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Structure and function of the soil microbial community in microhabitats of a heavy metal polluted soil

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Abstract Particle-size fractionation of a heavy metal polluted soil was performed to study the influence of environmental pollution on microbial community structure, microbial biomass, microbial residues and enzyme activities in microhabitats of a Calcaric Phaeocem. In 1987, the soil was experimentally contaminated with four heavy metal loads: (1) uncontaminated controls; (2) light (300 ppm Zn, 100 ppm Cu, 50 ppm Ni, 50 ppm V and 3 ppm Cd); (3) medium; and (4) heavy pollution (two- and threefold the light load, respectively). After 10 years of exposure, the highest concentrations of microbial ninhydrin-reactive nitrogen were found in the clay (2–0.1 μm) and silt fractions (63–2 μm), and the lowest were found in the coarse sand fraction (2,000–250 μm). The phospholipid fatty acid analyses (PLFA) and denaturing gradient gel electrophoresis (DGGE) separation of 16S rRNA gene fragments revealed that the microbial biomass within the clay fraction was predominantly due to soil bacteria. In contrast, a high percentage of fungal-derived PLFA 18:2ω6 was found in the coarse sand fraction. Bacterial residues such as muramic acid accumulated in the finer fractions in relation to fungal residues. The fractions also differed with respect to substrate utilization: Urease was located mainly in the <2 μm fraction, alkaline phosphatase and arylsulfatase in the 2–63 μm fraction, and xylanase activity was equally distributed in all fractions. Heavy metal pollution significantly decreased the concentration of ninhydrin-reactive nitrogen of soil microorganisms in the silt and clay fraction and thus in the bulk soil. Soil enzyme activity was reduced significantly in all fractions subjected to heavy metal pollution in the order arylsulfatase >phosphatase >urease >xylanase. Heavy metal pollution did not markedly change the similarity pattern of the DGGE profiles and amino sugar concentrations. Therefore, microbial biomass and enzyme activities seem to be more sensitive than 16S rRNA gene fragments and microbial amino-sugar-N to heavy metal treatment.

Keywords Soil microbial biomass · Soil enzymes · Particle-size fractions · Heavy metals · Phospholipid fatty acids

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Introduction

Heavy metals severely affect the growth, morphology and metabolism of microorganisms in bulk soils, through functional disturbance, protein denaturation or the destruction of the integrity of cell membranes (Leita et al. 1995). Several attempts have been made to describe the microenvironment of soil microorganisms and soil enzymes (Burns 1986; Foster 1988; Boyd and Mortland 1990; Robert and Chenu 1992; Nannipieri et al. 1996; Ruggiero et al. 1996; Stotzky 1986); however, limited information is available about heavy metal-in-

duced changes in the microhabitats of soil microorganisms (Ranjard et al. 2000).

Particle-size fractionation has been widely applied to distinguish pools of different soil organic matter quality and turnover time (e.g. Christensen 1996); however, no information is yet available concerning the soil microbial community structure of particle-size fractions. The few studies that have been carried out on soil microbial properties in particle-size fractions suggest that soil microorganisms are mainly associated with the silt and clay fractions (Ahmed and Oades 1984; Christensen and Bech-Anderson 1989; Jocteur Monrozier et al. 1991; Singh and Singh 1995; Ladd et al. 1996). In contrast, the enzyme activity of soil fractions depends largely on the enzyme investigated and the fractionation procedure used (Lensi et al. 1995; Ladd et al. 1996). Recently, Stemmer et al. (1998) proposed a particle-size fractionation procedure that allowed the complete recovery of organic carbon (C_{org}), total nitrogen (N_t), microbial biomass and several enzyme activities (Stemmer et al. 1998, 1999; Kandeler et al. 1999a,b). The procedure should thus be suitable for identifying microbial community structure in the different size fractions of soils.

The aim of the study was to test whether soil microorganisms occupy different microhabitats obtained by the particle-size fractionation procedure. We assessed microbial community structure and function within different size fractions using a range of approaches. The measurement of different soil enzyme activities in particle-size fractions should clarify whether the single enzymes were mainly associated with the clay fraction, which provides most of the surface available for interaction with microorganisms, or with larger plant debris not associated with the mineral fractions. The estimation of phospholipid fatty acid (PLFA) pattern should give information about the contribution of bacterial and fungal PLFAs within the soil microbial community (Frostegård et al. 1993a,b). A molecular approach using ribosomal DNA sequences should provide an analysis of bacterial community complexity in soils (Muyzer et al. 1993; Bruce et al. 2000). Different amino sugars were used to trace residues of different microbial origin in soils (Parsons 1981; Chantigny et al. 1997). The second aim of the study was to show whether the environmental pollution of the soil changed the structure and the functions of microbial communities in their microhabitats.

Materials and methods

Soil used

A Calcaric Phaeozem with the following chemical properties was selected for the experiment: 14.7% clay, 24.4% silt, 60.9% sand, 30% carbonate, 1.9% C_{org}, 0.13% N_t, pH 7.5. In spring 1987, Zn, Cu, Cd and Ni sulfate solutions and V oxide were added to the sieved soil (5 mm). The amounts of contamination corresponded to the following treatments: (1) control (no addition of heavy metals); (2) light pollution: 300 ppm Zn, 100 ppm Cu, 50 ppm Ni, 50 ppm V and 3 ppm Cd; (3) medium pollution: two-fold the light concentration; (4) heavy pollution: three-fold concentration. Contaminated soils and controls were placed in large containers (50 dm^3 pots) under field conditions. The crop rotation included spring barley/rape, spring wheat/rape, bush bean and lettuce. A detailed description of the experiment, of the plant uptake and the mobile fractions of heavy metals in the bulk soil can be obtained from Horak and Kamel (1990), Lummerstorfer (1993) and Kandeler et al. (1996). Using the physical fractionation procedure of Stemmer et al. (1998), the amounts of Cu and Ni (related to fraction dry weight) increased with diminishing particle size, V was equally distributed within the fractions, whereas Cd and Zn showed a bimodal distribution within the fractions (data not shown).

