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Assessing the Influence of Biota on Metal Mobility in a Multi-Species Soil System (MS·3)

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Multi-species soil systems (MS·3) are homogeneous soil columns that allow a combined assessment of chemical fate and effects on representative soil organisms. Theoretically, the presence of organisms can modify the movement of chemicals in the soil core. This influence was studied for copper and cadmium comparing the results on MS·3 with earthworms and two plant species versus *soil columns without organisms. Metals were applied on the top of the soil at three doses: low (3.4 g Cu/ha + 1.7 g Cd/ha), medium (8.5 g Cu/ha + 4.3 g Cd/ha) and high (17 g Cu/ha + 8.5 g Cd/ha). Three organic compounds (pentachlorophenol, 4-chlorophenol and chlorpyrifos) were applied. Toxicity and metal levels in biota followed dose-response relationships. Results showed higher metal concentrations in the depth layers of MS·3 than in the soil columns. The effect was higher for the lower dose, where organisms were less affected, than at the higher doses, where very severe toxicity was observed, confirming the role of organisms in the enhanced mobility.*

Keywords Soil column, microcosm, MS·3, Cu, Cd

Introduction

The assessment of the effect of chemicals on terrestrial systems has received significant attention in recent years. Introduction of organic or inorganic contaminants into the soil may result in toxicity effects on soil organisms as well as alter the natural soil chemistry, thereby resulting in changes in micro-and macroscale biotic communities. A significant amount of test methods on soil organisms is available (EC, 2000a) and several methods have been standardized by OECD, ISO, USEPA, etc. In generic assessments, three taxonomic groups are typically considered for toxicity testing: earthworms (ASTM, 1997; ISO, 1996; OECD, 1984a), plants (ASTM, 1998; ISO, 1995; OECD, 1984b), and microorganisms (OECD, 2000a, 2000b). The need for additional groups, such as soil dwelling arthropods, has been demanded (i.e. Fairbrother *et al*., 2002). For terrestrial ecosystems, higher tier studies at the ecosystem level can only be conducted as field or semi-field studies. However, specific concerns on soil related effects can be addressed using intermediate tier studies. Model soil systems, frequently described as Terrestrial Model Ecosystems (TME), have been described

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by various authors (Anderson, 1978; Giesy, 1980; Hagvar, 1988; Teuben and Roelofsma, 1990; Knacker and Morgan, 1994; EPA, 1996a), and proposals for standardization are under discussion. TMEs consist of encased intact (non-homogenized) soil-cores that are extracted from the site of interest (e.g. from a natural grassland). By this method, the disturbance of the natural vegetation, soil microflora and fauna, and the layering of the soil inside the cores are minimized (EC, 2000b).

The Laboratory for Ecotoxicology at INIA has developed an intermediate alternative, called Multi-Species Soil Systems (MS·3). MS·3s consist of columns of natural sieved soil, designed as a combination of the fate and effect soil assays, where soil dwelling macro-organisms are incorporated. The soil-air interface, water transport and degradation/sorption kinetics are reproduced in a better way than in the standard soil bioassays, while the use of homogeneous sieved soil and laboratory cultured macro-organisms facilitates the reproducibility of the results. The system also allows a realistic incorporation of the chemical, i.e., incorporated in the soil column (as for contaminated soils), incorporated into the 20–25 top cm representing the arable zone (fertilizer contaminants), deposited on the top surface (atmospheric contaminants, foliar pesticides), etc. Fate properties, including leaching, can be investigated, and the combination of toxicity tests on leachates allows the assessment of the expected overall effects including those related to the parent compound and its metabolites. The proposed design for the MS·3 does not resemble natural soils, which have a more complex biotic community and different architecture. However, they are suggested as reasonable simulators for arable soils (Montforts *et al*., 2003; Fernandez *et al*., 2004) where agricultural practices modify soil structure and biota; and a medium tier tool for assessing effects on soil organisms under 3-dimension conditions, offering a compromize between realism, reproducibility and cost.

