UPTAKE OF METAL IONS FROM SOLUTION BY INACTIVATED CELLS OF CYANOBACTERIA

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ABSTRACT Some species of cyanobacteria have the ability to grow under stress conditions that would kill many other bacteria. Solutions of 0.25 mM Cu^{2+} were inoculated with the cyanobacteria *Synechococcus sp*. PCC 7942 and *Synechocystis sp.* PCC 6803 with the idea that these cyanobacteria may develop certain defense mechanisms allowing their survival in such stressed environments. Transmission electron microscopy showed that *Synechocystis sp. g*rown in 0.25 mM copper(II) developed a filamentous sheath around the cell wall, which could be responsible for binding copper ions on the cell surface. The presence of copper adsorbed by *Synechocystis sp*. was corroborated using environmental scanning electron microscopy equipped with an x-ray energy-dispersive spectrometer. *Synechococcus* has the ability to grow in mass quantity under ideal conditions, providing usable biomass at a minimal effort. Using lyophilized biomass grown under normal conditions, *Synechococcus* was tested for its potential to bind copper(II), lead(II), and nickel(II) ions from solution. Batch experiments were performed to determine the optimum binding pH, time dependency, and metal binding capacities for copper(II), lead(II), and nickel(II), along with desorption of the metal bound. The biomass studied showed a high affinity for all metal ions as the pH increased from two to six with optimum binding occurring at pH 5. Time dependency studies showed that this cyanobacterium had rapid binding to all three metals. Capacity experiments showed that this cyanobacterium bound 11.3 mg of copper(II) per gram of biomass, 30.4 mg of lead(II) per gram of biomass, and 3.2 mg of nickel(II) per gram of biomass. More than 98% of the copper(II), lead(II) and nickel(II) adsorbed by the biomass was recovered when treated with 0.1M HCl. Cyanobacterial biomass can eventually be used as the source for a novel approach in using biosystems to remediate contaminants from solution.

KEYWORDS: *Synechococcus*, *Synechocystis*, cyanobacteria, heavy metal binding, bioremediation

INTRODUCTION

Few people doubt that environmental contamination has become an important source of concern. There exists a general belief that the major environmental threats faced by mankind can be corrected with some changes in technology and manufacturing practices. However, new environmentally-friendly processes need to be developed to clean the already contaminated areas of our environment. The first cells on earth were prokaryotic, i.e., they had no nucleus. These cells, which first appeared millions of years ago, gained the energy necessary for their metabolism by fermenting the limited supply of organic compounds then available. Simultaneously, inorganic molecules were uptaken into the biomass. Therefore, the biosphere was “cleaned” or “detoxified” of compounds such as hydrogen cyanide, carbon monoxide, and hydrogen sulfide-through the use of a natural process. Cells have also assisted in detoxification, using other natural methods.

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Heavy metals such as cadmium, copper, and lead were removed by the biomass by biomineralization and by passive accumulation in cells through surface binding with chemical functional groups. These chemical functional groups evolved most likely as a response mechanism to the metal toxicity.

Cyanobacteria (also known as blue-green algae) are oxygen evolving, photosynthetic prokaryotic organisms that respond to stress conditions such as light deprivation [1]. *Spirulina platensis*, a cyanobacterium, has been found to adapt to grow in cobalt and iodine-enriched media [2], and it has been determined that this cyanobacterium may be resistant to ammonia at high pH values [3]. Slotton, *et al.*, determined that commercially-grown *Spirulina* contains detectable levels of mercury and lead [4]. Their findings implied that the cyanobacteria was taking up the toxic heavy metal ions. Indeed, other investigators have found that various species of algae take up or adsorb metal ions [5-15]. Other reports have indicated that carboxyl groups on algal cell walls may be responsible for a great portion of metal binding to inactivated algal biomass [16]. In live algae, intracellular polyphosphate has been found to be also responsible for metal sequestration [17]. Other investigators have shown that algal extracellular polysaccharides also chelate or bind metal ions [18, 19]. Gordon, *et al.*, found that extracellular proteins are produced in copper-stressed cultures of *Vibrio alginolyticus* [20]. Production of extracellular metal-binding proteins has also been proposed as a resistance mechanism in bacteria such as *Pseudomonads* [21, 22]. In addition, ultrastructural changes in live *Dunaliella minuta* have been observed following acute and chronic exposure to copper and cadmium [23].

