Bacterial community structure and activity in different Cd-treated forest soils

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Abstract

In this study we compared indicators of Cd bioavailability (water extracts, Lakanen extracts, free ions) and ecotoxicity in forest soils with contrasting physico-chemical characteristics. Soil samples were treated with $CdCl_2$ solutions (0, 0.1, 1, 10 and 100 mM) and incubated for 30 days. Microbial activity indexes (acid phosphatase, β-glucosidase, basal respiration) and changes in bacterial community structure using terminal restriction fragment length polymorphism (T-RFLP) fingerprinting were investigated. The Cd concentrations measured ranged from 1% to 37% of the total additions in water extracts, to higher levels in Lakanen extracts. Effects of Cd were observed at bioavailable concentrations exceeding United Nations/European Economic Commission UN/ECE guidelines for total Cd in the soil solution. Basal respiration was the most affected index, while enzymatic activities showed variable responses to the Cd treatments. We also noticed that soils with pH higher than 6.7 and clay content higher than 50% showed inhibition of basal respiration but no marked shift in bacterial community structure. Soils with lower pH (pH <5.8) with less clay content ($<$ 50%) showed in addition strong changes in the bacterial community structure. Our results provide evidence for the importance of relating the effects of Cd on the soil communities to soil properties and to bioavailability.

Introduction

In the last century, the long-range atmospheric transport of heavy metals such as Cd, Pb and Hg has represented a concern due to the accumulation of these metals in pristine environments, also far away from the pollution source (Reinds *et al.*, 2001). Forest canopies are efficient in intercepting metal particles in the air and depositing them on the upper soil layers through litterfall (Hernandez *et al.*, 2003). The accumulation of metals in natural soils is further increased by the input of metals derived from the weathering of parent rock and from mobilization by plant roots (Huisman *et al.*, 1997; Blaser *et al.*, 2000; Starr *et al.*, 2003). These processes can cause a redistribution of metals in the soil horizons, and increase concentrations of metals in the soil water (Ukonmaanaho *et al.*, 2001).

Heavy metals show a high toxicity to organisms, and some, such as Cd, do not have any known biological function (Alloway, 1995). Toxic effects of metals in soil systems include alterations in soil activities and functions (Bååth, 1989; Khan & Scullion, 2000), as well as changes in the composition of the soil microbial communities (Pennanen *et al.*, 1996; Roane & Pepper, 2000). Recently, there was a United Nations (UN) and European Economic Commission (ECE) initiative to define strategies to regulate the emissions of heavy metals, which led to the development of provisory regulatory concentration target values (critical limits) that define the maximum atmospheric loads thought to cause no adverse effects on ecological receptors (Bak *et al.*, 1997; De Vries & Bakker, 1998). A further effort aims to relate heavy metal concentrations to the concept of bioavailability.

Total heavy metal concentrations are often poor indicators of toxicity in soils, as large metal fractions are present in biologically unavailable forms (i.e. bound to the soil matrix or complexed by organic matter) depending on a number of modifying factors such as pH, organic matter content, and clay content of soils (Sauvé *et al.*, 1998; Prokop *et al.*, 2003). These soil characteristics influence the solubility and mobility of the metal ions and therefore determine the actual bioavailability of metals to soil organisms (Lofts *et al.*, 2004). Generally bioavailable metals in soils are represented by the soluble fraction in the soil water and are measured

through chemical extractions of different extractive strength. In addition, chemical equilibrium models, such as the Windermere humic aqueous model (WHAM; Tipping, 1994), the free ion activity model (FIAM; Sunda, 1991) and the biotic ligand model (BLM; Di Toro *et al.*, 2001), have been developed for computing the distribution of the various metal species in a sample. Although these models were designed for aquatic systems, they have also been applied to calculate metal speciation in sediments or in soils, in which the site of interaction between the metal and organism is an aqueous environment such as the soil solution (Tipping, 1994; Parker & Pedler, 1997; Van Gestel & Koolhaas, 2004). For example, they have proven useful for estimating the concentration of the free ion form of the metal, which has been defined as the principle component of the soluble metal fraction in the soil water and as the form most toxic to organisms (Krishnamurti & Naidu, 2003; Vig *et al.*, 2003; Lofts *et al.*, 2004).

Soil microorganisms are good ecological receptors for the assessment of metal toxicity, as they are key organisms which constitute a major part of the biomass and regulate all nutrient cycles. Microbial activity is determined by studying responses in terms of CO_2 production (basal respiration) or enzymatic activities related to the major metabolic cycles (Klose *et al.*, 2003; Moreno *et al.*, 2003; Renella *et al.*, 2004). For example, acid phosphatase plays a very important role in the mineralization of organic P. It is a substrate-dependent extracellular enzyme, which is active mostly where conditions of low organic P prevail. β-glucosidase, on the other hand, is an extracellular enzyme at the beginning of the C cycle and catalyzes the hydrolysis of cellobiose. Enzymatic activities measured in previous studies have been shown however to vary in relation to soil properties and environmental conditions (Gianfreda & Bollag, 1994). In addition, heavy metals can affect enzymatic activities by directly binding with the substrate or with the enzyme (Moreno *et al.*, 2003; Stuczynski *et al.*, 2003), or indirectly by affecting the microorganisms involved in the activity (Kandeler *et al.*, 2000).

Bacterial community structure and diversity can be studied with either cultivation-dependent or cultivation-independent approaches. The former are limited to the study of the effects of contamination on the culturable organisms of the soil samples (Ellis *et al.*, 2003), while the latter take into account a much greater diversity and complexity of organisms, which would not be detected only using standard cultivation approaches. The use of biomarkers, such as phospholipid fatty acid analysis (PLFA), and marker genes for molecular genetic profiling methods, such as terminal restriction fragment length polymorphism (T-RFLP) or denaturing gradient gel electrophoresis (DGGE), has greatly facilitated the investigation of bacterial communities. In particular, T-RFLP has been shown to be a powerful tool for analyzing the structure and composition of soil bacterial communities in natural and contaminated soils in field and laboratory conditions (Sessitsch *et al.*, 2001; Pesaro *et al.*, 2004; Turpeinen *et al.*, 2004; Yrjälä *et al.*, 2004; Frey *et al.*, 2006). Finally, while field studies permit one to observe ecological processes, such as the effects of metal pollution, in nature, an alternative approach is the use of laboratory microcosm experiments. Microcosms are simple model ecosystems as close to the natural state as possible (Jessup *et al.*, 2004). They offer the advantage of high replication and of good control of external factors.

The current critical limits for total metal contents and for soluble metals in the soil solution are provisory and calculated from mathematical models on a limited set of data published in the literature, mainly referred to agricultural soils (De Vries *et al.*, 2001). They lack an ecotoxicological basis and still need to take into account different soil categories and different sensitivities of the biological communities in the different soils (De Vries *et al.*, 2001; Reinds *et al.*, 2001). Moreover, a uniform methodology to describe metal bioavailability and ecotoxicity in soil is still lacking (Vig *et al.*, 2003). The objective of this study was therefore to experimentally evaluate the current concentration guidelines for Cd in the soil solution (0.8 μg dissolved Cd L^{-1}; De Vries *et al.*, 2001) in relation to forest soils with contrasting physico-chemical characteristics. Cd is a major metal involved in atmospheric deposition. It is very mobile and can be taken up easily by plant roots (Ukonmaanaho *et al.*, 2001). Moreover, Cd is naturally present in the bedrock of many soils of northern and central Europe (P. Blaser, pers. commun.; Starr *et al.*, 2003). The soils were treated with solutions of $CdCl_2$, which is one of the major water-soluble chemical species involved in atmospheric deposition (Lum *et al.*, 1987). We hypothesized that, due to the high heterogeneity of the microbial communities in forest habitats (Hackl *et al.*, 2004), different soils would display both different Cd bioavailability and different reactions of the soil bacterial communities to Cd. We examined therefore the structure of the bacterial communities by T-RFLP profiling of the 16S rRNA gene, and combined it with the analysis of soil microbial activities such as basal respiration, acid phosphatase and β-glucosidase aiming at identifying the best indicators of Cd toxicity. We also measured the bioavailable pools of Cd with different methods in order to identify the best related to the toxicity data.

