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Functional activity and microbial community structure in soils amended with bimetallic sludges

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Abstract

Heavy metal availability, microbial biomass and respiration, bacterial diversity and enzyme activity were studied in soils from long-term field experiments contaminated with Mn–Zn- or Cd–Ni-rich sludge, incorporated into soils at two different rates. Soils that never received sludge were used as controls. Microbial biomass C content (B_C) and soil respiration (CO_2–C) were slightly reduced in soils amended with Mn–Zn at the higher incorporation rate whereas in soils receiving Cd–Ni-rich sludge B_C and respiration were unaffected. Metabolic quotient values (qCO_2) calculated by the B_C-to-CO_2-C ratio were not significantly different, regardless of the sludge type whereas the microbial biomass C-to-total organic C (B_C-to-TOC) ratios were significantly reduced in the soils receiving the higher rates of both sludge types. Phosphomonoesterase, β-glucosidase and arylsulfatase activities and hydrolase-to-B_C ratios, were significantly reduced in soils amended with Ni–Cd-sludge at both rates, whereas the Mn–Zn-sludge only reduced the arylsulfatase activity at the higher rate. Protease activity was generally higher in all the sludge-amended soils as compared to control soils whereas urease activity was unaffected by sludge amendments. The structure of the bacterial community, as determined by denaturing gradient gel electrophoresis (DGGE), was different in the sludge-amended soils as compared to the respective controls. The most important changes were observed in the soils amended with high-level Ni–Cd sludge. Because some of the adverse effects were observed at moderate contamination levels, our results indicate that the presence of certain heavy metal combinations can be a serious limitation for sludge disposal.

Keywords: Metal availability; Bacterial community structure; Hydrolase activities; Bimetallic sludge

1. Introduction

Incorporation of sewage sludge into agricultural soils has been proposed as a feasible, inexpensive and environmentally sound disposal practice, as generally such wastes can improve soil physical and chemical properties and contain nutrients beneficial to plants and microorganisms (Barker, 1997). For example, world-wide problems of arable lands deficient in Mn and Zn (Welch, 1995) might be alleviated by the use of Mn–Zn rich sludge. However, most of the research on the sludge use in agriculture aimed at quantifying the accumulation of heavy metals following sludge applications and at understanding the residual effects of heavy metals on soil biological properties. This is because to date in several EU countries, land application of sewage sludge is one of the largest single sources of toxic heavy metals in soils.

Generally, sludge contain different associated metals and their toxicity may depend on synergistic or antagonistic interactions which need to be assessed. For example, Zn and Cd may have antagonist or synergic effects depending on their respective concentrations and on the investigated target (Adriano, 2001). The presence of metals in combination may have greater adverse effects on soil microbial biomass and activity (Chander et al., 1995; Khan and Scullion, 1999) and soil hydrolase activities (Renella et al., 2003) than those caused by single metals at high concentrations.

It is well established that only a fraction of the soil total content of metals is available for plant uptake and may

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Table 1
Main chemical properties of sludges incorporated in the Ambarès and Louis Fargue soils

Sludge	C (g kg^{-1})	N (g kg^{-1})	P_2O_5 (g kg^{-1})	Ca (g kg^{-1})	Mg (g kg^{-1})	K (g kg^{-1})	Fe (g kg^{-1})	Cd (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Zn (mg kg^{-1})
Ambarés	29.2	2.71	4.2	4.8	0.41	0.16	3.29	31	85	230	6114	124	4900
Louis Fargue	16.8	1.26	4.2	10.6	0.29	0.12	3.65	1830	219	448	699	4071	3062

affect soil microorganisms. Several chemical extractants have been used for determination of the 'bioavailable' fraction of metals. The sludge-borne metals mainly exist in reactive forms on the surfaces of soil particles (McGrath and Cegarra, 1992). Therefore, good correlations have been reported between the amount of metals taken up by plants and the amount of soluble and exchangeable metals extracted by solutions of various electrolytes and chelating agents (Gerritse and van Driel, 1984; Norvell, 1991; Krishnamurti et al., 1997; Prueß, 1998). However, the relationship between these chemical fractions and the effects of more than one heavy metal on soil microflora and soil functions are less clear.

The aim of this work was to study the effects of past repeated land application of Mn–Zn or Cd–Ni rich sludges on soil microbial biomass and activity, microbial community structure and activity of hydrolytic enzymes. The measured biochemical parameters were compared to the amount of metals extracted by different chemical extractants. Innovative aspects of the research were: (i) the monitoring of the heavy metal mobility and microbial and biochemical parameters in soils sampled from unique field experiments where bimetallic sludge were applied for several years; (ii) the combination of classical and molecular techniques to study microbial biomass, hydrolase activity and bacterial diversity.

