4, 13, 20, 25, 32% respectively (Figure 3 & 4). The increase in CAT, PER, and PHE may be due to the increase in production of reactive oxygen species (ROS) such as hydroxyl radical (HO.), superoxide radical (O_2^-) or hydrogen peroxide (H_2O_2). Catalase enzyme production and extracellular release may be induced by exposure of the cells to elevated levels of hydrogen peroxide (Ercal et al., 2001). Pereira et al (2002) opined that the reactive oxygen species (ROS) induced by Cd are metabolised by CAT in the peroxisomes. Stimulation of CAT activity can be associated with effective antioxidant defense system acting against oxidative stress and/or compensating for the decrease in other antioxidant

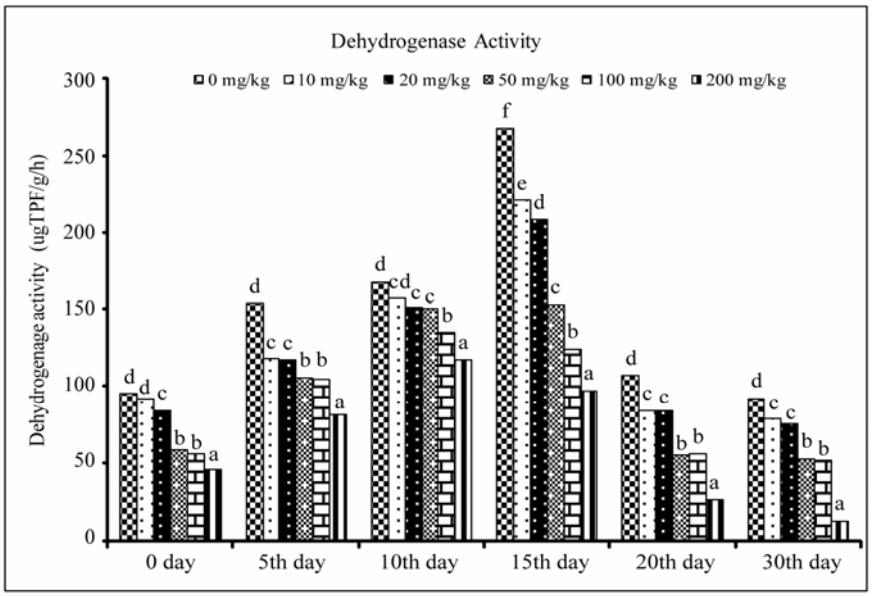

Figure 1.
Changes in dehydrogenase activity of soil added with Cd during the incubation period (*Within each incubation period, bars with the same alphabets are not significantly different at P < 0.05 level using LSD.)*

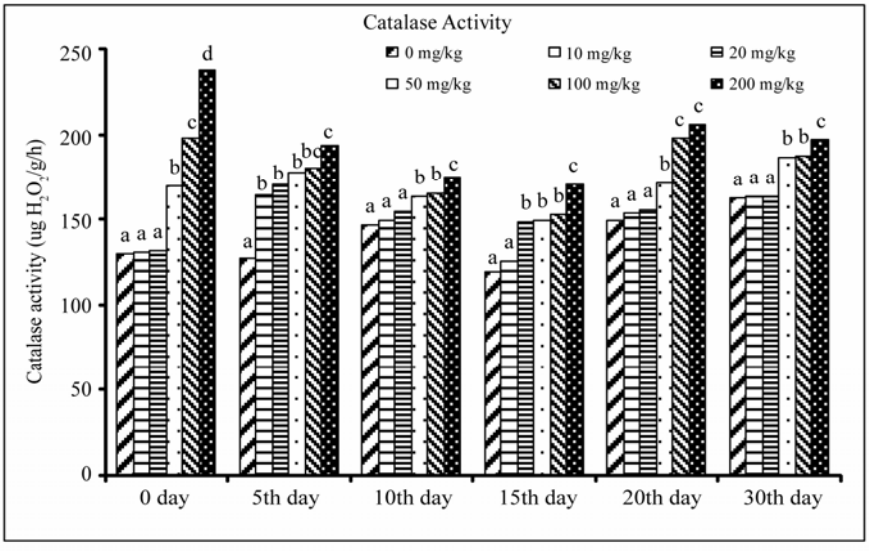

Figure 2.
Changes in catalase activity of soil added with Cd during the incubation period (*Within each incubation period bars with the same alphabets are not significantly different at P<0.05 level using LSD.)*

