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Soil Biology & Biochemistry 37 (2005) 133–139

Soil Biology & Biochemistry

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Microbial activity and hydrolase synthesis in long-term Cd-contaminated soils

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Received 8 July 2003; received in revised form 19 May 2004; accepted 3 June 2004

Abstract

Alkaline and acid phosphomonoesterase, β-glucosidase, arylsulfatase, protease and urease activities, CO_2–C evolution and ATP content were monitored in long-term Cd-contaminated (0–40 mg Cd kg^{-1} dry weight soil) sandy soils, kept under maize or 'set aside' regimes, amended with plant residues. The organic matter input increased soil respiration, ATP contents and hydrolase activities in all soils. However, the Cd-contaminated soils had significantly higher metabolic quotients (qCO_2), as calculated by the CO_2-to-ATP ratio, and significantly lower hydrolase activities and hydrolase activity-to-ATP ratios for alkaline phosphomonoesterase, arylsulfatase and protease activities, compared with the respective uncontaminated soils. The ratios between acid phosphomonoesterase, β-glucosidase and urease activities and ATP were unaffected. A significantly higher qCO_2/μ ratio, an expression of maintenance energy, was observed in most of the contaminated soils, indicating that more energy was required for microbial synthesis in the presence of high Cd concentrations. It was concluded that exposure to high Cd concentrations led to a less efficient metabolism, which was responsible for lower enzyme activity and synthesis and lower hydrolase activity-to-ATP ratios observed in these Cd-contaminated soils.

Keywords: Cadmium; Soil microbial biomass; Hydrolase synthesis

1. Introduction

The energy flow through soil microflora is regulated by biosynthesis and maintenance processes. Both may be affected by environmental factors such as the availability of nutrients and the presence of toxicants. Soil hydrolase activities are key factors controlling nutrient availability in soil (Nannipieri et al., 2002) and adverse effects of heavy metals on soil enzyme activity have long been recognised under both long-term field experiments and laboratory incubations (Tyler et al., 1989). Such effects have been quantified mainly by assaying soil enzyme activity after fresh additions of heavy metals (Tabatabai, 1977; Doelman and Haanstra, 1986; Eivazi and Tabatabai, 1990; Landi et al., 2000), or by calculation of the ecological dose (ED_{50}) value (Haanstra and Doelman, 1991; Speir et al., 1995; Moreno et al., 2001; Renella et al., 2003).

The microflora of heavy metal contaminated soils generally have altered microbial activities, manifested by higher metabolic quotients (qCO_2) (Brookes, 1995) and less microbial biomass synthesis per unit of organic substrate added (Chander and Brookes, 1991, 1992), compared with values in the respective unpolluted soils. Both of these features indicate a reduced metabolic efficiency in the conversion of substrates into biomass (Pirt, 1975).

Among heavy metals, Cd is considered to be particularly toxic and responsible for serious biological degradation of soils (Reber, 1992; Smith, 1996; Giller et al., 1998). However, severe adverse effects of Cd on soil microbial biomass, microbial growth and microbial activities such as enzyme activities have been generally detected only when Cd concentrations were particularly high (Dar, 1996; Moreno et al., 1998, 1999) and its effects on enzymes generally depend on the enzyme activity studied.

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0038-0717/$ - see front matter © 2004 Elsevier Ltd. All rights reserved.
doi:10.1016/j.soilbio.2004.06.015

Little information is available on the effects of heavy metals such as Cd on the synthesis of hydrolases induced by the availability of organic substrates. The rationale for this work was that stress due to high Cd concentrations in soil should also be reflected in a measurable effect on hydrolase production.

2. Materials and methods

2.1. Soil characteristics and treaments

Soils (Arenic Udifluvent) from the long-term AGIR (Table 1) Experiment (Unité d'Agronomie, INRA, Bordeaux, France) were contaminated between 1988 and 1990 with $Cd(NO_3)_2$ to give Cd concentrations of 0 (plots 16 and 17), 10 (plots 11 and 12), 20 (plots 6 and 7), and 40 mg kg^{-1} soil (plots 1 and 2). Soils from plots 16, 11, 6 and 1 were cultivated in maize from period the 1988 to 1990. Thereafter, grass (mainly *Festuca* spp.) was cultivated until the year 2000 when soils were cultivated with maize again. Soils from plots 17, 12, 7 and 2 were under set aside (SA) regime, kept barren by tilling (once a year). Soils were surface (0–20 cm) sampled in 2001, sieved (<2 mm) at field moisture, and kept at 50% WHC for 7 days at 25 °C prior to the analyses. After this pre-incubation, 1000 g soil samples were split into two subsamples: one was amended with 10 g kg^{-1} of dry-milled ryegrass (48.1% C, 3.4% N) and mixed thoroughly by hand to ensure homogenous distribution of the organic amendment; the other was mixed in the same way without organic amendment and these subsamples served as controls. Assays of all properties were carried out after 6 h (day 0), 4, 7, and 28 days after the soil amendments.

