INVESTIGATION OF METAL BINDING IN ALFALFA BIOMASS THROUGH CHEMICAL MODIFICATION OF AMINO AND SULFHYDRYL LIGANDS

J.L. Gardea-Torresdey, K.J. Tiemann, K. Dokken, G. Gamez

Department of Chemistry and Environmental Sciences and Engineering, University of Texas at El Paso, El Paso, Texas 79968; Phone: (915) 747-5359

ABSTRACT

Heavy metal pollution of the environment has become a serious problem, and as a result, there has been a great deal of attention given to new technologies for remediation of heavy metal ions from contaminated waters. Although alfalfa biomass has shown to be very effective at removing heavy metal ions form aqueous solutions, the mechanism of metal ion binding has yet to be fully understood. To better comprehend this mechanism, amino and sulfhydryl groups were chemically modified individually to block the metal binding ligands in order to determine their contribution to the metal uptake process by alfalfa biomass. Acetylation of the amino ligands on the alfalfa biomass resulted in a small reduction of metal binding for copper(II) and nickel(II), while no significant change was observed for lead(II) at pH 5.0. In addition, succination of the biomass was performed. This process adds a carboxylate group onto the possible nitrogen ligand. This resulted in an increase in binding for copper(II), nickel(II), and lead(II), indicating that carboxyl groups play an important role in metal binding. Also, this suggests that amino groups are indeed present but play a less significant role on the binding of these metals. Sulfhydryl ligands on the alfalfa biomass were chemically blocked resulting in no significant change in metal binding for copper(II), nickel(II), and lead(II). By determining the metal binding sites in the alfalfa biomass, a new treatment technology of heavy metal contaminated ground waters can be further understood and developed.

Key words: *bioremediation, functional diversity, phenanthrene, pyrene*

INTRODUCTION

Because of the severity of heavy metal contamination and the potential adverse health impact on the public, a tremendous effort has taken place to remediate lands and waters containing toxic metal ions. Traditional methods which have been employed, prove to be costly and prohibitive for low level waste remediation. Due to the economic barrier for remediation, there has been a great deal of attention to the use of both living and non-living biological materials for the economical remediation of toxic metal ions from industrial waste waters and mining effluents (Carvalho *et al.*, 1994; Volesky *et al.*, 1995; Romero *et al.*, 1987; Darnall *et al.*, 1989; Madgwick, 1991; Weider, 1990). Most recently, plant based biomaterials have been of interest. Unlike typical synthetic ion-exchange resins, plant based biomaterials do not require the use of toxic chemicals in their preparation. Therefore, plant based biomaterial systems may be effective for the removal of heavy metal ions from the environment in a cost effective fashion.

Biosorption or metal binding by inactivated biological materials occurs through the coordination of metal ions to different functional groups. Gardea-Torresdey *et al*. found that carboxyl groups were responsible for a great portion of copper(II) and aluminum(III) binding on algal cell walls as well as copper adsorption by sphagnum peat moss and its different humic substances (Gardea-Torresdey *et al.*, 1990; Gardea-Torresdey *et al.*, 1996a). Chemical analysis to determine metal site binding in *Datura innoxia* and algal biomass was performed by both Rayson *et al.*

and Majidi *et al*. using ^{113}Cd nuclear magnetic resonance (NMR) spectroscopy (Rayson *et al.*, 1992; Majidi *et al.*, 1990). Studies performed by Kuyucak *et al.* determined that carboxyl groups are responsible for the binding of cobalt to the nonliving biomass of the common seaweed *Ascophyllum nodosum* (Kuyucak *et al.*, 1989). Therefore, chemical functional groups have shown to be important to the binding of metal ions to biomaterials.

