Trace Metal Exposure of Soil Bacteria Depends on Their Position in the Soil Matrix

ÅSGEIR R. ALMÅS,* JAN MULDER, AND LARS R. BAKKEN

Department of Plant and Environmental Sciences, Norwegian University of Life Sciences, P.O. Box 5003, 1432 Ås, Norway

Micropores and biofilms of soils may protect bacteria against chemical stress, predation, and competition phenomena, explaining the great diversity and robustness of soil microbial communities and functions. We used sequential dispersion/density gradient centrifugation to separate free and loosely attached cells (FLA) from strongly attached cells (SA). The two fractions of the soils communities were investigated along a Zn and Cd pollution gradient, and the pollution-induced trace metal community tolerance (PICT) for SA and FLA was analyzed. FLA had developed a strong PICT in response to the 80 years of Zn and Cd pollution, whereas SA was virtually unaffected. It appears that the position of SA in biofilms and micropores has effectively protected them against toxic metal concentrations. The estimated free ion activity showed that the Cd activity was too low to reach toxic levels ($PICT_{Cd}$ was probably caused by Zn). In contrast, the estimated Zn ion activity was close to a critical level, and could have caused the observed $PICT_{Zn}$ in FLA, at least if temporal/spatial fluctuations of soil pH are taken into account. Such fluctuations could also explain the protection of SA as a result of diffusion constraints; which would be of little help under constant conditions because chemical equilibrium would be reached throughout.

Introduction

Soils contain a variety of toxic compounds, both natural and man-made. Many of these are bound to colloids, however, and are possibly only periodically exceeding critical concentrations for the microorganisms. One of the challenges in the study of soil pollution and its biological effects is to predict the biological availability of each compound, as determined by the physical chemistry of the system. Metal speciation in solutions can be calculated using complex equilibrium models such as the Windermere humic aqueous model, WHAM/Model VI (*1*) and the NICCA−Donnan model (*2*). We have previously successfully optimized WHAM/Model VI for soils to predict the solution concentrations and speciation of Cd, Cu, and Zn in metal-contaminated and noncontaminated soils (Almås et al., unpublished data).

An alternative way to explore the exposure of soil microorganisms to pollutants is to measure the microbial community tolerance to the toxic compound. Investigations of contaminated soils have demonstrated that the microbial community invariably develops an increased tolerance against the toxic compound when this compound exceeds a critical concentration (*3*). This phenomenon has been coined pollution-induced community tolerance, PICT (*4*). A recurring experience is that the measurable metabolic functions of the soil ecosystems are largely retained despite high levels of toxic compounds. However, a detectable increase in PICT demonstrates that the pollution has had its effects (*4*, *5*). Sometimes this is also seen as a slight decrease in diversity as measured with community DNA profiling (*6*). Hence, PICT provides a "report" from the microbial community, as an increase indicates that the toxic compound is or has been above a critical concentration (*3*). Several investigations have demonstrated a gradual increase in PICT with increasing pollution level (*7*−*11*). Thus, PICT can be considered a bioassay for toxic compounds; i.e., if the community tolerance is significantly raised in the polluted soil, it suggests that for some time the bioavailable level has exceeded a critical limit for a fraction of the community. In a previous study of Cd and Zn contaminated soils, we found a good relationship between measured and model-predicted trace metals in pore water on one hand and the PICT on the other, in a range of soils along a pollution gradient (*12*).

Soil is a complex matrix, and the positioning of organisms within the matrix may have an influence on their sensitivity for contaminants and their survival upon contamination. For instance, bacteria positioned within aggregates and cavities (micropores) will be better protected against predation and fluctuating chemical environments than their counterparts living in macropores and in free water (*13*). The same is true for organisms embedded in biofilms, be it in macro- or micropores. The spatial isolation of organisms within confined spaces in soils has also been proposed as a main reason for a high biodiversity, due to lack of competition among microorganisms (*14*). Thus, micropores may be a safe haven for fragile organisms otherwise unable to compete with others for survival. Micropores may also protect against toxic compounds, as demonstrated for mercury (*15*, *16*). An extreme example is chloroform fumigation, which rapidly kills bacteria in open spaces, but not those situated in narrow pores (*17*). A similar diffusion constraint may protect organisms against other transient pollution events, as well as naturally occurring toxins in the macropores.

Attempts to release bacteria from soil particles by dispersion and density gradient separation demostrate that the majority (>80−90%) are attached to surfaces, and that the strength of attachment varies over a wide range (*18*). The attachment strength of different functional groups seems inversely related to the group's growth or turnover rate. An extremely strong attachment was observed for ammonia-oxidizing bacteria (*19*) and for bacteria which oxidize atmospheric methane (*20*), which are both thought to be among the most slow growing ones in soil (*21*). Being strongly attached, either in pores, biofilms, or simply to a surface, appears to be a way to survive for soil bacteria.

We hypothesized that the position in the soil matrix will also influence the organism exposure to toxic levels of trace metals, either by diffusion constraints, by the metal sequestration onto soil colloids (providing a local environment with low chemical activity), or both. Neither of these mechanisms provides permanent protection if high metal concentrations occur in macropores for prolonged periods, but they would protect against transient peaks in macropore concentrations.