The date of soil sampling was chosen after a 1-year period of fallow to minimize the direct influence of plants. After 10 years of exposure, soil samples (0–20 cm) were taken in November 1997 from pots of each of the treatments which were replicated three times. From each pot, 12 subsamples were taken with a single-gouge auger (cores of 1.5 cm diameter), mixed and stored in plastic bags at –20 °C. After the storage period (up to 3 months), the samples were allowed to thaw at 4 °C for about 3 days. They were then sieved (< 2 mm), stored again in plastic bags at 4 °C and analysed within 2 weeks. Analyses of microbial biomass and enzyme activities were performed twice on each of the three replicate samples taken from the field. However, due to the high complexity of several techniques used (PLFAs, DGGE separation of 16S rRNA gene fragments, amino sugars), we had to select only those two treatments (control and medium pollution) that could be performed in duplicate for these techniques.

Physical fractionation of the soils

The procedure involved the dispersion of soil samples by a low-energy sonication and separation of particle-size fractions by a combination of wet sieving and centrifugation as described by Stemmer et al. (1998). Briefly, 35 g of field-moist soil was dispersed in 100 ml of cooled distilled water with a probe-type ultrasonic disaggregator (50 Js^{-1} for 120 s). Coarse and medium sand (2,000–250 μm) and fine sand (250–63 μm) were separated by manual wet sieving with about 400 ml of cooled distilled water. Silt-sized particles (63–2 μm) were separated from the clay fraction (< 2 μm) by centrifugation at approximately 150 *g* for 2.0 min at 15 °C. The pellets were resuspended in water and centrifuged three times under the above conditions to purify the silt fraction. The combined supernatants were centrifuged at 3,900 *g* for 30 min at 15 °C to yield clay-sized particles (2–0.1 μm, according to an equispherical diameter and a particle density of 2.65 g cm^{-3}). Results of the particle-size distribution using this method are given in Table 1. Compared with the particle-size method using sodium-pyrophosphate as a dispersion agent (see Kandeler et al. 1996), the particle-size distribution obtained after the fraction procedure yielded similar amounts of the sand fractions, but a higher amount of the 63–2 μm particles. The applied sonication energy thus did not disrupt microaggregates completely. Therefore, the fractionation procedure using the application of low energy sonication (0.2 kJ g^{-1} soil) prevented the release of the stable organic matter that is physically protected within microaggregates in natural sites (Stemmer et al. 1999).

Soil microbial biomass and enzyme activities

Ninhydrin-reactive N was measured according to a modified method of Amato and Ladd (1988). Briefly, 0.3–0.5 g of the moist fractions were fumigated with 0.1 ml of chloroform for 24 h at 25 °C. Subsequently, the chloroform in the samples was removed. Samples and unfumigated controls were extracted with 5.0 ml of 2 M KCl solution for 60 min on a shaker. After filtration, 2 ml of the filtrates were mixed with 0.5 ml of 0.4 M sodium citrate solu-

Table 1 Particle-size distribution of the soil used. Standard errors *in parentheses* ($n=3$)

Treatment	Particle-size distribution (%)				Recovery (%)
	2,000–250 μm	250–63 μm	63–2 μm	2–0.1 μm	
Control	30.7 (0.4)	25.4 (0.3)	32.2 (0.4)	10.4 (0.2)	98.7
Light pollution	29.8 (0.6)	25.3 (0.7)	32.0 (0.6)	10.8 (0.3)	97.9
Medium pollution	30.2 (0.4)	25.8 (0.7)	32.1 (0.6)	10.5 (0.2)	98.6
Heavy pollution	28.9 (0.3)	25.2 (0.4)	33.3 (0.1)	11.1 (0.1)	98.5

tion. Ninhydrin-reactive N was determined by a colorimetric procedure (Schinner et al. 1996).

For the determination of urease activity, 0.3–0.4 g of the moist fractions were incubated with 1.5 ml of a 79.9 mM urea solution for 2 h at 37 °C. Released ammonium was extracted with 13.5 ml of 2 M KCl solution and determined colorimetrically by a modified Berthelot reaction (Kandeler and Gerber 1988).

For the determination of xylanase activity, 0.5–1.0 g of the moist fraction was incubated with 5.0 ml of a substrate solution (1.7% w v^{-1} xylan from oat spelts suspended in 2 M acetate buffer, pH 5.5) and 5.0 ml of 2 M acetate buffer (pH 5.5) for 24 h at 50 °C. Before incubation, only the clay fractions were mixed for at least 1 min with 0.7 g of quartz to improve the dispersion of the suspension. Reducing sugars released during the incubation period reduced potassium hexacyanoferrate (III) in an alkaline solution. Potassium hexacyanoferrate (II) was measured colorimetrically according to the Prussian blue reaction (Schinner et al. 1996).

Alkaline phosphomonoesterase (alkaline phosphatase) activity was assayed using a modified disodium phenylphosphate method: 0.3–0.4 g of soil or soil fraction was incubated in 2.0 ml of 0.2 M borate buffer (pH 10.0) and 1.0 ml buffered phenylphosphate solution at 37 °C for 3 h; released phenol were estimated by a colour reaction (Hoffmann 1968).

For the determination of arylsulfatase, 0.3–0.4 g of soil or soil fraction were incubated with 1 ml of *p*-nitrophenylsulfate and 4 ml of acetate buffer (0.05 M, pH 5.8) for 1 h at 37 °C (Tabatabai and Bremner 1970). Released nitrophenol was determined photometrically at 420 nm. C_{org} and N_t were measured by dry combustion in a Leco 2000 CNS analyser.

Soil microbial community structure: PLFA analysis

Lipids were extracted from soil, fractionated and quantified using the procedure described by Bardgett et al. (1996) which is based on that of Bligh and Dyer (1959) as modified by White et al. (1979). Separated fatty acid methyl-esters were identified by chromatographic retention time and mass spectral comparison using standard qualitative bacterial acid methyl ester mix (Supelco) that ranged from C11 to C20. For each sample, the abundance of individual fatty acid methyl-esters was expressed on a dry weight basis per unit dry weight. Fatty acid nomenclature was used as described by Frostegård et al. (1993a,b). The fatty acids i15:0, a15:0, 15:0, i16:0, 17:0, i17:0, cy17:0, 18:1ω7 and cy19:0 were chosen to represent bacterial PLFAs (bactPLFAs) (Federle 1986; Tunlid et al. 1989; Frostegård et al. 1993a) and 18:2ω6 was used as an indicator of fungal biomass (Federle 1986). The ratio of 18:2ω6:bactPLFAs was taken to represent the ratio of fungal:bacterial biomass in the soil (Bardgett et al. 1996).