Alfalfa has shown to be very effective at the removal and recovery of several heavy metal ions from aqueous solutions (Gardea-Torresdey *et al.*, 1996b; 1996c; 1996d). Although much information has been gained on the binding of metal ions to the alfalfa biomass, relatively little information has been obtained in relation to the actual metal binding chemical groups. X-ray absorption spectroscopic studies previously performed indicated that some of the metal binding by alfalfa biomass may be occurring through an oxygen ligand (possibly carboxyl groups) for some metals, while other metals could be partially binding through nitrogen ligands (Tiemann *et al.*, 1997). Consequently the metal binding mechanism in the alfalfa biomass is not completely understood. Therefore, additional investigation is necessary to determine what chemical functional groups are responsible for binding of different heavy metal ions and in what proportions.

The objective of this study is to determine the extent of metal binding occurring through nitrogen and sulfhydryl ligands. This was accomplished by chemically modifying the available amino and sulfhydryl groups. Two methods of chemical modification were used to modify the available amino groups; acetylation of the biomass, and succination of the biomass. Through acetylation of the biomass, the available amino ligands were blocked through the addition of acetic anhydride, thereby reducing the metal binding to the available nitrogen ligands. In addition, succination of the biomass was performed which would add a carboxyl group onto the modified nitrogen ligand. Since carboxyl groups have been shown to play an important role in metal binding, succination of the biomass should increase the metal binding and indicate the metal binding ability of available modified amino groups. A comparison of the metal binding capacities by the acetylated biomass, succinated biomass, and control biomass, will show the percentage of metal binding by amino ligands in the alfalfa biomass for the different metal ions studied. Also, sulfhydryl ligands on the alfalfa biomass were chemically blocked through the addition of 2,2-dithiopyridine. By determining the difference in metal binding between unmodified and sulfhydryl modified biomass, the role of sulfhydryl ligands can be resolved for metal binding by alfalfa biomass. Through the determination of the metal binding sites in the alfalfa biomass, we will be able to gain a better understanding on the selectivity of the system and discern what other contaminants might also be remediated from polluted areas.

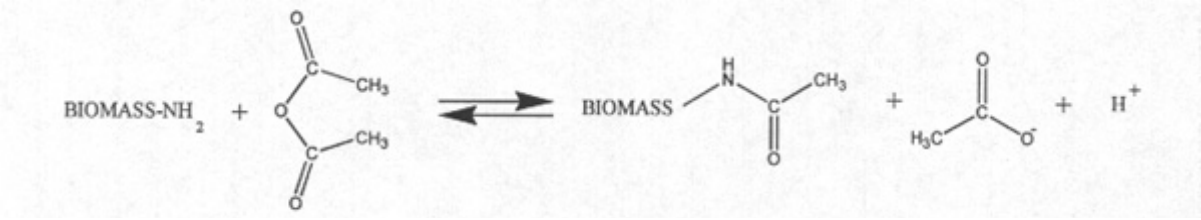
MATERIALS AND METHODS

Alfalfa Collection

The Malone population of alfalfa biomass was selected from previous studies for its abundance and metal binding abilities. The plant tissues were collected from controlled agricultural fields at New Mexico State University near Las Cruces, New Mexico. The plants were removed from the soils, washed throughly to remove any debris and the roots were separated from the shoots (stems and leaves). The samples were then oven dried at 90°C for one week. The dried samples were ground to pass through a 100-mesh screen by using a Wiley mill.

