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Hydrolase activity, microbial biomass and community structure in long-term Cd-contaminated soils

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Abstract

Long-term effects of high Cd concentrations on enzyme activities, microbial biomass and respiration and bacterial community structure of soils were assessed in sandy soils where Cd was added between 1988 and 1990 as $Cd(NO_3)_2$ to reach concentrations ranging from 0 to 0.36 mmol Cd kg^{-1} dry weight soil. Soils were mantained under maize and grass cultivation, or 'set-aside' regimes, for 1 year. Solubility of Cd and its bioavailability were measured by chemical extractions or by the BIOMET bacterial biosensor system. Cadmium solubility was very low, and Cd bioavailability was barely detectable even in soils polluted with 0.36 mmol Cd kg^{-1}. Soil microbial biomass carbon (B_C) was slightly decreased and respiration was increased significantly even at the lower Cd concentration and as a consequence the metabolic quotient (qCO_2) was increased, indicating a stressful condition for soil microflora. However, Cd-contaminated soils also had a lower total organic C (TOC) content and thus the microbial biomass C-to-TOC ratio was unaffected by Cd. Alkaline phosphomonoesterase, arylsulphatase and protease activities were significantly reduced in all Cd-contaminated soils whereas acid phosphomonoesterase, β-glucosidase and urease activites were unaffected by Cd. Neither changes in physiological groups of bacteria, nor of Cd resistant bacteria could be detected in numbers of the culturable bacterial community. Denaturing gradient gel electrophoresis analysis of the bacterial community showed slight changes in maize cropped soils containing 0.18 and 0.36 mmol Cd kg^{-1} soil as compared to the control. It was concluded that high Cd concentrations induced mainly physiological adaptations rather than selection for metal-resistant culturable soil microflora, regardless of Cd concentration, and that some biochemical parameters were more sensitive to stress than others.

Keywords: Cadmium; Bioavailability; Enzyme activities; Microbial community

1. Introduction

Cadmium and other heavy metals accumulate in soils as the result of wet and dry deposition, phosphatic fertilisation, manure inputs and incorporation of sewage sludge (Jensen and Bro-Rasmussen, 1992). Jones and Johnston (1989) reported that the atmospheric input of Cd into soils could be in the order of 3 g ha^{-1} yr^{-1}. As inputs into soil generally exceed losses (Moolenaar and Beltrami, 1998), the Cd concentration in soils is likely to increase and in a few decades exceed the EU mandatory limit for agricultural soils (CEC, 1986). Cadmium is considered to be the metal having the most adverse effects on microbial biomass and activity in heavy metal contaminated soils (Smith, 1996), as it is not required for any known biological process. It is also very mobile, due to its low affinity for soil colloids (Alloway, 1995). In heavy metal-contaminated soils, the specific toxicity of Cd is difficult to assess as it is often present as a co-contaminant with other heavy metals that may add to the toxic effects on soil microbial biomass and enzyme activities (Renella et al., 2003). Short-term laboratory incubations do not reflect the toxic effects after chronic exposure of soil microflora to heavy metals (Giller et al., 1998; Renella et al., 2002).

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Heavy metal contaminated soils may be cropped or left uncropped and set-aside. The latter involves taking soils out of agricultural production so as to reduce cropped areas in accordance with EU legislation. At present in Europe, about 8.5 million ha of agricultural land is set-aside and subjected to afforestation which may lead to increase of mobility of heavy metals (Andersen et al., 2002) and impact on the soil microflora and soil functions.

Solubility and bioavailability of heavy metals can be estimated by either single-solvent or sequential extractions using different extractants and extraction procedures (McBride, 1989). Although good correlations have been found between the chemically mobile pools of heavy metals such as Cd, Ni and Zn and their content in plants, the relationship between these pools and the responses of soil microflora is still unclear. The identification of bacterial determinants conferring heavy metal resistance and knowledge of their regulation has allowed the construction of bacterial biosensors containing *lux*-reporter genes (Paton et al., 1995; Ivask et al., 2002). Bacterial biosensors, created by transcriptional fusion of the promoterless *lux* operon (*lux*CDABE) of *Vibrio fischeri* and the metal-regulated promoter-operator of the *cnr* and *czc* operons of the heavy metal-resistant bacterium *Ralstonia metallidurans* CH34, were successfully used to assess the bioavailability Ni, Co, Cd and Zn in soil (Corbisier et al., 1999). To date, the bioavailability of heavy metals, as determined with these biosensors, has never been correlated with parameters reflecting microbial activities in contaminated soils.

Chronic exposures to elevated concentrations of heavy metals can change the composition of soil microflora as detected by DGGE analysis (Kozdroj and van Elsas, 2000) and can select for metal-resistant microorganisms (Mergeay, 2000). Such heavy metal-induced changes in microbial community structure may, in turn, cause alteration of some microbial functions in soil (Brookes, 1995). Adverse effects of elevated concentrations of heavy metals to soil enzyme activities have long been recognised under both field and laboratory conditions (Tyler et al., 1989; Kandeler et al., 1996; Landi et al., 2000). However, there is a little information from long-term field trials on the effects of Cd as the sole heavy metal on the composition of and the activity of the soil microflora.

