Impacts of heavy metal contamination and phytoremediation on a microbial community during a twelve-month microcosm experiment

Fabienne Gremion [a], Antonis Chatzinotas [a,*], Karin Kaufmann [a], William Von Sigler [b], Hauke Harms [a]

[a] *Swiss Federal Institute of Technology Lausanne (EPFL), ENAC, ISTE-Laboratory of Soil Science (LPE), CH-1015 Lausanne, Switzerland*
[b] *Department of Earth Ecological and Environmental Sciences, 2801 W. Bancroft MS 604, University of Toledo, Toledo, OH 43606, USA*

Abstract

The effects of heavy metals and phytoextraction practices on a soil microbial community were studied during 12 months using a hyperaccumulating plant (*Thlaspi caerulescens*) grown in an artificially contaminated soil. The 16S ribosomal RNA genes of the *Bacteria* and the β-*Proteobacteria* and the *amoA* gene (encoding the α-subunit of ammonia monooxygenase) were PCR-amplified and analysed by denaturing gradient gel electrophoresis (DGGE). Principal component analysis (PCA) of the DGGE data revealed that: (i) the heavy metals had the most drastic effects on the bacterial groups targeted, (ii) the plant induced changes which could be observed in the *amoA* and in the *Bacteria* 16S rRNA gene patterns, (iii) the changes observed during 12 months in the DGGE-patterns of the planted contaminated soil did not indicate recovery of the initial bacterial community present in the non-contaminated soil. The potential function of the microbial community was assessed recording community level physiological profiles (CLPP) and analysing them by PCA. The lower capability of the bacterial community to degrade the substrates provided in the BIOLOG plates, in particular the amino acids, amides and amines, as well as a delay in the average well colour development (AWCD) differentiated the bacterial community of the contaminated samples from that of the non-contaminated ones. However, the plant had a positive effect on substrate utilization as shown by the greater number of substrates used in all planted samples compared to unplanted ones. Finally, the measurement of the potential ammonia oxidation indicated that ammonia oxidising bacteria were completely inhibited in the contaminated soil. The stimulation of ammonia oxidation by the plant observed in the non-contaminated samples was surpassed by the inhibitory effect of the heavy metals in the contaminated soil. This study emphasises the combined use of culture-independent techniques with conventional methods to investigate the ecology of bacteria in their natural habitats.
© 2004 Federation of European Microbiological Societies. Published by Elsevier B.V. All rights reserved.

Keywords: Heavy metal contaminated soil; DGGE; CLPP; Potential ammonia-oxidation; *Thlaspi caerulescens*; Rhizosphere

1. Introduction

Heavy metal contamination of soils originating from agricultural (e.g., fertilizers and sewage sludge) or industrial activities (e.g., metal mining and smelting) is one of the major environmental problems in many parts of the world. The resulting damage is difficult to cure as metals cannot be chemically degraded [1]. Heavy metals affect all groups of organisms and ecosystem processes, including microbial activities [2–4]. Remediation approaches such as excavation and land fill, thermal treatment, electroreclamation and soil capping have been proposed depending on the extension, depth and kind of contamination, but all are expensive and environmentally destructive [5]. Phytoextraction, the use of plants to extract metals from soil, has been reported to be very efficient for cleaning up superficially contaminated soils [1,6–8]. This alternative remediation technique is promising as it is cheaper and less invasive than

traditional methods [9,10]. In particular, the hyperaccumulator plant *Thlaspi caerulescens* is a good candidate for the phytoextraction of Zn and Cd from contaminated soils as it can accumulate more than 1% Zn and 0.1% Cd in its shoots on a dry-weight basis [11–13]. The potential of microorganisms to enhance phytoremediation processes and the exact mechanism by which bacteria could enhance heavy metal accumulation in plants have recently received some attention [14,15]. Despite the great interest in improving the efficiency of metal hyperaccumulating plants, their influence on microorganisms has been rarely investigated [16–18]. Molecular fingerprinting techniques such as PCR in combination with denaturing gradient gel electrophoresis (DGGE) offer new approaches to the study of microorganisms in their habitat as they account for as yet uncultured organisms [19]. They are an important complement to conventional methods that require cultivation or that measure bacterial activities.

The aim of this study was to determine the impact of heavy metals and phytoextraction practices on microbial community structure at different levels of resolution and, more specifically, on a representative key soil function (ammonia oxidation). We were particularly interested in ammonia oxidising bacteria as these important catalysts of an essential step in the N cycle are known to be very sensitive to heavy metal pollution. For this purpose, we combined the two complementary fingerprinting techniques denaturing gradient gel electrophoresis (DGGE) and community level physiological profiles (CLPP) with the measurement of the potential ammonia oxidation rates. The resolution of the DGGE-approach was varied by targeting either the 16S rRNA gene (of the total bacterial community or the β-*Proteobacteria*) or the *amoA* gene (encoding the α-subunit of ammonia monooxygenase) as an alternative functional marker.

2. Materials and methods

2.1. Soil characteristics and sample preparation

The soil was kindly provided by the Swiss Federal Institute for Forest, Snow and Landscape Research (Birmensdorf, Switzerland). It was loamy with 15.1% clay, 49.4% silt and 35.5% sand. pH values and amounts of total and soluble heavy metals are presented in Table 1. Heavy metal dust (Zn [755 mg kg^{-1}], Cu [85 mg kg^{-1}], Cd [3 mg kg^{-1}]) was obtained from the air filters of a brass-smelter in Dornach (Switzerland) and mixed (3.7 g dust kg^{-1} soil) into one part of the sieved soil (<2 mm). The rest remained non-contaminated. Both soils were wetted to 80% of their water-holding capacity, stored at 10 °C in the dark and mixed weekly for three months.

Table 1
Mean values of pH and heavy metal concentrations in the contaminated soil and in the shoots of *Thlaspi caerulescens* used in the pot experiment with contaminated soil (X±SD)

Sampling time	pH$_{H2O}$ (±0.05)	Total concentrations in soil (HNO$_3$-extractable) (mg kg^{-1})			Soluble concentrations in soil (NaNO$_3$-extractable) (µg kg^{-1})			Total concentrations in shoots (mg kg^{-1})			Metal uptake by shoots[a] per kg soil (µg kg^{-1})		
		Zn$_{tot}$	Cd$_{tot}$	Cu$_{tot}$	Zn$_{sol}$	Cd$_{sol}$	Cu$_{sol}$	Zn	Cd	Cu	Zn	Cd	Cu
Time 0	n.d.[b]	3400±50	10.2±0.7	550±10	4900±200	21±2	370±11						
Unplanted													
3 months	7.2	n.d.	n.d.	n.d.	5100±500	27±6	670±50						
6 months	6.8	n.d.	n.d.	n.d.	6700±300	36±4	630±50						
9 months	7.2	n.d.	n.d.	n.d.	5100±100	32±2	800±190						
12 months	6.9	3300±100	8.8±0.7	560±10	6700±600	36±3	840±70						
Planted													
3 months	7.5	n.d.	n.d.	n.d.	3600±100	13±1	990±50	6400±1100	590±80	25±1	10000±1800	940±120	40±1
6 months	7.1	n.d.	n.d.	n.d.	4500±400	15±2	870±50	7000±180	1150±50	19±2	4500±100	750±30	12±1
9 months	7.5	n.d.	n.d.	n.d.	3700±250	13±1	990±200	5900±1800	870±440	18±5	3300±1000	480±240	10±3
12 months	7.3	3400±50	7.2±0.4	550±10	5000±350	16±2	940±50	6200±2000	680±370	18±2	1600±500	170±90	5±1

Total concentrations in shoots were further used for the calculation of metal uptake.
[a] Plant dry weight was 3.2 g at 3 months, 1.3 g at 6 months, 1.1 g at 9 months, 0.5 g at 12 months. Pots contained 2 kg soil.
[b] Not determined.