2. Materials and methods

2.1. Soil characteristics and field experiments design

Both Ambarès (A) and Louis Fargue (LF) field experiments are located at the Couhins Experimental Farm (Cadaujac, Gironde, France). At the beginning of the 20th century, the area was a vineyard, then an *Acacia* spp. forest. After the field trials were established, in 1974 for A and in 1976 for LF, the fields were cropped annually with maize (*Zea mays* L., cv. INRA 260), except in years 1997 (potato tubers), 2001 (lettuce), and 2002 (winter wheat).

The soil (Arenic Udifluvent) has developed on an alluvial material with a coarse sandy texture (clay 4.2%, silt 21.6%, sand 74.2%), with the following initial parameters: pH 5.3, C 1.57%, N 0.16%, and organic matter (OM) 2.8%.

Both A and LF experiments had the same treatments with five replicates in the field: control with inorganic fertilization (A 0 and LF 0) or sewage sludge disposal rate of 10 t DM ha^{-1} year^{-1} (S1) and 100 t DM ha^{-1} every 2 years (S2) (Weissenhorn et al., 1995; Sappin-Didier et al., 2002). The high sludge loading rate was used in the S2 treatment to reach soil metal concentrations significantly above the EU mandatory limits. At the A experiment, sludge applications terminated in 1993 and 1992 for S1 and S2 treatments, respectively, resulting in 200 (A S1) and 1000 (A S2) t dry matter (DM) ha^{-1} as total sludge inputs, whereas applications of LF sludge terminated in 1980 after cumulative inputs of 50 (LF S1) and 300 (LF S2) t DM ha^{-1}.

Sludges used in the A and LF experiments were anaerobically digested, floculated and dehydrated through a band filter (A sludge) or by heating under high pressure (Porteous process) (LF sludge), leading to a different sludge aggregation. Both A and LF sludges had a similar chemical composition and differed mainly for their Zn–Mn and Ni–Cd enrichment (Table 1). Therefore, although repeated sludge applications increased concentration of several heavy metals in the sampled soil layer (0–20 cm), soils A S1 and A S2 could be considered contaminated by Zn and Mn whereas the LF S1 and LF S2 soils were mainly contaminated by Cd and Ni (Table 2).

For each treatment, soils were sampled from five replicate plots from the 0–20 cm layer, mixed and bulked into pooled samples from where three subsamples

Table 2
Main soil properties

Soil	pH (H_2O)	CEC (cmol kg^{-1})	TOC (g kg^{-1})	N_{tot} (g kg^{-1})	Cd (mg kg^{-1})	Cr (mg kg^{-1})	Cu (mg kg^{-1})	Mn (mg kg^{-1})	Ni (mg kg^{-1})	Zn (mg kg^{-1})
A 0	6.4 (0.1)	3.4 (0.2)	7.4 (0.2)	0.73 (0.02)	0.3 (0.1)	6.7 (0.5)	12.4 (3.9)	44.6 (2.6)	1.7 (0.2)	24.1 (0.8)
A S1	6.5 (0.1)	3.6 (0.2)	8.7 (0.3)	0.87 (0.02)	1.1 (0.2)	12.2 (1.0)	17.8 (4.2)	222.3** (9.2)	5.5 (0.4)	219.2** (8.0)
A S2	6.3 (0.1)	7.2* (0.3)	14.8* (0.5)	1.21* (0.1)	3.9* (0.2)	25.4 (2.6)	58.9* (8.7)	1545** (76.2)	38.2** (1.2)	844.3** (21.3)
LF 0	6.4 (0.1)	3.0 (0.3)	6.8 (0.2)	0.64 (0.02)	1.7 (0.1)	9.3 (1.6)	8.8 (1.2)	29.2 (2.2)	14.4 (1.4)	16.2 (1.1)
LF S1	6.4 (0.2)	3.1 (0.2)	8.9 (0.3)	0.76 (0.02)	13.1* (1.1)	12.3 (1.2)	20.2 (2.9)	32.4 (2.4)	52.3 (4.2)	45.4 (3.9)
LF S2	6.5 (0.1)	5.1 (0.3)	11.5* (0.3)	1.12* (0.1)	70.6** (2.8)	21.2 (2.4)	38.7* (4.1)	51.0* (4.2)	138.0** (5.6)	140.4** (4.7)

Values in brackets are the standard error of the means ($n=3$). Symbols * and ** indicate significant differences at P levels of 0.05 and 0.01, respectively, between the sludge-treated and their respective control soils. TOC is the total organic C and N_{tot} is the total N.

(pseudoreplicates) were taken for analyses. This sampling strategy was adopted to reduce the number of samples to manage and transport during a large sampling campaign, although this could weaken the significance of the outcome. Soils were transported unsieved to minimize disturbance and sieved (<2 mm) after 1 week from the sampling date. After sieving, soils were moistened to 50% WHC and preincubated at 25 °C for 7 days prior to analyses, to stabilize microbial biomass and activity.

2.2. Heavy metal total content and availability

Total content of heavy metals was measured on 0.150 g of dry finely ground soil by microwave-assisted (Milestone 1200) acid digestion in HF:HNO_3 1:6 solution with increasing power (200–600 W for 24 min).