Materials and methods

Sampling sites

Seven forest soils with different physical and chemical properties were chosen from a soil profile database (Walthert *et al.*, 2004; Blaser *et al.*, 2005) of the Swiss Federal

Table 1. Coordinates, elevation, geology and vegetation types of the forest soils sampled

	Coordinates (*m*)*				
Location	*x* –	*y* –	Elevation	Soil type (m)	Main tree species
Lausanne	540 171	159 464	800	Dystric Cambisol	*Fagus sylvatica*, *Picea excelsa*
Burgdorf	612 080	209 700	625	Dystric Cambisol	*Fagus sylvatica*, *Abies alba*
Gerlafingen	608 975	224 412	477	Dystric Cambisol	*Fagus sylvatica*, *Carpinus betulus*
Piotta	694 750	151 925	1150	Dystric Cambisol	*Abies alba*, *Picea excelsa*
Schänis	723 464	225 068	730	Eutric Cambisol	*Fagus sylvatica*, *Fraxinus excelsior*
Laufen	602 200	249 150	678	Eutric/Gleyic Cambisol	*Fagus sylvatica*, *Quercus pubescens*
Sihlwald	685 375	233 775	503	Eutric/Gleyic Cambisol	*Fagus sylvatica*, *Abies alba*

Soil information from S. Zimmermann (pers. commun.).
*Swiss National Topographic System (*x*-coordinates, Easting; *y*-coordinates, Northing).

Table 2. Physico-chemical characteristics of the soils sampled

Location	pH (H_2O)	BS (%)	N (%)	C (%)	CEC ($cmol_c\,kg^{-1}$)	Sand (%)	Silt (%)	Clay (%)	Cd* ($mg\,Cd\,kg^{-1}$)
Lausanne	5.0	29	0.2	3.0	77	62	25	13	0.1
Burgdorf	4.5	51	0.1	2.0	66	66	23	11	0.1
Gerlafingen	4.6	41	0.3	1.0	100	36	40	24	3.1
Piotta	5.0	91	0.5	8.0	130	66	25	9	0.7
Schänis	5.7	96	0.3	3.0	118	43	35	22	0.4
Laufen	6.8	100	0.3	5.0	383	6	39	55	1.7
Sihlwald	7.4	100	0.3	4.0	297	14	33	53	0.2

*HNO_3-extractable Cd (total Cd), ICP-MS measurements.
BS, base saturation; CEC, cation exchange capacity.

Institute for Forest, Snow and Landscape Research (Tables 1 and 2). The locations 'Lausanne', 'Piotta', 'Sihlwald', 'Schänis', and 'Burgdorf' represent noncontaminated forest sites, while 'Laufen' is located on a Cd-containing bedrock, and 'Gerlafingen' is moderately contaminated by emissions from a nearby steel smelter processing scrap metals (Borer, 1997).

At each site, we sampled the soils from four to six zones in an area of 50 m^2. The litter layer was removed manually and soil from the top layer (0–10 cm, A horizon) was collected with a shovel in plastic bags. The A horizon was inspected for homogeneity, and care was taken to avoid collection of the upper organic and lower horizons. In the laboratory, the soil was stored at 4 °C until the following day, and then sieved through a 2 mm mesh. All the material from a given site was pooled together (Kelly *et al.*, 2003). The samples were stored at 10 °C until the experiment was started within one week of collection.

Cd treatments of soil microcosms

Four different Cd solutions (0.1, 1, 10 and 100 mM) were prepared by dissolving $CdCl_2$ (Sigma-Aldrich, St Louis, MO) in sterile double-deionized water (MQ water, Millipore, Billerica, MA). These concentrations were selected because they had been used in similar previous studies with Cd (Niklinska *et al.*, 1998; Renella *et al.*, 2003).

Sieved soil (80 g) was placed into a beaker, and 10 mL of each of the four Cd solutions or of double-deionized water (control treatment) were slowly added to the samples. The soils containing the added solution were gently mixed in order to obtain a homogeneous distribution of Cd (Rasmussen & Sørensen, 2001), thus resembling natural bioturbation processes which can cause redistribution of the metal in the soil (Anderson, 1988). Then the soils were transferred, in four replicates, into 250 mL plastic jars (Millipore Stericup filter systems), and covered with the supplied caps to maintain stable humidity conditions throughout the experiment, but permitting aeration. The microcosms were incubated at 20 °C under 50% of external humidity and at a water content ranging from 50–60% WHC for Laufen, Gerlafingen, Schänis and Lausanne to 70–80% WHC for Burgdorf, Piotta and Sihlwald. During the incubation the water content was monitored gravimetrically. Water loss was minimal, and, if necessary, was compensated for by the addition of sterile MQ water. The microcosms were harvested after 30 days of incubation. This incubation time has been often adopted in other microcosm studies (Ranjard *et al.*, 1997; Renella *et al.*, 2003; Tom-Petersen *et al.*, 2003). This time span is sufficient to visualize the effects of metal application on the microbial communities avoiding possible priming effects due to sieving, and avoiding long-term starving effects due to lack of carbon supply. Part of the soil was immediately used for basal respiration measurements,

while aliquots were frozen at −20 °C for enzymatic assays and in a DNA extraction buffer for DNA extraction. The remaining soil was oven-dried for 24 h at 105 °C for extractions of bioavailable Cd and dry weight estimation.

Soil chemical characteristics

As preliminary analyses showed low variability between replicates, all extractions were performed in triplicate with soils harvested from three of the four microcosms. Lakanen extracts were obtained using the method by Lakanen & Erviö (1971) and Blaser *et al.* (2000). Aliquots of 5 g of oven-dried soil were suspended at pH 4.65 in 50 mL solution containing ammonium acetate (0.5 M), Titriplex II (0.02 M) and acetic acid (0.5 M), shaken for 1 h at 20 °C on an overhead shaker, left to settle for 5 min and then filtered through paper filters (Nr. 0790 1/2, Schleicher & Schuell, Dassel, Germany). The Lakanen extracts were stored at 4 °C until measurement. Water extractions were performed by shaking 20 g of oven-dried soil mixed with 200 mL of MQ water for 16 h on an overhead shaker. The soil slurry was then centrifuged for 10 min at 1200 ***g*** in a Sigma 6-15 centrifuge (Sigma-Aldrich), and subsequently vacuum-filtered through a 0.45 μm membrane filter (Millipore Stericup systems). Each extract was divided into three portions for pH, dissolved organic carbon (DOC) and Cd measurements. For Cd analysis, the extract was acidified with 65% HNO_3 (*c.* 40 μL for 50 mL of water extract) and then stored at 4 °C until measurement. DOC concentrations in the water extracts were measured with a Shimadzu TOC-500 apparatus (Shimadzu, Kyoto, Japan), while pH was measured with an Orion 520A pHmeter (Thermo Electron, Waltham, MA).

Cd concentrations were measured within 4 weeks of extraction by atomic adsorption spectroscopy (AAS) using a spectrAA 300/400 (Varian, Palo Alto, CA). The values measured in the water extractions were converted from mg Cd L^{-1} to mg Cd kg^{-1} soil dry mass.

The Windermere Humic Aqueous Model (WHAM6; Tipping, 1994) was used to calculate the free ion (Cd^{2+}) concentration in the soil solution from the Cd concentrations (mg water-extractable Cd L^{-1}) of the water extracts. Input parameters included pH, fulvic acids (estimated from DOC measurements), and major cation and anion concentrations derived from the soil database.

Ecotoxicological tests

Basal soil respiration was measured using the method described by Zimmermann & Frey (2002) with some modifications. Aliquots of 10 g of fresh soil were weighed into plastic lipped tubes and incubated for 24 h at 20 °C in 250 mL flasks aerated with CO_2-free air and containing 20 mL of 0.025 M NaOH to trap pre-existing CO_2. After this step the tubes were transferred to clean CO_2-free flasks containing 20 mL of fresh 0.025 M NaOH and incubated again at 20 °C for 48 h. Na_2CO_3 was precipitated by successive addition of 1 mL of 1 M $BaCl_2$, while titration with HCl using phenolphthalein as an indicator permitted quantification of the CO_2 produced by the soil sample.