Pot experiments were carried out in plastic flower pots containing 2 kg of soil.

2.2. Plant growth conditions

Thlaspi caerulescens seeds were obtained from a population grown near an ancient Pb/Zn mine in Saint-Laurent-le Minier, Southern France. They were stored at 4 °C before use. The seeds were germinated on non-contaminated garden soil for 25 days. One seedling of *T. caerulescens* was subsequently transplanted into three pots containing contaminated and three containing non-contaminated soil. The plants were grown for three months in a climate chamber at 20 °C during daytime and 16 °C during night time with 16 h of light alternating with 8 h of darkness. Three unplanted pots were filled with contaminated soil and three with non-contaminated soil and also incubated in the climate chamber. The pots were watered with 100 ml deionized water every 4 days.

2.3. Heavy metal analysis

Total heavy metal content (extracted with 2 M HNO_3) and the soluble heavy metal fraction (extracted with 0.1 M $NaNO_3$) of the contaminated soil were measured according to the Swiss law recommendations [20]. Plant shoots were collected, oven-dried at 70 °C, weighed and ground in a tungsten Retsch mill (Haan, Germany). They were subsequently digested according to Hammer and Keller [21] to analyze heavy metal content. All soil and plant heavy metal concentrations were determined with Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Perkin–Elmer Plasma 2000) and all samples were run together with certified reference materials.

2.4. Sampling

Every 3 months, for 12 months, plants and soil were removed from pots and each plant was shaken carefully to remove the bulk soil. The soil still adhering to the roots, defined as rhizosphere soil, was separated from the roots by moderate agitation in 50 ml of sterile 0.9% NaCl solution for 5 min and then centrifuged at 8000*g* for 10 min [22]. Samples from unplanted pots were used as controls. Subsamples (0.6 g wet weight) were filled in 2-ml cryotubes and stored at –20 °C until further use. After sampling, pots were refilled with soil and and planted with fresh seedlings.

2.5. DNA extraction

Total soil DNA was extracted from 0.6 g (wet weight) samples of each of the three rhizosphere and unplanted soil samples from contaminated or non-contaminated pots using the protocol of Griffiths et al. [23] with some modifications. Briefly, 0.5 ml of CTAB buffer, 0.5 ml of phenol-chloroform-isoamylalcohol (25:24:1) and 0.75 g 0.1-mm glass beads were added to each sample. The mixture was shaken twice for 45 s in a Fastprep bead-beater (Bio 101) at 4 m s^{-1}. Samples were stored on ice for 1 min between each run. The lysed sample was then centrifuged (16,000*g*) for 5 min at 4 °C. An equal volume of chloroform-isoamylalcohol (24:1) was added to the aqueous phase to remove the remaining phenol. The supernatant was subsequently incubated for 2 h at room temperature with 2 volumes of the precipitation solution (30% polyethylene glycol 6000 in 1.6 M NaCl). DNA was pelleted by centrifugation at 18,000*g* for 10 min at 4 °C, washed once with ice cold 70% ethanol, and resuspended in 50 μl TE (pH 7.4). One microliter of the DNA solution was used as template for PCR amplification.

2.6. PCR amplification

PCR amplification of 16S ribosomal RNA genes was performed with a PTC-200 thermal cycler (MJ Research). The 50-μl PCR mixture contained 0.2 μM of each primer, 200 μM of each deoxynucleoside triphosphate, 1× PCR buffer (Qiagen), 2.5 mM $MgCl_2$, 4.95 mg ml^{-1} BSA, and 1 U Taq Polymerase (Qiagen). To minimize non-specific annealing of the primers to non-target DNA, Taq Polymerase was always added after an initial denaturing step at 94 °C for 5 min. Bacterial 16S rRNA genes were amplified with the primer combination 341f-GC and 907r according to Muyzer et al. [24] and the following program: 30 cycles at 94 °C for 1 min, 58 °C for 1 min, 72 °C for 2 min and a final extension at 72 °C for 10 min. A semi-nested PCR was used for amplification of 16S rRNA genes of β-*Proteobacteria* with initial amplification with (20 cycles) with primers 948f and 1492r [25] following the protocol of Gomes et al. [26] and reamplification with 1 μl of the first PCR amplification using the primer pair 984f-GC and 1492r according to Heuer et al. [27]. A nested PCR was applied for the amplification of *amoA* genes, with initial amplification with the primer set amoA-2f and amoA-5r [28] followed by a nested amplification using the primer set amoA-1f and amoA-2r [29], resulting in a 491-bp fragment. The thermal profiles used for amplification followed the protocols of Avrahami et al. [30]. Subsequently, the PCR products were quantified in a fluorimeter (Turner Designs, TD-700, Witec) using the PicoGreen nucleic acid dye (Molecular Probes, Inc.).

2.7. Denaturing gradient gel electrophoresis

DGGE analysis was performed with the D-Code system (Bio-Rad Laboratories) using the 16 cm × 16 cm × 1 mm plates for the 16S rRNA gene PCR-products of *Bacteria* and β-*Proteobacteria* and the 20 cm × 20

cm × 1 mm plates for *amoA* PCR-products. Four hundred nanograms of each PCR product was loaded onto 6% (wt/vol) polyacrylamide gels (acrylamide/bisacrylamide ratio [37.5:1]) (*Bacteria* and β-*Proteobacteria*) or onto 8% (wt/vol) polyacrylamide gels (*amoA*). Gels were poured at a flow rate of 14 ml min^{-1} using a two chamber gradient mixer linked to a peristaltic pump (Ismatec). The gradient of denaturant ranged from 35% to 58% (*Bacteria*), 43% to 58% (β-*Proteobacteria*) or 49% to 63% (*amoA*) where a 100% denaturing solution is defined as 7 M urea and 40% formamide. A 5-ml stacking gel (0% denaturant) was added to the top of the denaturing gel. Gels were run in 1× TAE buffer (pH 7.4) at 60 °C, for the first 15 min at 30 V, and subsequently for 14 h at 70 V. The reproducibility of the results and the analysis method was checked by loading PCR amplifications of three different extractions per treatment and triplicates within one extraction. To compare the patterns of all different treatments on a single denaturing gel, only one PCR product amplified from the DNA from one pot was finally loaded on the gel. Gels were then stained for 30 min with SYBR Green I nucleic acid gel stain (Molecular Probes) as specified by the manufacturer. The stained gels were immediately photographed on a UV transillumination table with a CCD camera (Syngene, Multigenius Bioimaging System, UK) using a short wavelength filter provided by the manufacturer. Digital images of the gels were further analysed by Quantity One image analysis software version 4.0 (Bio-Rad). A band of DNA was detected if it accounted for greater than 1% of the total lane intensity. The lanes were normalized to contain the same amount of total signal after background subtraction (rolling disc) and further used for statistical comparison by principal component analysis (PCA). We also analysed the data taking into account only the presence or absence of bands according to McCaig et al. [31].