Heavy metal availability was measured using different single-solvent extractions. Extractions with deionized H_2O were done by using a 1:10 w/v extraction ratio. Extraction with 1 M NH_4NO_3, were carried out using 20 g of soil in 50 ml 1 M NH_4NO_3 according to Prueß (1998). Extractions with 20 mM citric acid were carried out as described by Krishnamurti et al. (1997), and extractions in 40 mM Na_2-EDTA were done using a 1:10 w/v extraction ratio. All extractions were replicated three times using a reciprocal shaker for 2 h at 20 revolutions min^{-1}. All soil suspensions were filtered through Whatman 42 filter paper and extracts were acidified with 0.2 ml of concentrated HNO_3 before elemental analysis by flame or furnace atomic absorption (Perkin Elmer 5500).

2.3. Total organic C, microbial biomass C and respiration measurements

Total organic carbon content (TOC) was measured according to Walkley and Black (1934).

Soil respiration was measured by placing 100 g (dry weight equivalent) of soil in 1-l air-tight glass jars provided with 3-way valves and incubated at 25 °C in the dark for 7 days. Empty jars served as blanks accounting for the CO_2 background concentration. The CO_2 evolution was measured by sampling the head-space gas by an air-tight syringe and by injecting the gas samples in a gas-chromatograph (Hewlett-Packard 6890) equipped with a gas-sampling valve, a packed column (Poropack Q) and a thermal conductivity detector according to Blackmer and Bremner (1977). After respiration measurement, soils were used for determining the microbial biomass C (B_C) with the fumigation extraction technique (Vance et al., 1987).

2.4. Hydrolase activities measurement

Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969), the arylsulfatase activity and β-glucosidase activities according to Tabatabai (1982). Urease activity was measured by using 0.5 M urea as substrate and 0.1 M phosphate buffer at pH 7.1 (Ceccanti 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 for 1 h, with centrifugation of soil slurries at 6000*g* at 4 °C. Concentration of *p*-nitrophenol (*p*-NP) produced in the assays of β-glucosidase, arylsulfatase, 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 (FIAStar, Tecator, Sweden).

2.5. DGGE fingerprinting of soil bacterial community structure

The bacterial community structure was studied by a polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE) approach. Whole-community DNA was extracted from 0.5 g of triplicate fresh-sampled soil using a direct bead-beating extraction method by using the MO BIO UltraClean Soil DNA isolation kit (FastDNA SPIN Kit for soil, Bio 101 Inc., USA) according to manufacturer's instructions. Yield and quality of soil-extracted DNA were assessed by 0.7% agarose gel electrophoresis and staining with ethidium bromide (Sambrook et al., 1989).

The 16S rRNA genes from soil microbial communities were amplified by PCR by using the primer pair F984GC/R1378 described by Heuer et al. (1999). PCR was carried out with a thermal cycler PCR Express (Hybaid, Middlesex, UK). The 50-μl reaction mixture was: Stoffel buffer (10 mM Tris–HCl, pH 8.3, 10 mM KCl) (PE Applied Biosystems), 200 μM of each deoxy nucleotide triphosphate, 3.75 mM $MgCl_2$, 0.2 μM primer F984GC, 0.2 μM primer R1378, 2% (v/v) formamide, 0.25 μg/50 μl T4 gene 32 protein (Roche Diagnostics, Mannheim, D), 5 Units/50 μl of AmpliTaq® DNA Polymerase, Stoffel Fragment (PE Applied Biosystems), 1 μl of template DNA (ca. 10 ng), and sterile deionized-distilled H_2O to a final volume of 50 μl. PCR conditions for amplification of eubacterial 16S rRNA genes were those described by Gomes et al. (2001). Yield and quality of PCR-amplified DNA were assessed by 1.2% agarose gel electrophoresis and staining with ethidium bromide (Sambrook et al., 1989).

The DGGE was performed using a DCode™ Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, CA, USA). A 15-μl amount of PCR samples was loaded onto 6% (w/v) polyacrylamide gels containing a linear chemical gradient ranging from 40 to 58% denaturant (100% denaturant corresponds to 7 M urea plus 40% (v/v) of deionised formamide) in a 1× TAE buffer (40 mM Tris base, 20 mM acetic acid, and 1 mM

disodium EDTA, pH 8.3), which provided the best separation of the amplicons and the highest resolution for computer aided similarity analysis. This range of denaturant can improve resolution of high G+C bands (Schönfeld et al., 2003).

Electrophoresis was run in a 1× TAE buffer at 60 °C at a constant voltage of 100 V for 7 h. After the run, the gels were stained with SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed under UV light ($\lambda = 254$ nm) with a Polaroid MP-4 Land camera and a Polaroid 667 black-and-white printed film.

2.6. Statistics

Analysis of variance by the Tukey–Kramer test was used to assess the significance of differences ($P < 0.05$) of the means of three pseudoreplicates using STATVIEW5 computer programme (SAS Institute). Scanned banding patterns of DGGE profiles were analysed by the Diversity Database™ Fingerprinting software (Bio-Rad Laboratories) and similarity dendrograms (Dice coefficient of similarity) for both soils were generated via a Ward's clustering method (Rademaker et al., 1999).