enzymes (Radhakrishnan, 2009). Antioxidants are well known to play a prominent role in the defense against free radicals in plants. Catalase scavanges H_2O_2 by breaking it down directly to form water and oxygen while peroxidise decomposes H_2O_2 by oxidation of phenolic compounds. The increased activities of catalase and peroxidase suggest that soil biological systems depend on these antioxidative enzymes for elimination of H_2O_2 under Cd stress. Effect of Cd on the above soil enzymes is less found in literature; however, there are reports on such enzymes in plant system. Cui and Wang, (2006) reported an increase in leaf peroxidase activity with Cd treatments while leaf catalase activity decreased significantly. Similar declines in catalase activity were reported under Cd stress in rice, cabbage, bean, carrot, radish, and pea (Chaoui et al. 1997; Sandalio et al. 2001; Shah et al. 2001; Pandey and Sharma 2002). However, increased catalase activity was also observed in sunflower cotyledons and barley (Patra and Panda 1998; Gallego et al. 1999) under Cd stress. The effects of Cd on the growth and the activeties of the antioxidant enzymes, catalase, superoxide dismutase and glutathione reductase have been investigated in *Crotalaria juncea* seedlings, where the CAT activity did not exhibit any major variation in the roots following $CdCl_2$ treatment, however, 2 mM $CdCl_2$ induced a 6-fold increase in activity in the leaves when compared to the untreated control (Pereira et al., 2002).

Microbial Biomass, Respiration and Metabolic Quotient

The AMBC values significantly ($p < 0.05$) decreased with increasing level of Cd (Figure 5). The mean AMBC at 0, 10, 20, 50, 100, and 200 mg/kg Cd treatments were 580, 471, 396, 384, 362, and 310 mg/kg, respectively, which corresponds to 19, 32, 34, 38, and 47% AMBC reduction. The reduction in AMBC

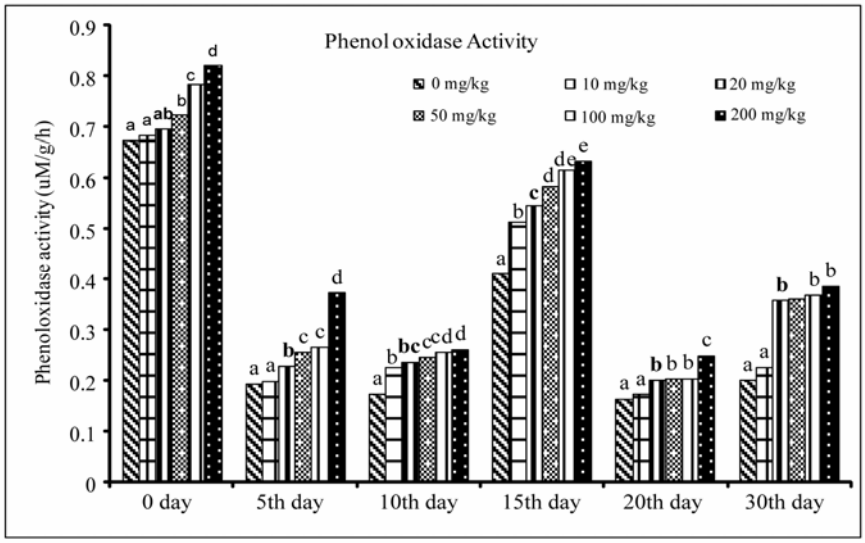

Figure 3.
Changes in phenol oxidase activity of soil added with Cd during the incubation period (Within each incubation period bars with the same alphabets are not significantly different at P<0.05 level using LSD.)

