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# Effects of Cd- and Zn-enriched sewage sludge on soil bacterial and fungal communities

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ABSTRACT

The effects of sewage sludge selectively enriched with Cd and Zn, both singly and in combination, on the bacterial, fungal, Alphaproteobacteria and Actinobacteria communities of a soil under arable or grassland management were studied with a PCR-DGGE approach. The effects of Cd and Zn were evaluated after a short time (7 d) when the Cd and Zn solubility were low and the C availability was high, and again after 180 d when the labile sludge C was mineralized and the effects of heavy metals predominated. In the arable soil all treatments induced significant short-term changes in the studied microbial groups, and long-term changes were observed in Actinobacteria and fungal communities. In the grassland soil, all treatments induced significant short-term changes in the studied microbial groups except for Alphaproteobacteria and fungi, and long-term effects on the actinobacteria and fungal communities. It was concluded that incorporation of Cd- and Zn-rich sludge into soils may have both short- and long-term effects on various bacterial phylogenetic groups whereas the metals may be better tolerated by the dominant soil fungi. In this study the impact was greater in arable than in grassland soil.



## 1. Introduction

Soils contaminated with heavy metals are found worldwide (Nriagu, 1996), due to dry and wet fall-out from the atmosphere of emitted dusts or the use of contaminated sewage sludge on agricultural lands. Because of their large production and use, Cd and Zn concentrations in soil have increased for decades (Jensen and Bro-Rasmussen, 1992a, 1992b). Although the legal limits restricting the use of contaminated waters and soil amendments in agriculture along with the control of emissions have limited the inputs, soil contamination and atmospheric deposition by Cd and Zn may rise again by improper disposal of electronic wastes (e.g. computers, TV sets, communication devices, etc.), which are often incinerated (Nordic Council of Ministers, 2003).

While most organic pollutants are eventually degraded by soil microorganisms, heavy metals are not, so they exert permanent selective pressure on soil microorganisms, depending on the extent of contamination and bioavailability. Molecular techniques based on the extraction and characterization of nucleic acids from soil have provided tools to analyze the composition of soil microbial communities (Heuer and Smalla, 1997; Torsvik et al., 1998). Reduction in microbial diversity in soils contaminated with heavy metals has been reported using both culture-dependent and molecular techniques (Nordgren et al., 1985; Bååth et al., 1998; Abaye et al., 2005). However, despite the high-resolution of fingerprinting techniques, the large richness of endogenous soil microbial communities generally results in complex fingerprintings, which cannot be quantitatively resolved, thus masking changes induced by heavy metals upon the soil microbial communities (Heuer and Smalla, 1997). The use of primer sets for specific bacterial groups can partially circumvent this bias, by reducing the complexity of DNA fingerprints and permitting detection of less abundant microbial populations (Heuer et al., 1997; Gomes et al., 2001). Generally, the Alphaproteobacteria and Actinobacteria are the most abundant and active bacterial taxonomic groups in acid arable (Courtois et al., 2001), grassland (Felske et al., 1998) and acidic forest (Lim et al., 2005) soils. However, different microbial groups may dominate in soils from different locations and managements including tropical soils (Nüsslein and Tiedje, 1999) and temperate soils under different managements (Buckley and Schmidt, 2001; MacCaig et al., 2001; Kent and Triplett, 2002; Girvan et al., 2003). The Alphaproteobacteria are generally abundant in the rhizosphere being plant-symbiotic, plant growth promoting or nitrogen fixing bacteria (McCaig et al., 1999; Gomes et al., 2001) whereas the Betaproteobacteria comprise ammonia oxidizer species involved in N

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dynamics in soils (Kowalchuk et al., 1997). Therefore, variations in the bacterial community composition may affect the plant community and soil fertility in general.

While the characterization of specific taxonomic groups of bacteria within the microbial community in soils contaminated with heavy metals may provide information about the effects of heavy metals on soil microbial community structure, knowledge is still poor. A predominance of Alphaproteobacteria in soils contaminated by Cd and Zn (Gremion et al., 2003) or Cu and Zn (Sandaa et al., 1999, 2001) has been reported previously. No effects of different heavy metals on Actinobacteria were reported by Griffiths et al. (1997) and Sandaa et al. (1999), whereas an increase in their relative abundance in Zn-polluted soils was reported by Moffett et al. (2003). Changes in various bacterial and fungal groups in soils after long-term disposal of Zn-rich sludge were reported by MacDonald et al. (2007).

Genes encoding multiple heavy metal resistance in bacteria are generally present on genetically mobile elements (e.g. plasmids, transposons), conferring metal co-resistance (Mergeay, 1995). Less information is available on the effects of metal-contaminated sludge on soil fungal communities. However, decreases in fungal diversity occur in metal-contaminated soils around smelters (Pennanen et al., 1996), whereas variable effects of heavy metals on soil fungi have been reported (Gadd, 1993; Anderson et al., 2008).

In some cases, exposure of soil microorganisms to one heavy metal may result in indirect selection of microorganisms having resistance to others. For this reason, multiple metal-contamination of soils from long-term field experiments may confound the specific effects of individual heavy metals on soil microbial community structure.

Our aim was to study the specific effects of sewage sludge selectively enriched with Cd and Zn singly and in combination on the bacterial and fungal communities and on Alphaproteobacteria and Actinobacteria. Because the dominance of different microbial phylogenetic groups is also influenced by the soil management, the effects of contaminated sludge were studied in a soil under either continuous wheat or permanent grass management. The effects of Cd and Zn were evaluated at a short time (7 d) after the incorporation and also after the labile sludge C was mineralized (180 d), as demonstrated by mineralization of the labile fraction of the organic matter was demonstrated by the constant respiration rate values of the soil respiration during the incubation reported by Renella et al. (2007), and the effects of the heavy metals predominated.