3. Results

3.1. Heavy metal total content and availability

Total heavy metal content of soils is reported in Table 2. In the A S2 soils the Cd content was close to- and Zn was about 5-times the maximum upper EU limit for agricultural sludge-amended soils (Commission of the European Communities (CEC), 1986), whereas concentrations of Ni and other metals (e.g. Cr and Cu) were below such limits (Table 2).

In LF S1 and LF S2 soils, Cd concentrations were at about 3- and 25-times the EU limits, respectively, whereas Ni were below and 2-times the upper EU limit, respectively; other measured metals were all below the EU limits (Table 2).

Extractants used mobilized different amounts of heavy metals from the soils. Deionized H_2O extracted generally low amounts of metals, often below the detection limit for Cd and Cr in the A soils (Table 3). Higher amount of Cd was extracted by 1 M NH_4NO_3 from the LF soils than from the A soils, reflecting sludge-borne Cd inputs (Table 1). The 1 M NH_4NO_3-excheangeable Mn was higher in the A S1 and S2 soils, than in the LF soils, whereas the 1 M NH_4NO_3-extractable Zn was highest only in the A S2. The 1 M NH_4NO_3-extractable Ni increased in LF soils according to

Table 3
Heavy metal availability estimated by single extractions with deionized H_2O, 1 M NH_4NO_3, 0.02 M citric acid and 0.02 M EDTA

Extractant	Heavy metals availability (mg Me kg^{-1} soil)					
	Cd	Cr	Cu	Mn	Ni	Zn
H_2O						
A 0	BDL	BDL	BDL	2.1 (0.4)	BDL	0.2 (0.08)
A S1	BDL	BDL	0.4 (0.02)	7.4 (0.6)	0.1 (0.03)	0.8 (0.02)
A S2	BDL	BDL	1.0 (0.06)	12.5 (1.2)	0.4 (0.1)	2.1 (0.07)
LF 0	BDL	BDL	BDL	0.2 (0.03)	BDL	BDL
LF S1	0.2 (0.02)	BDL	0.6 (0.04)	0.3 (0.03)	0.6 (0.1)	0.3 (0.01)
LF S2	0.5 (0.03)	BDL	0.5 (0.02)	0.6 (0.04)	1.6 (0.4)	0.5 (0.04)
NH_4NO_3						
A 0	0.1 (0.01)	0.5 (0.04)	0.4 (0.04)	0.3 (0.06)	0.1 (0.02)	0.2 (0.03)
A S1	0.1 (0.01)	0.3 (0.01)	0.6 (0.02)	1.9 (0.1)	0.2 (0.03)	1.1 (0.1)
A S2	0.4 (0.02)	0.3 (0.05)	0.9 (0.02)	5.2 (0.7)	0.5 (0.02)	15.3 (1.2)
LF 0	0.2 (0.02)	0.3 (0.03)	0.1 (0.02)	0.3 (0.01)	0.1 (0.01)	0.3 (0.03)
LF S1	2.1 (0.2)	0.4 (0.02)	0.2 (0.02)	0.8 (0.1)	2.2 (0.3)	0.9 (0.1)
LF S2	2.6 (0.2)	0.5 (0.03)	0.6 (0.02)	1.2 (0.07)	4.0 (0.9)	1.1 (0.2)
Citric acid						
A 0	0.2 (0.03)	0.3 (0.1)	2.0 (0.2)	4.7 (0.6)	0.2 (0.02)	5.8 (0.02)
A S1	0.4 (0.05)	0.7 (0.2)	2.6 (0.2)	34.5 (3.3)	0.4 (0.07)	71.1 (7.7)
A S2	0.7 (0.09)	1.1 (0.4)	9.2 (0.9)	60.9 (5.9)	1.1 (0.1)	223.7 (9.3)
LF 0	0.2 (0.03)	0.4 (0.03)	1.1 (0.4)	1.9 (0.02)	0.7 (0.2)	2.6 (0.3)
LF S1	7.4 (1.2)	0.5 (0.06)	1.7 (0.1)	3.7 (0.7)	11.9 (1.1)	10.1 (1.1)
LF S2	31.9 (2.8)	0.9 (0.04)	6.1 (0.7)	5.1 (0.7)	30.1 (3.0)	44.1 (4.4)
EDTA						
A 0	0.2 (0.02)	0.4 (0.01)	6.7 (1.9)	5.9 (0.7)	0.1 (0.1)	6.3 (0.9)
A S1	0.6 (0.04)	0.6 (0.02)	11.9 (0.02)	41.4 (3.3)	0.5 (0.3)	53.9 (6.7)
A S2	0.9 (0.07)	1.0 (0.2)	39.0 (3.9)	71.3 (6.3)	1.2 (0.2)	315.7 (21.5)
LF 0	0.2 (0.08)	0.4 (0.02)	1.8 (0.7)	2.5 (0.1)	0.7 (0.3)	1.3 (0.3)
LF S1	8.3 (1.1)	0.5 (0.04)	7.2 (0.9)	4.1 (0.5)	20.2 (2.6)	10.3 (1.1)
LF S2	41.0 (5.7)	1.2 (0.3)	12.8 (2.7)	6.4 (0.7)	42.5 (5.5)	47.5 (4.8)

Values in brackets are the standard error of the means ($n = 3$). BDL, concentrations below detection limit.