2.2. Soil respiration and ATP content determinations

Soil samples were placed in 1 l air-tight conical flasks provided with three-way valves and incubated at 25 °C in the dark, with empty flasks used as blank to account for the background CO_2 concentration. Respiration of both amended and unamended soils was measured by sampling the head-space gas, using an air-tight syringe and injection into a gas-chromatograph (Hewlett-Packard 6890), equipped with a gas-sampling valve, a packed column (Poropack Q) and a thermal conductivity detector (Blackmer and Bremner, 1977). After soil respiration measurements, samples were assayed for the remaining biochemical parameters.

The ATP content of both amended and unamended soils was measured by the phosphoric acid method of Webster et al. (1984) as modified by Ciardi and Nannipieri (1990).

2.3. Soil hydrolase activities measurements

At each selected incubation time, soil hydrolase activities were measured on both amended and unamended soils. Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969), arylsuphatase activity as reported by Tabatabai and Bremner (1970) and β-glucosidase activity according to Tabatabai (1982). Urease activity was measured by the method of Nannipieri et al. (1978), and protease activity by hydrolysis of *N*-benzoylargininamide (*N*-BAA) according to Ladd and Butler (1972). All enzyme assays were carried out at 37 °C, using a 1 h incubation, followed by centrifugation of soil slurries (6000*g*) at 4 °C. The concentration of *p*-nitrophenol (*p*-NP) produced in the assays of β-glucosidase, arylsulfatase, and acid and alkaline phosphomonoesterase activities was calculated from a *p*-NP calibration curve after subtraction of the absorbance of the controls at 400 nm wavelength. The NH_4^+ produced by urease and *N*-BAA hydrolysing activities was determined by a Flow Injection Analyzer (FIAS 300-Perkin Elmer) coupled to a spectrophotometer Lambda 2 (Perkin Elmer).

2.4. Microbial physiological indices and statistics

The metabolic quotient (qCO_2) values were calculated by the CO_2–C-to-ATP ratio. The qCO_2/μ ratio, the slope of

Table 1
Main physico-chemical characteristics of the AGIR soils

Soil	pH (H_2O)	Clay (%)	Silt (%)	Sand (%)	CEC (cmol kg^{-1})	TOC (%)	N tot (%)	Total Cd (mg kg^{-1})
Maize-cropped soils								
Plot 16	7.4	17.5	15.0	67.5	14.2	0.65	0.10	0.57
Plot 11	7.5	17.5	15.0	67.5	14.5	0.55	0.03	10.9
Plot 6	7.1	17.5	15.0	67.5	15.1	0.47	0.04	17.4
Plot 1	7.1	17.5	15.0	67.5	14.8	0.49	0.04	38.2
Set aside soils								
Plot 17	7.5	17.5	15.0	67.5	14.6	0.64	0.08	1.07
Plot 12	7.0	17.5	15.0	67.5	14.7	0.43	0.02	8.33
Plot 7	7.1	17.5	15.0	67.5	14.9	0.43	0.03	17.2
Plot 2	7.1	17.5	15.0	67.5	14.5	0.49	0.04	39.1

the regression line between the metabolic quotient (qCO_2) and the growth rate (μ), is an expression of the microbial maintenance energy (Pirt, 1975). The (μ) value was calculated from the following relationship: $[\ln(ATP_2)-\ln(ATP_1)]/(\Delta T)$, where ATP_1 and ATP_2 are the ATP values at T_1 (day 0) and T_2 (7 days) and ΔT (T_2-T_1) is the time interval (in hours); thus, dimensionally, μ was expressed as μg ATP h^{-1}.

All measurements were carried out in triplicate. The standard error and the least significant differences (LSD) of the means by the Tukey–Kramer test (P level <0.05) were calculated using STATVIEW 5 computer programme (SAS Institute).

3. Results

The addition of plant residues increased the soil respiration, the ATP content and hydrolase activities in all soils. Net cumulative respiration rates, net hydrolase activity values, and net ATP content of the maize-cropped (MC) and SA soils were calculated by subtracting the values of the control soils from those of the respective ryegrass-amended soils.

