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Biochemical parameters and bacterial species richness in soils contaminated by sludge-borne metals and remediated with inorganic soil amendments

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Amendments (coal fly ash, zerovalent-Fe iron grit), reduced labile fractions of Cd and Ni in contaminated soils and restored the activity of key soil hydrolases.

Abstract

The effectiveness of two amendments for the in situ remediation of a Cd- and Ni-contaminated soil in the Louis Fargue long-term field experiment was assessed. In April 1995, one replicate plot (S1) was amended with 5% w/w of beringite (B), a coal fly ash (treatment S1 + B), and a second plot with 1% w/w zerovalent-Fe iron grit (SS) (treatment S1+SS), with the aim of increasing metal sorption and attenuating metal impacts. Long-term responses of daily respiration rates, microbial biomass, bacterial species richness and the activities of key soil enzymes (acid and alkaline phosphatase, arylsulfatase, β-glucosidase, urease and protease activities) were studied in relation to soil metal extractability. Seven years after initial amendments, the labile fractions of Cd and Ni in both the S1 + B and S1 + SS soils were reduced to various extents depending on the metal and fractions considered. The soil microbial biomass and respiration rate were not affected by metal contamination and amendments in the S1 + B and S1 + SS soils, whereas the activity of different soil enzymes was restored. The SS treatment was more effective in reducing labile pools of Cd and Ni and led to a greater recovery of soil enzyme activities than the B treatment. Bacterial species richness in the S1 soil did not alter with either treatment. It was concluded that monitoring of the composition and activity of the soil microbial community is important in evaluating the effectiveness of soil remediation practices.

Keywords: Coal fly ash; Metal; Soil hydrolase; Soil microbial community; Zerovalent-Fe iron grit

1. Introduction

Cumulative applications of sewage sludge have been a major source of trace elements to agricultural soils before the establishment of statutory guidelines aiming to prevent the build-up of toxic element concentrations in soils (Commission of the European Communities (CEC), 1986). Many studies have aimed at understanding the residual effects of sludge-borne trace elements on soil biological properties (Giller et al., 1998). The presence of high metal concentrations can have significant adverse effects on whole soil microbial biomass and activity (Chander et al., 1995; Khan and Scullion, 1999; Preston et al., 2000) and soil hydrolase activities (Renella et al., 2003, 2004, 2005a,b). Moreover, these may change

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the composition of the soil microflora, and select for metal-resistant microorganisms and/or alter the bacteria-to-fungi ratio, as fungi can generally better tolerate elevated metal concentrations (Mergeay, 1995).

Sludge-borne Cd and Ni accumulated in soils at the Louis Fargue Experiment, a sewage sludge field trial started in 1976 (Juste and Mench, 1992). In April 1995, one replicate block of the Louis Fargue Experiment was amended with a coal fly ash (beringite, B, 5% by soil dry weight) and another with zerovalent-Fe (iron grit, SS, 1%) to increase metal sorption and to ameliorate metal impacts on crops (Mench et al., 2000, 2002). Data showed the beneficial effects of SS addition to the sludge-treated soils on plant yield and trace element concentrations in crops, confirming previous reports (Mench et al., 2003a). In general, the growth of Zn-tolerant grass varieties in combination with soil additives such as beringite rehabilitated the soil ecosystem and reduced metal bioavailability (Bouwman et al., 2001). In a 30-month lysimeter study, microbial biomass, dehydrogenase, aryl-sulphatase and protease activities were positively correlated with soil pH and negatively correlated with soluble trace element concentration. These were considered to be better indicators of the progress of soil remediation than β-glucosidase and acid-phosphatase activities (Pérez de Mora et al., 2005).

