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Microbial immobilization of cadmium released from CdO in the soil

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Abstract. The effect of microorganisms on the fate of Cd introduced into the soil as cadmium oxide (CdO) was investigated. Cadmium oxide (875 μg Cd per gram of soil) was added to γ-irradiated (sterile) and non-sterile soils. The soils were incubated for 90 days at 18 °C under aerobic conditions with moisture kept at 60% of water-holding capacity. Half of the samples in each treatment were supplemented with starch (0.5%, w/w) in order to stimulate microbial growth in the non-sterile soil. After various time intervals (7- or 10-day), soil samples from each treatment were extracted with deionized distilled water (ratio 1:40) or 0.25 M $CaCl_2$ (ratio 1:5). The results indicated that during the incubation period the amount of Cd extracted from the non-sterile soil with either solvent was markedly lower than that extracted from the γ-irradiated sterile control. The addition of starch to the non-sterile soil reduced the concentration of Cd in the 0.25 M $CaCl_2$ extracts without affecting the Cd-content in the water extracts. Short-term experiments in which Cd was added to the soil as a solution of $Cd(NO_3)_2$ indicated that irradiation did not affect the sorption of Cd to the soil. The addition of bacterial mass (1 mg of dry weight g^{-1} soil) decreased the amount of Cd extracted with water as well as that extracted with 0.25 M $CaCl_2$. Under sterile conditions the solubility of CdO in soil extracts was higher than in the other extractants. The addition of glucose (0.5%, w/w) or a glucose/starch mixture (0.5%, w/w of each) to the sterile soil increased the amount of extractable Cd after a short incubation (18 h at 18 °C). The obtained results suggest that primarily physicochemical reactions are involved in dissolving CdO in the soil but that microbial activity may be responsible for the immobilization of the released metal.

Introduction

Cadmium (Cd) is an important environmental contaminant released into the environment as a by-product of various industrial processes. The mean global emission rates of Cd to the atmosphere from natural sources has been estimated at 5400 t per year and – on the average – the anthropogenic emission of Cd is about double that of this element from natural sources (Nriagu and Pacyna 1988). Cadmium enters agricultural soils through aerial deposition, phosphate fertilizers, and land application of sewage sludge (Kabata-Pendias and Pendias 1992). Hansen and Tjell (1979) have estimated a relative contribution of these processes to the input of Cd to agricultural land in Denmark with application of sewage sludge as fertilizer contributing 5%, aerial deposition resulting in 70% and inorganic fertilizers (phosphates) contributing 25% of the entering Cd. Because of a relatively high

vapor pressure of Cd, volatilization of this element from the non-ferrous metal industry accounts for the largest fraction of anthropogenic Cd. Emitted into the atmosphere, this heavy metal can be transferred to plants, soil, and waters by wet or dry depositions (Alloway 1990). Soils normally contain 0.1–1.0 mg of $Cd\,kg^{-1}$, while soils in industrial areas may accumulate up to 100 mg of $Cd\,kg^{-1}$ (Kabata-Pendias and Pendias 1999). Cadmium is readily absorbed and its accumulation in plants grown on contaminated soil provides a mechanism by which Cd enters into the food chain (Welch and Norvell 1999). The metal has been determined to be mutagenic, exhibiting toxic effects on animals and humans (Newhook et al. 1994), as well as on plants by rapidly inhibiting photosynthesis (Krupa 1999), and thus its fate in the environment is of great concern.

From an ecological standpoint, the concentration of Cd in the aqueous phase of the soil is of primary importance since it is in this phase that the metal can be taken up by plants. Therefore, it is necessary to understand the soil properties which influence the concentration of Cd in the soil aqueous phase. These properties include: pH, redox potential, soil texture, mineral composition (including proportion of clays, iron, and manganese oxides), cation-exchange capacity, amount and type of organic compounds present in the soil and soil aqueous phase, heavy metal composition (other heavy metals may compete for adsorption sites), temperature, moisture content, and the numbers and types of microorganisms inhabiting the soil. Indeed, microbial growth can also affect many of the physico-chemical soil properties (Huang and Germida 2002).