* Corresponding author tel: +47 64965572; e-mail: asgeir.almas@umb.no.

10.1021/es048113w CCC: $30.25
Published on Web 07/12/2005

We used a set of Zn- and Cd-contaminated soils to explore the relationship between the binding strength of microorganisms to surfaces and their decreased PICT. We measured the PICT of strongly attached versus free/loosely attached bacteria as separated from each other by sequential dispersion and density gradient centrifugation. We expected the strongly attached cells to be more protected against toxic exposure than the free/loosely attached bacteria, since their resistance against dispersion would partly be due to being positioned in micropores and biofilms. Thus, we expected the PICT to be low or absent in strongly attached cells compared to that of the free or loosely attached cells from the same soil.

Materials and Methods

Soil Sampling. Soil samples were taken from cultivated and noncultivated fields along the Sørfjorden fjord near Odda, Hordaland county, southwest Norway. The soil sampling area is influenced by atmospherically transported pollution from a local zinc smelter. There is no doubt that the zinc smelter, established in 1924, is the dominating Cd and Zn source. For the present study, four soil samples with similar soil texture but with a range in Cd and Zn contents were selected out of a larger set of 10 samples from a previous study of the metal tolerance of soil microbial communities (*12*). Soil samples were collected at distances increasing from 0.5 to 12.3 km from the smelter. All samples were collected from the topsoil layer (2−5 cm depth) and stored moist and cold (at 4 °C) after sampling. Some of the most relevant soil quality data for the present study are shown in Table 1.

Analysis of Soils and Pore Water. Procedures for chemical analysis of soil parameters, including soil pH_{H2O} (1:2.5), content of soil organic carbon (using a LECO CHN-1000 analyzer), concentration of exchangeable base cations and exchangeable acidity (extracted by 1.0 M NH_4NO_3), texture, Aqua Regia (AR) extractable metals and pore water compositions, have been described in detail in Almås et al. (*12*). Pore water was collected after moistening fresh soils (250 mL of soil) to field capacity with ultrapure water (electric resistance greater than 18.2 MΩ·cm). The added water was equilibrated with the soil for 2 d in closed containers. After 2 d of equilibration, the equilibrated pore water was collected through a drain in the bottom after adding ultrapure water equivalent to the estimated pore volume at the top. The collected water samples were filtered through 0.45-μm membrane filters.

Graphite-furnace atomic adsorption spectrometry (GFAAS) and inductively coupled plasma (ICP) were used to determine concentrations of Cd and Zn, respectively, in the AR extracts. Metal concentrations in the pore water were determined using quadropole inductively coupled plasma mass spectrometry (ICP-MS) (Perkin-Elmer Elan 6000). A Shimadzu TOC-5000 analyzer (Shimadzu Scientific, Columbia, MD) was used to determine the concentration of dissolved organic carbon (DOC) in pore water. An atomic absorption spectrophotometer (AAS) was used to determine the exchangeable cation concentrations. The exchangeable acidity was determined by titration to pH 7.00.

Calculation of the free ion Cd and Zn activity, referred to as $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$, was carried out using WHAM/Model VI, version 6.0 for waters (*1*). Input parameters and values are shown in Table 2. Acid groups on dissolved organic matter (DOM) bind H^+ and metal cations, and we assumed DOM to have the ion binding properties of "default" fulvic acid, FA, as defined in WHAM/Model VI. Furthermore, we assumed that DOM is 50% carbon by weight, and that 50% of the acid groups of FA are active in proton/metal binding. Total dissolved Fe was assumed to be present as FeIII. Partial pressure of CO_2 was 3.4 10^{-4} atm, and the temperature was 293 K.

TABLE 1. Selected Important Properties of the Soils (All Sandy Loams) from the 4 Sampling Sites Arranged with Decreasing Metal Concentration in Pore Water

site no.	cultivation/ vegetation cover	distance and direction[a] from smelter		pH H_2O	DOC[b] (mg L^{-1})	organic C in soil (%)	CEC[c] (cmol kg^{-1})	content in soil (mol kg^{-1})		concn in pore water (mol L^{-1})			
		km						Cd_{AR}[e]	Zn_{AR}[e]	Cd_{tot}	$\{Cd^{2+}\}$[f]	Zn_{tot}	$[Zn^{2+}]$[f]
3	young orchard with permanent grass cover	1.0	SW	5.4	16.5	7.7	12.9	57.0×10^{-6}	15.0×10^{-3}	4.3×10^{-8}	27.0×10^{-9}	210.0×10^{-7}	140.0×10^{-7}
1	old orchard with permanent grass cover	12.3	N	5.1	8.2	4.2	6.6	6.0×10^{-6}	2.2×10^{-3}	1.8×10^{-8}	12.0×10^{-9}	43.0×10^{-7}	31.0×10^{-7}
8	garden lawn	0.9	NW	6.6	10.2	6.3	27.1	70.0×10^{-6}	15.0×10^{-3}	1.1×10^{-8}	6.8×10^{-9}	16.0×10^{-7}	11.0×10^{-7}
4	meadow, intensive grass production and partly pasture	5.0	W	6.4	10.2	2.0	15.9	3.0×10^{-6}	1.8×10^{-3}	1.3×10^{-8}	7.3×10^{-9}	5.9×10^{-7}	3.6×10^{-7}

[a] North is toward the mouth of the fjord, whereas South is further into the fjord and up the valley behind. [b] Dissolved organic carbon (DOC). [c] Cation exchange capacity (CEC). Determined by extracting soils with 1.0 M NH_4NO_3. [d] nd: not detectable. [e] AR: aqua regia extractable (total content in soil). [f] Calculated using WHAM/Model VI, v. 6.0.

