REMOVAL AND SELECTIVE RECOVERY OF HEAVY METAL IONS FROM INDUSTRIAL WASTE WATERS

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ABSTRACT

The influence of hard-water components (calcium(II) and magnesium(II)) on metal-ion binding to different algal biomasses was investigated. Concentrations as high as 10,000 ppm of calcium and magnesium ions were virtually without effect on the binding of copper(II), aluminum(III), gold(III), and mercury(II) ions to harvested cells of *Spirulina* or *Cyanidium.* Slight inhibition of cadmium(II), nickel(II), and zinc(II) ion binding was observed for both algal species.

Different algal-silica polymers showed good copper binding properties when exposed to an authentic copper-plating bath sample. However, various algal polymers exhibited substantial variations in performance under comparable conditions.

The algae, *Spirulina* and *Cyanidium*, were cultured under different nitrogen concentrations. Metal-ion binding experiments with the resultant biomass indicated that the nitrogen concentration present during growth of *Spirulina* had no impact on its metal-ion binding capacity. Conversely, the metal-ion binding capacity of *Cyanidium* was decreased in biomass grown at nitrogen levels below those found in the normal growth medium; however, growth medium nitrogen concentrations above normal may cause increased expression of high affinity gold binding sites. The copper binding capacity of *Cyanidium* biomass was found to increase, when cells were cultured in medium containing elevated levels of copper ion.

The mechanism of metal-ion binding to algae was studied by modifying algal chemical functional groups. These modifications showed that carboxyl groups are primary binding sites. Amino and sulfhydryl groups also play a role in algal metal-ion binding, but to a lesser extent.

Key words: water treatment, algae, biomass, heavy metal ions

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PROJECT OBJECTIVE

Accumulation of toxic metal ions in water supplies is a matter of increasingly grave concern. Primarily the undesirable by-products of mining and industrial activity, these ions can cause acute and chronic illnesses in humans and other animals. In an effort to limit further contamination, state and federal laws have been implemented, requiring industries to install expensive pollution-control systems. Development of efficient, widely applicable, low-cost methods for removal of heavy metal ions from waters, therefore, deserves high priority.

One new method that has allowed both the removal and recovery of metal ions from water has been the utilization of microorganisms such as algae. This metal-ion sorption process is based upon the natural, very strong affinity of the cell walls of algae for heavy metal ions. The algal cells have been immobilized in a silica polymer and this preparation has been used much as ion-exchange resins are used to remove heavy metal ions from water. There appear to be, however, distinct advantages of the immobilized algal system over other technology currently used for heavy metal-ion cleanup from waste waters. The goals of this project were: 1) to examine the effects of calcium(II) and magnesium(II) on transition metal binding to the algae, 2) to test the immobilized silica-algal polymers for removal of metal ions from electroplating plant waste waters, 3) to evaluate the effects of culturing conditions on the metal binding capacity of the resulting biomass and 4) to investigate the mechanism of metal-ion binding to different algae.

BACKGROUND

Algae are widespread microorganisms, comprising approximately 70% of the earth's biomass, and are organized into about 21,000 different algal species (Robinson, Mak, and Trevan 1986) with over 100,000 strains (Parkinson 1987). Metal-ion binding by algae and the application of this phenomenon in a water-treatment scheme are rapidly growing areas of interest.

A survey of the scientific literature for metal binding by algae discloses two different approaches: 1) use of living organisms, and 2) use of nonviable biomass. Metal-ion binding to living microorganisms can take place either through surface adsorption or intracellular accumulation. This postulate is supported by the work of Khummangkol, Canterford, and Fryer (1982), who showed that uptake of cadmium by live cultures of *Chlorella vulgaris* can not be accurately explained by a model which assumes adsorption only. In living algal cells, trace nutrient heavy metals (such as cobalt, molybdenum, calcium, and magnesium) are accumulated intracellularly by active biological transport, whereas toxic heavy metal ions may be sequestered by the cytoplasm of algal cells in at least one of the following three manners: (1) accretion or precipitation of the heavy metals on the cell wall surface, (2) intracellular chelation by biological polymers (Wood and Wang 1983), or (3) by adsorptive surface binding to various cell wall chemical functional groups. Adsorptive surface binding (sometimes referred to as biosorption) does not necessarily require biological activity and may result in sequestering of both essential and toxic metal ions into the cell surface. At this time, adsorption is the only process reported in which metal ions are bound to nonviable algal cells. Jennet, Hassett, and Smith (1977) studied representative species of living green algae (*Chlamydomonas, Chlorella pyrenoidosa* and *Chlorotyllium*) and blue-green algae (*Geotrichia, Nostoc, Oscillatoria rubenscens and Ulothrix*) for their ability to remove heavy metal pollutants. They observed at pH 5.0 cadmium was removed rapidly by *Geotrichia, Nostoc, Chlorella, and Chlorotyllium* whereas no discernible removal was achieved at pH 6.5 or 8.0. This study pointed out the drawback of using living algae since at pH 5.0, blue-green algae will grow only marginally. Becker (1983) showed that metal removal by means of live algae is not feasible in practice. The investigator demonstrated that nitrogen-fixing blue-green algae (such as *Anabaena cylindrica*) can be used for biological sewage treatment only at very low mercury and cadmium concentrations in the medium. At metal concentrations higher than 1 ppm, the growth of *Anabaena* was inhibited.

Methods for water-treatment that utilize non-viable algae are not complicated by the problem of trying to maintain growth under adverse conditions. In fact, Horikoshi, Nakajima, and Sakaguchi (1979) determined that heat-killed cells of *Chlorella regularis* display a binding

capacity for uranium(VI) three times greater than that measured for living alga. Also, Hwang, Wang, and Lii (1980) found that spray-dried *Chlorella sp.* could accumulate mercury(II) much faster than the living algal cells. The inactivated algal biomass is utilized in a fashion similar to ion-exchange resins. The biosorption, or binding, of metal ions by the biomass arises from the coordination of the ions to different functional groups in or on the cell. These coordinating groups (provided by proteins, lipids, and carbohydrates) include amino, thioether, sulfhydryl, carboxyl, carbonyl, imidazole, phosphate, phenolic, hydroxyl, and amide moieties (Crist et al. 1981; Schecher, Hassett, and Driscoll 1982). Ferguson and Bubela (1974) studied the biosorption of lead(II), copper(II), and zinc(II) to frozen or freeze-dried preparations of the green algae *Ulothrix, Chlamydomonas, and Chlorella vulgaris.* The amount of binding observed was greater at pH 7.0 than at pH 3.0. Salts such as sodium chloride inhibited only the binding of zinc, suggesting that selective adsorption of lead(II) or copper(II) was conceivable.

Nakajima, Horikashi, and Sakagusi (1981) examined the binding of various ions to heat killed *Chlorella regularis.* They observed selective accumulation of ions from an equimolar solution (0.1 mM of each ion at pH 5.0) which decreased in the order:

$$U(VI) >> Cu(II) >> Zn(II) > Co(II) > Cd(II) > Sr(II)$$

They concluded that the selectivity of heavy metal-ion uptake by *Chlorella regularis* is due mainly to the strength of coupling between nearby metal ions and cell components, especially proteins.

Greene et al. (1984) reported that dried *Chlorella vulgaris,* when resuspended in solution, will strongly and rapidly adsorb a variety of metal ions. These metal ions include aluminum(III), cadmium(II), cobalt(II), chromium(III), copper(II), nickel(II), lead(II), uranium(VI), and zinc(II). The metal ions were bound to the algal surface in a pH-dependent manner, with the binding increasing between pH 2 and 6. Most metals could be desorbed from the algae at pH 2.

The pH dependence for metal-ion binding to *Chlorella vulgaris* was studied by Darnall et al., (1986a) and Greene (1985). The investigators determined that metal ions can be divided into three classes based upon the pH dependence of binding to the alga. The first class is comprised of metal ions which are tightly bound at pH 5 and which can be stripped (or are not bound) at pH 2. Many ions fall into this class: aluminum(III), copper(II), lead(II), chromium(III), cadmium(II), nickel(II), cobalt(II), zinc(II), iron(II), beryllium(II), and uranium(VI). The second class is comprised of metallic anions which display the oppposite behavior of class I metal ions, i.e., they are bound strongly at pH 2 and bound weakly at pH values near 5. Ions in

class II include $PtCl_4^{2-}$, CrO_4^{2-} and SeO_4^{2-}. The third class of metal ions includes those metal ions for which there is no discernible pH dependence for binding between pH 1 and pH 6. Metal ions in this class include silver(I), mercury(II) and the chloride complex of gold(III). Of all the metal ions tested, these were bound the strongest.

The accumulation of gold by dried *Chlorella vulgaris* was studied by Greene et al. (1985, 1986). They found under certain conditions, *Chlorella* can accumulate gold to a level close to 10% of the organism's dry weight. Tetrachloroaurate(III) and gold(I) thiomalate were adsorbed rapidly by algal cells over a wide pH range. In contrast, dicyanoaurate(I) was adsorbed slowly and in a highly pH-dependent manner, with maximum binding observed near pH 3. Algal-bound gold(III) was observed to be reduced slowly to gold(0), but gold could be reoxidized and liberated from the alga in the presence of thiourea and oxygen.

Hosea et al. (1986) also investigated the accumulation of gold(0) by lyophilized preparations of *Chlorella vulgaris*. Gold was bound to the alga upon suspending dry algal cells in solutions containing hydrogen tetrachloroaurate(III). The relative amounts of ionic and atomic algal-bound gold were determined by thiourea extraction, both in the presence and absence of oxygen. They found the amount of algal-bound atomic gold produced from ionic gold increased with time. Three different classes of sites for binding and reducing gold were proposed by the investigators. One class, composed of weak-binding sites, provides an environment which permits the facile reduction of bound gold(I) to gold(0). A second class, associated with strong-binding sites does not permit gold reduction. The third class, presumably of intermediate binding strength, does permit gold reduction but only after elemental gold has accumulated elsewhere on the algal cell.

In addition to algae, other naturally occurring microorganisms such as fungi and bacteria have shown to be good adsorbents for metal ions. Fungal biomass has been demonstrated to be an effective adsorbent, especially for certain radionuclides such as uranium (Treen-Sears et al. 1984; Tsezos 1984; Tsezos and Noh 1984) and thorium (Tsezos and Volesky 1981; Tsezos and Volesky 1982). The uptake of other metal ions such as lanthanum(III), cadmium(II), lead(II) and silver(I) by fungal cells has been studied also (Friis and Myers-Keith 1986; Tobin, Cooper, and Neufold 1987). The biosorption of lead and chromium by *Penicillium* has been examined (Siegel et al. 1986a; Siegel et al. 1986b). The adsorption of metal ions by bacterial cells such as *Klebsiella aerogenes* (Sterritt and Lester 1986), and *Bacillus subtilis* (Hancock 1986) has been reported.

Although bacteria, fungi and algae have all been shown to be capable of biosorption of metal ions, algae have been demonstrated to possess the greatest selectivity and diversity for metal-ion binding. Furthermore, at this point in time, algae are the most economically amenable of all the microorganisms to incorporation into a bioresin, which will permit repeated metal binding-metal release recycling of the adsorptive material through either simple pH changes or the use of an eluting ligand.

METHODOLOGY

Metal-Binding Assays

Two different procedures are employed currently to assess the metal-binding properties of algal preparations. One is a "batch" technique; the other is a "column" or chromatographic, technique. In the batch procedure, a known quantity of lyophilized (freeze-dried) algal biomass is washed two or three times by suspension and centrifugation in 0.01 M HCl at pH 2.0. This treatment removes soluble biomolecules which could bind metal ions and complicate the interpretation of results. The washed material is then resuspended in a known volume of the solution of interest. After a sufficient contact time, the sample is centrifuged and the supernatant liquid is analyzed for residual metal ions. The difference between the initial and final metal-ion concentrations represents the amount of metal ion bound to the algae.

The batch configuration is often employed in preliminary experiments because of the ease with which solution parameters can be manipulated. For pH studies, aliquots of the algal suspension can be adjusted readily to the desired pH prior to metal-ion introduction. The degree of interference of metal-ion binding caused by the other ions is easily ascertained by adding various amounts of the species in question to otherwise identical aliquots of an algal suspension.

It would be useful to pack algae directly into a column to make an "algae filter" through which waters containing metal ions could be passed. Unfortunately, the algae pack so tightly that water will not flow through a column containing only algal biomass. Therefore, it is necessary to immobilize the algal cells in a support medium to obtain acceptable flow characteristics. The resulting material is granulated and sieved to obtain a preparation having particle sizes between 40- and 100-mesh. This material functions extremely well as a chromatographic matrix. The "column" methodology is useful for investigating certain questions relevant to the use of algae in water-treatment applications, since it bears a closer resemblance to a practical contact system than does the batch system.

Metal-ion Analysis

Determination of metal ions was performed by atomic absorption spectrophotometry (AAS) using air-acetylene or nitrous oxide-acetylene flames or graphite furnace. A Perkin-Elmer model 3030B atomic absorption/atomic emission spectrophotometer was used. The instrument was equipped with an automatic sampler and an HGA-600 graphite furnace. All instrumental conditions were optimized for maximum sensitivity as described by the manufacturer. When measuring metal ions, matrix matching of standards and samples was utilized. The mean and standard deviation of five AAS measurements were recorded in every sample analysis.

Algal Culturing Conditions

Spirulina platensis, a blue-green alga was cultivated indoors in growth chambers consisting of 20-liter polycarbonate flasks. The growth medium was Zaroux's medium (Zaroux 1966) prepared in deionized water. The amount of nitrogen source ($NaNO_3$) was varied according to the medium formulation. The pH was maintained between 9 and 10 and the culture was kept under red and blue fluorescent light at a temperature of 25°C. The algal cells were harvested by centrifugation on a Beckman model J2-21 centrifuge, and dialyzed against deionized water (to remove salts). Subsequently, the algal cells were lyophilized in a Labconco freeze-dryer.

Cyanidium caldarium, a red alga was cultivated indoors in growth chambers consisting of 20-liter polycarbonate flasks. The normal growth medium contained sulfuric acid (0.1M), calcium chloride (0.1M), potassium biphosphate (0.2 M), magnesium sulfate (0.1M), micronutrients according to Ford (1979) and ammonium chloride (0.01 M). The ammonium chloride concentration was varied according to the medium formulation. The pH of the growth medium was approximately 1.9 and the culture was kept under red and blue fluorescent light at a temperature of 45°C. Carbon dioxide (6%) and air were bubbled through the medium constantly. Harvesting conditions were the same as for *Spirulina platensis.*

Chemical Modification of Algal Biomass

Chemical modification of amino groups with acetic anhydride was performed by resuspending 3.2 grams of the washed algal biomass in 120 mL of buffer at pH 7.2 (0.1M sodium phosphate/1.0M sodium acetate). Acetic anhydride (16 mL) was then added, and the sample was stirred continuously for one hour while maintaining the pH at 7.2 by addition of NaOH. After centrifugation, the biomass was washed sequentially with deionized water, 1M hydroxylamine, 0.1 M H_2SO_4, and water, and then was lyophilized in a Labconco freeze-dryer.

Chemical modification of algal amino groups with succinic anhydride was performed first by washing the algal biomass (4.0 grams) with 0.1M H_2SO_4, and then with 1.0 M $NaHCO_3$. Subsequently, the material was resuspended in 1 M $NaHCO_3$ and maintained at a pH of 8.0 to 8.3 with NaOH while six, four-gram additions of succinic anhydride were made at fifteen-minute intervals. After being washed with 0.1 M H_2SO_4, then deionized water, the treated algal biomass was lyophilized in a Labconco freeze-dryer.

The ninhydrin reaction was used to determine the extent to which samples of algal biomass had been modified by treatment with either acetic or succinic anhydride. This experiment was performed by suspending the algal biomass (both modified and unmodified) at a concentration of 1 mg/mL in distilled-deionized water. One milliliter of $SnCl_2$ reagent (0.17g $SnCl_2 \cdot 2H_20$ in 100 mL of 5.8 M acetic acid at pH 5.5) and 1.0 mL of ninhydrin reagent (4% (w/v) ninhydrin

in methyl cellosolve) were then added. After incubation for twenty minutes in a boiling water bath, 5.0 mL of 50% aqueous ethanol was added to each sample. Upon cooling to room temperature, the absorbance of the supernatant fraction of each sample was measured spectrophotometrically at 570 nm. The difference in the readings between the modified and control samples was divided by the reading of the control sample in order to calculate the fraction of total amino groups modified.

Modification of algal carboxyl groups with acidic methanol was performed as follows. Nine grams of algal biomass (sieved to less than 100 mesh) was suspended in 633 mL of 99.9% methanol and 5.4 mL of concentrated hydrochloric acid (0.1M HCl final concentration) with continuous agitation. Aliquots were removed from the stirred suspension after 6, 12, 24, and 48 hours at room temperature. The reactions were quenched by addition of a large volume of cold distilled deionized water. The samples were then dialyzed exhaustively against 0.001 M HCl to eliminate unreacted methanol, using Baxter dialysis bags (6-8000 d) at room temperature, then lyophilized to reduce volume.

The degree of esterification of carboxyl groups with methanol was determined by gas chromatography after base hydrolysis of the modified biomass. Base hydrolysis was performed by suspending the algal biomass (modified and unmodified) with 1.25 mL of 0.005 M Citrate in 0.1 M NaCl at pH 5.0. Subsequently, 0.10 mL of 1.0 M NaOH was added to the suspension. Following a five minute agitation period, samples were sealed with parafilm and were incubated overnight at 4°C. Then, samples were hand-mixed until an even suspension was obtained and were centrifuged at 2,500 rpm for fifteen minutes in a Sorvall GLC-2B centrifuge at room temperature. The supernatant fractions were transferred to a 1.0 mL volumetric flask. Then, 0.15 mL of 0.082 M Citrate at pH 3.0 was added and the final volume was brought up to 1.0 mL with deionized water when necessary. Samples were then placed in 1.5 mL Wheaton GC vials with teflon caps. Before injecton into the gas chromatograph, the samples were spiked with 0.03 mM 2-propanol as an internal standard. One µL of the resulting solution was then injected into a Hewlett-Packard 5880A gas chromatograph equipped with an automated splitless injector, a flame ionization detector and a 20 meter, RSL-500 polar column. The conditions for GC analysis were as follows: injector temperature, 250°C; detector temperature, 275°C; carrier gas N_2; carrier gas velocity, 3 mL/minute. Samples were run isothermally at 40°C for three minutes.

Chemical modification of algal sulfhydryl groups was performed by suspending 1.0 gram of the dry algal biomass in 100 mL of the 2,2'-dithiodipyridine reagent (0.001M). This reagent was prepared by dissolving 0.220g of 2,2'-dithodipyridine (Sigma reagent grade) in 2.0 mL of concentrated HCl, and bringing the volume up to 1L with 0.1M sodium acetate at pH 5.0. The reaction was carried out while stirring at room temperature. At appropriate times, aliquots

from the modified algal biomass were withdrawn from the reaction vessel. These biomasses were then lyophilized in a Labconco freeze-dryer.

The extent of sulfhydryl modification was determined by following the course of the reaction spectrophotometrically. Grassetti and Murray (1967) found that 2,2'-dithiodipyridine reacts with sulfhydryl groups forming a disulfide linkage and a by-product, 2-thiopyridone. Thus, one can follow this reaction by the appearance of 2-thiopyridone at 343 nm. This was performed by using a Coleman 124 double-beam spectrophotometer. Determination of algal sulfhydryl groups was carried out by comparing absorption values of 2-thiopyridone from the supernatant fractions of the modified algae at different times, to absorbances from a cysteine calibration curve. These curves were obtained by adding increasing amounts of cysteine (0.5-2.5 µmoles) to a 10 mL solution of (0.001 M) 2,2'-dithiodipyridine. The absorbances of the by-product 2-thiopyridone at 343 nm were then plotted against cysteine concentration.

RESULTS

Algal biomass possesses several properties which make it potentially useful for the treatment of industrial effluents contaminated with toxic metal ions. For example, it exhibits high affinities and high binding capacities, for a broad range of metal ions (Darnall et al. 1986a). The goal of this project was to examine further the applicability of algal biomass to water-treatment processes. The specific objectives of this project were: 1) to examine the effects of calcium(II) and magnesium(II) on transition metal binding to algal biomass, 2) to test immobilized silica algal polymers for removal of metal ions from electroplating plant waste water, 3) to evaluate the effects of culturing conditions on the metal binding capacity of the resultant biomass and 4) to investigate the mechanism of metal-ion binding to different algal biomass. Results and discussion of these experiments are presented in this section.

Effect of Calcium(II) and Magnesium(II) on Transition Metal Binding to Algal Biomass

The influence of high concentrations of the hard-water cations, calcium(II) and magnesium(II), on metal-ion binding to dry biomasses of *Cyanidium caldarium* and *Spirulina platensis* was investigated. The metal ions evaluated were: copper(II), zinc(II), cadmium(II), aluminum(III), gold(III), nickel(II), and mercury(II). In these experiments, washed algal biomass (5 mg/mL) was resuspended in a predetermined concentration of the metal ion at either pH 5.0 or pH 6.0. Sodium acetate (0.05 M)was used to buffer solutions at pH 5.0, while 0.05 M MES (2-N, morpholinoethanesulfonic acid) was used at pH 6.0. Calcium(II) or magnesium(II) were added at different concentrations as the nitrate salts, and sodium nitrate was added to samples as necessary to maintain uniform ionic strength throughout a series of samples. In all cases, equilibrium between the biomass and metal-ion solutions was allowed to proceed for one hour at room temperature. At the end of this contact period, the algal suspensions were centrifuged, and the supernatant solution was analyzed for residual metal ion by atomic absorption spectroscopy.