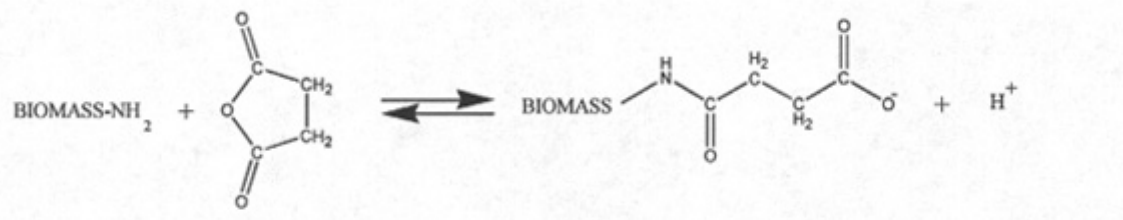
Chemical Modification

Acetylation of the amino groups on the alfalfa shoot biomass was achieved by washing 12.0 grams of Malone alfalfa shoot biomass first in 0.1M HCl to remove any debris, followed by washing in sodium phosphate / sodium acetate buffer (0.1M Na3PO4 / 1.0M Na C2H3O2) at pH 7.2. The biomass was reacted with 64 mL of acetic anhydride and stirred while maintaining the pH of 7.2 for 1 hour. This will cause the amino acetylation of the available amino groups by the following mechanism:



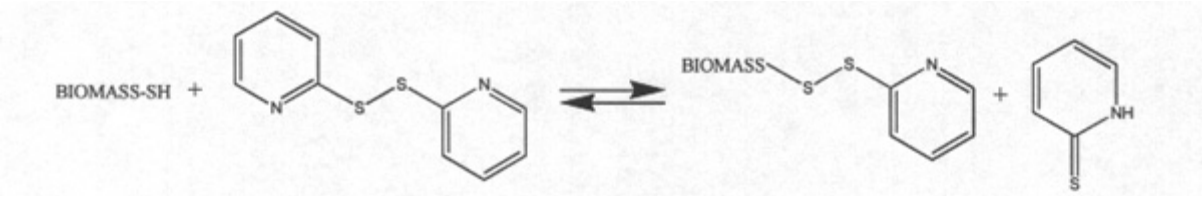
The acetylated biomass was next centrifuged for 5 minutes at 3,000 rpm. After removing the supernatant, the biomass was washed with de-ionized water, and then resuspended in 1M hydroxylamine to remove O-acetyl groups. The biomass was then washed with 0.1 M HCl to remove any more soluble materials and finally was washed with de-ionized water. The biomass was then lyophilized in a Labconco freeze dryer. The amino groups should now be neutralized and reduce the metal binding for metals that bind to amino ligands.

Succination of the amino groups on the alfalfa shoot biomass was achieved by washing 12.0 grams of Malone alfalfa shoot biomass first in 0.1M HCl to remove any debris, followed by washing in 0.1M sodium acetate at pH 8.0. The alfalfa biomass was then resuspended in 500 mL of 1.0 M Na C2H3O2*H2O at pH 8.0. Sixteen grams of succinic anhydride were added to the suspended biomass. An additional 16 g of succinic anhydride were added after 15 minute intervals for the next one and a half hours (6 additions of 16 g succinic anhydride to the biomass). This will cause the amino succination of the available amino groups by the following mechanism:



The biomass was then washed with 0.1M HCl, centrifuged, and washed again with de-ionized water. The biomass was then lyophilized in a Labconco freeze dryer. Although the amino group is neutralized, it now forms an additional carboxyl group. By the addition of a carboxylate group, there should be an enhancement of metal binding by those metals that bind to carboxyl ligands. The percentage increase in metal binding will indicate the quantity of modified amino groups.

Chemical modification of available sulfhydryl ligands on the alfalfa biomass was performed using a solution of 0.001M dithiodipyridine which was prepared by dissolving 0.22 g of 2,2 - dithiopyridine in 2 mL of concentrated HCl and diluted to 1 liter with 0.1M sodium acetate at pH 5.0 and at room temperature. Next, the biomass was prepared by washing 12.0 grams of Malone alfalfa shoot biomass (oven dried and 100 mesh) first in 0.1M HCl to remove any debris, followed by washing in sodium acetate (0.01 M Na C2H3O2) at pH 5.0. Chemical modification of the available sulfhydryl groups on the alfalfa shoot biomass was achieved by suspending the 12 grams of washed biomass in one liter of the 0.001M dithiodipyridine solution at pH 5.0 at room temperature and stirring for one hour. The modification of the available sulfhydryl groups on the alfalfa biomass proceeds according to the following reaction:



The sulfhydryl modified biomass was then washed with 0.1M HCl, centrifuged, and washed again with de-ionized water. The biomass was then lyophilized in a Labconco freeze dryer.

