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Sorption of Heavy Metals by Intact Microorganisms, Cell Walls, and Clay-Wall Composites

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Abstract

Sorption of Ag^{+}, Cd^{2+}, Cu^{2+}, and La^{3+} from solution by four bacteria, *Bacillus cereus, B. subtilis, Escherichia coli,* and *Pseudomonas aeruginosa,* and two fungi, *Aspergillus niger* and *Mucor rouxii,* was examined. Metal sorption was assessed using Freundlich adsorption isotherms to partitioning of these metals between the solution and microbial biomass phases. Precipitation of Ag and La by the bacteria precluded the use of the Freundlich isotherm for these metals. Freundlich K values for bacterial sorption of Cd and Cu ranged from 0.389 to 1.067 and 2.188 to 4.150, respectively. The affinity series for bacterial sorption of these metals decreased in the order Ag > La > Cu > Cd. Mean K values for fungal metal sorption were 2.235, 0.098, 0.818, and 4.290 for Ag, Cd, Cu, and La, respectively. The fungal affinity series was La > Ag > Cu > Cd.

To further define toxic heavy metal sorption by bacterial surfaces, walls from representative gram-negative (*E. coli*) (E) and gram-positive (*B. subtilis*) (B) bacteria were isolated and purified, and compared to smectite (S) and kaolinite (K) with regards to metal binding capacity. Metal binding decreased in the order B > E > S > K for a group of metals consisting of Ag, Cu, Ni, Cd, Pb, Zn, and Cr. High levels of metal immobilization in B and E were the result of surface-associated heavy metal precipitates. Adsorption isotherms were constructed for wall-clay interactions and clearly showed that there were strong interactions between each clay and each wall type to form composite aggregates. The composites utilized a variable proportion of the innate reactive sites available to heavy metal ions and resulted in lower concentrations of immobilized metals

Binding capacity was in the order B+S > B+K > E+S > E+K and it was apparent that the biological constituents dominated the immobilization process. Experiments were designed to remobilize three bound metals (Ag, Cu and Cr) and relied on several parameters -- pH fluctuation, metal chelation by outside agents (EDTA), metal complexation by natural organic acids (fulvic acid), competition for binding sites by non-toxic metal ions (Ca^{2+}), and enzyme hydrolysis of the wall fabric (lysozyme). The results of these experiments suggested that there was no easily observed trend for remobilization; each particulate component, each composite, and each metal had a distinct influence on the ease of heavy metal remobilization. Increased knowledge of the metal sorption capacity of microbial cells and their cell walls should enable us to better predict the fate of metals introduced into the environment, and may also be of value for enhanced utilization of microbial cells in renovation and metal recovery from municipal and industrial wastewaters.

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Background

Adsorption-desorption processes regulate the extent of binding that exists between a given solid surface and a chemical species in solution. Adsorption refers to a process whereby solutes such as metal ions adhere to a solid surface such as that presented by microbial cells. In the environment, mineral and organic surfaces in soils and sediments are the principal sites of metal ion adsorption. One important organic surface category that has not been extensively studied is that possessed by microorganisms. The cell surfaces of bacteria and fungi have chemical properties that could play important roles in the adsorption and mass partitioning of metal ions. To conduct a risk assessment of potential heavy metal contamination of terrestrial and aquatic ecosystems, it is necessary to quantitate the magnitude of metal adsorption to biological surfaces.

The information available on metal adsorption by microbial surfaces is rather limited, and most of it has been published in the last 20 years. Bacteria behave as colloidal particles in aqueous systems and have a pH-dependent net negative surface charge[1]. Metal binding studies of bacterial cell walls have demonstrated that these surfaces are capable of removing appreciable quantities of a variety of metals [2, 3]. Isolated cell walls of the gram-positive bacteria *B. subtilis* and *B. licheniformis* bound larger quantities than cell envelopes of the gram-negative bacterium *E. coli* [2]. Metal ion uptake by the fungus *Rhizopus arrhizus* was found to be directly related to the ionic radii of some ten metals tested, and it was concluded that adsorption of the metals occurred at sites in and on the cell that contained phosphate and carboxylate groups [4].