Acid phosphatase activity was measured according to the protocol for soils rich in organic matter by Schneider *et al.* (2000). Subsamples of 1 g of frozen soil were incubated with *p*-nitrophenyl phosphate substrate (PNPP, Merck, Darmstadt, Germany) and buffer (MUB pH 6.5) for 30 min at 30 °C. The reaction was stopped by adding 2 M $CaCl_2$ and 0.2 M NaOH. The product (*p*-nitrophenol, PNP) was determined photometrically at 410 nm. For β-glucosidase we used the method described by Tabatabai (1994), in which 5 g of fresh soil is incubated in a 2 M acetate buffer (pH 6.2) and 35 mM substrate (salicin, Merck) for 3 h at 37 °C. After incubation, the product saligenin was recovered by filtration. The concentration of saligenin was determined by mixing the filtrate with 0.2 M boric acid buffer (pH 10.0) and 6.68 mM 2, 6-dibromoquinon-4-chloroimid as colour reagent. After a further 1 h incubation in the dark at room temperature, the solution was analyzed photometrically at 578 nm. All the ecotoxicological tests were performed in triplicate.

DNA extraction

For DNA extraction from soils, a modification of the bead-beating method described by Bürgmann *et al.* (2001) was used. Immediately after harvesting the microcosms, 500 mg of fresh soil were stored frozen in 1.3 mL of extraction buffer [2% hexadecyl trimethyl ammonium chloride (CTAB); 20 mM EDTA pH 8; 2 M NaCl; 100 mM tris hydroxymethylaminomethane pH 8; 2% polyvinylpyrrolidone (PVP-40)] and 750 mg of glass beads of 0.10–0.11 mm diameter (Merck). Following thawing, the samples were extracted by bead-beating for 40 s at a speed of 5.5 m s^{-1} with a Thermosavant FastPrep FP120 (BIO 101, MP Biomedicals, Irvine, CA) bead beater and centrifugation for 5 min at 13 000 ***g*** in a 5415D microcentrifuge (Vaudaux-Eppendorf, Schönenbuch, Switzerland). The extraction procedure was repeated three times. Combined extracts were extracted with 2 mL chloroform: isoamylalcohol (24 : 1), followed by precipitation of the supernatant with 3 mL of isopropanol for 1 h at 37 °C. After centrifugation at 12 000 ***g*** for 15 min with a Sorvall RC5B centrifuge (Du Pont instruments, Wilmington, DE), the isopropanol supernatant was discarded, and the remaining pellet was washed in 1.5 mL of 70% ethanol and centrifuged for 2 min at 10 000 ***g***. The pellet was air dried and redissolved in AE buffer (1 mL for 1 g soil dry weight).

DNA was quantified using the method described by Hartmann *et al.* (2005). An aliquot of DNA was previously

diluted 1 : 25 with MQ water. A dye solution was prepared separately by diluting an aliquot of a fluorescent dye stock (Pico Green®, Molecular Probes, Basel, Switzerland) 1 : 200 with AE buffer. Twenty-five microliters of diluted DNA were added to 25 μL of the dye solution in an optical plate (Applied Biosystems, Rotkreuz, Switzerland). In parallel, DNA standards were prepared from herring sperm DNA (Invitrogen, San Diego, CA) representing a concentration range of 0–750 ng mL^{-1}. Fluorescence signals at 520 nm excitation wavelength were detected with an ABI PRISM® 7700 Sequence Detection System (Applied Biosystems).

PCR reaction and digestion

For PCR reactions, 5 ng μL^{-1} of DNA were pretreated with 3 μg μL^{-1} of BSA for 2 min at 95 °C. Ten to 50 ng of pretreated DNA were then added as a template for the PCR reaction, containing 1 × PCR buffer, 0.5 mM $MgCl_2$, 400 μM of dNTP mixture (Catalys, Wallisellen, Switzerland), 0.3 μg μL^{-1} BSA, 0.2 μM of each primer (Microsynth, Balgach, Switzerland), 2 U of HotStarTaq® polymerase (Qiagen, Hilden, Germany) in a 50 μL volume. The bacterial 16S rRNA gene was amplified with primers 27F (AGA GTT TGA TCM TGG CTC AG, 5′ labelled with 6-FAM) and 1378R (CGG TGT GTA CAA GGC CCG GGA ACG), which amplify a region of *c.* 1350 bp of the bacterial 16S rRNA gene domain. The reactions were carried out in a PTC-100 thermocycler (MJ Research, Waltham, MA). PCR conditions applied an initial activating step for HotStarTaq® polymerase (15 min at 95 °C), followed by 35 cycles with denaturation at 94 °C for 45 s, annealing at 48 °C for 45 s and extension at 72 °C for 2 min. The PCR amplification was ended by an additional final extension step at 72 °C for 5 min.

The PCR product was digested with an equal volume of digestion mix, consisting of 10 U per reaction (each) of the restriction enzymes *Hha*I and *Hae*III in 1% Y^+ Tango buffer for 3 h at 37 °C (Fermentas, Burlington, ON). This enzyme pair was chosen because preliminary experiments that had also included other tetrameric enzymes (e.g. *Msp*I and/or *Hae* III) and other studies (Sessitsch *et al.*, 2001, Frey *et al.*, 2006) showed that it produced the largest number of fragments. Finally, a clean-up reaction of the digestions was performed with Montage single-sample PCR clean-up microspin columns (Millipore, Billerica, MA), according to the manufacturer's instructions, in order to eliminate excess salts.

T-RFLP

Digestion products were prepared for analysis by capillary electrophoresis as follows: 2 μL of the digestion products were added to 11.4 μL of HIDI formamide (Applied Biosystems) and 0.6 μL of ROX500 DNA fragment length standard (Applied Biosystems). The samples were then denatured for 2 min at 95 °C and immediately chilled on ice. Electrophoresis was performed for 30 min at 60 °C with an ABI Genetic Analyzer 310 (Applied Biosystems). The lengths of fluorescently labelled T-RFs were determined with internal standards using GeneScan 3.1. and Genotyper softwares (Applied Biosystems). Operational taxonomic units (OTU) were defined as peaks with a size of $x \pm 0.5$ relative migration units (rmu) and a height of at least 150 fluorescence units in all the replicates of at least one of the treatments. In this study we referred only to the changes in the community structure, considering that distinct phylotypes could produce T-RFs of the same size and that estimation of diversity would therefore not be accurate (Hartmann *et al.*, 2005, Widmer *et al.*, 2006).

Statistical analysis

Normality of the data was evaluated with the Shapiro–Wilk test using Origin 7 software. One-way ANOVA analysis corrected with the Tukey post hoc test, or the Kruskal–Wallis analysis of variance, was performed. Regression and correlation analysis were performed with the $\log_{10}$-transformed Cd and ecotoxicological data. ANOVA and regression analysis were performed with SYSTAT 10 (Statsoft Inc., Tulsa, OK).

The raw data from the electropherogram (T-RFLP) profiles were normalized by converting the peak height of each OTU above the threshold limit of 150 fluorescence units to the percentage value of the total peak heights in the profile. Average ($n = 4$) relative abundances (i.e. peak height) of each OTU in each Cd treatment were then compared for significant differences with the controls by one-way ANOVA corrected with the Tukey post hoc test (SYSTAT 10). The data from the T-RFLP profiles were further treated using Fisher's Z transformation. Principal component analysis (PCA) based on covariance was performed on the transformed data using CANOCO 4.5 software (Microcomputer power, Ithaca, NY).

Results

Soil properties

The largest amount of DNA was extracted from samples of Laufen and Piotta soil (> 30 μg DNA g^{-1}), while in the other soils the average amounts varied in a range between 10 and 27 μg DNA g^{-1}. In general, Cd treatments had no significant effect on the extracted DNA contents (Table 3).

The pH measured in the soil aqueous extracts of the microcosms (Table 3) showed that the most acidic soils were from Lausanne, Burgdorf, Piotta and Gerlafingen (pH 4.5–5), followed by Schänis (pH 5.7). A higher soil pH (pH > 6.7) was found at Sihlwald and Laufen. The pH values significantly decreased with increasing Cd addition.