When evaluating the efficiency of an in situ immobilisation strategy, the following important questions need to be answered: (i) are microorganisms with the desired characteristics and activities present at the remediated site and at what densities, (ii) what is their activity level, (iii) and how is the composition and function of the microbial community influenced by changes in environmental parameters (Geets et al., 2003)? Although there is a general consensus that efficiency of soil remediation also depends on the presence and activity of soil microorganisms, the ecological consequences of inorganic amendments for these features of the microflora of metal contaminated soils have received little attention. Mench et al. (2003b) showed that SS addition to a sludge-treated soil (S1), mainly contaminated by Cd and Ni due to a total sludge input of 50 t DM ha^{-1} between 1976–1980, restored the *Rhizobium* nodule count on roots of dwarf beans from 0 to 33% compared to the control soil, after soil re-inoculation with a bean rhizospheric solution. Metal availability, microbial biomass and respiration, bacterial species richness and hydrolase activity were previously studied in the control and sludge-treated Louis Fargue soils 20 years after the last sludge application (Renella et al., 2005a). Microbial biomass C content (B_C), soil respiration (CO_2-C), and the metabolic quotient values (qCO_2) were unaffected, whereas phosphomonoesterase, β-glucosidase and arylsulfatase activities, and bacterial species richness were reduced in sludge-borne Ni-Cd contaminated soils (Renella et al., 2005a). Here we report the responses of soil microbial biomass and respiration, enzyme activities and bacterial species richness in sludge-borne Ni-Cd contaminated Louis Fargue soils 7 years after initial amendment with either beringite or zerovalent-Fe. The goal of this work was the evaluation of the soil remediation through the assessment of metal extractability and measurement of microbial and biochemical parameters using both classical and molecular techniques to study soil hydrolase activity and bacterial species richness.

2. Materials and methods

2.1. Soil characteristics and field experiments design

The Louis Fargue (LF) field experiment is located at the Couhins Experimental Farm (Villenave d'Ornon, Gironde, France). At the beginning of the 20th century, the area was a vineyard, then an *Acacia* sp. forest. 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%. The field trial was established in 1976 (Juste and Mench, 1992; Weissenhorn et al., 1995). Plots (3 m × 6 m) were replicated 5 times and randomised in a Latin square. Control soils received inorganic fertilisers whereas sludge-treated soil (S1) received a total sludge input of 50 t DM ha^{-1} between 1976–1980. Sludges were anaerobically digested, flocculated and dehydrated by heating under high pressure (Porteus process) (Juste and Mench, 1992). The chemical composition of sludges showed high Ni and Cd concentrations (Table 1), therefore, although repeated sludge applications increased concentrations of several trace elements in the sampled soil layer (0–20 cm), soil S1 was mainly contaminated by Cd and Ni (Table 2). In April 1995, one S1 plot replicate of the LF experiment was amended with 5% (w/w) beringite (B), which is a coal fly ash, and another with 1% (w/w) zerovalent-Fe iron grit (SS) (Boisson et al., 1998). Treated plots were labelled as S1 amended with B (S1 + B), and S1 amended with SS (S1 + SS). Plots were annually cropped with maize (*Zea mays* L., cv. INRA 260) during the course of the experiment (1976–present), except in the years 1997 (potato tubers), 2001 (lettuce), and 2002 (winter wheat).

Five soil samples were collected from the 0–20 cm layer in each plot, mixed and bulked into pooled samples from where three sub-samples (pseudo-replicates) were taken for analysis. This sampling strategy was adopted to reduce the number of samples to manage and transport during a large sampling campaign, although it was recognised that this could weaken the significance of the results. Soils were transported unsieved to minimise disturbance and sieved at field moisture (<2 mm) one week after sampling. The sieved soils were moistened to 50% water holding capacity (WHC) and pre-incubated at 25 °C for 7 days prior to analysis. Water addition to soils results in some dissolved organic matter. This becomes available C, capable of triggering a microbial metabolic response, which is usually erratic for the first 4–10 days after the addition. A 7-day stabilization period after soil rehydration was necessary prior to measuring the potential microbial biomass and its activity in soils.

2.2. Total metal content and extractability

Total metal content was measured following microwave-assisted (Milestone 1200) acid digestion of 0.15 g of dry finely ground soil in a 1:6 solution of HF:HNO_3 with increasing power (200–600 W for 24 min).