## 2. Materials and methods

### 2.1. Soils and sludge treatments

A silty clay loam Aquic Paleudalf soils (USDA, 1992) was sampled from the Highfield Long-Term Ley Arable Experiment at Rothamsted (UK), under either continuous wheat or permanent grass ley. The soil contained 27% clay ($<2$ μm soil particles) and had a pH in $H_2O$ (1:2.5) of 5.8 (ISO 10390, 1994). The main chemical characteristics of the untreated and treated soils are given in Table 1. Soils sieved were ($<2$ mm) immediately after sampling at field moisture, to minimize the impact of soil handling on soil microbial communities, moistened to 50% water holding capacity (WHC) and pre-incubated at 25 °C for 10 d prior to sludge treatments to stabilize the microbial activity prior to treatments.

An anaerobically digested sewage sludge, containing 36% organic C, 6% total N, and 2.6 Cd, 49 Cr, 588 Cu, 555 Zn (in mg $kg^{-1}$ oven dry weight), was added with deionized water or Cd, Zn or Cd+Zn solutions, dried and milled to $<1$ mm diameter. Soils amended with no metal sludge (S), Cd, Zn and Cd+Zn enriched sludge were mixed manually with the soils to achieve an even distribution of the sludge with the soil particles. Twenty grams of dried sludge per kg of soil were mixed for all treatments to simulate a final addition rate equivalent to 40 t sludge dry solids per hectare in the 0–20 cm depth, and total soil metal concentrations of: 12 Cd, 300 Zn or 12 Cd+300 mg Zn $kg^{-1}$. The unamended soils were mixed similarly. We used a relatively high sludge application rate and Cd and Zn concentrations in the sludge higher than sludge commonly produced, to detect the eventual changes in the microbial communities within the six months of incubation. Soils were incubated in triplicate for each treatment at 25 °C, in 1 L jars with separate vials containing deionized water and 1 M NaOH. After 7 and 180 d, three independent replicates for each treatment were destructively sampled and 20 g of soil from each replicate were immediately frozen (−20 °C) prior to DNA extraction.

Soil total organic C (TOC) and total N ($N_{tot}$) were measured according to Walkley & Black (1934) and a Perkin-Elmer NA 1500 elemental analyzer, respectively. The soluble P was determined according to Olsen and Sommers, 1982, and the cation exchange capacity (CEC) with the cobaltihexamine method (Orsini and Rémy, 1976). Total heavy metal content of soils and sludge were measured by microwave-assisted (Milestone 900) aqua regia digestion ($HNO_3$:HCl 1:3 ratio) at 600 W for 24 min on 0.250 g of dry, finely ground soil. Exchangeable Cd and Zn after 0, 7, 28, 70, 140 and 180 d of incubation were estimated by extraction of air dried soils with 1 M $NH_4NO_3$ (Prueß, 1998).

### 2.2. DNA extraction

Whole soil DNA was extracted using a bead beating method (FastDNA SPIN Kit for soil, Bio 101, Inc., USA) according to the manufacturer's instructions. The amount of extracted DNA was determined by fluorometry (Hoefer DyNA Quante 200) using bis-benzimide-dye (Hoechst H 33258). The quality of the extracted DNA was checked on agarose gel; high-molecular weight DNA was generally obtained.

### 2.3. Polymerase chain reaction (PCR) amplification of 16S RNA gene fragments

The PCR was performed with a DNA Thermal Cycler 480 (Perkin-Elmer Cetus, Norwalk, USA). The bacterial specific primers F984 and R1378 (Heuer et al., 1997) were used for amplification of 16S rRNA gene fragments. A GC-rich sequence was attached to the primer F984 to prevent complete melting of the PCR products during separation in the denaturating gradient gel. The PCR reaction mixture was: 1 μl template DNA (20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 4% (w/v) acetamide, 100 nM F984-GC, 100 nM R1378 and 1 U taq DNA polymerase (Stoffel fragment, Perkin-Elmer Cetus) in each 25 μl reaction vial. The PCR temperature program was: 5 min of denaturation at 94 °C, 30 cycles of 1 min at 94 °C, 1 min at 53 °C and 2 min at 72 °C; the PCR was completed by an extension step at 72 °C for 10 min.

### 2.4. Bacterial group-specific PCR

The Alphaproteobacteria 16 S rRNA gene fragment was amplified with the primer set F203α and R1494 according to Gomes et al. (2001). The PCR reaction mixture was: 1 μl template DNA (20 ng), Stoffel buffer (10 mM KCl, 10 mM

**Table 1**
Main chemical properties of the arable and grassland soils.

| Treatments | Arable soil | | | | | Grassland soil | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | TOC (%) | $N_{tot}$ (%) | Soluble P (mg $kg^{-1}$) | pH | CEC (cmol $kg^{-1}$) | TOC (%) | $N_{tot}$ (%) | Soluble P (mg $kg^{-1}$) | pH | CEC (cmol $kg^{-1}$) |
| S | 1.64[a] | 0.16[a] | 5.44[a] | 5.85[a] | 8.7[a] | 3.41[a] | 0.38[a] | 7.73[a] | 5.89[a] | 14.9[a] |
| Sl | 3.05[b] | 0.19[a] | 6.62[a] | 6.06[a] | 17.5[b] | 4.82[b] | 0.42[a] | 8.69[a] | 6.11[a] | 21.8[b] |
| Cd | 3.09[b] | 0.20[a] | 6.16[a] | 5.89[a] | 16.7[b] | 4.69[b] | 0.40[a] | 8.35[a] | 5.81[a] | 22.4[b] |
| Zn | 2.89[b] | 0.18[a] | 5.83[a] | 5.96[a] | 18.3[b] | 4.38[b] | 0.40[a] | 8.58[a] | 5.86[a] | 21.0[b] |
| Cd+Zn | 2.92[b] | 0.19[a] | 5.67[a] | 5.92[a] | 17.6[b] | 4.55[b] | 0.41[a] | 8.42[a] | 5.90[a] | 23.3[b] |

Values with different superscripts indicate significant differences ($P<0.05$) between mean values in columns.
S=soil; Sl=soil+non metal sludge; Cd=soil+Cd spiked sludge; Zn=soil+Zn spiked sludge; Cd+Zn=soil+Cd–Zn spiked sludge.