Information on partitioning of heavy metals in terrestrial and aquatic ecosystems is not complete. Increased knowledge of heavy metal partition coefficients between aqueous solutions and solid surfaces will enable us to more accurately predict heavy metal behavior in the environment and, thus, more carefully assess potential toxic heavy metal exposure. Specifically, this information will aid in the development of risk assessment models. It will aid in our understanding of the mobility and fate of metals in both surface and groundwaters. Land application of municipal sewage sludge and disposal of heavy metal-containing hazardous wastes are also areas where information regarding metal partition coefficients is crucial. An additional area where heavy metal adsorption to bacteria and other biomass is particularly important is in the understanding of metal partitioning in municipal and industrial wastewater treatment facilities and their evaluation relative to metal removal efficiency.

Laboratory Procedures

Intact Microbial Cell Studies

Bacterial cells of *B. cereus* strain ATCC 11778, *P. aeruginosa* strain ATCC 14886, *B. subtilis* 168 and *E. coli* K-12 strain AB264 were maintained and cultured as previously described [5]. The fungi examined were *A. niger* ATCC 34467 and *M. rouxii* ATCC 24905. Fungal cells were harvested by vacuum filtration and washed with 3 volumes of cold, 10 mM $Ca(NO_3)_2$ solution. Portions of moist fungal biomass were weighed, placed in 10-mL polypropylene tubes, and 8 mL of $Ca(NO_3)_2$ were added. The cells were stored at 5°C for approximately 2 h prior to use. Moisture determinations were done on six subsamples to determine the dry weight added to the tubes.

Nitrate salts of Ag^+, Cd^{2+}, Cu^{2+}, and La^{3+} were used. All metal solutions were made in pH 4, 10 mM $Ca(NO_3)_2$ solutions to minimize precipitation of metals and differences in ionic strength across metal concentrations. Initial metal concentrations for the bacterial experiments were: 1, 0.1, 0.01, and 0.001 mM Cd^{2+}; 1, 0.1, 0.01 and 0.005 mM Cu^{2+}; and 10, 1, 0.1, and 0.01 mM for Ag+ and La^{3+}. Equilibrium concentrations of both Ag and La were typically below detection limits at initial concentrations of 0.01 mM or less. For fungal sorption experiments, initial concentrations of all metals were 1, 0.1, 0.01, and 0.005 mM.

Bacterial metal sorption was determined by equilibrating cells in the metal solutions at a concentration of 2 to 3 mg dry wt mL^{-1}. The cell suspensions for all microorganisms were equilibrated for 2 h at 5°C on a rotating shaker. Timed equilibration experiments indicated that metal sorption was relatively constant within the 2 h time. After equilibration, bacterial cells were removed from solution by centrifugation and fungal cells were removed by filtration through 0.45-µm membrane filters. Concentrations of metal in solution were determined by inductively coupled argon plasma spectroscopy. Sorption is defined as the removal of metal from solution by microorganisms by one or more processes, such as adsorption, precipitation, or uptake. Where applicable, sorption isotherms were evaluated using the logarithmic form of the Freundlich adsorption equation.

$$\log S = \log K + n \log C \qquad (1)$$

where S is the metal sorbed in µmol g dry wt^{-1}, C is the equilibrium solution concentration in µmol L^{-1}, and K and n are constants [6]. Isotherms were constructed using the methods outlined by Dao et al.[7]

Isolated Bacterial Wall, Clay, and Wall-Clay Composite Studies

Walls from *B. subtilis* 168 and from E. coli K-12 strain AB264 were isolated and purified according to Walker et al.[8] Na-smectite (montmorillonite, SWY-1 Crook County, WY) and Na-kaolinite (KGA-1, Washington County, GA) were obtained from the Source Clays Repository of the Clay Minerals Society.

Adsorption isotherms of the wall-clay composites were determined by reacting 1 mg mL^{-1} of clays in distilled water with 0, 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 or 1.0 mg dry weight mg mL^{-1} of walls at circumneutral pH for 10 min at 22°C. The experiments relied on the natural buffering capacity of the particulate material. Centrifugation at 12000 x g for 30 min into a 60% (w/v) sucrose cushion separated unabsorbed walls from the clay and the clay-wall composite and allowed adsorption efficiency to be estimated.