Bonilla, *et al.*, studied the interaction of boron and calcium with live cyanobacteria species such as *Anabaena* and *Synechococcus* [24]. Kanamaru, *et al.*, found a copper-transporting P-type ATPase in the thylakoid membrane of the cyanobacterium *Synechococcus* sp. PCC7942 [25]. On the other hand, other investigators isolated a strain of *Synechococcus cedrorum* 1191 tolerant to heavy metals and pesticides [26]. Although the uptake of some heavy metal ions to several live species of cyanobacteria has been studied, no reports have yet appeared to relate the uptake of heavy metals to inactivated biomass of cyanobacteria, specifically *Synechococcus spp.* In addition, the possible morphological alterations that cyanobacteria undergo, when cultured in high concentrations of copper ions, have not been studied.

The purpose of this work was to study the binding or uptake of copper(II), lead(II), and nickel(II) ions from solution by inactivated biomass of the cyanobacterium *Synechococcus sp.* PCC 7942 cultured under normal conditions. Batch laboratory experiments were carried out in order to determine optimal metal binding pH. We also determined optimal metal binding times and the capacity of the *Synechococcus* biomass to adsorb each metal ion. The possibility of desorbing the metal ions adsorbed by the biomass was studied. Additionally, transmission electron microscopy (TEM) and environmental scanning electron microscopy (ESEM) were used to determine any morphological changes that could occur when the cyanobacterium *Synechocystis sp.* PCC 6803 was cultured under high copper(II) levels. Also, we wished to investigate (using ESEM) if copper(II) ions were being taken up when the live cultures of cyanobacteria were grown under high copper levels.

PROCEDURES

Cyanobacterial strains and culture conditions

Synechococcus sp. PCC 7942 was cultured under normal conditions using liquid BG-11 media, which is utilized for growing unicellular blue-green algae on plates [27]. This media contains only trace concentrations of copper(II) ions (0.3 μM copper). *Synechocystis sp.* PCC 6803 was also cultured using the BG-11 media but also in the presence of 0.25 mM copper(II). The resulting biomasses were washed with sterile distilled water, freeze-dried (in a Labconco freeze-dryer), ground and sieved to pass a 100-mesh screen.

Electron microscopy

Transmission electron microscopy (TEM) of *Synechocystis sp.* PCC 6803 biomasses grown under normal copper levels (control) and grown under high copper levels (0.25 mM, experimental) were used to determine morphological changes in the cells. The biomass pellets (control and experimental), after centrifugation, were immobilized in 10% agarose. Sections of immobilized cyanobacteria were prepared by fixing them with 2% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.2) for 40 min. Glutaraldehyde was removed, and the samples were rinsed twice with 0.1 M sodium cacodylate buffer (pH 7.2) for 1 min. Samples were incubated in 2% OsO_4 in 0.1 M sodium cacodylate buffer for 1 hr at 4ºC. After OsO_4 was removed, the samples were rinsed with sodium cacodylate buffer for 5 min. The buffer was removed, and 0.5% aqueous uranyl acetate was added and incubated for 40 minutes at 4ºC. After incubation the samples were rinsed with distilled water for 5 min. The samples were dehydrated with 75%, 95%, and 100% ethanol for five minutes each. Then, the ethanol was removed and 100% acetone was added for 15 min, twice. The samples were embedded in Poly/Bed 812 resin plastic. Samples were sectioned and examined in a Zeiss EM 10 transmission electron microscope operating at 60 kV. Environmental scanning electron microscopy (ESEM) was used to try to determine if the copper was being taken up by *Synechocystis sp.* PCC 6803. The uncoated *Synechocystis* freeze-dried biomass was placed in the ESEM sample chamber and run in a water vapor atmosphere at three torr in an Electroscan ESEM model 2020, equipped with an EDAX power MX system.

pH profile for copper(II), lead(II), and nickel(II) uptake

A batch laboratory method was utilized for the pH profile studies of metal binding to inactivated cells of *Synechococcus sp.* PCC 7942. The cyanobacterial biomass (250 mg) was washed twice with 0.01 M HCl. It was then centrifuged and the washings were collected, dried, and weighed to account for biomass weight loss. The rest of the experiment for the pH profile for copper(II) binding is similar to that reported before [28]. A comparable method was used for the pH profile for lead(II) and nickel(II) binding to the *Synechococcus* inactivated biomass. Concentrations of 0.1 mM lead(II) (as lead nitrate) and of 0.1 mM nickel(II) (as nickel nitrate) were used.