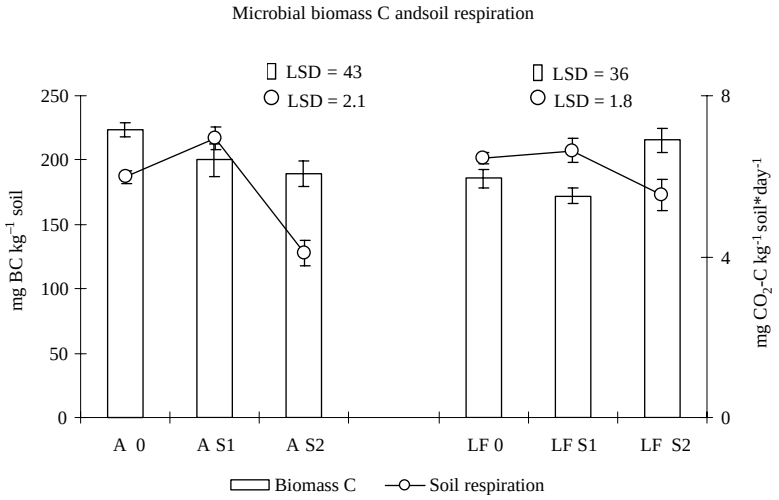

Fig. 1. Microbial biomass C (B_C) content (bars) and CO_2–C evolution rates (lines) of the Ambarès (A) and Louis Fargue (LF) soils. Error bars are the standard error of the means ($n=3$). LSD values are the least significant differences calculated by the Tukey–Kramer test.

sludge-borne metal inputs (Table 3). No particular trends were noted for 1 M NH_4NO_3-extractable Cr and Cu (Table 3).

Citric acid extracted more Cd from the LF soils and more Zn and Mn from the A soils, with trends consistent with the amount of sludge-borne metals applied to soils (Table 3). Higher amounts of Ni and Cd were extracted by citric acid in LF soils with high sludge loading as compared to A soils (Table 3). The EDTA was the most effective extractant for all the studied metals. Compared to other extractants, EDTA extracted more Cd and Ni from the LF soils and more Zn and Mn from the A soils, with trends consistent with the input of sludge-borne metal to the soils (Table 3). Compared to other extractants, EDTA had no appreciable effects on Cr solubility, whereas more Cu was extracted from both the A and LF soils (Table 3).

3.2. Soil respiration, microbial biomass C and TOC content

Soil respiration was lower in A S2 than A 0 soil, whereas it was less affected by sludge and metal loads in the LF soils (Fig. 1).

Similarly, in the A soils the B_C was lower in A S2 than the A 0 soil, whereas in the LF soils the B_C values was unaffected by sludge amendment and soil total metal content (Fig. 1).

The TOC of soils was significantly higher in soils receiving sludge at the higher rates (Table 2).

3.3. Enzyme activities

In the A soils, phosphomonoesterase, β-glucosidase and urease activities were not significantly affected by sludge and metal loads, whereas the arylsulfatase activity was significantly reduced ($P<0.05$) and the protease activity was significantly ($P<0.01$) increased (Fig. 2) at the higher sludge inputs. In the LF soils, phosphomonoesterase,