Sorption of Ag, Cu, Ni, Cd, Zn, Pb and Cr nitrate salts was accomplished in 5 mM metal solutions at a clay, wall or clay-wall concentration of 1 mg dry weight mL^{-1} for 10 min at 22°C. The particulates were washed five times in distilled water to remove unbound metal. The metals were analyzed by atomic absorption spectrophotometry. In addition, location of metal concentration was monitored by transmission electron microscopy and energy dispersive X-

ray spectroscopy. More experimental details can be found in Walker et al.[8]

Remobilization Experiments

Ag^{+}, Cu,$^{+}$ or Cr^{+} loaded *B. subtilis* and *E. coli* walls, clays, and wall-clay composites (as outlined in section 2) were used in this study. Fulvic acid (10 to 120 mg L^{-1}), Ca^{2+} (0 to 160 mg L^{-1}), EDTA (0 to 500 M), H^{+} (pH 3 to 9). and lysozyme (40 to 160 mg L^{-1}) were used as remobilization agents and were interacted with the heavy metal-loaded particulates for 48 h at 22°C. Particulates were separated from the fluid phase by centrifugation (18000 x g for 30 min) and the supernatant was analyzed for remobilized metal. The amount of cell wall hydrolysis by lysozyme was estimated by the acid ninhydrin test [9].

Results and Discussion

Intact Microbial Cell Studies

Constants for sorption of Cd and Cu from solution by the four bacteria are given in Table 1. *B. subtilis* was the least efficient bacterium for sorption of Cd and *B. cereus* was the least efficient for Cu sorption, with K values of 0.147 and 2.188, respectively. *E. coli* and *B. subtilis* were the most efficient for Cd and Cu sorption with K values of 1.067 and 4.150, respectively. The slopes of the isotherms were all less than one and were generally different among bacteria within metals. Because the slopes were not equal, the differences in affinities for the metals by the bacteria predicted by K at an equilibrium concentration of 1 µM may not hold at higher concentrations. For example, *P. aeruginosa* removed the most Cd and Cu from solution when the initial concentration was 1 mM. Figure 1 presents the actual Cd sorption isotherms for *B. cereus* and P. aeruginosa.

Bacterial sorption of Ag and La did not conform to the Freundlich equation because of precipitation of these metals by the bacteria. On average, 99% of the total Ag^{+} and 89% of the total La^{3+} were removed from the 0.1 mM solutions by the bacteria. Electron microscopy and energy dispersive X-ray analysis indicated that Ag precipitation was likely a reductive process with the formation of colloidal Ag aggregates, whereas La precipitates were crystalline and probably La-oxides or -hydroxides [5]. The affinity series for bacterial sorption of these metals decreased in the order Ag > La > Cu > Cd.

Freundlich constants for sorption of all four metals by the filamentous fungi are given in Table 2. The isotherms adequately described the removal of the metals by the fungi, although some precipitation of Ag may still be occurring as Ag sorption was much greater than La sorption (188.3 versus 48.6 µmol g^{-1} at an initial concentration of 1 mM). Based on K values, *M. rouxii* was more efficient at Ag and La sorption, whereas *A. niger* removed the most Cd and Cu from solution. Because of the differences in isotherm slopes, however, *A. niger* was more efficient than *M. rouxii* for sorption of Ag and La from 1-mM solutions. The K values indicated that fungal affinity for these metals decreased in the order La > Ag > Cu > Cd. Although not statistically comparable, the bacteria in this study generally

Figure 1. Freundlich isotherms for cadmium sorption by *B. cereus* and *P. aeruginosa*. The dotted lines represent 95% confidence intervals about the isotherms. Reproduced from Applied and Environmental Microbiology 55: (in press), 1989 by permission of the American Society for Microbiology and the authors.

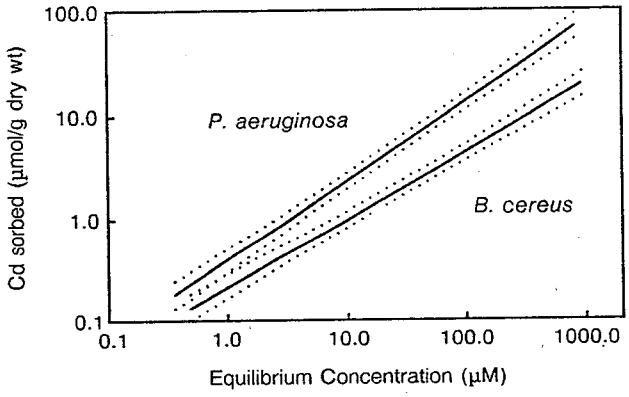

Table 1. Freundlich isotherms for sorption of Cd^{2+} and Cu^{2+} by bacteria[a]