DNA isolation

The DNA was isolated following the method of J. Newton and K. Hodder (personal communication). Soil (200 mg) was treated with 800 μl of sodium phosphate buffer (200 mM, pH 8.0) and 100 μl GES solution (guanidium thiocyanate (5 M), EDTA (0.1 M) and sarkosyl (30% v/v)) (Pitcher et al. 1989); 100 mg glass beads (0.17–0.18 mm diameter, Braun Biotech, Melsungen, Germany) were added and the samples were homogenized using a bead-beater (Braun Biotech) for 30 s. Samples were heated to 70 °C for 20 min and immediately placed on ice for a further 20 min. Soil, beads and other debris were pelleted by centrifugation at 13,000 *g* for 5 min. The supernatant was transferred to a fresh microfuge tube and NaCl (to a final concentration of 0.5 M) and polyethylene glycol (to a final concentration of 15%) were added. Samples were left to precipitate for 1 h at 4 °C. DNA was pelleted by centrifugation at 13,000 *g* for 5 min, with the pellet resuspended in 300 μl sterile distilled water. Aquaphenol (0.3 ml, buffered-saturated phenol; Appligene Oncor, Watford, UK) was added to each sample before the tubes were shaken by vortex. Following centrifugation (13,000 *g* for 5 min), supernatants were transferred to fresh microfuge tubes. An aquaphenol (pH 8.0): chloroform:isoamyl alcohol (25:24:1) mixture (0.3 ml) was added and shaken by vortex. Following centrifugation at 13,000 *g* for 5 min, the supernatants were precipitated, using an equal volume of isopropanol and 1:10 volume of 10 M ammonium acetate, for 1 h at –20 °C. The pellets obtained after centrifugation at 13,000 *g* for 5 min were washed twice in 70% ethanol. After drying the pellets, DNA was resuspended in 100 μl sterile distilled water.

PCR amplification

Following the procedure described by Muyzer et al. (1993), 16S rRNA gene fragments were amplified from the extracted DNA. The oligonucleotide primers used were Primer 2 5′ ATT ACC GCG GCT GCT GG 3′ and Primer 3 5′ CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG 3′ and were used at a concentration of 20 pmol per 100 μl PCR reaction. In addition to both primers, each PCR reaction contained 200 μmol of each deoxyribonucleoside triphosphate, 10 μl of 10*x* reaction buffer, 2.5 units of *Taq* DNA polymerase (Advanced Biotechnology, Epsom, UK), and sterile distilled water. Cycling conditions used to amplify the 16S rRNA gene fragment were 95 °C for 4 min, followed by 28 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 2 min. A final extension period of 72 °C for 10 min was used. PCR products were checked prior to DGGE by electrophoresis in 1.4% (wt/vol) agarose gels stained with ethidium bromide.

The DGGE separation of 16S rRNA gene fragments

The 16S rRNA gene fragments were separated using the D-Gene system (Biorad, Hemel Hempstead, UK) on 8% (w/v) polyacrylamide gels (bisacrylamide acrylamide gel stock solution; Severn Biotech, Kidderminster, UK) containing a linear gradient from 15% to 55% denaturant solution [where 100% denaturant solution is 7 M urea and 40% (v/v) formamide]. Following polymerization with TEMED (tetramethylethylendiamine) and ammonium persulfate and sample loading, the electrophoresis conditions used were 2 h 50 min at 180 V and 60 °C in TAE buffer [40 mM Tris, 20 mM sodium acetate and 1 mM EDTA (pH 7.4)]. Gels were stained for 45 min in SYBR green dye (10 μl in 200 ml TAE buffer) following the manufacturer's instructions (Flowgen, Lichfield, UK) and scanned using a Storm 860 set at 100 μ and 800 V

scan (Molecular Dynamics, Chesham, UK). Images were generated using ImageQuant software (version 4.1).

Amino sugars

Different amino sugars were estimated to trace residues of different microbial origin. Briefly, after hydrolysis with 6 M HCl (8 h, 105 °C) and subsequent purification, amino sugars were converted into aldononitrile acetate derivatives, separated by capillary gas chromatography, and detected by a flame ionization detector (Zhang and Amelung 1996). As considerable amounts of dissolved lime in the samples interfered with the formation of derivatives, samples were pretreated with cold 0.5 M trifluoroacetic acid for lime dissolution. Thereafter the supernatant was removed by centrifugation, and the samples were washed twice with distilled water prior to hydrolysis with hot HCl. Preliminary experiments (W. Amelung, unpublished results) have shown that this pretreatment does not remove amino sugars from soil on a significant scale.

Statistical analyses

All soil microbial measurements were calculated on the basis of the oven-dry weight (105 °C) of soil. Statistical procedures were carried out with the software package SPSS for Windows. The effect of heavy metal contamination on soil microbial biomass and enzymatic activity (biomass N, urease, xylanase, phosphatase, arylsulfatase) was tested by simple-factorial analysis of variance using the Student-Newman-Keuls test. Differences of biomass and enzyme activities in the particle-size fractions were examined by the same method. Untransformed data sets were used for all statistical analyses. Significance was accepted at the $P<0.05$ level of probability. For the 16S rRNA gene fragments, gels were analysed using Phoretix 1D Advanced software, version 4.0 (Phoretix, Newcastle, UK). Dendrograms (unweighted pair-group method using arithmetic average – UPGMA) format were generated based on the similarities of individual lanes matched to a reference lane containing a composite of each band observed.

Results and discussion

C_{org}, N_t and soil microbial biomass of particle-size fractions

We found that C_{org} and N_t (related to fraction weight) increased with decreasing particle size of the soil. The mean recovery of C_{org} and N_t after the fractionation procedure was 93.4 and 99.5%, respectively. Heavy metal pollution decreased the content of C_{org} and N_t in the coarse sand fraction (Table 2). Since plant growth was reduced due to heavy metal pollution (Lummerstorfer 1993), a low input of plant and root litter is likely to have caused the decrease in C_{org} of the particle-size fractions of polluted soils.