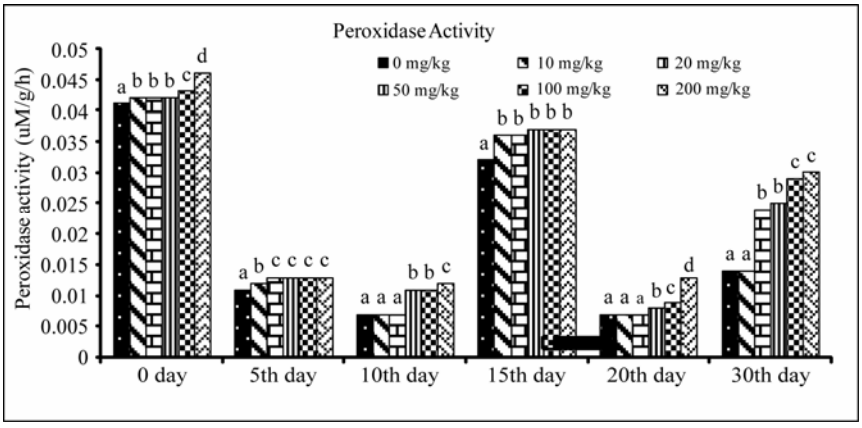

Figure 4.
Changes in peroxidase activity of soil added with Cd during the incubation period (Within each incubation period bars with the same alphabets are not significantly different at P<0.05 level using LSD.)