Tris–HCl, pH 8.3), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 5% (w/v) DMSO, 100 nM of the forward group-specific primer, 100 nM R1494 and 1 U taq DNA polymerase (Stoffel fragment, Perkin-Elmer Cetus) in each 25 μl reaction mix. The PCR temperature program was: 5 min of denaturation at 94 °C followed by 20 cycles of 1 min at 94 °C, 1 min at 56 °C and 2 min at 72 °C, followed by a final extension step at 72 °C for 10 min.

The actinobacterial 16S rRNA gene fragment was amplified with the primer set F243HGC and R1494 (see above), as described by Heuer et al. (1997). The PCR reaction mixture was: 1 μl template DNA (20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 5% (w/v) DMSO, 100 nM F243HGC, 100 nM R1494 and 1 U taq DNA polymerase (Stoffel fragment, Perkin-Elmer Cetus). The PCR temperature program was: 5 min denaturation at 94 °C followed by 20 cycles of 1 min at 94 °C, 1 min at 63 °C and 2 min at 72 °C, followed by a final extension step at 72 °C for 10 min.

Bovine serum albumin (0.1 mg $ml^{-1}$) was added to all group-specific PCR reactions and a hot start was performed for actinobacterial PCR. A GC clamp was added to bacterial group specific amplicons by a second amplification with bacterial primers F984-GC and R1378 (Heuer and Smalla, 1997; Heuer et al., 1997).

### 2.5. Fungal-specific PCR

The fungal 18S rRNA gene fragment was amplified using the primer set NS1 and FR1-GC for amplification of 18S rRNA fragments of 1650 bp and further fingerprint analysis was performed (Vainio and Hantula, 2000). The reaction mixture was: 1 μl template DNA (ca. 20 ng), Stoffel buffer (10 mM KCl, 10 mM Tris–HCl, pH 8.3), 0.2 mM dNTPs, 3.75 mM $MgCl_2$, 2% (w/v) DMSO (dimethyl sulfoxide), 0.2 μM of each primer (NS1 and FR1-GC) and 2 U taq DNA polymerase (Stoffel fragment, Applied Biosystems, Foster City, CA) in 25 μl reaction vial. A GC-rich sequence was attached to the primer FR1 (indicated by –GC) to prevent complete melting of PCR products during separation in the denaturing gradient gel. The DMSO was added to the reaction mixture to improve the PCR specificity and facilitate the amplification of GC-rich templates (Varadaraj and Skinner, 1994). The PCR temperature program was: 8 min denaturation at 94 °C, followed by 35 cycles of 30 s at 94 °C, 45 s at 48 °C and 3 min at 72 °C, followed by an extension step at 72 °C for 10 min.

### 2.6. Denaturing gradient gel electrophoresis (DGGE)

The DGGE of the amplified bacterial and fungal amplicons was performed using the Dcode System (Universal Mutation Detection System, Biorad). Separation of the bacterial amplicons fragments was done onto a double gradient polyacrylamide gel containing 6–9% acrylamide with a denaturant gradient comprising 27.5–56% of denaturant, in 1x Tris–acetate–EDTA buffer at constant temperature (58 °C) and voltage (220 V) for 6 h. Separation of the fungal amplicons was done onto a 7.5% polyacrylamide gel containing a denaturant gradient of 18–43% of denaturant, in 1x Tris–acetate–EDTA buffer at constant temperature (58 °C) and voltage (180 V) for 18 h. The amplicons were silver stained according to Heuer et al. (2001).

### 2.7. Analysis of DGGE community profiles

Analysis of bacterial and fungal community profiles was performed with the software package Gelcompar 4.0 program (Applied Maths, Gent, Belgium). Background was subtracted using a rolling disk method with an intensity of 10 (relative units), and the lanes were normalized. Dendrograms were constructed by the Pearson correlation index for each pair of lanes within a gel and cluster analysis by the unweighted pair group method using arithmetic averages (UPGMA). In addition, multivariate analyses of DGGE profiles of each treatment were performed based on the calculated Bray–Curtis similarities, using analysis of similarities (ANOSIM) to assess the significance of separation between microbial communities from different treatments (Ramette, 2007). The $R$ value in ANOSIM ranges from 0 to 1, where $R > 0.7$ indicates significant differences, $R > 0.5$ moderate separation and $R < 0.25$ high similarity.

## 3. Results

The soil TOC, $N_{tot}$, available P and CEC values of the control and sludge-amended soils are reported in Table 1. The total aqua regia extractable metal concentrations (mg $kg^{-1}$) of the unamended arable soil were Cd 0.51(±0.1), Cr 41(±3), Cu 25(±2), Ni 29(±1), Pb 40(±4) and Zn 80(±6), whereas those of the grassland soil were Cd 0.51(±0.1), Cr 34(±3), Cu 17(±1), Ni 26(±1), Pb 44(±3) and Zn 84(±5). The total aqua regia extractable metal concentrations (mg $kg^{-1}$) in the sludge-amended arable soil were Cd 11.0(±2) in the Cd treatment, Zn 398(±43) in the Zn treatment, Cd 13.9(±4) and Zn 412(±34) in the Cd+Zn treatment. The total aqua regia extractable metal concentrations (mg $kg^{-1}$) in the sludge-amended arable soil were Cd 13.1(±3) in the Cd treatment, Zn 382(±37) in the Zn treatment, Cd 11.3(±4) and Zn 366(±28) in the Cd+Zn treatment, respectively.

In soils amended with Cd- and Zn-enriched sludge, the $NH_4NO_3$ exchangeable Cd and Zn increased during incubation, being greater in the arable than in the grassland soil, particularly in the Cd+Zn treatment (Table 2). In the unamended soils, and soils amended with unspiked sludge, exchangeable Cd and Zn concentrations were nearly constant throughout the incubation period. In the Cd+Zn treatments, exchangeable Cd increased in the presence of Zn, whereas exchangeable Zn concentrations in the presence of Cd were similar in both the arable and grassland soils (Table 2).