This study aimed to assess the specific effects of long-term field exposure to elevated concentrations of Cd as the sole contaminant on microbial composition and microbial activity in sandy soils under either maize or set-aside regimes. Traditional assays such as single-solvent metal extractions, determination of soil microbial biomass and respiration, plate counts, and enzyme activities were combined with molecular techniques and the use of a bacterial biosensor, to determine Cd bioavailability in soils from plots treated with different Cd concentrations.

2. Materials and methods

2.1. Field experimental design and soil sampling

Soils (sandy-clay, Arenic Udifluvent) were sampled (0–20 cm) from field plots at the long-term AGIR Experiment managed by Unité d'Agronomie, INRA, Bordeaux, France in September 2001. Cadmium contamination of the soil was achieved as described below. The density of the 0–30 cm soil layer was determined and it was calculated the as dry soil (fine fraction <2 mm) per ha and per m^2. The different plots (6 m × 3 m) were designed at the experimental site. Then, the amount of $Cd(NO_3)_2$ (Rectapur Quality, Prolabo) to be added to each plot was calculated so as to contaminate the 0–30 cm soil layer at Cd concentrations of 0, 0.09, 0.18, and 0.36 mmol kg^{-1} soil; it was spread on the soil surface and mixed by rototilling at 0–30 cm depth in the 1988–1990 period. The soil was ploughed at 0–30 cm depth through the years to ensure an even distribution of Cd within the soil layer. The chemical analyses demonstrated that the target Cd contaminations were reached. The background Cd concentration in the control soil was $\approx$0.006 mmol kg^{-1}. The soil have been kept under maize cultivation (plots 16, 11, 6 and 1) or set-aside regime (plots 17, 12, 7, 2). The soils were sampled at three points within each plot by using a stainless steel spade. Soil samples were placed in sealed plastic bags, placed in a cooled box, transported to the analytical laboratories unsieved and kept at field moisture to preserve the microbial community. In the laboratory, the soils were sieved ($<$ 2 mm), moistened to 50% of the water holding capacity and pre-incubated for 7 days at 25 °C before the analyses.

2.2. Cadmium solubility and bioavailability

Cadmium solubility was estimated by extractions with deionised water (1:2 w/v extraction ratio), or with 1 M NH_4NO_3, according to Preuss (1998). Soil slurries were filtered through Watman 42 filter paper and Cd was estimated by atomic absorption spectrophotometry (Perkin Elmer 1100B). Cadmium bioavailability was estimated by using the BIOMET biosensor based on *R. metallidurans* AE1433 (ex *Alcaligenes eutrophus*) containing the *czc* gene that codes for Cd, Zn and Co resistance, in transcriptional fusion with the *lux*CDABE operon of *V. fischeri* (Corbisier et al., 1999). When the intracellular Cd or Zn concentration exceedes a threshold, expression of the *czc* operon is induced and the *lux* gene is co-expressed with production of light, which is detectable in a luminometer. The luminometry assay

was carried out using an ANTHOS LUCY1 luminometer (Anthos Labtech b.v., Heerhugowaard, The Netherlands) at 23 °C, as previously described (Corbisier et al., 1999). For metal standards, duplicate samples (20 μl) were set up per microtitre assay. As negative controls, eight reaction samples containing deionised water were included in the test. Soils suspensions were made by adding 5 g soil to 35 ml reconstitutional medium (RM), and 20 μl of undiluted, two and four times diluted soil suspensions were added to the wells of a 96-well microtitre plate. Subsequently, 180 μl of a diluted culture (final OD660 of 0.1 in RM) of the AE1433 biosensor strain was added to each well. The bioluminescence emitted (ALU) and the optical density ($OD_{620\ nm}$) of the culture was measured over 16 h at 30 min intervals, and processed using the MIKROWIN software, as previously described (Corbisier et al., 1999). A constitutively light emitting strain (*R. metallidurans* AE864) was used as a positive control to account for both toxicity and eventual quenching of light. The induction of bioluminescence (presented as the signal to noise (*S*/*N*) ratio) is calculated as the light production value (*S*) found for the soils tested, divided by the light production value found for the uncontaminated control soil (*N*). A *S*/*N* value below 1 indicates toxicity, while induction was considered significant when the *S*/*N* ratio was greater than 1.5. A calibration curve set up for each microtitre plate allowed the calculation of the amount of bioavailable Cd.

2.3. Microbial biomass and respiration

Soil microbial biomass was estimated by the fumigation–extraction method (Vance et al., 1987). Soil (25 g dry weight) was fumigated with ethanol-free chloroform for 24 h and immediately extracted with 0.5 M K_2SO_4 by using 1 l plastic bottles; soil slurries were then filtered using Watman 42 filter paper. The unfumigated soils were extracted in the same way and the extracts were frozen prior to analysis. The C content of the extracts was determined by the dichromate oxidation method and the microbial biomass C was calculated according to Vance et al. (1987).