2.8. Community level physiological profiles

CLPP was performed in BIOLOG-GN2 plates (Oxoid). Five grams of soil (wet weight) of each treatment was mixed with 12.5 ml autoclaved water and 3 g of 3 mm-glass beads. The mixture was shaken at 180 rpm for 30 min at room temperature and subsequently centrifuged at 500*g* for 1 min to remove larger suspended soil particles. To adjust the cell density of the inoculum in each well, total cell counts were determined. Briefly, 100 μl of the soil suspension was filtered on a polycarbonate filter and subsequently dehydrated by sucking increasing ethanol concentrations through the system and finally stained 15 min in the dark with 15 μl of SYBR Green II (5×10^{-3} dilution of the stock) [32]. Filters were mounted with Citifluor solution AF1 (Citifluor Ltd., London, UK) and preparations were examined with an Olympus BX-60 microscope equip-

ped for epifluorescence with a high pressure mercury bulb (100 W) and filter set HQ-EGFP (AHF Analysentechnik AG, Germany). Each of the 96 wells of the BIOLOG-GN2 plates was inoculated with 125 μl of a 1:100 dilution of the initial soil suspension (5×10^6 cells ml^{-1}). BIOLOG-analysis was performed with three replicate plates inoculated with a dilution originating from the same initial soil suspension. Plates were incubated at 20 °C in the dark and absorbance was measured at 590 nm every 12 h with a plate reader (Dynex Technologies Inc. MRX II, Chantilly, USA) for 111 h. For further analysis, the optical density (OD) of the control well was subtracted from the OD of each of the substrate-containing wells in order to obtain blanked absorbance values. The rate of substrate utilization was determined by the average well colour development (AWCD) which was calculated as the mean of the blanked absorbance values for all 95 response wells per reading time [33]. The area under the resulting curve of each substrate, using the readings between 0 and 111 h for each plate, was calculated with the trapezium rule as described in Hackett and Griffiths [34] and subsequently used for statistical analysis (PCA). Substrate richness, defined as the number of substrates utilized, was determined as the number of background-corrected absorbance values higher than 0.25 after 111 h incubation [33].

2.9. Determination of the potential ammonia oxidation activity

Five grams of planted and unplanted soil (contaminated or non-contaminated) was incubated at 25 °C in a test medium for 6 h according to the international standard draft ISO/DIS 15685 [35]. Hourly samples of the soil slurries were taken and one volume of 4 M KCl was added to stop ammonia oxidation. Nitrite accumulated was subsequently determined by colorimetry. Ammonia oxidation rates were calculated by linear regression of accumulated nitrite over time.

2.10. Statistical analysis

All experiments were performed in triplicate. Student *t*-tests were carried out to determine significant differences ($p < 0.01$) between planted and unplanted pots at different sampling times. Data obtained by DGGE and CLPP were interpreted by principal component analysis (PCA) using SPSS 10.0.5 for Windows applying a covariance matrix and no rotation. With this method, the number of variables (16S rRNA gene bands and 95 substrates) was reduced to a few axes (PCs) which explain more variance than randomly regressed variables. The first two PCs were subsequently plotted to visualize the results.

3. Results

3.1. Effects of Thlaspi caerulescens on heavy metal content and soil properties

At any sampling time, the concentrations of $NaNO_3$-extractable Cd and Zn were significantly lower ($p < 0.05$) in soil recovered from planted pots than from unplanted pots (Table 1). In contrast, concentrations of soluble Cu were significantly increased in planted pots for 6 months ($p < 0.05$). After 9 and 12 months soluble Cu was also increased in unplanted pots. Analysis of the plants revealed that Cu did not accumulate in the shoots. Regarding the total heavy metals, only Cd was significantly reduced after 12 months ($p < 0.05$). The plant was able to take up between 1.55–10.3 g Zn and 0.17–0.936 g Cd kg^{-1} soil (Table 1). Accordingly, the plant removed up to 7 times more Zn and up to 67 times more Cd than the difference observed between the soluble concentrations of planted and unplanted pots. An increase in available heavy metals and a simultaneous decrease in soil pH were observed in all pots of month 6 and 12 compared to months 3 and 9. During the whole period of the experiment, the pH in the planted contaminated soil was between 0.24 and 0.30 units higher than in the unplanted contaminated soil ($p < 0.01$). A similar trend was observed in the non-contaminated soil (pH 6.7–6.9 in planted soil; pH 6.5–6.8 in unplanted soil). Total C and N contents were 1.5% and 0.14%, and did not change during the 12 months.

3.2. Effects of heavy metals and Thlaspi caerulescens on microbial communities

3.2.1. Potential ammonia oxidation activity

The rates of nitrite production were 335 ± 51 and 190 ± 9 ng g^{-1} dry soil h^{-1} in planted and unplanted non-contaminated soil, respectively. No nitrite was produced in planted or unplanted contaminated soil.

3.2.2. Microbial genetic diversity

PCR-amplification was performed using the different primer pairs on DNA-extracts from the four pot experiments (contaminated–non-contaminated, rhizosphere–unplanted soil) sampled at 3, 6, 9 and 12 months. Amplification products were analysed by DGGE and patterns resulting from replicate DNA-extractions obtained from one pot at a given sampling time were highly reproducible, as were those from DNA-extractions from the rhizospheres of different plants. For all sampling times and both treatments (contaminated–non-contaminated), 43 different bands positions were observed for *Bacteria* patterns (24–31 bands per PCR-product), 32 different bands for the β-*Proteobacteria* (13–22 bands per PCR-product) and 19 different bands for the *amoA* pattern (10–18 bands per PCR-product).

With each of the primer pairs used, the DGGE-patterns derived from contaminated and non-contaminated soils clearly differed. Within one treatment, the rhizosphere and unplanted soil patterns from the different sampling times were very similar but could still be differentiated by the presence of weak bands and changes in band intensities. Subsequently, DGGE gels were interpreted using principal component analysis (PCA) in which data were transformed in two ways, taking into account either the relative intensity or the presence of bands. Using the intensity data, the first two principal components (PC1 and PC2) were sufficient to explain 65.7% of the variance for the *Bacteria*, 71.7% for the β-*Proteobacteria* and 69.2% for the *amoA* PCR-products (Figs. 1–3). Regardless of the target groups, the first axis (PC1) separated contaminated and non-contaminated samples. DGGE-profiles of PCR-products of β-*Proteobacteria* were most stable over the experimental period as shown by tight clustering in PCA plots. In contrast, DGGE profiles of *Bacteria* 16S rRNA gene PCR-products and *amoA* gene PCR-products formed more open clusters. Clusters of the former drifted apart with incubation time in the contaminated soil samples, whereas this tendency for *amoA*-clusters was more pronounced in non-contaminated samples. PCA considering only the presence or the absence of bands resulted in similar plots.