An important aspect in this development is the assessment of the effects of macro-organisms on the mobility and leaching potential of the chemicals. This paper presents the influence of plants and earthworms on the mobility of two metals, copper and cadmium. MS·3 and equivalent soil columns, without macro-organisms, were run in parallel allowing direct comparisons. In order to understand the phenomena, different doses, including those heavily affecting the organisms, were studied. The metal concentration in the biota was also measured.

Materials and Methods

Soil and Organisms

Soil from the top 5–10 cm layer was collected from an untreated grassland area in Madrid (Spain). Soil was characterized for pH (6.99), residual humidity (1.86%), oxydizable organic mater (2.20%), oxidizable organic carbon (1.28%), following BSSS methods (British Society of Soil Science) (http://www.soil.org.uk). All MS·3 were filled with 6, 25 kg of sieved (mesh size: 2 mm) and homogenized soil.

The soil organisms used in this study were the invertebrate *Eisenia fetida* (kept in culture by several generations in our lab) and seeds of the vascular plants *Triticum aestivum* and *Avena sativa*, kindly provided for National Seed Service of Spain. Ten adult worms were deposited on top of the soil and 10 seeds of each plant were sown in each MS·3.

Experimental Design

MS·3 consisted of two PVC semi-cylinders that were jointed lengthwise, constructing a PVC tube (diameter 15 cm, height 40 cm). At the bottom of each MS·3, a funnel and a

Table 1
Levels and rate of applications to the five compounds mixed with the MS·3 soil

	Metal amount per MS·3			Application rate		
	Level 1 (mg/MS·3)	Level 2 (mg/MS·3)	Level 3 (mg/MS·3)	Apl. rate 1 (g/ha)	Apl. rate 2 (g/ha)	Apl. rate 3 (g/ha)
Copper	60	150	300	3.4	8.5	17
Cadmium	30	75	150	1.7	4.3	8.5
PCP*	5	12.5	25	0.28	0.70	1.42
4-CP*	8	20	40	0.45	1.13	2.26
Chlorpyrifos	10	25	50	0.56	1.42	2.83

*PCP: Pentachlorophenol and 4-CP:4-Chlorophenol.

bottle were placed for collecting the leachates. MS·3s were saturated by a constant flow of spring water; then macro-organisms were introduced. Soil columns were treated in the same way as MS·3 but without introducing the earthworms and the seeds.

MS·3s and soil columns were consecutively sprayed with five chemicals, two metals (Cu and Cd as dilutions of certified solutions of the metal in nitric acid) and three organic compounds (pentachlorophenol, 4-chlorophenol and chlorpyrifos), at three different levels described as the low (1×), the intermediate (2.5×) and the high (5×) rate (Table 1). Application rates were selected according to the available toxicity data, trying to cover from no/low up to very high lethality in earthworms. The metal amounts per column (containing 6.25 kg of soil fresh weight) were 60, 150, 300 mg Cu and 30, 75 and 150 mg Cd, respectively, representing the following application rates: low (3.4 g Cu/ha + 1.7 g Cd/ha), medium (8.5 g Cu/ha + 4.3 g Cd/ha), and high (17 g Cu/ha + 8.5 g Cd/ha). The respective application rates for the organic chemicals were 5, 12.5 and 25 mg/column for pentachlorophenol; 8, 20 and 40 mg/column for 4-chlorophenol; and 10, 25 and 50 mg/column for chlorpyrifos. A non treated control was also included. All assays were run in duplicates. Limitations in the analytical methods for these organic chemicals in soil hampered the assessment of their fate in the MS·3 and, therefore, the data presented in this paper focuses on metal mobility. The co-application of these organic chemicals when assessing metal mobility is assumed to be irrelevant regarding physical-chemical processes, as the maximum amount or organic material was 115 mg for a column of over 6 kg of soil with 2.2% of organic matter. However, this co-application was expected to contribute to the observed toxicity, as explained below.