Table 3. Total Cd added to the soils, proportions of soluble Cd retrieved in Lakanen and water extracts, predicted Cd free ion, dissolved organic carbon (DOC), DNA amounts extracted from the soils, and pH values measured in the water extracts

Location	Treatment (mM $CdCl_2$)	Cd addition (mg Cd kg^{-1} dry weight soil)	Cd Lakanen extracts (mg Cd kg^{-1} dry wt. soil)	Cd water extracts (mg Cd kg^{-1} dry weight soil)	Cd free ion (mg Cd kg^{-1} dry weight soil)	DOC (mg C kg^{-1} dry weight soil)	DNA content (mg DNA kg^{-1} dry weight soil)	pH
Lausanne	0	0	0.13 ± 0.04a	0.01 ± 0.00a	0.01 ± 0.00a	84.0 ± 4.0b	24 ± 1a	5.0 ± 0.0b
	0.1	1.9	1.80 ± 0.10b	0.09 ± 0.01b	0.09 ± 0.01b	81.0 ± 2.0b	15 ± 2a	4.7 ± 0.0a
	1	19	20.01 ± 0.29c	1.12 ± 0.07c	0.86 ± 0.08c	80.0 ± 6.0b	20 ± 2a	4.8 ± 0.0a
	10	190	179.97 ± 4.48d	11.24 ± 1.69d	8.54 ± 0.81d	86.0 ± 3.0b	22 ± 0a	4.8 ± 0.0a
	100	1900	2059.91 ± 177.38e	562.05 ± 40.80e	83.63 ± 6.12e	68.0 ± 1.0a	22 ± 3a	4.8 ± 0.0a
Burgdorf	0	0	0.14 ± 0.02a	0.02 ± 0.00a	0.02 ± 0.00a	360.0 ± 7.0a	11 ± 3a	4.5 ± 0.0b
	0.1	2.5	2.05 ± 0.02b	0.25 ± 0.00b	0.22 ± 0.00b	372.0 ± 7.0a	14 ± 2a	4.5 ± 0.0b
	1	25	20.23 ± 0.34c	3.26 ± 0.08c	2.47 ± 0.06c	373.0 ± 1.0a	15 ± 1a	4.5 ± 0.0b
	10	247	164.01 ± 40.58d	40.13 ± 1.11d	17.08 ± 0.42d	383.0 ± 0.0a	18 ± 2a	4.4 ± 0.0b
	100	2473	1241.01 ± 38.44e	1000.79 ± 12.81e	152.76 ± 1.92e	372.0 ± 0.0a	12 ± 1a	4.2 ± 0.0a
Gerlafingen	0	0	0.28 ± 0.02a	0.02 ± 0.01a	0.02 ± 0.01a	287.0 ± 3.0a	19 ± 2a	4.6 ± 0.0b
	0.1	2.1	1.96 ± 0.22b	0.31 ± 0.06b	0.28 ± 0.05b	284.0 ± 2.0a	20 ± 1a	4.6 ± 0.0b
	1	21	20.68 ± 0.22c	4.07 ± 0.28c	3.59 ± 0.24c	279.0 ± 2.0a	18 ± 3a	4.6 ± 0.0b
	10	214	197.84 ± 5.05d	46.42 ± 4.27d	18.54 ± 1.91d	286.0 ± 3.0a	17 ± 2a	4.5 ± 0.0b
	100	2136	1265.96 ± 28.55e	926.37 ± 22.03e	138.93 ± 3.30e	272.0 ± 6.0a	16 ± 0a	4.2 ± 0.0a
Piotta	0	0	0.37 ± 0.02a	0.01 ± 0.01a	0.01 ± 0.01a	201.0 ± 3.0b	32 ± 1a	5.0 ± 0.0a
	0.1	2.6	2.47 ± 0.13b	0.03 ± 0.01a	0.02 ± 0.01a	195.0 ± 2.0b	36 ± 1a	5.2 ± 0.0a
	1	26	22.82 ± 0.13c	0.34 ± 0.02b	0.21 ± 0.01b	182.0 ± 1.0b	37 ± 1a	4.9 ± 0.0a
	10	258	211.21 ± 5.51d	0.56 ± 0.11c	2.41 ± 0.47c	190.0 ± 1.0b	37 ± 0a	4.9 ± 0.0a
	100	2585	2103.64 ± 38.55e	427.38 ± 88.80d	71.27 ± 13.32d	166.0 ± 3.0a	36 ± 1a	4.9 ± 0.0a
Schänis	0	0	0.03 ± 0.01a	0.04 ± 0.04a	0.03 ± 0.03a	135.0 ± 3.0b	19 ± 0a	5.7 ± 0.0a
	0.1	2.1	0.43 ± 0.01b	0.05 ± 0.01a	0.04 ± 0.01a	136.0 ± 3.0b	21 ± 1a	5.8 ± 0.0a
	1	21	3.71 ± 0.06c	0.47 ± 0.04b	0.37 ± 0.04b	131.0 ± 5.0b	20 ± 1a	5.6 ± 0.1a
	10	214	38.44 ± 0.45d	6.74 ± 0.58c	2.72 ± 0.23c	139.0 ± 3.0b	18 ± 1a	5.7 ± 0.1a
	100	2136	364.65 ± 7.19e	283.16 ± 2.58d	42.49 ± 0.39d	114.0 ± 2.0a	21 ± 0a	5.8 ± 0.1a
Laufen	0	0	1.01 ± 0.04a	0.02 ± 0.00a	0.01 ± 0.00a	315.0 ± 2.0b	36 ± 0a	6.8 ± 0.1a
	0.1	2.2	2.36 ± 0.01b	0.12 ± 0.01b	0.11 ± 0.00b	323.0 ± 2.0b	36 ± 0a	6.8 ± 0.0a
	1	22	16.75 ± 0.39c	0.31 ± 0.07c	0.23 ± 0.05c	320.0 ± 3.0b	28 ± 8a	6.8 ± 0.1a
	10	225	142.08 ± 1.91d	0.46 ± 0.06d	0.17 ± 0.02d	315.0 ± 3.0b	34 ± 1a	6.9 ± 0.1a
	100	2248	1034.85 ± 39.79e	77.23 ± 1.01e	11.57 ± 0.15e	297.0 ± 11a	34 ± 2a	6.8 ± 0.0a
Sihlwald	0	0	0.03 ± 0.01a	0.02 ± 0.00a	0.01 ± 0.00a	128.0 ± 6.0a	27 ± 5a	7.4 ± 0.0b
	0.1	2.4	0.47 ± 0.01b	0.08 ± 0.01b	0.07 ± 0.01b	137.0 ± 6.0a	14 ± 7a	7.5 ± 0.0b
	1	24	4.38 ± 0.02c	0.12 ± 0.01c	0.09 ± 0.01c	134.0 ± 6.0a	13 ± 5a	7.5 ± 0.0b
	10	236	39.01 ± 0.52d	1.82 ± 0.08d	0.73 ± 0.03d	118.0 ± 7.0a	18 ± 2a	7.3 ± 0.0b
	100	2360	331.72 ± 5.62e	52.27 ± 9.21e	7.87 ± 1.36e	107.0 ± 3.0a	21 ± 3a	6.9 ± 0.0a

Values are means ± SE; $n = 3$ for Cd measurements and DOC; $n = 4$ for DNA measurements.
Letters indicate significance values ($P < 0.05$) calculated with Kruskal–Wallis variance analysis or one-way ANOVA between the controls and the different Cd treatments.

At the 100 mM Cd treatment, pH was 0.2–0.5 U lower in the soil samples from Lausanne and Burgdorf ($P < 0.05$), and from Gerlafingen and Sihlwald ($P < 0.01$) than in the controls.

The average concentrations of DOC measured in the water extracts (Table 3) ranged from values around 80 μg C g^{-1} soil dry weight in the Lausanne soil to concentrations exceeding 300 μg C g^{-1} soil dry weight in the Burgdorf and Laufen soils. The 100 mM Cd treatment decreased DOC significantly in the soils from Lausanne and Piotta ($P < 0.01$) and from Laufen and Schänis ($P < 0.05$).

Lakanen-extractable Cd tended to decrease with the Cd treatments in all soils except for Lausanne (Table 3). In the soil from Lausanne, the amount of Lakanen-extractable Cd to the total additions was always close to 100%. In Schänis and Sihlwald soils, Lakanen-extractable Cd was less than 20% of the total additions throughout all the Cd treatment levels. In Burgdorf, Piotta, Gerlafingen and Laufen soils it ranged from 95% in the lowest Cd treatments (0.1 mM Cd) to 40% in the highest (100 mM Cd). Lakanen extracts correlated significantly with the total Cd additions ($R^2 = 0.86$, $P < 0.01$, Fig. 1a).