3.2.3. Potential microbial functional diversity

The potential capacity for substrate utilization of the soil microbial communities was investigated using BIOLOG-GN2 plates providing 95 different substrates. This method clearly differentiated contaminated and non-contaminated soil samples (Fig. 4). Average well colours of plates inoculated with non-contaminated
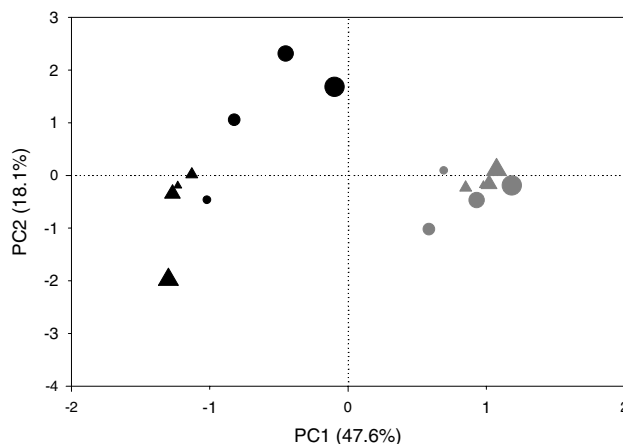

Fig. 1. Principal component analysis (PCA) on the DGGE data of the *Bacteria* 16S rDNA PCR-products. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲, unplanted contaminated; ●, rhizospheric contaminated; ▲, unplanted non-contaminated and ●, rhizospheric non-contaminated.
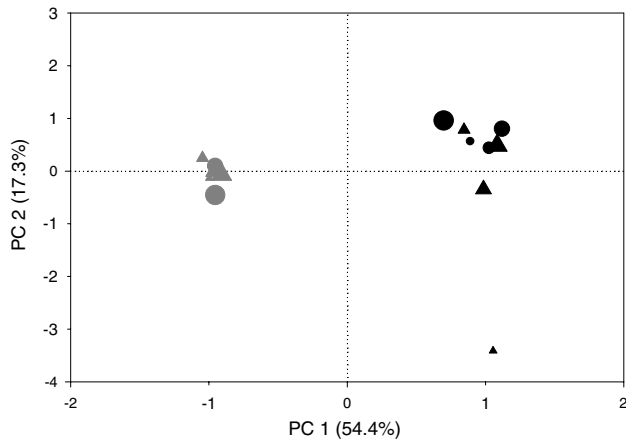
Fig. 2. Principal component analysis (PCA) on the DGGE data of the β-*Proteobacteria* 16S rDNA PCR-products. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲, unplanted contaminated; ●, rhizospheric contaminated, ▲, unplanted non-contaminated and ●, rhizospheric non-contaminated.

Fig. 4. Average well colour development (AWCD) of the non-contaminated (grey) and contaminated (black) soil samples during the 12 months experiment. ——, planted; –––, unplanted; ■, 3 months; ▲, 6 months; *, 9 months and ◆, 12 months.
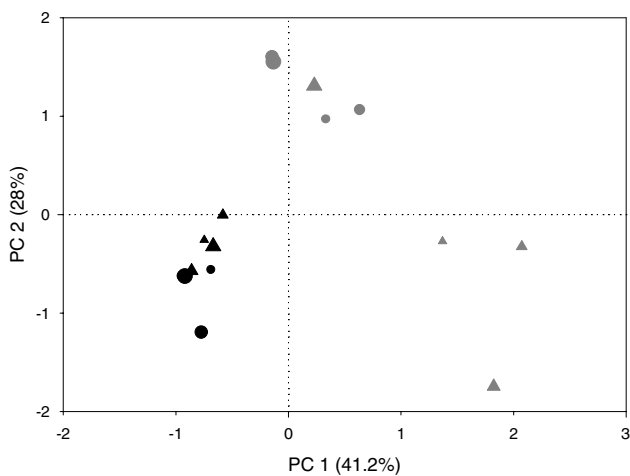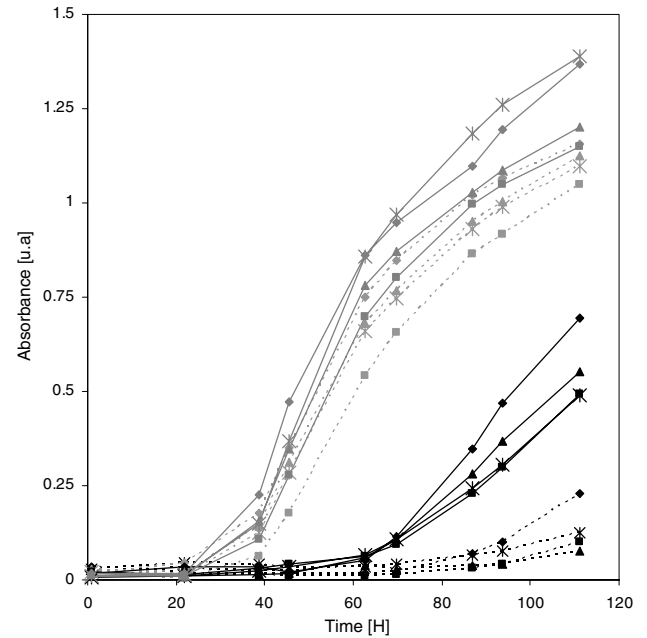

Fig. 3. Principal component analysis (PCA) on the DGGE data of the *amoA* gene PCR-products. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲, unplanted contaminated; ●, rhizospheric contaminated; ▲, unplanted non-contaminated and ●, rhizospheric non-contaminated.

Table 2
Number of substrates metabolised by the microbial communities after incubation for 111 h

Sampling time	Non-contaminated soil		Contaminated soil	
	Planted	Unplanted	Planted	Unplanted
3 months	67 ± 4	65 ± 5	39 ± 4	8 ± 3
6 months	72 ± 3	66 ± 3	40 ± 6	6 ± 2
9 months	77 ± 3	65 ± 5	35 ± 2	9 ± 2
12 months	83 ± 4	67 ± 1	57 ± 4	19 ± 4

Only the mean values (background-corrected, OD > 0.25) of each substrate of the three replicate plates for each soil treatment were taken into account (mean ± standard deviation).

samples increased rapidly after 39 h and reached significantly higher values within 111 h than plates with inocula from contaminated soil samples. The AWCD for both pristine soils was sigmoidal, the absorbance measured for the rhizosphere soil being generally higher than that of the unplanted soil. Utilization of substrates in plates with inocula from contaminated soil started only after approximately 70 h. In particular utilization rates for the unplanted contaminated samples remained very low. Soil samples were also compared at a fixed incubation time (111 h) with respect to substrate richness, i.e., the number of substrates metabolized (Table 2). BIOLOG plates with the highest AWCD values, i.e.,