3.1. Soil cumulative respiration and ATP content

About 40% of the organic C added with plant material evolved as CO_2–C during the 28 days of incubation from both the MC and SA soils, with no significant differences due to Cd content or soil management (Fig. 1).

The ATP content measured generally increased throughout after the soil amendment (Fig. 1). At the end of the incubation period, the ATP contents in all of the MC soil treatments were similar, whereas they were significantly lower in SA soils containing 10 mg Cd kg^{-1} soil (Fig. 1).

3.2. Hydrolase activities of soils

All hydrolase activities were stimulated by soil amendment with plant material. The alkaline phosphomonoesterase activity peaked after 7 days of incubation in all soils, whereas acid phosphomonoesterase activity, β-glucosidase, arylsulfatase, protease and urease activities displayed the highest value after 28 days in both the MC and SA soils (Figs. 2 and 3).

After 28 days of incubation, the activity of alkaline phosphomonoesterase was significantly lower in the MC soils containing 20 and 40 mg Cd kg^{-1} soil than the respective soil containing 0 and 10 mg Cd kg^{-1}. Its activity was also significantly lower in all Cd-contaminated SA soils compared with the uncontaminated SA soil (Fig. 2). The activity of acid phosphomonoesterase was significantly reduced in the MC soil containing 40 mg Cd kg^{-1} and in all Cd-contaminated SA soils, compared with the respective control soils (Fig. 2).

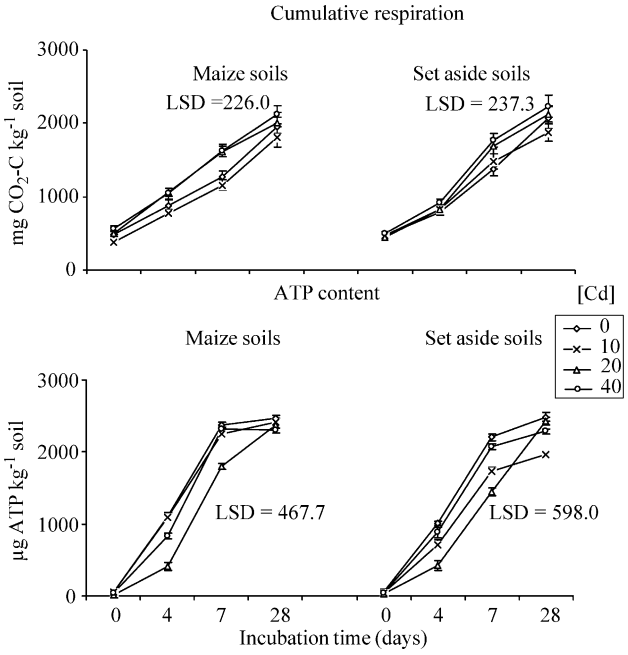

Fig. 1. Net cumulative respiration and net ATP content of maize and set aside soils amended with plant residues. The error bars represent the standard errors of the means ($n=3$). The least significant difference values (LSD) were calculated with the Tukey–Kramer test ($P=0.05$) for day 28 only.

The activity of β-glucosidase was inhibited in all Cd-contaminated MC soils and in SA soils containing 20 and 40 mg Cd kg^{-1} (Fig. 2) whereas the activity of arylsulfatase was strongly inhibited in all Cd-contaminated MC soils and in SA soils containing 20 and 40 mg Cd kg^{-1} (Fig. 2).

The activity of urease was not inhibited by Cd in both MC or SA soils whereas the activity of protease was significantly inhibited in MC soils and in SA soils containing 20 and 40 mg Cd kg^{-1} (Fig. 2).

Significantly lower hydrolase activity-to-ATP ratios after 28 days of incubation were found in MC soils for alkaline phosphomonoesterase activity (20 and 40 mg Cd kg^{-1} soil) and for arylsulfatase activity (10, 20 and 40 mg Cd kg^{-1} soil) (Table 2). In SA soils, the hydrolase activity-to-ATP ratios after 28 days of incubation were lower for alkaline phosphomonoesterase and arylsuphatase activities in the presence of 40 mg Cd kg^{-1} soil (Table 2).

3.3. Microbial physiological indices

The metabolic quotient (qCO_2), as calculated by the CO_2–C-to-ATP ratio was significantly increased in MC soils with Cd concentrations of 20 and 40 mg Cd kg^{-1} soil and in the SA soil with 40 mg Cd kg^{-1} soil (Table 3).