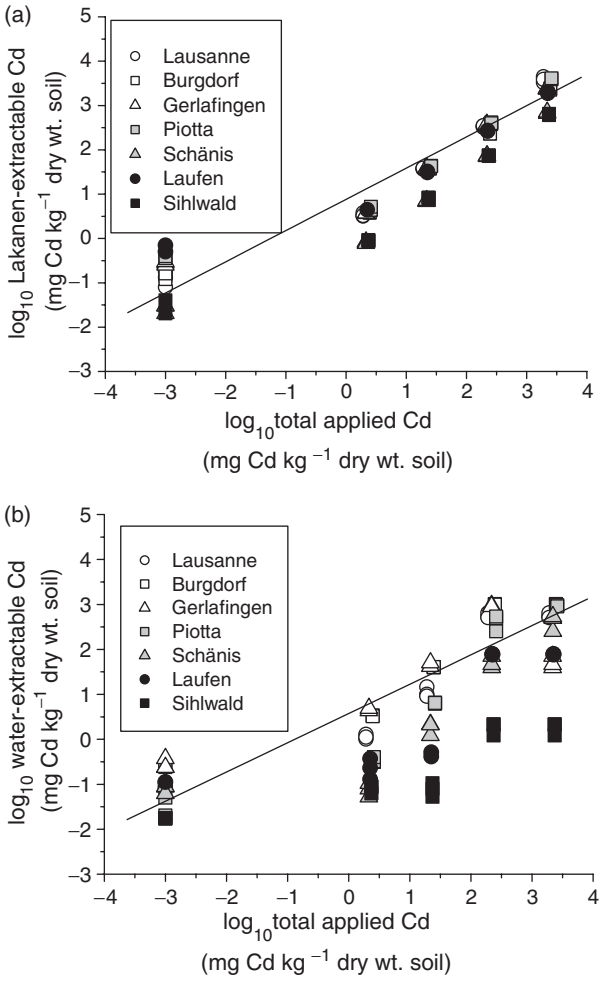

Fig. 1. (a) Correlations ($R^2 = 0.86$, $P < 0.01$) between $\log_{10}$-transformed Lakanen-extractable concentrations of Cd and $\log_{10}$-transformed initially applied Cd ($n = 105$). The 0 mM Cd addition was set as 0.001 mg Cd kg^{-1} dry weight soil. (b) Correlations ($R^2 = 0.62$, $P < 0.01$) between $\log_{10}$-transformed water-extractable Cd and $\log_{10}$-transformed initially applied Cd ($n = 105$) 0 mM Cd addition was set as 0.001 mg Cd kg^{-1} dry weight soil.

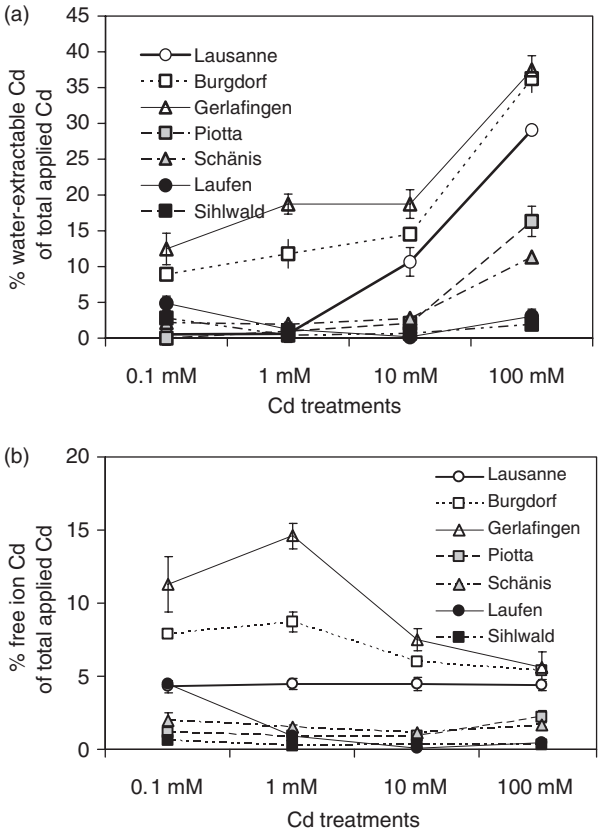

Fig. 2. (a) Percentages of water-extractable Cd to initially applied Cd. Bars represent SE ($n = 3$). (b) Percentages of free cations of Cd in the soil solution modelled with WHAM6 (Tipping, 1994) to initially applied Cd. Bars represent SE ($n = 3$). Lausanne, Burgdorf, Gerlafingen, Piotta, Schänis: pH < 5.8, clay content < 50%; Laufen, Sihlwald: pH > 6.7, clay content > 50%.

The amounts of Cd extracted with water to the Cd added ranged from 1% to 37% and increased with the treatment level (Fig. 2a). Detectable amounts of water-extractable Cd were observed in the water controls in all soils (Table 3). Water extracts correlated with the total Cd additions weakly but significantly ($R^2 = 0.62$, $P < 0.01$, Fig. 1b). The free ion concentrations modelled with WHAM6 decreased from 90% of the water-extractable Cd in the treatment with 0.1 mM Cd to 15% in the treatment with 100 mM Cd. Other important species were $CdCl^+$, $CdOH^+$ and $CdHCO_3^+$. In all soils and in all treatments the free ion represents less than 20% of the total additions (Fig. 2b). The free ion concentrations also correlated ($R^2 = 0.96$, $P < 0.001$, data not shown) with the Cd concentrations measured in the water extracts.

Ecotoxicological tests

Significant effects on β-glucosidase activity were observed only in the soil samples from Gerlafingen ($P < 0.05$) and Laufen ($P < 0.05$). In these soils the enzymatic activity was reduced at the highest Cd treatment (100 mM), as compared to the control. At the same high Cd treatment acid phosphatase was inhibited ($P < 0.05$) in the samples from Sihlwald, Gerlafingen and Schänis, but increased ($P < 0.05$) in those from Piotta.

Basal respiration was significantly inhibited (> 50%) in the highest Cd treatment (100 mM) in the soils from Piotta and Burgdorf ($P < 0.05$) and in the soil from Sihlwald (42% inhibition, $P < 0.05$). Inhibition in soils from Schänis (61%), Lausanne, Gerlafingen (41–51%) and Laufen (< 30%) was not significant. An initial increase, but not significant, in respiration rates was observed in three soils (Sihlwald, Laufen, Burgdorf) between the 0.1 mM and the 1 mM Cd treatments, corresponding to water-extractable concentrations up to *c.* 3.3 mg Cd kg^{-1} soil dry weight.

As correlations between the ecotoxicological variables and the different bioavailable Cd concentrations are very similar one to each other, only data obtained from correlations between the ecotoxicological variables and water-extractable Cd concentrations are shown (Figs 3a–c). In general, neither acid phosphatase nor β-glucosidase significantly correlated with total Cd additions (data not shown) or with the different bioavailable Cd pools (correlations with water extracts are shown in Figs 3a and b). Basal respiration was weakly but significantly correlated with all the Cd concentrations examined: water extracts ($R^2 = 0.25$, $P < 0.01$, Fig. 3c), free ion predictions ($R^2 = 0.21$, $P < 0.01$), total additions ($R^2 = 0.20$, $P < 0.01$) and Lakanen extracts ($R^2 = 0.14$, $P < 0.01$).

Bacterial communities analyzed by T-RFLP profiles

Variability within replicate T-RFLP profiles was relatively small, and OTU abundance and OTU size were always similar. The structures of the bacterial communities analyzed by T-RFLP analysis varied instead considerably from soil to soil. The largest number of OTUs scored was 76 in the soil from Lausanne, followed by the soils from Burgdorf (69), Laufen (69), Sihlwald (62), Gerlafingen (61), Schänis (60) and Piotta (58) (Table 4).

Marked structural changes in the bacterial communities due to Cd treatment were evident in the T-RFLP profiles of the Burgdorf and Lausanne soils: in the soil from Burgdorf (Fig. 4, Table 4), overall 86% of the OTUs differed significantly in abundance (five OTUs at 1 mM treatment, 19 at 10 mM, 59 at the 100 mM treatment). In the samples from Lausanne, 75% of the OTUs responded significantly to the Cd treatments (15 OTUs at the 10 mM treatment). In these soils, the OTUs increased or decreased similarly in abundance with treatment levels from 1 to 100 mM.