plates inoculated with the two pristine soils, also showed significantly higher utilization of substrates than plates inoculated with contaminated soil (Table 2). Independently of the soil treatment, the number of substrates degraded increased significantly during this one-year experiment in the planted samples ($p < 0.01$). As seen in Table 2, the effect of *T. caerulescens* was significantly more pronounced for plates with contaminated soil samples ($p < 0.001$). The areas under the curves, summarizing statistically the substrate utilization during 111 h incubation, were used for principal component analysis (PCA). Using areas instead of endpoint optical density (OD) values enabled different parameters to be taken into account, such as lag phase, the rate of increase and the maximum absorbance obtained during the incubation time [36]. PCA discriminated all four soil
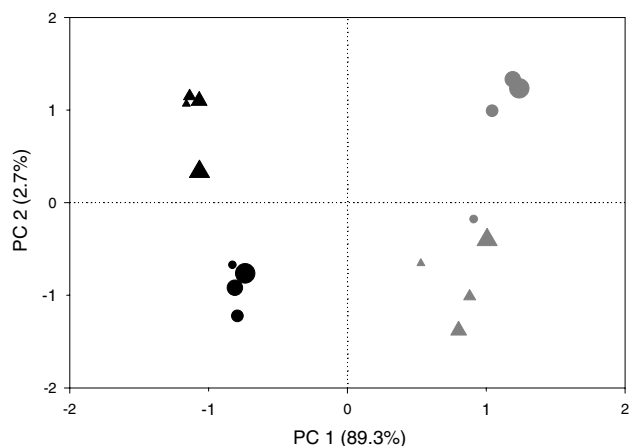
Fig. 5. Principal component analysis (PCA) on the Biolog data after 111 h incubation. The increase in size of the symbols represents the different sampling points (3, 6, 9 and 12 months). ▲, unplanted contaminated; ●, planted contaminated; ▲, unplanted non-contaminated and ●, planted non-contaminated.

types and 92% of the variance in the BIOLOG data was explained by the first two axes (PC1 and PC2) (Fig. 5). The first principal component had the greatest power of separation as it accounted for 89.3% of the variance. This can be explained by the high number of substrates used in the non-contaminated samples, mainly carbohydrates, carboxylic acids, amino acids, amides, and amines. Seventy-two per cent of these substrates was highly correlated to the first axis (Pearson coefficient >0.9; $p < 0.05$). The planted samples were separated from the unplanted ones along the second axis.

4. Discussion

In this study, we used an approach combining various complementary methods to determine the effect of heavy metal contamination followed by phytoextraction on indigenous soil microbial populations. During a one year pot experiment, four consecutive harvests of *T. caerulescens* resulted in a decrease in the soluble, bioavailable Zn- and Cd-concentration by 30% and 60%, respectively. The amounts of Zn and Cd in the shoots were higher than the depletion of the soluble heavy metal pool. It appeared that uptake of Cd and Zn from the soluble pool was partly compensated by replenishment with heavy metals from initially less available pools. It is unclear if this is due to passive re-equilibration of the heavy metal pools in soil, or actively driven by the plant, which may have, e.g., access to non-$NaNO_3$-extractable Zn and Cd fractions [21,37–39]. Observed variations in the available pool of heavy metals in both planted and unplanted samples during the experiment were concomitant with pH changes of the soil solution. An increase of pH-values is usually

considered to be the major factor for a decrease in the concentration of available heavy metals [40]. However, our data indicate that the driving force for the observed decrease in extractable Zn and Cd is mainly the heavy metal uptake by the plant.

The impact of the heavy metals could be seen in the results obtained with all three approaches, i.e., DGGE, CLPP and potential ammonia oxidation activity measurement. These approaches account for different parts of the community. DGGE analysis was performed with two different molecular markers, i.e., genes encoding for the 16S rRNA and the α-subunit of ammonia mono-oxygenase (*amoA*). DGGE using the 16S rRNA gene marker was done at two levels of resolution with different primer pairs. This approach potentially reduces the complexity of DGGE-banding patterns usually observed after PCR-amplification with primers targeting the total bacterial community. In addition, primers specific for selected populations allow analysis of less abundant groups that may be outcompeted in the PCR reaction using the *Bacterial* primer pair. Nested PCR has been shown to be necessary for the detection of minor but ecological significant populations which make up less than 0.01% of the total bacterial soil community [41]. Primers targeting 16S rRNA genes of ammonia oxidising bacteria (AOB) have been widely used to recover sequence information from these bacteria in terrestrial environments (see [42] and references therein). In a few cases, such as for the AOB affiliated to the β-*Proteobacteria*, group-specific primers may allow conclusions to be drawn about processes potentially carried out by the organisms targeted. AOB are important key-players in the nitrogen cycle since they are responsible for the rate-limiting step in the nitrification process [42]. However, a 16S rRNA based DGGE approach to assess the molecular diversity of AOB in complex environmental systems may be prone to the co-amplification of non-target sequences [29,42,43]. Targeting *amoA* as a functional marker for the analysis of ammonia oxidising bacteria is a promising alternative [28–30], as the higher degree of sequence variation within *amoA* sequences allows a more sensitive analysis of the genetic diversity within the AOB of the β-*Proteobacteria*. Normalised DGGE-profiles were used to perform statistical analysis by PCA based on number of bands or on number and intensity of bands. It is widely accepted that the number and intensity of bands in a DGGE-profile should be regarded as semi-quantitative information due to different PCR-biases [44] and to the possibility of similar melting behaviour of 16S rRNA gene fragments from organisms with different ribotypes [19]. Independently of the marker gene used, multivariate analysis of the DGGE data clearly revealed consistent differences between contaminated and non-contaminated samples. This is in accordance with other studies which detected shifts in the bacterial community caused by heavy metal

contamination using DGGE and other molecular techniques [45,46]. Therefore, we conclude that the application of group-specific primers does not offer any additional resolution power if the only goal is to distinguish a heavy metal-polluted soil from a pristine soil. DGGE-analysis of 16S rRNA gene sequences also revealed gradual changes in microbial community structure in the contaminated, unplanted soil during 12 months in contrast to the community structure in the non-contaminated, unplanted soil, which remained stable. A possible explanation for the changes at the two levels of resolution (i.e., *Bacteria* and β-*Proteobacteria*) in the contaminated, unplanted soil is that the microbial community was initially disturbed by the addition of the heavy metal dust and that community shifts towards a new steady state were not finished within the adaptation period of three months and the experimental period. In contrast to the contaminated soils, PCA of the *amoA* DGGE-profiles indicated some separation of the *amoA* DGGE-patterns between the non-contaminated planted and unplanted soils. Since this tendency was not observed to that extent with DGGE-profiles of the β-*Proteobacteria* and the total bacterial community, one could assume that *amoA* might be a more sensitive indicator of the impact of the rhizosphere in pristine environments. Kowalchuk et al. [47] have shown that shifts in dominant ammonia oxidising populations in chalk grassland soils occurred in a background of general stability in the dominant bacterial populations as determined by DGGE using a *Bacterial* primer pair. Our observation corroborates also with the ammonia oxidation activity measurements. In the non-contaminated soil, the potential activity of the AOB was significantly higher in planted pots than in the unplanted samples. It has been shown that plant roots may stimulate growth of AOB [48,49]. Assuming that *T. caerulescens* took up preferentially nitrate instead of ammonium, as indicated by an increase in pH [50], the AOB would be less in competition with the plant for this substrate.