Ninhydrin-reactive N was recovered completely after the fractionation procedure. The summed amount of ninhydrin-reactive N of several soil samples was considerably greater than that of the bulk soil: 1.23-fold (heavy polluted soil) to 1.93-fold (medium polluted soils) of the amount of the unfractionated bulk soil were measured (data not shown). It is not completely clear from these data whether chloroform fumigation of soil fractions destroyed more soil microorganisms than the fumigation of the bulk soil; Badalucco et al. (1997) revealed that the efficiency of chloroform fumigation in lysing microbial cellsis was stongly influenced by soil structural properties. Ultrastructural studies of Foster (1988) showed that microorganisms can survive chloroform fumigation in mucigel deposits or deep in the interior of micropores. Therefore, in our experiment, microorganisms of particle-size fractions were probably more accessible to chloroform fumigation than cells from the bulk soil.

The highest concentration of ninhydrin-reactive N was found in the silt (63–2 μm) and clay (2–0.1 μm) fractions, whereas the lowest amount was in the fine sand particles (250–63 μm) (Fig. 1). This result is in accordance with several studies of microbial biomass in particle-size fractions (Christensen and Bech-Anderson 1989; Jocteur Monrozier et al. 1991; Singh and Singh 1995; Ladd et al. 1996; Kandeler et al. 1999a,b) and can be explained by the differences of organic matter content and quality of the size fractions (Van Gestel et al. 1996). The three levels of soil contamination showed a trend for lower values of ninhydrin-reactive N of the bulk soil in comparison with the uncontaminated control (Fig. 1). The decrease in ninhydrin-reactive N was mainly due to a significantly lower biomass of soil microorganisms in the silt and clay fractions of the polluted soils (Table 3). Therefore, the adsorption of soil microorganisms onto organo-mineral microaggregates

Table 2 The response of organic carbon (C_{org}) and total nitrogen (N_t) in bulk soil and particle-size fractions to different heavy metal pollution. Standard errors *in parentheses* ($n=3$). Mean recovery of C_{org} and N_t of particle-size fractions were 93.4 and 99.5%, respectively

Soil property	Treatment	Particle size (μm)				
		Bulk soil	2,000–250 μm	250–63 μm	63–2 μm	2–0.1 μm
C_{org} (mg g^{-1})	Control	20.1 (0.9)	9.9 (0.6)	9.9 (0.6)	25.0 (0.5)	61.1 (0.5)
	Light pollution	19.7 (0.2)	4.4 (0.7)	7.7 (1.4)	23.0 (0.6)	63.3 (0.9)
	Medium pollution	17.9 (0.6)	6.3 (1.1)	5.9 (3.8)	21.9 (1.4)	60.1 (0.3)
	High pollution	19.9 (0.4)	8.4 (0.8)	7.1 (5.1)	19.9 (2.0)	60.3 (0.2)
N_t (mg g^{-1})	Control	1.52 (0.01)	0.28 (0.06)	0.47 (0.06)	2.23 (0.04)	5.51 (0.01)
	Light pollution	1.43 (0.03)	0.14 (0.02)	0.39 (0.09)	2.04 (0.05)	5.49 (0.03)
	Medium pollution	1.37 (0.06)	0.18 (0.03)	0.48 (0.02)	1.96 (0.01)	5.30 (0.03)
	High pollution	1.41 (0.05)	0.15 (0.01)	0.42 (0.01)	1.97 (0.00)	5.23 (0.03)