Controlled light cycles of 16 hours light (8000 lux) and 8 hours darkness were used. MS·3 and soil columns were irrigated with artificial rainfall spring water with 3 applications per week simulating 1000-mm rainfall/year. The water quality characteristics were pH (7.6), alkalinity (3.2 mg$CaCO_3$/L), hardness (408 mg$CaCO_3$/L), conductivity (433.9 μS/cm), bicarbonates (5.04 mg $NaCO_3H$/L), Calcium (100.2 mg Ca/L), Sodium (47.42 mg Na/L), total Copper (0.45 μg Cu/L), total Cadmium (0.017 μg/L).

On day 25, MS·3 and soil columns were opened and the soil-cores were cut in seven equal sections ("profiles") of 5 cm each. Earthworms and plants were removed and counted, and the soil was homogenized and stored frozen for chemical analysis. Seed germination, earthworm mortality and plant and earthworm biomass (dry weight) were the measured toxicity endpoints. Metals were analyzed in soil, leachates and organisms.

Analytical Methods

For total metal analysis, soils were extracted by acid digestion (with HNO_3 suprapur) following EPA Method 3051 (EPA, 1994a). The water extractable metal fraction was analyzed after water extraction at 10:1 water:soil ratio (water at the same pH as soil), mechanically shaken for 2 hours, centrifuged and membrane-filtered (0.45 μm). Schultz *et al.* (2004) suggested the use of water extracts for assessing the metal bioavailability. The leachates and organisms were analyzed after digestion in concentrated HNO_3 using the EPA Method 3015 and EPA Method 3052, respectively (EPA, 1994b, 1996b).

Metal ion concentration was measured by Atomic Absorption Spectrometry (Graphite Furnace AAS; Perkin Elmer Model Analyst 800) with Zeeman-effect background correction. The data were tested statistically using a one-way analysis of variance (ANOVA). Pairwise comparisons to determine which means were significantly different from which others were made using a Multi Range Test. Data normalization (raw data distributions transformed in distributions with a mean value of 0 and standard deviation of 1) was used to achieve homogeneous values when needed. Non-parametric Kruskal-Wallis Test was applied when the assumptions of the parametric test were not met. All the statistical analyses were performed using STATGRAPHICS plus (version 4.1).

Results

Effects on Soil Organisms

Toxicity cannot be related exclusively to copper and cadmium application rates, as three additional chemicals were included in the treatment. Toxicity results are, however, presented here because they are essential to understanding the effect of biota on metal mobility.

Effects on Invertebrates (Eisenia fetida)

Earthworm lethality reached 50% in the systems dosed with the low application rate and over 75% at the medium and high application rates (Table 2). Total biomass and average weight were also affected by the treatments.

Effects on Plants (Triticum aestivum)

Seed germination of *Avena sativa* was very low at all treatments including controls, therefore phytotoxic effects were only studied on wheat (*Triticum aestivum*).

Wheat emregence was reduced in the highest application rate (Table 2). Root biomass showed a concentration (rate)-related reduction, which was statistically significant ($p \leq 0.05$). The biomass of the aerial part was increased at the lowest rate ($p \leq 0.05$) and reduced at the highest one.

Total Metal Concentration in Soil

Control MS·3 and soil columns showed a uniform distribution of total copper (3.54–12.76 mg/kg dry weight) and total cadmium (0.05–0.25 mg/kg dry weight) all along the columns. As expected, treated systems presented higher concentrations in topsoil cores (0–5, 5–10 cm), with concentrations directly related to the applied amount (Figures 1 and 2).