Table 1
Composition of sludge and additives applied to the Louis Fargue soils

	C	N	P_2O_5	Ca	Mg	K	Fe
	g kg^{-1}						
Sludge	16.8	1.26	4.2	10.6	0.29	0.12	3.65
Beringite				24.6	8.9	1.9	32.8
Iron grit	10						990
	Cd	Cr	Cu	Mn	Ni	Zn	
	mg kg^{-1}						
Sludge	1830	219	448	699	4071	3062	
Beringite	9	950	120	1100	120	630	
Iron grit	<10	3500	2000	8000	1150	105	

Table 2
Main properties of control, sludge-contaminated and amendment-treated soils from the Louis Fargue field experiment

Soil	pH (H_2O)	CEC cmol kg^{-1}	TOCg kg^{-1}	N_{tot} g kg^{-1}	B_C mg C kg^{-1}	Soil respiration mg CO_2-C kg^{-1}*d^{-1}	Cd	Cr	Cu	Fe	Mn	Ni	Zn
							mg kg^{-1}						
Control	6.4 (0.1)	3.4 (0.2)	7.4 (0.2)	0.73 (0.02)	186.1 (5.9)	6.0 (0.2)	0.3 (0.1)	6.7 (0.5)	12.4 (3.9)	1687	44.6 (2.6)	1.7 (0.2)	24.1 (0.8)
S1	6.4 (0.2)	3.1 (0.2)	8.9 (0.3)	0.76 (0.02)	172.4 (13.0)	6.9 (0.3)	13.1 (1.1)*	12.3 (1.2)	20.2 (2.9)	2604	32.4 (2.4)	52.3 (4.2)	45.4 (3.9)
S1 + B	7.0 (0.3)	4.4 (0.4)	12.1 (1.3)*	0.91 (0.11)	159.1 (10.2)	5.7 (0.3)	18.8 (2.1)*	16.2 (1.4)*	17.9 (1.2)	5936	125.8 (8.9)***	67.2 (6.0)*	89.1 (2.5)*
S1 + SS	6.4 (0.2)	3.9 (.05)	9.8 (1.7)	0.82 (0.21)	180 (16.6)	5.8 (0.4)	16.7 (2.4)*	23.1 (2.1)*	10.6 (1.7)	8924	112.4 (6.7)***	41.7 (5.4)	37.3 (1.8)

Values in brackets are the standard error of the means ($n = 3$). Symbols *, ** and *** indicate significant differences at P levels of 0.05, 0.01 and 0.001 respectively between the sludge and the amended soils and the control soil. TOC is the total organic C and N_{tot} is the total N. B_C is the soil microbial biomass C content.

Metal extractability was measured using different single-solvent extractions. Extractions with deionized H_2O were made using a 1:10 w/v extraction ratio. Extractions with 1 M NH_4NO_3 were carried out using 20 g of soil in 50 ml 1 M NH_4NO_3 according to Prüeß (1997). Extractions with 20 mM citric acid were carried out as described by Krishnamurti et al. (1997), and extractions with 40 mM Na_2-EDTA were done using a 1:10 w/v extraction ratio. Triplicate extracts were made by reciprocal shaking of soils plus extractants for 2 h at 20 turns min^{-1}. All resulting soil suspensions were filtered through Whatman 42 filter paper and extracts were acidified with 0.2 mL of 14 M HNO_3 prior to element analysis by flame or furnace atomic absorption (Perkin Elmer 5500). Water-soluble, 1 M NH_4NO_3 extractable (soluble and exchangeable), 2×10^{-2}M citric acid extractable (acido-soluble), and 4×10^{-2} M EDTA extractable (acido-soluble and chelated) fractions were thus operationally defined.