The qCO_2/μ ratio, the slope of the regression line between the metabolic quotient (qCO_2) and growth rate (μ) which represents the maintenance energy of microorganisms (Pirt, 1975), was higher in both MC and SA soils containing 20 and 40 mg Cd kg^{-1} (Fig. 4).

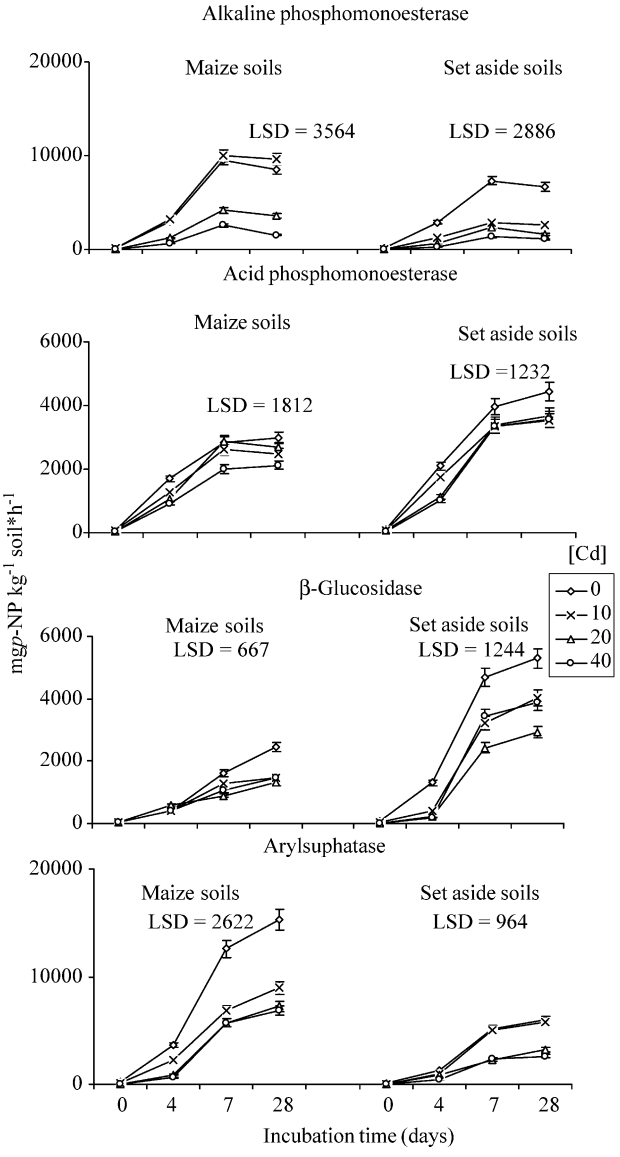

Fig. 2. Net alkaline and acid phosphomonoesterase, β-glucosidase and arylsulphatase activities in maize and set aside soils amended with plant residues. The error bars represent the standard errors of the means ($n=3$). The least significant difference values (LSD) were calculated with the Tukey–Kramer test ($P=0.05$) for day 28 only.

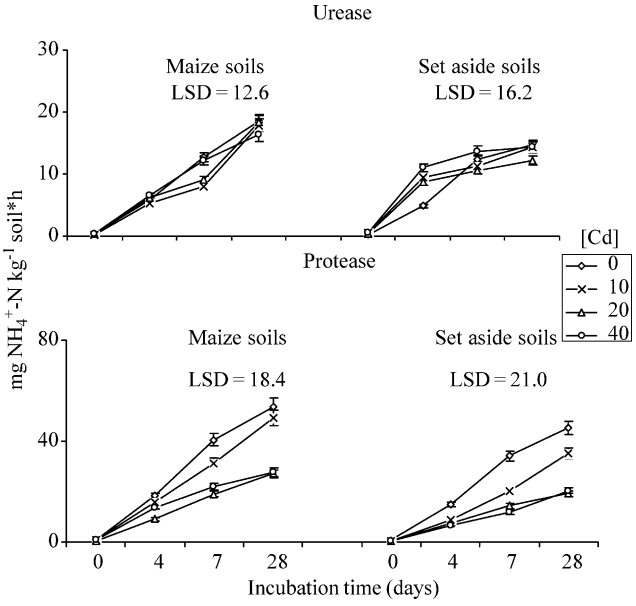

Fig. 3. Net urease and protease activities in maize and set aside soils amended with plant residues. The error bars represent the standard errors of the means ($n=3$). The least significant difference values (LSD) were calculated with the Tukey–Kramer test ($P=0.05$) for day 28 only.