Although growth of *T. caerulescens* in contaminated soil resulted in changes in the DGGE-profiles of the total bacterial community, the plant did not exert any positive influence on the potential ammonia oxidation activity in the contaminated soil as generally no activity was detected in contaminated soil samples, indicating complete inhibition of the AOB. Ammonia oxidation is a microbial process very sensitive to soil pollution [51,52]. Although the plant removed in our study a significant part of the soluble Zn and Cd, no recovery of the nitrification activity in the contaminated planted samples was observed. Whether this is due to the Zn and Cd remaining in the soil solution or the unchanged soluble Cu fraction is not clear. This result, together with the DGGE-profiling, indicates that the AOB populations present in the non-contaminated samples may have been replaced in the contaminated soil by a heavy metal resistant but probably ineffective population [53]. Our results are also in accordance with the observations of Stephen et al. [54], who studied the ammonia oxidiser community structure and nitrogen turnover in metal-treated microcosms. Population structure analysis based on *amoA* clone libraries as well as the ammonia concentration in the microcosms were significantly affected by heavy metal exposure. Their data suggest that ammonia oxidising bacteria representing a certain group of *amoA* sequences (i.e., cluster C) [54] had a selective advantage over other AOB in heavy metal-contaminated soil. Since we did not aim to sequence bands from the DGGE gels, it remains open if these cluster C sequences are also present in our soil.

Community level physiological profiles (CLPP) reflect the potential activity of that fraction of the bacterial community that is able to grow on the substrates provided on BIOLOG microtitre plates. CLPP is not necessarily related to the functional potential of the most abundant bacteria in soil [55], and is prone to the biases inherent to methods measuring diversity under culture conditions [56]. Yet, it has been shown useful as a rapid technique in providing habitat-specific patterns. One can expect that substrates in the plant rhizosphere were present in lower concentrations than the substrates in the wells. Although the bacteria grown in the BIOLOG plates were not further analysed with molecular methods, it is most likely that fast growing r-selected populations which were not necessarily dominant in the inoculum accounted for the colour development in the wells. As already shown in other studies, the potential degradation capabilities of the microbial community were drastically reduced in the heavy metal-contaminated soil [57–60]. The rate of colour development was lower and delayed in the contaminated samples as compared to the non-contaminated ones. As the inoculum size was previously adjusted in the wells, the differences in the average well colour development must be due to the heavy metal contamination [58]. In contrast to the contaminated samples, we observed a positive association of non-contaminated samples with PC1. The C-sources which were also positively correlated to this axis should consequently be metabolized at a higher rate by the non-contaminated samples [61]. Interestingly, the majority of the amino acids, amines and amides were correlated positively as well to PC1. The lower utilization of these compounds in the contaminated samples indicates that bacterial adaptation to heavy metals is probably maintained at the expense of specific or rare degradative abilities [62–65]. For instance, heavy metal contamination has been shown to delay the degradation of several compounds such as starch, cellulose, glutamic acid and casamino acids [2,66]. The higher number of substrates used in the planted samples compared to the unplanted samples suggests a stimulating effect of the plant, probably due to the root exudates [67]. Most in-

teresting, however, is the observation that the phytoextraction practice involving *T. caerulescens* had a positive impact on some potential functional abilities of rhizosphere microbial communities in the heavy metal contaminated soil. Kojdroz et al. showed that artificial root exudates supported the development of bacterial populations in heavy metal contaminated soils [68]. Campbell et al. [69] suggested use of carbon sources reported as constituents of root exudates as BIOLOG substrates. The authors argued that the carbon sources present in exudates represent a more diverse set of substrates than the carbon sources used in the BIOLOG GN plates and consequently also select for the slower growing soil organisms. Although the commercially available microtitre plates sufficiently separated the planted from the unplanted samples, we cannot exclude that the use of plates with root exudates might provide some additional information. If we take also into account the observed changes in the DGGE-profiles of the total rhizosphere bacterial community in the contaminated soil, we could speculate that in contrast to ammonia oxidation, other microbial processes may be stimulated. Since these microorganisms may be important for the recovery of the vegetation in a soil following reduction of the pollutant, we believe that more studies should focus on the effect of metal remediation on functional diversity in soils undergoing phytoremediation. Among rhizosphere organisms deserving more attention due to their important interactions with plants are arbuscular mycorrhizal (AM) fungi. Colonization of plants by AM fungi has in particular been observed when plants are exposed to diverse stress conditions such as exposure to heavy metals [70]. However, *Thlaspi* belongs to the few plant families which are believed not to form symbiosis with AM fungi or are at best poorly colonized [71].

Although the duration of our experiment does not allow prediction of microbial genetic diversity and potential functional abilities after a longer period of phytoextraction, we can draw the following conclusions: Firstly, exposing soil to heavy metals changed the microbial community structure as measured by DGGE analysis of 16S rRNA and *amoA* gene sequences representing dominant but also minor populations. Secondly, bacterial functional abilities were heavily affected by the heavy metal contamination. Thirdly, growth of the hyperaccumulator *T. caerulescens* in the contaminated soil did not allow the potential ammonia oxidising activity in the rhizosphere to recover. Fourthly, phytoextraction with *T. caerulescens* not only resulted in some changes in the rhizosphere microbial community structure, but fifthly clearly improved the potential functional abilities as compared to the contaminated unplanted soil. Further studies are needed to predict if, in particular, the functional abilities of soil microbial communities will be fully re-established after the endpoint of phytoremediation has been reached.

References

[1] Salt, D.E., Blaylock, M., Kumar, N.P.B.A., Dushenkov, V., Ensley, B.D., Chet, I. and Raskin, I. (1995) Phytoremediation: a novel strategy for the removal of toxic metals from the environment using plants. Biotechnology 13, 468–474.

[2] Babich, H. and Stotzky, G. (1985) Heavy metal toxicity to microbe-mediated ecologic processes: A review and potential application to regulatory policies. Environ. Res. 36, 111–137.

[3] Baath, E. (1989) Effects of heavy metals in soil microbial processes and populations (a review). Water. Air Soil Pollut. 47, 335–379.

[4] Giller, K.E., Witter, E. and McGrath, S.P. (1998) Toxicity of heavy metals to microorganisms and microbial processes in agricultural soils: a review. Soil Biol. Biochem. 30, 1389–1414.

[5] Vangronsveld, J. and Cunningham, S.D. (1998) Introduction to the concepts. In: Metal-Contaminated Soils: In Situ Inactivation and Phytorestoration (Vangronsveld, J. and Cunningham, S., Eds.), pp. 1–15. Springer, New York.

[6] Robinson, B.H., Leblanc, M., Petit, D., Brooks, R.R., Kirkman, J.H. and Gregg, P.E.H. (1998) The potential of *Thlaspi caerulescens* for phytoremediation of contaminated soils. Plant Soil 203, 47–56.

[7] Baker, A.J.M., McGrath, S.P., Sidoli, C.D.M. and Reeves, R.D. (1994) The possibility of in situ heavy metal decontamination of polluted soils using crops of metal-accumulating plants. Resour. Conser. Recy. 11, 41–49.

[8] Garbisu, C. and Alkorta, I. (2001) Phytoextraction: a cost-effective plant-based technology for the removal of metals from the environment. Bioresour. Technol. 77, 229–236.

[9] Glass, D.J. (2000) Economic potential of phytoremediation. In: Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment (Raskin, I. and Ensley, B., Eds.), pp. 15–31. Wiley, New York.

[10] Mulligan, C.N., Yong, R.N. and Gibbs, B.F. (2001) Remediation technologies for metal-contaminated soils and groundwater: an evaluation. Eng. Geol. 60, 193–207.

[11] Baker, A.J.M., Reeves, R.D. and Hajar, A.S.M. (1994) Heavy metal accumulation and tolerance in british populations of the metallophyte *Thlaspi caerulescens* J. &C Presl (Brassicaceae). New Phytol. 127, 61–63.