TABLE 2. IC_{50} Values (mol L^{-1}) for Cd and Zn and for the FLA and SA Communities Together with the Free Ion Activity of Cd and Zn in Pore Water

		IC_{50} Cd			IC_{50} Zn	
site	$\{Cd^{2+}\}$	FLA	SA	$\{Zn^{2+}\}$	FLA	SA
3	27.0×10^{-9}	12.7×10^{-3}	0.1×10^{-3}	140.0×10^{-7}	7.6×10^{-3}	0.7×10^{-3}
1	12.0×10^{-9}	4.5×10^{-3}	0.2×10^{-3}	31.0×10^{-7}	2.3×10^{-3}	0.7×10^{-3}
8	6.8×10^{-9}	0.1×10^{-3}	0.1×10^{-3}	11.0×10^{-7}	1.8×10^{-3}	0.4×10^{-3}
4	7.3×10^{-9}	0.1×10^{-3}	0.1×10^{-3}	3.6×10^{-7}	0.1×10^{-3}	0.6×10^{-3}

Separation of Loosely and Strongly Attached Cells. A protocol was set up to separate soil bacteria that are strongly attached cells (**SA**) from those cells that are free and loosely attached (**FLA**), from soil particles. The protocol is based on experiences with separation of cells from soils by various dispersion, centrifugation, and density gradient techniques (*18*, *22*). For relatively mild soil dispersion, we used a Waring blender (model 32BL80, Dynamic Corporation of America) for 3 min, as is the standard procedure for extraction of bacteria (*22*). To obtain a stronger dispersion, so as to release more strongly attached, SA, cells, we used an UltraTurax T8 dispersion rod (IKA Labortechnik, Germany) on a small volume (10 mL) containing soil from which FLA had already been extracted by the first dispersion-gradient centrifugation. The Ultra Turax dispersion was shown by Bakken (*18*) to be substantially more efficient than the Waring blender dispersion. To separate liberated cells from those still attached after each dispersion step, we used the Nycodenz (Nycomed Pharma AS, Norway) density centrifugation method (*22*), which is based on the difference in buoyant density between clean cells and soil particles (with attached cells (*18*)).

In detail, the protocol starts with a 3-min dispersion of 5 g of soil in 40 mL of filter-sterilized ultrapure water in a Waring blender (continuously cooled by a cooling jacket). The suspension was transferred to 50-mL centrifugation tubes (two tubes, 20 mL in each), and a cushion of high-density Nycodenz solution (0.8 g of Nycodenz per mL of distilled water, resulting in a density of 1.3 g/cm^3) was placed beneath the suspension (syringe with a long needle). The tubes were centrifuged at 10000*g* for 2 h (4 °C), and the bacteria floating on top of the Nycodenz cushion were siphoned off and stored at 4 °C for later experiments, which were done within 24 h. This bacterial fraction is hereafter called the free and loosely attached (FLA). Next, the bottom pellet below the Nycodenz cushions, which contains cells not released by the first dispersion, were redispersed (after first removing the Nycodenz cushion) in 10 mL of filtered sterilized distilled water using the Ultraturax at full speed for 3 min. The dispersion was done in the centrifugation tubes, which were continuously cooled by keeping them in crushed ice. A Nycodenz cushion was placed underneath, followed by implementation of high-speed centrifugation (10000*g*, 2 h) and harvesting of cells on the cushion surface as in the first extraction. This bacterial fraction will be called strongly attached (SA). The different bacterial fractions (FLA and SA) were subjected to the [H^3] thymidine incorporation test as described below.