Soil respiration was measured by gas-chromatography, according to Blackmer and Bremner (1977). Soils (25 g oven dry equivalent) were placed in 250 ml Quickfit flasks and incubated at 25 °C in the dark. Empty flasks incubated in the same way were used as blanks for correction of background CO_2 concentration. The CO_2-C evolution was measured by sampling the headspace by using an air-tight syringe and injected into a gas-chromatoghraph (Hewlett-Packard 6890), equipped with a gas-sampling valve, a packed column (Poropack Q) and a thermal conductivity detector, and run in isothermal mode. Both microbial biomass and respiration measurements were carried out in triplicate.

2.4. Soil hydrolase activities measurements

Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969), arylsuphatase activity as reported by Tabatabai and Bremner (1972) and β-glucosidase activity according to Tabatabai (1982). Urease activity was measured by the method of Nannipieri et al. (1974) by using 0.5 g of soil and 2 ml of 0.1 M phoshate buffer rather than 5 g of soil and 10 ml of 0.1 M phoshate buffer. Protease activity was determined by hydrolysis of *N*-benzoylargininamide (BAA) according to Ladd and Butler (1972). All enzyme assays involved incubations at 37 °C for 1 h, with subsequent centrifugation of soil slurries at 6000*g* at 4 °C. The concentration of *p*-nitrophenol (*p*-NP) produced in the assays of β-glucosidase, arylsulphatase, 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 BAA-hydrolysing activities was determined by a Flow Injection Analyzer (FIAS 300-Perkin Elmer) coupled with a spectrophotometer Lambda 2 (Perkin Elmer). Because we have no information on the clay types of this soil, NH_4^+-fixing capability of soil was evaluated by measuring the recovery of NH_4^+ solutions shaken with soil for 1 h at 37 °C and then extracted with 2 M KCl. We used a NH_4^+ concentration range compatible with product concentrations of both urease and protease activities. The recovery was always greater than 98% (data not shown). Soil hydrolase activity measurements were carried out in triplicate.

2.5. Bacterial community structure

Total culturable bacterial community and bacterial resistance to Cd, Ni and Zn were monitored according to Mergeay (1995). Bacteria were extracted from soils and serially diluted and plated on rich media, and minimal media containing glucose or gluconate as C sources, with or without heavy metals (Cd, Ni or Zn). Cadmium concentrations in the plates were 0.2, 0.4, and 0.8 mmol, whereas Ni and Zn concentrations were 1 and 2 mmol, respectively. Plates were then kept at 37 °C for 7 days.

The total bacterial community structure, including both culturable and non culturable microorganisms, was monitored by denaturing gradient gel electrophoresis (DGGE) after amplification of the 16S rDNA by polymerase chain reaction (PCR). Whole-community DNA was extracted from 0.5 g of soil using a bead-beating method (FastDNA SPIN Kit for soil, Bio 101, Inc., USA) according to the manufacturer's instructions. The amount of extracted DNA was calculated by fluorometer (Hoefer™ DyNA Quant™ 200) using bisbenzimide-dye (Hoechst H 33258). The community eubacterial 16S rDNA was then amplified using the primers GC-968f: 5′-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCA-CGGGGGGAACGCGAAGAACCTTA-3′ and 1401r:

5′-GCGTGTGTACAAGACCC-3′ (Felske et al., 1997). The DNA was amplified with 2.5 U μl^{-1} PolyTaq (Polymed), 12.5 pM of each primer, 12.5 mM of each deoxynucleoside triphosphate, 75 mM $MgCl_2$, BSA (500 μg/ml) and reaction buffer 50 × (Polymed, without $MgCl_2$) in a final reaction volume of 50 μl. The PCR was performed with a Perkin Elmer 2400 thermocycler with reaction conditions of 94 °C for 90 s followed by 33 cycles of 95 °C for 20 s, 56 °C for 30 s, 72 °C for 45 s, and final annealing at 72 °C for 7 min. The PCR products were checked on 1% agarose gel and the amount of amplified DNA was calculated by comparing the relative band intensities with those of Mass Ruler™, DNA Ladder Mix (Fermentas). DGGE of the amplified 16S rDNA sequences was performed by using the Dcode System (Universal Mutation Detection System, Biorad) loading 300 ng of DNA onto a 6% polyacrylamide gel with a denaturant gradient of 46–56%. Electrophoresis conditions were: 60 °C, 75 V for 16 h followed by a 2 h coloration in SybrGreen I (FMC BioProducts, Rockland, ME, USA). Bands were detected manually from digitals images (Polaroid Gel Cam, Elect; Polaroid Type 667 Film ISO 3000) by UV light transillumination (254/497 nm). Soil DNA extractions, PCR and fingerprintings were carried out in triplicate.

2.6. Statistics

Analysis of variance by the Tukey–Kramer test was used to assess the significance of differences (P level <0.05) of the means ($n = 3$) using the STATVIEW 5 computer program (SAS Institute).