Table 2
Toxicity end-points for soil organisms used in this study. Mortality and biomass (dry weight) of earthworm *Eisenia fetida*. Emergence and biomass (dry weight) of wheat—*Triticum aestivum*

	Eisenia fetida			Triticum aestivum				
		Biomass (mg)					Biomass Whole plant	
	Survival	Total	Average	Emergence	Total aerial Biomass	Total root Biomass	Total	Average
Control A	10	0.134	0.013	8	0.091	0.063	0.153	0.019
Control B	10	0.085	0.009	6	0.077	0.040	0.117	0.020
Application 1 A	4	0.029	0.007	9	0.137	0.046	0.183	0.020
Application 1 B	6	0.066	0.011	9	0.150	0.043	0.194	0.022
Application 2 A	1	0.005	0.005	7	0.068	0.030	0.098	0.014
Application 2 B	0	0	0	7	0.119	0.034	0.153	0.022
Application 3 A	2	0.012	0.006	4	0.034	0.014	0.048	0.012
Application 3 B	3	0.025	0.008	5	0.052	0.026	0.079	0.016

Biomass; g (dry weight)

Comparisons on metal mobility were conducted on normalized data, using soil core and treatment as grouping parameters. Statistically significant differences between the MS·3 and the soil columns (Kruskal-Wallis test, $p < 0.01$) were found. The analysis of individual cores detected significant differences for the intermediate cores. Mass balance estimations justify the lack of differences in the top layers. Metal amounts mobilized below the top 10 cm were too low, when compared to total application rates, for allowing detectable changes. Comparisons among application rates indicated an inverse relationship. Differences between

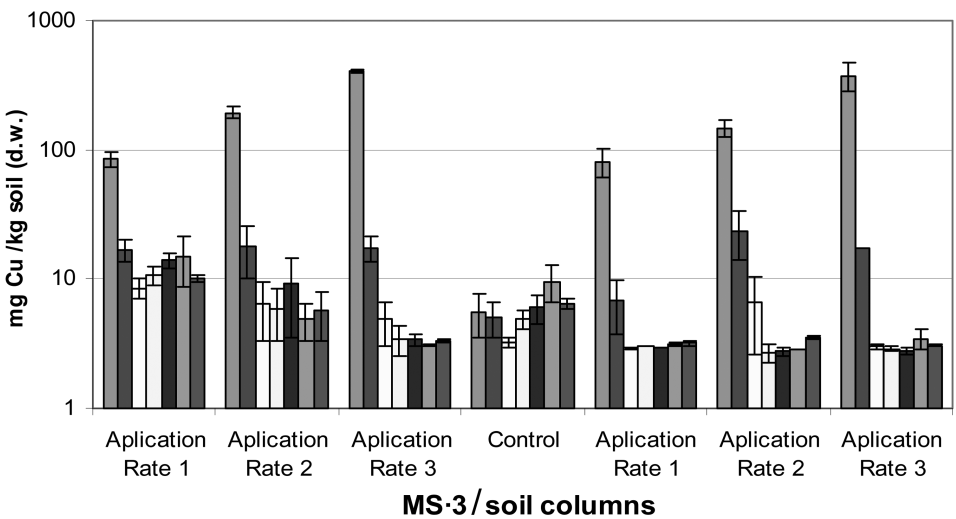

Figure 1. Copper concentration (mg of Cu/kg of soil-dry weight-) all along the MS·3s and soil columns (1–7 profiles). See applications rates.