Metal	Bacterium	log K ± SE	n ± SE	K	r^2
Cd	*B.cereus*	−0.673 ± 0.083	0.657 ± 0.043	0.212	0.962
	B. subtilis	−0.833 ± 0.027	0.857 ± 0.014	0.147	0.998
	E. coli	0.028 ± 0.066	0.497 ± 0.033	1.067	0.966
	P. aeruginosa	−0.410 ± 0.043	0.770 ± 0.023	0.389	0.992
Cu	*B. cereus*	0.340 ± 0.064	0.482 ± 0.036	2.188	0.952
	B. subtilis	0.618 ± 0.032	0.521 ± 0.019	4.150	0.988
	E. coli	0.411 ± 0.049	0.574 ± 0.029	2.576	0.977
	P. aeruginosa	0.399 ± 0.140	0.677 ± 0.091	2.506	0.860

[a] Log K is the intercept and n is the slope of the regression line. The constant K represents the amount of metal sorbed in µmol g^{-1} at an equilibrium concentration of 1 µM (log C = 0).

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appeared to bind more metal than the fungi on a µmol g dry wt^{-1} basis.

The disparity between predicted efficiencies of different microorganisms at low and high concentrations for a given metal may be indicative of differences in total binding sites per gram dry weight and/or affinities of individual binding sites on the bacteria for the metals. For example, *B. subtilis* may have sites with a higher affinity for Cu than does *P. aeruginosa*, with *P. aeruginosa* having more total sites available on a dry weight basis. This may explain *P. aeruginosa* binding more Cu at high concentrations and *B. subtilis* binding more at low concentrations.

These data indicate that microorganism-metal interactions may be amenable to equilibrium modeling, particularly when precipitation of metal is not a major factor. Such modeling may allow for the eventual inclusion of biological surfaces in equilibrium solution chemistry codes, for example MINTEQ.

Isolated Bacterial Wall, Clay, and Wall-Clay Composite Studies

Of all bacterial structures, cell walls are the most resilient and are virtually indestructible unless either degraded by specific enzymes (muramidases) or hydrolyzed by extreme pH conditions.[10] Frequently, once a bacterial cell dies, uncontrolled autolysis ensues, the cell lyses, and the wall is degraded into small fragments. Yet, the very wall enzymes that are responsible for this phenomenon are easily inactivated by dilute heavy metals, such as Fe, Cu, Cr and Ag, within the cell's aqueous environment. Because bacteria are ubiquitous to natural soils, sediments, and groundwater systems, it is very possible that they can affect the migration of toxic heavy metals throughout these natural environments; indeed, these metals may actually increase the residence time of bacterial walls within waters, soils and sediments thereby making them a major force in the determination of toxic metal mobility. For this reason, it was important to study exactly how representative bacterial wall types modified heavy metal migration patterns in simple soil simulations, such as clay suspensions in the laboratory.

The metallic ion adsorption capacity of soils is controlled by aluminosilicate clay minerals, metal oxides/-hydroxides and organic matter. For this reason, our simulation experiments used two clays, smectite (montmorillonite) and kaolinite, that at circumneutral pH carry a net negative charge and function as cation exchangers. Cell walls of *B. subtilis* (representative of a gram-positive bacterium) and *E. coli* (representative of a gram-negative bacterium) were used as the biological component of the system. At neutral pH, the metal ion sorption capacity of these walls is dominated by ionized carboxyl and phosphoryl groups.[2] Previous experimentation has shown that these walls can immobilize large quantities of soluble metal cations and act as nucleation sites for the production of various minerals.[2]

Before laboratory simulations using clay-bacterial wall composites could be performed, it was necessary to establish the heavy metal sorption capacity of each of the single components (Table 3); it was already apparent that the order of reactivity was *B. subtilis* (B) > *E. coli* (E) > smectite (S) > kaolinite (K). Further experimentation revealed that each of the clays also was capable of binding to the bacterial walls, presumably through polynuclear aluminohydroxide bridging,[11] to make organo-clay composites.[8] Kinetic analysis of these adsorption isotherms revealed that saturation occurred at approximately a 1:1 stoichiometry of clay-to-wall masses (Figure 2). Metal immobilization experiments revealed that a proportion of the reactive sites of each component (wall and clay) were apparently used in composite production and, consequently, were not available for metal binding yielding a reduced capacity for heavy metal immobilization (see calculated versus observed binding values in Table 4 and following discussion). Yet, it was apparent that the biological components of the clay-wall composites continued to dominate the system (Table 5). The order of reactivity for the composites was B+S > B+K > E+S > E+K.