**Table 2**
Exchangeable Cd and Zn concentrations in the arable and grassland soils.

| | Arable soil | | | | | Grassland soil | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Exchangeable Cd (mg $kg^{-1}$) Treatments | | | | | | | | | |
| Incubation time (d) | S | Sl | Cd | Zn | Cd+Zn | S | Sl | Cd | Zn | Cd+Zn |
| 0 | 0.020[a] | 0.012[a] | 0.54[a] | 0.024[a] | 0.57[a] | 0.004[a] | 0.008[a] | 0.072[a] | 0.011[a] | 0.17[a] |
| 7 | 0.022[a] | 0.015[a] | 0.58[a] | 0.023[a] | 0.71[a] | 0.006[a] | 0.003[a] | 0.095[a] | 0.009[a] | 0.19[a] |
| 28 | 0.025[a] | 0.023[a] | 1.10[b] | 0.019[a] | 1.10[b] | 0.005[a] | 0.009[a] | 0.200[c] | 0.013[a] | 0.29[b] |
| 70 | 0.014[a] | 0.022[a] | 1.02[b] | 0.033[a] | 0.99[b] | 0.008[a] | 0.006[a] | 0.171[b] | 0.020[a] | 0.45[c] |
| 140 | 0.011[a] | 0.018[a] | 1.08[b] | 0.035[a] | 1.12[b] | 0.003[a] | 0.006[a] | 0.221[c] | 0.017[a] | 0.46[c] |
| 180 | 0.016[a] | 0.019[a] | 1.16[b] | 0.029[a] | 1.14[b] | 0.004[a] | 0.008[a] | 0.256[c] | 0.019[a] | 0.47[c] |
| | Exchangeable Zn (mg $kg^{-1}$) Treatments | | | | | | | | | |
| Incubation time (d) | S | Sl | Cd | Zn | Cd+Zn | S | Sl | Cd | Zn | Cd+Zn |
| 0 | 0.331[a] | 0.176[a] | 0.252[a] | 18.2[a] | 16.7[a] | 0.014[a] | 0.084[a] | 0.092[a] | 2.88[a] | 2.77[a] |
| 7 | 0.315[a] | 0.165[a] | 0.339[a] | 14.0[a] | 15.7[a] | 0.031[a] | 0.082[a] | 0.090[a] | 4.14[b] | 3.81[a] |
| 28 | 0.328[a] | 0.476[b] | 0.284[a] | 19.1[a] | 19.6[a] | 0.037[a] | 0.084[a] | 0.189[a] | 5.72[b] | 5.53[b] |
| 70 | 0.403[a] | 0.474[b] | 0.262[a] | 24.1[b] | 25.7[b] | 0.055[a] | 0.089[a] | 0.195[a] | 8.97[c] | 10.6[c] |
| 140 | 0.439[a] | 0.572[b] | 0.311[a] | 30.8[b] | 38.1[c] | 0.071[a] | 0.247[c] | 0.225[a] | 11.6[c] | 14.9[d] |
| 180 | 0.411[a] | 0.564[b] | 0.302[a] | 34.8[c] | 42.2[c] | 0.025[a] | 0.162[b] | 0.209[a] | 15.4[d] | 17.2[d] |

Values with different superscripts indicate significant differences ($P < 0.05$) between mean values in columns.
S=soil; Sl=soil+non metal sludge; Cd=soil+Cd spiked sludge; Zn=soil+Zn spiked sludge; Cd+Zn=soil+Cd–Zn spiked sludge.

Extracted soil DNA was generally of high molecular weight and its DNA concentration and microbial biomass C were significantly ($r^2=0.911$; $P<0.01$) correlated.

The 16S rRNA gene fragments were amplified from all community DNA samples. Both the arable and grassland community profiles were characterized by a complex pattern, with a high number of equally abundant bands.

### 3.1. Analysis of the bacterial communities

The profiles of the bacterial community in the non-amended arable soil at 7 d after the soil amendments clustered separately, whereas there were no differences between the control and the treatments after 180 d (Fig. 1). The ANOSIM revealed that all treatment induced significant differences in the profiles after 7 d, whereas after 180 d significant differences remained between the unamended soil and Zn and Cd+Zn rich sludge treatment (Table 3).

The profiles of the bacterial community of the non-amended grassland soil clustered separately from those of all other treatments after 7 and 180 d (Fig. 1). The ANOSIM revealed that all treatments induced significant differences in the profiles after 7 d. After 180 d, the differences between the unamended soil and no metal and metal sludge were still significant, whereas the differences between the unamended soil and the Cd+Zn treatment and between the different metal-enriched sludge were not significant (Table 4).

**Table 3**
Analysis of similarities (ANOSIM) of Bray–Curtis similarity measures (R) of bacterial and fungal community fingerprints of the unamended and sludge amended arable soil after 7 and 180 d of incubation. Values in bold indicate significant differences between treatment pairs.

| Microbial groups<br>Treatment pairs | Bacteria | Alphaproteobacteria | Actinobacteria | Fungi |
|---|---|---|---|---|
| *7 d* | | | | |
| S–Sl | **1** | **0.815** | **1** | **1** |
| S–Cd | **1** | **0.963** | **1** | **1** |
| S–Zn | **1** | **0.889** | **1** | **1** |
| S–Cd+Zn | **1** | **1** | **1** | 0.556 |
| Sl–Cd | 0.370 | **0.741** | 0.519 | 0.481 |
| Sl–Zn | 0.185 | **0.778** | **0.750** | **0.963** |
| Sl–Cd+Zn | 0.222 | **0.741** | 0.481 | 0.444 |
| Cd–Zn | 0.519 | **0.852** | 0.417 | 0.556 |
| Cd–Cd+Zn | 0.667 | **0.852** | 0.222 | 0.037 |
| Zn–Cd+Zn | 0 | 0.333 | 0 | 0.333 |
| *180 d* | | | | |
| S–Sl | 0.630 | 0 | **0.778** | 0.667 |
| S–Cd | 0.556 | 0 | **0.852** | 0.083 |
| S–Zn | **0.704** | 0.519 | **1** | **0.917** |
| S–Cd+Zn | **0.926** | 0.481 | **0.963** | **0.750** |
| Sl–Cd | **0.778** | 0 | 0.593 | 0 |
| Sl–Zn | 0.556 | 0.222 | 0.556 | 0.333 |
| Sl–Cd+Zn | 0.444 | 0.296 | 0.519 | 0.185 |
| Cd–Zn | 0.667 | 0.037 | **1** | 0.111 |
| Cd–Cd+Zn | 0.667 | 0 | **1** | 0 |
| Zn–Cd+Zn | 0.556 | 0.296 | **0.741** | 0.519 |

S=soil; Sl=soil+uncontaminatd sludge; Cd=soil+Cd spiked sludge; Zn=soil+Zn spiked sludge; Cd+Zn=soil+Cd–Zn spiked sludge.