In the other soils major effects were found mainly at the highest Cd treatments (100 mM). More than 50% of the OTUs differed significantly at this concentration in soil samples from Piotta and Schänis, while soils from Gerlafingen (36%), Sihlwald (30.6%) and Laufen (10.6%) appeared less affected.

The patterns of changes in the T-RFLP profiles were similar in all the reacting soils (data not shown). For example, in the soil samples from Burgdorf and Lausanne, up to 16 OTUs disappeared from the profile with increasing levels of Cd treatments. Moreover, the 100 mM Cd treatment increased four OTUs in the size ranges from 63 to 75 rmu and from 200 to 205 rmu. In the other soils the major changes in OTU abundance were also detected in the two regions from 65 to 100 rmu and from 190 to 270 rmu. In the soil samples from Laufen and Sihlwald, however, changes in the T-RFLP profiles were not as pronounced as in the other soils, and no OTU strongly dominated the T-RFLP profile of the 100 mM Cd treatments.

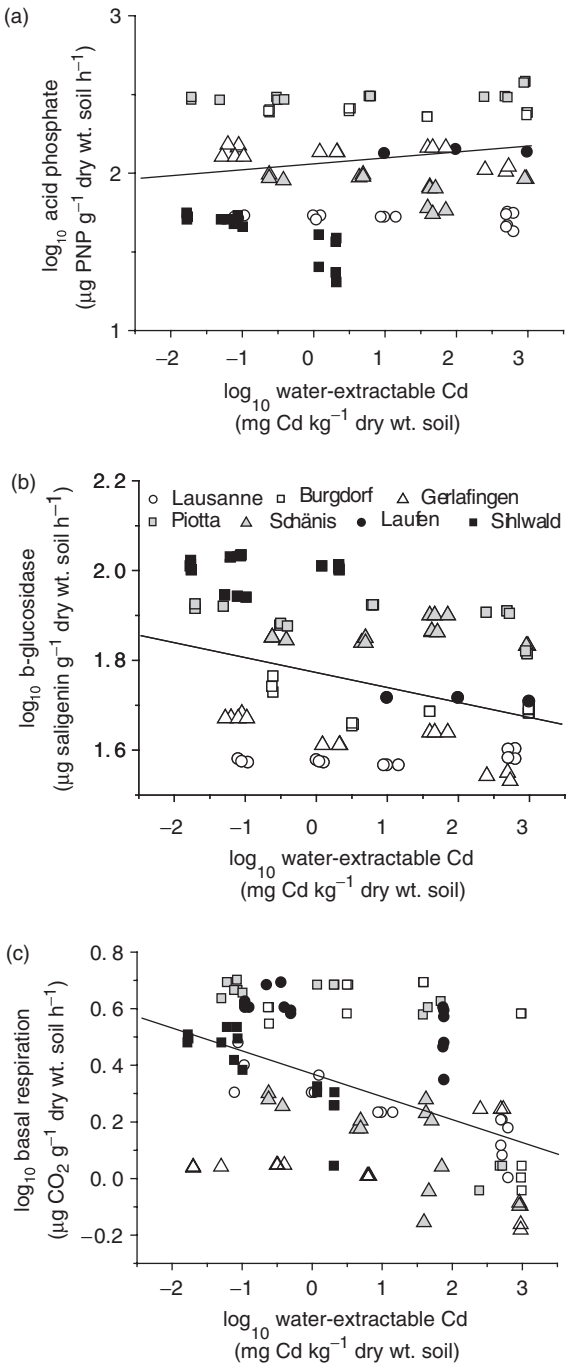

Fig. 3. (a) Relationship between $\log_{10}$-transformed acid phosphatase activity (μg PNP g^{-1} dry weight soil h^{-1}) and $\log_{10}$-transformed water-extractable concentrations of Cd ($R^2 < 0.1$, $P > 0.05$; $n = 105$). (b) Relationship between $\log_{10}$-transformed of β-glucosidase activity (μg saligenin g^{-1} dry weight soil h^{-1}) and $\log_{10}$-transformed water-extractable concentrations of Cd ($R^2 = 0.13$, $P > 0.05$; $n = 105$). (c) Relationship between $\log_{10}$-transformed basal respiration (μg CO_2 g^{-1} dry weight soil h^{-1}) and $\log_{10}$-transformed water-extractable Cd ($R^2 = 0.25$, $P < 0.01$; $n = 105$).

Table 4. Species richness detected in the T-RFLP profiles of the water control (0 mM Cd) samples of the different soils tested, and number of OTUs significantly ($P < 0.05$) decreasing and increasing in the different Cd treatments compared to the control

	0 mM Cd	0.1 mM Cd		1 mM Cd		10 mM Cd		100 mM Cd	
Location	Total OTUs	OTUs increasing	OTUs decreasing	OTUs increasing	OTUs decreasing	OTUs increasing	OTUs decreasing	OTUs increasing	OTUs decreasing
Lausanne	76	0	0	4	3	11	4	12	45
Burgdorf	69	0	0	1	4	10	9	18	41
Gerlafingen	61	0	3	0	3	3	5	6	16
Piotta	58	0	0	0	0	1	8	14	18
Schänis	60	3	0	3	0	3	2	13	18
Laufen	69	0	0	2	0	4	0	1	6
Sihlwald	62	0	1	4	1	8	1	8	11

Lausanne, Burgdorf, Gerlafingen, Piotta, Schänis: pH < 5.8, clay content < 50%.
Laufen, Sihlwald: pH > 6.7, clay content > 50%.

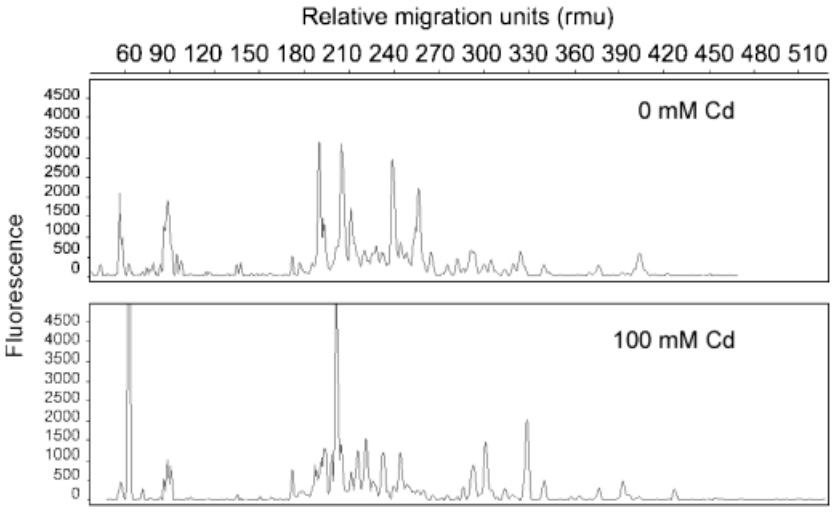

Fig. 4. T-RFLP profile of a sample from (a) 0 mM Cd (water treatment) and (b) 100 mM Cd treatment of the soil from Burgdorf.

PCA permitted one to visualize the different distributions of the Cd treatments between acidic and calcareous soils. In the soils from Lausanne and Burgdorf (Figs 5a and b), the 100 mM Cd treatment forms a separate cluster from the other treatments on the first principal component (PC1), explaining 62.4% and 66.8% of the variation, respectively. The 10 mM Cd treatment appears also slightly separated along the second component (PC2). The clustering of the 100 mM Cd treatment appears also in the soils from Gerlafingen, Piotta and Schänis (Figs 5c–e), but no clear separation is evident in the soils from Laufen and Sihlwald (Figs 5f and g).

Discussion

The objective of this study was to determine the effects of increasing Cd concentrations on the bacterial communities of different forest soils. We tested microbial activity variables in soils and examined the bacterial community structure in relation to the total and different (Lakanen-extractable, water-extractable, free ion) Cd pools to estimate which could better reflect Cd bioavailability in forest soils.