Batch Laboratory pH Experiments

After the biomass was lyophilized, ten milligrams of biomass was weighed and placed into clean tubes (30 tubes per metal studied) in order to obtain a biomass concentration of 5 mg biomass (dry weight) per milliliter of solution. The weighed biomass was washed again with 0.1M HCl to remove any soluble materials that might have been left following the chemical modification. The biomass was next washed with de-ionized water followed by centrifugation. The biomass pellets were then suspended in two milliliters of 0.1 mM metal solution at either pH 2.0 or pH 5.0 to give a

final biomass concentration of 5 mg biomass per milliliter of metal solution. The experiment was performed in triplicate at each pH with each metal studied and controls were kept for each metal at each pH value. The biomass was equilibrated by rocking for one hour and following centrifugation, the supernatants were separated from the biomass for metal analysis by flame atomic absorption.

Batch Laboratory Binding Capacity Experiments

Binding capacity experiments were performed with the chemically modified biomass as well as controls for each metal studied. Ten milligrams of biomass were weighed and placed into clean tubes and washed as described above. Separate metal solutions were made of 0.3 mM copper (from CuSO4), 0.3 mM lead (from Pb(NO3)2), and 0.3 mM nickel (from Ni(NO3)2). Each of the metal solutions were adjusted to pH 5.0 using sodium hydroxide (NaOH). Two milliliters of the metal solution was added to each tube containing biomass as well as the controls and equilibrated by rocking for ten minutes (biomass concentration of 5 mg biomass (dry weight) per milliliter). The biomass was then centrifuged for five minutes at 3,000 rpm. The supernatants were separated from the pellets and kept for analysis while the biomass was again reacted with fresh 0.3 mM metal solution. This process was continued for ten cycles or until the biomass became saturated and was no longer able to bind more metal ions from the solution. The experiment was performed in triplicate to maintain quality control.

Metal Analysis

Each metal studied was analyzed with a Perkin Elmer model 3110 flame atomic absorption spectrometer. The methods used for metal analysis were obtained from the Perkin Elmer model 3110 Atomic Absorption Spectrometer manual. The wavelengths used for the analysis of the metals in this study were as follows: 327.4 nm for copper, 283.3 nm for lead, and 352.5 nm for nickel. The instrument was calibrated within the linear range of analysis and a correlation coefficient of 0.98 or greater was obtained for the calibration curve. The instrument was periodically checked throughout the analysis with known standards. Three readings were obtained for each sample, and a mean value was computed along with standard deviations for each sample. The amount bound on the biomass was assumed to be the difference between the initial metal concentration and that found in the supernatant.

RESULTS AND DISCUSSION

As previously reported, alfalfa has the ability to bind a considerable amount of heavy metal ions from aqueous solution (Gardea-Torresdey *et al.*, 1996b; 1996c; 1996d). The inactivated plant tissues of the alfalfa biomass offer a number of potential sites for metal binding. Individual binding sites may be investigated through chemical modification, where the potential site is altered to either block the site from metal binding or enhanced by introducing a functional group that may have higher affinity for metal binding. By acetylation of the available amino groups on the alfalfa