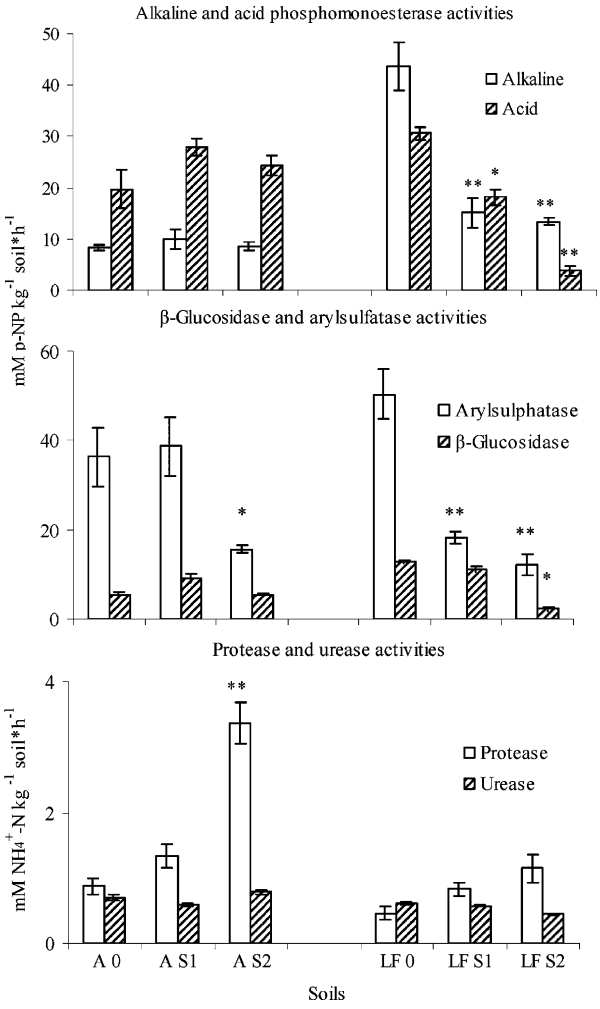

Fig. 2. Hydrolase activities of the Ambarès (A) and Louis Fargue (LF) soils. Error bars are the standard error of the means ($n=3$). Symbols * and ** indicate significant differences at levels of $P<0.05$ and $P<0.01$, respectively, between the sludge-treated and their respective control soils.

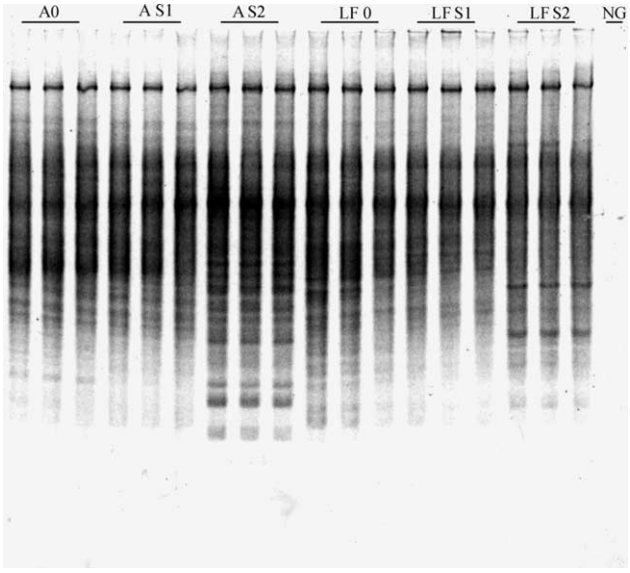

Fig. 3. DGGE patterns of 16S rDNA fragments from Ambarès (A) and Louis Fargue (LF) soils treated with increasing amounts (0, 10 and 100 t DM ha^{-1}) of different sludge. The NG lane is PCR negative control.

β-glucosidase and arylsulfatase activities were significantly reduced by sludge incorporation, whereas protease and urease activities were not significantly affected (Fig. 2).

3.4. Bacterial community structure

The DGGE fingerprinting of PCR-amplified 16S rDNA extracted from the A and LF soils, is shown in Fig. 3. In A soils, the DGGE profiles revealed a shift in soil bacterial community structure consistent with the amount of sludge and metal loads, with a greater complexity of banding pattern in A S2 soils than A 0 and A S1 soils (Fig. 3), providing an evidence that amendment with increasing amounts of Mn–Zn-rich sludge promoted a greater diversity of soil bacterial microflora. In A 0 and A S1 soils, the majority of bands were localised in the middle part of the gel, whereas in A S2 soil, more bands appeared in the lower part of the gel under higher denaturing conditions, resolving high-G+C bands typical for Actinomycetes and Gram-positive bacteria (Fig. 3). In the LF soils, sludge amendments caused a slight decrease in soil bacterial diversity, as less bands were detectable in LF S1 and LF S2 soils as compared to LF 0 soil, suggesting that past applications of Cd–Ni-rich sludge decreased bacterial diversity in soil (Fig. 3).

Similarity dendrograms, generated by the image analysis of DGGE coupled with the Dice similarity coefficient, showed that DGGE profiles of S1 and S2 soils could be well discriminated from those of the respective control soils (Fig. 4).