This study was conducted to determine the net effect of clay sorption on the metal binding capacity of bacterial walls. The results indicated that metal binding was substantially reduced in wall-clay aggregates, a reduction

Table 2. Freundlich constants for metal sorption by filamentous fungi[a]

Metal	Fungus	K	n	r^2
Ag	*A. niger*	1.096	0.892	0.953
	M. rouxii	3.373	0.641	0.806
Cd	*A. niger*	0.156	0.679	0.861
	M. rouxii	0.039	0.875	0.994
Cu	*A. niger*	0.889	0.495	0.921
	M. rouxii	0.746	0.551	0.963
La	*A. niger*	2.877	0.426	0.971
	M. rouxii	5.702	0.314	0.968

[a] The constant K represents the amount of metal sorbed in µmol g^{-1} at an equilibrium concentration of 1 µM and n is the slope of the log transformed isotherm.

Table 3. Metal bound by native *Bacillus subtilis* walls, *Escherichia coli* envelopes, kaol;inite and smectite.[a]

	µMole Metal Bound/Gram Dry Weight			
Metal	Wall	Envelope	Kaolinite	Smectite
Ag	423 ± 15	176 ± 3	0.46 ± 0.02	43 ± 0.3
Cu	530 ± 13	172 ± 9	5 ± 0.03	197 ± 4
Ni	654 ± 25	190 ± 3	4 ± 0.2	173 ± 10
Cd	683 ± 19	221 ± 6	6 ± 0.2	1 ± 0.02
Pb	543 ± 11	254 ± 5	3 ± 0.2	118 ± 6
Zn	973 ± 13	529 ± 32	37 ± 1	65 ± 2
Cr	435 ± 37	102 ± 2	8 ± 0.5	39 ± 5

[a] Each component was suspended for 10 min. at 22° C in a 5 mM metal nitrate solution and washed 5 times by centrifugation to remove unbound metal. Metals were analyzed by atomic absorption spectrophotometry. The data represents the average of 3-5 determinations for each sample ± standard error.

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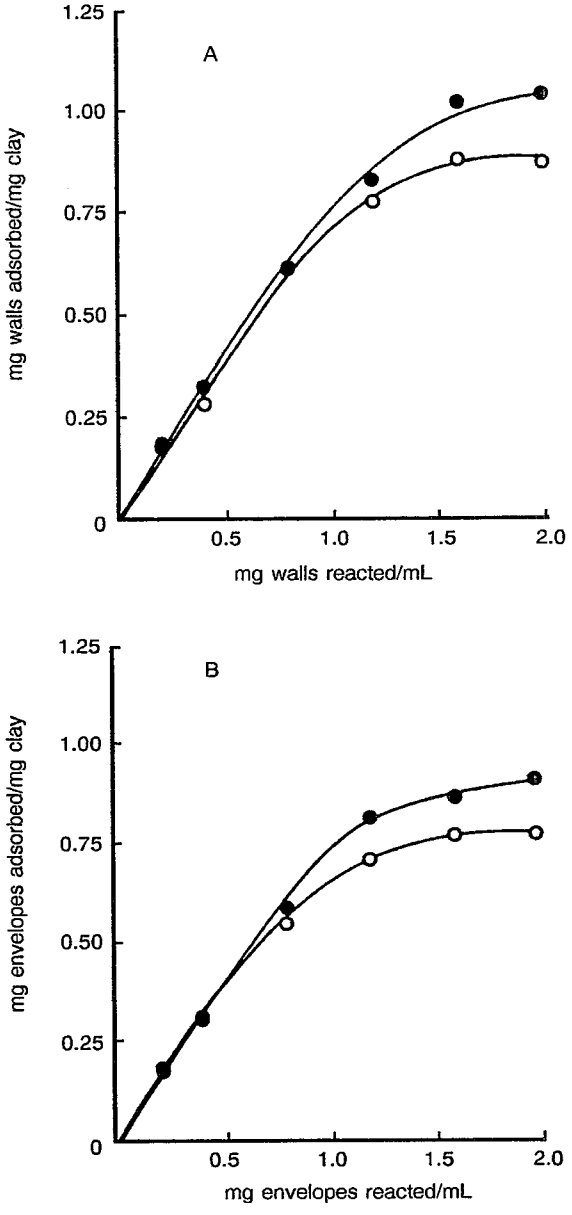
attributed to a physical blocking of negatively charged sites in the cell walls and envelopes by the sorbed clay particles. The contribution of the clays to heavy metal binding was small in comparison to that of the organic constituents of these composites (Table 5). These results suggest that small remnants of bacterial walls in soils or sediments, adsorbed to clay particles, would substantially increase the metal binding capacity of the soil. An important question which should be addressed in future research regards the stability and immobility of metals bound by wall-clay complexes compared to metals bound to the walls or clays alone.