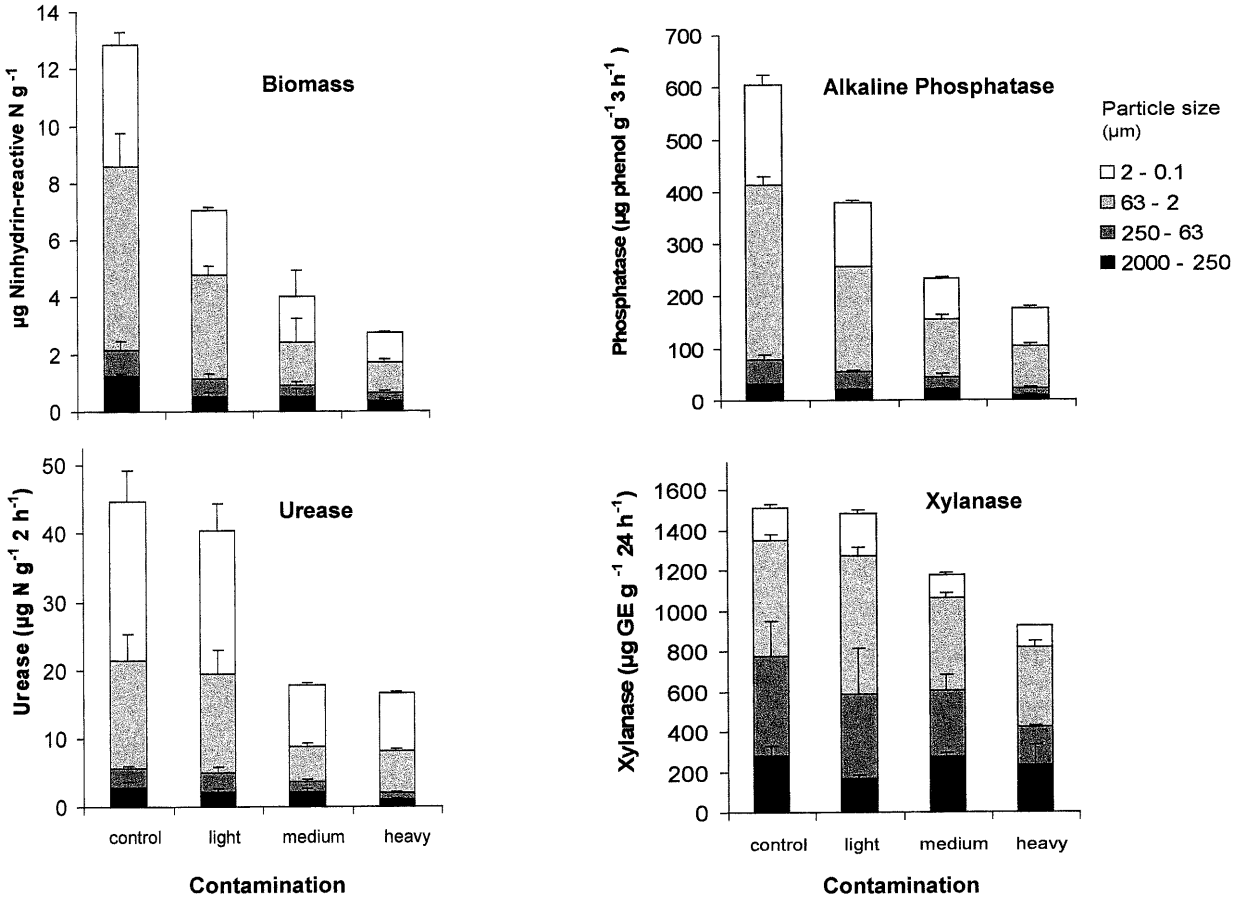

Fig. 1 Effects of heavy metal pollution on the distribution of ninhydrin-reactive N (CFE), alkaline phosphatase, xylanase and urease activity within the particle-size fractions. Data are given as means of three replicate samples, *bars* indicate standard error. Results of xylanase are expressed as µg glucose equivalents (GE) g^{-1} of the bulk soil

did not protect against the heavy metal pollution. Microorganisms and heavy metals seem to occupy the same binding sites and spatial separation of toxic heavy metals from soil microorganisms did not occur. Further investigation should clarify the localization of heavy metals and soil microorganisms at the nano- and pico-scale.

Soil enzyme activities of particle-size fractions

The recovery of the enzyme activities was in the range 84.9–100.0%. The measurement of xylanase activities includes mainly extracellular enzyme activity, since the high-molecular substrate (xylan) cannot be taken up by soil microorganisms. In contrast, alkaline phosphatase, urease and arylsulfatase can be attributed to cellular and extracellar enzyme activities, respectively. The distribution of xylanase, urease, alkaline phosphatase and arylsulfatase activities in particle-size fractions varied with the enzyme assayed (Fig. 1, Table 4). Urease activity was mainly located in the 2–0.1 µm fraction, alkaline phosphatase and arylsulfatase activities in the 63–2 µm

Table 3 Statistical differences (*F*-values and significance level) among means of variables computed by a one-way variance analyses of the bulk soil and soil fractions with the factor heavy metal pollution ($df=2$)

Variable	Bulk soil	Particle-size fractions			
		2,000–250 µm	250–63 µm	63–2 µm	2–0.1 µm
Ninhydrin-reactive N	2.26 n.s.	1.19 n.s.	1.95 n.s.	21.8^{***}	40.6^{***}
Urease	6.79^{*}	1.52 n.s.	3.23 n.s.	4.14^{*}	6.77^{*}
Xylanase	6.09^{*}	0.61 n.s.	0.82 n.s.	16.9^{***}	10.1^{**}
Phosphatase	207.1^{***}	32.9^{***}	6.52^{*}	78.5^{***}	41.4^{***}
Arylsulfatase	76.9^{***}	94.2^{***}	69.6^{***}	80.7^{***}	54.3^{***}

$^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$, *n.s.* not significant

Table 4 The response of arylsulfatase in bulk soil and particle-size fractions to different heavy metal pollution. Arylsulfatase activity was calculated on the basis of soil dry matter. Standard errors ($n=3$) *in parentheses*. Mean recovery of arylsulfatase activity of particle-size fractions was 88.7%

Soil property	Treatment	Particle size				
		Bulk soil	2,000–250 μm	250–63 μm	63–2 μm	2–0.1 μm
Arylsulfatase (μg nitrophenol g^{-1} h^{-1})	Control	60.24 (5.77)	24.02 (1.28)	28.46 (3.10)	84.84 (8.77)	150.95 (17.74)
	Light pollution	11.48 (1.77)	1.34 (0.39)	4.26 (0.59)	11.60 (1.80)	61.52 (1.42)
	Medium pollution	6.94 (1.72)	4.36 (1.89)	2.17 (0.44)	2.51 (0.91)	18.46 (4.21)
	Heavy pollution	n.d.[a]	n.d.	n.d.	n.d.	n.d.

[a] No arylsufatase activity could be detected in the bulk soil and particle-size fractions of heavy polluted soils

and 2–0.1 μm fractions. Urease, phosphatase and arylsulfatase were located in fractions being enriched with microbial biomass and C_{org} (Stemmer et al. 1998, 1999). Xylanase activity was almost equally distributed among the particle-size fractions, with a slightly lower activity in the clay fractions (Fig. 1). Since our results are in agreement with several earlier studies (Tabatabai 1973; Mateos and Carcedo 1987; Kandeler et al. 1999a,b), this would suggest that the predominance of enzyme activities in the different size fractions is a constant characteristic of soils and is independent of soil type, soil management and soil pollution.

Soil contamination with 300 ppm Zn, 100 ppm Cu, 50 ppm Ni, 50 ppm V and 3 ppm Cd significantly reduced enzyme activities involved in C-, N-, P- and S-cycling (xylanase, urease, alkaline phosphatase, arylsulfatase) (Fig. 1, Tables 3, 4). At the low contamination level, xylanase and urease activities were least affected, whereas phosphatase and arylsulfatase significantly decreased their activities. Arylsulfatase activity in the sand and silt fractions decreased to a few percent of its activity in the corresponding unpolluted control, whereas the arylsulfatase in the clay fraction of the least polluted soil decreased only to 40.8 percent of that measured in the control (Table 4). The decrease in arylsulfatase and phosphatase activities in polluted soils was detected in all particle-size fractions, although the smaller fractions were enriched in Cu, Ni, Zn and Cd. These results provide strong evidence that the decrease of arylsulfatase and phosphatase activities in particle-size fractions was caused by a heavy-metal induced reduction of the enzyme synthesis by soil microorganisms, rather than by a heavy-metal induced inhibition of the enzyme activities. Contamination of soils did not affect xylanase activity in the sand-sized fractions, probably because a higher C requirement was necessary for the repair and maintenance of soil microorganisms in polluted soils (Bardgett and Saggar 1994) or because the microbial community changed from bacterial to fungal dominance.