Time-dependence studies for metal ions binding

A batch laboratory method was used for the time dependence studies of metal binding to inactivated cells of *Synechococcus sp.* PCC 7942. The cyanobacterial biomass (250 mg) was washed twice with 0.01 M HCl. It was then centrifuged and the washings were collected, dried, and weighed to account for

biomass weight loss. The following part of the experiment is analogous to that reported previously [29]. However, the metal ion concentrations were 0.3 mM copper(II), 0.3 mM lead(II), and 0.3 mM nickel(II), and each one of the metal ion solutions was at pH 5 in 0.01 M sodium acetate buffer. The sampling time intervals were 5, 10, 15, 30, 60, 90, and 120 min.

Metal ion binding capacity studies

A batch laboratory method was used for the binding capacity experiments of metal binding to inactivated cells of *Synechococcus sp.* PCC 7942. The cyanobacterial biomass (50 mg) was washed twice with 0.01 M HCl. It was then centrifuged and the washings were collected, dried, and weighed to account for biomass weight loss. The following part of the experiment is similar to that reported before for copper(II) binding to *Mucor rouxii* [28]. A comparable method was used to determine the binding capacity for lead(II) and nickel(II) binding to the *Synechococcus* inactivated biomass. Concentrations of 0.3 mM lead(II) (as lead nitrate) and of 0.3 mM nickel(II) (as nickel nitrate) were used.

Recovery of metal ions adsorbed

In an attempt to recover or desorb the bound metal ions, the biomasses loaded to capacity with the metal ions were exposed twice to 2 ml of 0.1 M HCl, reacted by agitation for 5 min and centrifuged. After, centrifugation the supernatants were transferred into test tubes and analyzed for copper, lead, and nickel content by flame atomic absorption spectroscopy.

Metal analyses by flame atomic absorption spectroscopy (FAAS)

All metal analyses were performed by FAAS using a Perkin Elmer model 3110 atomic absorption spectrometer with deuterium background subtraction. Analytical wavelengths used for the various metals were as follows: copper—327.4 nm; lead—217 nm; and nickel—352.5 nm. An impact bead was used to improve the sensitivity, samples were read three times, and the mean value was computed. The calibration was performed within the calibration range of each metal, and the correlation coefficients for the calibration curves were 0.98 or better. Controls of each one of the metal solutions were run to detect any possible metal precipitation or contamination.

RESULTS AND DISCUSSION

Transmission electron microscopy was used to observe the possible morphological effects on the cyanobacterium *Synechocystis sp.* PCC 6803 after culturing the cells under trace and high copper concentrations. Figure 1 (A and B) shows transmission electron micrographs of cells of *Synechocystis sp.* PCC 6803. Figure 1A shows the cells grown at trace copper levels (normal conditions, 0.3 μM copper), while Figure 1B shows the cells grown at high copper concentrations (0.25 mM copper). As observed in Figure 1, the copper-stressed cells developed some type of filamentous structure outside the cell wall as a response to the exposure to copper(II) ions. These filamentous projections, which seem to originate at the cell membrane and extend through the cell wall could be fimbriae, also called common pili. Fimbriae are frequently found in large numbers projecting from the entire surface of freshly isolated gram-negative bacteria [30]. They are made of a single protein whose molecules are arranged in a helix to form a filament and their function is not very clear, but there is mounting evidence that they may play a role in facilitating the adherence of bacterial cells to other surfaces. Our results may indicate that these