Microorganisms can influence the solubility of metal oxides, not only by changing the physical and chemical properties of the soil, but also by direct enzymatic action on these compounds which can serve as terminal electron acceptors (Ehrlich 2002) as well as by synthesis of metabolites having chelating characteristics (Francis 1998). Little information is available on the fate of Cd introduced into the soil as insoluble CdO. A study by Khan and Frankland (1983a,b) demonstrated that after a 1000-h incubation of a mixture of non-sterile soil and CdO, the concentration of Cd in the soil solution was similar to that obtained when this heavy metal was introduced into the soil as soluble $CdCl_2$. Likewise Fritze et al. (2000) also found that there was no difference in Cd concentration or its effect on microbial activity in the humus from a Scots pine stand supplemented with $CdCl_2$ or CdO after 60-day incubation. These results suggest that both CdO and $CdCl_2$ may cause ecological problems. The purpose of this study was to determine the effect of microorganisms on the fate of Cd, introduced into the soil as an insoluble compound (CdO).

Materials and methods

Deionized distilled water purified by a Milli-Q water system (Millipore Corp., MA) was used throughout this study. All glassware and plastic containers were soaked for at least 2 h in 7.5 M HNO_3 and rinsed thoroughly with deionized distilled water before use.

Soil

Morrison loam soil was used in the study. An aqueous soil suspension (1:1, soil:-H_2O) had a pH of 6.4, organic matter content of 3.7%, total nitrogen content of 0.22%, cation exchange capacity of 13.3 cmol $(NH^+_4)\,kg^{-1}$, exchangeable phosphorus content of 81.9 mg kg^{-1}, total Cd content of 0.13 mg kg^{-1}, and a composition of 34% sand, 44% silt and 22% clay. The soil was passed through a 2-mm sieve prior to use.

Each treatment throughout this study was performed in triplicate and each experiment was run three times.

Cadmium treatment of soil

Air-dried soil (5 g) was mixed with CdO (Cd concentration of 875 μg g^{-1} soil), and sterile Milli-Q water was added to moisten the soil to 60% of its water-holding capacity. The samples were incubated under aerobic conditions at 18 °C for 90 days and the moisture was kept at a constant level.

Experimental design

For this study soil samples were incubated under the following conditions: (1) unamended sterile soil; (2) sterile soil supplemented with 0.5% (w/w) of starch; (3) non-sterile soil; and (4) non-sterile soil supplemented with 0.5% (w/w) of starch. Dry mixtures of soil and CdO were sterilized by γ-irradiation, using ^{60}Co as a radiation source (2.5×10^2 C kg^{-1}). Water and starch solutions were sterilized by autoclaving.

Soil sampling and analysis

On day 0 and at 7- or 10-day intervals, three samples (5 g) of soil incubated under all conditions were extracted with Milli-Q water (ratio 1:40) by shaking for 30 min (to determine water-soluble Cd), or extracted with 0.25 M $CaCl_2$ (ratio 1:5) according to the method of Zhan (1986) to measure exchangeable Cd. The soil suspensions were then centrifuged at 18,000 × *g* for 30 min and the supernatants decanted and analyzed for Cd. The pH water and $CaCl_2$ extracts was also measured.

Cadmium analysis

To determine the total Cd concentration in water or $CaCl_2$ soil extracts, the samples (10 ml) were evaporated to dryness on a hot plate at 95 °C and digested in concentrated HNO_3. After evaporation of the acid, 10 ml of 5% HNO_3 were added to each sample. The Cd concentration in the aqueous phase was then determined using an atomic absorption spectrophotometer (Instruments Laboratory Model 751, Wilmington, MA) with a H^3 background corrector. Prior to analysis of the samples,

the instrument was calibrated with five standard Cd solutions [0.2, 0.5, 0.75, 1.0, and 2.0 $\mu g\,ml^{-1}$ of Cd as $Cd(NO_3)_2$] prepared in 5% HNO_3.

Amylase activity determination

Amylase activity in the soil was determined as follows: 5 g of air-dried soil were placed in a 50-ml Erlenmeyer flask and 1.5 ml of toluene were added. The mixtures were shaken and allowed to stand for 15 min; then 5 ml of a 2% (w/v) solution of soluble starch were added and the final mixture was incubated for 5 h at 37 °C. After incubation, 15 ml of water were added to the flask. The contents were mixed and 10 ml samples of each suspension were centrifuged at 18,000 × *g* for 30 min. One ml of clear supernatant was then analyzed for reducing sugars using a modified Somogyi–Nelson's method as described by Nelson (1944). Soil samples incubated with 5 ml of water in place of the starch solution were used as controls.