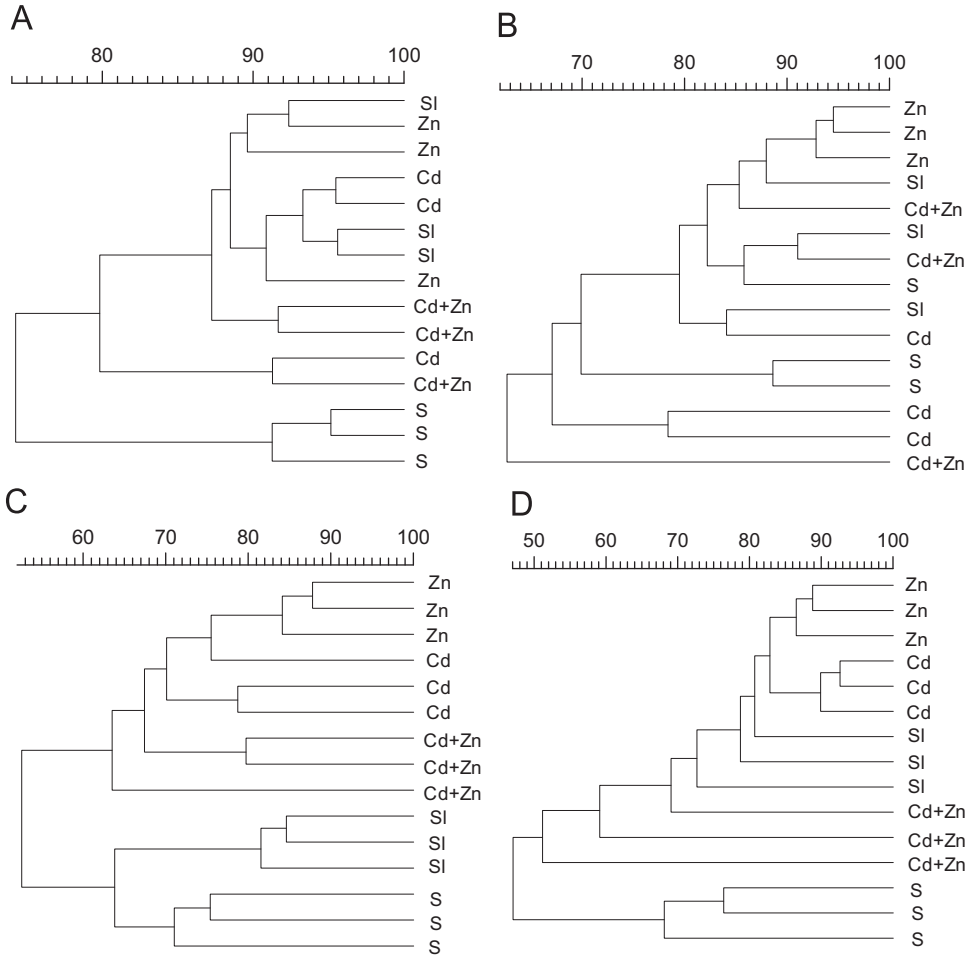


**Fig. 1.** Cluster analysis of the Bacterial DGGE profiles of the arable and grassland soils treated or not with sludge after 7 and 180 d of incubation. A and B are the profiles of the arable soil after 7 and 180 d, and C and D are the profiles of the grassland soil after 7 and 180 d, respectively.

**Table 4**
Analysis of similarities (ANOSIM) of Bray–Curtis similarity measures (R) of bacterial and fungal community fingerprints of the unamended and sludge amended grassland soil after 7 and 180 d of incubation. Values in bold indicate significant differences between treatment pairs.

| Microbial groups<br>Treatment pairs | Bacteria | Alphaproteobacteria | Actinobacteria | Fungi |
|---|---|---|---|---|
| *7 d* | | | | |
| S–Sl | **1** | 0.407 | **0.926** | 0 |
| S–Cd | **1** | 0.556 | **0.833** | 0.296 |
| S–Zn | **1** | 0.111 | **0.852** | **0.852** |
| S–Cd+Zn | **1** | 0 | **0.963** | 0.296 |
| Sl–Cd | **0.926** | **0.741** | 0.583 | 0.222 |
| Sl–Zn | **0.778** | 0.222 | 0.519 | 0 |
| Sl–Cd+Zn | **1** | 0.593 | **1** | 0 |
| Cd–Zn | **0.963** | 0 | **1** | 0.259 |
| Cd–Cd+Zn | 0 | 0.407 | **0.917** | 0.148 |
| Zn–Cd+Zn | **0.926** | 0.074 | **1** | 0 |
| *180 d* | | | | |
| S–Sl | **0.852** | **0.852** | **0.889** | 0.333 |
| S–Cd | **1** | 0.333 | **1** | 0.074 |
| S–Zn | **1** | 0.333 | **1** | **0.778** |
| S–Cd+Zn | 0.593 | 0.222 | **1** | 0.556 |
| Sl–Cd | 0.167 | 0.407 | **0.926** | 0.296 |
| Sl–Zn | 0.259 | 0.111 | 0.444 | 0.481 |
| Sl–Cd+Zn | 0.444 | 0.074 | **0.815** | 0.037 |
| Cd–Zn | 0 | 0.111 | 0.667 | 0.444 |
| Cd–Cd+Zn | 0.083 | 0.148 | **0.926** | 0.148 |
| Zn–Cd+Zn | 0 | 0 | 0.630 | 0.111 |

S=soil; Sl=soil+non metal sludge; Cd=soil+Cd spiked sludge; Zn=soil+Zn spiked sludge; Cd+Zn=soil+Cd–Zn spiked sludge.