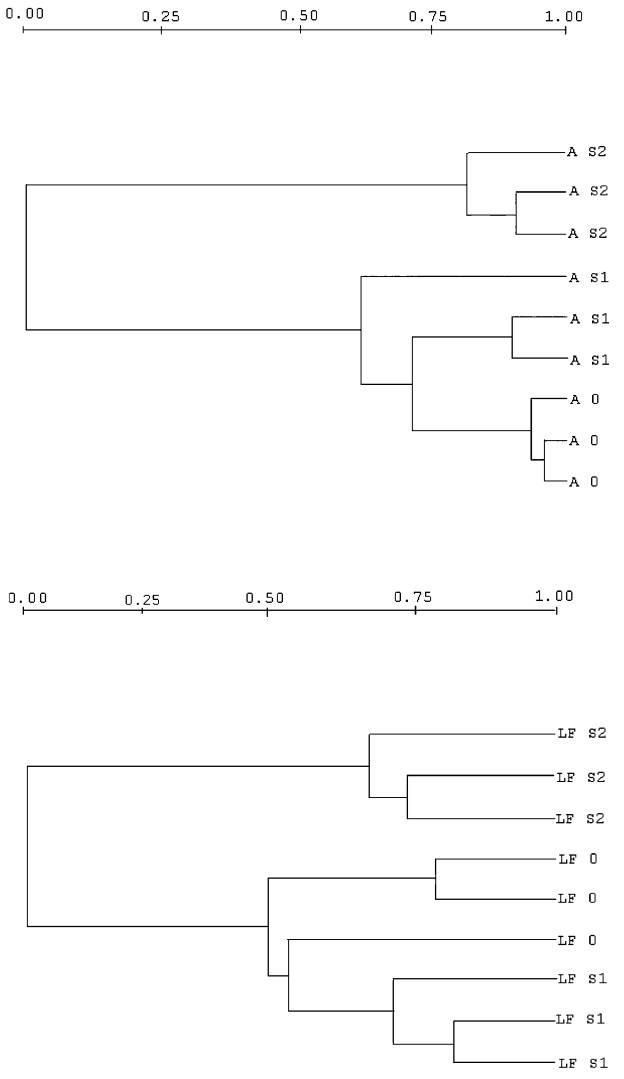

Fig. 4. Similarity dendrograms (Ward's Dice coefficient of similarity) of banding patterns generated by PCR-DGGE of 16S rDNA fragments from Ambarès (A) and Louis Fargue (LF) soils. Scale (0–1) indicates the similarity level.

4. Discussion

Data reported in Table 2 show that TOC and total N contents were the main soil characteristic differing between A S2 and LF S2 and their respective control soils. Furthermore, main characteristics of A S2 and LF S2 soils were similar and therefore, the observed differences in the studied biochemical and microbiological parameters could be ascribed to the different heavy metal contents and availabilities.

Metal fractions analysed in this work represent soluble and exchangeable forms of metals (H_2O and NH_4NO_3) and acido-soluble metal forms (citric acid and EDTA) more strongly sorbed or complexed in soil. Low molecular weight organic acids form a stabile pool of the soil organic matter, resulting mainly from rhizodeposition and microbial metabolism during soil organic matter decomposition (Stevenson, 1994) and can induce mobilization of heavy metals through complexation (Krishnamurti et al., 1997) increasing their toxicity to soil microflora (Norvell, 1991).

Our results showed that coarse sandy soils contaminated with high levels of sludge-borne Mn–Zn had no or little effects on soil biochemical parameters and bacterial diversity, although the metal mobilities increased in high sludge loaded soils, and notwithstanding total Cd concentration was little above the EU mandatory upper limit (Table 2). Previous studies on Zn speciation in A S2 soil based on EXAFS technique, showed that sludge-borne Mn can form chalcophanite (a phyllomanganate) in these soils (Manceau et al., 1998). The low amounts of soluble and exchangeable Zn and its higher mobilization by citric acid and EDTA (Table 3) could be explained by Zn sorption exerted by chalcophanite.

Differently, applications of high Cd–Ni-containing sludge reduced phosphomonoesterase, arylsulfatase and β-glucosidase activities significantly. In our study, these enzyme activities were significantly inhibited by Cd and Ni at concentrations of 13.1 and 52.3 mg kg^{-1}, respectively, (Fig. 2). These values were lower than those previously reported in experiments where sludge enriched with single metals were used. For example, Dar (1996) reported that Cd decreased soil enzyme activities at concentration of 50 mg kg^{-1} whereas Moreno et al. (1999) reported that Cd concentrations as high as 800 mg kg^{-1} affected the dehydrogenase activity but not the β-glucosidase, urease and phosphomonoesterase activities. Karaca et al. (2002) reported significant reductions of soil hydrolase activities by Cd-enriched (50 mg kg^{-1}) sewage sludge but urease activity was not inhibited. Arylsulfatase activity was the most sensitive enzyme activity as it was negatively and significantly correlated with the mobility of Cd, Ni, Mn and Zn, whereas urease and benzoyl-argininamide hydrolysing activities were the most resistant ones (Fig. 2). Moreno et al. (2003) reported that the phosphomonoesterase activity was more sensitive to contamination by Cd and Ni than urease and protease activities, with Cd being more inhibitory than Ni. Different effects of metals on enzyme activities might be due to the fact that divalent metal ions such as Mn^{2+}, Zn^{2+} or Ni^{2+}, are functional cofactors for glycosylhydrolase (Bouma et al., 1997), phosphohydrolase (Coleman, 1992), and amidohydrolase enzymes (Holm and Sander, 1997), whereas Cd^{2+} is not required for any known biochemical reaction.