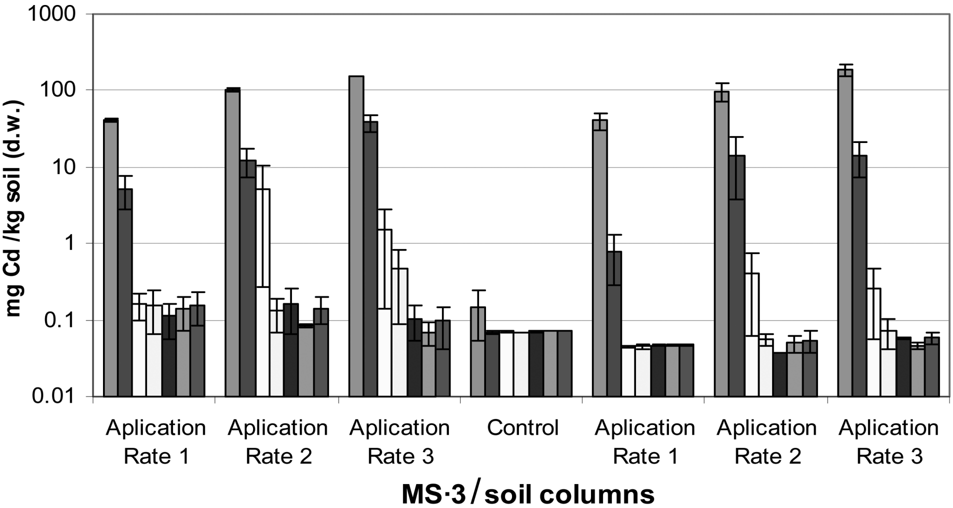

Figure 2. Cadmium concentration (mg of Cd/kg of soil-dry weight-) all along the MS·3s and soil columns (1–7 profiles). See applications rates.

MS·3 and soil columns were greater at the low application rate ($p \leq 0.01$) than at the medium ($p = 0.015$ for Cu; $p = 0.0029$ for Cd) and high ($p = 0.075$ for Cu, $p = 0.12$ for Cd) rates.

Potentially Bioavailable Metals Concentrations in Soil

A very good linear correlation (Figure 3) between total and water extractable soil metal concentrations was observed for copper (R^2: 0.99) and cadmium (R^2: 0.98). There is a statistically significant relationship between bioavailability and total metal at the 99% confidence level. Bioavailable metals represented 1/60 and 1/100 of total metal for copper and cadmium, respectively.

Metals Concentration in Leachates

Total metal amounts in the leachates collected during the 25 days represented less than 0.2% of the applied amounts in all cases. No significant differences between MS·3 and soil columns were observed.

Metals Concentration in Biota

Metal concentrations in control earthworms were 19,7 ± 1,87 and 2,76 ± 0,10 mg/kg (dry weight) for copper and cadmium, respectively. Levels in surviving exposed earthworms were 23.4 ± 0.23 mg Cu/kg (dw) and 10.48 ± 5.6 mg Cd/kg (dw) for the application rate 1 and 30.93 ± 4.2 mg Cu/kg (dw) and 8.34 ± 2.64 mg Cd/kg (dw) for the application rate 3. No data are presented for the application rate 2 due to the massive mortality.

Metal concentrations in plants were dose related. Figure 4 shows the measured copper and cadmium levels in plant tissues. The concentrations of both metals, copper and cadmium, in roots were rising as the application rates were increasing. Cadmium concentrations in wheat leaves showed a similar trend, while copper levels were only significantly higher ($p < 0.05$) at the maximum application rate.