The effect of abiotic factors on CdO solubility

The solubility of CdO was determined in filter-sterilized Milli-Q water; 0.25 M $CaCl_2$, and in the corresponding soil extracts (air-dried soil samples were extracted with either Milli-Q water or with 0.25 M $CaCl_2$, as described above). CdO was first sterilized by washing with ethanol. Twenty ml of the sterile soil extract or corresponding extracting solvent were mixed with 4 mg of CdO and incubated under shaking for 3 days at 18 °C. After this period of time the samples were centrifuged at 18,000 × *g* for 30 min and the Cd concentration of the supernatant was measured by atomic absorption spectrometry.

A subsequent experiment was performed under sterile conditions in order to examine the effect of glucose and a glucose-starch mixture on extractability of Cd added to the soil as CdO. The γ-irradiated air-dried soil supplemented with CdO (875 $\mu g\,g^{-1}$ of Cd) was incubated with either a glucose solution (5 $mg\,g^{-1}$ soil) or a mixture of glucose and starch (5 $mg\,g^{-1}$ soil of each compound). The samples were incubated for 18 h at 18 °C, and the soil was then extracted with sterile Milli-Q water or with sterile 0.25 M $CaCl_2$, as previously described.

The influence of biomass on Cd sorption to the soil

A mixed soil microbial culture adapted to grow in the presence of CdO was incubated for 2 days at 28 °C in the medium as described by Babich and Stotzky (1977). The culture was centrifuged at 18,000 × *g* for 30 min, the cells were washed twice with deionized distilled water and resuspended in deionized distilled water or in filter-sterilized spent medium. The cells were dried at 80 °C to obtain dry weight measurements. The air-dried soil was mixed with the bacterial suspension (1 mg dry weight of cells g^{-1} soil), and the mixture was allowed to stand at room temperature until all the liquid had evaporated. After sorption of the cells to the soil,

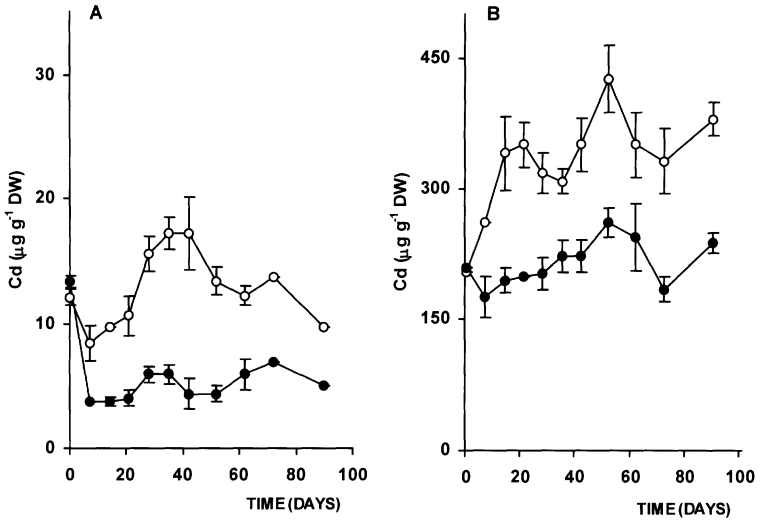

Figure 1. Extractable cadmium in the soil amended with CdO. A: Extracted with water; B: Extracted with $CaCl_2$; ○—○: γ-Irradiated sterile soil; ●——●: Native, non-sterile soil.

water solution of $Cd(NO_3)_2$ was added to give the final concentration of 300 μg g^{-1} of Cd. Soil moisture was adjusted to 60% of the soil's water-holding capacity, and the samples were incubated for 48 h at 18 °C. Following the incubation, Cd was extracted from the soil with deionized distilled water or with 0.25 M $CaCl_2$ solution, as described above.