### 3.2. Analysis of the Alphaproteobacteria and Actinobacteria

The analysis of the Alphaproteobacterial community of the arable soil showed that all treatments had different effects after 7 d, as all treatments clustered separately, whilst the similarity of the profiles increased after 180 d (Fig. 2). The ANOSIM confirmed that all treatments induced significant differences in the profiles of Alphaproteobacterial communities immediately after the treatments whereas no significant differences were found after 180 d (Table 3).

In the grassland soil the treatments did not affect the Alphaproteobacterial community after 7 and 180 d (Fig. 2). However, the ANOSIM revealed significant differences between the profiles of the unamended soil and that of the Cd treatment after 7 d and between the fingerprints of the unamended soil and that of soil amended with uncontaminated sludge after 180 d (Table 4).

All treatments affected the actinobacterial community of the arable soil after 7 and 180 d, as all treatments clustered separately compared to the unamended soil (Fig. 3). The ANOSIM confirmed that differences between the unamended soil and all sludge-amended soils were significant after 7 and 180 d, with a significant effect of Zn after 7 d as compared to the no metal sludge and significant differences between Cd and Zn singly or in combination after 180 d (Table 3).

In the grassland soil, the actinobacterial community was influenced by all treatments after 7 and 180 d, as all treatments clustered separately from the unamended soil (Fig. 3). The ANOSIM confirmed that differences between the unamended soil

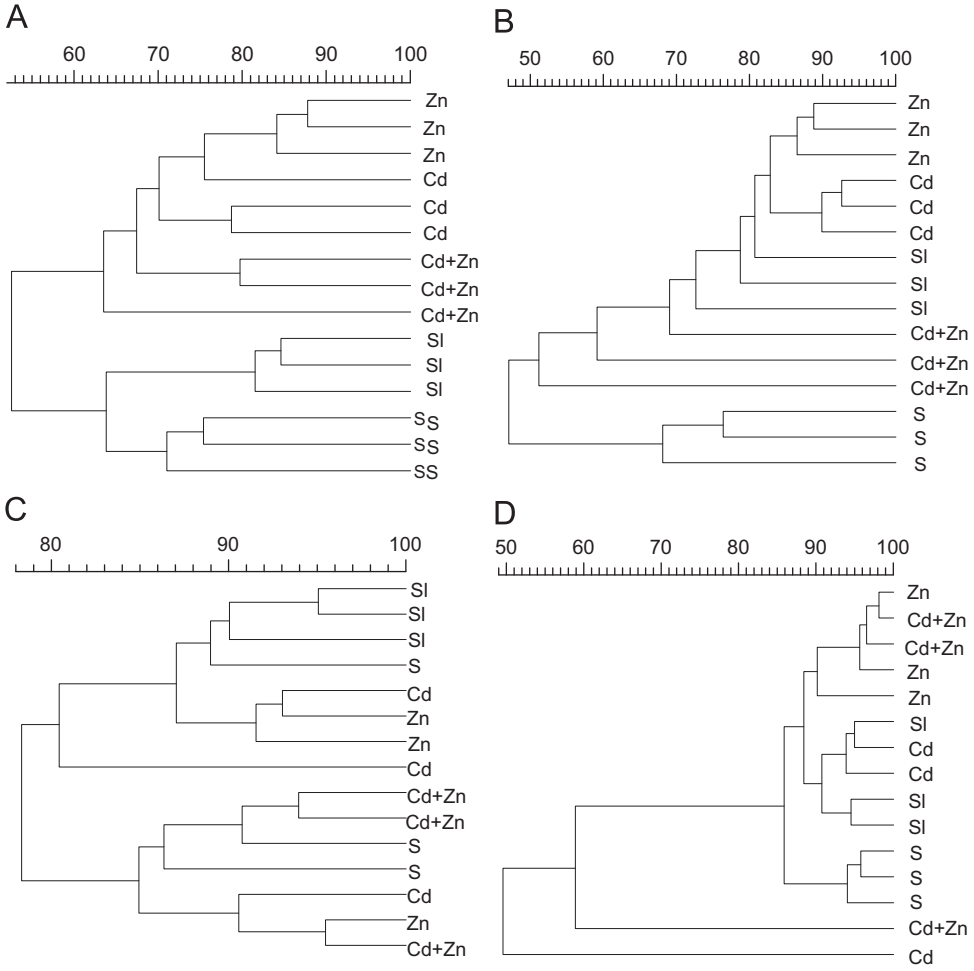


**Fig. 2.** Cluster analysis of the Alphaproteobacterial DGGE profiles of the arable and grassland soils treated or not with sludge after 7 and 180 d of incubation. A and B are the profiles of the arable soil after 7 and 180 d, and C and D are the profiles of the grassland soil after 7 and 180 d, respectively.