biomass, the amino groups should be neutralized and reduce the metal binding for metals that bind to amino ligands. Conversely, through succination of the available amino groups, an additional carboxyl group will be added and there should be an enhancement of metal binding by those metals that bind to carboxyl ligands. Since carboxyl groups have shown to play an important role in metal binding by alfalfa biomass, and the succination of the biomass should add a carboxyl group onto the modified nitrogen ligand, metal binding experiments were carried out at pH 2.0 and pH 5.0 (Gardea-Torresdey *et al.*, 1996b; 1997). This would allow for comparison of metal binding while the carboxyl groups are protonated at pH 2.0 and de-protonated at pH 5.0. The difference in the metal binding at pH 2.0 and 5.0 will aid in determining the approximate amount of available amino ligands that were modified. Table 1 shows the percentage of metal binding by modified and unmodified (acetylated and succinated) alfalfa shoot biomass after being equilibrated with 0.1 mM metal solution at pH 2.0 for one hour. From Table 1, it can be seen that at pH 2.0, there is only a slight reduction in the binding of copper(II) to the acetylated biomass. There was not a great difference in the binding of nickel(II) or lead(II) to the acetylated biomass as compared to the control. Because the carboxyl ligands are protonated at pH 2.0, we did not expect to see a great change in the binding of copper(II), nickel(II), or lead(II) by the succinated biomass as compared to the control. On the other hand, Table 2 shows quite different results. Table 2 displays the percentage of metal binding by modified and unmodified alfalfa shoot biomass after being equilibrated with 0.1 mM metal solution at pH 5.0 for one hour. As can be seen from the Table, there was an increase in metal binding by the succinated alfalfa biomass. This is due to the addition of carboxyl ligands to the biomass which lead to a greater percentage of metal binding for each metal studied. The average percentage increase for all the metals was approximately fifteen percent. This indicates that there were a considerable number of available amino ligands which were chemically modified. On the other hand, copper(II) and nickel(II) only showed a small reduction in metal binding ability by the acetylated alfalfa biomass. This corresponds to the data already observed by X-ray absorption spectroscopic studies (data not shown) which indicate that some of the metal binding by alfalfa shoot biomass may be occurring through an oxygen ligand (possibly carboxyl groups) while a small percentage of metal binding for some metals could be through nitrogen ligands. Table 3 shows the results from metal binding adsorption capacity experiments, where 0.3mM metal solutions were equilibrated with the inactivated alfalfa shoot biomass at pH 5.0. After ten minutes, the supernatants were removed and fresh metal solution was added. This cycle was repeated ten times or until the biomass became saturated. As seen in Table 3, the results of the binding capacity experiment for lead(II) showed a significant decrease between the control and the acetylated biomass, but the succinated biomass showed approximable a 50% increase in metal binding when compared to the control biomass. Both copper(II) and nickel(II) showed a decrease in binding by the acetylated alfalfa biomass when compared to the control, while the succinated biomass showed

a significant increase when compared to the control for both metals. The increase in metal binding through the addition of carboxyl groups onto the available amino ligands indicate that the amino ligands were chemically modified. In addition, these results demonstrate that amino groups play only a minor role in the binding of copper(II), nickel(II), and lead(II) by alfalfa biomass. Furthermore, the increase in metal binding by succinated alfalfa shoot biomass also indicates indirectly that carboxyl groups must play an important role in metal binding by alfalfa.

In order to determine the extent of metal binding occurring through sulfhydryl ligands, the available sulfhydryl groups of the alfalfa biomass were chemically modified. By the addition of 2,2-dithiopyridine to the biomass, the available sulfhydryl groups should be chemically blocked and converted to thiol groups (see methods section). Because thiopyridine is bound to the sulfhydryl ligand, there should be no binding of metal ions through the blocked groups on the alfalfa biomass. By comparing the amount of binding prior to sulfhydryl modification and after, the amount of binding through sulfhydryl ligands can be determined. Since at pH 2.0, the carboxylate groups should be protonated and if the available sulfhydryl groups are blocked, then metal ion binding should only be binding through other groups such as amino ligands. Table 4 shows the percentage of each metal ion bound by the unmodified and sulfhydryl modified alfalfa biomass. There was a decrease in binding by the sulfhydryl modified biomass for lead(II) and nickel(II). However, by modifying the available sulfhydryl ligands, there was an insignificant increase in the binding of copper(II). Nevertheless, as seen in Table 5, a totally different trend in metal binding is observed at pH 5.0. Overall, there was a slight increase in metal binding by the sulfhydryl modified biomass for lead(II) and nickel(II), while no significant change occurred for copper(II) binding by the alfalfa biomass. These data suggests that sulfhydryl groups may not play a significant role in the binding copper(II), lead(II), and nickel(II) by alfalfa biomass. Also, the increased nickel(II) and lead(II) binding by the modified biomass could be due to metal coordination to the thiol groups. We are currently investigating these results further. Batch binding capacities were performed with the sulfhydryl modified biomass and the results of these experiments are shown in Table 6. From the Table, is can be seen that nickel(II) binding was slightly enhanced by the sulfhydryl modification of the alfalfa biomass. This corresponds to the results of the pH 5.0 binding experiments. In addition, there was very little difference in binding of copper(II), and lead(II) between the sulfhydryl modified alfalfa biomass and the unmodified alfalfa biomass. Therefore, the binding of copper(II), nickel(II) and lead(II) by the alfalfa biomass may not be dependent on sulfhydryl ligands. The information obtained from this study will be useful for the development of an innovative phytofiltration method for the removal and recovery of heavy metal ions from contaminated waters.