[12] Brown, S.L., Chaney, R.L., Angle, J.S. and Baker, A.J.M. (1994) Phytoremediation potential of *Thlaspi caerulescens* and Bladder Campion for Zinc- and cadmium-contaminated soil. J. Environ. Qual. 23, 1151–1157.

[13] Reeves, R.D. and Baker, A.J.M. (2000) Metal-accumulating plants. In: Phytoremediation of Toxic Metals: Using Plants to Clean Up the Environment (Raskin, I. and Ensley, B.D., Eds.), pp. 193–229. Wiley, New York.

[14] De Souza, M.P., Huang, C.P., Chee, N. and Terry, N. (1999) Rhizosphere bacteria enhance the accumulation of selenium and mercury in wetland plants. Planta 209, 259–263.

[15] Whiting, S.N., de Souza, M.P. and Terry, N. (2001) Rhizosphere bacteria mobilize Zn for hyperaccumulation by *Thlaspi caerulescens*. Environ. Sci. Technol. 35, 3144–3150.

[16] Pawlowska, T.E., Chaney, R.L., Chin, M. and Charvat, I. (2000) Effects of metal phytoextraction practices on the indigenous community of arbuscular mycorrhizal fungi at a metal-contaminated landfill. Appl. Environ. Microbiol. 66, 2526–2530.

[17] Delorme, T.A., Galiardi, J.V., Angle, J.S. and Chaney, R.L. (2001) Influence of the zinc hyperaccumulator Thlaspi caerulescens J. & C. Presl. and the nonmetal accumulator Trifolium pratense L. on soil microbial populations. Can. J. Microbiol. 47, 773–776.

[18] Gremion, F., Chatzinotas, A. and Harms, H. (2003) Comparative 16S rDNA and 16S rRNA sequence analysis indicates that Actinobacteria might be a dominant part of the metabolically active bacteria in heavy metal-contaminated bulk and rhizosphere soil. Environ. Microbiol. 5, 896–907.

[19] Muyzer, G. and Smalla, K. (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Anton. Leeuw. Int. J.G. 73, 127–141.

[20] OIS (1998). Swiss Ordinance relating to Impacts on the Soil; 1st July 1998. SR 814.12, Switzerland.

[21] Hammer, D. and Keller, C. (2002) Changes in the rhizosphere of metal-accumulating plants evidenced by chemical extractions. J. Environ. Qual. 31, 1561–1569.

[22] Marilley, L., Vogt, G., Blanc, M. and Aragno, M. (1998) Bacterial diversity in the bulk soil and rhizosphere fractions of Lolium perenne and Trifolium repens as revealed by PCR restriction analysis of 16S rDNA. Plant Soil 198, 219–224.

[23] Griffiths, R.I., Whiteley, A.S., O'Donnell, A.G. and Bailey, M.J. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. Appl. Environ. Microbiol. 66, 5488–5491.

[24] Muyzer, G.S.H., Teske, A. and Wawer, C. (1996) Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA: a new molecular approach to analyze the genetic diversity of mixed microbial communities. In: Molecular Microbial Ecology Manual (Akkermans, A.D.L., Van Elsas, J.D. and De Bruijn, F., Eds.), pp. 3.4.4/1–3.4.4/23. Kluwer Academic Publishers, Dordrecht, The Netherlands.

[25] Lane, D.J. (1991) 16S/23S rRNA sequencing. In: Nucleic Acid Techniques in Bacterial Systematics (Stackebrandt, E. and Goodfellow, M., Eds.), pp. 115–175. Wiley, New York.

[26] Gomes, N.C.M., Heuer, H., Schönfeld, J., Costa, R., Mendonça-Hagler, L. and Smalla, K. (2001) Bacterial diversity of the rhizosphere of maize (Zea mays) grown in tropical soil studied by temperature gradient gel electrophoresis. Plant Soil 232, 167–180.

[27] Heuer, H., Kresk, M., Baker, P., Smalla, K. and Wellington, E.M.H. (1997) Analysis of Actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl. Environ. Microbiol. 63, 3233–3241.

[28] Webster, G., Embley, T.M. and Prosser, J.I. (2002) Grassland management regimens reduce small-scale heterogeneity and species diversity of β ammonia oxidizer populations. Appl. Environ. Microbiol. 68, 20–30.

[29] Rotthauwe, J.-H., Witzel, K.-P. and Liesack, W. (1997) The ammonia monooxygenase structural gene amoA as a functional marker: molecular fine-scale analysis of natural ammonia-oxidizing populations. Appl. Environ. Microbiol. 63, 4704–4712.

[30] Avrahami, S., Liesack, W. and Conrad, R. (2003) Effects of temperature and fertilizer on activity and community structure of soil ammonia oxidizers. Environ. Microbiol. 5, 691–705.

[31] McCaig, A.E., Glover, A.L. and Prosser, J.I. (2001) Numerical analysis of grassland bacterial community structure under different land management regimens by using 16S ribosomal DNA sequence data and denaturing gradient gel electrophoresis banding patterns. Appl. Environ. Microbiol. 67, 4554–4559.

[32] Weinbauer, M.G., Beckmann, C. and Höfle, M.G. (1998) Utility of green fluorescent nucleic acid dyes and aluminum oxide membrane filters for rapid epifluorescence enumeration of soil and sediment bacteria. Appl. Environ. Microbiol. 64, 5000–5003.

[33] Garland, J.L. (1996) Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. Soil Biol. Biochem. 28, 213–221.

[34] Hackett, C.A. and Griffiths, B.S. (1997) Statistical analysis of the time-course of Biolog substrate utilization. J. Microbiol. Meth. 30, 63–69.

[35] International Organization for Standardisation (ISO) (1999). Ammonium Oxidation, A Rapid Method to Test Potential Nitrification in Soil. ISO/CD 15685. Geneva, Switzerland.

[36] Guckert, J.B., Carr, G.J., Johnson, T.D., Hamm, B.G., Davidson, D.H. and Kumagai, Y. (1996) Community analysis by Biolog: curve integration for statistical analysis of activated sludge microbial habitats. J. Microbiol. Meth. 27, 183–197.

[37] Knight, B., Zhao, F.J. and Shen, Z.G. (1997) Zinc and cadmium uptake by the hyperaccumulator Thlaspi caerulescens in contaminated soils and its effects on the concentration and chemical speciation of metals in soil solution. Plant Soil 197, 71–78.

[38] McGrath, S.P., Shen, Z.G. and Zhao, F.J. (1997) Heavy metal uptake and chemical changes in the rhizosphere of Thlaspi caerulescens and Thlaspi ochroleucum grown in contaminated soils. Plant Soil 188, 153–159.

[39] Whiting, S.N., Leake, J.R., McGrath, S.P. and Baker, A.J.M. (2001) Assessment of Zn mobilization in the rhizosphere of Thlaspi caerulescens by bioassays with non-accumulator plants and soil extraction. Plant Soil, 147–156.

[40] Hornburg, V. and Brümmer, G.W. (1993) Behaviour of heavy metals in soil 1. Investigations on heavy metal mobility (In German). Z. Pflanz. Bodenkunde 156, 467–477.