Soil microbial community structure: PLFA analysis

The content of the PLFA was in the range 4.35–476.6 nmol kg^{-1} and the recovery rate of PLFA content was in the range 41.8–77.7% (Table 5). The relatively low amounts of PLFA in bulk soils (Frostegård et al. 1993b; Bardgett et al. 1999) could be due to an incomplete extraction of the PLFAs of the soil containing 30% of carbonate. Total bacterial PLFAs increased with diminishing particle size, whereas fungal PLFA decreased (Table 5). Therefore, the ratio of fungal to bacterial PLFAs decreased in the smaller particle-size fractions of the soils. These data suggest that fungi and bacteria occupy two separate soil microenvironments;

Table 5 Total PLFAs, bacterial and fungal PLFA contents (nmol kg^{-1}) in bulk soil and in the soil fractions of the control and the medium polluted soil. Results are means of two replicates (mean absolute deviation *in parentheses*)

Soil property	Treatment	Bulk soil		Particle size								Recovery (%)
				2,000–250 μm		250–63 μm		63–2 μm		2–0.1 μm		
PLFA (nmol kg^{-1})	Control	134.11	(31.30)	14.29	(6.72)	13.33	(0.86)	83.16	(5.78)	476.56	(58.86)	61.9
	Medium pollution	137.67	(35.54)	9.69	(1.27)	4.35	(2.51)	36.44	(9.50)	425.96	(16.49)	44.2
Bacterial PLFA (nmol kg^{-1})	Control	124.95	(26.12)	1.95	(1.95)	5.68	(2.41)	77.44	(5.91)	470.00	(57.58)	59.9
	Medium pollution	128.65	(32.28)	n.d.[a]		0.12	(0.12)	29.71	(6.47)	217.57	(15.28)	41.8
Fungal PLFA (nmol kg^{-1})	Control	9.14	(5.17)	12.34	(4.77)	7.65	(1.54)	5.72	(0.13)	6.57	(1.28)	89.6
	Medium pollution	9.02	(3.26)	9.69	(1.27)	4.23	(2.38)	6.73	(3.03)	8.39	(1.21)	77.7
Fungal:bacterial ratio[b]	Control	0.07		6.33		1.35		0.07		0.01		
	Medium pollution	0.07		n.d.		34.66		0.23		0.02		

[a] Not detectable
[b] Ratios have dimension 1

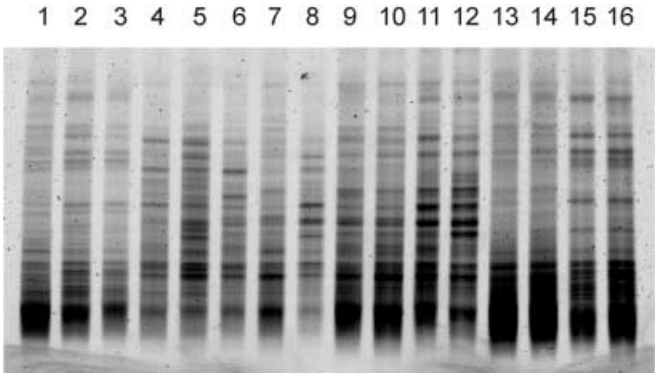

Fig. 2 The DGGE profiles of 16S rRNA gene fragments amplified from DNA extracted from different particle-size fractions of the control and medium polluted soil. *Lane 1* Bulk soil, control. *Lanes 2 and 3* Bulk soil, pollution. *Lane 4* Coarse sand control. *Lane 5* Coarse sand pollution. *Lanes 6 and 7* Fine sand, control. *Lane 8* Fine sand, pollution. *Lanes 9 and 10* Silt, control. *Lanes 11 and 12* Silt, pollution. *Lanes 13 and 14* Clay, control. *Lanes 15 and 16* Clay, pollution. PCR products stained using SYBR green 1 and scanned using Storm 860

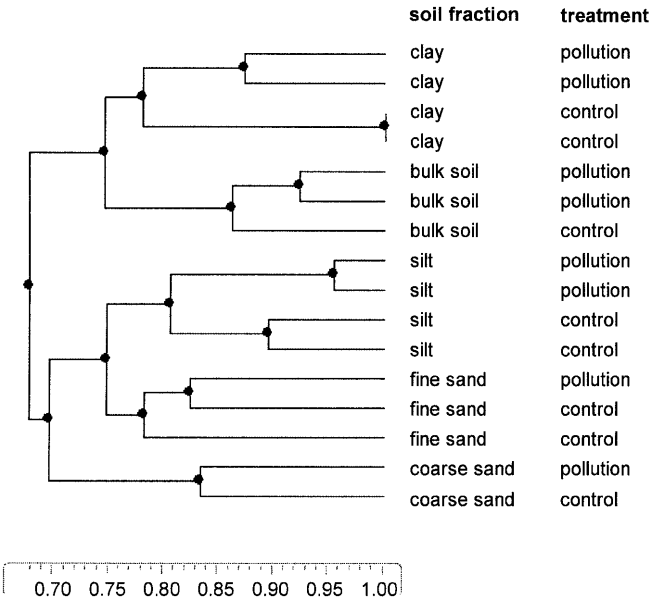

Fig. 3 The UPGMA dendrogram generated from gel image data using DGGE profiles of 16S rRNA gene fragments amplified from DNA extracted from different particle-size fractions of the control and medium polluted soil. These banding patterns were matched with the data analysed directly using Phoretix software. *Scale* indicates the similarity level (scale 0–1)

fungi prefer the particulate organic matter of the coarse sand fraction, whereas bacteria are mainly sorbed onto the clay fraction.

Heavy metal contamination induced a shift of the ratio of 18:2ω6:bactPLFAs (Table 5). The higher ratio of fungal to bacterial PLFAs in the fine sand and silt fractions of the medium polluted soils clearly demonstrated that heavy metal-resistant fungi can survive in the medium polluted soil. This notion is supported by the results of Frostegård et al. (1996) who showed a strong increase in 18:2ω6 due to Zn contamination in arable soils. However, a higher tolerance of fungi towards heavy metals could not be detected in several forest soils (Pennanen et al. 1996; Bååth et al. 1998).