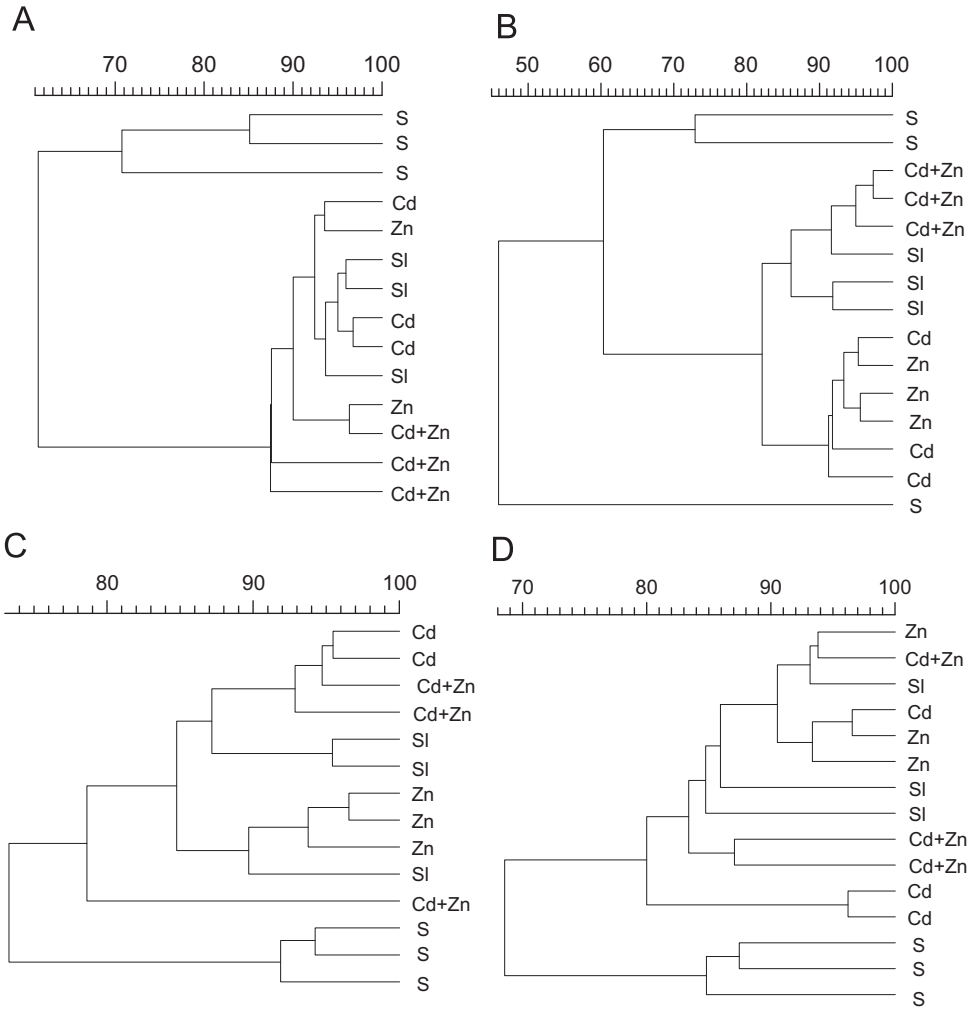


**Fig. 3.** Cluster analysis of the actinobacterial DGGE profiles of the arable and grassland soils treated or not with sludge after 7 and 180 d of incubation. A and B are the profiles of the arable soil after 7 and 180 d, and C and D are the profiles of the grassland soil after 7 and 180 d, respectively.

and all sludge-amended soils were significant, with significant heavy metal effects in most treatments after 7 d, and with Cd after 180 d (Table 4).

### 3.3. *Analysis of the fungal community*

The fungal communities of the arable soil after 7 d were affected by the no metal sludge, Cd and Zn treatments (Fig. 4). The ANOSIM revealed significant differences in the no metal sludge, Cd and Zn treatments, whereas the difference between the unamended soils and the Cd+Zn treatments was not significant (Table 3). Significant effects of Zn were observed either after 7 or 180 d as compared to the unamended soil (Table 3). In the grassland soil, the fungal community was less influenced by all the treatments after 7 and 180 d (Fig. 4), although significant effects of Zn after 7 and 180 d were revealed by the ANOSIM, compared to the profiles of the unamended soil (Table 4).

## 4. Discussion

The DGGE profiles of the unamended soils after 7 and 180 d showed high similarity, demonstrating that the incubation conditions did not significantly affect the microbial communities and that the complexity of the microbial communities inhabiting grassland soils is higher than in arable soil. This is in accordance with previous research on soil microbial communities under different management (Girvan et al., 2003; Garbeva et al., 2003). We also found a short-term (7 d) impact of the unspiked sludge on selected microbial groups (S vs. Sl treatments, Tables 3 and 4) in both soils, and a permanent effect of the unspiked sludge on the microbial communities of the grassland soil, confirming the importance of organic matter addition on soil microbial communities (Watson et al., 2002). The effects of the sludge-borne organic matter is mostly related to the beneficial effects on the soil physical conditions (Epstein, 1975) and at moderate contamination levels the effects induced by C and nutrient supply dominate possible toxicity effects (Griffiths et al., 2005). More research on gene expression in soil microbial communities is needed to verify this hypothesis.

The taxon-specific primer sets allowed the evaluation of the effects of Cd and Zn added with sludge on specific bacterial populations. The ANOSIM showed that in the arable soil the studied microbial groups were significantly impacted more by the sludge amendments than in the grassland soil, either in the short-term or in the long-term (Tables 3 and 4). The Alphaproteobacteria were affected differently by all treatments whereas the Actinobacteria and fungi were affected by the Zn-rich sludge (Table 3). The Alphaproteobacteria have been suggested to be involved in soil bacterial community adaptation to heavy metal