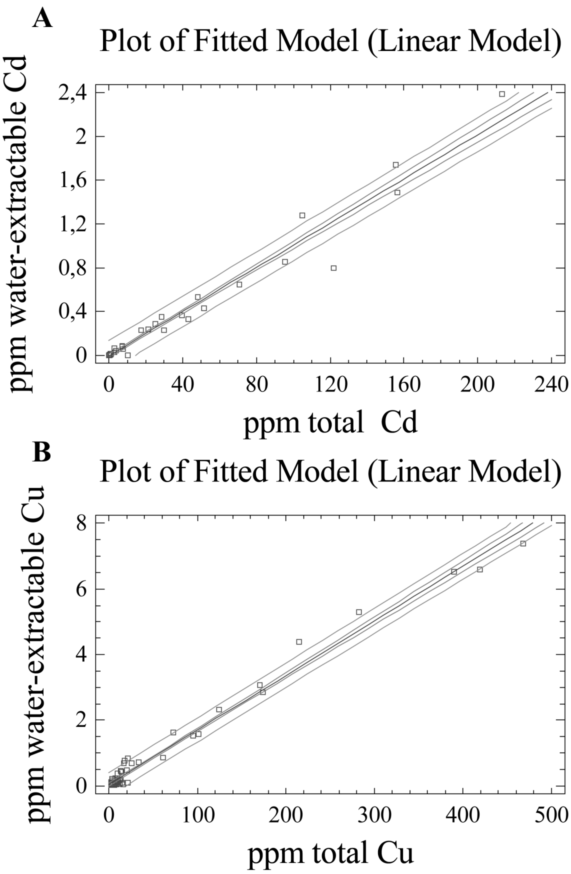

Figure 3. Correlations between total and potentially water extractable metal. A: Results of fitting a linear model to describe the relationship between total and water extractable Cu (correlation coefficient = 0.993) and B between total and water extractable Cd (correlation coefficient: 0.987). ppm (mg metal/kg soil-dry weight-).

Discussion

The need of new effect assessment tools for terrestrial systems is receiving significant attention (EC, 2000a), and covering not only single-species tests (see the enlargement of ISO and OECD guidelines) and higher tier soil microcosms such as the Terrestrial Model Ecosystems (Knacker *et al.*, 2004) The MS·3s represent a medium tier artificial assemblage, which offer a higher level of realism that single-species standard tests and a lower complexity than soil microcosms based on real soil cores. They are particularly suitable for testing effects on arable soil where soil structure is destroyed by agricultural practices producing homogenization of the top soil core (Fernandez *et al.*, 2004) and offer good possibilities for assessing the effects of agrochemicals reaching agricultural soils through fertilization processes (Montforts *et al.*, 2003).

In soil microcosms, different fate properties, such as mobilization and leaching, can be measured simultaneously with toxic effects. Similar to real situations, the fate processes can be affected by the presence of soil macroorganisms; the objective of this study was to address this hypothesis experimentally. The results showed that the organisms placed into