It has been determined that *Chlorella vulgaris* displays relatively little affinity for the hard-water ions calcium(II) and magnesium(II) (Darnall et al. 1986a). Likewise it was determined that algal biomasses of *Cyanidium caldarium* and *Spirulina platensis* have little affinity for calcium(II) and magnesium(II) (Figures 1 and 2, respectively). The extent of uptake is low in both cases and, for all practical purposes, identical over the entire concentra

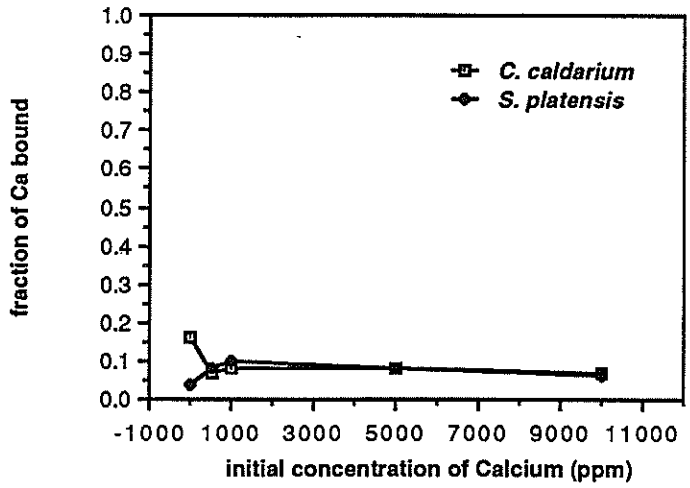


Figure 1: **Binding of Calcium(II) to *C. caldarium* and *S. platensis* at pH 5.0**

Lyophilized *Cyanidium caldarium* or dried *Spirulina platensis* was washed three times in 0.01 M HCl at pH 2 and once in 0.01 M sodium acetate at pH 5.0. The algae were then resuspended at a concentration of 5 mg/mL in 0.05 M sodium acetate at pH 5.0, and differing concentrations of calcium, with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate at room temperature with agitation for one hour. After twenty minutes, the pH of the samples was checked and adjusted back to pH 5.0 with 1.0 M NaOH or 1.0 M HCl as required. At the end of the equilibration period, the samples were centrifuged and the supernatant fraction was analyzed for calcium(II) by flame atomic absorption at 422.7 nm.

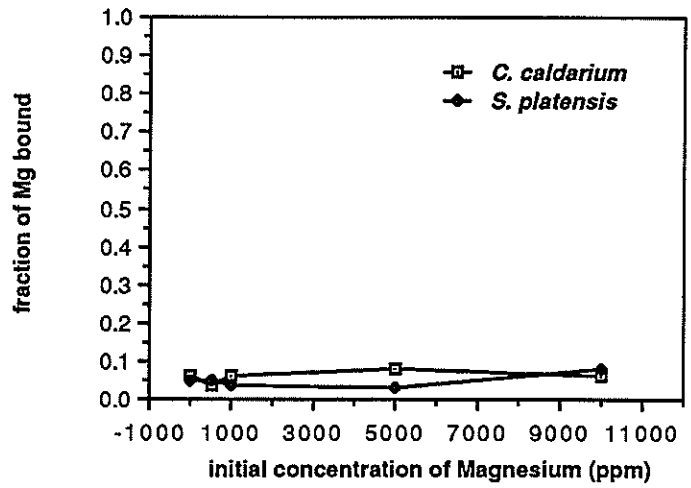


Figure 2: **Binding of Magnesium(II) to *C. caldarium* and *S. platensis* at pH 5.0**

Lyophilized *Cyanidium caldarium* and dried *Spirulina platensis* were washed three times in 0.01 M HCl and once in 0.01 M sodium acetate at pH 5.0. The algae were then resuspended, at a concentration of 5 mg/mL, in 0.05 M sodium acetate at pH 5.0 and differing concentrations of magnesium, with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate at room temperature with agitation for one hour. After twenty minutes, the pH of the samples was checked, and adjusted back to pH 5.0 with 1.0 M NaOH or 1.0 M HCl as required. At the end of the equilibration period, the samples were centrifuged and the supernatant fraction was analyzed for magnesium(II) by flame atomic absorption at 285.2 nm.

tion range. This suggests that these algal biomasses might be well-suited for removal of heavy metal ions from hard waters. This hypothesis was largely confirmed by experiments (presented below) which determined the binding of different metal ions in the presence of increasing levels of calcium(II) and magnesium(II).

Figure 3, displays the effect of calcium(II) on copper(II) binding to dry biomass of *Cyanidium caldarium* or *Spirulina platensis* at pH 5.0. *Cyanidium* displays a marginally higher binding capacity, but uptake is diminished by less than 10% in either case. Also, high concentrations of magnesium(II) did not affect copper(II) binding by *Cyanidium caldarium* (Figure 4)

The effect of calcium(II) on zinc(II) uptake by *C. caldarium* and *S. platensis* at pH 6.0 is shown in Figure 5. Binding capacities are similar for both species. No significant effect on zinc(II) binding is observed. Figure 6 displays the effect of magnesium(II) on zinc(II) binding at pH 6.0 by *Spirulina* or *Cyanidium* biomass. A decrease in binding of approximately 25% is observed for the *Spirulina* biomass, at the highest magnesium(II) concentration employed indicating that magnesium ions slightly inhibit zinc uptake by *Spirulina*. In contrast, a decrease in zinc(II) binding of only approximately 10% was observed for the *Cyanidium* biomass.

Figure 7 illustrates the effect of calcium(II) on cadmium(II) binding at pH 6.0. In this case, *Cyanidium* shows a markedly greater capacity for cadmium ions. Moreover, the uptake of cadmium(II) by *Cyanidium* is significantly less susceptible to interference by calcium ions. The relatively low inhibition for cadmium(II) binding by *Spirulina* and *Cyanidium* biomass observed at high magnesium(II) concentrations is shown in Figure 8. When present at 10,000 ppm, magnesium(II) decreases the binding of cadmium(II) by only approximately 15% in both biomasses. Since the uptake of cadmium(II) is extremely sensitive to small variations in pH in the region of 5.0, it was decided to conduct the hard metal-ion interference studies for cadmium(II) at pH 6.0.

The effect of high calcium(II) and magnesium(II) concentrations on aluminum(III) uptake by *C. caldarium* and *S. platensis* was examined. Experiments were performed as described previously except the concentration of aluminum(III) employed was 1.0×10^{-3} M, the sodium acetate concentration was 0.05M, and the pH of each sample was measured after the first twenty minutes of equilibration and readjusted to 5.0 with sodium hydroxide if necessary. Figure 9 displays the effect of calcium(II) and magnesium(II) competition on aluminum (III) uptake by *Spirulina platensis*. Clearly, calcium and magnesium ions have minimal effect on the binding of aluminum(III) by *Spirulina* biomass. Similarly, minimal effect on aluminum binding by *Cyanidium caldarium* was observed at high calcium(II) and magnesium(II) concentrations at pH 5.0 (Figure 10).

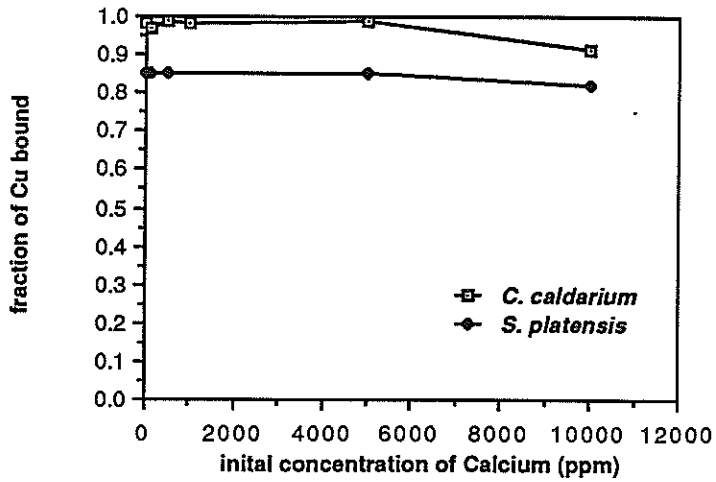


Figure 3: **Effect of Calcium(II) on the Binding of Copper(II) by *C. caldarium* and *S. platensis* at pH 5.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed three times in 0.01 M HCl and once in 0.01 M sodium acetate at pH 5.0. The pellet from the last wash was resuspended at a concentration of 5 mg/mL in 0.01 M sodium acetate at pH 5.0 containing 0.1 mM copper (II) and variable concentrations of calcium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 5.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for copper using flame atomic absorption at 324.8 nm.

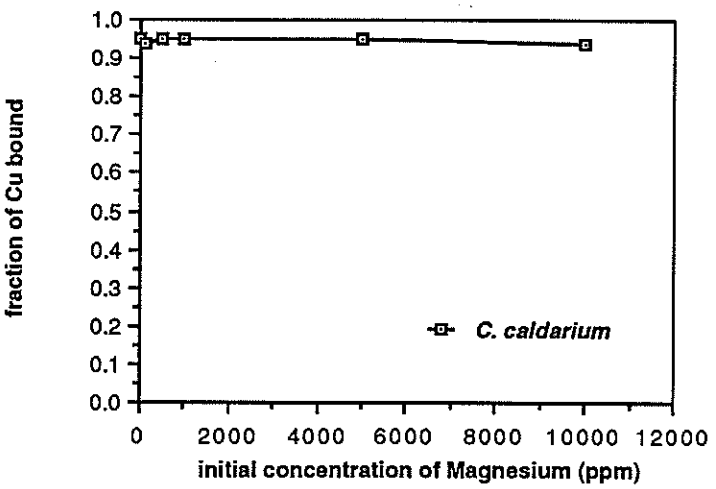


Figure 4: **Effect of Magnesium(II) on the Binding of Copper(II) to C. *caldarium* at pH 5.0**

Lyophilized *C. caldarium* was washed three times in 0.01 M HCl and once in 0.01 M sodium acetate at pH 5.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.1 mM copper(II) in 0.05 M sodium acetate at pH 5.0 containing variable concentrations of magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 5.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, the samples were centrifuged and the supernatant fractions were analyzed for copper(II) by flame atomic absorption at 285.2 nm.

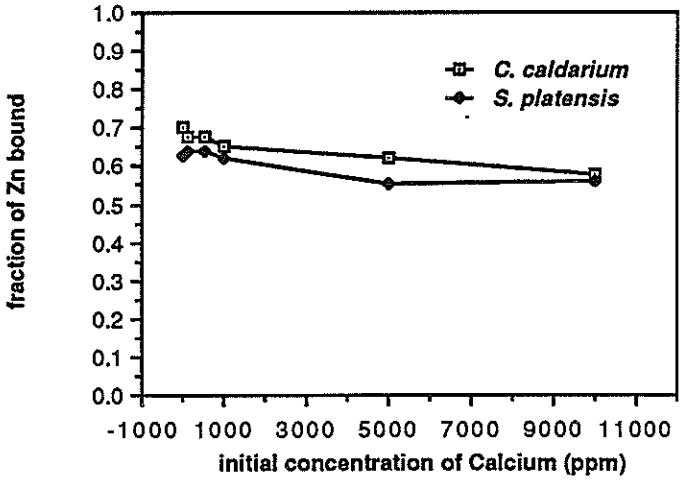


Figure 5: **Effect of Calcium(II) on the Binding of Zinc(II) by *C. caldarium* and *S. platensis* at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M nitric acid and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL in 0.02 M MES at pH 6.0 containing 0.1 mM zinc(II) and variable concentrations of calcium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for zinc using flame atomic absorption at 213.9 nm.

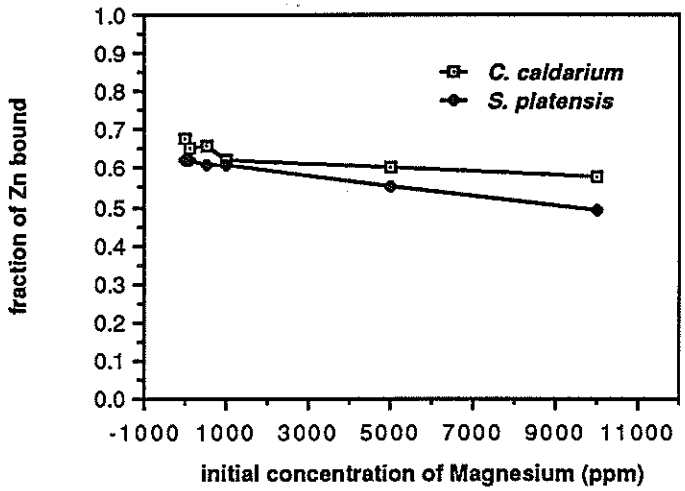


Figure 6: **Effect of Magnesium(II) on the Binding of Zinc(II) by *C. caldarium* and *S. platensis* at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended at a concentration of 5 mg/mL in 0.02 M MES at pH 6.0 containing 0.1 mM zinc(II) and variable concentrations of magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for zinc using flame atomic absorption at 213.9 nm.

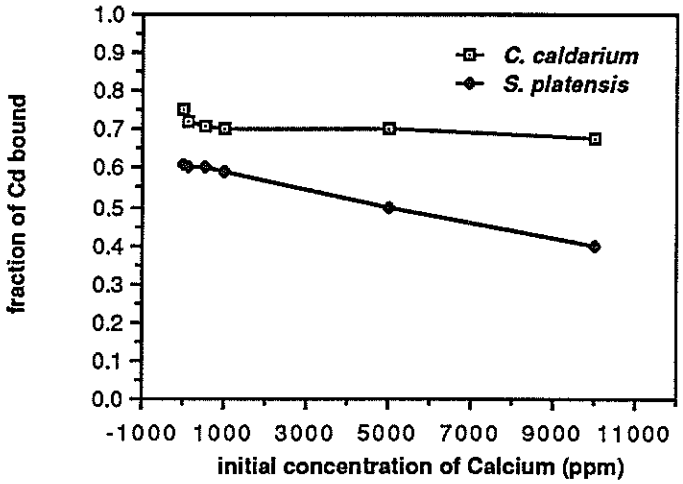


Figure 7: **Effect of Calcium(II) on the Binding of Cadmium(II) by *C. caldarium* and S. *platensis* at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended at a concentration of 5 mg/mL in 0.02 M MES at pH 6.0 containing 0.1 mM cadmium(II) and variable concentrations of calcium(II), with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for cadmium using flame atomic absorption at 228.8 nm.

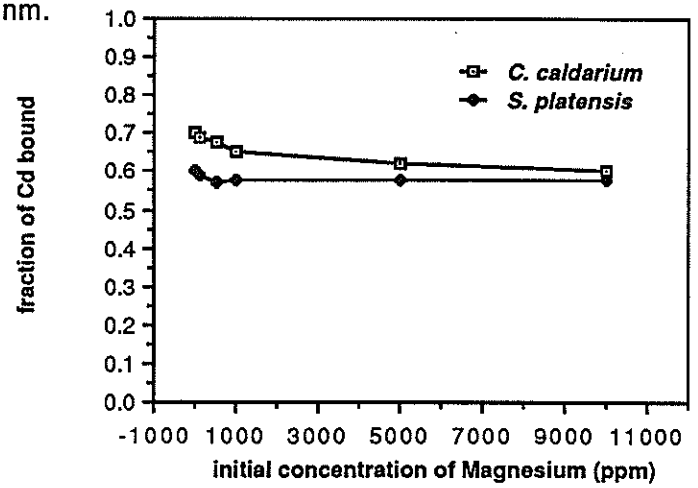


Figure 8: **Effect of Magnesium(II) on the Binding of Cadmium(II) by *C. caldarium* and *S.platensis*.at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01M HCl and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended at a concentration of 5 mg/mL in 0.02 M MES at pH 6.0 containing 0.1 mM cadmium and variable concentrations of magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for cadmium using flame atomic absorption at 228.8 nm.

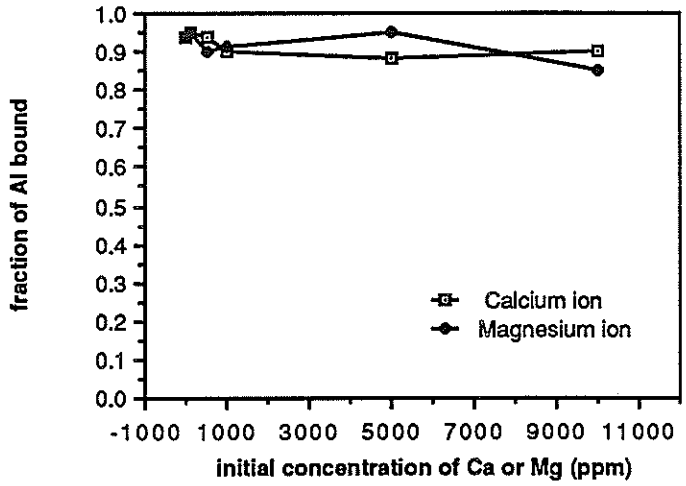


Figure 9: **Effect of Calcium(II) or Magnesium(II) on the Binding of Aluminum(III) to *S. platensis* at pH 5.0**

Dried *S. platensis* was washed twice in 0.01 M HCl and once in 0.05 M sodium acetate at pH 5.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.05 M sodium acetate at pH 5.0 containing 1.0 M aluminum(III) and variable concentrations of calcium(II) or magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) or magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for aluminum using flame atomic absorption at 303.9 nm.

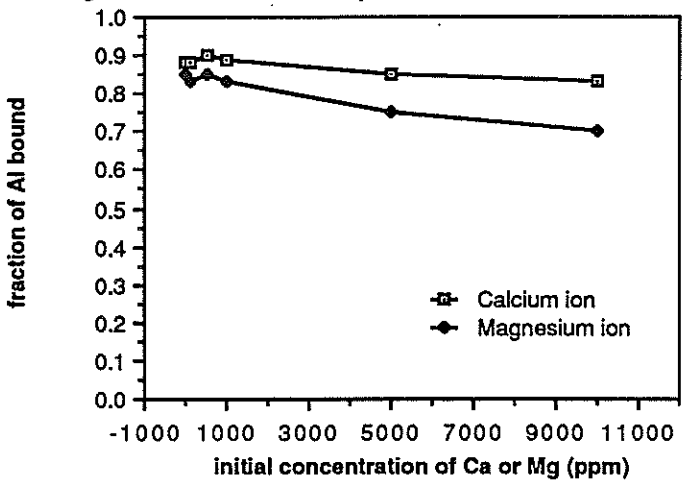


Figure 10: **Effect of Calcium(II) or Magnesium(II) on the Binding of Aluminum(III) to *C. caldarium* at pH 5.0**

Lyophilized *C. caldarium* was washed twice in 0.01 M HCl and once in 0.05 M sodium acetate at pH 5.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.05 M sodium acetate at pH 6.0 containing 1.0 mM aluminum(III) and variable concentrations of calcium(II) and magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) and magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M NaOH or 1.0 M HCl as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for aluminum using flame atomic absorption at 303.9 nm.

Experiments with gold(III) (as the tetrachloroaurate(III) complex) were conducted at pH 2.0 in 0.01M sodium acetate. Figure 11 demonstrates that at pH 2.0, biomasses from *Cyanidium caldarium* and *Spirulina platensis* have identical binding capacities for gold(III). Calcium ions do not inhibit uptake in either case and in fact, appear to cause increased uptake at higher concentrations. The effect of magnesium ions on gold(III) uptake by either *Cyanidium* or *Spirulina* biomass is shown in Figure 12. Magnesium ions, even at high concentrations, do not seem to interfere with gold(III) binding.

Figure 13 displays the variation of nickel(II) uptake at pH 6.0 by either *Cyanidium caldarium* or *Spirulia platensis* biomass as a function of calcium ion concentration. Neither species has a very high capacity for nickel ions, and the presence of 10,000 ppm of calcium(II) causes roughly a 30% decrease in binding. Figure 14 exhibits the change of nickel(II) uptake at pH 6.0 by either *Cyanidium* or *Spirulina* biomass as a function of magnesium ion concentration. The uptake of nickel(II) is inhibited to a similar extent, with 10,000 ppm of magnesium(II) causing a 20% reduction in binding.

The effect of calcium(II) or magnesium(II) ions on the adsorption of mercury(II) by *Spirulina platensis* biomass at pH 2.0 was also studied. The results of these experiments, performed at an initial mercury(II) concentration of 100 ppm, are presented in Figure 15. These results demonstrate that high calcium(II) or magnesium(II) concentrations have virtually no influence on mercury(II) binding to *Spirulina* biomass.

Effectiveness of Algal-Based Polymers for the Removal of Metal Ions from Electroplating Waste Waters

One objective of this project was to evaluate the effectiveness of algal-based silica polymers for the removal of metal ions from electroplating waste waters. Therefore, copper-bearing waste samples were obtained from various electroplating operations and were used to test the performance of algal-silica preparations. For these studies, dry algal-silica polymers were ground, sized, acid-washed, and then packed into a 0.7 cm (internal diameter) glass column. The column was rinsed with deionized water, adjusted to the appropriate pH with a buffer solution, then rinsed once more with deionized water prior to use. A simulated solution from an electroplating rinse tank (known in the electroplating field as "drag-out" solution) was prepared by diluting an authentic copper-plating bath sample. This solution was passed through the column while effluent samples were collected and analyzed for the presence of copper. A binding curve (known as a "breakthrough" curve) was then obtained by plotting the copper concentration of the effluent versus total effluent volume (usually plotted as "bed volumes"). It

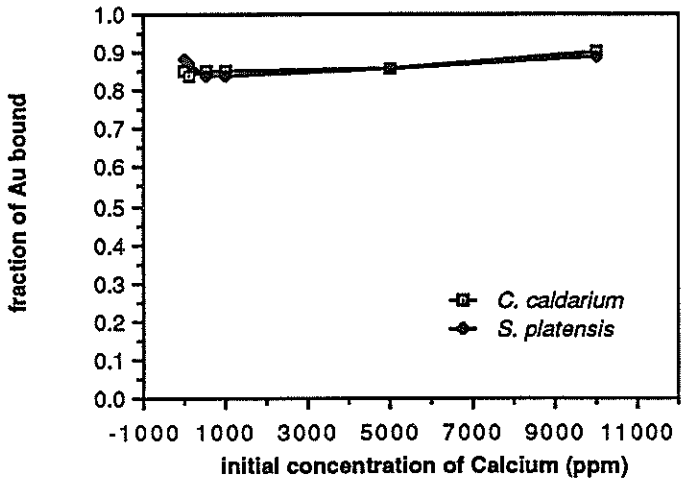


Figure 11: **Effect of Calcium(II) on the Binding of Gold(III) by *C. caldarium* and *S. platensis* at pH 2.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl at pH 2.0 and once in 0.01 M sodium acetate at pH 2.0. The pellet from the last wash was resuspended at a concentration of 5 mg/mL in 0.01 M sodium acetate at pH 2.0 containing 0.1 mM gold(III) and variable concentrations of calcium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 2.0 with 1.0 M HCl or 1.0 M NaOH as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for gold(III) using flame atomic absorption at 242.8 nm.