4. Discussion

Previous studies have shown that when soil microflora are exposed for a long time to high metal concentrations, its qCO_2 increases, indicating a greater energy requirement for maintenance (Brookes, 1995). Our results confirm these findings, because the qCO_2/μ ratio values, expressing the maintenance energy (Pirt, 1975), showed that more CO_2–C was respired per unit of ATP formed as the amount of Cd in soil increased (Fig. 4). A lower metabolic efficiency of soil microflora in the presence of high Cd concentrations might be due to energy diversion into physiological adaptations necessary to tolerate heavy metals, such as synthesis of intra- and extracellular metal-sequestering proteins or saccharides, and biochemical reactions to precipitate or trap metals onto microbial surfaces (Hughes and Poole, 1991; Gadd, 1993). In fact, any adaptation mechanism is energy-demanding, thus enhancing the energy requirements for growth and/or reducing the conversion of substrates into new microbial biomass and its use for synthesis of enzymes, which represent a large portion of total cellular proteins. This might explain the lower synthesis of some hydrolases following the addition of readily mineralizable organic matter in Cd-contaminated soils (Table 2). Indications of greater energy requirements for biomass synthesis in heavy metal contaminated than in uncontaminated soils, have been reported previously (Killham, 1985; Chander and Brookes, 1991, 1992; Chander and Joergensen, 2001).

Another mechanism that could account for the lower enzyme activity during the microbial growth is the synthesis of different enzyme forms with different kinetic parameters. From in vitro studies, it has been showed that, in some microrganisms, genes encoding sulfur-rich isozymes are specifically repressed during exposure to Cd, whereas genes encoding sulfur-poor isozymes are specifically induced (Fauchon et al., 2002).

Variable effects of heavy metal contamination on soil enzyme activities due to the metal type, the contamination mode and the contact time have been reported previously. For example, Dar (1996) found that Cd as sole contaminant decreased the soil enzyme activities when its concentration was as high as 50 mg kg^{-1}. Moreno et al. (1999) reported that high Cd concentrations (>800 mg Cd kg^{-1}) affected dehydrogenase activity, but not β-glucosidase urease

Table 2
Hydrolase activity-to-ATP ratios in control (incubation time 0 days) and ryegrass-amended soils (incubation times 7 and 28 days) during the incubation

Cd content (mg kg^{-1})	Hydrolase activity-to-ATP ratios (U/mg ATP^{-1})[a]					
	Maize-cropped soils			Set aside soils		
	Incubation time (days)					
	0	7	28	0	7	28
Alkaline phosphomonoesterase						
0	1.22	0.42	0.37	1.08	0.26	0.20
10	1.58	0.43	0.36	0.60	0.23	0.17
20	1.57	0.38	0.21	1.10	0.34	0.11
40	2.26	0.18	0.16	1.38	0.09	0.07
LSD	0.42	0.16	0.15	0.56	0.12	0.12
Acid phosphomonoesterase						
0	0.51	0.15	0.14	0.46	0.13	0.11
10	0.64	0.14	0.12	0.48	0.23	0.18
20	0.91	0.24	0.13	0.72	0.33	0.12
40	1.58	0.13	0.14	1.57	0.16	0.13
LSD	0.46	0.09	0.04	0.67	0.14	0.07
Arylsulphatase						
0	2.24	0.57	0.59	0.54	0.16	0.16
10	1.51	0.33	0.33	0.75	0.35	0.30
20	1.95	0.43	0.29	0.88	0.30	0.12
40	2.61	0.26	0.29	1.52	0.13	0.10
LSD	0.53	0.27	0.18	0.41	0.25	0.05
β-glucosidase						
0	0.10	0.13	0.13	0.11	0.10	0.09
10	0.16	0.05	0.07	0.16	0.08	0.09
20	0.31	0.10	0.08	0.16	0.17	0.07
40	0.40	0.07	0.08	0.29	0.11	0.10
LSD	0.37	0.09	0.06	0.14	0.07	0.01
Urease						
0	0.02	0.01	0.01	0.02	0.00	0.00
10	0.03	0.00	0.01	0.02	0.01	0.01
20	0.02	0.00	0.01	0.03	0.01	0.00
40	0.07	0.01	0.01	0.11	0.01	0.01
LSD	0.012	0.003	0.001	0.021	0.001	0.001
Protease						
0	0.08	0.02	0.02	0.11	0.02	0.01
10	0.12	0.02	0.02	0.07	0.02	0.02
20	0.11	0.02	0.01	0.09	0.02	0.01
40	0.21	0.02	0.02	0.19	0.01	0.01
LSD	0.018	0.001	0.002	0.016	0.003	0.002

The LSD value is least significant difference calculated for each incubation time with the Tukey–Kramer test ($P=0.05$).