DGGE separation of 16S rRNA gene fragments

The comparison of soil bacterial communities can be obtained by DGGE of ribosomal RNA gene fragments (Bruce et al. 2000). The 16S rRNA gene fragment PCR products were amplified from DNA that had been extracted directly from bulk soil and the different particle-size fractions (Fig. 2). No PCR products were observed in reagent controls (i.e. samples with sterile water in place of soil but processed identically to the test material; data not shown). Profiles containing on average ca. 20 bands were resolved by DGGE for each lane, combining common features and also regions of variability in banding pattern (Fig. 2). Extracted DNA electrophoresed higher than 12 kb in size and showed little or no sign of degradation. The yield parameters fell within the range of values that we get from different soil types. Again, they showed no sign of being affected by heavy metal pollution. Duplicate samples were found to match closely to another track showing similar profile complexity and band composition (Fig. 2). This was most marked for lanes 2 and 3 (replicates of polluted bulk soil), lanes 9 and 10 (replicates of control silt fraction), lanes 11 and 12 (replicates of polluted silt fraction), lanes 13 and 14 (replicates of control clay fraction) and lanes 15 and 16 (replicates of polluted clay fraction). A dendrogram generated from the banding pattern showed the same clustering of profiles of the bulk soil or soil fraction as had been identified visually (Fig. 3). Repeated measurements were clustered together, indicating high reproducibility of the PCR procedure. Since all PCR amplifications generated PCR products that were within a factor of two, there is no evidence that DGGE profiles of the heavy metal polluted soil were biased technically by the PCR amplification. In contrast, Brim et al. (1999) and Wintzingerode et al. (1997) reported pitfalls in PCR-based analyses in polluted and unpolluted soils. Our results showed that the bulk soil was clustered more closely with the clay than with other fractions. Therefore, the bacterial community of the clay fraction, which contained the majority of microbial biomass measured by the fumigation extraction method (Fig. 1), induced the similarity between the clay fraction and the bulk soil. Most studies in the past, using DGGE or the closely-related temperature gradient gel electrophoresis (TGGE) approach, have concentrated on the extraction of 16S rRNA gene fragments from different bulk soils (Felske et al. 1996; Hungate et al. 1996; Felske and Akkermans 1998). Only a preliminary experiment on the distribution of bacterial communities over soil aggregate fractions revealed that similar bacterial types were distributed over soil aggregates of different sizes

by wet sieving (Gelsomino et al. 1999). Several authors have concluded that a limited number of dominant, ubiquitous and ecological recalcitrant bacteria types are being targeted by the bacterial PCR–DGGE system (Duarte et al. 1998; Gelsomino et al. 1999). Nevertheless, the results of the DGGE separation of 16S rRNA gene fragments confirmed the distribution pattern found by the measurement of the bacterial PLFA. Therefore, we conclude that the soil bacterial community in the clay fraction showed higher genetic diversity than the other particle-size fractions. The sand fractions showed greater DGGE profile heterogeneity than other fractions, mainly due to the variable contribution of particulate organic matter. Similarly, there was also high variability in measures of microbial biomass N and several enzyme activities in the coarse sand fraction (Kandeler et al. 1999a,b; Stemmer et al. 1999).

Differences between samples can be seen either in terms of the presence/absence of individual DGGE bands or in terms of the intensity of co-migrating DGGE bands. Little variation was observed for the examples of replicated samples. However, some differences were observed between the control and polluted soil samples. Whilst these could be identified even in the bulk soil, the extent to which samples differed increased in the smaller fractions. On the basis of similarities between rRNA-based bacterial diversity profiles, we can conclude that microbial biomass and enzyme activities seem to be more sensitive to heavy metals than the bacterial diversity measured by DGGE profiles of 16S rRNA gene fragments. Although DGGE is a sensitive method, it is possible that rarer ecological significant species are at the limit of detection which may have been affected, but not detected (Muyzer et al. 1993; Murray et al. 1996; Bruce et al. 2000). This problem could be solved by using DGGE with group-specific primers as proposed by Heuer et al. (1997).

Amino sugars

The bulk soils contained 800–1,200 mg amino sugars kg^{-1} soil, corresponding to an amino sugar-N proportion of ca. 5% of total N. After fractionation, the recovery of amino sugars slightly exceeded 100% of the amount in the bulk soil. A similar result has been obtained for ninhydrin-reactive N; however, more samples need to be analysed to investigate whether soft dispersion of bulk soil favours amino sugar extraction. The distribution of amino sugars among size fractions and between sites paralleled differences in organic matter contents (Tables 2, 6). For a better understanding of amino sugar dynamics, both absolute contents and N-related concentrations were considered.

The different distribution of individual amino sugars among size fractions suggested that the microbial N of these fractions consisted of different compounds (Zhang et al. 1998). The maximum amino sugar contents occurred in the clay fractions. The ratios of glucosamine to muramic acid and of glucosamine to galactosamine were lower for the clay fractions than for the bulk soil (Table 6). Apparently, the high proportions of bacterial biomass in this fraction as indicated by PFLA and DGGE analysis resulted in a preferential enrichment of bacterial cell wall residues containing muramic acid (Zhang et al. 1998) and of capsular and extracellular polysaccharides containing galactosamine, the latter also being common in actinomycetes (Sharon 1965;

Table 6 The response of amino sugars in bulk soil and particle-size fractions to different heavy metal pollution. Mean absolute deviation *in parentheses* ($n=2$). Mean recovery of total amino sugars in particle-size fractions was 99% for the control and 122% for the medium polluted soil