In the A and LF soils, bacterial diversity was affected by higher rates of sludge in different ways. In the A soils, higher rates of Mn–Zn-rich sludge increased the diversity in A soils (Fig. 3) and the DGGE profiles of the different treatments were more related (Fig. 4), probably as a combined effect of the nutrient input and lack of metal toxicity. Differently, in the LF soils the Cd–Ni-rich sludge decreased the bacterial diversity in LF soils (Fig. 3) and DGGE profiles similarity (Fig. 4), possibly due to higher metal availability and toxicity (Table 3). Bååth et al. (1998) reported changes in the microbial community structure in soils amended with Zn- and Ni-rich sludge at concentrations of 359 and 89 mg kg^{-1} soil, respectively, without significant changes in soil respiration and B_C content.

Significantly lower enzyme activity-to-B_C (EA/B_C) ratios were found in the LF S2 soils (Table 4), in accordance with studies on different metal-contaminated soils (Tscherko and Kandeler, 1997; Aoyama and Nagumo, 1997). This might be due to both a lower synthesis and/or release of extracellular enzymes by soil microorganisms or to inhibition of extracellular enzymes. It is difficult to interpret the effect of any pollutant on soil enzyme activity because the meaning of these measurements are not clear, due to the different locations of enzymes in soils; for example, the present enzyme assays do not discriminate between extra- and intracellular enzyme activity (Nannipieri et al., 2003).

Microflora of metal-contaminated soils can have a lower metabolic efficiency compared with microflora of the respective unpolluted soils; this is generally reflected by an increase of the qCO_2 values (Brookes, 1995). However, in this case, no increase of qCO_2 values were observed as responses to the different metal total contents and availabilities and to changes in bacterial diversity (Table 4). No effects of single-metal sludge soil

Table 4
Ecophysiological indices calculated for the Ambares (A) and Louis Fargue (LF) soils

Soils	Ecophysiological indices							
	qCO_2 (mg CO_2–C mg B_C d^{-1})	B_C/TOC (%)	Hydrolase activity-to-B_C ratios					
			Alk phosph	Ac phosph	Arylsulfatase	β-Glucosidase	Protease	Urease
A 0	0.14 (0.01)	3.0 (0.2)	0.62 (0.06)	1.57 (0.09)	2.71 (0.15)	0.44 (0.08)	0.07 (0.01)	0.05 (0.02)
A S1	0.12 (0.01)	2.3 (0.1)	0.83 (0.18)	2.30 (0.12)	3.16 (0.16)	0.77 (0.06)	0.11 (0.02)	0.05 (0.01)
A S2	0.09 (0.01)	1.5 (0.1)	0.76 (0.07)	2.14 (0.16)	1.38* (0.11)	0.48 (0.10)	0.30 (0.02)	0.07 (0.01)
LF 0	0.14 (0.01)	2.7 (0.1)	3.91 (0.17)	2.74 (1.2)	4.11 (0.21)	1.15 (0.07)	0.05 (0.01)	0.06 (0.01)
LF S1	0.10 (0.01)	1.9 (0.1)	1.46* (0.08)	1.75* (0.09)	1.77* (0.14)	1.08 (0.08)	0.08 (0.01)	0.06 (0.02)
LF S2	0.08 (0.01)	1.8 (0.1)	1.09* (0.07)	0.31** (0.05)	0.99** (0.11)	0.18* (0.02)	0.09 (0.02)	0.09 (0.02)

The hydrolase activity-to-B_C ratios were calculated by dividing the enzyme units by the biomass C content of soils. One enzyme unit (EU) will release 1 μM min^{-1} of *p*-nitrophenol for phosphomonoesterase, arylsulfatase and β-glucosidase activities and μM min^{-1} of $NH_4^+ - N$ for urease and protease activities at 37 °C and at optimal pH. Values in brackets are the standard errors of the means ($n=3$). Alk phosph and Ac phosph are the alkaline and acid phosphomonoesterase, respectively. Symbols * and ** indicate significant differences at levels of $P<0.05$ and $P<0.01$, respectively, between the sludge-treated and their respective control soils. The qCO_2 is the metabolic quotient; B_C is the microbial biomass C; TOC is the total organic C.

incorporation on the qCO_2 values were reported by Filcheva et al. (1996), whereas lower qCO_2 values in metal-contaminated soils were reported by Tyler et al. (1989).

The B_C/TOC ratio has been considered a long-term indicator of the effects of heavy metals on microorganisms in soils amended with sewage sludge (Fließbach et al., 1994). In our study, a significant reduction of B_C/TOC ratio was found in soils with the higher rates of sludge application (Table 4), probably as result of the inhibition of degradation of soil organic matter (Said and Lewis, 1991), or increase of organic C from sludge.

In conclusion, our study showed that addition of sludge rich in Mn and Zn may not impact key soil biochemical functions even at high addition rates. Differently, sludge with relatively high contents of Ni and Cd at comparable rates, showed adverse effects at soil metal concentrations lower than those generally reported in literature for single-metal sludge.

Simultaneous use of molecular and classical techniques revealed that the use of both the sludge types induced some 'functionally silent' changes in the structure of bacterial communities of these soils that may in turn weaken the resistance of soil functions to further stressing factors. These results also confirm that the relationship between metal availability, microbial community composition and soil functions is only partially understood and deserves more studies.

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