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. Evolution of CO_2 was measured by sampling the head-space gas with an air-tight syringe then injecting the gas samples into a gas-chromatograph (Hewlett-Packard 6890) equipped with a gas-sampling valve, a packed column (Poropack Q) and a thermal conductivity detector (Blackmer and Bremner, 1977). After respiration measurement, the microbial biomass C content of soils (B_C) was determined using the fumigation extraction technique (Vance et al., 1987).

2.4. Measurement of hydrolase activity

Acid and alkaline phosphomonoesterase activities were assayed according to Tabatabai and Bremner (1969), and the arylsulfatase and β-glucosidase activities according to Tabatabai (1982). Urease activity was measured using 0.5M urea as substrate in 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. Concentrations of p-nitrophenol (*p*-NP) produced in the assays of β-glucosidase, arylsulfatase, acid and alkaline phosphomonoesterase activities were calculated from a *p*-NP calibration curve after subtracting the absorbance of the controls at 400 nm wavelength. The NH_4^+-N produced by urease and N-BAA hydrolysing activities were determined using a flow injection analyzer (FIAStar, Tecator, S).

2.5. DGGE fingerprinting of soil bacterial community structure

Bacterial community structure was studied using 16S rDNA-based polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). Whole-community DNA was extracted from 0.5 g of triplicate fresh-sampled soil using a direct bead-beating extraction method (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 using the primer pair F984GC/R1378 (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 each of deoxynucleotide triphosphate at concentration of 2.5 mM each, 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 give 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 aliquot of each PCR sample was loaded onto a 6% (w/v) polyacrylamide gel 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 carried out in 1 × TAE buffer at 60 °C at a constant voltage of 100 V for 7 h. After the run, gels were stained with SYBR® Gold nucleic acid gel stain (Molecular Probes, Eugene, OR, USA) and photographed under UV light (λ = 254 nm) using a Polaroid MP-4 Land camera with Polaroid 667 black-and-white printed film.

2.6. Data analysis

Analysis of variance using the Tukey–Kramer test to assess the significance of differences ($P < 0.05$) of the means ($n = 3$) was carried out using the STATVIEW 5 computer programme (SAS Institute). Scanned banding patterns of triplicate 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 and discussion

Cadmium concentration in the S1 soil was about 12 times the current European Union (EU) statutory limit for Cd in soil with a pH range of 5–7, and just exceeded the intervention value (Table 2). Nickel concentration in the S1 soil was 1.7 times the current EU maximum value of Ni in soil. Other measured metals were all below EU limits. Different single extractants mobilised different concentrations of Cd and Ni from the tested soils (Table 3). Sludge-borne metal inputs resulted in higher extractable Cd and Ni concentrations in the S1 soil compared to the control soil (Table 3). Extractable Cd and Ni concentrations generally increased in the following order: H_2O < 1 M NH_4NO_3 < citric acid, EDTA. The acidity and complexation properties of EDTA resulted in highest extractable Cd and Ni concentrations in the S1 soil. Deionized H_2O generally extracted the lowest metal concentrations; water-extractable Cd and Ni concentrations represented 1.5% and 1.1%, respectively, of the total metal content of the S1 soil (Table 3). Beringite amendment of the S1 soil decreased both H_2O- and 1 M NH_4NO_3-extractable Cd concentrations. The Cd concentration numerically increased in citric acid and EDTA extracts from the S1 + B soil compared to the S1 soil. However, all extractable Cd concentrations, expressed as relative values, decreased because total Cd content was higher in this plot (Table 3), probably as a result of lateral contamination from adjacent plots (Boisson et al., 1998). Beringite has low Cd and Ni concentrations, but adding it 5% w/w may have also slightly contributed to soil contamination. Beringite is an alkaline material containing several minerals such as illite, ettringite, magnetite, and hematite (Vangronsveld et al., 1996; Mench et al., 2000); its incorporation into soil increased both pH and CEC in the S1 + B soil (Table 3). This may have contributed to the decrease in soluble and exchangeable Cd soil fractions. Our data confirmed previous reports showing decrease in water-soluble and exchangeable fractions of metals such as Cd and Zn in contaminated soils amended with B (Vangronsveld et al., 1996). However such pH-dependant sorption mechanisms may be less efficient in reducing acid-soluble soil fractions, as seen in the case of the extractions with citric acid and EDTA. In contrast, less Ni was extracted with citric acid and EDTA from the S1 + B soil compared with the S1 soil, whereas the H_2O- and 1 M NH_4NO_3 extractable Ni concentrations were similar. Due to the thermal desiccation of the sludge by up to 50% DM in the Porteus process, some sludge particles (<0.5 cm) were still present at sampling time, 22 years after the last sludge application to the soil; these particles may continue to contribute to soluble, exchangeable, and colloidal soil metal fractions despite application of the amendments.