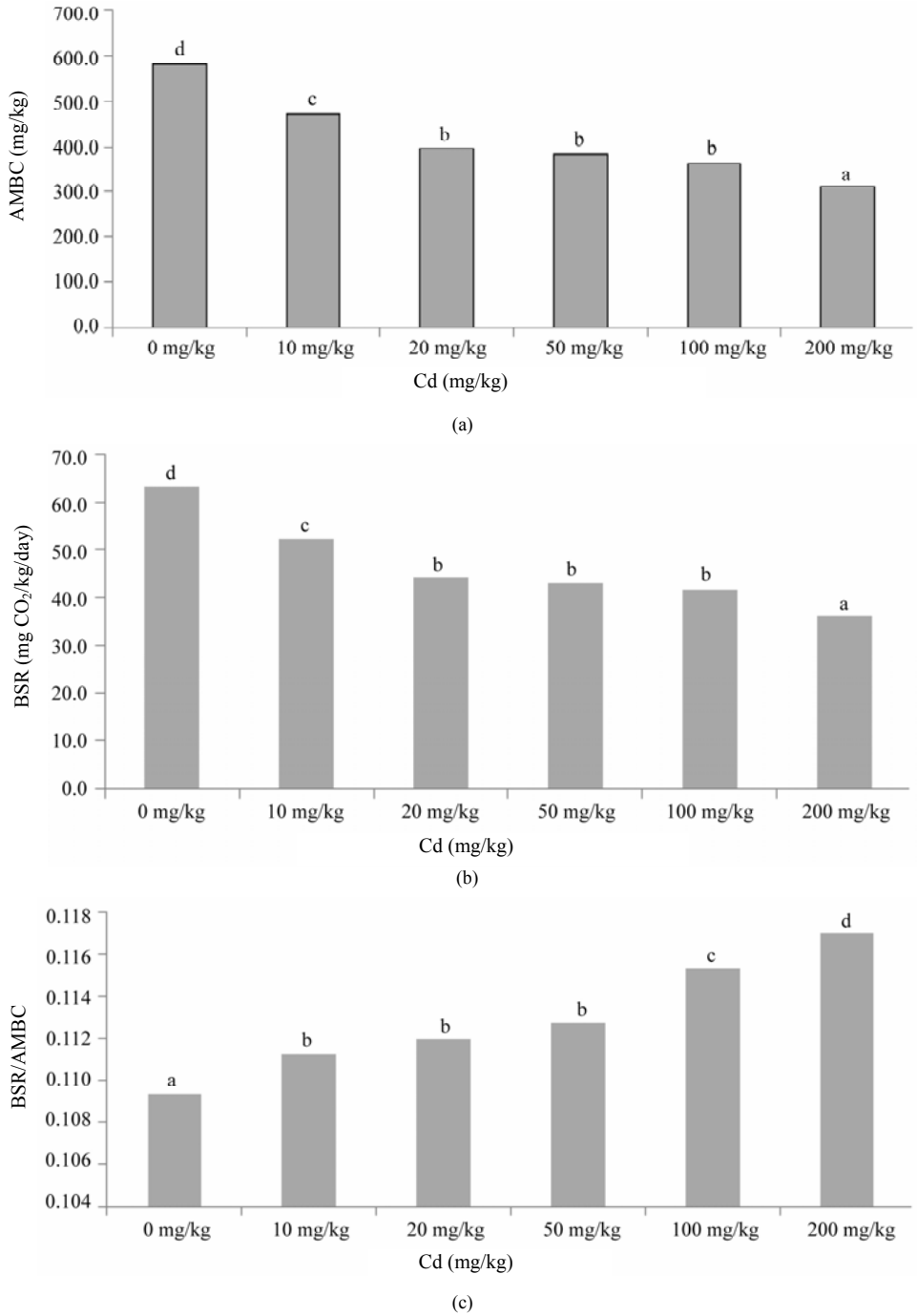

Figure 5.
Effect of Cd on (a) Active microbial biomass carbon, (b) Basal soil respiration, (c) Metabolic quotient (bars with the same alphabets are not significantly different at P < 0.05 level using LSD.)

is probably due to the decreased conversion of substrate into new microbial biomass in the Cd contaminated soils, i.e reduced primary production and resultant lower input of energy (Chander & Brookes, 1991). Giller et al. (1997) expressed that micro organisms differ in their sensitivity to metal toxicity and sufficient metal exposure will result in immediate death of cells due to disruption of essential functions, and to more gradual changes in population sizes due to changes in viability or competitive ability. According to Zhang et al. (2008), MBC decreased with increasing Cd concentration in soil. These results are in agreement with the findings of previous studies (Bhattacharyya et al. 2008; Khan et al., 2010). But results of Fritze et al., (2000) and Landi et al., (2000) showed that even at high Cd contamination up to 1000 mg Cd/ kg microbial biomass C was

not found to be negatively affected. Results from another study conducted in a laboratory on an agricultural sandy loam showed a significantly lower biomass C in a Cd polluted soil at the very low contamination level of 0.001799 mg Cd/kg (Griffiths et al., 1997). Giller et al. (1998) reported that the microbial biomass C in agricultural soils under long- term metal stress is reduced in comparison to an unpolluted site. This shows that the comparison of these results is very difficult because the different soil types, time frames, and metal concentrations, lead to different bioavailable fractions of the metals. Moreover Cd is not an essential element and so cannot have a direct positive influence on the soil microbes. There may be an indirect effect of Cd on the availability of other essential micronutrients. The different soil types and locations contain different microbial communities which may not have the same sensitivity to Cd toxicity.

Microbial biomass alone does not provide information on microbial activity. Some measure of microbial biomass turnover, such as BSR, is required for this assessment (Anderson and Domsch, 1986; Sparling & Ross, 1993). The basal respiration decreased by 18, 30, 32, 34, and 43% under 10, 20, 50, 100 and 200 mg/kg Cd treatments respectively (Figure 5). The reduction in BSR may be due to the adverse effects of Cd on soil microflora, which appeared to increase the accumulation of organic matter as the heavy metal content increased, probably because the biomass was less effective in mineralizing soil organic matter under these conditions. Soil respiration studies on forest soils showed a decreasing trend with Cd contamination level. (Landi et al., 2000). The microbial metabolic quotient (respiration-to-biomass ratio) or qCO_2 is increasingly being used as an index of ecosystem development (during which it supposedly declines) and disturbance (due to which it supposedly increases) (Wardle & Ghani, 1995). The mean qCO_2 at 0, 10, 20, 50, 100, and 200 mg/kg Cd treatments were 0.109, 0.111, 0.112, 0.113, 0.115, and 0.117 mg/kg/day respectively. The metabolic quotient increased by 2, 2.7, 3.4, 5.8 and 7.4% under 10, 20, 50, 100, and 200 mg/kg Cd treatments respectively (Figure 5). This may be due to the fact that under stress, the soil micro organisms need to expend more energy to survive. The greater demand for energy by microorganisms in order to cope with the toxicity of Cd was also confirmed by the increase in metabolic quotient (qCO_2). Chander and Brookes (1991) and Bardgett and Saggar (1994) reported a doubling of qCO_2 upon heavy metal contamination. An increased qCO_2 indicates shifting of energy from growth to maintenance in an ecosystem. Biomass synthesis is less efficient under heavy metal stress and biomass reduction in heavy metal contaminated soils is mainly due to inefficient biomass synthesis. The shift towards catabolic processes is often better reflected by increased metabolic quotients (qCO_2), i.e. the ratio of CO_2 production rate to microbial biomass.