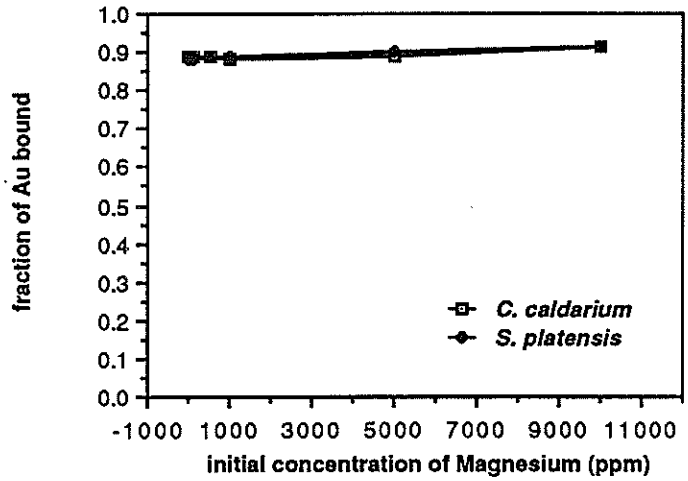


Figure 12: **Effect of Magnesium(II) on the Binding of Gold(III) by C. *caldarium* and S. *platensis* at pH 2.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl at pH 2.0 and once in 0.01 M sodium acetate at pH 2.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.01 M sodium acetate at pH 2.0 containing 0.1 mM gold(III) and variable concentrations of magnesium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 2.0 with 1.0 M HCl or 1.0 M NaOH as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for gold(III) using flame atomic absorption at 242.8 nm.

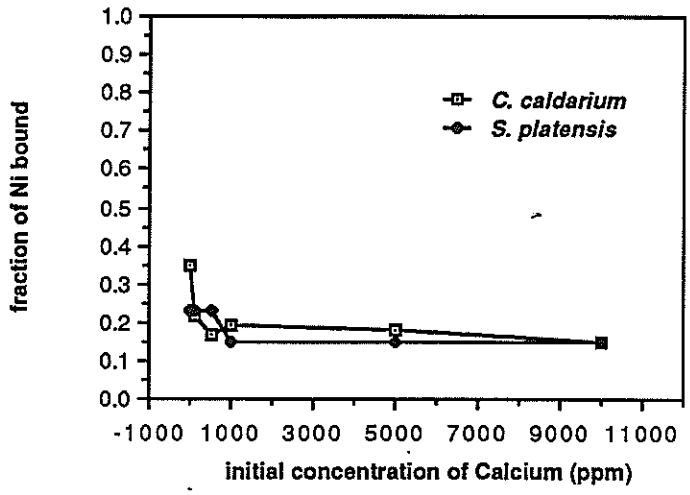


Figure 13: **Effect of Calcium(II) on the Binding of Nickel(II) by C. *caldarium* and *S. platensis* at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl at pH 2.0 and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.02 M MES at pH 6.0 containing 0.1 mM nickel(II) and variable concentrations of calcium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M HCl or 1.0 M NaOH as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for nickel using flame atomic absorption at 232.0 nm.

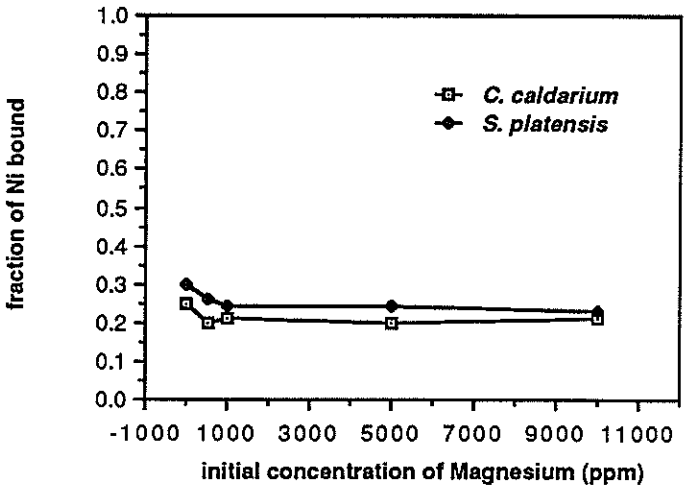


Figure 14: **Effect of Magnesium(II) on the Binding of Nickel(II) by *C. caldarium* and *S. platensis* at pH 6.0**

Lyophilized *C. caldarium* and dried *S. platensis* were washed twice in 0.01 M HCl at pH 2.0 and once in 0.02 M MES at pH 6.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.02 M MES at pH 6.0 containing 0.1 mM nickel and variable concentrations of calcium(II) with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 6.0 with 1.0 M HCl or 1.0 M NaOH as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for nickel using flame atomic absorption at 232.0 nm.

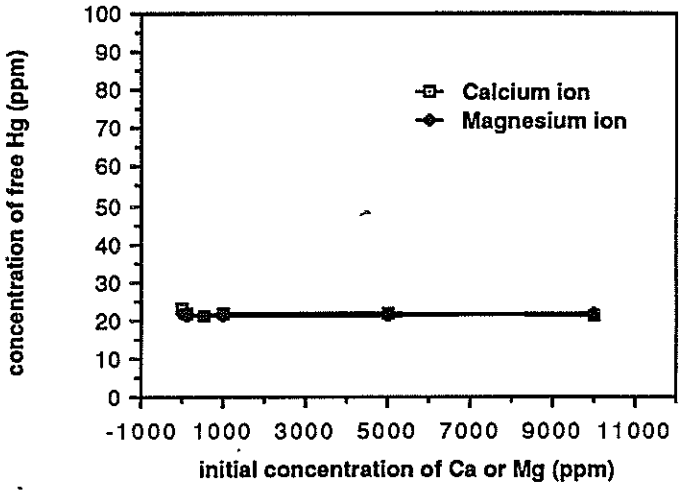


Figure 15: **Effect of Calcium(II) and Magnesium(II) on the Binding of Mercury(II) by *S. platensis* at pH 2.0**

Lyophilized, dried *S. platensis* was washed twice in 0.01M nitric acid, and once in 0.01 M sodium acetate at pH 2.0. The pellet from the last wash was resuspended, at a concentration of 5 mg/mL, in 0.01 M sodium acetate at pH 2.0 containing 100 ppm mercury(II) and variable concentrations of calcium(II) or magnesium(II), with sufficient sodium nitrate to achieve an ionic strength equivalent to that of the 10,000 ppm calcium(II) or magnesium(II) sample. The suspensions were allowed to equilibrate for one hour at room temperature. After twenty minutes, the pH of the samples was checked and adjusted back to pH 2.0 with 1.0 M HCl or 1.0 M NaOH as required. After equilibration was complete, samples were centrifuged and the supernatant fractions were analyzed for mercury(II) using flame atomic absorption at 253.7 nm.

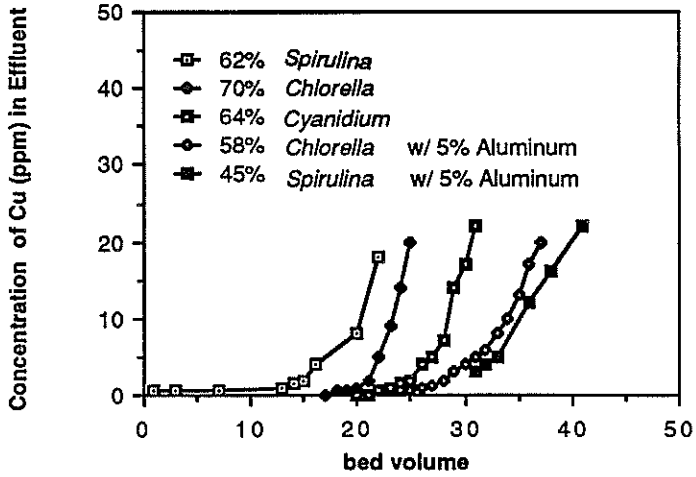


Figure 16: **Breakthrough Curves for Various Algal-Silica Polymers Employing a Simulated Alkaline Drag-Out Solution**

A sample of ammoniacal etch-solution was diluted to simulate a drag-out solution containing 68-84 ppm of copper at pH 8.0-8.3. The algal-silica preparations contained 70% *Chlorella* (-◆-), 58% *Chlorella* with 5% aluminum (-◆-), 62% *Spirulina* (-■-), 45% *Spirulina* with 5% aluminum (-■-), and 64% *Cyanidium* (-■-). The *Chlorella* and *Cyanidium* columns had bed volumes of 3 mL and operated at pH 8.3, using 68 ppm of copper. The *Spirulina* columns had bed volumes of 6 mL and were operated at pH 8.0, employing 75 ppm of copper for the 5% aluminum prep and 84 ppm of copper for the preparation lacking aluminum. All flow rates were 1 mL/min.

is important to point out that one bed volume corresponds to the total volume of the algal-polymer packed in the column.

Breakthrough curves for several different algal-silica preparations containing either *Chlorella pyrenoidosa*, *Spirulina platensis*, or *Cyanidium caldarium* are displayed in Figure 16. It is quite apparent that all algal preparations are capable of removing copper ions from the electroplating solution containing copper as the tetraammine complex at pHs between 8.0 and 8.3. However, different algal polymers show substantial variations in performance under comparable conditions with the 45% *Spirulina* polymer (with 5% aluminum) possessing the highest copper(II) binding capacity and the 62% *Spirulina* (without aluminum) having the lowest. Thus, preparations in which aluminum ion has been incorporated appear to perform better than those in which aluminum was omitted, even though the former contain significantly less algal material on a weight to weight basis. The poor performance exhibited by the *Spirulina* polymer lacking aluminum may be due to decomposition of the polymer at high pH (pH approximately 8.3). Alkaline solutions are quite detrimental to the physical integrity of this preparation, due to the propensity of silica gel to hydrolyze in alkaline solution. The addition of aluminum ion represents one approach for overcoming the instability of this algal-silica polymer at high pH.

Another important factor to consider in the application of algal polymers to the removal of metal ions from electroplating waste waters is their durability or recyclability. It is important to know whether or not the algal polymers can stand up to repeated cycles of binding and elution. Breakthrough curves for three cycles of binding and elution (with 1 M sulfuric acid) are presented in Figure 17 for a *Chlorella* algal-silica polymer containing aluminum ion. It is important to point out the electroplating waste water used in this experiment contained copper(II) as the tetraammino complex and the experiment was performed at pH 8.0. Consistent performance is exhibited for all three cycles. Similar experiments are presented in Figure 18 for the *Spirulina*-based polymer containing aluminum. There is a significant loss of binding capacity for copper (as the ammine complex) upon recycling the *Spirulina*-based polymer. Thus, it was decided to compare the recycling ability of algal polymers containing aluminum to those from which aluminum was omitted. To evaluate these algal preparations, an acidic copper (as copper sulfate) etchant waste water was chosen, and the experiments were conducted at pH 5.0. Results of this comparison are displayed in Figures 19 and 20 (*Spirulina*-based polymers) and 21 and 22 (*Chlorella*-based polymers). It is clear that the substantial improvement in binding-capacity exhibited by algal preparations containing aluminum is lost after the first cycle when the waste water contains acidic copper. However, the algal polymers containing no added aluminum demonstrate consistent performance throughout the three binding and elution cycles.

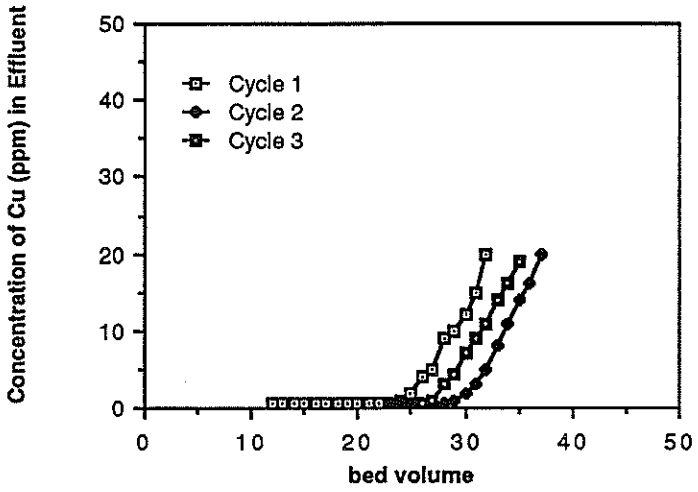


Figure 17: **Recycling of *Chlorella*-Based Algal-Silica Polymer**

A column of Chlorella-based polymer prepared with 5% aluminum was used to remove copper from an ammoniacal solution containing 68 ppm of copper at pH 8.3. The column was adjusted to pH 8.3 with 0.1M sodium phosphate buffer, then rinsed with 20 mL of deionized water before exposure to the simulated copper waste water. Bound copper was eluted from the column with 1 M sulfuric acid. All flow rates were 1 mL/min, and the bed volume was 3 mL.

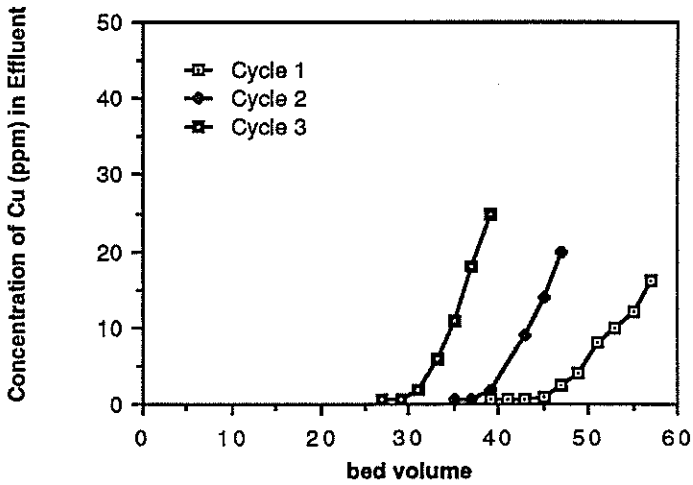


Figure 18: **Recycling of *Spirulina*-Based Algal-Silica**

A column of *Spirulina*-based polymer containing 5% aluminum was used to remove copper from an ammoniacal solution at pH 8.0. The copper level was 75 ppm in cycles 1 and 3 and 81 ppm in cycle 2. The column was adjusted to pH 8.0 by passing 0.5 M sodium phosphate buffer then rinsed with 20 mL of deionized water. Bound copper was eluted from the column with 1 M sulfuric acid. All flow rates were 1 mL/min., and the bed volume was 6 mL.

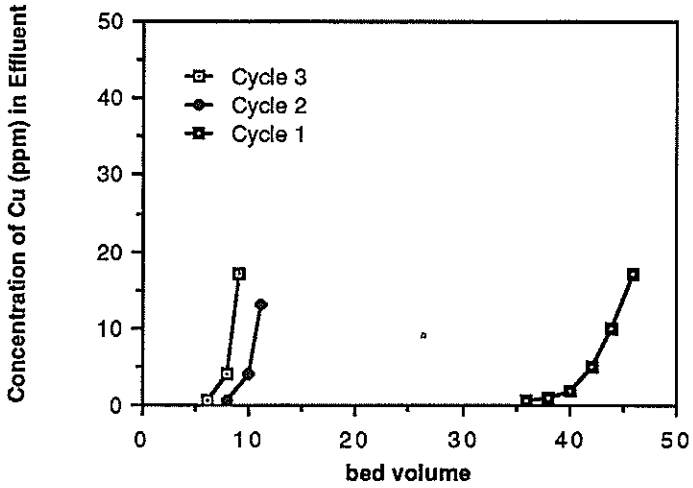


Figure 19 : **Recycling of *Spirulina*-based Algal-Silica Prepared with Added Aluminum**

Spirulina-based polymers made in the presence of aluminum ion were used to treat a simulated rinse water containing approximately 110 ppm of copper. The simulated solution was prepared by diluting an acidic (sulfuric acid) peroxide etch bath obtained from an electroplating operation. The polymers were adjusted to pH 5.0 with 0.1 M sodium acetate and rinsed with deionized water prior to use. The pH of the simulated rinse water was 5.0. Bound copper was eluted with 0.3 M Sulfuric acid. Column bed volumes were 3 mL, and all flow rates were 1 mL/min.

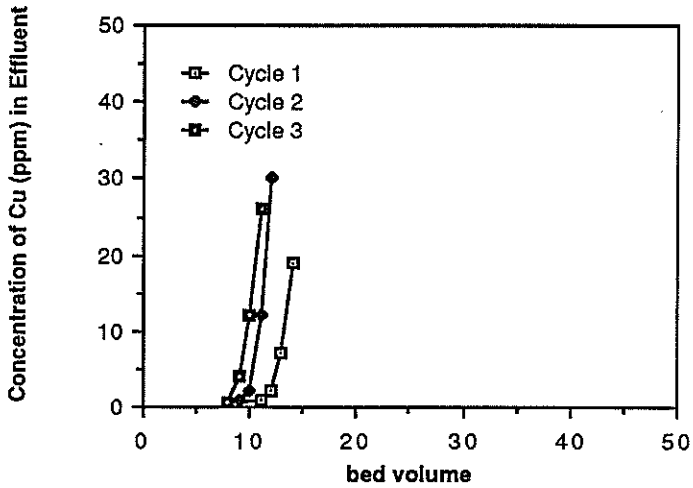


Figure 20: **Recycling of *Spirulina*-based Algal-Silica Prepared in Absence of Added Aluminum**

Spirulina-based polymers made in the absence of aluminum ion were used to treat a simulated rinse water containing approximately 110 ppm of copper. The simulated solution was prepared by diluting an acidic (sulfuric acid) peroxide etch bath obtained from an electroplating operation. The polymers were adjusted to pH 5.0 with 0.1 M sodium acetate and rinsed with deionized water prior to use. The pH of the simulated rinse water was 5.0. Bound copper was eluted with 0.3 M sulfuric acid. Column bed volumes were 3 mL, and all flow rates were 1 mL/min.

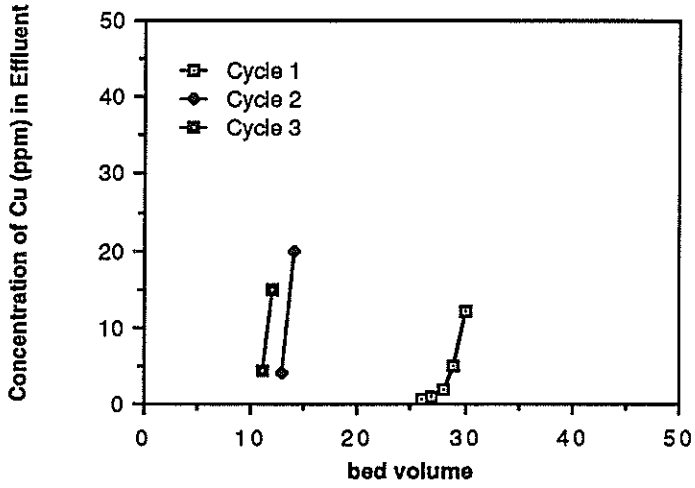


Figure 21: **Recycling of *Chlorella*-based Algal-Silica Prepared with Added Aluminum**

Chlorella-based polymers prepared in the presence of added aluminum ion were used to remove copper from a simulated rinse water. The rinse solution was prepared by diluting an acidic (sulfuric acid) peroxide etch solution to approximately 110 ppm. The pH of the solution was 5.0. The polymer was adjusted to pH 5.0 with 0.1 M sodium acetate then rinsed with deionized water. Bound copper was eluted with 0.3 M sulfuric acid. Column bed volumes were 3 mL, and flow rates were 1 mL/min.

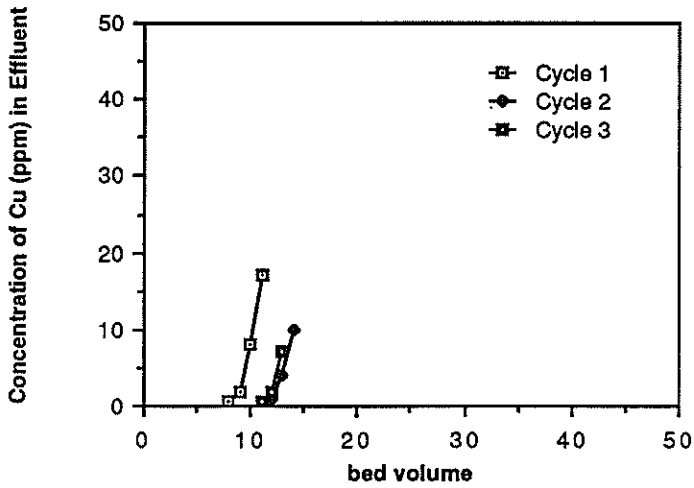


Figure 22: **Recycling of *Chlorella*-based Algal-Silica Prepared in Absence of Added Aluminum**

Chlorella-based polymers prepared in the absence of added aluminum ion were used to remove copper from a simulated rinse water. The rinse solution was prepared by diluting an acidic (sulfuric acid) peroxide etch solution to approximately 110 ppm. The pH of the solution was 5.0. The polymer was adjusted to pH 5.0 with 0.1 M sodium acetate then rinsed with deionized water. Bound copper was eluted with 0.3 M Sulfuric acid. Column bed volumes were 3 mL, and flow rates were 1 mL/min.