Table 3
Concentrations of metals (mg kg^{-1}) extracted from control, sludge-contaminated and amendment-treated soils from the Louis Fargue field experiment

Extractants	H_2O	1 M NH_4NO_3	2 10^{-2}M citric acid	4 10^{-2}M EDTA
	Cd			
Control	BDL* (–)	0.2 (66)[a]	0.2 (66)[a]	0.2 (66)[a]
S1	0.2 (1.5)[a]	2.1 (16)[b]	7.4 (56)[b]	8.3 (63)[b]
S1 + B	0.1 (0.4)[a]	0.6 (2.5)[a]	11.4 (49)[b]	9.6 (41)[b]
S1 + SS	0.1 (0.6)[a]	0.3 (1.8)[a]	5.2 (31)[c]	4.7 (28)[c]
	Ni			
Control	BDL*	0.1 (0.4)[a]	0.7 (2.9)[a]	1.3 (5.3)[a]
S1	0.6 (1.1)[a]	2.2 (4.2)[b]	11.9 (23)[b]	20.2 (38)[b]
S1 + B	0.7 (1.1)[a]	3.8 (6.4)[c]	10.5 (18)[b]	14.7 (25)[c]
S1 + SS	0.6 (1.4)[a]	1.9 (4.5)[b]	9.5 (23)[b]	10.3 (25)[c]

*Concentrations below instrumental detection limit 0.02 and 0.1 μg L^{-1} for Cd and Ni, respectively; values in brackets are the % of total metal content. Different superscripts indicate mean values significantly different ($P < 0.05$).

The SS treatment was more efficient than B in reducing extractable Cd and Ni concentrations (Table 3). Cadmium concentrations fell by 60%, 89%, 45%, and 56% respectively in the H_2O, 1 M NH_4NO_3, citric acid, and EDTA extracts from the S1 + SS soil in relation to total soil Cd content, when compared with the S1 soil. Extractable Cd decreased in the S1 + SS soil probably as a result of two cooperative processes. Firstly, steel contains a number of metallic impurities, including Cu, Mn, Ni and Zn (Table 1). When steel shots corrode in soil, the presence of these impurities results in the creation of numerous small electrical fields. Where distances between these are small, their presence can alter the electrochemical potential gradient in the matrix and can have local effects on the rate at which charged particles diffuse. Newly formed Fe and Mn oxides after SS corrosion in soil sorb thereafter Cd (Mench et al., 2000). Recovery of hydrolase activities due to newly formed protective surfaces might also enhance hydrolysis of organic phosphates, then precipitation of Cd by phosphates, and consequent Cd redistribution in residual fractions. Nickel concentration was only 35% less in EDTA

extracts from the S1 + SS soil compared to the S1 soil (Table 3). Adding SS resulted in additional Ni input to the soil (Table 1) that may help to maintain Ni concentrations in both soluble and exchangeable fractions (Table 3). The decrease in EDTA-extractable Ni content in the S1 + SS soil cannot be easily explained. It might reflect a re-sorption by a soil fraction or Ni redistribution in residual fraction, insolubilisation with phosphates probably being involved as well. The reductions in Ni and Cd concentrations in the acid-soluble fractions of the S1 + SS soil were beneficial in terms of potential metal remobilisation due to rhizosphere acidification.