ACKNOWLEDGMENTS

The authors acknowledge the financial support from the National Institutes of Health (NIH), (grant # GM 08012-25) and the financial support from the University of Texas at El Paso's Center for Environmental Resource Management (CERM) through funding from the HBCU/MI Environmental Technology Consortium which is funded by the Department of Energy and the Office of Exploratory Research of the U.S. Environmental Protection Agency (cooperative agreement CR-819849 - 01 - 4). Also, Gerardo Gamez would like to acknowledge the financial assistance of the William L. Staley grant from the College of Science at the University of Texas at El Paso.

REFERENCES

Carvalho, R.P., K.H. Chong, B.Volesky, 1994, Effects of Leached Alginate on Metal Biosorption, Biotechnology Letters, 16, 8, pp. 875-880.

Darnall, D.W., R. M. McPherson, J.L. Gardea-Torresdey, 1989,

Biohydrometallurgy, Proceedings of the International Symposium held at Jackson Hole, Wyoming, Edited by J. Salley, R.L. McCready, P.L. Wichlacz, pp. 341-348.

Gardea-Torresdey, J.L., M.K. Becker-Hapak, J. M. Hosea, D.W. Darnall, 1990, Effect of Chemical Modification of Algal Carboxyl Groups on Metal Ion Binding, Environmental Science and Technology, 24,pp. 1372-1378.

Gardea-Torresdey, J. L., L. Tang, J.M. Salvador, 1996a, Copper adsorption by esterified and unesterified fractions of Sphagnum peat moss and its different humic substances, Journal of Hazardous Materials, 48, pp. 191-206.

Gardea-Torresdey, J.L., K.J. Tiemann, J.H. Gonzalez, J.A. Henning, and M.S.Towsend, 1996b, Ability of Silica-Immobilized Medicago sativa (Alfalfa) to Remove Copper ions from Solution, J. of Hazardous Materials, 48, pp.181-190.

Gardea-Torresdey, J.L., K.J. Tiemann, J.H. Gonzalez, J.A. Henning, M.S. Townsend, I. Canno-Aguilera, 1996c, Removal of Nickel Ions from Aqueous Solution by Biomass and Silica - Immobilized Biomass of Medicago sativa (Alfalfa). J. of Hazardous Materials, 49, pp. 205-223.

Gardea-Torresdey J. L. , K.J. Tiemann, J.H. Gonzalez, O. Rodriguez, 1996d, Biosorption of Cadmium, Chromium, Lead, and Zinc, by Biomass of Medicago sativa (Alfalfa), Proceedings of the 11th Annual Conference on Hazardous Waste Research., Edited by L.E. Erickson, D.L. Tillison, S.C. Grant and J.P. McDonald, Kansas State Univ., Manhattan, KS, pp. 209-214.