Effects of Algal Culturing Conditions

While algal cells are killed or inactivated in the process of either immobilizing or drying, the metal binding characteristics of the resultant biomass seem to be dependent upon the manner in which the cells were cultured. It is well known that culture conditions affect the chemical composition of algal cells (Naes and Post 1988). Variations in nitrogen concentration, addition or deletion of nutrients, and the addition of toxic or subtoxic concentrations of heavy metal ions can affect the cell composition (Aliotta and Pollio 1982). Specific enzyme systems can be repressed, derepressed, or induced depending on the environment of the culture. Metal binding properties may be influenced strongly by growth conditions for a particular biomass. Thus, the purpose of the experiments in this section was to examine the effects of various algal culture medium modifications on the metal-ion binding affinities of the resultant biomass. Evaluated were: (A) the effect of different nitrogen concentrations and (B) the influence of varying copper levels. Metal-ion binding properties of the resultant biomasses were examined and the results of these studies are presented.

(A) Influence of Nitrogen Level in Algal Culture. The objective of this experiment was to determine whether changes in the fixed nitrogen concentration of the culture medium would alter the metal-binding characteristics of the resultant biomass. Studies were performed with both *Cyanidium caldarium* (employing NH_4Cl as fixed nitrogen source) and *Spirulina platensis* (using $NaNO_3$ as fixed nitrogen source).

Spirulina platensis was grown at five different nitrogen levels. These levels corresponded to: 50%, 75%, 100%, 125% and 150% of that found in the normal growth medium formulation for the alga. The biomass was collected by centrifugation and tested for copper(II) and gold(III) binding capacities. Determination of copper(II) binding capacity was made at initial copper(II) concentrations of both 0.5 ppm and 100 ppm, to identify changes in populations of high-affinity and low-affinity sites, respectively. The results of these studies with three different harvests of *Spirulina* are displayed in Figures 23 and 24. Determination of gold(III) binding capacities was conducted at initial gold(III) concentrations of both 0.8 ppm and 130 ppm, to estimate variations in populations of high-affinity and low-affinity binding sites, respectively. The results of the experiments carried out with three different *Spirulina* harvests are shown in Figures 25 and 26. These experiments clearly show that changes in relative nitrogen concentration had no significant effect on copper(II) or gold(III) binding properties.

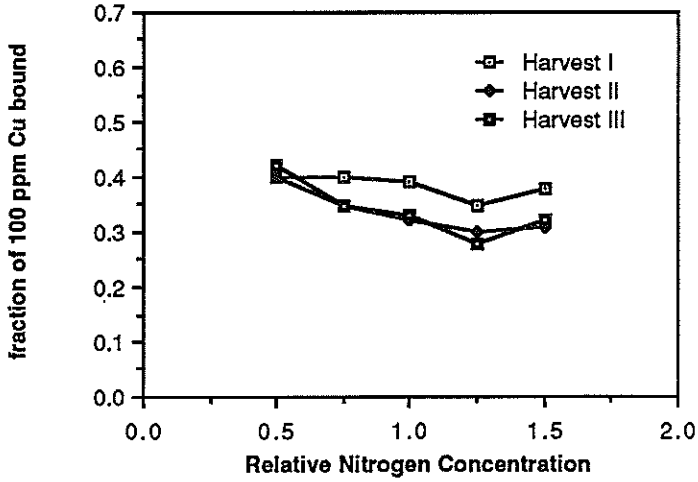


Figure 23: **Effect of Cell Culture Nitrogen Level on Low-Affinity Copper(II) Binding Sites of *S. platensis***

Spirulina platensis was grown at five different levels of fixed nitrogen supplied as sodium nitrate. The resultant biomass was resuspended at 5.0 mg/mL in 0.05 M sodium acetate at pH 5.0 containing 100 ppm copper(II). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual copper(II). Three different algal harvests were examined.

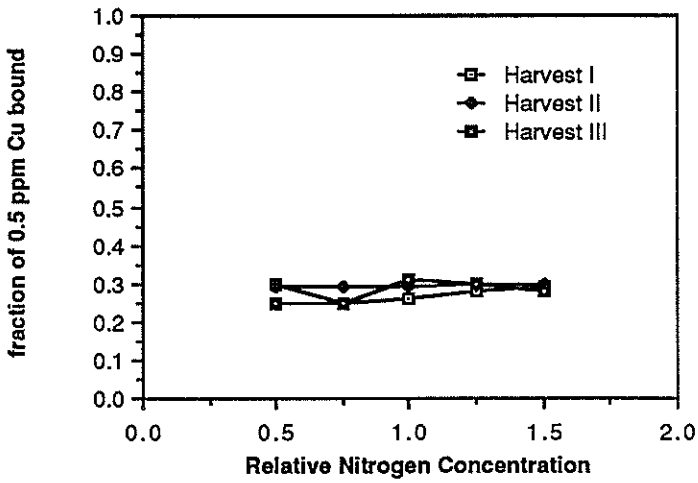


Figure 24: **Effect of Cell Culture Nitrogen Level on High-Affinity Copper(II) Binding Sites of *S. platensis***

Spirulina platensis was grown at five different levels of fixed nitrogen supplied as sodium nitrate. The resultant biomass was resuspended at 0.08 mg/mL, in 0.05 M sodium acetate at pH 5.0 containing 0.5 ppm copper(II). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual copper(II). Three different algal harvests were examined.

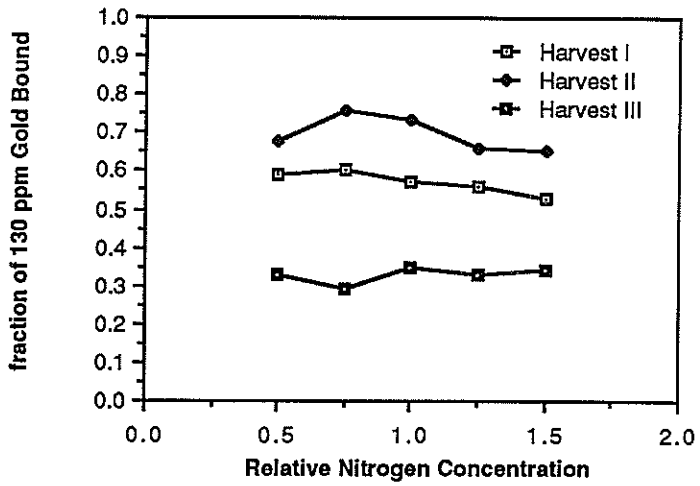


Figure 25: **Effect of Cell Culture Nitrogen Level on Low-Affinity Gold(III) Binding Sites of *S. platensis***

Spirulina platensis was grown at five different levels of fixed nitrogen supplied as sodium nitrate. The resultant biomass was resuspended at 1.0 mg/mL, in 0.01 M HCl at pH 2.0 containing 130 ppm of gold (tetrachloroaurate). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual gold(III). Three different algal harvests were examined.

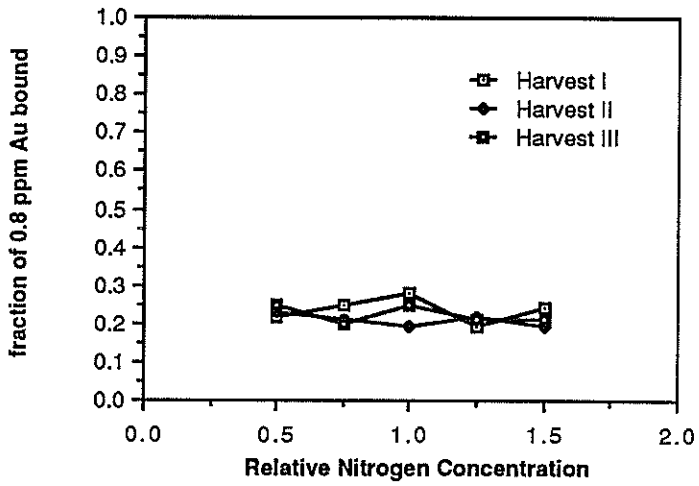


Figure 26: **Effect of Cell Culture Nitrogen Level on High-Affinity Gold(III) Binding Sites of *S. platensis***

Spirulina platensis was grown at five different levels of fixed nitrogen supplied as sodium nitrate. The resultant biomass was resuspended at 0.02 mg/mL in 0.01 M HCl at pH 2.0 containing 0.8 ppm of gold (tetrachloroaurate). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual gold(III). Three different algal harvests were examined.

Cyanidium caldarium was grown at eight different nitrogen concentrations. These concentrations represented: 50%, 75%, 100%, 125%, 150%, 175%, 200%, and 250% of that found in the normal growth medium formulation for the alga. The biomass was collected by centrifugation and tested for copper(II) and gold(III) binding capacities. Determination of copper(II) and gold(III) binding capacities of *Cyanidium* biomass were performed under conditions similar to those described previously for *Spirulina*. Figure 27 exhibits the results for low-affinity copper(II) binding from three different harvests of *Cyanidium*. Figure 28 shows the effect on high-affinity copper(II) binding sites of varying nitrogen levels during growth of *Cyanidium*. In Figures 29 and 30 the results of experiments monitoring both low and high-affinity gold(III) binding sites, respectively are shown for three different *Cyanidium* harvests. From these results, there is no clear effect on copper(II) binding at either high- or low-affinity binding sites, when relative nitrogen levels are increased. However, the affinity for gold(III) seems to increase with increasing nitrogen levels at the high-affinity binding sites.

Experiments were conducted to determine the effect of culture time on high- and low-affinity copper(II) and gold(III) binding sites. Samples of biomass grown in medium containing the same levels of nitrogen discussed in the above study were harvested after five, eight and fifteen days and tested for both high- and low-level copper(II) and gold(III) binding. The results of these experiments (not shown) indicated no systematic variation in gold(III) or copper(II) binding properties could be attributed to the length of time that cells were cultured. Thus, the length of exposure of *Cyanidium* cells to elevated nitrogen levels does not have an effect on the metal-binding properties of the resultant biomass.

Growth curves obtained with *Cyanidium caldarium* at various fixed nitrogen levels were examined. These results are presented in Figure 31. All cultures exhibited identical doubling times, at low-cell densities. However, higher nitrogen levels support growth to significantly higher densities. Maximum cell numbers are observed in the culture containing 1.5 times the standard concentration of NH_4Cl. Slightly lower densities were observed in the cultures containing still higher concentrations of fixed nitrogen. This decrease in cell density may reflect the onset of ammonia toxicity.

(B) Copper(II) Levels in Culture and their Influence on Copper Binding. It is known that less than 1 ppm of copper ion is extremely toxic to most microorganisms (Bowen 1966). At least one exception is known to exist. The red alga, *Cyanidium caldarium* is known to grow in up to 4000 ppm of copper ions (Aliotta and Pollio 1982). Thus, experiments were undertaken to determine whether elevated levels of copper(II) during growth would increase the copper(II) binding capacity of the resultant biomass. *Cyanidium caldarium* was grown in medium containing different concentrations of copper ion. After five days of growth, the cells were harvested

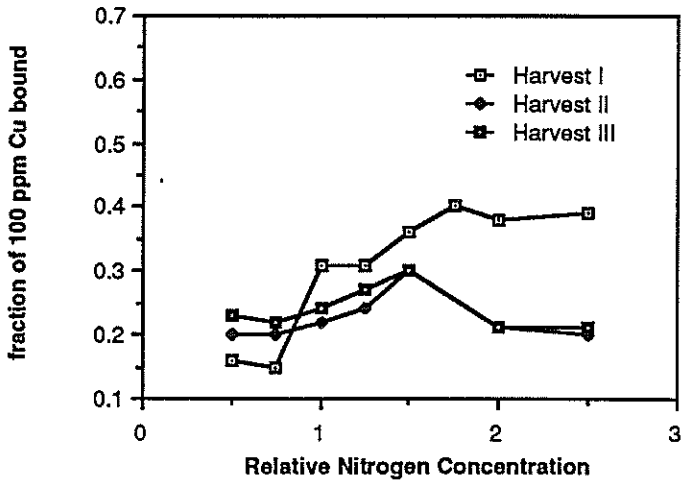


Figure 27: **Effect of Cell Culture Nitrogen Level on Low-Affinity Copper(II) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown at five different levels of fixed nitrogen supplied as ammonium chloride. The resultant biomass was resuspended, at 5.0 mg/mL in 0.05 M sodium acetate at pH 5.0 containing 100 ppm copper(II). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual copper(II). Three different algal harvests were examined.

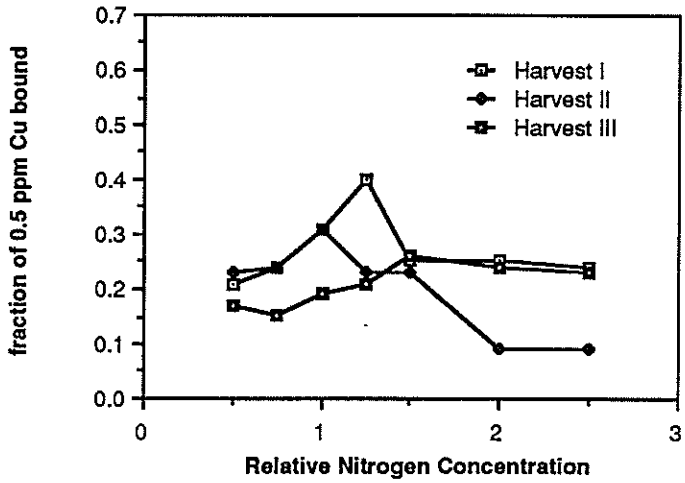


Figure 28: **Effect of Cell Culture Nitrogen Level on High-Affinity Copper(II) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown at five different levels of fixed nitrogen supplied as ammonium chloride. The resultant biomass was resuspended, at 0.08 mg/mL in 0.05 M sodium acetate at pH 5.0 containing 0.5 ppm cooper(II). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual copper(II). Three different algal harvests were examined.

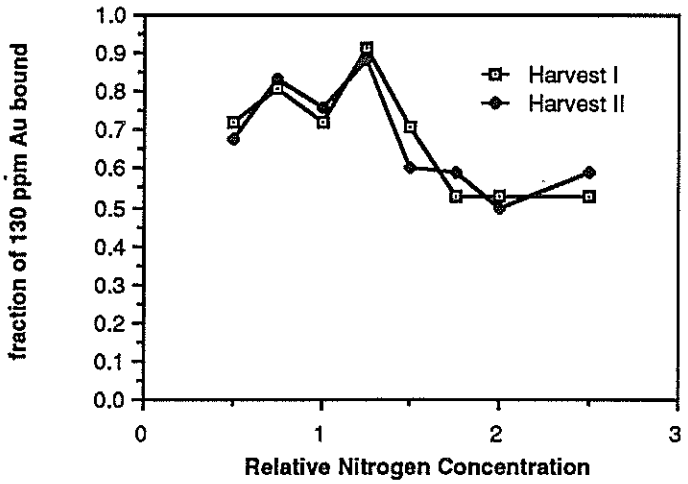


Figure 29: **Effect of Cell Culture Nitrogen Level on Low-Affinity Gold(III) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown at five different levels of fixed nitrogen supplied as ammonium chloride. The resultant biomass was resuspended, at 1.0 mg/mL in 0.01 M HCl at pH 2.0 containing 130 ppm of gold (tetrachloroaurate). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual gold(III). Three different algal harvests were examined.

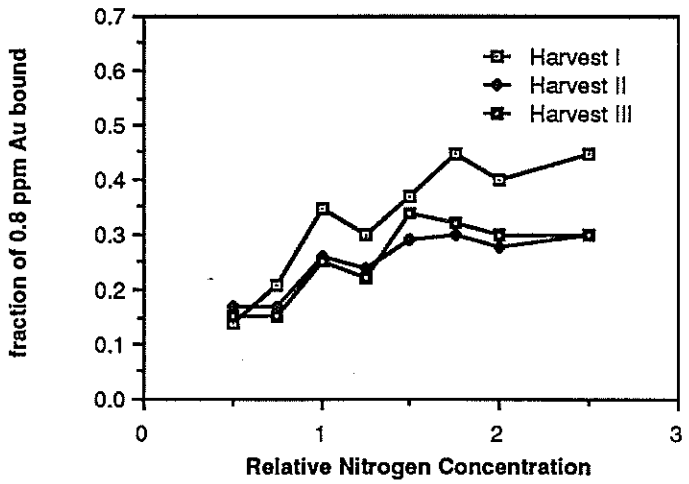


Figure 30: **Effect of Cell Culture Nitrogen Level on High-Affinity Gold(III) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown at five different levels of fixed nitrogen supplied as ammonium chloride. The resultant biomass was resuspended at 0.2 mg/mL in 0.01 M HCl at pH 2.0 containing 0.8 ppm of gold (tetrachloroaurate). After thirty minutes, the samples were centrifuged and the supernatant solutions were analyzed for residual gold(III). Three different algal harvests were examined.

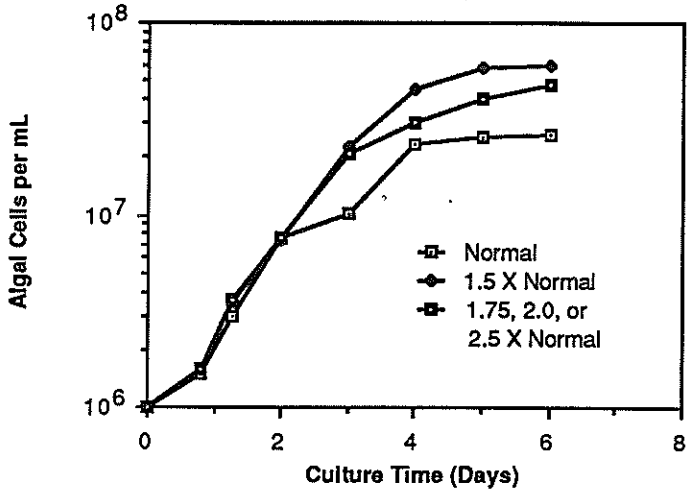


Figure 31: **Growth Rate of *Cyanidium caldarium* Cultured at Different Cell Culture Nitrogen Levels**

Cyanidium caldarium was grown at five different concentrations of fixed nitrogen either at the standard concentration (-■-) at 1.5 times the standard concentration (-◆-), or at 1.75, 2.0, or 2.5 times the standard concentration (-■-). The cultures were maintained at 45°C at pH 2.6 and were sparged continuously with 6% CO_2 in air. Illumination of 650 foot candles was provided by a mixture of cool-white, red, and blue fluorescent lamps. Cell numbers were measured at the times indicated with a hemocytometer or Klett spectrophotometer.

and lyophilized. Experiments to determine the copper(II) binding properties of the biomass were performed at initial copper(II) concentrations of 0.5 ppm and 100 ppm to identify changes in populations of high-affinity and low-affinity sites, respectively. Results of monitoring high-affinity binding sites are shown in Figure 32, while Figure 33 displays data indicative of low-affinity sites. The results of these experiments suggest strongly that exposure to levels of up to 2000 ppm copper(II) during growth, causes increased expression of high-affinity copper ion binding sites in *Cyanidium* biomass. Copper binding at low-affinity sites seems to increase in biomass from cells cultured at copper concentrations of up to 200 or 300 ppm. At growth medium copper levels higher than 300 ppm, the production of low-affinity copper binding sites seems to be inhibited.

In addition to copper-binding capacity, it was decided to examine what relationship, if any, might exist between the growth rate of algal cells and the concentration of copper ion in the culture medium. Growth curves obtained for *Cyanidium* cultured at various copper concentrations are shown in Figure 34. Copper ion concentrations of 50 to 300 ppm caused a slight depression in growth rate as compared to the control, and when grown in severely high concentrations of copper (2000 ppm), the alga's growth rate is extremely depressed. The slight depression in growth rate is accompanied by an increase in low-affinity copper binding capacity in the resultant biomass. Severe growth rate depression is accompanied by a decrease in high-affinity binding capacity. It is interesting to note, however, that weak site inhibition occurs at much lower growth medium copper concentrations (300 ppm) and strong site enhancement is evident at much higher growth medium copper concentrations (2000 ppm). This difference in behavior suggests that there are major differences in the chemical nature and biological function of weak and strong copper binding sites.

Mechanism of Binding of Metal ions to Different Algae

Algal biomass have high-affinity binding sites for hard metal ions such as aluminum(III) and for soft metal ions such as silver(I) and gold(III). It binds cations as well as anions, depending on pH and other conditions. To use algal biomass most effectively for water purification and metal reclamation, it is important to understand the chemical nature of the metal binding process. Therefore, it is necessary to determine which chemical groups on the cell wall are responsible for binding different metal ions. One approach to gaining this information is chemical modification of the metal binding functional groups on the algal surface. For example, if the binding of copper(II) occurs through interaction with carboxyl groups, then chemical modification which renders the carboxyl groups unavailable for metal complexation should cause a marked reduction in copper(II) binding. Therefore, experiments were undertaken to modify the various algal chemical functional groups of five different algal species. The func

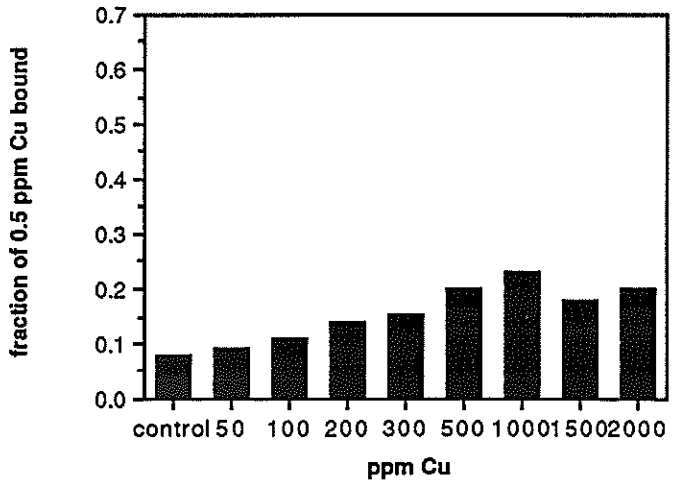


Figure 32: **Effect of Cell Culture Copper(II) Level on High-Affinity Copper(II) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown in copper concentrations of 50, 100, 200, 300, 500, 1,000, 1,500, and 2,000 ppm copper(II), while otherwise maintaining the standard growth media and conditions described in figure 31. Copper(II) binding experiments were preceded by two washes in 0.3 M sulfuric acid and one wash in distilled-deionized water. The alga was suspended at a concentration of 0.08 mg/mL in 0.5 ppm Cu in 0.05 M sodium acetate at pH 5.0. The suspension was allowed to equilibrate for thirty minutes at room temperature. After equilibration, the samples were centrifuged in a Beckman J2-21 centrifuge using a JA-10 rotor at 8,500 rpm (11,000 x g) for twenty minutes. The supernatant fractions were analyzed for copper(II) by flame atomic absorption at 324.8 nm.