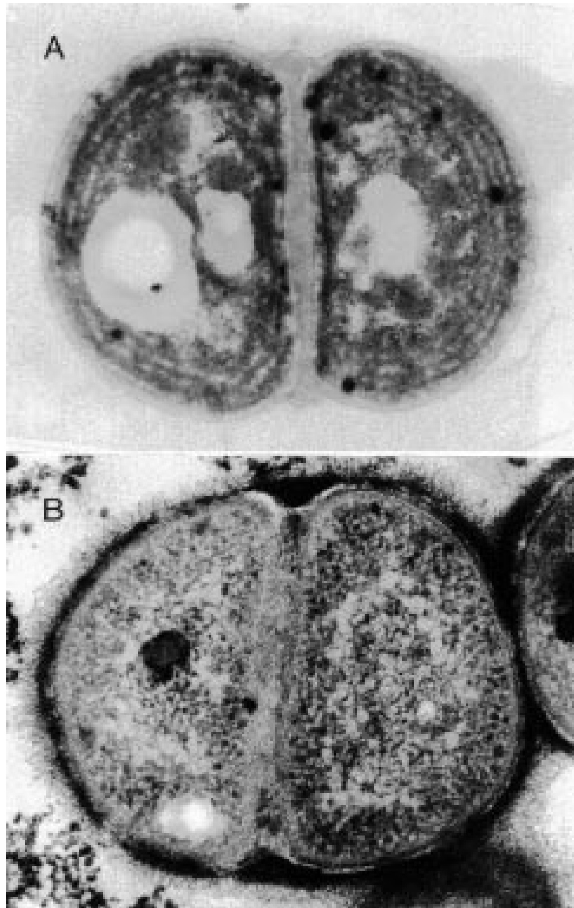

FIGURE 1. TRANSMISSION ELECTRON MICROGRAPHS OF CELLS OF *SYNECHOCYSTIS SPP.* PCC 6803. (A) STRAIN CULTURED AT TRACE COPPER CONCENTRATIONS (0.3 μM). NOTE THE NORMAL CELL STRUCTURE. (B) STRAIN CULTURED AT HIGH COPPER CONCENTRATIONS (0.25 mM). NOTE THE APPEARANCE OF A FILAMENTOUS ENVELOPE ON THE CELL WALL. THE MAGNIFICATION USED WAS 138,600X.

projections result as a defense mechanism against copper toxicity. The metal ions may be complexed or chelated outside the cells by the fimbriae's chemical functional groups, thus blocking the penetration of the metal ions through the cell wall. In addition to fimbriae, these filamentous projections could be an envelope outside the outer lipopolysaccharide (LPS) layer of cyanobacteria [31]. This envelope is variously called the sheath, glycocalyx, capsule, or merely gel, mucilage, or slime, depending on the consistency. This sheath is composed of polysaccharides and its function is unknown, but it has been suggested that it could be a protection mechanism from high light intensity. Thus, this sheath could also be used as a protection mechanism against metal ion toxicity. Polysaccharides such as galacturonic acid (present in the sheath) could provide good metal binding sites such as carboxyl groups.

Environmental scanning electron microscopy (ESEM) equipped with an energy-dispersive spectrometer was used to determine the possibility of copper adsorption or uptake by the live cells of *Synechocystis sp.* PCC 6803, when the cells were cultured at high copper concentrations. Scanning electron micrographs of the cells of *Synechocystis* cells (Figure 2) grown at 0.25 mM copper showed abnormal development. In addition, their growth rate was very slow. Figure 3 shows an x-ray map for copper of the scanning electron micrograph (Figure 2) taken with NIH image acquisition software [32]. This image (Figure 3) shows the copper detected in the scanning micrograph as bright spots or pixels. In order to verify that the bright spots were indeed copper, energy-dispersive spectroscopy (EDS) was utilized. In EDS, as primary beam electrons enter the sample bulk, they lose energy in the sample through the ionization of an inner shell electron of a sample atom. The resulting inner shell vacancy is filled when an outer shell electron drops into the vacancy. These electron transitions between shells of different energies result in the production of an x-ray photon of a characteristic energy. EDS detects the x-ray photons and it measures their energies or their wavelengths, and these characteristic energies are assigned to particular elements [33]. Figure 4