Figure 2. Adsorption of *Bacillus subtilis* walls (A) and *Escherichia coli* envelopes (B) to smectite (*) and kaolinite (o) clays. Reproduced from Applied and Environmental Microbiology 55: (in press), 1989 by permission of the American Society for Microbiology and the authors.



Remobilization Experiments

Once toxic heavy metals have been concentrated by walls, clays, and composites in a natural environmental setting, they would be subject to a range of chemical and enzymatic agents that could leach the particulates of their bound metal. For instance, low pH frequently has the capability of remobilizing metal precipitates.[12] To test this reaction, four different pHs (pH 9, 7. 5 and 3) were used on our single and multicomponent systems (Figure 3); Ag was remobilized best, whereas Cr was little affected.

It is also possible that competing, non-toxic, naturally occurring counter-ions such as Ca^{2+} could prove effective at displacing bound heavy metals. In this instance,, laboratory tests showed that Cu was remobilized best (Figure 4) followed by Ag and Cr. Fulvic acid, a natural complexing agent, proved to be not as effective at remobilization as Ca^{2+} (cf. Figure 4 and 5), whereas EDTA, which has a high binding constant for these metals and which forms true chemical chelates, had a profound effect on Cu but not on Ag or Cr (data not shown).

Lysozyme is a muramidase that cleaves the covalent bonds holding the glycan chains of a major bacterial wall constituent, peptidoglycan, together[10] and, consequently, should solubilize the wall fabric away from the bound metal. Interestingly, there was a highly variable response to this enzyme and Cr was barely remoblized at all. Previous work suggested that heavy metal ions can inactivate native muramidases (autolysins) within the wall[13], and it is possible that a variable proportion of the lysozyme used in our study was denatured by the heavy metals liberated during initial wall digestion. We currently are conducting studies to confirm this hypothesis.

Summary and Future Research

It is clear from the studies outlined in this report that microorganisms and their surfaces can have a profound effect on the immobilization of toxic heavy metals in aqueous solution. Furthermore, they are capable of chemically interacting with aluminosilicate minerals to produce composites in which the microbial component dominates the aggregate binding of metals. Frequently, the remobilization of these bound metals is difficult and does not follow a set pattern. Clearly, microbial complexation with metals in soils, sediments, and pore waters is important and must be taken into account when modeling the transport patterns and ultimate fate of toxic heavy metals in natural systems.

Future research should include the evaluation of other environmentally relevant metals and microorganisms, and their physiological processes related to metal dynamics. For example, microorganisms produce extracellular compounds that assist in Fe transport[14], and these compounds also may act as complexing agents for a full range of metals.[15] The role of microbial metal complexing agents in metal transport is poorly understood and requires careful assessment if we are to develop complete mathematical models that accurately predict metal movement in the soil, surface, and ground water.

Table 4. Comparison of the metal binding capacities of clay-wall and clay-envelope mixtures with predicted values as calculated from Table 3

Metals	µMole Metal Bound/Gram Dry Weight							
	Wall + Smectite		Wall + Kaolinite		Envelope + Smectite		Envelope + Kaolinite	
	Calculated[a]	Observed[b]	Calculated	Observed	Calculated	Observed	Calculated	Observed
Ag	233 ± 8	115 ± 2	212 ± 8	107 ± 2	110 ± 8	19 ± 0.2	87 ± 2	30 ± 2
Cu	364 ± 8	263 ± 4	268 ± 7	181 ± 10	364 ± 9	100 ± 2	89 ± 6	49 ± 2
Ni	414 ± 18	148 ± 2	329 ± 13	176 ± 0.3	414 ± 18	37 ± 2	97 ± 2	28 ± 0.6
Cd	342 ± 10	689 ± 13	345 ± 10	299 ± 8	342 ± 10	141 ± 5	114 ± 3	57 ± 1
Pb	331 ± 8	148 ± 5	273 ± 6	271 ± 5	331 ± 9	134 ± 6	129 ± 3	27 ± 2
Zn	519 ± 7	464 ± 15	505 ± 7	367 ± 7	519 ± 8	92 ± 6	283 ± 17	82 ± 2
Cr	237 ± 21	122 ± 22	222 ± 19	122 ± 16	237 ± 21	19 ± 1	55 ± 13	28 ± 4

[a] Calculated from average uptake for each component of the mixture in Table 3.
[b] The same metal binding conditions as outlined in Table 3 were used and the data represents averages from 3-5 determinations ± standard error.