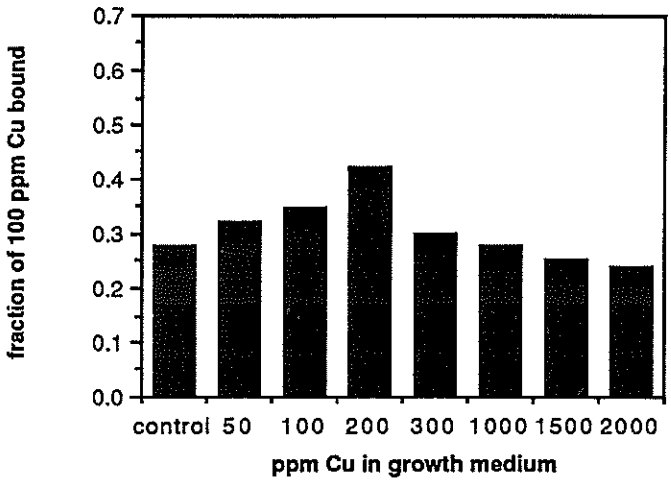


Figure 33: **Effect of Cell Culture Copper(II) Level on Low-Affinity Copper(II) Binding Sites of *C. caldarium***

Cyanidium caldarium was grown in copper concentrations of 50, 100, 200, 300, 500, 1,000, 1,500, and 2,000 ppm copper(II), while otherwise maintaining the standard growth media and conditions described in figure 31. Copper(II) binding experiments were preceded by two washes in 0.3 M sulfuric acid and one wash in distilled-deionized water. The alga was suspended at a concentration of 5.0 mg/mL in 100 ppm copper(II) in 0.05 M sodium acetate at pH 5.0. The suspension was allowed to equilibrate for thirty minutes at room temperature. After equilibration, the samples were centrifuged in a Beckman J2-21 centrifuge using a JA-21

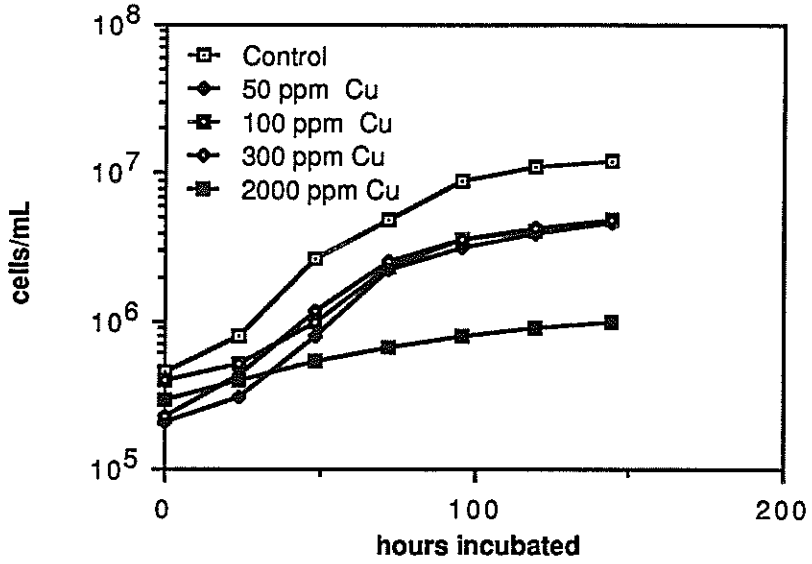
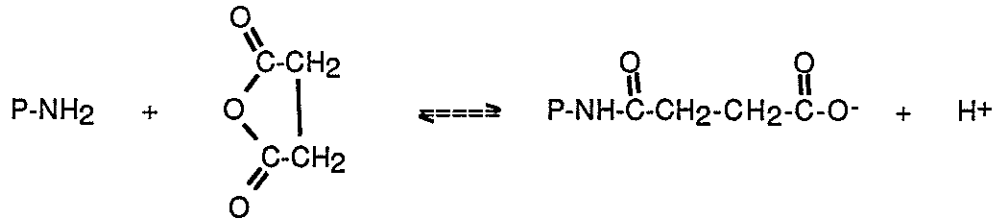


Figure 34: **Growth Rate Curves for *Cyanidium caldarium* Grown Under Copper Stress**

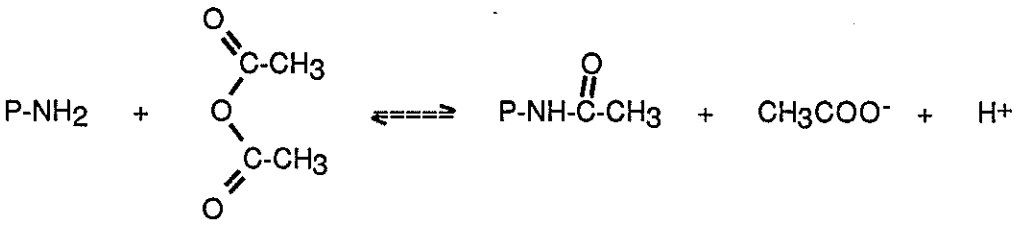
Cyanidium caldarium was grown in copper concentrations of 50, 100, 300, and 2,000 ppm copper(II), while otherwise maintaining the standard growth media and conditions described in figure 31.

tional groups targeted for modification on the algal cells were: (A) amino groups, (B) carboxyl groups, and (C) sulfhydryl groups. The chemically modified algal biomasses were examined for metal-ion binding and the results of these experiments are presented in this section.

(A) Chemical Modification of Algal Amino Groups. Succinic anhydride will react with nucleophiles, primarily amino groups according to the chemical reaction:



Also, acetic anhydride will react with amino groups according to the chemical reaction:



Thus, to modify algal amino groups, dried cells of different algal species were reacted with acetic and succinic anhydride.

To determine the extent to which samples of algal biomass were modified by treatment with either acetic or succinic anhydride, the ninhydrin reaction was employed. This reaction is widely used in the quantitation of amino groups. Upon heating, an amino group reacts with two molecules of ninhydrin to produce an intensely colored product that can be assayed spectrophotometrically. Several samples of modified algal biomass were reacted with ninhydrin at 100°C as described in the Methodology section. The results, displayed in Figures 35 and 36, are presented as the fractional decrease in reactivity with ninhydrin relative to control samples of unmodified biomass. With the exception of the *Laminaria japonica* (known commercially as "Kelp") biomass, treatment with either acetic or succinic anhydride results in substantial conversion of free amino groups. In every instance, a higher degree of modification was achieved with succinic anhydride. Exposure of *Laminaria* to either succinic or acetic anhydride

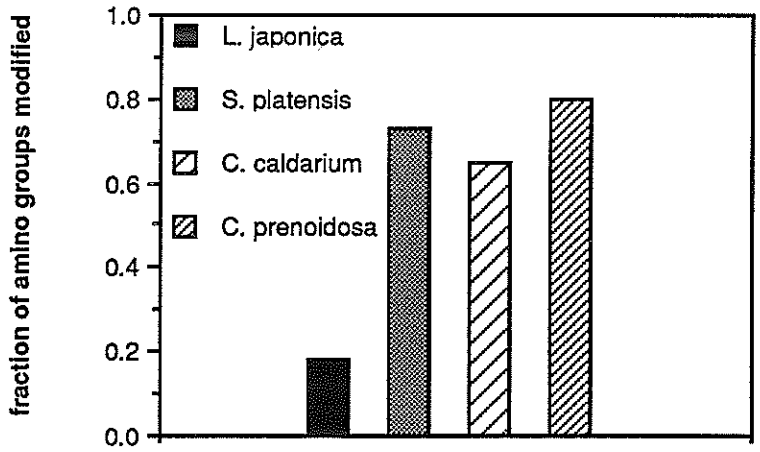


Figure 35: **Chemical Modification of Amino Groups by Succinic Anhydride Monitored By the Ninhydrin Reaction**

Laminaria japonica, Spirulina platensis, Cyanidium caldarium, and *Chlorella pyrenoidosa* were modified with succinic anhydride by washing 4.0 g of dried cells in 0.1 M sulfuric acid, then with 1.0 M sodium bicarbonate. The biomass was resuspended in 1.0 M sodium bicarbonate and maintained at a pH of 8.0 to 8.3 with NaOH. Six, four-gram additions of succinic anhydride were made at fifteen minute intervals. The algae were washed again in 0.1 M sulfuric acid and then with deionized water. After washing, the biomass was lyophilized on a Labconco freeze-dryer. After the algae were completely dry, they were resuspended in distilled-deionized water at 1 mg/mL. One mL of tin chloride reagent (0.17g tin chloride dihydrate in 100 mL of 5.8 M acetic acid at pH 5.5) and 1.0 mL of ninhydrin reagent (4% (w/v) ninhydrin in methyl cellosolve) were added to 1 mL of the algal suspension and the reaction was allowed to take place in capped 16 mL culture tubes for twenty minutes in a boiling water bath. After the incubation period, 5.0 mL of 50% (v/v) ethanol was added to each sample to quench the reaction. Upon cooling to room temperature, the samples were centrifuged and the absorbance of the supernatant fraction of each sample was measured spectrophotometrically at 570 nm. The difference in absorbance between the modified and the control samples was divided by the absorbance of the control sample to calculate the fraction of total amino groups modified.

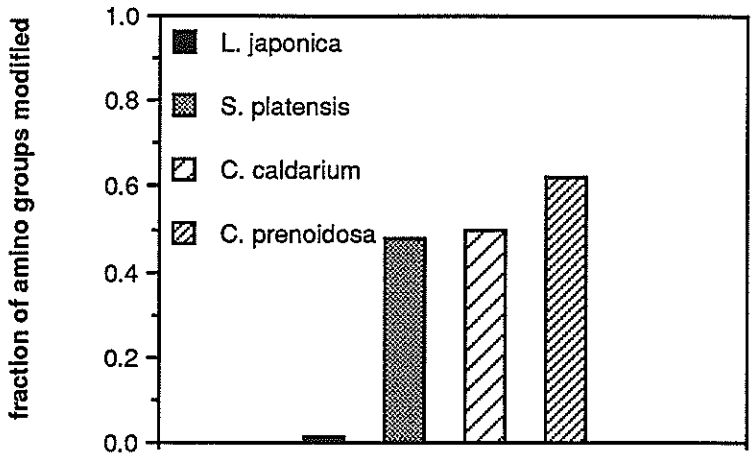


Figure 36: **Chemical Modification of Amino Groups by Acetic Anhydride Monitored By the Ninhydrin Reaction**

Laminaria japonica, Spirulina platensis, Cyanidium caldarium, and *Chlorella pyrenoidosa* were modified with acetic anhydride by washing 4.0 g of dried cells in 0.1 M sulfuric acid, then with 1.0 M sodium bicarbonate. The biomass was resuspended in 1.0 M sodium bicarbonate and maintained at a pH of 8.0 to 8.3 with NaOH. Six, four-gram additions of acetic anhydride were made at fifteen minute intervals. The algae were washed again in 0.1 M sulfuric acid and then with deionized water. After washing, the biomass was lyophilized on a Labconco freeze-dryer. After the algae were completely dry, they were resuspended in distilled-deionized water at 1 mg/mL. One mL of tin chloride reagent (0.17g tin chloride dihydrate in 100 mL of 5.8 M acetic acid at pH 5.5) and 1.0 mL of ninhydrin reagent (4% (w/v) ninhydrin in methyl cellosolve) were added to 1 mL of the algal suspension and the reaction was allowed to take place in capped 16 mL culture tubes for twenty minutes in a boiling water bath. After the incubation period, 5.0 mL of 50% (v/v) ethanol was added to each sample to quench the reaction. Upon cooling to room temperature, the samples were centrifuged and the absorbance of the supernatant fraction of each sample was measured spectrophotometrically at 570 nm. The difference in absorbance between the modified and the control samples was divided by the absorbance of the control sample to calculate the fraction of total amino groups modified.

results in little or no modification. This was expected since the cell wall of *Laminaria* contains very few amino groups (Bird and Haas 1931; Siegel and Siegel 1973).

Experiments were then conducted to determine whether the modification of amino groups had altered the metal-ion binding properties of the algal cells. Results for samples modified with succinic anhydride are presented first. The relative levels of copper(II) binding observed with modified and control biomass are displayed in Figure 37; the relative levels of gold binding for the same samples are displayed in Figure 38. At these high metal-ion concentrations (100 ppm copper(II) or 125 ppm of gold(III)), data should reflect differences in metal binding due to modification of low-affinity binding sites. Our results indicate that, with the exception of *Laminaria japonica*, treatment of algal biomass with succinic anhydride increases the binding capacity for copper ion, while decreasing the capacity for gold. Copper and gold ion binding experiments performed at high metal-ion concentrations with algal biomass modified with acetic anhydride are exhibited in Figures 39 and 40. For the most part, the results parallel those observed following succinic anhydride treatment. With the exception of *Laminaria*, treatment of algal biomass with acetic anhydride results in increased binding of copper ion and correspondingly diminished binding of gold(III). In contrast to the previous study, however, the modified sample of *Spirulina platensis* exhibits a slight increase in capacity for gold(III) relative to the control.

These results suggest that at higher concentrations (approximately 100 ppm), copper ion is bound more effectively to oxygen-containing groups (e.g. carboxyls), while gold(III) is more effectively coordinated by nitrogen-containing functional groups, since anhydride treatment results in the replacement of amino groups with carboxyl or acetyl moieties. In contrast to the other biomass samples, modification of *Laminaria* with either acetic or succinic anhydride has little effect on the material's binding capacities for gold(III) and copper(II). The relatively low content of accessible amino groups of this algal biomass indicated in Figures 35 and 36 may account for this behavior. The intrinsically low capacity of *Laminaria* for gold ion is certainly consistent with this hypothesis. If in fact, the concentration of amino groups is low, then treatment with the anhydrides would be expected to cause at most, a modest alteration of binding properties. The results of ninhydrin assays (see Figures 35 and 36) correlate well with ion-binding results. High levels of modification, as determined by the ninhydrin reaction, were accompanied by large changes in the copper(II) and gold(III) binding capacities. Conversely, the ninhydrin assay indicated relatively little modification of *Laminaria* biomass, consistent with the insignificant changes in copper(II) and gold(III) binding properties.

The effect of modification of algal amino groups with either succinic or acetic anhydride on high-affinity metal-ion binding sites was also investigated. Modified and control samples of biomass were equilibrated with solutions containing either 0.5 ppm copper(II) or 0.8 ppm

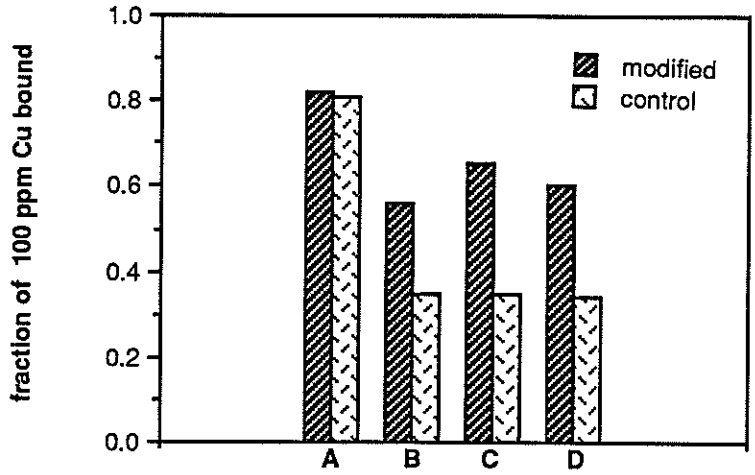


Figure 37: **Effect of Succinic Anhydride Modification on Low-Affinity Copper(II) Binding of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Four grams of the succinic anhydride modified, lyophilized algal biomass was suspended at 5.0 mg/mL in 100 ppm copper(II) in 0.05 M sodium acetate at pH 5.0. Following a thirty minute equilibration period, the suspension was centrifuged, and the supernatant was analyzed for residual copper by flame atomic absorption at 324.8 nm. Control samples which were not modified with succinic anhydride, were treated identically.

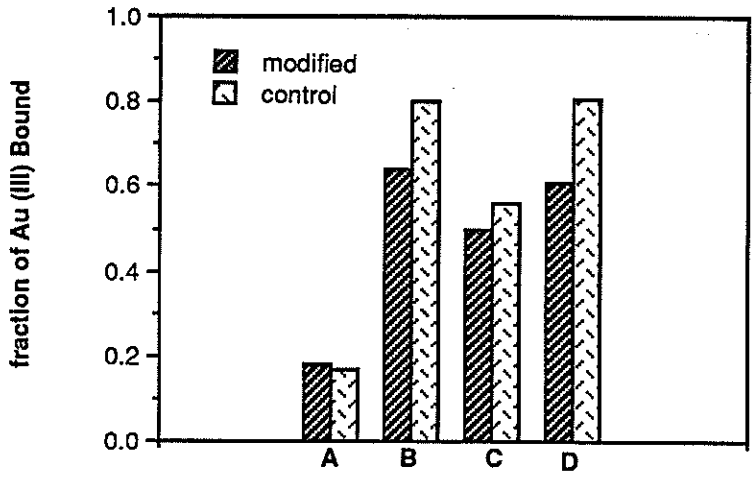


Figure 38: **Effect of Succinic Anhydride Modification on Low-Affinity Gold(III) Binding of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *.Cyanidium caldarium,* and (D.) *Chlorella pyrenoidosa***

Four grams of succinic anhydride modified, lyophilized algal biomass was suspended at 1.0 mg/mL in 130 ppm of gold (tetrachloroaurate) in 0.01 M HCl at pH 2.0. After an equilibration period of thirty minutes, the suspensions were centrifuged and the supernatants were analyzed for residual gold by flame atomic absorption at 242.8 nm Control samples which were not modified with succinic anhydride, were treated identically

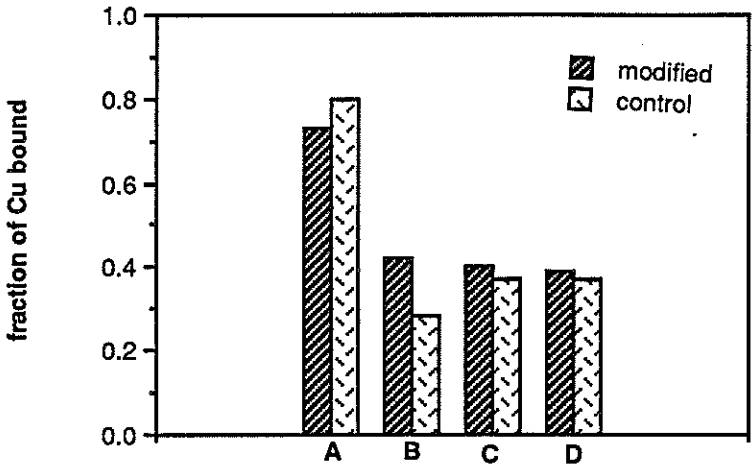


Figure 39: **Effect of Acetic Anhydride Modification on Low-Affinity Copper(II) Binding of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Washed algal biomass (3.2g) was resuspended in 120 mL of buffer (0.1 M sodium phosphate/1.0 M sodium acetate) at pH 7.2. Sixteen mL of acetic anhydride was then added, and the sample was stirred continuously for 1 hour, while maintaining the pH at 7.2 by addition of NaOH. After centrifugation, the pellet was washed, and centrifuged, sequentially with deionized water, 1 M hydroxylamine, 0.1 M H_2SO_4, and deionized water. The final water washed pellet was then lyophilized. For binding experiments, samples of the lyophilized biomass were resuspended, at 5.0 mg/mL, in 100 ppm copper(II) in 0.05 M sodium acetate at pH 5.0. After a thirty minute contact period, the suspensions was centrifuged, and the supernatant fraction was analyzed for copper by flame atomic absorption at 324.8 nm. Control samples which were not modified with acetic anhydride, were treated identically.

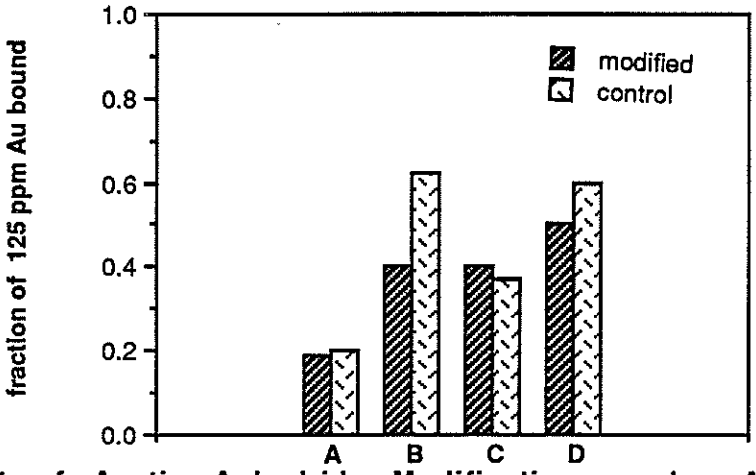


Figure 40: **Effect of Acetic Anhydride Modification on Low-Affinity Gold(III) Binding of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Algae were modified as described in figure 36. The washed, freeze-dried material was then adjusted to pH 2.0 with HCl and resuspended, at 1.0 mg/mL in 125 ppm of gold (tetrachloroaurate) in 0.01 M HCl at pH 2.0. After a thirty minute equilibration period, the suspensions were centrifuged, and the supernatants were analyzed for gold(III) by flame atomic absorption at 242.8 nm Control samples which were not modified with acetic anhydride, were treated identically.

gold(III). Figures 41 and 42 compare the uptake of copper(II) and gold(III) by samples of succinic-anhydride modified and unmodified algal biomass. Figures 43 and 44 present the corresponding data for samples of biomass modified with acetic anhydride.