Thymidine Incorporation. Metal tolerance in all fractions was tested by a slightly simplified version of the procedure of Bååth (*23*) as presented in Bååth et al. (*24*). The [^{3}H] thymidine is incorporated into the active bacteria in suspension, and used as an indicator of microbial activity responding to the added Cd and Zn. The [^{3}H] thymidine incorporation was carried out on sub-samples obtained from the Nycodenz separation experiment with contaminated soils. Increasing concentrations of $CdSO_4$ or $ZnSO_4$ were added to parallel sample series (one for testing Cd and the other for testing Zn tolerance) together with [^{3}H] thymidine. As indicated above, 10 equal subsamples of 1350 μL were taken from all cell fractions prepared (extracted cell suspensions), and transferred to 2-mL eppendorf tubes to carry out the test on each fraction. Aliquots of 150 μL containing either ultrapure water (controls) or increasing concentrations of $CdSO_4$ or $ZnSO_4$ (10^{-7} to 10^{-1} M) were added to each sub sample together with 15 μL of [^{3}H] thymidine (final thymidine concentration = 100 nM). One control was added with 75 μL of cold 100% TCA (trichloracetic acid) prior to labeled thymidine, to inactivate all living cells (zero-time control). This control was prepared to check whether significant amounts of [^{3}H] thymidine would be adsorbed to the soil matrix by mechanisms other than active uptake. All samples, including the blanks, were incubated for 2 h at 20 °C, and the incubation was terminated by adding 75 μL of cold 100% TCA. Washing of samples and preparation for scintillation counting were carried out the same way as described in Almås et al. (*12*), by a series of washings using different reagents, mixing by vortexing, centrifugation at 15000*g*, and removal of supernatant (waste) by suction as follows: (1) centrifugation of microorganisms, removal of supernatant; (2) addition of 1.5 mL of cold 5% TCA followed by vortexing, centrifugation, and removal of supernatant; (3) addition of 1.5 mL of ice cold 80% ethanol, vortexing, centrifugation, and removal of supernatant; and (4) addition of 0.2 mL of 1 M NaOH, vortexing, and heating at 90 °C in a water bath for 1 h.

The treatment with 1 M NaOH at 90 °C dissolves macromolecules in samples. The supernatant was transferred directly into scintillation vials containing the scintillation liquid by suction (vacuum), and the ^{3}H activity was thereafter measured by liquid scintillation.

The IC_{50} values (i.e., the metal concentration which inhibits 50% of the activity) were estimated by fitting a nonlinear regression curve as in Díaz-Raviña et al. (*25*), to the relative inhibition (%) of [H^3] thymidine incorporation.

Results

Characteristics of Soils and Pore Water. The soil samples, all collected within a radius of <13 km from the zinc smelter, comprise a variety of vegetation types (Table 1), including intensively cultivated grassland (site 4), orchards (sites 1 and 3), and a lawn (site 8). The sites shown in Table 1 are arranged with decreasing total metal concentrations in pore water.

Soils taken from sites 3 and 8 contain high amounts of total Cd and Zn (AR extractable) whereas soils from sites 1 and 4 contain lower amounts (Table 1). The soils are all sandy loams. Probably due to different vegetation cover and land use, the content of organic C and cation exchange capacity (CEC), as well as soil pH, vary (Table 1). The content of organic C is relatively high in most of the soils, and the soils are near-neutral to slightly acidic with soil pH_{H2O} varying from 5.1 to 6.6.

The calculated free ion activities of Cd and Zn are shown in Table 1, and it can be seen that the pore water from soil 3 has the greatest $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$, whereas the $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$ is small in solution from soil 8. The $\{Cd^{2+}\}$ decreases in the order $3 > 1 > 4 \approx 8$, whereas $\{Zn^{2+}\}$ decreases in the order $3 > 1 > 8 > 4$.

Tolerance of FLA and SA Fractions for to Cd and Zn. In Figure 1a,b, the relative thymidine incorporation is plotted

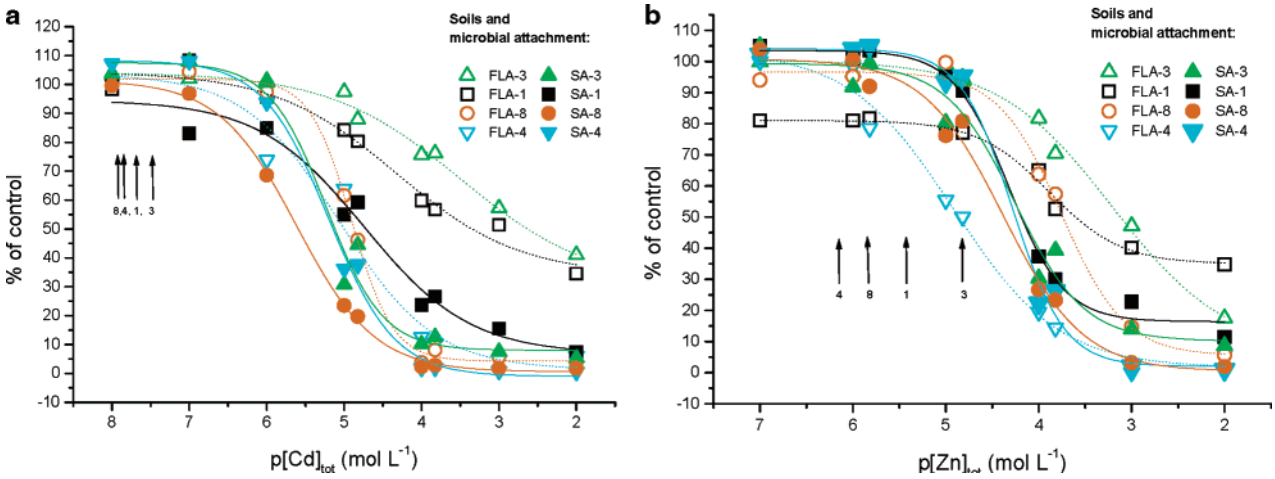

FIGURE 1. Percent [^{3}H] thymidine inhibition with increasing concentrations of Cd and Zn. The metal concentrations, in mol L^{-1}, are shown as pCd and pZn (−log[metal]). The open symbols refers to the free and loosely attached (FLA) communities, whereas the filled symbols refer to the strongly attached (SA) communities. Arrows indicate measured metal concentration in pore water of soils tested.