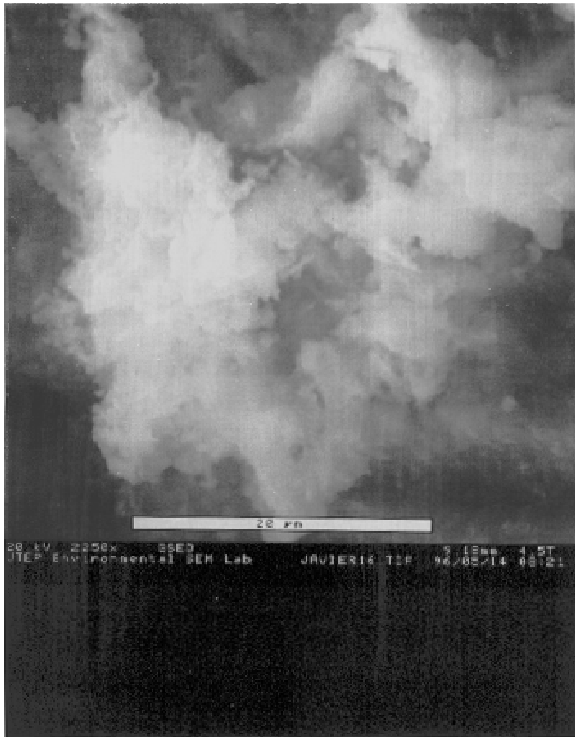

Figure 2. Environmental scanning electron micrographs of cells of *Synechocystis sp.* PCC 6803 grown at high copper concentrations. Note the abnormal morphology of the cells.

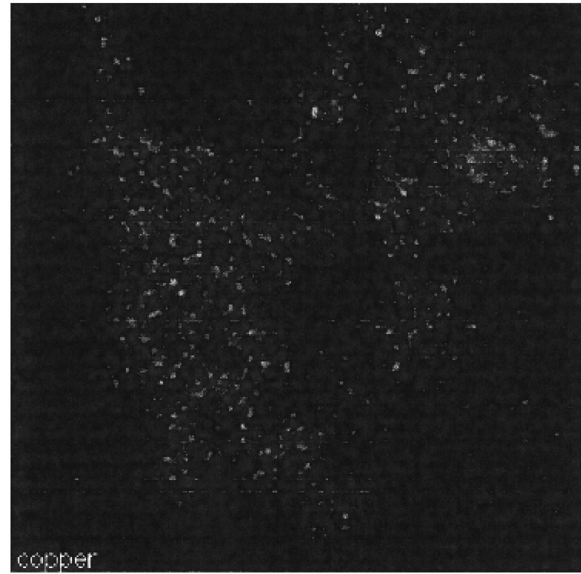

Figure 3. X-ray map for copper of the environmental scanning electron micrograph on Figure 2. The bright spots or pixels indicate the presence of copper. This x-ray map of copper was taken with NIH image acquisition software [see reference 32].

shows the EDS spectrum of the *Synechocystis sp.* cells (Figure 2) grown at 0.25 mM copper concentrations. The presence of copper is verified by the appearance of the copper energy line (0.9) in the x-axis. As observed in Figure 4, the energies of carbon, oxygen, and sodium were also detected. Since this image was collected at low voltage, this could approximate a surface map, meaning that the copper ions may be bound or adsorbed to the cell wall of *Synechocystis* cells. Hashemi and collaborators have also used EDS to investigate the binding of copper to *Anabaena variabilis* [34].

The inactivated biomass of the cyanobacterial species *Synechococcus sp.* PCC 7942, cultured under normal conditions, was screened for the effect of pH on uptake of copper(II), lead(II), and cadmium(II) ions. The pH profile for copper(II), lead(II), and nickel(II) binding to the *Synechococcus* biomass is shown in Figure 5. It is evident from this graph that increased sorption of lead(II) and nickel(II) was observed as the pH increased from 2 to 6. On the other hand, *Synechococcus* biomass bound lead(II) over a relatively wide pH range in a pH-independent manner. The pH profile for copper(II) and nickel(II) binding is consistent with the metal cation being the species which is bound to ligands on the *Synechococcus* cell walls, with more adsorption at higher pHs than at lower pHs. The pH dependence for copper binding to *Synechococcus* biomass suggests an electrostatic kind of interaction, probably metal binding to carboxyl groups [28, 29]. Since pH can affect the charge on the