Kuyucak, N., B. Volesky, 1989, The Mechanism of Cobalt Biosorption, Biotechnology and Bioengineering, 23, pp. 823-831.

Madgwick, J.C., 1991, Biological Sorption and Uptake of Toxic Metal fromWastewaters, Australasian Biotechnology, 4, pp. 292-297.

Majidi, V., D.A. Laude, A. Holcombe, 1990, Investigation of the Metal-Algae Binding Site with ^{113}Cd nuclear Magnetic Resonance, Environmental Science and Technology, 24, pp. 162-173.

Rayson, G.D., D.Ke. Huey-Yang, 1992, Characterization of Cd Binding sites on Datura Innoxia Using ^{113}Cd NMR Spectrometery, Environmental Science and Technology, 26, pp. 137-145.

Romero,M., M. Gnassia-Barelli, C. Carre, 1987, Trace metals:Cd, Cu, Pb, and Zn in Gelatinous Macroplankton from the Northwester Mediterranean, Water Research, 21,10, pp. 1287-1292.

Tiemann, K.J., Gardea-Torresdey, J.L., Renner, M.W., Sias, S., Gamez, G., and Rodriguez, O., 1997, "Study of the Ligands Involved in Metal Binding to Alfalfa Biomass." Proceedings of the 12th Annual Conference on Hazardous Waste Research,"Building Partnerships for Innovative Technologies," Edited by L.E.Erickson, M.M. Rankin, S.C. Grant and J.P. McDonald, Kansas State Univ., Manhattan, KS., pp.33-49.

Volesky, B., H.A. May-Phillips, 1995, Biosorption of Heavy metals by Saccharomyces cervisae, Appl. Microbiol. Biotechnol., 42, pp. 797-806.

Wieder, R. K., 1990, Metal Cation Binding to Sphagnum Peat and Sawdust: Relation to Wetland treatment of Metal-Polluted Waters, Water, Air, and Soil Pollution, 53, pp. 391- 400.

Table 1. Percentage of metal bound by inactivated alfalfa shoot biomass at pH 2.0.

	Cu(II)	Ni(II)	Pb(II)
Acetylated	9.8	17.2	60.1
Succinated	13.1	21.7	58.7
Unmodified Control	13.0	16.3	57.6

NOTE: The biomass was equilibrated with a 0.1mM metal solution at pH 2.0 for one hour.

Table 2. Percentage of metal bound by inactivated alfalfa shoot biomass at pH 5.0.

	Cu(II)	Ni(II)	Pb(II)
Acetylated	86.2	64.7	88.1
Succinated	94.8	89.6	96.1
Unmodified Control	83.4	70.0	86.8

NOTE: The biomass was equilibrated with a 0.1mM metal solution at pH 5.0 for one hour.

Table 3. Adsorption capacities for modified and unmodified alfalfa biomass (mg metal / g biomass).

	Cu(II)	Ni(II)	Pb(II)
Acetylated	8.6 mg/g	4.3 mg/g	21.0 mg/g
Succinated	13.5 mg/g	8.5 mg/g	41.3 mg/g
Unmodified Control	10.9 mg/g	6.0 mg/g	31.9 mg/g

NOTE: The experiments were performed at pH 5.0.

Table 4. Percentage of metal bound by sulfhydryl-modified and unmodified alfalfa shoot biomass at pH 2.0.

	Cu(II)	Ni(II)	Pb(II)
Modified	13.7	8.3	46.3
Unmodified	13.0	16.3	58.7

NOTE: The biomass was equilibrated with a 0.1mM metal solution at pH 2.0 for one hour.

Table 5. Percentage of metal bound by sulfhydryl-modified and unmodified alfalfa shoot biomass at pH 5.0.

	Cu(II)	Ni(II)	Pb(II)
Modified	83.2	82.1	91.2
Unmodified	83.4	70.0	86.8

NOTE: The biomass was equilibrated with a 0.1mM metal solution at pH 5.0 for one hour.