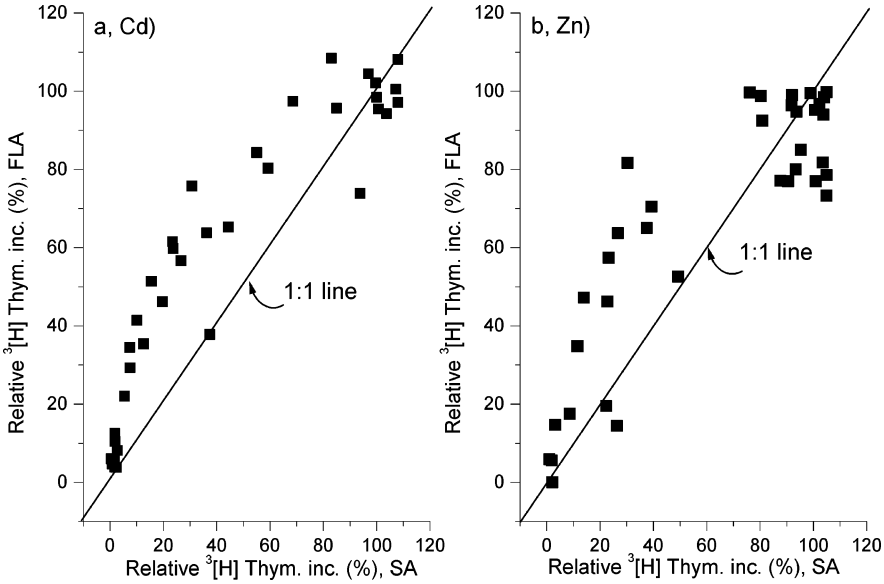

FIGURE 2. Contrasts between the strongly attached (SA) and free and loosely attached (FLA) populations through all soils. The relative thymidine incorporation rate for each soil and metal are plotted against each other in a 1:1 plot. Each point represents the metal treatment effect on the thymidine incorporation for each of the two fractions.

against the concentration of Zn and Cd showing the tolerance of FLA and SA cells from the four soils.

For the sake of comparison, the measured pore water concentrations of Zn and Cd in the four soils are indicated in the same figures. These concentrations are invariably much lower than the concentrations giving a detectable inhibition of the thymidine incorporation. The figure shows that the FLA from the most polluted soils tolerated much higher metal concentrations than their SA counterparts.

The contrast between the two populations is summarized graphically (through all soils) in Figure 2, where the relative thymidine incorporation rate in SA and FLA for each soil and each single metal concentration are plotted against each other. At each metal concentration (treatment) added during the test, the effect on relative thymidine incorporation between the fractions can be compared. There is naturally no difference between the two fractions when the incorporation is 0 and 100%, whereas between, the incorporation is substantially higher for the FLA compared to the SA fractions. There is a significantly positive relationship between IC_{50} values for the FLA fractions (Table 2) and the $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$ in pore water. The IC_{50} values for SA are much lower than those for FLA, and they are generally not correlated with the $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$. In other words, FLA has a high metal tolerance compared to that of SA, and it is positively correlated with $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$ in pore water ($r^2 = 0.98$ and 0.99 for FLA-IC_{50} values against the $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$, respectively). In contrast, SA shows no such sign of PICT. Another remarkable feature with these data is that the in situ metal activities (in soil pore water, Table 2, Figure 1) are several orders of magnitude lower than the lowest IC_{50} values (soil 4).

Discussion

Soil and Soil Solution Chemistry. The total metal concentration in the soil is a poor estimator of what the organisms experience, since the pore water concentrations and the free ion activities of metals are strongly controlled by the quality of soil organic matter, clays, and oxides, soil pH, and the activity of inorganic anions in solution (*26*, *27*). For instance, soil 8 has a high content of Cd and Zn, but due to the near-neutral soil pH, the solubility of Cd and Zn is small, explaining its low concentration and ion activity in the pore water (Table 1). On the basis of a batch titration experiment carried out

TABLE 3. Total Element Concentrations in Pore Water Obtained Used as Input Values for the WHAM Modeling

		mol L^{-1}							
site[a]	colloidal FA (g L^{-1})	Al	Ca	Fe	Cu	Cl^-	SO_4^{2-}	Cd	Zn
3	3.2×10^{-2}	7.0×10^{-6}	3.9×10^{-4}	1.3×10^{-6}	1.7×10^{-7}	2.1×10^{-4}	5.6×10^{-4}	4.3×10^{-8}	210.0×10^{-7}
1	2.3×10^{-2}	8.2×10^{-6}	2.4×10^{-4}	9.5×10^{-6}	1.6×10^{-7}	2.8×10^{-4}	1.2×10^{-4}	1.8×10^{-8}	43.0×10^{-7}
8	1.0×10^{-2}	4.2×10^{-6}	2.9×10^{-4}	3.4×10^{-6}	7.4×10^{-7}	1.6×10^{-4}	1.9×10^{-4}	1.1×10^{-8}	16.0×10^{-7}
4	1.8×10^{-2}	4.8×10^{-6}	32×10^{-4}	2.4×10^{-6}	2.5×10^{-7}	2.7×10^{-4}	1.5×10^{-4}	1.3×10^{-8}	5.9×10^{-7}