Soil microbiota have been shown to respond to high metal concentrations (Khan & Scullion, 2002). In the case of forest soils, the presence of large amounts of organic matter can reduce metal bioavailability and toxicity to microorganisms (Hattori, 1996). In accordance with these observations, the total Cd amounts added to our soils were spanning a wide range of concentrations from environmentally relevant concentrations up to levels of extreme pollution, and always exceeding median yearly atmospheric deposition values, which have been currently estimated in Switzerland as *c.* $0.75 - 1\,\mathrm{g\,ha^{-1}\,year^{-1}}$ (BUWAL report, 2002).

The soils chosen in this study represent a range of soil types in Switzerland which vary in their physico-chemical characteristics (Table 2). Water-extractable background concentrations in all soils are in the same concentration range as reported by Andersen *et al.* (2002) for soil solution Cd in different forest soils. The results from the different Cd measurements generally revealed the important role of soil

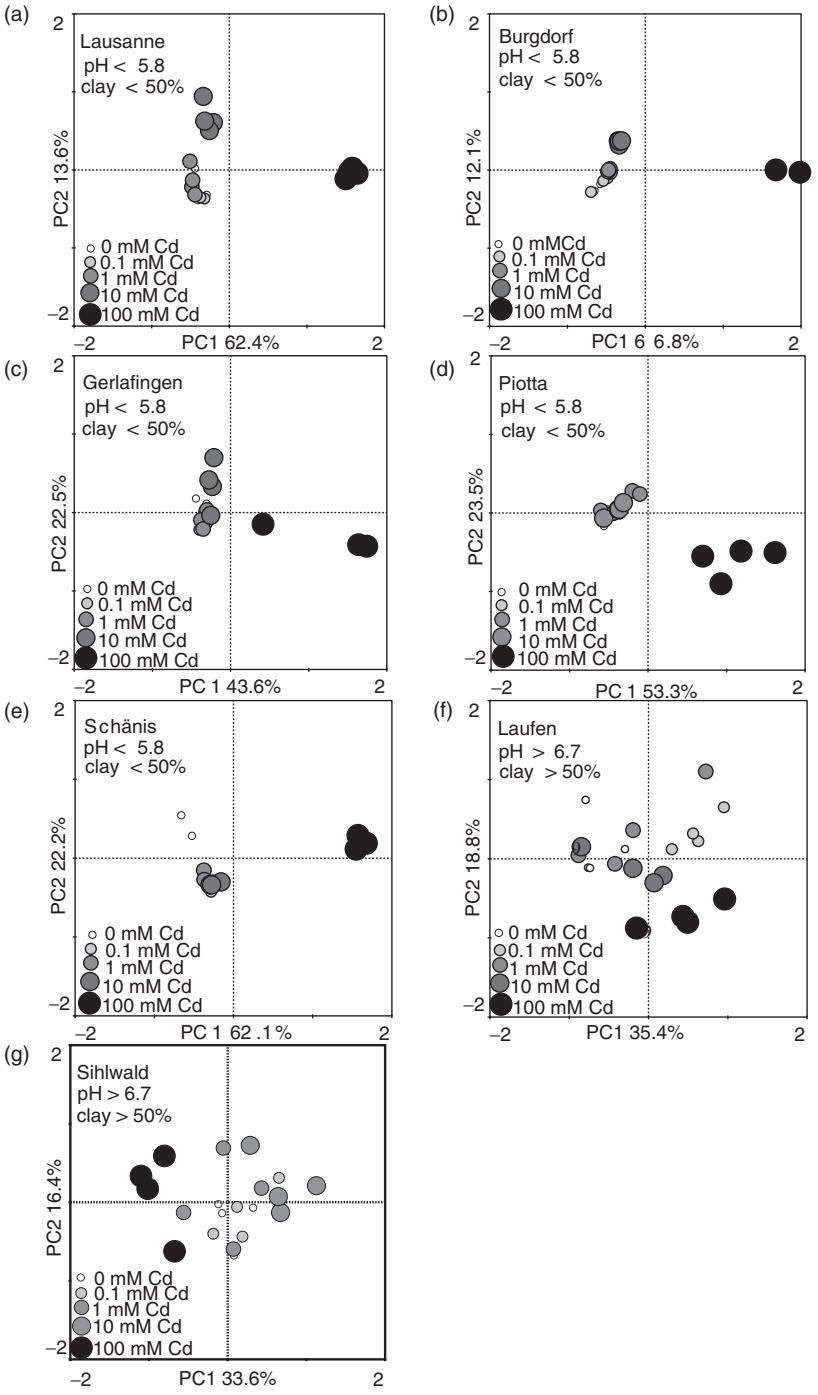

Fig. 5. PCA ordination diagrams from the T-RFLP profiles of the soil from (a) Lausanne, (b) Burgdorf, (c) Gerlafingen, (d) Piotta, (e) Schänis, (f) Laufen and (g) Sihlwald, treated with increasing Cd concentrations.

factors such as pH and clay content in determining the chemical behaviour of Cd. A higher amount of Cd was retrieved in Lakanen extracts than in water extracts, showing the extractive strength of this method. In water extracts, Cd was always retained more effectively in soils with a higher pH (Laufen, Sihlwald, Schänis) rather than in soils with a lower pH, such as those from Lausanne and Burgdorf. Interestingly, we did not observe any difference in water-extractable Cd between the soils from Gerlafingen and Laufen, which were subjected to anthropogenic or geogenic long-term Cd contamination, and the other soils, without Cd contamination. Thus, we hypothesize that in the soils

from Gerlafingen and Laufen, the total (HNO_3-extractable) background Cd content may not be in a readily bioavailable form.

The fraction of water-soluble Cd increased with increasing total added concentrations, as has also been reported by Niklinska *et al.* (1998) and Prokop *et al.* (2003). Cd binding in the soils decreased little up to a concentration of 10 mM, but considerably at the highest concentration. Stronger sorption properties of forest soils have been reported by Renella *et al.* (2003), who retrieved $< 2\%$ of total Cd in water and DTPA extracts, and by Landi *et al.* (2000), who measured in NH_4NO_3 extracts a range from 0% to 0.24% of various Cd additions.

We also characterized DOC in the soil water extracts, as DOC has been reported to play an important role in metal binding mechanisms and in determining metal bioavailability (Antoniadis & Alloway, 2002; Kiikilä *et al.*, 2002). In the soils from Lausanne, Piotta and Laufen, DOC was significantly decreased ($P < 0.05$) in the highest Cd treatments. Soil DOC concentrations are the results of a variety of different processes. For example, DOC is produced by the decomposing activity of the soil microbes, or by C inputs from microbial biomass. On the other hand, the DOC pool can also be decreased by microbial activity (Kalbitz *et al.*, 2000). In soils treated with heavy metals, DOC is generally known to decrease initially, due to a lowering of the microbial activity, but it can also increase, in the long-term, due to the accumulation of decomposing residues which are not readily utilized (Merckx *et al.*, 2001). The decrease of DOC observed in some of the soils of our short-term microcosm experiment may reflect a decreased decomposition rate of soil organic matter. In the soils in which DOC did not vary, it could be assumed that the processes of DOC production and DOC decomposition were affected to a similar extent by the contamination.

In our study, the toxic effects of Cd concentrations could not be defined clearly by the enzymatic activities. Both enzymes tested generally exhibited a variable response in samples treated with different Cd concentrations. In addition, they did not correlate significantly with any of the Cd pools investigated. The variability noted among different soils in both enzymes may be due to physiological adaptations to heavy metals (Renella *et al.*, 2004) at low bioavailable concentrations, or to progressive modifications of the bacterial community structure at higher bioavailable Cd concentrations. Moreover, the physico-chemical properties of the soil can influence the enzyme's activity. Soil enzymes can be adsorbed, for example, on the soil surface or interact with organic matter (Gianfreda & Bollag, 1994; Nannipieri *et al.*, 2003).