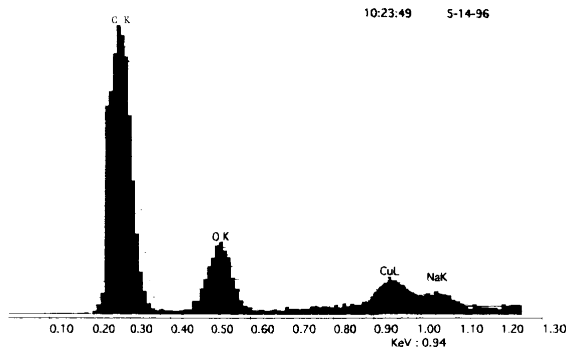

FIGURE 4. X-RAY ENERGY DISPERSIVE SPECTRUM OF THE *SYNECHOCYSTIS SP.* PCC 6803 GROWN AT HIGH COPPER CONCENTRATIONS. MAJOR IDENTIFIABLE PEAKS ARE COPPER (0.94 KeV), SODIUM (1.05 KeV), OXYGEN (0.55 KeV), AND CARBON (0.27 KeV). NOTE THE DEFINITE PRESENCE OF COPPER IN THE SPECTRUM.

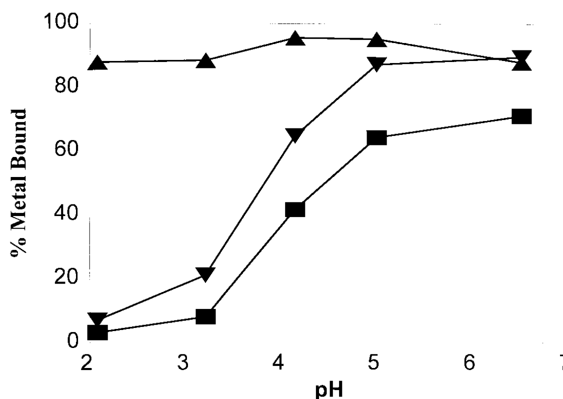

FIGURE 5. PERCENT COPPER(▼), LEAD(▲), AND NICKEL (■) REMOVED FROM SOLUTION AS A FUNCTION OF pH BY INACTIVATED CELLS OF *SYNECHOCOCCUS*. BIOMASS (5 mg/ml) WAS REACTED FOR 1 HOUR AT THE APPROPRIATE pH WITH 0.1 mM COPPER(II). THE SAME PROCEDURE WAS REPEATED WITH 0.1 mM LEAD(II) AND 0.1 mM NICKEL(II).

Synechococcus biomass surface, the lack of pH dependence in lead(II) binding suggests a covalent rather than electrostatic kind of interaction. Experiments were performed to determine the possibility of copper(II), lead(II), and nickel(II) precipitation by soluble *Synechococcus* material at various solution pHs. For this the reacted supernatants with the biomass at every pH were equilibrated with each of the metal ions. No metal ions precipitation was observed from the soluble *Synechococcus* material (data not shown).

In order to make sure that sufficient time had elapsed for the metal ion/*Synechococcus* system to come to equilibrium, experiments were performed to determine the time-dependence of copper(II), lead(II), and nickel(II) binding to *Synechococcus* biomass. Washed biomass was suspended at 5 mg/ml in 0.3 mM copper(II), 0.3 mM lead(II), and 0.3 mM nickel(II) in 0.01 M sodium acetate at pH 5 (independently). The supernatant solutions were analyzed for the metal ions, and these results are shown in Figure 6. Under these experimental conditions, lead(II) and nickel(II) were completely adsorbed by *Synechococcus* biomass within the few minutes it took to mix the biomass and the metal ions and to centrifuge. In contrast, adsorption of copper(II) occurred more slowly, requiring about 20 minutes for equilibrium to be reached under these conditions.