[a] Sites are arranged with decreasing metal concentrations in pore water (see Table 1).

with soils from that same sampling area (Almås et al., unpublished data), we optimized the WHAM/Model VI (for soils) and simulated the concentrations of Zn and Cd in the supernatants assuming the Aqua Regia-extractable metals ("total" metal contents in soil) to comprise the "active" pools. The model predicted the trends in dissolved metals well over a pH range from <4.5 to >7.5. In the present work, the free ion activity was calculated based on measured total pore water composition of metals, and organic and inorganic anions (Table 3). The $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$ were 50−60% of the measured $[Cd]_{tot}$ and $[Zn]_{tot}$ in pore water (Table 1). It appears likely that the calculated $\{Cd^{2+}\}$ and $\{Zn^{2+}\}$ are reasonable estimates of the concentrations experienced by bacteria in the bulk soil at the four sites. It is important to stress, however, that this is the estimated concentration in bulk soil without plants present, which was the condition prior to the pore water extraction. In the rhizosphere, on the other hand, the situation can be radically different. Plant roots may transiently lower the pH of their immediate surroundings when cation uptake dominates over anion uptake (*28*, *29*) resulting in substantially higher activities than that measured in bulk soil: a pH reduction of one unit is not unlikely during uptake of NH_4^+ (*30*), and based on the previous batch titration experiment with these soils, such a reduction in soil pH may increase the pore water concentration of Zn and Cd 1 order of magnitude (Almås et al., unpublished data). Such potential transient upshots in Zn and Cd metal concentrations are important for the interpretations of the present results (see below).

During the metal tolerance test, Zn and Cd are spiked at increasing concentrations. According to our calculations, Zn is thermodynamically unstable for $\{Zn^{2+}\}$ above 10^{-3} M final concentration at pH = 7.0 (K_{so} $Zn(OH)_2$ is $10^{-16.9}$, (*31*)). The experimental solution will not be exceeded with respect to $Cd(OH)_2$ (K_{so} $Cd(OH)_2$ is $10^{-14.4}$, (*33*)). However, due to the short exposure time (2 h) the effect of precipitation is uncertain. From Figure 1b, a slight decrease in $[^3H]$ thymidine incorporation can still be noticed even at $[Zn]_{tot}$ above 10^{-3} M, indicating that the highest additions may have affected the cells negatively.

Community Tolerance versus Soil Chemistry. The community tolerance to Cd, as tested with the thymidine incorporation, showed that even the most sensitive communities (SA for the least contaminated soils, Figure 1a,b) would hardly be affected by the measured Cd concentrations/activities in the pore waters of the bulk soil samples; the lowest IC_{50} values (0.1×10^{-3} M, Table 2) were more than 1000 times higher than the highest estimated pore water activity (2.7×10^{-8} M for site 3, Table 2). Judging from the data in Figure 1a, it seems unlikely, therefore, that the observed increase in Cd tolerance (for FLA Table 2) is due to a direct effect of Cd on the microbial communities, even if we take into account the possible upshots in activity due to transient pH reductions in rhizospheres. A more plausible explanation to this apparent PICT is that Zn, which reached more than 100 times higher concentrations, has selected for organisms that are also tolerant to Cd. Such cotolerance (*4*) is not uncommon; other research (*25*, *32*) found that Cu contamination selected conferred increased tolerance to Zn, Ag, and Ni; Rusk et al. (*33*) found that Zn exposure of soil conferred increased tolerance to Pb (nitrifying activity).

In contrast, the Zn activities in the pore water for the most contaminated soils appeared to approach a minimum inhibiting concentration, as judged from Figure 1b. It appears therefore, that the observed PICT (Table 2) was a true response to a selection pressure by the Zn contamination, at least if we take into account that transient pH reductions in rhizospheres could increase the concentrations substantially (see above).

Contrast between Free and Attached Cells. There was a remarkable difference between the strongly attached (SA) and the free or loosely attached (FLA) communities in terms of tolerance to both Cd and Zn. The pollution (soils 1 and 3) conferred a substantial increased tolerance to both Cd and Zn, whereas SA did not respond at all (Table 2). This result strongly supports the theory that exposure to trace metals is fundamentally different for free cells compared to cells hidden within biofilms and micropores in the soil matrix. The problem with this theory is that trace metal contamination has often been long lasting (at the present sites contamination had accumulated over the last 8 decades), hence there should be plenty of time for chemical steady-state to establish throughout the entire soil volume (including even the most inaccessible micropores), resulting in the same chemical activity of Zn experienced by all the organisms (including those situated at the surfaces of cation-exchanging clays (*34*, *35*)). This would only be true, however, in the absence of any perturbations of the soil. As already mentioned, roots are known to be able to reduce the local pH substantially, but transiently. This would spike the Zn (and Cd) solution concentrations, and these transient increases would probably be confined to the macropores where the roots exist (micropores would be protected by diffusion constraints). This phenomenon would probably suffice to "save" our explanation of the lacking PICT in the strongly attached fraction of the microbial community.