Metal ions and their ligands are commonly classified as "hard" or "soft" Lewis acids and bases, (Pearson 1963). Hard acids have the greatest affinity for hard bases, while soft acids have the greatest affinity for soft bases. In this scheme, gold(III) is classified a soft acid and is expected to coordinate preferentially to sulfur-containing ligands. If sulfur containing ligands are responsible for strong site gold binding, then anhydride treatment which modifies amino groups, would produce no significant decrease in binding of gold(III) at low levels of the ion. Data shown in Figures 42 and 44 indicate that anhydride modification has very little impact on gold(III) uptake by algal biomass.

(B) Chemical Modification of Algal Carboxyl Groups. Two popular means of carboxyl group modification are the use of water soluble carbodiimides and esterification via acidic methanol (Lundblad and Noyes 1984). While using water soluble carbodiimides is advantageous because of its high degree of specificity, it is unattractive due to its high cost, both in reagents and equipment required to complete the modification. In addition, this method does not provide a practical way of monitoring the degree of modification. Use of acidic methanol to esterify carboxyl groups, on the other hand, is relatively inexpensive. In addition, base hydrolysis of the modified sample, and analysis of the released methanol allow quantification of the modification reaction.

To modify carboxyl groups with acidic methanol, an adaptation of the procedure used by Wilcox (1972) was utilized. The basic chemical reaction is shown below:

$$A\text{-}\overset{\overset{O}{\|}}{C}\text{-}OH \quad + \quad CH_3OH \quad \xrightarrow{\text{dil HCL}} \quad A\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}CH_3 \quad + \quad H_2O$$

Esterification was achieved by reacting five different algal biomasses, (*Cyanidium caldarium, Spirulina platensis, Eisenia bicyclis, Laminaria japonica and Chlorella pyrenoidosa)*, with acidic methanol for periods of 6, 12, 24, or 48 hours at room temperature. Following extensive dialysis to eliminate excess methanol, the samples were lyophilized, and experiments were conducted on the freeze-dried material. The degree of esterification was monitored by two means: first, by examining the modified algal biomass' copper(II) binding ability at intermediate levels of copper(II) at pH 5.0 and pH 2.0; and second, by base hydrolysis of the modified biomass and subsequent analysis of the released methanol by gas chromatography.

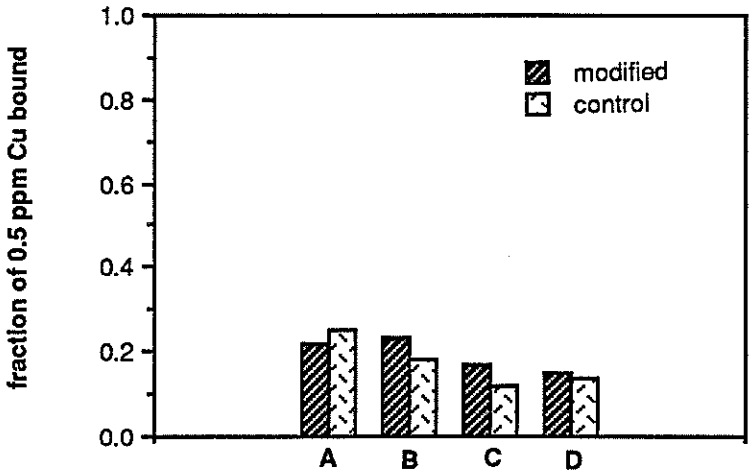


Figure 41: **Effect of Succinic Anhydride Modification on High-Affinity Copper(II) Binding Sites of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Algae were modified with succinic anhydride as described earlier. The freeze-dried material was washed, then resuspended at 0.08 mg/mL in 0.5 ppm copper(II) in 0.05 M sodium acetate at pH 5.0. After thirty minutes, the samples were centrifuged, and the supernatant solutions were analyzed for residual copper(II).

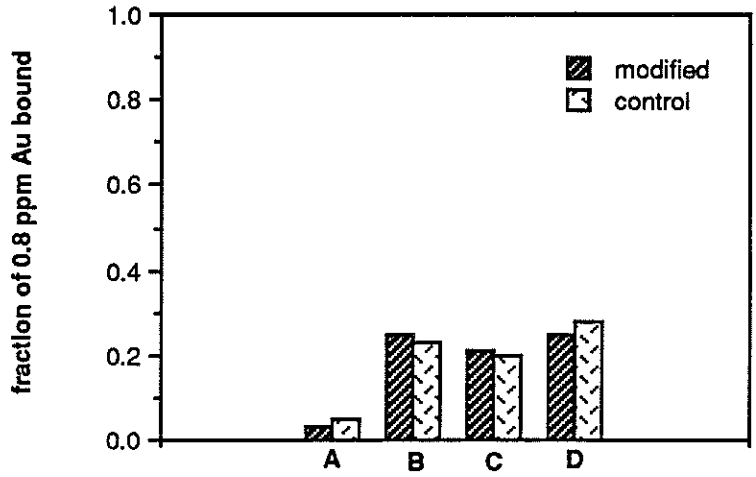


Figure 42: **Effect of Succinic Anhydride Modification on High-Affinity Gold(III) Binding Sites of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Algae were modified with succinic anhydride as described earlier. The resulting washed biomass was resuspended at 0.02 mg/mL in 0.8 ppm of gold (tetrachloroaurate) at pH 2.0. Following a thirty minute equilibration period, the samples were centrifuged and the supernatants were analyzed for gold(III) by flame atomic absorption at 242.8 nm.

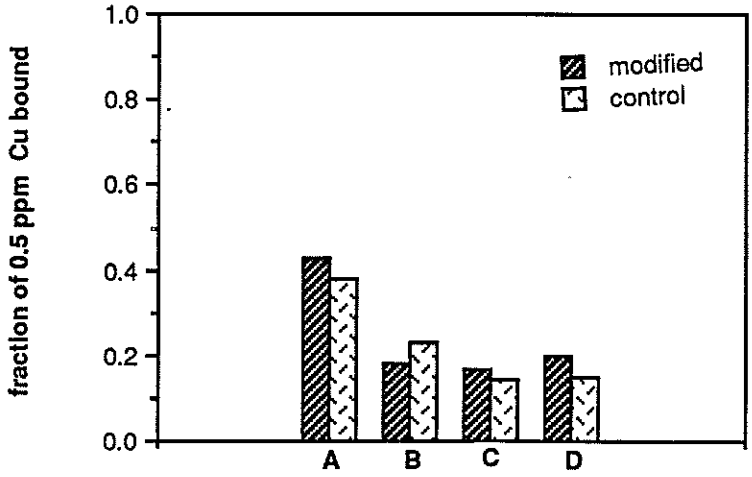


Figure 43: **Effect of Acetic Anhydride Modification on High-Affinity Copper(II) Binding Sites of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Algal biomass was modified with acetic anhydride as described earlier. The freeze-dried material was washed, then resuspended at 0.08 mg/mL in 0.5 ppm copper(II) in 0.05 M sodium acetate at pH 5.0. After thirty minutes, the samples were centrifuged, and the supernatant solutions were analyzed for residual copper(II).

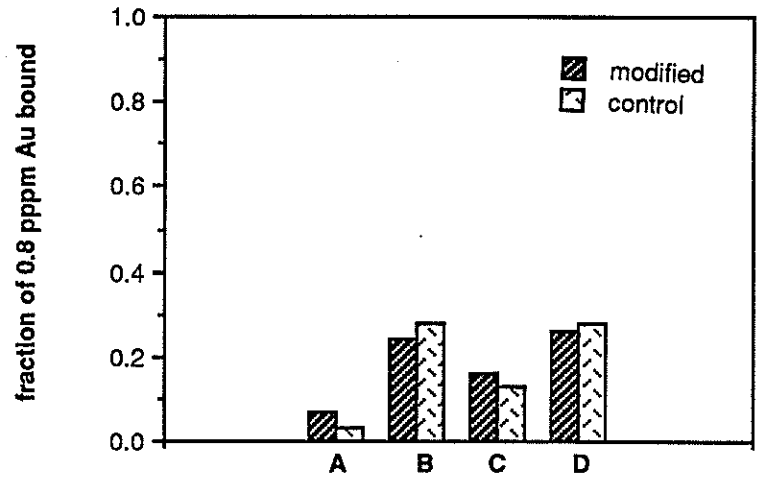


Figure 44: **Effect of Acetic Anhydride Modification on High-Affinity Gold(III) Binding Sites of (A). *Laminaria japonica,* (B). *Spirulina platensis,* (C). *Cyanidium caldarium,* and (D). *Chlorella pyrenoidosa***

Algal biomass was modified with acetic anhydride as described earlier. The resulting washed biomass was resuspended at 0.02 mg/mL in 0.8 ppm of gold (tetrachloroaurate) at pH 2.0. Following a thirty minute equilibration period, the samples were centrifuged and the supernatants were analyzed for gold(III) by flame atomic absorption at 242.8 nm.

Experiments were conducted on the methanol-modified biomass to determine whether modification had altered copper(II) binding characteristics. These studies were performed at pH 5.0 and pH 2.0 using an intermediate copper(II) concentration of 50 ppm. Figures 45A, 46A, 47A, 48A, and 49A display the results of binding copper(II) at pH 5.0, while Figures 45B, 46B, 47B, 48B, and 49B display the results of binding copper(II) at pH 2.0. At pH 5.0, uptake of copper(II) by the algal biomasses is decreased dramatically by incubation with methanol, and the loss of binding capacity is proportional to the length of the incubation period. The biomasses of *Cyanidium, Spirulina, Eisenia, Laminaria,* and *Chlorella* esterified for 48 hours exhibit a 46%, 57%, 27%, 40%, and 66% decrease in copper(II) binding, respectively. Other evidence (not shown) indicates that a substantial portion of the metal-ion uptake by algal biomass at pH 5.0 is the result of electrostatic interactions between the ions and carboxylate groups on the algal surface. A reduction in copper binding at pH 5.0 after esterification of algal biomass is clearly consistent with that hypothesis. However, the decrease in copper binding of esterified samples, as observed, is species-dependent. It has been observed previously (Darnall et al. 1986b) that copper(II) uptake by algal biomass is reduced significantly at pH 2.0, presumably because most carboxyl groups are protonated, and therefore uncharged, at this pH. It is likely that the residual copper(II) binding at pH 2.0 is occurring at groups other than carboxyls. This hypothesis is supported by esterification data in that esterification has little or no effect on the extent of copper ion binding at pH 2.0 by different algal species (Figures 45B, 46B, 47B, 48B, and 49B).

Base hydrolysis of modified algal biomass followed by gas chromatographic analysis of released methanol also was used to quantifiy the degree of biomass esterification. Samples of the methylated biomass, as well as an untreated control, were hydrolyzed with NaOH to release methanol. Following the hydrolysis, samples were neutralized by addition of citric acid, 2-propanol was added as an internal standard, and the samples were analyzed by gas chromatography. Representative gas chromatograms of hydrolysates of *Cyanidium caldarium* samples modified for 6, 12, 24, and 48 hours, as well as an unmodified control are presented in Figure 50. The methanol peak (first peak on Figure 50), which elutes with a retention time of 1.6 minutes, increases in peak area from the 6-hour sample to the 48-hour sample. No methanol was found in the hydrolysate of the untreated control (Figure 50). Methanol quantification was performed by comparison to appropriate standards. Table 1 displays the concentrations of methanol determined in hydrolysates of the various algal biomasses. Briefly stated, these results indicate a good correlation between the loss of copper(II) binding capacity and the amount of methanol released by hydrolysis. As copper(II) binding decreases the concentration of methanol found in the respective hydrolysates increases for all modified algal biomasses.

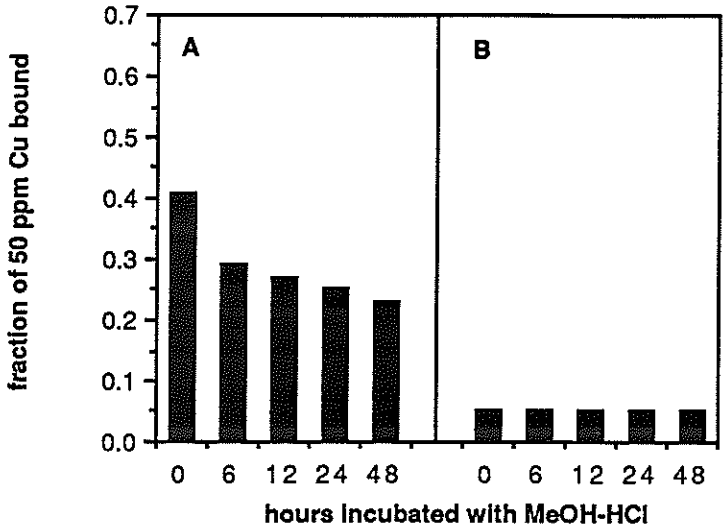


Figure 45: **(A). Effect of Acidic Methanol Modification of *Cyanidium caldarium* on Copper(II) Binding at pH 5.0**

Nine grams of lyophilized *C. caldarium* was suspended in 633 mL of 99.9% methanol and 5.4 mL of concentrated hydrochloric acid at room temperature with continuous agitation. Aliquots were removed from the stirred suspension after 6, 12, 24 and 48 hours. The reactions were quenched by the addition of a large volume of cold distilled-deionized water. The samples were dialyzed exhaustively against 0.001 M HCl, using Baxter dialysis bags, (6-8,000 d) at room temperature to remove unreacted methanol, then lyophilized to reduce volume. For binding experiments, samples of the modified and unmodified biomass were washed twice in 0.3 M sulfuric acid and once in distilled-deionized water. After washing, all samples were resuspended at 5.0 mg/mL in 0.05 M sodium acetate at pH 5.0. Copper(II) binding experiments with the algal biomass were performed by equilibrating the samples in 50 ppm copper(II) in 0.05 M sodium acetate at pH 5 for sixty minutes at room temperature. After the equilibration period, the suspension was centrifuged in a Sorvall GLC-2B benchtop centrifuge, at 2,500 rpm, for fifteen minutes at room temperature. The supernatant fraction was analyzed for copper by flame atomic absorption spectroscopy at 324.8 nm.

(B). Effect of Acidic Methanol Modification of *Cyanidium caldarium* on Copper(II) Binding at pH 2.0

C. caldarium was modified as previously described. Both modified and unmodified samples were treated identically. Samples were washed twice in 0.3 M sulfuric acid, once in distilled-deionized water and finally in 0.05 M sodium acetate at pH 2.0. Binding experiments were performed as discussed in the binding experiments at pH 5.0, except samples were resuspended in 50 ppm copper(II) in 0.05 M sodium acetate at pH 2.0.

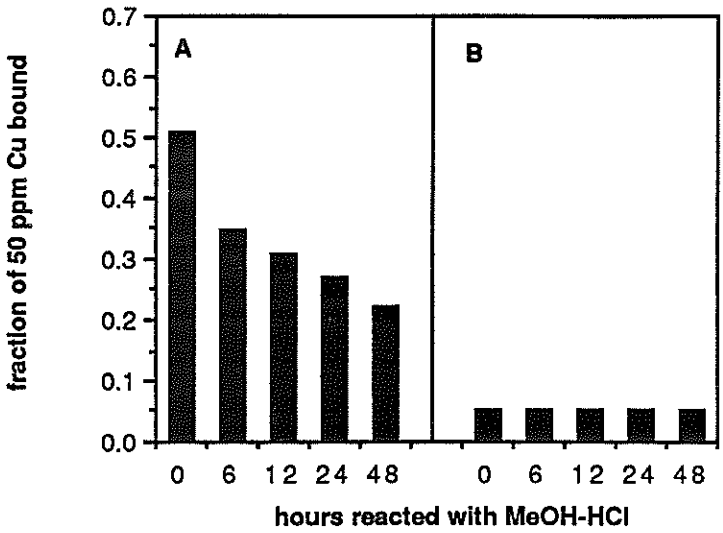


Figure 46: **(A). Effect of Acidic Methanol Modification of *Spirulina platensis* onCopper(II) Binding at pH 5.0**

S. platensis was modified and equilibrated with copper(II) in the same manner as described in figure 45 A.

(B). Effect of Acidic Methanol Modification of *Spirulina platensis* on Copper(II) Binding at pH 2.0

S. platensis was modified and equilibrated with copper(II) in the same manner as described in figure 45 B.

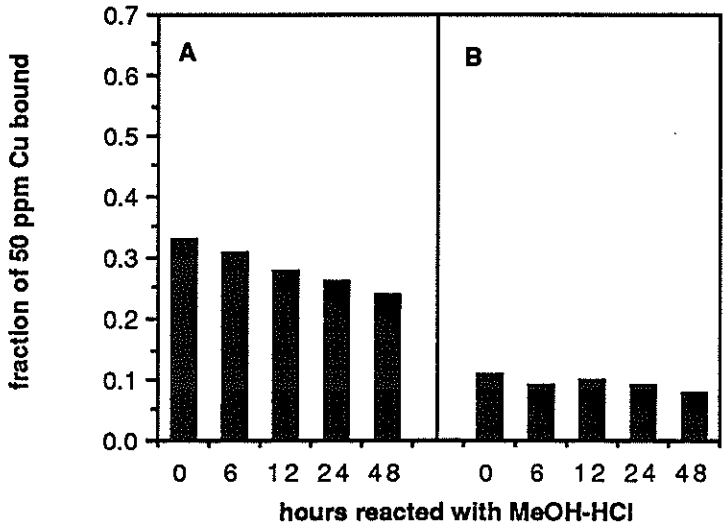


Figure 47: **(A). Effect of Acidic Methanol Modification of *Eisenia bicyclis* on Copper(II) Binding at pH 5.0**

E. bicyclis was modified and equilibrated with copper(II) in the same manner as described in figure 45 A.

(B). Effect of Acidic Methanol Modification of *Eisenia bicyclis* on Copper(II) Binding at pH 2.0

E. bicyclis was modified and equilibrated with copper(II) in the same manner as described in figure 45 B.

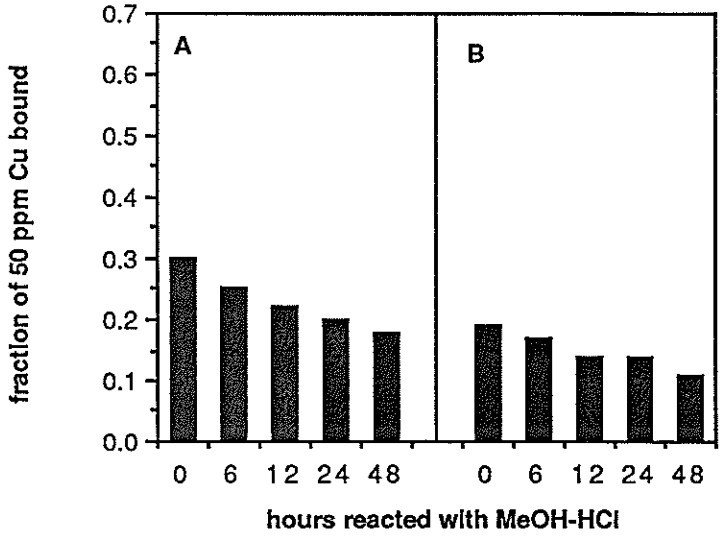


Figure 48: **(A). Effect of Acidic Methanol Modification of *Laminaria japonica* on Copper(II) Binding at pH 5.0**

L. japonica was modified and equilibrated with copper(II) in the same manner as described in figure 45 A.

(B). Effect of Acidic Methanol Modification of *Laminaria japonica* on Copper(II) Binding at pH 2.0

L. japonica was modified and equilibrated with copper(II) in the same manner as described in figure 45 B.

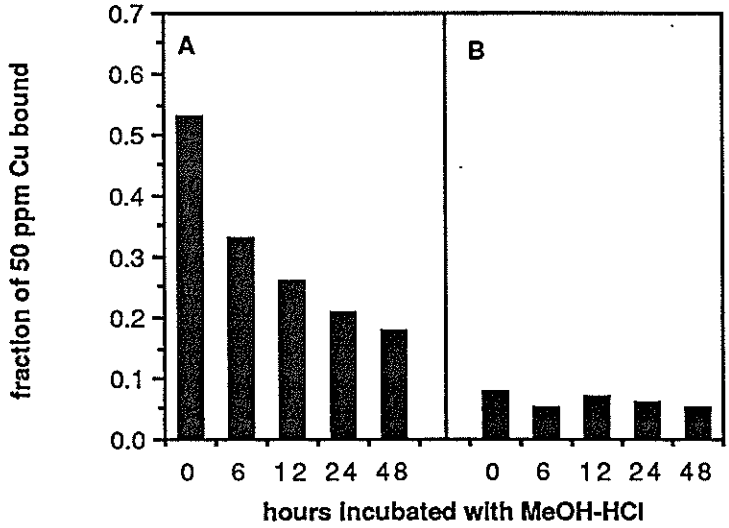


Figure 49: **(A). Effect of Acidic Methanol Modification of *Chlorella pyrenoidosa* on Copper(II) Binding at pH 5.0**

C. pyrenoidosa was modified and equilibrated with copper(II) in the same manner as described in figure 45 A.

(B). Effect of Acidic Methanol Modification of *Chlorella pyrenoidosa* on Copper(II) Binding at pH 2.0