[a] The ratios were calculated by dividing the enzyme units by the ATP content of soils. One enzyme unit will release 1 μM/min of *p*-nitrophenol for alkaline and acid phosphomonoesterase, arylsuphatase and β-glucosidase activities and μM/min of NH_4^+–N for urease and protease activities at 37 °C and optimal pH.

Table 3
Metabolic quotient (qCO_2) of the soils with increasing Cd concentrations

qCO_2 (mg CO_2–C μg ATP^{-1} day^{-1})									
Maize-cropped soils					Set aside soils				
Plot	Unamended	Ryegrass-amended incubation time (days)			Plot	Unamended	Ryegrass-amended incubation time (days)		
		4	7	28			4	7	28
16	0.09	0.70	0.51	0.75	17	0.12	1.16	0.85	0.95
11	0.09	2.62	0.89	0.85	12	0.11	1.92	1.17	0.88
6	0.13	1.25	0.70	0.92	7	0.13	1.04	0.85	0.97
1	0.18	0.79	0.53	0.79	2	0.21	0.79	0.62	0.84
LSD	0.03	0.32	0.26	0.19	LSD	0.05	0.48	0.32	0.22

The LSD value is least significant difference calculated with the Tukey–Kramer test ($P=0.05$) and it is referred to each incubation time.

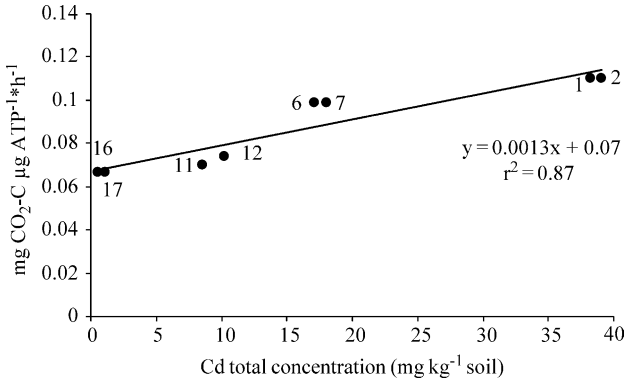

Fig. 4. Linear regression between qCO_2/μ ratio values and soil total Cd contentration. The lables of the points represent the plots number. For calculations of qCO_2 and μ values, see Section 2.

and phosphomonoesterase activities. Karaca et al. (2002) reported significant reductions of several hydrolase activities by Cd-enriched (50 mg kg^{-1}) sewage sludge, but urease activity was not inhibited. Most of the above mentioned studies investigated the effects of incorporation of multi-metal contaminated sewage sludge into soil and the possiblity that the results obtained with this approach could be due to additive toxicity of different heavy metals cannot be excluded (Renella, 2003). The approach we have adopted in this study is different because it involves the addition of plant residues to soils where Cd is the only contaminant rather than the incorporation of metal-contaminated organic residues to soils. However, plant material can bind heavy metals due to the presence of reactive functional groups, thereby reducing their availability (Tiemann et al., 1999) and this effect should be taken into account in assessing the adverse effects of Cd in the contaminated soils, where the addition of plant material might have reduced the heavy metal availability.

A change in the soil functionality might also be caused by changes in composition of the microbial community induced by heavy metals (Sandaa et al., 2001; Kozdroj and van Elsas, 2001). However, Renella et al. (2004) reported no shifts in the dominant members of the bacterial community in the same soils. Reductions of soil enzyme activities in heavy metal contaminated soils without changes in the soil microbial community structure were previously reported by Kandeler et al. (2000).

Our results suggest that negative effects on nutrient turnover in Cd-contaminated soils could be due to reductions in enzyme activity. However, such effects are difficult to predict because, although it has been frequently postulated that the regulation of nutrient cycling in soils should involve both enzyme synthesis and enzyme activity, there is a lack of data on the relationship between enzyme synthesis and organic matter mineralization in heavy metal contaminated soils.

In conclusion our results show that long term exposure of soil microflora to high Cd concentrations leads to lower hydrolase synthesis and reduced metabolic efficiency, possibly as the result of a physiological adaptation of soil microflora to high cadmium concentrations.

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