Our main focus is the Cd and Zn effect on microbial communities in natural terrestrial ecosystems. We see two major results from this study, namely that there is a clear difference in pollution-induced community tolerance (PICT) for Cd and Zn between the FLA and the SA communities. There was hardly any difference in IC_{50} values between SA communities obtained from the different sites, whereas the IC_{50} values for the FLA communities are strongly correlated with the pore water concentrations of Cd and Zn. Second, we found strong indications that the observed increase in Cd tolerance could not have been induced by the actual Cd in situ concentrations, suggesting a co-selection of Cd tolerance by the Zn toxicity. These findings illustrate the importance in acknowledging the chemical, physical, and biological heterogeneity in soil. For instance, the apparent lack in metal tolerance for bacterial cells identified as being strongly attached may be important when assessing the impact of metal contamination on microbial functions. Metal

tolerant organisms may very well maintain the microbial metabolic functions, but these results indicate that this may not be the only explanation for the apparent robustness often seen in studies of microbial functions in metal contaminated soils.

Literature Cited

(1) Tipping, E. Humic ion-binding Model VI: An improved description of the interactions of protons and metal ions with humic substances. *Aquat. Geochem.* **1998**, *4*, 3−48.

(2) Kinniburgh, D. G.; Milne, C. J.; Benedetti, M. F.; Pinheiro, J. P.; Filius, J.; Koopal, L. K.; Van Riemsdijk, W. H. Metal ion binding by humic acid: Application of the Nica−Donnan Model. *Environ. Sci. Technol.* **1996**, *30*, 1687−1698.

(3) Diaz-Ravina, M.; Baath, E. Response of soil bacterial communities pre-exposed to different metals and reinoculated in an unpolluted soil. *Soil Biol. Biochem.* **2001**, *33*, 241−248.

(4) Blanck, H. A critical review of procedures and approaches used for assessing pollution-induced community tolerance (PICT) in biotic communities. *Hum. Ecol. Risk Assess.* **2002**, *8*, 1003−1034.

(5) Dahlin, S.; Witter, E.; Martensson, A.; Turner, A.; Baath, E. Where's the limit? Changes in the microbiological properties of agricultural soils at low levels of metal contamination. *Soil Biol. Biochem.* **1997**, *29*, 11405−1415.

(6) Muller, A. K.; Westergaard, K.; Christensen, S.; Sorensen, S. J. The diversity and function of soil microbial communities exposed to different disturbances. *Microb. Ecol.* **2002**, *44*, 49−58.

(7) Bååth, E.; Frostegård, Å.; Díaz-Raviña, M.; Tunlid, A. Microbial community-based measurements to estimate heavy metal effects in soil: The use of phospholipid fatty acid patterns and bacterial community tolerance. *AMBIO* **1998**, *27*, 58−61.

(8) Davis, M. R. H.; Zhao, F. J.; McGrath, S. P. Pollution-induced community tolerance of soil microbes in response to a zinc gradient. *Environ. Toxicol. Chem.* **2004**, *23*, 2665−2672.

(9) Müller, A. K.; Rasmussen, L. D.; Sørensen, S. J. Adaptation of the bacterial community to mercury contamination. *FEMS Microbiol. Ecol.* **2001**, *204*, 49−53.

(10) Salminen, J.; van Gestel, C. A. M.; Oksanen, J. Pollution-induced community tolerance and functional redundancy in a decomposer food web in metal-stressed soil. *Environ. Toxicol. Chem.* **2001**, *20*, 2287−2295.

(11) van Beelen, P.; Wouterse, M.; Posthuma, L.; Rutgers, M. Location-specific ecotoxicological risk assessment of metal-polluted soils. *Environ. Toxicol. Chem.* **2004**, *23*, 2769−2779.

(12) Almås, Å. R.; Bakken, L. R.; Mulder, J. Changes in tolerance of soil microbial communities in Zn and Cd contaminated soils. *Soil Biol. Biochem.* **2004**, *36*, 805−813.

(13) Wright, D. A.; Killham, K.; Glover, L. A.; Prosser, J. I. Role of Pore-Size Location in Determining Bacterial-Activity During Predation by Protozoa in Soil. *Appl. Environ. Microbiol.* **1995**, *61*, 3537−3543.

(14) Zhou, J. Z.; Xia, B. C.; Treves, D. S.; Wu, L. Y.; Marsh, T. L.; O'Neill, R. V.; Palumbo, A. V.; Tiedje, J. M. Spatial and resource factors influencing high microbial diversity in soil. *Appl. Environ. Microbiol.* **2002**, *68*, 326−334.