The soil TOC was higher in sludged soils, particularly in soil amended with B (Table 1), whilst the daily respiration rate and B_C were not significantly affected by sludge loads nor by B or SS amendments (Table 1). The activities of five out of six enzymes were lower in the S1 soil than in the control soil, although only reductions in alkaline and acid phosphatase and arylsulfatase activities were statistically significant (Table 4). Alkaline and acid phosphatase, arylsulfatase, β-glucosidase, and protease activities were reduced by 65%, 44%, 64%, 13%, and 7%, respectively, in the sludge-treated soil. Therefore the sludge-borne metals had a long-term impact on soil hydrolase activities after the termination of LF sludge applications. In contrast, urease activity increased by 81%. Karaca et al. (2002) reported reductions of soil hydrolase activities by Cd-enriched (50 mg kg^{-1}) sewage sludge whereas urease activity was not inhibited. Both soil treatments resulted in changes in hydrolase activity in the amended soils (Table 4). Amendment of the S1 soil with B increased alkaline and acid phosphatase, arylsulfatase, β-glucosidase, and protease activities by 12%, 19%, 14%, 2%, and 3% respectively, whereas urease activity decreased by 7%. This confirms beneficial effects on soil microflora and nematodes reported after beringite addition to a metal-contaminated soil (Bouwman et al., 2001; Bouwman and Vangronsveld, 2004). The SS treatment was even more efficient in restoration of hydrolase activity as it increased alkaline and acid phosphatase, arylsulfatase, β-glucosidase, and protease activities by 32%, 40%, 51%, 19%, and 11%, whereas its application reduced urease activity by 15% (Table 4). Decrease in enzyme activities in the S1 soil may be due to both a lower synthesis and/or release of extracellular enzymes by soil microorganisms (Renella et al., 2005a), or to direct metal inhibition of extracellular enzymes. In fact, different extracellular glycosylhydrolases, phosphohydrolases and amidohydrolases generally require divalent metals such as Mn^{2+}, Zn^{2+} or Ni^{2+} as functional cofactors (Coleman, 1992; Bouma et al., 1997; Holm and Sander, 1997), whereas Cd^{2+} is not required for any known biochemical reaction. Therefore, the recovery of the hydrolase activities observed in the B and SS amended soils could be related to the reduction of labile (e.g. H_2O-soluble and exchangeable) Cd pools. Moreno et al. (2003) found greater sensitivity of phosphomonoesterase activity than either urease or protease activity in relation to soil Cd and Ni contamination. In Fig. 1, enzyme activities were plotted against the sum of NH_4NO_3-extractable Cd and Ni. Acid and alkaline phosphatases and arylsulfatase activities decreased in relation to extractable (Cd + Ni) concentrations, with arylsulfatase activity being the most sensitive. Curves were fitted using second order polynomial equations. Additional mechanisms, not investigated in this study, may also be involved. Zerovalent iron grit added Mn to the soil (Table 1). Consequently Mn oxides were newly formed following SS corrosion and both water-soluble Mn in soil and Mn concentrations in plants increased in S1 + SS plots (Mench et al., 2000, 2002). An increase in available Mn in the S1 + SS soil could enhance Mn competition with metals (Cd, Ni) in uptake processes and as enzyme cofactors, and alleviate oxidative stress in cells. The microflora is a major extracellular hydrolase source in soils effective in N, P, and S bioconversion. The recovery of hydrolase activities in the amended S1 soils would coincide with increase in the breakdown of soil organic matter and in soluble C concentrations. The cycle of nutrients (C, N, S, P) would consequently be enhanced, elements being more available to plants. For example the hydrolysis of organic phosphate monoesters by phosphomonoesterases can account for 30–80% of P taken up by plants (Gilbert et al., 1999). In addition, SS may have produced new protective surfaces for extracellular soil enzymes during corrosion. Huang and Shindo (2000) reported that an acid phosphatase adsorbed onto Fe-oxides was not inhibited by Zn^{2+} ions.