[41] Phillips, C.J., Harris, D., Dollhopf, S.L., Gross, K.L., Prosser, J.I. and Paul, E.A. (2000) Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. Appl. Environ. Microbiol. 66, 5410–5418.

[42] Kowalchuk, G.A. and Stephen, J.R. (2001) Ammonia-oxidizing bacteria: a model for molecular microbial ecology. Annu. Rev. Microbiol. 55, 485–529.

[43] Kowalchuk, G.A., Bodelier, P.L.E., Heilig, G.H.J., Stephen, J.R. and Laanbroek, H.J. (1998) Community analysis of ammonia-oxidising bacteria, in relation to oxygen, availability in soils and root-oxygenated sediments, using PCR, DGGE and oligonucleotide probe hybridisation. FEMS Microbiol. Ecol. 27, 339–350.

[44] von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E. (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol. Rev. 21, 213–229.

[45] Müller, A.K., Westergaard, K., Christensen, H. and Sorensen, S.J. (2001) The effect of long-term mercury pollution on the soil microbial community. FEMS Microbiol. Ecol. 36, 11–19.

[46] Sandaa, R.A., Enger, O. and Torsvik, V. (1999) Abundance and diversity of Archaea in heavy-metal-contaminated soil. Appl. Environ. Microbiol. 65, 3293–3297.

[47] Kowalchuk, G., Stienstra, A., Heilig, G., Stephen, J. and Woldendorp, J. (2000) Changes in the community structure of ammonia-oxidizing bacteria during secondary succession of calcareous grasslands. Environ. Microbiol. 2, 99–110.

[48] Klemedtsson, L., Berg, P., Clarholm, M., Schnurer, J. and Rosswall, T. (1987) Microbial Nitrogen Transformations in the Root Environment of Barley. Soil Biol Biochem 19, 551–558.

[49] Briones, A.M., Okabe, S., Umemiya, Y., Ramsing, N.B., Reichardt, W. and Okuyama, H. (2003) Ammonia-oxidizing bacteria on root biofilms and their possible contribution to N use efficiency of different rice cultivars. Plant Soil 250, 335–348.

[50] Luo, Y.M., Christie, P. and Baker, A.J.M. (2000) Soil solution Zn and pH dynamics in non-rhizosphere soil and in the rhizosphere of grown in a Zn/Cd-contaminated soil. Chemosphere 41, 161–164.

[51] Lee, Y.W., Ong, S.K. and Sato, C. (1997) Effects of heavy metals on nitrifying bacteria. Water Sci. Technol. 36, 69–74.

[52] Sauve, S., Dumestre, A., McBride, M., Gillett, J.W., Berthelin, J. and Hendershot, W. (1999) Nitrification potential in field-collected soils contaminated with Pb or Cu. Appl. Soil Ecol. 12, 29–39.

[53] Gong, P., Siciliano, S.D., Srivastava, S., Greer, C.W. and Sunahara, G.I. (2002) Assessment of pollution-induced microbial community tolerance to heavy metals in soil using ammonia-oxidizing bacteria and Biolog assay. Hum. Ecol. Risk Assess. 8, 1067–1081.

[54] Stephen, J.R., Chang, Y.-J, Macnaughton, S.J., Kowalchuk, G.A., Leung, K.T., Fleming, C.A. and White, D.C. (1999) Effect of toxic metals on indigenous soil β-proteobacterium ammonia oxidizer community structure and protection against toxicity by inoculated metal-resistant bacteria. Appl. Environ. Microbiol. 65, 95–101.

[55] Smalla, K., Wachtendorf, U., Heuer, H., Liu, W.T. and Forney, L. (1998) Analysis of BIOLOG GN substrate utilization patterns by microbial communities. Appl. Environ. Microbiol. 64, 1220–1225.

[56] Preston-Mafham, J., Boddy, L. and Randerson, P.F. (2002) Analysis of microbial community functional diversity using sole-carbon-source utilisation profiles - a critique. FEMS Microbiol. Ecol. 42, 1–14.

[57] Knight, B.P., McGrath, S.P. and Chaudri, A.M. (1997) Biomass carbon measurements and substrate utilization patterns of microbial populations from soils amended with cadmium, copper, or zinc. Appl. Environ. Microbiol. 63, 39–43.

[58] Kelly, J.J. and Tate, R.L. (1998) Effects of heavy metal contamination and remediation on soil microbial communities in the vicinity of a zinc smelter. J. Environ. Qual. 27, 609–617.

[59] Dobler, R., Saner, M. and Bachofen, R. (2000) Population changes of soil microbial communities induced by hydrocarbon and heavy metal contamination. Biorem. J. 4, 41–56.

[60] Ellis, R.J., Neish, B., Trett, M.W., Best, J.G., Weightman, A.J., Morgan, P. and Fry, J.C. (2001) Comparison of microbial and meiofaunal community analyses for determining impact of heavy metal contamination. J. Microbiol. Meth. 45, 171–185.

[61] Garland, J.L. and Mills, A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. Appl. Environ. Microbiol. 57, 2351–2359.

[62] Reber, H.H. (1992) Simultaneous estimates of the diversity and the degradative capability of heavy-metal-affected soil bacterial communities. Soil Biol. Biochem. 13, 181–186.

[63] Doelman, P., Jansen, E., Michels, M. and van Til, M. (1994) Effects of heavy metals in soil on microbial diversity and activity as shown by the sensitivity-resistance index, an ecologically relevant parameter. Biol. Fert. Soils 17, 177–184.

[64] Wenderoth, D.F. and Reber, H.H. (1999) Correlation between structural diversity and catabolic versatility of metal-affected prototrophic bacteria in soil. Soil Biol. Biochem. 31, 345–352.

[65] Wenderoth, D.F., Stackebrandt, E. and Reber, H.H. (2001) Metal stress selects for bacterial ARDRA-types with a reduced catabolic versatility. Soil Biol. Biochem. 33, 667–670.

[66] Obbard, J.P. and Jones, K.C. (1993) The use of the cotton-strip assay to assess cellulose decomposition in heavy metal-contaminated sewage sludge-amended soils. Environ. Pollut. 81, 173–178.

[67] Heinonsalo, J., Jorgensen, K.S., Haahtela, K. and Sen, R. (2000) Effects of *Pinus sylvestris* rooth growth and mycorrhizosphere development on bacterial carbon source utilization and hydrocarbon oxidation in forest and petroleum-contaminated soils. Can. J. Microbiol. 46, 451–464.

[68] Kozdrój, J. and van Elsas, J.D. (2000) Response of the bacterial community to root exudates in soil polluted with heavy metal assessed by molecular and cultural approaches. Soil Biol. Biochem. 32, 1405–1417.

[69] Campbell, C.D., Grayston, S.J. and Hirst, D.J. (1997) Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. J. Microbiol. Meth. 30, 33–41.

[70] Smith, S.E. and Read, D.J. (1997) Mycorrhizal Symbiosis, second ed. Academic Press, San Diego, London.

[71] Regvar, M., Vogel, K., Irgel, N., Wraber, T., Hildebrandt, U., Wilde, P. and Bothe, H. (2003) Colonization of pennycresses (*Thlaspi* spp.) of the Brassicaceae by arbuscular mycorrhizal fungi. J. Plant Physiol. 160, 615–626.