Correlations and Cluster Analysis

Significant negative correlation ($P < 0.05$) was observed between DHA and PHE/ CAT (Table 2), however, positive relation ($P < 0.01$) was observed between DHA and AMBC/BSR. PHE had significant positive correlation with CAT, and negative relation with AMBC/BSR. Negative correlation was observed between CAT and AMBC/BSR, and positive correlation with qCO2. AMBC had a significant positive correlation with BSR. The responses to Cd amendment by different soil pa-

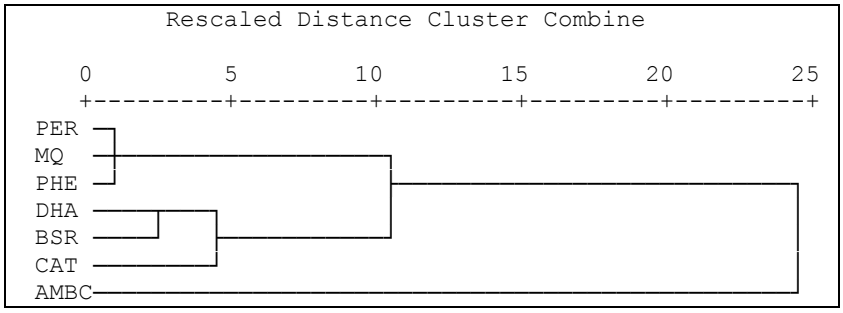

Figure 6.
Hierarchical dendrogram for soil parameters obtained by Ward's hierarchical clustering method.

Table 2.
Correlations between the parameters of soil contaminated with Cd.

	DHA	PHE	PER	CAT	AMBC	BSR	qCO_2
DHA	1.00	–0.966**	–0.704	–0.992**	0.933**	0.923**	–0.799
PHE		1.00	0.662	0.961**	–0.974**	–0.969**	0.761
PER			1.00	0.724	–0.549	–0.539	0.632
CAT				1.00	–0.901*	–0.889*	0.854*
AMBC					1.00	0.999**	–0.660
BSR						1.00	–0.637
qCO_2							1.00

*Correlation is significant at the 0.05 level (2-tailed); **Correlation is significant at the 0.01 level (2-tailed).

rameters were further classified by cluster analysis. Relatively homogeneous groups of variables were identified by hierarchical cluster analysis, using an algorithm that starts with each variable in a separate cluster and combines clusters. This was done by Ward's method, with Euclidean distances as the criterion to form the clusters. For Cd amended soils, Figure 6 shows three clusters: 1) PER-PHE-qCO_2 2) DHA-BSR-CAT, 3) AMBC. However, the clusters 1 & 2 could be joined together at a relatively high level. The group of AMBC was remarkably different from the other parameters in terms of Euclidian distances in cluster analysis. Judging from these results, the AMBC appears to be the sensitive indicator for the effects of Cd on the soil quality. Microbial biomass is well established as an early indicator of gross changes in C input caused by pollution (Brookes and McGrath, 1984). In ecotoxicological studies, the microbial biomass has been proposed as a sensitive indicator to define the impact of contaminants such as metals on soil biological functioning (Brookes, 1995; Dahlin et al., 1997; Giller et al., 1998).

Conclusion

The soil biological and biochemical activities significantly altered with the exposure of Cd as evidenced by the reduction in the AMBC, BSR, and DHA activity; and increase in the activities of CAT, PHE, PER and microbial quotient. AMBC appears to be sensitive soil indicator for the effects that are resulting from Cd contamination. Further studies involving different types of soils along the natural Cd contamination gradient are needed to clarify the trends detected in this study.

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