The DGGE fingerprinting of PCR-amplified 16S rDNA extracted from the soils is shown in Fig. 2. Sludge inputs caused a slight decrease in bacterial species richness, as fewer bands are detectable in the S1 soil compared to the control soil. Renella et al. (2005a) also reported a decrease in bacterial species richness related to cumulative sludge applications in soils in the Louis Fargue Experiment. Comparison of the eubacterial community composition and activity is useful to evaluate the effectiveness of a soil remediation procedure (Geets et al., 2003). Changes in the microbial community structure in soils amended with sludge-borne Zn- and Ni (359 mg Zn and 89 mg Ni kg^{-1} soil) have been reported (Bååth et al., 1998). Similarity dendrograms, generated by the image analysis of DGGE

Table 4
Hydrolase activity in control, sludge-contaminated and amendment-treated soils from the Louis Fargue field experiment

Soils	Alkaline phosphatase	Acid phosphatase	Arylsulfatase	β-glucosidase	Urease	Protease
	mg *p*-nitrophenol kg^{-1} soil × h^{-1}				NH_4^+-N kg^{-1} soil × h^{-1}	
Control	5718.8[a]	4003.8[a]	6600.4[a]	1676.9[a]	8.2[a]	11.1[a]
S1	1982.7[d]	2242.8[c]	2391.9[d]	1460.7[a]	14.9[b]	10.3[a]
S1 + B	2220.7[c]	2668.9[c]	2726.8[c]	1489.9[a]	13.8[b]	10.6[a]
S1 + SS	2617.2[b]	3139.9[b]	3611.8[b]	1738.2[a]	12.6[b]	11.4[a]

Different superscripts indicate mean values significantly different ($P < 0.05$).

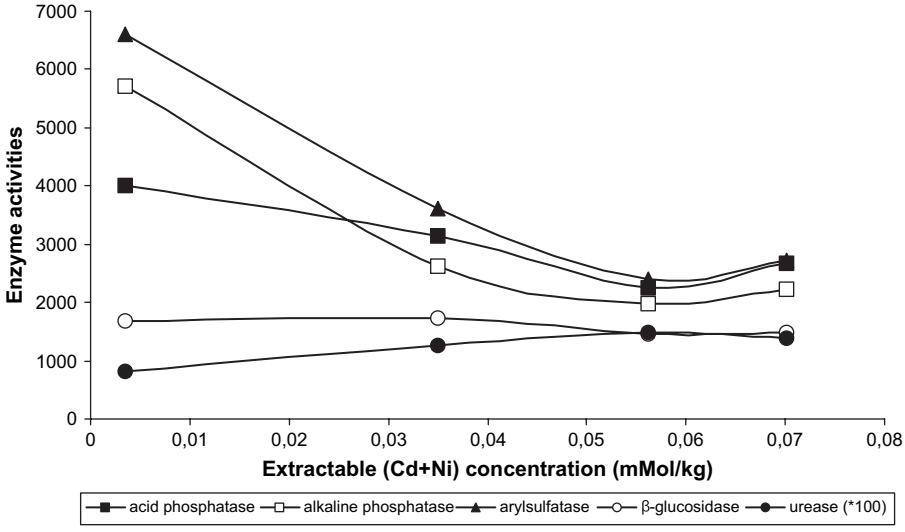

Fig. 1. Hydrolase activities in relation to the addition of extractable Cd and Ni concentrations in the soil.