3. Results

3.1. Cadmium availability

The amounts of water- and 1 M NH_4NO_3-extractable Cd were both low compared to the total Cd concentrations, indicating a low availability of Cd. The available Cd increased gradually but not in proportion to total Cd content of the soils. Cadmium solubility was significanlty higher in soils from plots 1 and 2 both containing 0.36 mmol Cd kg^{-1} soil, compared to control soils (Fig. 1). The percentage of Cd that was water-soluble ranged between 7.9 and 0.8 in maize cropped soils (plots 16 and 1, respectively) and from 9.5 to 0.5 in set-aside soils (plots 17 and 2, respectively). The percentages of NH_4NO_3-extractable Cd in maize soils ranged between 11.5 and 1.8 (plots 16 and 1, respectively) and from 5.4 to 1.2 in set-aside soils (plots 17 and 2, respectively) (Table 1).

No bioavailable Cd was detected by the BIOMET biosensor in the control, or soils amended with 0.09 and 0.18 mmol Cd kg^{-1}, whereas a response was detected in soils from plots 1 and 2 containing 0.36 mmol Cd kg^{-1} soil, indicating that only in these soils was enough Cd bioavailable to evoke a bacterial response encoded by a specific resistance mechanism (Fig. 1).

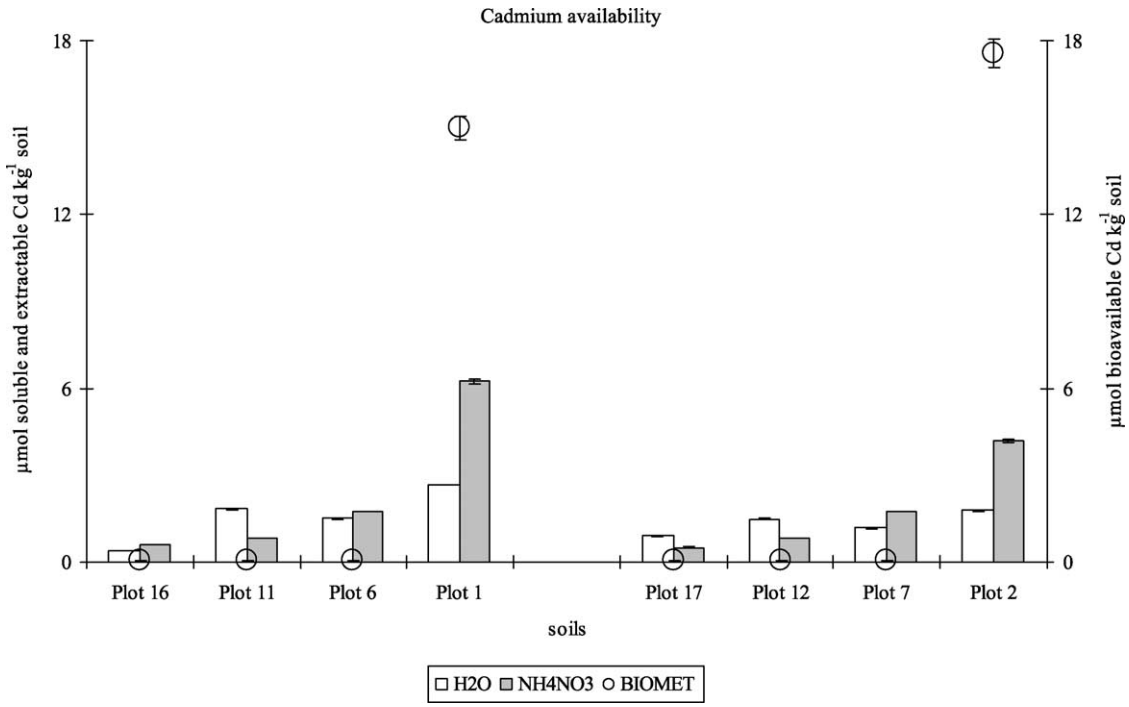

Fig. 1. Water-soluble and NH_4NO_3-extractable and bioavailable Cd as estimated by BIOMET. The error bar is the standard error of the means ($n = 3$).

Table 1
Soil properties

Soil	pH (H_2O)	Clay (%)	Silt (%)	Sand (%)	TOC (%)	N tot (%)	Total Cd (μmol kg^{-1})
Maize soils							
Plot 16	7.4	17.5	15.0	67.5	0.85	0.10	0.57
Plot 11	7.5	17.5	15.0	67.5	0.55	0.03	97.0
Plot 6	7.1	17.5	15.0	67.5	0.47*	0.04	154.8
Plot 1	7.1	17.5	15.0	67.5	0.49*	0.04	339.8
Set aside soils							
Plot 17	7.5	17.5	15.0	67.5	0.64	0.08	9.52
Plot 12	7.0	17.5	15.0	67.5	0.43	0.02	74.1
Plot 7	7.1	17.5	15.0	67.5	0.43*	0.03	153.0
Plot 2	7.1	17.5	15.0	67.5	0.49*	0.04	347.8

*Symbols indicate significant differences at $P < 0.05$ level, as compared to respective control soils (plots 16 and 17).