C. pyrenoidosa was modified and equilibrated with copper(II) in the same manner as described in figure 45 B.

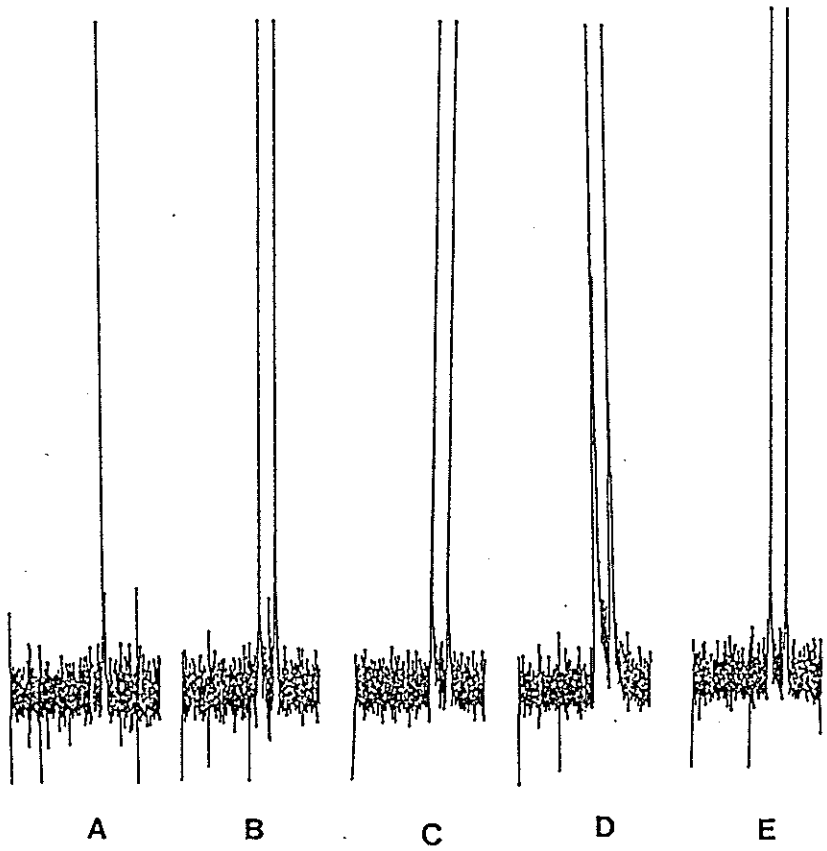


Figure 50: **Gas Chromatographic Analysis of Base Hydrolyzed *Cyanidium caldarium***

Fifty mg of unesterified and esterified *C. caldarium* {**(A.)** untreated *C. caldarium*, **(B.)** 6 hr modified, **(C.)** 12 hr. modified, **(D.)** 24 hr. modified, **(E.)** 48 hr. modified} was suspended in 0.05 M citrate at pH 5.0 in 0.1 M NaCl. After vortexing, 0.1 mL of 1.0 M NaOH was added. Samples were then sealed with parafilm and hand shaken for five minutes. Following an overnight incubation period at 4°C, the samples were hand shaken until an even suspension was obtained. Algal hydrolysates were centrifuged at 2,500 rpm for fifteen minutes in a Sorvall GLC-2B centrifuge at room temperature. The supernatant fractions were transferred into 1.0 mL volumetric flasks, 0.15 mL of 0.082 M Citrate at pH 3.0 was added and the final volume was brought up to 1.0 mL with deionized water when necessary. Algal hydrolysates were placed in 1.5 mL Wheaton vials equipped with teflon caps. Before injection into the HP 5880A Gas Chromatograph, hydrolysates were spiked with 0.1 mL of 0.03 mM 2-propanol as an internal standard. One µL of the resulting solution was injected into the GC which was equipped with an automated splitless injector, flame ionization detector, and a 20 meter RSL 500 polar capillary column. The GC operating conditions were as follows: injector temperature, 250°C; detector temperature, 275°C; carrier gas , nitrogen at 3 mL/min. Samples were run isothermally at 40°C for three minutes at an attenuation value of 2 0.

Table 1: **Gas Chromatographic Analysis of Methanol Released From Esterified Algae**

Algal species	hours modified	MeOH released (ppm)
C. caldarium	0	0.00
	6	0.36
	12	0.58
	24	0.71
	48	0.71
C. pyrenoidosa	0	0.00
	6	0.39
	12	0.56
	24	0.59
	48	0.65
E. bicyclis	0	0.00
	6	0.20
	12	0.48
	24	0.65
	48	0.90
S. platensis	0	0.00
	6	0.50
	12	0.61
	24	0.65
	48	0.76
L. japonica	0	0.00
	6	0.10
	12	0.32
	24	0.76
	48	0.85

Experimental procedure was the same as described in figure 50 for each algal species.

The copper(II) binding capacities of the 48-hour esterified algal biomasses (and corresponding controls) were determined. These determinations were performed at initial copper(II) concentrations of both 0.5 ppm and 100 ppm, to identify changes in populations of high-affinity and low-affinity copper binding sites, respectively. The results of these experiments are shown in Figures 51 and 52. At low copper levels, all biomasses showed decreased copper binding (as compared to the controls), with *Eisenia bicyclis* displaying the smallest change (13% decrease). Interestingly, the modified biomass of *Chlorella pyrenoidosa* did not bind copper at all, indicating that its high-affinity copper binding sites had been totally blocked. At high copper concentrations (testing of low-affinity binding sites) a dramatic decrease in copper binding by the 48-hour modified algal biomasses is again observed. The modified biomass of *Eisenia* shows a 48% decrease, while *Chlorella* displays a 70% decrease. These studies suggest that carboxyl groups are integrally involved in both low- and high-affinity copper binding. However, the fact that low-affintiy sites seem more affected by carboxyl group modification than high-affintiy sites (except in the case of *Chlorella*) indicates that carboxyl group activity is more important at weak-binding sites than at strong-binding sites.

The gold-binding capacities of 48-hour esterified algal biomasses and corresponding controls were also investigated. These experiments were performed at initial gold(III) concentrations of 0.8 ppm and 130 ppm, to identify changes in populations both of high and low-affinity gold(III) binding sites, respectively. The results of these experiments are shown in Figures 53 and 54. Interestingly, gold (III) binding increased after esterification in all cases. These results were not expected and may indicate some unexplained electrostatic or steric interactions.

(C) Chemical Modification Of Sulfhydryl Groups. Sulfhydryl groups are another potential metal-ion binding moiety which may be incorporated into the algal cell wall. Grassetti and Murray (1967) found that 2-2'-dithiodipyridine reacts specifically with sulfhydryl groups forming a disulfide linkage and 2-thiopyridone according to the following reaction:

An attempt was made to modify sulfhydryl groups by reacting biomasses from different algal species with 2,2'-dithiodipyridine for periods of 30, 60, and 120 minutes at room

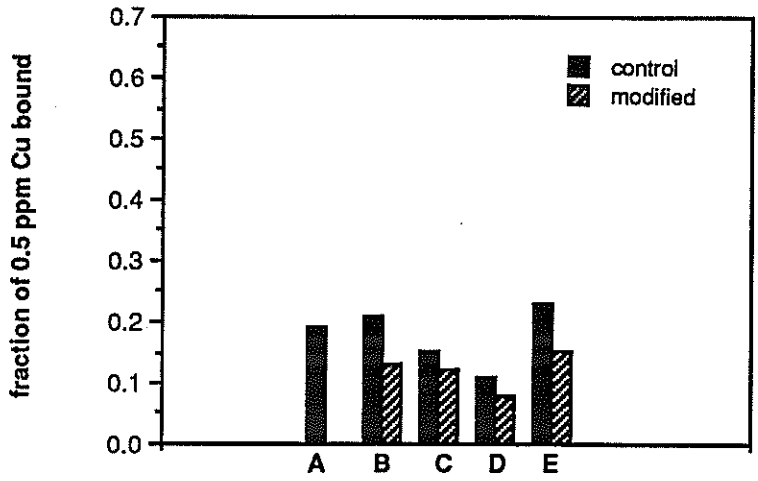


Figure 51: **Effect of Acidic Methanol Modification on High-Affinity Copper(II) Binding**

A 0.04 gram sample of lyophilized, modified (48 hours) and unmodified algal biomass {**(A)**. *Chlorella pyrenoidosa*, **(B)**. *Spirulina platensis*, **(C)**. *Eisenia bicyclis*, **(D)**. *Laminaria japonica*, **(E)**. *Cyanidium caldarium*} was washed twice in 0.3 M sulfuric acid and once in distilled-deionized water. Between each wash, the suspensions were centrifuged in a Beckman model J2-21 centrifuge, using a JA-20 rotor at 15,000 rpm (27,000 x g) for eight minutes at 25°C. The pellet from the water wash was resuspended in 2.0 mL of 0.05 M sodium acetate at pH 5.0. One mL of this suspension was transferred into each of two 400 mL centrifuge bottles. To each bottle, 249 mL of 0.5 ppm copper(II) in 0.05 M sodium acetate at pH 5.0 was added. The suspensions were equilibrated for thirty minutes at room temperature on a Burrell "wrist-action" shaker. The samples were then centrifuged in a JA-10 rotor for twenty minutes at 8,500 rpm (13,000 x g) at 25°C. The supernatant fractions were analyzed for copper by flame atomic absorption spectroscopy at 324.8 nm. The pH of each sample was recorded. Each bar represents the mean of two determinations.

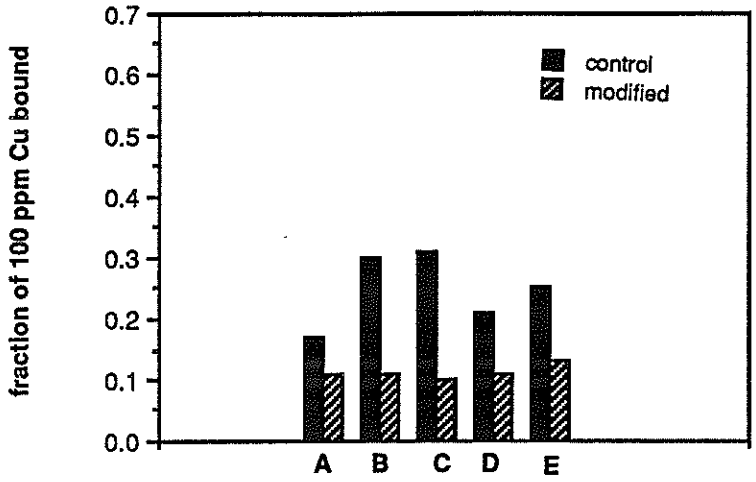


Figure 52: **Effect of Acidic Methanol Modification on Low-Affinity Copper(II) Binding**

A 0.1 gram sample of lyophilized, modified (48 hours) and unmodified algal biomass {**(A).** *Laminaria iaponica*, **(B).** *Spirulina platensis*, **(C).** *Chlorella pyrenoidosa* **(D).** *Eisenia bicyclis* **(E).** *Cyanidium caldarium*} was washed twice in 0.3 M sulfuric acid and once in distilled-deionized water. Between each wash, samples were centrifuged in a Beckman model J2-21 centrifuge, using the JA-20 rotor at 15,000 rpm (27,000 X g) for eight minutes at 25 ^{o}C. After the water wash, the pellets were resuspended in 20 mL of 0.05 M sodium acetate at pH 5.0. Five, 4.0 mL aliquots were distributed into 5.0 mL capped culture tubes. These samples were centrifuged in a Sorvall GLC-2B benchtop centrifuge, at 2,500 rpm for 10 minutes at room temperature. The pellets were equilibrated for sixty minutes with 4.0 mL of 100 ppm copper(II) in 0.05 M sodium acetate at pH 5. After equilibration, the samples were again centrifuged at 2,500 rpm for 10 minutes at room temperature. The supernatant fractions were analyzed for copper by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded.

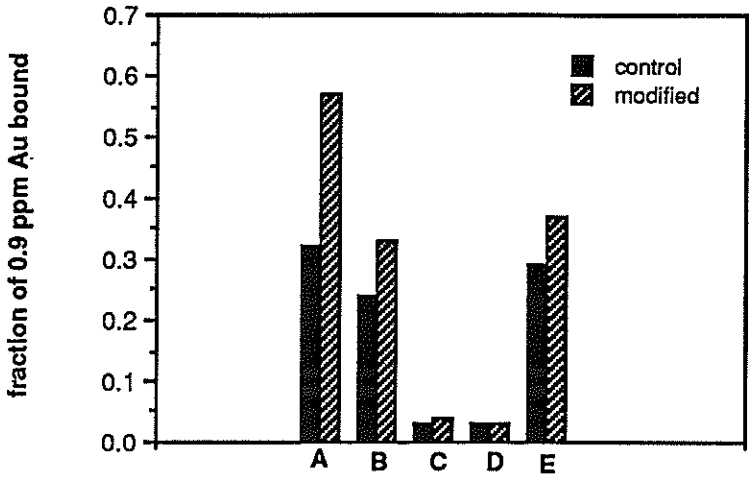


Figure 53: **Effect of Acidic Methanol Modification on High-Affinity Gold(III) Binding Sites**

A 0.01 gram sample of lyophilized modified (48 hours) and unmodified algal biomass {**(A).** *Chlorella pyrenoidosa*, **(B).** *Spirulina platensis*, **(C).** *Eisenia bicyclis*, **(D).** *Laminaria japonica*, **(E).** *Cyanidium caldarium*} was washed twice in 0.3 M sulfuric acid and once in 0.01 M HCl at pH 2.0. Between each washing, the samples were centrifuged in a Beckman model J2-21 centrifuge using the JA-20 rotor at 15,000 rpm (27,000 x g) for eight minutes at 25°C. The pellet was resuspended in 2.0 mL of distilled-deionized water adjusted to pH 2.0 with concentrated HCl. One mL was transferred into each of two 400 mL centrifuge bottles. Added to this 1.0 mL suspension was 249 mL of 0.9 ppm of gold (tetrachloroaurate) in 0.01 M HCl at pH 2.0 (0.02 mg algae/mL). These bottles were then equilibrated at room temperature for thirty minutes on a Burrell "wrist-action" shaker. After equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge, using a JA-10 rotor at 8,500 rpm (13,000 x g) for twenty minutes at 25°C. The supernatant fractions were analyzed for Au by flame atomic absorption spectroscopy at 242.8 nm. The pH of each sample was recorded. Each bar represents the mean of two determinations.

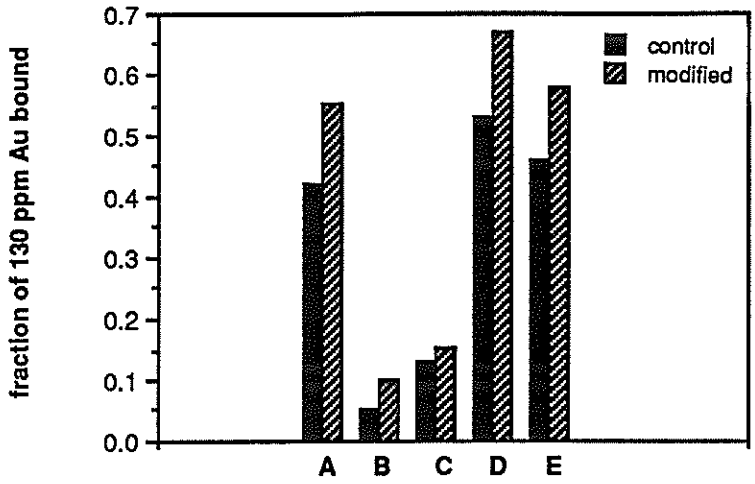


Figure 54: **Effect of Acidic Methanol Modification on Low-Affinity Gold(III) Binding Sites**

A 0.02 gram sample of modified (48 hours) and unmodified algal biomass {**(A).** *Chlorella pyrenoidosa*, **(B).** *Spirulina platensis*, **(C).** *Eisenia bicyclis*, **(D).** *Laminaria japonica*, **(E).** *Cyanidium caldarium*) was washed twice in 0.3 M sulfuric acid. Between each wash the algal suspensions were centrifuged in a Beckman model J2-21 centrifuge, using a JA-20 rotor at 15,000 rpm (27,000 x g) for eight minutes at 25°C. The pellet was resuspended in 20 mL of distilled-deionized water at pH 2.0. Five, 4.0 mL aliquots of the suspension were distributed into 5.0 mL capped culture tubes. These were centrifuged in a Sorvall GLC-2B benchtop centrifuge at 2,500 rpm for 10 minutes at room temperature. The pellets were resuspended in 4.0 mL of 130 ppm (Tetrachloroaurate) in 0.01 M HCl at pH 2.0. The suspension was allowed to equilibrate for sixty minutes at room temperature. After equilibration, the samples were again centrifuged at 2,500 rpm for ten minutes at room temperature. The supernatant fractions were analyzed for gold by flame atomic absorption spectroscopy at 242.8 nm. The pH of a representative sample was recorded.

temperature (other conditions described in the Methodology section). Following washing with 0.01 M HCl, the samples were lyophilized and experiments were conducted on the freeze-dried biomass. The degree of sulfhydryl modification was determined by two means: 1) by examining copper binding ability at low copper(II) levels (pH 5.0) and 2) by monitoring spectrophotometrically the appearance of the by-product 2-thiopyridone at 343 nm.

Copper binding experiments were performed with algal biomass which had been modified for 30, 60, and 120 minutes to evaluate alterations in copper(II) binding properties. The experiments were carried out at pH 5.0 and at a copper concentration of 0.5 ppm. The results are shown in Figures 55 through 58. For the most part, these results suggest that biomasses of *Chlorella pyrenoidosa, Cyanidium caldarium,* and *Spirulina platensis* were modified within the 30 minute time period. The decrease in copper binding of these samples is the same at either 30 or 120 minutes. In contrast, *Eisenia biyclis* did not show any decrease in copper binding at any of the time periods tested. This may indicate that either this alga had not been modified or that it contains no accessible sulfhydryl groups.

To determine the content of sulfhydryl groups of these four different algae, and thus determine whether or not the algae had been modified, absorption values were obtained for the by-product, 2-thiopyridone, from the supernatant solutions of modified algal fractions. These absorbance values were compared to calibration curves which were made by reacting 2,2'-dithiodipyridone with cysteine. The sulfhydryl content found for *Chlorella, Cyanidium, Spirulina,* and *Eisenia* were respectively, 0.016, 0.015, 0.006, and 0.002 mmol SH/gram of biomass. The lowest sulfhydryl content for *Eisenia* correlates with its invariant copper binding behavior upon modification, as compared to the control. The appearance of the modification reaction by-product, 2-thiopryidone, was monitored in all sample supernatants. The data obtained from these absorption measurements (not shown) showed that there was no significant increase in the by-product concentration after 30 minutes of reaction time for the *Chlorella, Cyanidium,* and *Spirulina* samples. This data correlates well with the copper binding experiments which show nearly identical modification at 30, 60, and 120 minutes.

The gold-binding properties of the 120-minute modified algal biomasses and corresponding unmodified controls were evaluated. These studies were carried out at initial gold(III) concentrations of both 0.8 ppm and 130 ppm to identify variations in populations of high-affinity and low-affinity gold(III) binding sites, respectively. The results of these experiments are displayed in Figures 59 and 60. At low gold concentrations, all modified biomasses except *Eisenia* showed a decrease in gold binding, with *Chlorella* having the greatest change. The relatively higher sulfhydryl content of *Chlorella* accounts for its larger change in gold binding at low gold ion concentrations. In contrast, at high gold levels no significant change in gold(III) binding is observed in any of these samples when compared to the control.