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Table 5. Metal Proportion (%) associated with the cellular constituents in the clay-wall/envelope components[a]

Metal	Wall-Kaolinite	Wall-Smectite	Envelope-Kaolinite	Envelope-Smectite
Ag	100	91	100	80
Cu	99	73	97	47
Ni	99	79	98	52
Cd	99	100	97	100
Pb	99	82	99	68
Zn	99	94	99	89
Cr	99	92	93	72

[a]Calculated from the data in Table 3.

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Figure 3. **Effect of decreasing pH on the remobilization of bound metal. B = *B. subtilis* walls, E = *E. coli* envelopes, S = smectite, and K = kaolinite. The pHs are arranged in groups of four and, from left to right, are pH = 9,7, 5 and 3.**

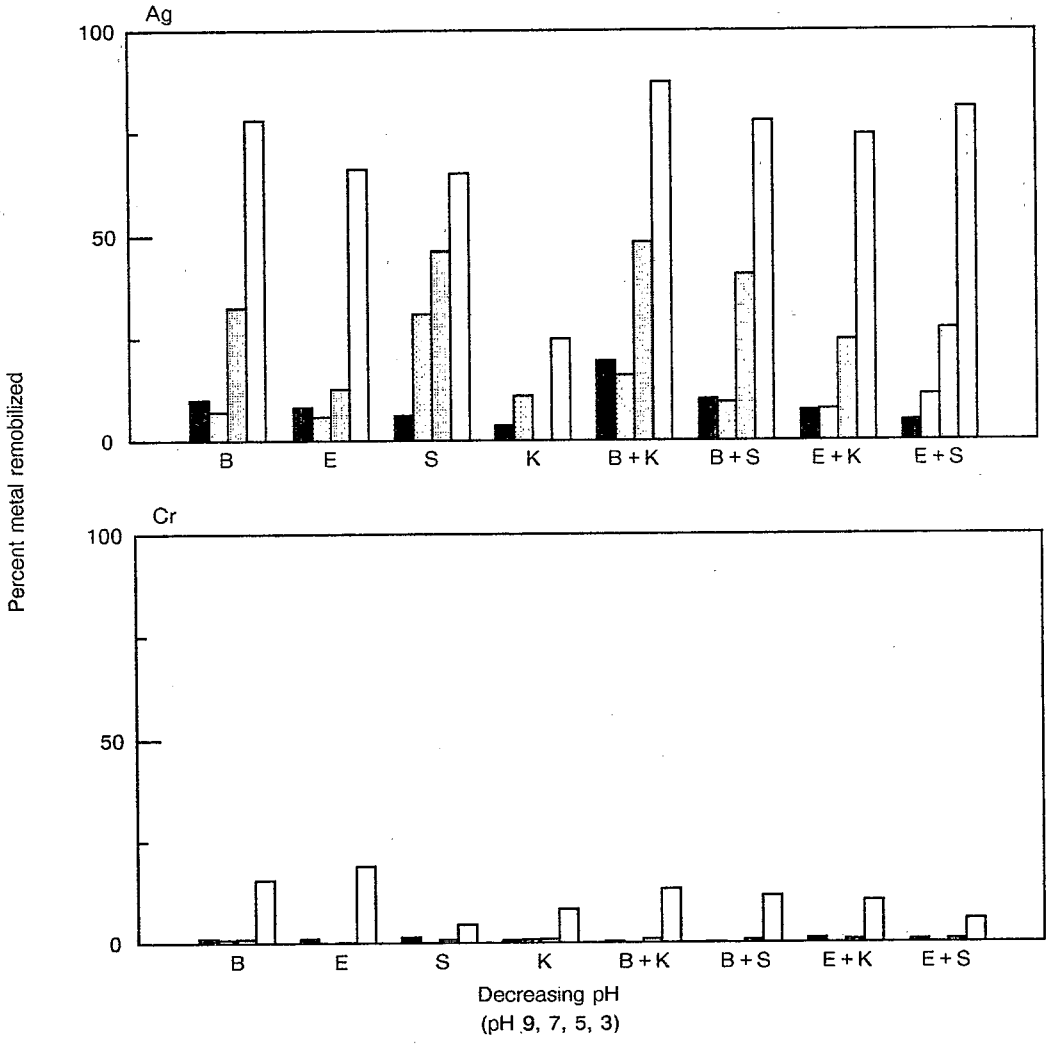


Figure 4. Effect of increasing Ca concentrations on the remobilization of Cu. The Ca concentrations are arranged in groups of four and are Ca = 0, 40, 80, and 160 mg L^{-1}.