3.2. Soil microbial biomass, respiration and ecophysiological parameters

Soil microbial biomass (B_C) was lower in Cd-contaminated than in control soils, but the differences were not significant (Table 2). Soil respiration was higher in all Cd-contaminated soils as compared to control soils (Table 2) and differences were significant in all Cd-contaminated maize soils and in the set-aside soil containing 0.36 mmol Cd kg^{-1}. Set-aside soils always displayed lower B_C and respiration than maize cropped soils (Table 2), but differences between soils polluted to the same Cd concentration and under different management were not significant. The metabolic quotient (qCO_2), calculated by the CO_2-C/B_C ratio was significantly higher in maize soils with Cd concentrations of 0.18 and 0.36 mmol Cd kg^{-1} of soil (plots 6 and 1, respectively) and in the set-aside soil (plot 2) containing 0.36 mmol Cd kg^{-1} of soil (Table 2). The B_C-to-TOC ratio was unaffected in all soils regardless of management and Cd content (Table 2).

3.3. Soil hydrolase activities

In maize soils the alkaline phosphomonoesterase, arylsulphatase and protease activities were significantly reduced for all Cd concentrations whereas acid phosphomonoesterase, β-glucosidase and urease activites were unaffected by Cd (Figs. 2 and 3). In set-aside soils the alkaline phosphomonoesterase was significantly reduced by all Cd concentrations whereas, arylsulphatase and protease activities were significanlty lower in soils containing 0.18 and 0.36 mmol Cd kg^{-1}, respectively (Figs. 2 and 3). In set-aside soils the acid phosphomonoesterase, β-glucosidase and urease activites were unaffected by Cd (Figs. 2 and 3).

3.4. Culturable bacteria screening

No significant changes were detected in the bacteria colony forming units (CFU) grown on rich media (Table 3), whereas a decrease in CFUs of bacteria grown on minimal medium plates with glucose as sole C sources was observed for the high Cd soils containing 0.18 and 0.36 mmol Cd kg^{-1} soil (Table 3). No bacteria were able to grow on minimal glucose or gluconate plates supplemented with Zn, whereas a large number of bacteria were able to grow on minimal plates supplemented with Ni (data not shown). Few bacteria were able to grow on glucose and gluconate plates supplemented with 0.2 and 0.4 mmol Cd but the percentage of bacteria able to grow on Cd supplemented minimal medium plates increased when they were extracted from the most contaminated soils (Table 3). No bacterial growth was observed on plates containing either glucose or gluconate and 0.8 mmol Cd.

3.5. PCR-DGGE analysis of total soil DNA

The DGGE analysis was performed on the total DNA extracted directly from soil. The DGGE analysis showed that the DNA profiles of all samples were similar in terms of number and intensity of migrating bands (Fig. 4A and B). However, an additional band appeared in the maize soils containing 0.09 and 0.18 mmol Cd kg^{-1} soil, indicated by the lower arrows in Fig. 4A (lanes 7–9 and 10–12, respectively). One more band appeared in the DNA profiles of maize soils containing 0.18 and 0.36 mmol Cd kg^{-1} soil

Table 2
Microbial Biomass C (B_C), soil respiration and ecophysiological parameters (q_{CO_2} and B_C/TOC ratio) of the soils

Soil	B_C (mg C kg^{-1} soil)	Soil respiration (mg CO_2-C kg^{-1} soil × day)	q_{CO_2} (mg CO_2-C mg B_C^{-1} × day)	B_C/TOC (%)
Maize soils				
Plot 16	340.9	9.6	0.0014	4.01
Plot 11	235.8	20.4*	0.0031	4.29
Plot 6	190.3	22.8*	0.0050*	4.05
Plot 1	264.9	29.0**	0.0046*	5.41
Set aside soils				
Plot 17	314.4	7.7	0.0010	4.91
Plot 12	192.9	14.6	0.0030	4.49
Plot 7	190.1	12.0	0.0026	4.42
Plot 2	180.4	15.5*	0.0036*	5.01

* And ** symbols indicate significant differences at $P < 0.05$ and < 0.01 levels, respectively, as compared to respective control soils.