(15) Nazaret, S.; Brothier, E.; Ranjard, L. Shifts in diversity and microscale distribution of the adapted bacterial phenotypes due to Hg(II) spiking in soil. *Microb. Ecol.* **2003**, *45*, 259−269.

(16) Ranjard, L.; Brothier, E.; Nazaret, S. Sequencing bands of ribosomal intergenic spacer analysis fingerprints for characterization and microscale distribution of soil bacterium populations responding to mercury spiking. *Appl. Environ. Microbiol.* **2000**, *66*, 5334−5339.

(17) White, D.; Wright, D. A.; Glover, L. A.; Prosser, J. I.; Atkinson, D.; Killham, K. A Partial Chloroform-Fumigation Technique to Characterize the Spatial Location of Bacteria Introduced Into Soil. *Biol. Fertil. Soils* **1994**, *17*, 191−195.

(18) Bakken, L. R. Separation and purification of bacteria from soil. *Appl. Environ. Microbiol.* **1985**, *49*, 1482−1487.

(19) Aakra, Å.; Hesselsoe, M.; Bakken, L. R. Surface attachment of ammonia-oxidizing bacteria in soil. *Microb. Ecol.* **2000**, *39*, 222−235.

(20) Prieme, A.; Sitaula, J. I. B.; Klemedtsson, A. K.; Bakken, L. R. Extraction of methane-oxidizing bacteria from soil particles. *FEMS Microbiol. Ecol.* **1996**, *21*, 59−68.

(21) Prieme, A.; Christensen, S.; Dobbie, K. E.; Smith, K. A. Slow increase in rate of methane oxidation in soils with time following land use change from arable agriculture to woodland. *Soil Biol. Biochem.* **1997**, *29*, 1269−1273.

(22) Bakken, L. R.; Lindahl, V. Recovery of bacterial cells from soil. In *Nucleic Acids in the Environment, Methods and Applications*; van Elsas, J. D., Trevors, J. T., Eds.; Springer-Verlag; Berlin, 1995; pp 9−26.

(23) Bååth, E. Measurements of heavy metal tolerance of soil bacteria using thymidine incorporation into bacteria extraced after homogenization-centrifugation. *Soil Biol. Biochem.* **1992**, *24*, 1167−1172.

(24) Bååth, E.; Pettersson, M.; Söderberg, K. H. Adaptation of a rapid and economical microcentrifugation method to measure thymidine and leucine incorporation by soil bacteria. *Soil Biol. Biochem.* **2001**, *33*, 1571−1574.

(25) Díaz-Raviña, M.; Bååth, E.; Frostegård, Å. Multiple heavy-metal tolerances of soil bacterial communities and its measurement by a thymidine incorporation technique. *Appl. Environ. Microbiol.* **1994**, *60*, 2238−2247.

(26) Tipping, E.; Woof, C.; Kelly, M.; Bradshaw, K.; Rowe, J. E. Solid-solution distributions of radionuclides in acid soils—application of the WHAM chemical speciation model. *Environ. Sci. Technol.* **1995**, *29*, 1365−1372.

(27) Turner, D. R. Problems in trace metal speciation modeling. In *Metal Speciation and Biavailability in Aquatic Systems*; Tessier, A., Turner, D. R., Eds.; John Wiley & Sons: Chichester, 1995; pp 149−203

(28) Bloom, A. J. Interactions between inorganic nitrogen nutrition and root development. *Z. Pflanzenernaehr. Bodenkd.* **1997**, *160*, 253−259.

(29) Bolan, N. S.; Hedley, M. J.; White, R. E. Processes of Soil Acidification During Nitrogen Cycling with Emphasis on Legume Based Pastures. *Plant Soil* **1991**, *134*, 53−63.

(30) Calba, H.; Firdaus, Cazevieille, P.; Thee, C.; Poss, R.; Jaillard, B. The dynamics of protons, aluminium, and calcium in the rhizosphere of maize cultivated in tropical acid soils: experimental study and modelling. *Plant Soil* **2004**, *260*, 33−46.

(31) McBride, M. B. *Environmental Chemistry of Soils*; Oxford University Press: New York, 1994,

(32) Bååth, E.; Díaz-Raviña, M.; Frostegård, Å.; Campbell, C. D. Effect of metal-rich sludge amendments on the soil microbial community. *Appl. Environ. Microbiol.* **1998**, *64*, 238−245.

(33) Rusk, J. A.; Hamon, R. E.; Stevens, D. P.; McLaughlin, M. J. Adaptation of soil biological nitrification to heavy metals. *Environ. Sci. Technol.* **2004**, *38*, 3092−3097.

(34) Singh, U.; Uehara, G. Electrochemistry of the double layer: principles and applications to soils. In *Soil Physical Chemistry*; Sparks, D. L., Ed.; CRC Press: Boca Raton, FL, 1998; pp 1−46

(35) van Loosdrecht, M. C. M.; Lyklema, J.; Norde, W.; Zehnder, A. J. B. Influence of interfaces on microbial activity. *Microb. Rev.* **1990**, *54*, 75−87.

Received for review November 30, 2004. Revised manuscript received April 21, 2005. Accepted May 6, 2005.

ES048113W