Results

Mobilization of Cd from CdO in the soil

During a 90-day incubation of the γ-irradiated soil, changes in Cd concentration were observed in water-soil extracts. Cd levels from the sterile soil extracts increased between days 21 and 52. However, after 90-day incubation, the concentration of water-extractable cadmium decreased to slightly less than that at the starting time. In the non-sterile soils, the concentration of Cd extracted with water markedly decreased from 13.3 μg g^{-1} at time 0 to 5.0 μg g^{-1} after 90 days. The greatest reduction in the amount of water-soluble Cd occurred during the first 7 days (to 3.5 μg g^{-1}) which was followed by a period in which Cd levels slowly increased (Figure 1(A)).

The effect of microbial growth on the $CaCl_2$-exctractable Cd fraction in the soil was also examined (Figure 1(B)). The amount of Cd extracted with this solvent from the γ-irradiated soil gradually increased from 206 μg g^{-1} at time 0 to 380 μg g^{-1} after 90 days. The amount of metal extracted with $CaCl_2$ from the non-sterile soil was only slightly greater at the end of the 90-day incubation period than at time 0.

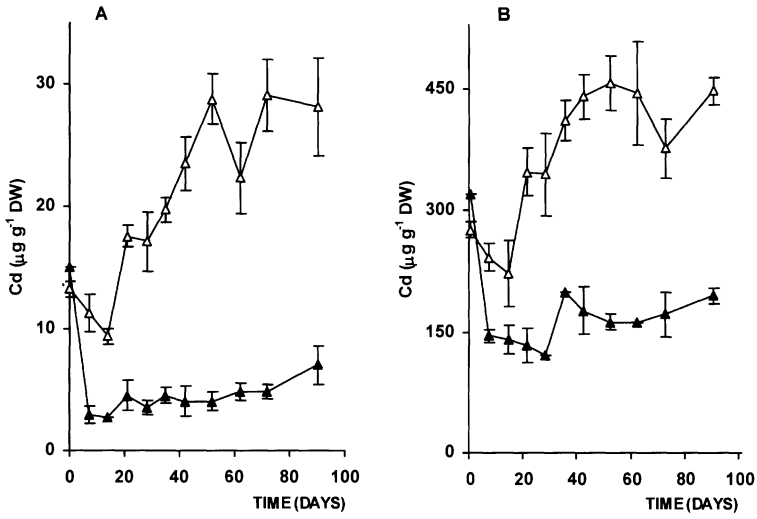

Figure 2. Extractable Cd from the soil amended with starch and CdO. A: Extracted with water; B: Extracted with $CaCl_2$; Δ—Δ: γ-Irradiated, sterile soil; ▲——▲: Native, non-sterile soil.

However, the concentration of exchangeable Cd in the soil fluctuated during the incubation period.

Addition of starch had a different effect on extractable Cd levels in γ-irradiated and non-sterile soils. In the γ-irradiated soil supplemented with starch, the Cd concentration extracted with water as well as with 0.25 M $CaCl_2$ considerably increased after a 90-day incubation, while the Cd concentration extracted with water or with $CaCl_2$ in the non-sterile soil markedly decreased from 15 to 7 $\mu g\, g^{-1}$, or from 320 to 195 $\mu g\, g^{-1}$, respectively (Figure 2(A and B)).

Factors affecting solubility of CdO and concentration of extractable Cd in the soil

The high concentration of Cd in the extracts of the γ-irradiated soil was unrelated to microbial growth, as plate counts performed on days 50 and 90 gave no indication of viable microorganisms. Moreover, the experiments showed that the amylase was still active in the γ-irradiated soil incubated with CdO and as a result of this enzyme activity in the γ-irradiated soil supplemented with starch, glucose accumulated to high levels, which indicated the lack of microbial growth (Figure 3).

All these findings suggested that the increased Cd concentration in extracts of sterile soil, especially in that enriched with starch, might be caused by factors other than microbial proliferation, while Cd concentration in extracts of non-sterile soils was connected with microbial growth.