Soil property	Treatment	Bulk soil		Particle size								Recovery[a] (%)
				2,000–250 μm		250–63 μm		63–2 μm		2–0.1 μm		
Glucosamine	Control	707	(41)	354	(47)	125	(56)	1050	(37)	2130	(3)	99
(mg kg^{-1})	Medium pollution	528	(34)	337	(10)	129	(5)	936	(45)	2000	(51)	122
Mannosamine	Control	18	(0)	n.d.[b]		12	(8)	42	(17)	63	(3)	130
(mg kg^{-1})	Medium pollution	18	(7)	n.d.		22	(12)	29	(0)	67	(7)	122
Galactosamine	Control	347	(44)	94	(14)	33	(17)	471	(7)	1330	(39)	95
(mg kg^{-1})	Medium pollution	251	(1)	61	(2)	29	(4)	428	(9)	1220	(122)	116
Muramic acid	Control	36	(3)	36	(9)	7	(2)	43	(2)	173	(25)	124
(mg kg^{-1})	Medium pollution	27	(7)	28	(3)	25	(2)	34	(1)	163	(4)	156
Sum	Control	1100	(87)	484	(41)	178	(83)	1600	(61)	3690	(63)	99
(mg kg^{-1})	Medium pollution	824	(35)	425	(9)	205	(20)	1430	(56)	3450	(176)	122
Sum	Control	738	(57.9)	1560	(255)	352	(150)	714	(19)	672	(11)	99
(g kg^{-1} N)	Medium pollution	602	(25.8)	2670	(110)	424	(46)	730	(23)	645	(39)	122
GluN/GalN[c]	Control	2.1	(0.1)	3.9	(1.1)	3.9	(0.3)	2.2	(0.0)	1.6	(0.0)	
	Medium pollution	2.1	(0.1)	5.6	(0.0)	4.5	(0.4)	2.2	(0.1)	1.6	(0.1)	
GluN/Mur[c]	Control	20	(0.5)	10	(1.2)	16	(3.0)	25	(1.9)	12	(1.7)	
	Medium pollution	19	(3.8)	12	(1.9)	5.3	(0.5)	27	(0.3)	12	(0.6)	

[a] Recovered from the sum of fractions relative to the bulk soil. The analytical recovery of the surrogate standard averaged 97% of initial spike level

[b] Not detected

[c] Ratios have dimension 1

Parsons 1981). Mannosamine, derived from bacteria (Kenne and Lindburg 1983) and perhaps from fungal melanine (Coelho et al. 1997), was enriched in the finest fractions as well. The contribution of amino sugars to N content, however, was highest for the coarse sand fraction (Table 6), possibly indicating a higher relevance of microbes to N transformations in the labile organic matter pool of the fraction. Nevertheless, for the sand fractions, the results obtained by different biomarker methods were less consistent. A relatively low glucosamine:muramic acid ratio in the coarse sand fraction contradicted the findings from PFLA analysis, whereas changes in the glucosamine:galactosamine ratio as an alternative marker of fungal:bacterial residues (Kögel and Bochter 1985) was more in accordance with that obtained from PFLA analysis (Tables 5, 6). Apparently, galactosamine related more closely to extractable bacterial derived PFLAs than it did to muramic acid, but more data will be required to ascertain this trend. Differences between methods were also apparent for the bulk soil. Here, PFLA analysis estimated only a minor proportion of fungal compounds, while amino sugar analysis suggested dominance of fungal residues (Table 5, 6).

The usefulness of muramic acid or glucosamine measurement as an indicator for bacterial and fungal biomass in soils, respectively, has been critically discussed (Millar and Casida 1970; Parsons 1981; Hicks and Newell 1984; Zelles et al. 1990; Amelung 2000). Our data clearly show that a reduction of microbial biomass N, PFLA contents and enzyme activity (Fig. 1; Tables 3, 4, 5) in the heavy metal polluted samples did not relate to changes in amino sugar contents (Table 6). This agrees with findings by Nannipieri et al. (1979), who did not find a correlation between phosphatase or urease activity and the hexosamine content. Our data therefore confirm that amino sugars indicated dead rather than living microbial residues, not being related to living soil microbial biomass (Chantigny et al. 1997), and being relatively stable against microbial transformations, possibly due to interactions with the mineral soil matrix (Amelung 2000).

The amino sugar N proportions of the coarse sand fractions were higher in the medium polluted samples than in the control (Table 6). As the amino sugar contents (g kg^{-1} coarse sand) were similar for both the control and medium polluted soil (data not shown), we suggest that the losses of total N of the polluted soil (Table 2) did not include losses of amino sugar N. Whether heavy metal pollution caused a stress-induced immobilization of N as amino sugar N in the coarse sand fraction might warrant further attention. Moreover, it remains unclear whether the higher muramic acid concentration in the fine sand fraction of the medium polluted soil compared to the control (Table 6) was the result of a selective preservation of muramic acid during the incubation period (due to the formation of complexes of its carboxylic group with heavy metals), or whether it indicated a shift of Gram-negative to Gram-positive bacteria in this fraction after heavy metal amendment.

Conclusions

We conclude that physical fractionation of the soil according to particle size, combined with different chemical and microbiological methods, has advanced our understanding of the distribution of soil microorganisms in their micro-environment, and their response to heavy metal pollution. Several independent methods for charactering the microbial biomass and soil microbial community structure revealed that living and dead soil bacteria were mainly associated with the silt and clay fraction, whereas fungi and their exoenzymes, involved in the decay of complex organic compounds, were found associated with the particulate organic matter in the coarse sand fraction.

Heavy metal pollution influenced the structure and function of the microbial community in the bulk soil and in particle-size fractions. The increase of fungal:bacteria ratio in polluted soils suggests that heavy metal resistant fungi could establish after long-term heavy metal pollution of the soil. Nevertheless, microbial biomass decreased significantly in the silt and clay fraction that was enriched with heavy metals. Consequently, the microbial community produced fewer enzymes (urease, phosphatase and arylsulfatase) in the small fractions of polluted soils. A reduction of enzyme activity due to heavy metal pollution did not result in a significant depletion of amino sugar concentration, suggesting that enzyme activity is not related to the concentration of microbial cell wall residues in soil. The majority of amino sugars in the clay and silt-sized fractions remained unchanged during the incubation period, suggesting that they comprise an amino sugar fraction stabilized by minerals, not responding to long-term environmental stress. On the basis of similarities between rRNA-based bacterial diversity profiles, we can conclude that microbial biomass and enzyme activities seem to be more sensitive to heavy metals than the analyses of bacterial composition based on DGGE of 16S rRNA gene fragments. Further studies should clarify whether heavy metal pollution shifts the numerical abundance of the target molecules, suggesting that certain (dominant) bacteria contain greater amounts of rRNA than others.

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