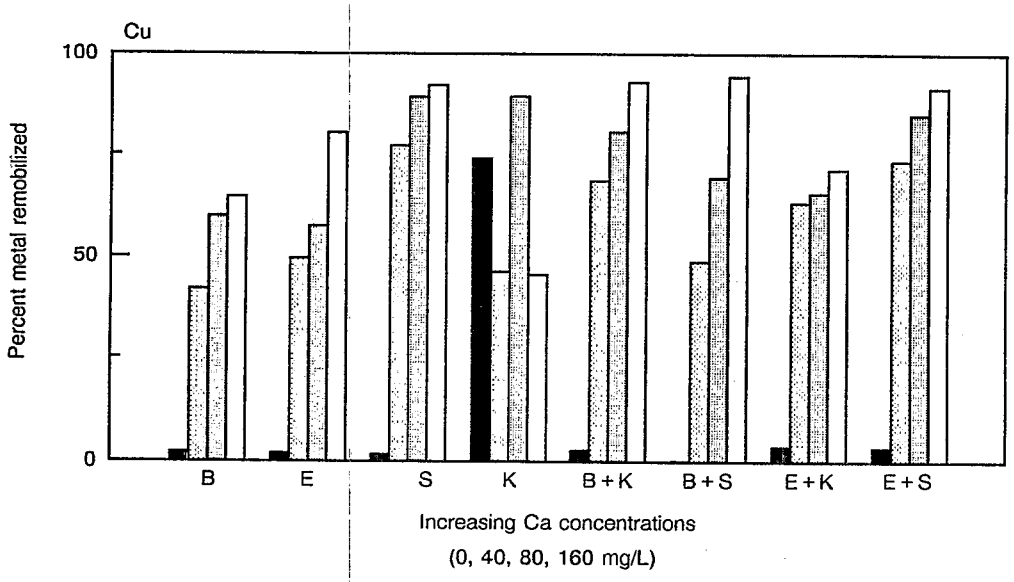
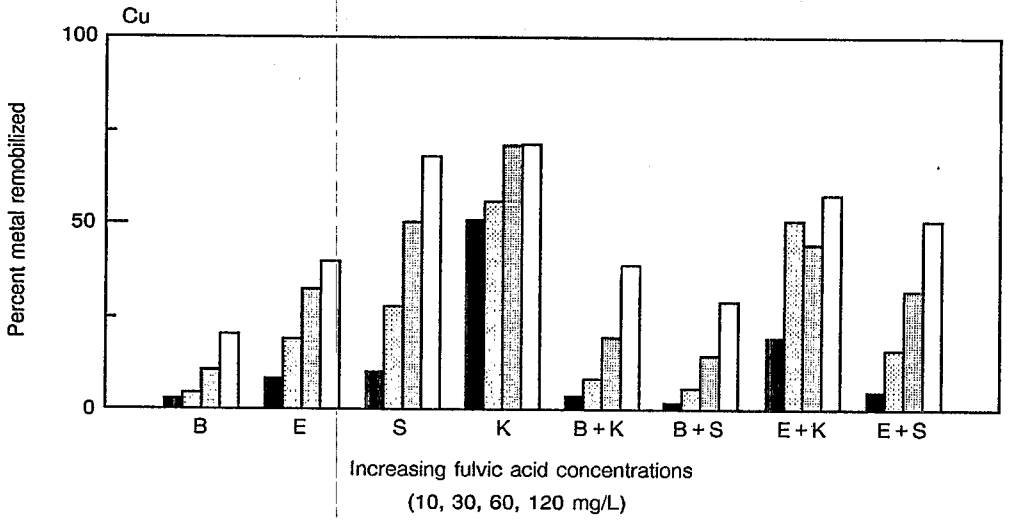


Figure 5. Effect of fulvic acid on Cu remobilization. Fulvic acid concentrations = 10, 30, 60, and 120 mg L^{-1}.



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