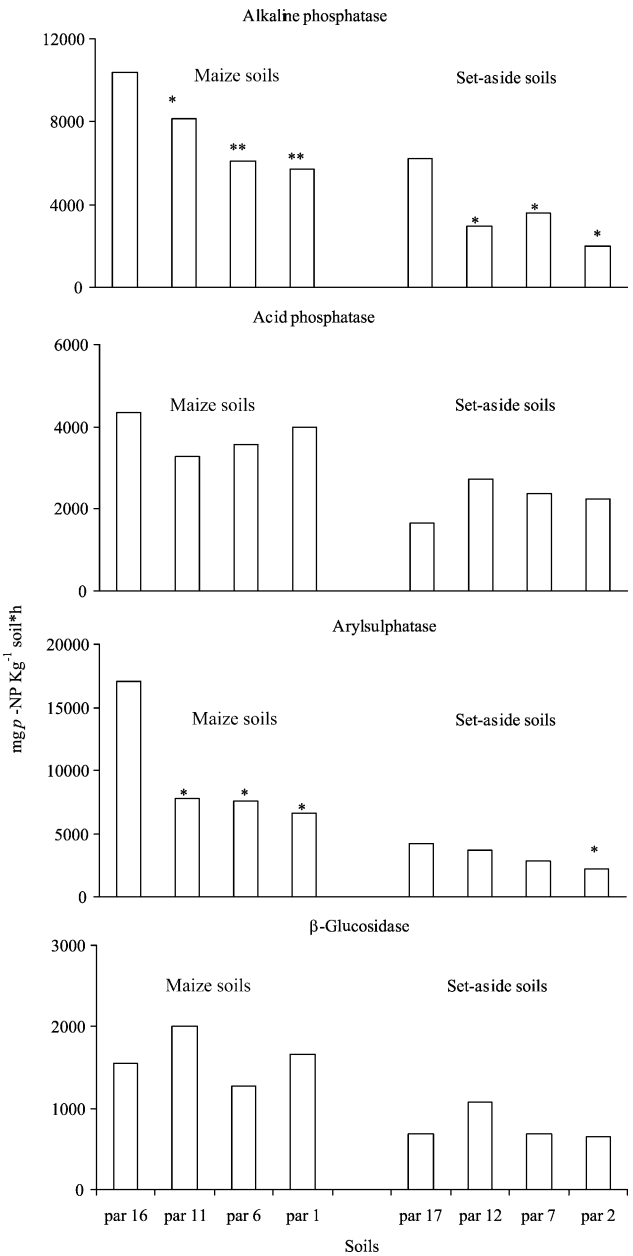

Fig. 2. Alkaline and acid phosphatase, arylsulphatase and glucosidase activities in soils with increasing Cd concentrations. * and ** symbols indicate significant differences at $P < 0.05$ and <0.01 levels, respectively compared to respective control soils.

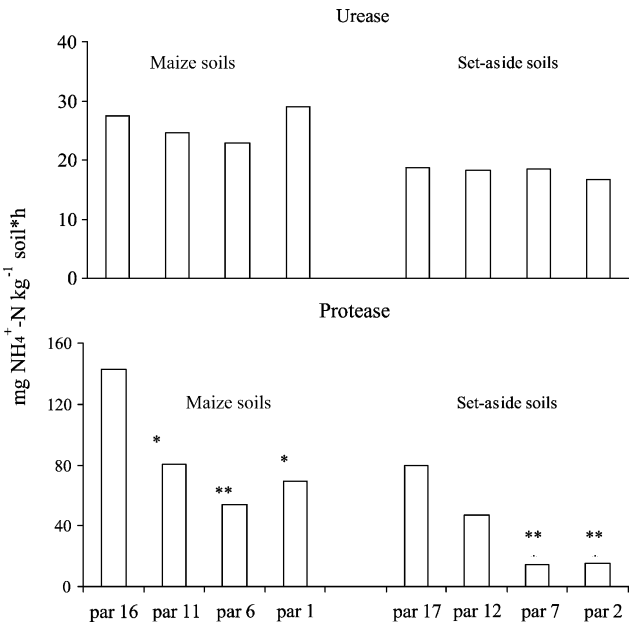

Fig. 3. Protease and urease activities in soils with increasing Cd concentrations. * and ** symbols indicate significant differences at $P < 0.05$ and <0.01 levels, respectively.

indicated by the arrow in Fig. 4A (lanes 10–12). The DNA profiles of the Cd-contaminated set aside soils did not show the corresponding new band (Fig. 4B).

4. Discussion

The amounts of water-soluble and NH_4NO_3-extractable Cd were both very low when compared to total Cd concentrations indicating a low availability of Cd. Nolan et al. (2003) reported that in agricultural heavy metal-contaminated Australian soils, soluble Cd ranged from 22 to 86%, of total soil Cd, whereas Smolders et al. (1999) reported that isotopically exchangeable Cd in contaminated Belgian soils ranged from 62 to 90%. In contrast, Pandeya et al. (1998) noted that the water-soluble and exchangeable fraction of Cd in different soils accounted for 4.2–7.2% of total Cd and that these fractions were not correlated with the isotopic distribution coefficient (K_d). Ahnstrom and Parker (1999) reported that Cd in the soluble-exchangeable fraction from a specific sequential extraction procedure accounted for less than 10% of total Cd. Such differences should be ascribed to differences in soil properties and to the different techniques used for extraction of Cd (Harter and Naidu, 2001). Heavy metal mobility in soils depends on several factors including adsorption onto soil colloids, precipitation and interaction with organic ligands (McBride, 1989). The Cd concentration in soil solution is generally reduced at neutral or alkaline pH (Adriano, 1986). Moreover, organic matter of no-tilled soils is richer in water-soluble organic compounds than ploughed soils (Arshad et al., 1990). Complexation by low molecular weight organic acids can increase Cd solubility (Krishnamurti et al., 1997; Collins et al., 2003). This might, at least in part explain the higher Cd solubility in set aside than maize cropped soils (Fig. 1). McGrath et al. (1999) reported that heavy metal toxicity, as determined by biosensors, is correlated with the free ion metal concentration. The weak responses of the Cd/Zn BIOMET biosensor confirmed that bioavailability of Cd in these soils was low (Fig. 1).