Previous studies of enzyme activities with forest soils have yielded similar variable results. However, because these have often been derived from different Cd extraction procedures, a comparison with the different bioavailable pools is difficult and in many cases we can only relate our results to the total (HNO_3-extractable) Cd concentrations reported. For example, Stuczynski *et al.* (2003) and Renella *et al.* (2003) found no significant response of acid phosphatase to increasing amounts of Cd added to forest soils, exceeding in some cases 500 mg total Cd kg^{-1} soil (Stuczynski *et al.*, 2003). On the other hand, inhibition of phosphatase activity was found by Moreno *et al.* (2003), with sludge-amended soils at Cd concentrations between 25 and 8000 mg total Cd kg^{-1} soil. Also Effron *et al.* (2004) reported, in a native forest soil, up to 51% inhibition at a total Cd concentration ranging from 20 to 500 mg total Cd kg^{-1}. A significant reduction in the activity of acid phosphatase has also been reported at lower water-extractable concentrations (10–30 mg Cd kg^{-1}) in a forest soil (Landi *et al.*, 2000) and in a sandy loam (Karaca *et al.*, 2002). These concentrations fall approximately in between the two highest water-extractable Cd concentrations we measured, at levels approximating the point where we also sometimes observed effects on the enzyme. However, due to the variability of our results and those of others, we cannot relate the effects specifically to a precise range of Cd concentrations.

β-glucosidase in nonforest soils has not been found to be sensitive to Cd at both low concentrations (10–40 mg total Cd kg^{-1} soil; Renella *et al.*, 2004) and much higher concentrations (up to 8000 mg total Cd kg^{-1} soil; Moreno *et al.*, 2003). Also, in forest soils β-glucosidase showed no effect at a Cd addition range of 20–500 mg total Cd kg^{-1} (Effron *et al.*, 2004). These findings are in accordance with our data, in which the inhibition of this enzyme was significant only in two of the seven soils, and always remained below 35%. As these soils are contrasting in properties and in concentrations at which effects were seen, we can hypothesize that environmental or experimental factors such as soil properties or quality and concentration of organic matter, more than Cd, may have caused the inhibition observed.

Among the ecotoxicological tests we considered, soil basal respiration showed a clearer response to Cd concentrations. In the two soils from Sihlwald and Laufen, basal respiration was inhibited in a range between 52.27 and 77.23 mg water-extractable Cd kg^{-1}, while in the other five soils it was generally inhibited at water-extractable concentrations higher than 283.16 mg Cd kg^{-1}.

At lower Cd concentrations than those reported above, we sometimes noted an increase in respiration rates. The increase in respiration after a moderate Cd treatment has also been reported by Renella *et al.* (2004) in a sandy soil artificially amended with Cd solutions, and related to the increasing demand for maintenance energy. In addition, Landi *et al.* (2000) assumed that this effect could be due to the decomposition of the dead cells by surviving

microorganisms. At moderate concentrations, not very pronounced changes in respiration rates were observed by Khan & Scullion (2002) for a soil treated with Cd-enriched (0.7–75 mg total Cd kg^{-1} soil) sludge. At higher Cd concentrations, however, decrease in CO_2 production in forest soils was generally reported. For example, Niklinska *et al.* (1998) and Landi *et al.* (2000) found a marked decrease in respiration on forest litter and on a forest soil spiked with, respectively, 400 and 500 mg total Cd kg^{-1} soil. For comparison, these concentrations are in the range between the two highest levels of Cd treatments in our experiment, and are in agreement with our findings. Basal respiration indicates the combination of bacterial and fungal activity (Rajapaksha *et al.*, 2004). Although generally fungal communities have been often found to be more tolerant to metals than the bacterial communities (Fritze *et al.*, 2000; Rajapaksha *et al.*, 2004), also metal-sensitive fungal groups have been identified by PLFA analysis of soils in the vicinity of smelters (Pennanen *et al.*, 1996). The effects of heavy metals on basal respiration reflect therefore the responses of metal-sensitive and metal-tolerant microbial groups together.

In addition to the effects on basal respiration rates, the highest bioavailable concentrations (>283.16 mg water-extractable Cd kg^{-1} soil dry weight) also caused significant changes in the bacterial community structure in five of the seven soils (Fig. 5a–e), which were generally characterized by the appearance and dominance of some OTUs over the whole community profile, as well as reduction in abundance and in a few cases a complete disappearance of other OTUs (Table 4). Heavy metals have been shown to decrease microbial diversity (Rasmussen and Sørensen, 2001) and in some cases caused the disappearance of OTUs in T-RFLP profiles (Turpeinen *et al.*, 2004; Mengoni *et al.*, 2005). In addition, however, it was often observed that the metal-induced effects on community structure are also characterized by the dominance of particular groups. Dominance has been shown in long-term contaminated sites (Turpeinen *et al.*, 2004; Frey *et al.*, 2006; Widmer *et al.*, 2006), and also after short-term incubations of soils spiked with different heavy metals, such as Cu (Tom-Petersen *et al.*, 2003), Hg (Ranjard *et al.*, 2000), Zn (Kelly *et al.*, 1999), or various heavy metals including Cd (Ranjard *et al.*, 2006). These reactions may reflect the selective pressure of metals on the soil community, which leads to the death or reduction of sensitive populations but at the same time also to the survival and growth of populations adapting to the metal contamination (Bååth *et al.*, 1998), and finally to the change of community composition.

Interestingly, we did not observe a marked shift in the bacterial community composition in the soils from Sihlwald and from Laufen (Figs 5f and g), which are the only two soils in this study characterized by a pH > 6.7 and a high clay content (>50%), and where basal respiration was inhibited at the lowest water-extractable Cd concentrations (55.27–77.23 mg Cd kg^{-1} soil). The low bioavailability of Cd in these two soils may not have been sufficient to induce a change in the bacterial populations, although causing a general reduction on basal respiration due to physiological adaptations of the communities. In fact, Renella *et al.* (2004) also found that water-extractable Cd concentrations of less than 0.3 mg Cd kg^{-1} soil, much lower than those we measured, affected basal respiration and enzymatic activities but not the structure of the bacterial community in agricultural soils with a pH > 7. Moreover, Hattori (1992) and Ranjard *et al.* (1997) related the changes in the structure of the bacterial communities to the different physico-chemical properties of the soils, and in particular to the characteristic of the microenvironment where the bacteria are located. It has in fact been shown that bacteria associated with macropores are more heavily exposed to heavy metal fluxes in the soil water than those living in micropores (Ranjard *et al.*, 1997). A more pronounced increase in metal-adapted populations in more sandy soils characterized by larger soil aggregates (Doelman *et al.*, 1994), than in clayey soils such as Sihlwald and Laufen, could therefore be expected.

Although our microcosm experiment is a short-term study, and does not allow prediction of the toxic effects of Cd on the structure of the soil communities in a long timescale (Renella *et al.*, 2002), we observed in general that, after 30 days of incubation in the microcosm, no effects of Cd on the soil microbial activity, referred here especially as basal respiration, and on the bacterial community structure, as visualized by T-RFLP, were observable up to the second highest Cd treatment (10 mM). In terms of Cd bioavailability, no-effect concentrations corresponded to values ranging from 0.09–0.86 mg Cd^{2+} kg^{-1} soil, 0.12–1.12 mg water-extractable Cd kg^{-1} soil, and 4.38–20.01 mg Lakanen-extractable Cd kg^{-1} soil. All these concentration ranges always far exceeded the UN/ECE soil limits for total Cd in the soil solution of 0.8 μg Cd L^{-1} (De Vries *et al.*, 2001). Also, background values were often approaching these guidelines (Table 3). Our results suggest that these guidelines ensure protection for a wide range of soil types, although the background concentrations that we found in the soil water would imply that, according to these limit values, no further accumulation of Cd in our soils should be allowed. In a realistic policy for risk assessment, the great heterogeneity of forest soils, which could influence metal bioavailability, and also the reaction mechanisms of the microbial communities to the metals, should therefore be strongly considered. In conclusion, our model microcosm system provided insight on indicators of bioavailability in different soils, combined with a set of ecotoxicological tests which were also evaluated. We concluded that while water extractions and free ion concentrations are good predictors of Cd bioavailability,

Lakanen extractions may be too strong and often resemble total concentrations. Among the ecotoxicological tests, enzymatic activities do not provide a clear index of Cd toxicity, but basal respiration and T-RFLP fingerprinting are more sensitive tools. We also identified in particular a possible common pattern of reaction distinguishing forest soils with a high pH (pH > 6.7) and a considerable clay content (>50%) from more acidic (pH < 5.8) forest soils with a greater sandy fraction. Further studies would help to elucidate on the different degrees of vulnerability of the microbial communities of forest soils, in both the short and long term, to the different bioavailable Cd pools.

Acknowledgements

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