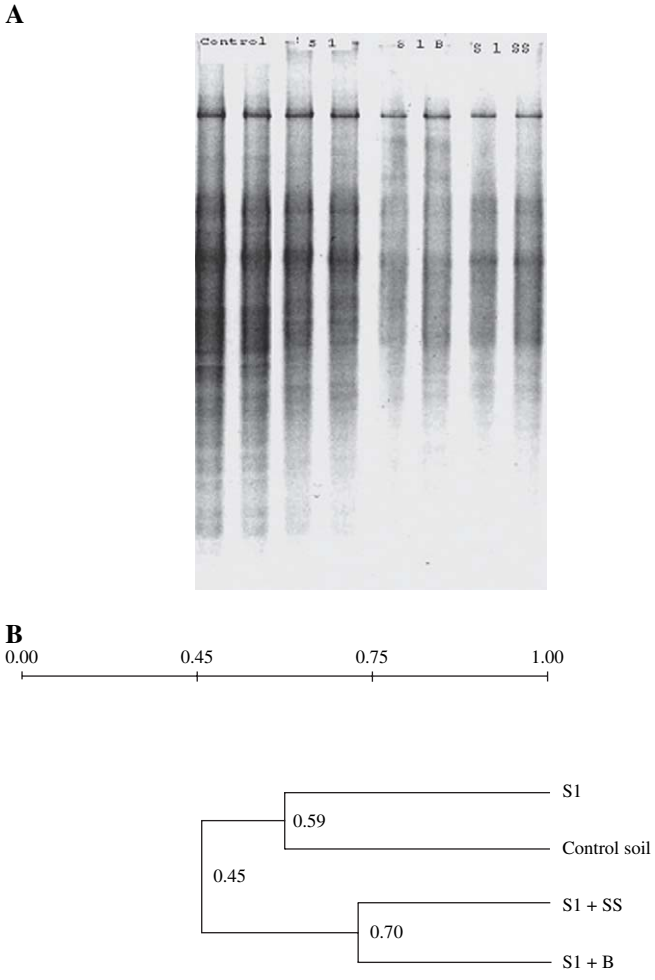

Fig. 2. Eubacterial DGGE profiles (A) and similarity dendrogram (Ward's, Dice coefficient) (B) of the control, sludged (S1) and amended (S1 + SS, S1 + B) Louis Fargue soils.

coupled with the Dice similarity coefficient, revealed that samples from control and S1 soils clustered together at 59% similarity level whereas S1 + B and S1 + SS soils were grouped on a separate cluster, being related to each other at 70% similarity (Fig. 2), so bacterial species richness seems to be unaffected by both treatments. Mench et al. (2003b) found *Rhizobium* nodules on dwarf bean roots after the reinoculation of the S1 + SS soil, whereas none occurred in plants grown in reinoculated untreated S1 soil, indicating eventual beneficial effects of SS amendment on the microflora. The lack of major differences between the DGGE profiles of the different soils was also probably due to the fact that all soils were cultivated with the same crops for the preceding 7 years. However, the characterisation of the fungal communities, the use of primers specific for bacterial sub-groups, as well as plating on different selective media suitable for cultivable sub-populations and monitoring of metal resistant microorganisms using metal-enriched culture media may provide further insights in more subtle changes within the soil microflora.

4. Conclusions

Monitoring the soil microbial community and its activity is an important management tool for evaluating the efficiency of any strategy of soil remediation, especially when applying in situ soil treatments. Untreated and remediated metal-contaminated soils from a long-term sewage sludge field experiment were investigated 7 years after soil remediation. Our data demonstrated the efficiency of in situ soil remediation using biochemical and molecular techniques. Several hydrolase activities, reduced in the Cd-Ni-contaminated soil (Renella et al., 2005a), recovered after the incorporation of inorganic amendments, in particular zerovalent-Fe iron grit. Decreases in the labile pools of Cd and, to a lesser extent, extractable Ni, which was less of a problem, 7 years after the in situ soil treatments were suggested as driving forces behind the

recovery of enzyme activities. Bacterial species richness was not affected by either remediation treatment. While the measurement of enzyme activity can be considered a direct bioassay, responding quickly to changes in labile metal pools, the composition of the eubacterial communities may respond more slowly to soil remediation practices, as this depends on complex ecological interactions. Restoration of full bacterial species richness following decrease in metal exposure and re-establishment of the fundamental ecological interactions may take time. Development of monitoring molecular tools will help to monitor spatial and temporary changes in microbial community composition and function during remediation processes (Geets et al., 2003).

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