The pH values in water extracts of γ-irradiated soils were slightly higher than those obtained from non-sterile soils. $CaCl_2$ extracts of γ-irradiated and non-sterile

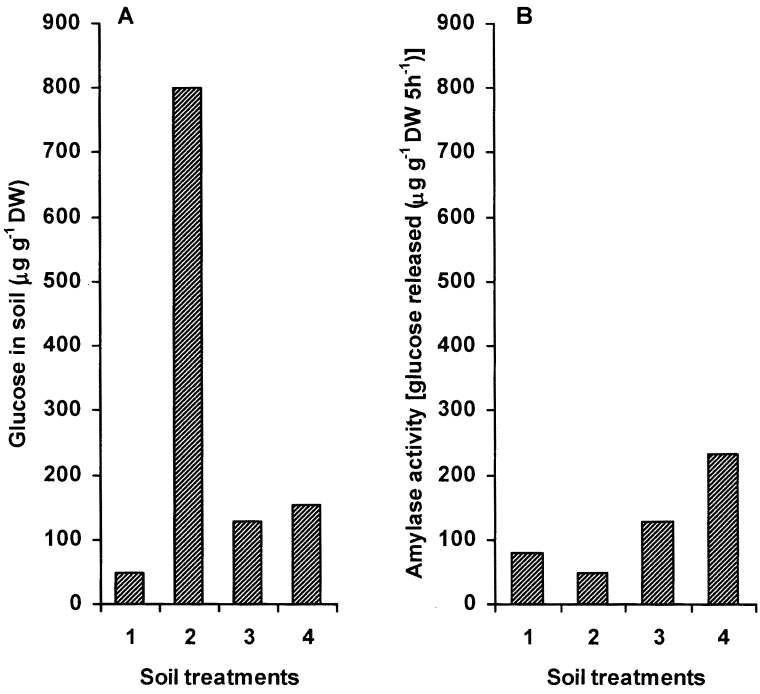

Figure 3. Glucose contents (A) and amylase activity (B) in γ-irradiated or non-sterile soils amended with CdO after a 90-day incubation period. 1. γ-Irradiated soil; 2. γ-Irradiated soil amended with starch; 3. Non-sterile soil; 4. Non-sterile soil amended with starch.

soils exhibited similar pH values over the 90-day incubation period. Addition of starch to the γ-irradiated soil lowered the pH by 0.2 on average in water extracts and 0.1 in $CaCl_2$ extracts (data not shown). Therefore, differences in pH cannot account for such a big increase in water-soluble and $CaCl_2$-extractable fractions of Cd in the γ-irradiated soil.

Metal oxides are usually insoluble or of low solubility in water; inorganic and organic compounds present in the aqueous phase of soil, however, can have a significant effect on their solubility. Experiments performed with sterile solvents indicated that chemical composition of the solution influenced the solubility of CdO (Table 1). For instance, CdO solubility was two-fold greater in the sterile water-soil extract than in Milli-Q water. Likewise, a sterile $CaCl_2$-soil extract was a better solvent than sterile $CaCl_2$ solution alone. These results suggest that changes in the chemical composition of the soil solution can affect CdO solubility in the γ-irradiated soil.

Because amylase activity and the lack of microbial proliferation were found to be the reasons for the high glucose concentrations in the γ-irradiated soil samples supplemented with starch, additional experiments were performed to evaluate the effect of glucose and starch on the extractability of Cd from CdO-amended soils.

Table 1. Effect of soil factors on solubility of CdO under sterile conditions.

Solvent solutions	pH		Soluble Cd ($\mu g\,ml^{-1}$)
	0 day	3 days	
H_2O	6.0	6.4	7.4 ± 0.09
H_2O-soil extract	5.5	6.5	18.6 ± 0.08
0.25 M $CaCl_2$	5.8	6.5	93.0 ± 0.79
0.25 M $CaCl_2$-soil extract	5.3	7.5	124.5 ± 1.19

$n = 9$.

Table 2. Effect of carbohydrate addition on CdO solubility in sterile soil.

Treatment	Cd extracted with H_2O ($\mu g\,g^{-1}$)	Cd extracted with 0.25 M $CaCl_2$ ($\mu g\,g^{-1}$)	pH	
			In H_2O soil extract	In 0.25 M $CaCl_2$ soil extract
Control	17.8 ± 0.75	372.0 ± 12.4	5.9	6.0
Glucose[1]	22.0 ± 2.68	399.0 ± 29.7	6.4	6.0
Glucose + starch mixture[2]	22.6 ± 2.13	445.5 ± 29.25	6.4	6.0

[1]Glucose – 5 $mg\,g^{-1}$.
[2]Glucose and starch mixture – 5 $mg\,g^{-1}$ of each compound.
The values are the average from two experiments. Each of them was carried out in triplicate.