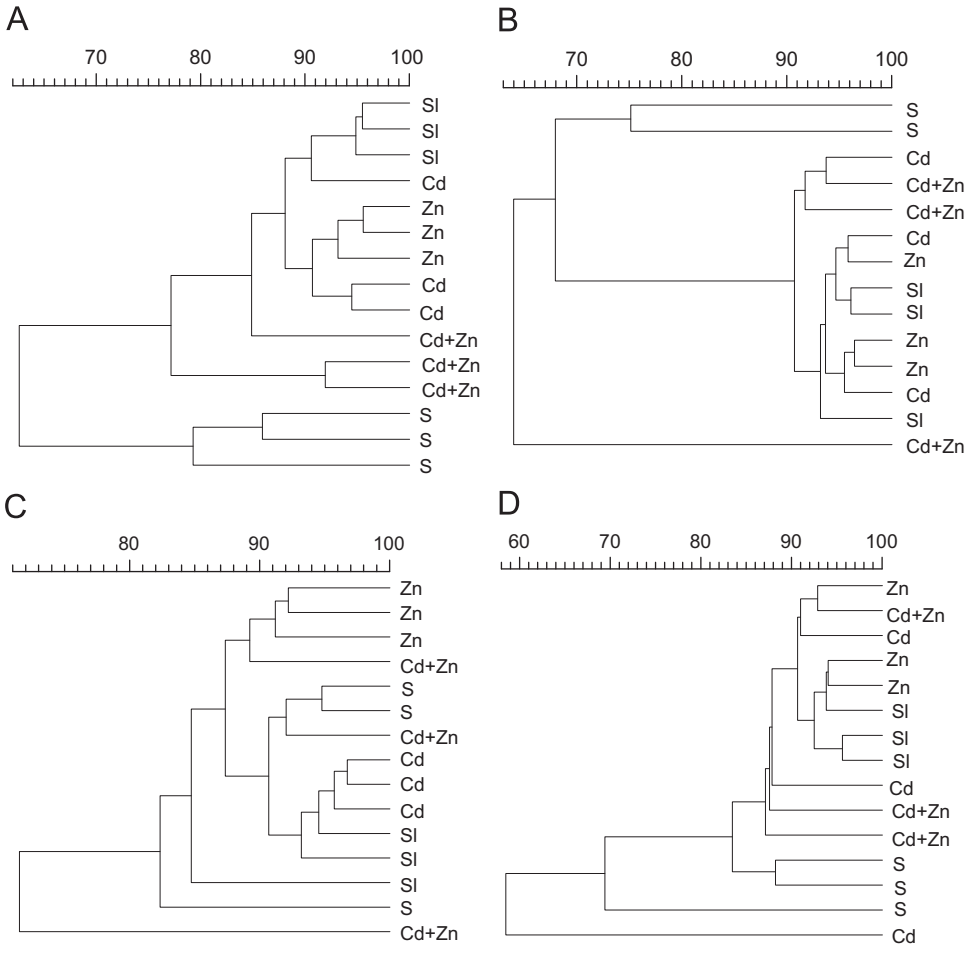


**Fig. 4.** Cluster analysis of the fungal DGGE profiles of the arable and grassland soils treated or not with sludge after 7 and 180 d of incubation. A and B are the profiles of the arable soil after 7 and 180 d, and C and D are the profiles of the grassland soil after 7 and 180 d, respectively.

contamination (Sandaa et al., 1999, 2001; Nazarét et al., 2003). However, Griffiths et al. (1997) and Sandaa et al. (1999) reported no effects on the profiles of the actinobacterial community of soils contaminated by metal-rich sludge incorporation. Despite this, our results do not exclude the possibility that actinobacteria species might be metabolically active in high-metal soils, as reported by Gremion et al. (2003) and Moffett et al. (2003). Wenderoth and Reber (1999) reported that after long-term application of high-metal sewage sludge to arable soils, Gram-negative bacteria dominated over Gram-positive ones, but there was a loss of catabolic activity towards selected organic compounds, compared to isolates from uncontaminated soils.

Slight changes between control soil and arable and grassland soils amended with no metal sludge, and the lack of differences between fungal communities induced by specific heavy metals showed that the fungal community composition was influenced by the quality and amount of sludge soil applications, but not by Cd and Zn at the concentrations applied by us. Greater effects on the soil fungal community of Zn-enriched sludge application compared to the bacterial communities were reported by MacDonald et al. (2007), whilst greater effects of Cu than Cd and Zn on fungal communities of sludge-amended soils were reported by Anderson et al. (2008). We added comparatively small concentrations of heavy metals, other than the intended concentrations of Cd and Zn to the soils, present as contaminants in the sludges (e.g. Cu 23 mg $kg^{-1}$ soil; Pb 3 mg $kg^{-1}$ soil). On the whole, our results showed that Cd at higher concentrations and Zn at maximum allowed soil concentration did not affect the composition of the soil fungal communities.

Incorporation of Cd- and Zn-enriched sewage sludge caused microbial stress (i.e. higher $qCO_2$), with no decrease in soil microbial biomass (Renella et al., 2007). It has been postulated that stress or changes in ecosystems may result in changes in biodiversity (Kennedy and Smith, 1995). Changes in soil microbial community structure associated with indications of microbial stress determined from respiration measurements in soils amended with high metal sewage sludge were reported by Witter et al. (2000).

The specific effects of Cd and Zn on the studied microbial groups may be related to the exchangeable, i.e. available, of these elements in the studied soils. In both soils amended with Cd- and Zn-enriched sludge the $NH_4NO_3$ exchangeable Cd and Zn increased during incubation, particularly in the Cd+Zn treatment (Table 2). Trace elements in sludge are mostly associated with the surfaces of soil particles (McGrath and Cegarra, 1992). Therefore, the increase in exchangeable Cd and Zn during incubation may be due to their release upon the mineralization of the organic matter in the sludge and possibly due to the effects of the solubilizing activity of the microbial metabolites (Majewska et al., 2007). However, while significant correlations have been reported between the concentrations of metals taken up by plants and the exchangeable heavy metals in soils (Prueß, 1998), the effects of metals on specific microbial groups are less clear. The fact that the other soil properties did not change was also important because, in moderately contaminated soils, differences in initial soil properties, such as pH can affect the mobility of the elements,

thus influencing the soil microbial activity and community structure (Dahlin et al., 1997). Changes in the microbial community structure of soils amended with sludge containing Zn at similar concentrations of our study have been reported (Bååth et al., 1998), whereas Cd, as the sole contaminant, was reported to have few effects on the bacterial community (Renella et al., 2005).

## 5. Conclusions

Our work showed that although the incorporation of sludge into soil is an acceptable agricultural practice from the viewpoint of nutrient recycling in the agro-ecosystems, highly metal-contaminated sludge application may affect the soil microbial communities through increased inputs of potentially heavy metals. In particular, our results suggest that amendment of soils with sludge contaminated by Cd and Zn may have both short- and long-term effects on various bacterial phylogenetic groups whereas the soil fungi are less affected.

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## Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2010.07.027.

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