The media used for the screening of culturable bacteria provide indications on the shifts in dominance of *Pseudomonas*-like bacteria, which are able to grow using glucose as sole C source and *Ralstonia*-like bacteria which are able to grow using gluconate. Indeed, *Pseudomonas*-like bacteria

Table 3
Total culturable bacteria, glucose and gluconate metabolising bacteria and percentage of populations resistant to Cd. The percentage of growth refers to the respective media

Soils	Total culturable bacteria	CFU		CFU (%)[a]			
		Glucose metabolising bacteria	Gluconate metabolising bacteria	Selective culture media			
				Glucose (mmol Cd)		Gluconate (mmol Cd)	
				0.2	0.4	0.2	0.4
Maize soils							
Plot 16	3.4×10^5	1.7×10^5	1.7×10^5	10.0	0.12	14.9	0.40
Plot 11	3.0×10^5	2.3×10^5	1.8×10^5	7.1	0.13	13.0	0.49
Plot 6	4.5×10^5	4.1×10^4	3.0×10^5	41.5*	0.49	102.2**	4.62
Plot 1	4.7×10^5	3.9×10^4	4.9×10^5	53.3*	0.51	99.2*	7.89*
Set aside soils							
Plot 17	1.1×10^5	1.4×10^5	0.5×10^5	11.1	0.42	6.0	3.44
Plot 12	5.4×10^5	4.9×10^5	2.5×10^5	2.8	0.08	10.2	0.63
Plot 7	1.9×10^5	7.9×10^4	3.9×10^5	22.8	0.76	73.6*	6.09
Plot 2	4.4×10^5	2.4×10^4	2.9×10^5	75.0*	3.72*	90.8*	11.1*

[a] The percentage of CFU was calculated from the formula $(X/Y)100$, where X was the CFU from Cd-amended plates (figures not shown) and Y the CFU grown on glucose and gluconate with no Cd added (second and third column of this table, respectively).

are more sensitive to heavy metals than *Ralstonia*-like bacteria and tend to disappear in the heavy metal contaminated soils (Mergeay, 1995). The screening of the culturable bacterial community indicated that in these soils Cd did not affect the ratio between the *Pseudomonas*-like and *Ralstonia*-like subpopulations of the culturable bacterial community. None of the bacteria extracted from any of the soils was able to grow on either 0.8 mmol Cd or 2 mmol Zn thus no genetically determined resistance mechanisms (e.g. plasmid-mediated *Ralstonia*-like resistance) could be identified from the screening of the culturable bacterial populations (Table 3). This result could be ascribed to the low levels of soluble and bioavailable Cd in all the investigated soils (Fig. 1). Similar results were reported by Angle et al. (1993). Indeed, selection of genetically determined metal-resistant bacterial populations either due to plasmids encoding for membrane antiport efflux systems such as the *czc*, or the P-type heavy metal efflux ATP-ases has been only detected in heavily contaminated soils or soils in mining areas (Mergeay, 2000). Bacterial selection might also result from the synergistic effects of Cd with other metals such as Zn, Ni, Pb and Cu, and/or other soil factors (e.g. acidification, lower nutrient inputs in the rhizosphere). However, in the most contaminated soils the proportion of bacteria growing on selective media containing 0.2 and 0.4 mmol of Cd significantly increased compared to the control soils (Table 3) and this result was correlated with the water-soluble and NH_4NO_3-exchangeable Cd (Table 4). It is possible that the large inputs of Cd in these soils mainly induced physiological adaptations rather than bacterial selection. Microbial physiological adaptations to heavy metals rely on several mechanisms, such as precipitation of metals as phosphates, carbonates, and sulphides, physical exclusion by exopolymers, and intracellular sequestration