Experiments were carried out to determine the copper(II), lead(II), and nickel(II) binding capacities of *Synechococcus* biomass at optimum binding pH. To determine the metal ion binding capacities, washed *Synechococcus* biomass was suspended at 5 mg/ml for 20-minute intervals in 0.3 mM copper(II) solution. After centrifugation, the supernatants were saved for analysis and the cyanobacterial pellets were transferred to fresh copper solutions. This method was repeated until no significant additional copper binding could be detected. This point indicated an apparent saturation under these experimental

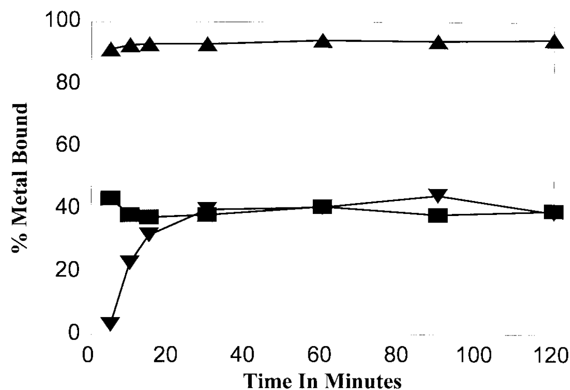

FIGURE 6. PERCENT COPPER(▼), LEAD(▲), AND NICKEL(■) REMOVED FROM SOLUTION AT DIFFERENT REACTION TIMES BY INACTIVATED CELLS OF *SYNECHOCOCCUS*. BIOMASS (5 mg/ml) WAS REACTED FOR APPROPRIATE TIMES WITH 0.3 mM COPPER(II) IN 0.01 M SODIUM ACETATE BUFFER AT pH 5.0. THE SAME PROCEDURE WAS REPEATED WITH 0.3 mM LEAD(II) AND 0.3 mM NICKEL(II).

conditions. The same procedure was repeated for lead(II) and nickel(II). Table 1 displays the numerical values of the binding capacities for the three metal ions by *Synechococcus* biomass. These results indicate a much larger binding capacity for lead(II), followed by copper(II), and then nickel(II).

Experiments were performed to determine the possibility of recovering metal ions bound to *Synechococcus* biomass. Studies on the effect of pH on the binding of copper(II) and nickel(II) and, to a lesser extent, lead(II) showed that the binding of these metal ions is favored at higher pH. This may suggest that metal binding at high pH might be reversed at lower pH. Therefore, experiments were performed by suspending *Synechococcus* biomass with metal ions bound to capacity in 0.1 M HCl and agitating them for 20 min. The supernatant solutions were analyzed for each metal ion, and the results are shown in Table 2. Very efficient copper(II), lead(II), and nickel(II) removal was observed (better than 98.5%).

TABLE 1. METAL ION BINDING CAPACITIES OF INACTIVATED CELLS OF *SYNECHOCOCCUS* BIOMASS.

Metal Ion Used	mg Metal/g Biomass
copper(II)	11.3
lead(II)	30.4
nickel(II)	3.2

Note: Each data represent the mean of 3 replicates.

CONCLUSIONS

TEM studies of the cyanobacterium *Synechocystis sp.* PCC 6803 grown at high copper concentrations have shown the presence of a filamentous sheath on the cyanobacterial cell wall. This sheath or projection might be the defense mechanism against copper ion toxicity. This sheath may not allow the copper ions to enter the cyanobacterial cell since the functional chemical groups on the sheath would complex or chelate the copper ions. ESEM with EDS capabilities corroborated the presence of copper, probably adsorbed on the *Synechocystis* cell surface. These studies also give preliminary results for the use of

TABLE 2. PERCENT OF METAL ION RECOVERY FROM INACTIVATED CELLS OF *SYNECHOCOCCUS* BIOMASS BY TREATMENT WITH 0.1 M HCl.

Metal Ion	% Metal Ion Recovered
copper(II)	98.45
lead(II)	99
nickel(II)	99

Note: Each data represent the mean of 3 replicates.

inactivated biomass of the cyanobacterium *Synechococcus sp.* PCC 7942 for the removal and recovery of copper(II), lead(II), and nickel(II) ions from contaminated waters. Batch laboratory experiments have shown that *Synechococcus* biomass has a very high capacity to bind lead(II) ions and, to some extent, copper(II) and nickel(II) ions. Additional experiments need to be performed in order to determine the application of these cyanobacteria in metal recovery processes and to investigate the mechanism involved in metal binding by cyanobacterial biomass.

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