Enrichment of the γ-irradiated soil with glucose as well as with a mixture of glucose and starch resulted in an increase in the levels of Cd extracted from these CdO-treated soils during short-term incubation (Table 2). These results suggest that the presence of carbohydrates might affect the extractable Cd in γ-irradiated soil samples during long-term incubation.

γ-Irradiation may induce changes in the composition of the treated soil and thereby affect either the solubility of CdO and/or the sorption of Cd to soil particles. To distinguish between these two possibilities, γ-irradiated and non-sterile soils were incubated with a $Cd(NO_3)_2$ solution for 48 h; Cd was then extracted from the soils with either water or $CaCl_2$. No differences in the amounts of extractable Cd were observed between γ-irradiated and non-sterile soils either in water or $CaCl_2$ extracts (Table 3). Therefore, γ-irradiation does not appear to affect the sorption of Cd to the soil.

To determine the influence of microbial growth on Cd sorption, a biomass of a mixed microbial culture, suspended in water or spent medium, was added to γ-irradiated and non-sterile soils. Addition of these microorganisms significantly decreased Cd concentrations in both extracts of the two soils (Table 3). This result suggests that differences in extractable Cd concentrations observed between γ-irradiated and non-sterile soils may be partly due to the lack of microbial growth

Table 3. Effect of biomass addition on cadmium sorption to the soil.

Treatment of soil	Cells added[1]	Cd extracted with H_2O ($\mu g\,g^{-1}$)	Cd extracted with 0.25 M $CaCl_2$ ($\mu g\,g^{-1}$)	pH	
				In H_2O extract	In 0.25 M $CaCl_2$ extract
Non sterile	None	6.0	156.0	6.0	5.7
γ-Irradiated	None	6.8	152.0	6.1	5.8
Non-sterile	In H_2O	4.8*	139.5*	6.3	5.8
γ-Irradiated	In H_2O	4.1*	126.0*	6.4	6.0
Non-sterile	In spent medium	4.5*	129.0*	6.3	5.9
γ-Irradiated	In spent medium	5.8*	139.5*	6.3	6.0

[1] 1 mg of dry weight g^{-1} soil.
*The values were significantly different at $p = 0.01$.

(lower biomass content and different pH values and chemical composition of the soil) in the γ-irradiated soil.

Discussion

The land disposal of Cd-containing solid waste and deposition of insoluble CdO emitted to the atmosphere potentially result in the leaching of the metal to surface and ground waters. Because of the toxicity of Cd, an understanding of the fate of this heavy metal in the environment is of great importance.

While the effects of different soil factors on the amount of bioavailable Cd in the soil are well known (Boeckhold et al. 1993; Harter and Naidu 1995; Madrid and Diaz-Barrientos 1998; Kabata-Pendias and Pendias 1999), the role of microorganisms in transformations (solubilization, mobilization and immobilization) of heavy metals in the soil is poorly understood. The lack of research in this field may be due to the difficulty in obtaining soil samples that are sterile yet unchanged with respect to their physiochemical properties. All methods of sterilization cause changes in the chemical and physical properties of the soil. However, γ-irradiation seems to bring on the least number of disturbances (Lotrario et al. 1995), killing the microorganisms without affecting soil enzymes (Peterson and Alloway 1979). Therefore, in the studies presented here, γ-irradiation was used as a method of soil sterilization.

The amount of bioavailable heavy metal can be estimated by determining metal concentrations in aqueous and exchangeable fractions of soil. These fractions have often been correlated to the metal uptake by plants (Berti and Jacobs 1996). In our study, the water-soluble form of Cd was extracted with deionized distilled water and the exchangeable fraction of Cd was contained in $CaCl_2$ extracts. During long-term aerobic incubation, the amount of cadmium extracted from the non-sterile soil