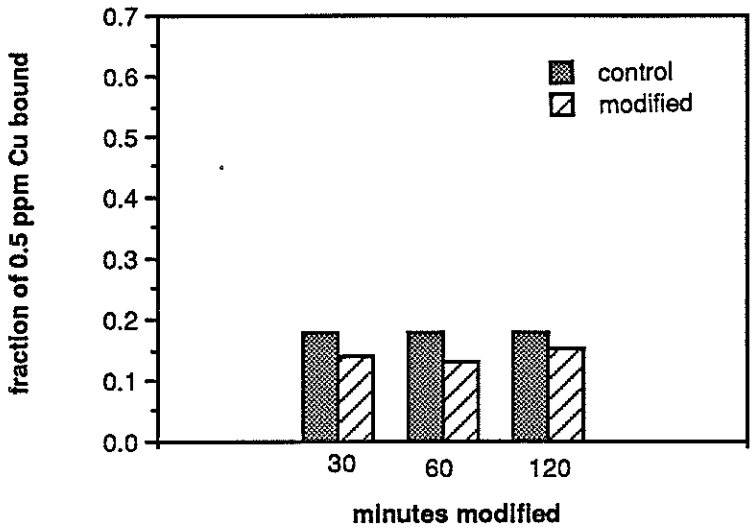


Figure 55: **Effect of 2,2'-Dithiodipyridine Modification of *Chlorella pyrenoidosa* on High-Affinity Copper Binding Sites**

Duplicate 0.032 g samples of lyophilized algal biomass modified for 30, 60, or 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and the corresponding unmodified controls were washed twice with 0.15 M sulfuric acid, once with distilled-deionized water and then equilibrated for five minutes in 0.05 M sodium acetate at pH 5. After each washing and equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor. The pellets were then resuspended at a concentration of 0.08 mg/mL in 400 mL of 0.5 ppm Copper(II) in 0.05 M sodium acetate at pH 5 and agitated in 500 mL centrifuge bottles for thirty minutes on a Burrell "wrist action" shaker. The suspensions were then centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for Cu by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

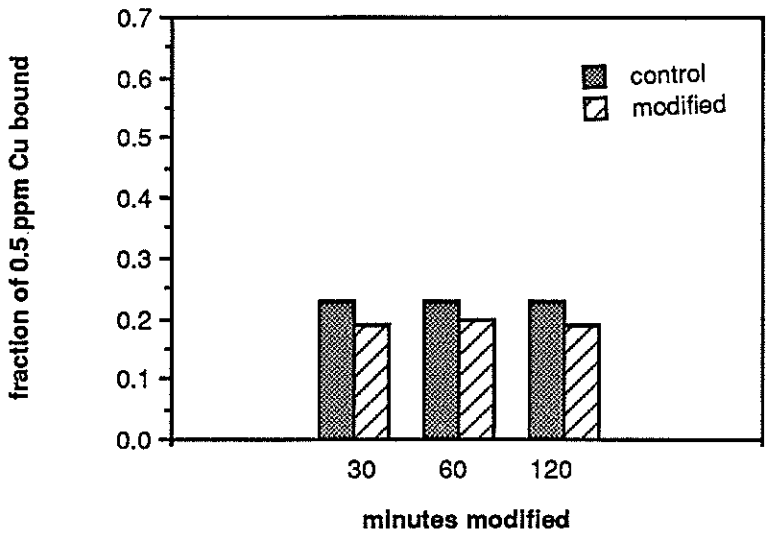


Figure 56: **Effect of 2,2'-Dithiodipyridine Modification of *Cyanidium caldarium* on High-Affinity Copper Binding Sites**

Duplicate 0.032 g samples of lyophilized algal biomass modified for 30, 60, or 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and the corresponding unmodified controls were washed twice with 0.15 M sulfuric acid, once with distilled-deionized water and then equilibrated for five minutes in 0.05 M sodium acetate at pH 5. After each washing and equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor. The pellets were then resuspended at a concentration of 0.08 mg/mL in 400 mL of 0.5 ppm Copper(II) in 0.05 M sodium acetate at pH 5 and agitated in 500 mL centrifuge bottles for thirty minutes on a Burrell "wrist action" shaker. The suspensions were then centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for copper(II) by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

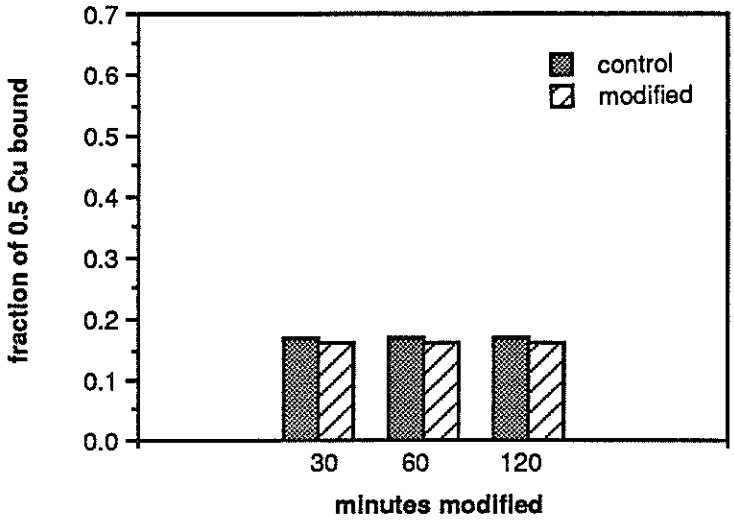


Figure 57: **Effect of 2,2'-Dithiodipyridine Modification of *Spirulina platensis* on High-Affinity Copper Binding Sites**

Duplicate 0.032 g samples of lyophilized algal biomass modified for 30, 60, or 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and the corresponding unmodified controls were washed twice with 0.15 M sulfuric acid, once with distilled-deionized water and then equilibrated for five minutes in 0.05 M sodium acetate at pH 5. After each washing and equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor. The pellets were then resuspended at a concentration of 0.08 mg/mL in 400 mL of 0.5 ppm copper(II) in 0.05 M sodium acetate at pH 5 and agitated in 500 mL centrifuge bottles for thirty minutes on a Burrell "wrist action" shaker. The suspensions were then centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for Cu by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

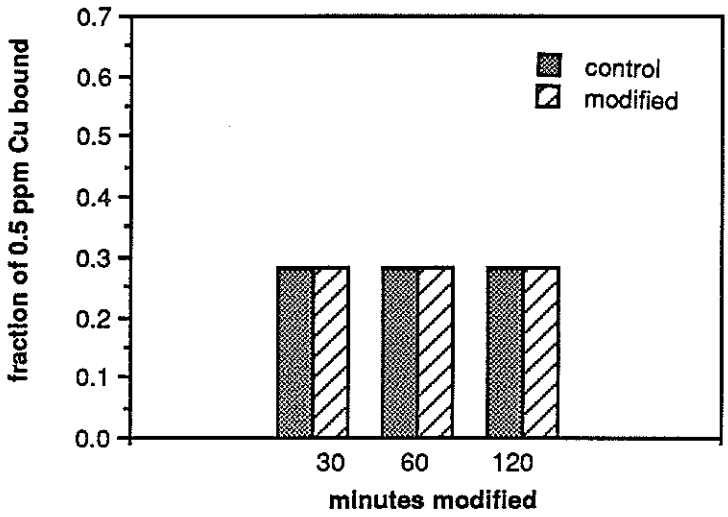


Figure 58: **Effect of 2,2'-Dithiodipyridine Modification of *Eisenia bicyclis* on High-Affinity Copper Binding Sites**

Duplicate 0.032 g samples of lyophilized algal biomass modified for 30, 60, or 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and the corresponding unmodified controls were washed twice with 0.15 M sulfuric acid, once with distilled-deionized water and then equilibrated for five minutes in 0.05 M sodium acetate at pH 5. After each washing and equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor. The pellets were then resuspended at a concentration of 0.08 mg/mL in 400 mL of 0.5 ppm Copper(II) in 0.05 M sodium acetate at pH 5 and agitated in 500 mL centrifuge bottles for thirty minutes on a Burrell "wrist action" shaker. The suspensions were then centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for Copper(II)by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

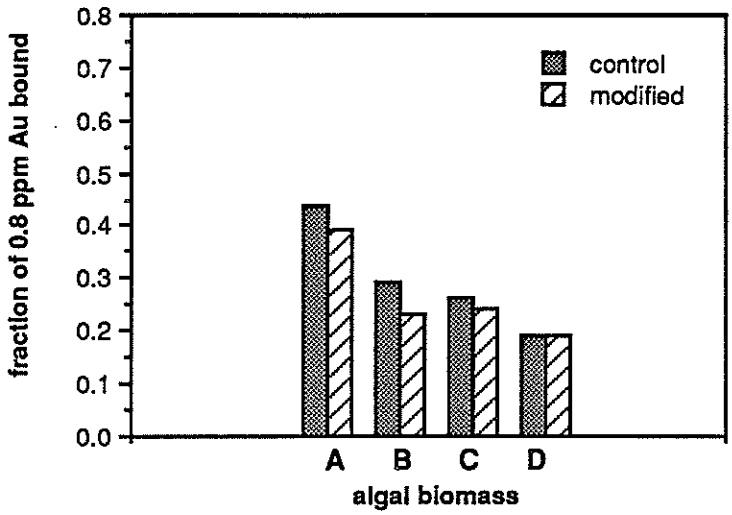


Figure 59. **Effect of 2,2'-Dithiodipyridine Modification of (A). *Chlorella pyrenoidosa*, (B). *Cyanidium caldarium*, (C). *Spirulina platensis*, (D). *Eisenia bicyclis* on High-Affinity Gold Binding Sites**

Duplicate 0.008 g samples of lyophilized algal biomass modified for 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and a 120 minute unmodified control were washed twice with 0.15 M sulfuric acid and once with 0.01 M hydrochloric acid at pH 2. After each washing, the samples were centrifuged at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor in a Beckman model J2-21 centrifuge. The pellets were then resuspended at a concentration of 0.02 mg/mL in 400 mL of 0.8 ppm gold(III) in 0.01 M hydrochloric acid at pH 2 and agitated in 500 mL centrifuge bottles with a Burrell "wrist action " shaker for thirty minutes. The suspensions were then centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for gold by flame atomic absorption spectroscopy at 242.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

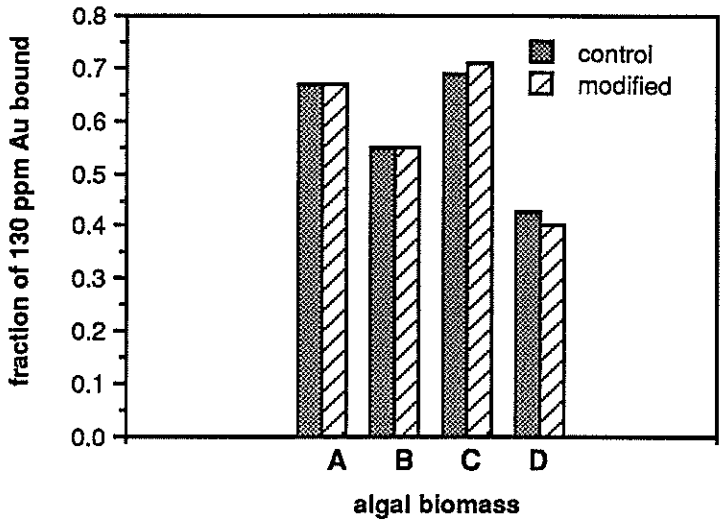


Figure 60. **Effect of 2,2'-Dithiodipyridine Modification of (A). *Chlorella pyrenoidosa*, (B). *Cyanidium caldarium*, (C). *Spirulina platensis*, (D). *Eisenia bicyclis* on Low-Affinity Gold Binding Sites**

Duplicate 0.020 g samples of lyophilized algal biomass modified for 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and a 120 minute unmodified control were washed twice with 0.15 M sulfuric acid. After each washing, the samples were centrifuged at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor in a Beckman model J2-21 centrifuge. The pellets were then mixed into 20 mL of 0.01 M hydrochloric acid at pH 2 until a homogeneous mixture was obtained, and separated into five, 4 mL aliquots in 16 mL culture tubes. The aliquots were then centrifuged at 8,000 rpm for ten minutes at 25°C using a JA-20.1 rotor. The pellets were then resuspended at a concentration of 1 mg/mL, in 4 mL of 130 ppm gold(III) in 0.01 M hydrochloric acid at pH 2 and equilibrated for thirty minutes. The suspensions were then centrifuged and the supernatant fractions were analyzed for gold by flame atomic absorption spectroscopy at 242.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

Gold(III) is classified as a soft acid and is expected, at low levels, to coordinate preferentially to sulfur-containing ligands. Sulfhydryl modification results correlate well with this hypothesis. At high gold concentrations, one would not expect significant variations in gold ion-binding by the modified biomass since sulfhydryl groups are not expected to be involved in weak gold binding. In addition, the relatively low sulfhydryl content of the algal biomasses tested would not represent a significant amount of binding capacity at high gold concentrations.

The copper-binding properties of the 120 minute modified algal samples were also investigated. These experiments were performed at initial copper(II) concentrations of both 0.5 and 100 ppm to identify changes in populations of high and low-affinity binding sites, respectively. The results of these studies are displayed in Figures 61 and 62. Results for copper(II) binding appear to be very similar to those seen for gold(III) binding. At low copper levels, all modified biomasses except *Eisenia* show a decrease in copper(II) binding. Conversely, at high copper(II) concentrations, no significant change is observed in any of the biomasses.

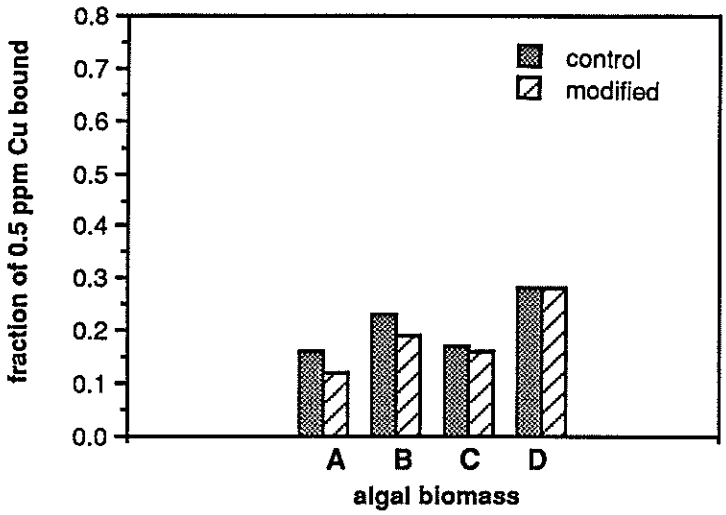


Figure 61. **Effect of 2,2'-Dithiodipyridine Modification of (A). *Chlorella pyrenoidosa*, (B). *Cyanidium caldarium*, (C). *Spirulina platensis*, (D). *Eisenia bicyclis* on High-Affinity Copper Binding Sites**

Duplicate 0.032 g samples of lyophilized algal biomass modified for 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and a 120 minute unmodified control were washed twice with 0.15 M sulfuric acid, once with distilled-deionized water then equilibrated for five minutes in 0.05 M sodium acetate at pH 5. After each washing and equilibration, the samples were centrifuged in a Beckman model J2-21 centrifuge at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor. The pellets were then resuspended at a concentration of 0.08 mg/mL in 400 mL of 0.5 ppm copper(II) in 0.05 M sodium acetate at pH 5 and agitated in 500 mL centrifuge bottles for thirty minutes on a Burrell "wrist action" shaker. The suspensions were centrifuged at 8,500 rpm for twenty minutes at 25°C using a JA-10 rotor and the supernatant fractions were analyzed for copper(II) by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

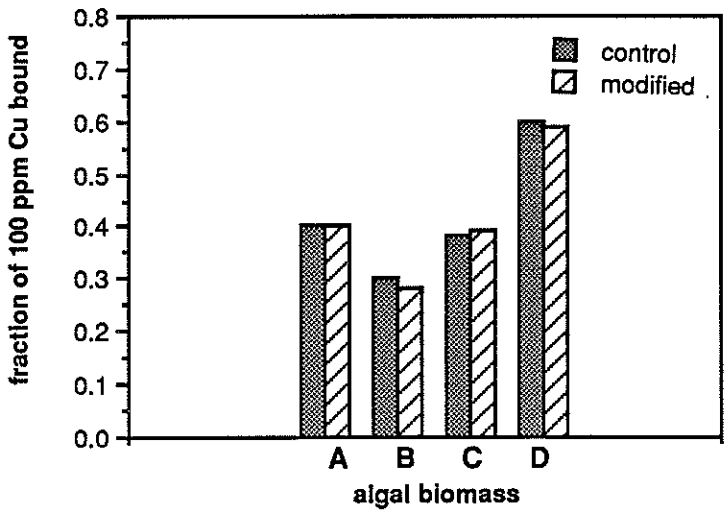


Figure 62. **Effect of 2,2'-Dithiodipyridine Modification of (A). *Chlorella pyrenoidosa*, (B). *Cyanidium caldarium*, (C). *Spirulina platensis*, (D). *Eisenia bicyclis* on Low-Affinity Copper Binding Sites**

Duplicate 0.100 g samples of lyophilized algal biomass modified for 120 minutes with 0.001 M 2,2'-dithiodipyridine in a pH 5 HCl/acetate buffer and a 120 minute unmodified control were washed twice with 0.15 M sulfuric acid and once with distilled-deionized water. After each washing step, the samples were centrifuged at 15,000 rpm for ten minutes at 25°C using a JA-20 rotor in a Beckman model J2-21 centrifuge. The pellets were then equilibrated with 20 mL of 0.05 M sodium acetate at pH 5 for five minutes and separated into five, 4 mL aliquots in 16 mL culture tubes. The aliquots were then centrifuged at 8,000 rpm for ten minutes at 25°C using a JA-20.1 rotor. The pellets were then resuspended, at a concentration of 5 mg/mL, in 4 mL of 100 ppm copper(II) in 0.05 M sodium acetate at pH 5 and equilibrated for thirty minutes. The suspensions were then centrifuged as before and the supernatant fractions analyzed for copper by flame atomic absorption spectroscopy at 324.8 nm. The pH of a representative sample was recorded. Control biomass was treated for the same periods of time and at the same concentration (10 mg/mL) in the pH 5 (0.024 M HCl/0.10 M sodium acetate) buffer only.

CONCLUSIONS

This investigation provided additional evidence that algal biomass has several attributes which make it an excellent material for use in water-treatment processes. In addition, valuable information has been gained concerning the mechanism of metal-ion binding to algal biomass.

1. Calcium and magnesium ions (hard-water components) in high concentrations were found not to interfere significantly with the binding of certain metal ions to algal biomass. Concentrations as high as 10,000 ppm of calcium or magnesium ions had virtually no effect on the binding of copper, aluminum, gold, or mercury ions to *Spirulina platensis* or *Cyanidium caldarium*, as long as ionic strength remained constant. Slight inhibition of cadmium, nickel, and zinc ion binding was observed for both algal species. These experiments suggested that the algal system would perform well in removing heavy metal ions from hard waters, in contrast to other water treatment methods such as conventional ion-exchange.
2. Algal biomass immobilized in a silica polymer was capable of removing metal ions from electroplating waste waters. Certain algal polymers showed good copper(II) binding properties when an authentic copper-plating bath sample was used. However, different algal polymers exhibited substantial variations in performance under comparable conditions.
3. The metal-ion binding properties of algae may be strongly influenced by the culture conditions for a particular biomass. Algae were cultured under different nitrogen concentrations and the metal-ion binding properties of the resultant biomass were examined. Results indicate that the nitrogen concentration present during growth of *Spirulina platensis* has no impact on its metal-ion binding capacity. Conversely, the metal-ion binding capacity of *Cyanidium caldarium* is decreased in biomass grown at nitrogen levels below those found in the normal growth medium. However, at nitrogen concentrations above normal, the metal-ion binding capacity of high-affinity gold binding sites on *Cyanidium* may increase.
4. *Cyanidium caldarium* is the only organism known to grow in medium containing high copper concentrations. Therefore, it was decided to determine whether or not elevated levels of copper(II) in the growth medium would increase the copper(II) binding capacity of the *Cyanidium* biomass. Results strongly suggest that exposure of *Cyanidium* to high levels of copper(II) during growth, causes increased expression of high- and low-affinity binding sites for the copper ion. Low-affinity binding is enhanced by growth medium copper(II) concentrations of up to 200 ppm. Higher copper(II) levels however, tend to result in reduced low-

affinity binding. High-affinity binding is enhanced by growth medium copper concentrations of up to 2000 ppm.

5. It is important to determine the mechanism of metal-ion binding to algal biomass. One approach to gaining this information is modification of chemical groups on the algal cell surface. Amine-containing algal groups were modified using acetic and succinic anhydrides. Metal-ion binding experiments with modified biomass indicated that treatment of *Chlorella, Cyanidium* and *Spirulina* biomass with these anhydrides increased the copper binding capacity while decreasing the gold binding capacity. This suggested that amino groups on algal cell walls play a significant role in the binding of metal ions. Carboxyl groups on algal cell walls were modified using acidic methanol. Copper binding experiments with methanol-modified biomasses of *Chlorella, Cyanidium, Spirulina, Laminaria* and *Eisenia* exhibited major decreases in copper binding (*Chlorella* displayed a 100% inhibition). However, gold binding capacities in all the methanol-modified biomasses slightly increased. These results imply that carboxyl groups on algal cells are responsible for a great portion of copper binding, and that they play only a minor role in gold binding. The content of sulhydryl groups on algal biomass was found to be relatively low. Nevertheless, these scarce algal sulfhydryl groups were modified using the reagent 2,2'-dithiodipyridine. Metal-ion binding experiments with the modified biomass indicated that treatment of *Chlorella, Cyanidium, Spirulina,* and *Eisenia* with the reagent, slightly decreased the gold and copper binding capacities at low ion concentrations. However, no change in binding capacity was observed at high metal-ion levels. These results suggest that sulfhydryl groups play only a minor role in low-affinity metal-ion binding by algal biomass.

RECOMMENDATIONS

Biomass from different algae have demonstrated a strong potential for use in the removal and recovery of metal ions from waste waters. Recommendations regarding additional development of this technology and further understanding of metal-ion interactions with algal cells are listed below.

Effects of Calcium(II) and Magnesium(II) on Transition Metal Binding to Algae

Work with non-living algae indicates that the alkaline earth metals (calcium(II) and magnesium(II)) interfered minimally with binding of toxic metal ions to *Cyanidium* and *Spirulina.* Studies should be undertaken using other algal species and contaminated ground waters (which are generally hard-waters) containing toxic heavy metal ions.

New Algal Polymers for the Removal of Metal ions

Although the alga-silica material has proven to be successful in removing heavy metal ions, other algal immobilization methods may help to increase the metal-ion binding capacities of the biomass.

Removal of Heavy Metal ions from Electroplating Waste Waters

Removing copper(II) ions from authentic electroplating baths proved successful in certain alga-silica polymers. Further research should be undertaken, testing the algal-silica material on other heavy metal ions such as lead(II) and nickel(II).

Effects of Culturing Conditions on the Metal Binding Capacity of the Resultant Biomass

This investigation has shown evidence indicating that changes in certain algal nutrient compositions and/or growth time intervals may have strong effects on the metal-ion binding properties of the resultant biomass. This work should be continued and *Cyanidium caldarium* as well as other algal species such as *Chlorella acidophilia* should be cultured in the presence of toxic heavy metal ions (i.e. silver(I), nickel(II), molybdenum(II), and cobalt(II)) to evaluate the effects of these metal ions on the binding properties of the resultant biomass. This information would prove useful in the development of algal strains with markedly improved metal-ion binding properties for use in water purification.

Mechanism of Binding of Metal ions to Algae

Results indicate that carboxyl, amino, and sulfhydryl groups play signficantly different roles in heavy metal-ion binding to algae. Further investigation of these functional groups is warranted. Other algal functional groups such as hydroxyl and phosphate moieties also remain to be investigated. It would also be worthwhile to compare electron micrographs of modified and unmodified biomass in order to determine possible locations on the cell wall where metal-ion binding has been altered by chemical modification. A more concise knowledge of the binding mechanism would allow more judicious selection of the proper algal biomass for purifying waters of known chemical composition.

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