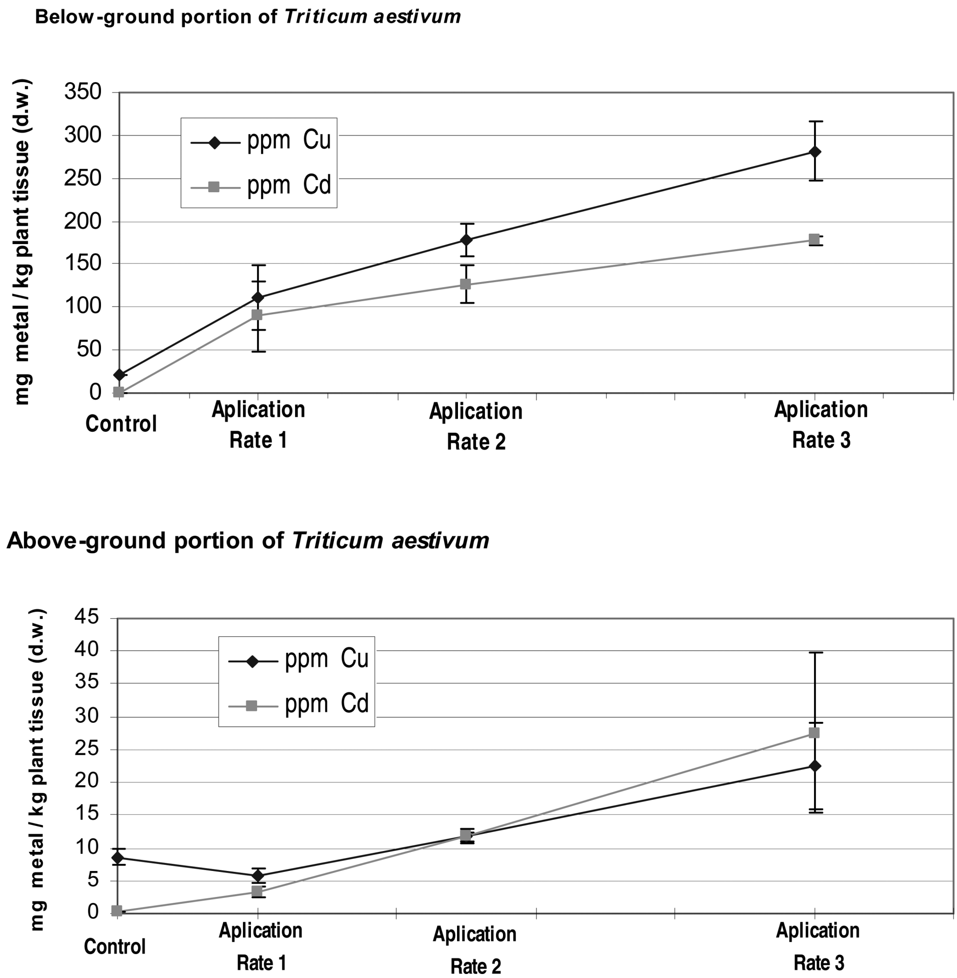

Figure 4. Measurement of Copper and Cadmium levels in *Triticum aestivum*. A: below-ground portions B: above-ground portions. See applications rates.

the MS·3 favored the vertical movement of copper and cadmium along the soil columns. A significantly uneven distribution of metals into the MS·3 with and without organisms occurred only in the MS·3 exposed to the lowest application rate, while in the MS·3 exposed to medium and high rates the differences were not statistically significant. These results are consistent with the assumption of a role of macroorganisms, and particularly earthworms, in the increased movement. The increased organism-induced mobility decreased at contamination rates, producing dramatic effects on soil macroorganisms. Statistical analysis of normalized data confirmed a higher mobilization of metals down the soil columns in the presence of macroorganisms. The effect was clear, resulting in higher metal concentrations in the bottom part of the columns treated with the low dose when compared not only with the macroorganisms free column, but also with MS·3 treated with higher metal amounts but showing less activity due to earthworm mortality and lower plant emergence. The effects were the same within the soil column; no significant differences among the leacheates were observed. These results confirm that the influence of soil macroorganisms on metal fate can be reproduced in small experimental systems.

The mobility of the earthworms within the column could explain discrepancies between the application rate and the actual exposure level; for example avoidance behavior (Hund-Rinke and Wiechering, 2001) could be expected for the very high concentrations reached on the top portion of the columns receiving the largest metal amounts. This explanation is consistent with mortality observations, as mortality was higher at the medium than at the highest rate. Cu and Cd concentrations in *Triticum aestivum* (Figure 4) were clearly related to the applied amount of metal. Obviously, avoidance behavior is not relevant for plants, whose exposure level is expected to be mostly related to soil pore water concentrations in the top part of the column. As expected, concentrations in roots were higher than in the above ground part for both metals (Pinto *et al*., 2004). Relative root-to-shoot translocation was higher for copper; in agreement with the suggestion from Harris and Taylor (2004) indicating that cadmium uptake in this commercial plant species could be controlled by restriction mechanisms for root-to-shoot translocation.

Conclusion

Studies conducted in our laboratory show that the presence of organisms in the Multi-Species Soil Systems (MS·3) produce significant changes in the metal mobilization patterns. Results suggest a direct relation between mobilization of the metals to the lowest parts of the soil column and biological activity, particularly for earthworms; while no effects were observed on leachate concentrations. The production of macropores, but also the direct transfer of soil particles during migration and feeding, are assumed to be the basic mechanisms for these effects. The observed effects indicated a higher realism of the MS·3 when compared to standardized soil leaching columns, suggesting that the mobilization and leaching behavior measured in the MS·3 systems could be related to an intermediate level assay, located between the soil leaching columns and the liximeter studies.

In addition, pollutant levels in biota, plants and soil invertebrates can be measured simultaneously.

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