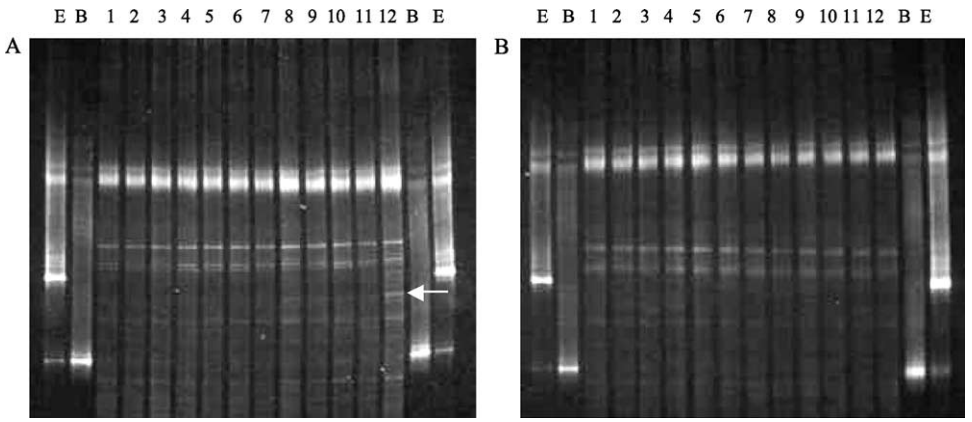

Fig. 4. DGGE profiles of 16S rDNA amplicons from whole community DNA extracted from maize (A) and set aside soils (B). Legend: (A) Lanes 1–3: Plot 16, lanes 4–6: Plot 11, lanes 7–9: Plot 6, lanes 10–12: Plot 1. (B) Lanes 1–3: Plot 17, lanes 4–6: Plot 12, lanes 7–9: Plot 7, lanes 10–12: Plot 2. Lines B = B. subtilis-marker, lanes E = *E. Coli*- marker.

Table 4
Correlation matrix between water-and NH_4NO_3-extractable Cd and CFU on Cd-amended plates

Extractant	Culture media (mmol Cd)							
	0.2				0.4			
	Glucose		Gluconate		Glucose		Gluconate	
	r^2	P	r^2	P	r^2	P	r^2	P
H_2O	0.48	0.24	0.75	0.03	-0.06	0.89	0.72	0.04
NH_4NO_3	0.86	0.004	0.99	<0.0001	0.67	0.88	0.94	<0.0001

with low molecular weight cysteine-rich proteins (Gadd, 1993). Such cellular mechanisms are energy-demanding, increase the maintenance energy and reduce the conversion of substrate into new microbial biomass and other metabolic processes (Pirt, 1975). This might explain the reduction of microbial biomass and the increase of the qCO_2 values in the high Cd soils (Table 2), thus confirming earlier reports (Brookes, 1995). Chander and Brookes (1991b) reported that lower nutrient inputs in the rhizosphere could induce a decrease of soil microbial biomass in heavy metal contaminated soils. Soils with high Cd contents and soils from set-aside plots had lower TOC contents but the B_C-to-TOC ratio of soils was not significantly different across all plots (Table 2). The B_C-to-TOC ratio has been considered a sensitive ecophysiological parameter indicating an environmental pressure suppressing the soil microbial biomass or diversity of soil microflora (Anderson and Domsch, 1989).

The above mentioned hypothesis might also explain the reduction of enzyme activities observed in the Cd-contaminated soils. Reductions of soil enzyme activities without changes in the soil microbial community structure were also reported by Kandeler et al. (2000). However, changes in the strucuture of the soil microflora and decreases in the microbial diversity determined by nucleic acid or fatty acid methyl ester (FAMEs) profiles induced by contamination of soil with heavy metals have been reported (Pennanen et al., 1996; Kozdroj and van Elsas, 2000; Sandaa et al., 2001). The PCR-DGGE technique has become a widespread molecular technique to monitor changes in soil microflora due to pollution and changes in agricultural practices (Nannipieri et al., 2003). The DGGE analysis of the AGIR soils revealed a higher diversity in the maize than in set-aside soils and the presence of an additional band was noted in maize soils containing 0.18 and 0.36 mmol Cd kg^{-1} soil. Identification of bacterial populations related to the new bands would require sequencing. Moreover, although no such changes were detected in set aside soils, changes in more sensitive subpopulations can not be excluded.

The observed effects on soil microbial biomass, predominant bacterial species and hydrolase activities in soils, with Cd concentrations much lower than those used in our investigation, might be due to the additive toxicity of other heavy metals and/or organic pollutants, as polluted soils are usually contaminated by more than a single heavy metal or other pollutant. Chander and Brookes (1993) reported that amendement of soil with single-metal (Cu, Ni, and Zn) sludge had only minor effects on soil microbial biomass, but in plots receiving bimetallic sludge (Ni + Zn or Cu + Zn) the microbial biomass was reduced to 50% of that in control soils. In addition, Chaudri et al. (1993) reported that in soils polluted with different metals, high Zn and not Cd concentrations was responsible for the decline in rhizobia number. Moreover, elevated concentration of Cu and Zn can potentiate toxicity of Cd (Renella et al., 2003). Future research should address mechanisms responsible for the additive toxic effects of different heavy metals on the composition and the activity of microflora of heavy metal contaminated soils.

In conclusion, this study showed that low amounts of bioavailable Cd are present even in high Cd-contaminated soils and that such low concentrations of bioavailable Cd do not affect the structure of the most representative eubacterial species of the total soil microbial community. Effects were observed on microbial functions such as respiration and enzyme activities. This may have been due to physiological adaptation of soil microflora rather than permanent changes in the composition of the bacterial community.

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