with the two extraction solvents was significantly lower than that extracted from the corresponding γ-irradiated soil. An increased immobilization of Cd in the non-sterile soil was not apparently related to changes in pH of the soil extracts. This finding agrees with the studies conducted by Chanmugathas and Bollag (1987, 1988) and suggests that microbial proliferation plays a primary role in immobilization of Cd solubilized from CdO in the soil. Short-term experiments indicated that an increase in the number of microbial cells present in the soil resulted in a significant decrease in Cd concentration of mobile fractions. These observations are in agreement with the previous results obtained in our laboratory indicating that soil microorganisms – alive or dead – are capable of removing Cd from a liquid medium and a soil suspension (Chanmugathas and Bollag 1987; Kurek 1989). The ability of microorganisms to immobilize heavy metals introduced into the soil has also been demonstrated by Wollum (1973) for Cd, and by Zamani et al. (1984) for Zn and Mn. Microorganisms may act in several ways to decrease the metal concentration in a liquid; the metal may be (1) bound to the cell wall of the organisms (McLean et al. 2002); (2) accumulated and stored via energy-dependent mechanisms (Blaude et al. 2000; Ngu et al. 1998); (3) complexed with polyanionic biopolymers such as acid capsular polysaccharides (Schlekat et al. 1998); and (4) precipitated after reaction with metabolites such as polyphosphates, phosphates or sulfides (White et al. 1998; Macaskie et al. 1987).

Earlier studies have been conducted to determine the kinetics of microbial transformation of Cd in liquid media, soil suspension, and the continuously leached acid sandy soil (pH 4.0) (Keefer et al. 1984; Chanmugathas and Bollag 1987, 1988; Kurek et al. 1991). In these experiments it has been shown that Cd can be initially immobilized and subsequently mobilized (Keefer et al. 1984; Chanmugathas and Bollag 1987), or initially mobilized and then immobilized (Hansen and Tjell 1979; Kurek and Majewska 1998), or else the metal can be continuously mobilized during leaching into water (Chanmugathas and Bollag 1988). We observed no mobilization of Cd in the non-sterile loamy soil (pH 6.4) samples. Addition of starch to the non-sterile soil did not affect the concentration of Cd in the water extract. This result suggests that immobilization of a soluble metal might be a result of the activity of autochthonous soil microorganisms, which grow on indigenous soil organic matter but do not generally respond to nutrient additions (Lynch 1982; Stotzky 1997). However, addition of starch increased immobilization of Cd in the exchangeable fraction of non-sterile soil. In the soil, zymogenous microorganisms represent a subpopulation of chemoorganotrophs exhibiting a rapid response in the presence of high nutrient levels, but grow poorly in low-nutrient habitats (Lynch 1982; Stotzky 1997). This group of microorganisms could be involved in immobilization of the exchangeable fraction of cadmium.

The γ-irradiated soil supplemented with starch exhibited the greatest increase in concentration of mobile Cd during the incubation period. This increase may be caused by a decrease in Cd sorption and increase in its solubility in γ-irradiated soils, as a consequence of the absence of microbial growth (Kurek et al. 1982, 1991). Alternatively it may be caused by an increase in solubility of CdO brought on by a change in chemical composition of the soil solution caused by the activity of γ-irradiation-resistant soil enzymes (Peterson 1962). An *Arthrobacter* sp. strain

resistant to Cd ($100\,\mu g\,ml^{-1}$) able to synthesize extracellular protein compound forming a precipitate with cadmium contained in the solution was isolated from the same soil (Kurek et al. 1991). Amylase activity we found in the γ-irradiated soil suggests that other enzymes involved in the transformation of starch could also be active in this soil. Brusseau et al. (1997) reported that some compounds formed as a result of starch transformations by action of extracellular bacterial enzymes could form soluble complexes, exhibiting little reactivity with the soil with cationic heavy metals. Carboxymethyl β-cyclodextrin is a compound with this property. Cyclodextrins are ring structures consisting of 6–8 glucose residues linked by α-1,4bonds, formed from starch by the action of cyclomaltodextrin glucanotransferase. Because of lack of microbial growth in the γ-irradiated soil samples, starch that had been added could be transformed by active soil enzymes into compounds responsible for the increased amount of mobile Cd fractions in this soil.

Our results suggest that microbial activity plays an essential role in controlling Cd mobility in the soil. Microorganisms influence metal immobilization by acting directly as biosorbents or by forming extracellular metabolites, by complexing metal or by forming insoluble precipitates. Changes pH and in the chemical composition of soil, especially in the soluble fraction of organic matter resulting from microbial activity, may also affect the solubility or immobilization of Cd in the soil.

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