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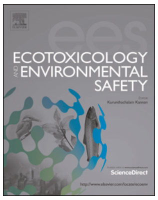

Role of Cu and pb on Ni bioaccumulation by *Chlamydomonas reinhardtii:* Validation of the biotic ligand model in binary metal Mixtures

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ABSTRACT

Ni, Pb and Cu uptake by *Chlamydomonas reinhardtii* has been quantified in single and binary metal systems in order to test some of the key assumptions of the biotic ligand model (BLM). Experiments were performed in solutions containing 5×10^{-7} M of free metal at 30 °C and pH 6. Nickel internalization fluxes (J_{int}) were measured in the presence of various concentrations of lead or copper from 5×10^{-8} M to 5×10^{-6} M at pH 6.0. Competition experiments did not show a straightforward antagonistic competition, as would be predicted by BLM. Synergistic and antagonistic effects were observed in binary metal systems which implies that bioaccumulation process is much more dynamic than assumed in the equilibrium models. Ni uptake decreased significantly in the presence of Cu^{2+} concentrations higher than 5×10^{-7} M. However, a maximum value of Ni uptake was observed at 5×10^{-7} M Pb^{2+}. Cu^{2+} was shown to compete strongly with Ni for uptake, having a higher binding affinity to Ni transport sites ($K_{\mathrm{Cu}\text{-}Rs} = 10^{6.95}\ \mathrm{M}^{-1}$) than to Cu transport sites ($K_{\mathrm{Cu}\text{-}Rs'} = 10^{6.22}\ \mathrm{M}^{-1}$). In contrast, the effect of Pb^{2+} on Ni uptake could not be explained by a simple competitive equilibrium with the transport sites of Ni such as the BLM. On the other hand, internalization fluxes of Cu and Pb were nearly constant in the absence and in the presence of Ni, implying that nickel had no effect on the uptake of copper or lead. The calculated affinity constant of Cu to Ni transport sites in the presence of Ni was similar to that obtained in the absence of Ni ($K'_{\mathrm{Cu}\text{-}Rs'} = 10^{6.22}\ \mathrm{M}^{-1}$). It was further concluded that Cu and Pb did not interact with the same active sites on the cell surface. Ni and Cu were shown to interfere with Mg and Na transporters, while Pb uptake was thought to occur by the Ca pathway.

1. Introduction

Trace metals are important pollutants in the aquatic environment. Many studies have been undertaken to investigate the bioavailability and toxicity of metal pollutants on a range of organisms, and toxicity testing protocols have been set up to provide data for formulating environmental quality standards (Fairbrother et al., 2007). Algae have been commonly used as test organisms because of their ecological importance as primary producers of most aquatic food chains. Moreover, results of algal toxicity tests are relatively reliable and repeatable (Borgmann et al., 2010; Duangrat et al., 2002; Franklin et al., 2002; Schiewer and Volesky, 2000; Soeprobowati and Hariyati, 2013). Almost all the ecotoxicological studies to date have focused on the effect of single metals on an algal species, despite the fact that toxic metals rarely occur in isolation in natural and wastewaters. Under environmental conditions, aquatic organisms are often exposed to a mixture of metals rather than a single element. Responses of aquatic organisms exposed to several metals simultaneously require consideration of the interactions of their effects on the organisms. The interpretation of bioaccumulation or toxicity results from metal mixtures is complex because there are chemical interactions with constituents in the media, interactions with physiological processes and interactions at the site(s) of toxicity. The subsequent fate of the trace metal depends on the particular physiology of the organisms, as to whether the metal is used for an essential metabolic purpose, excreted, stored in the body or even gains access to the wrong biomolecule and thus exerts a toxic effect (Rainbow, 2002). Many trace metals, such as Ni, Cu and Zn, cannot be immediately excreted or detoxified, for they are required in certain quantities to play essential roles in cellular metabolism. Any further accumulation of these essential trace metals in metabolically available form, however, has the potential to be

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toxic. Non-essential metal, like As, Hg, Cd and Pb, would have no required minimum concentration and need to be detoxified or excreted forthwith (Rainbow, 2002). Additive joint action of more than one metal on target organisms must therefore be taken into consideration when developing toxicologically relevant water quality criteria. There is clearly no consensus in the toxicology literature, on the effects of metal mixtures: additive, synergetic, and antagonistic effects have been observed with respect to the metals (Borgmann et al., 2010, 2008; Chen et al., 2010; Flouty and Estephane 2012; Komjarova and Blust, 2009; Abboud and Wilkinson, 2013). Essential and non-essential metals may share common uptake routes and interact with each other affecting uptake, bioaccumulation and toxicity. The results of such interactions are highly variable ranging from antagonism to synergism depending on the metal, its external concentration and exposure scenario, length of exposure, studied species and examined organs (Norwood et al., 2003). Access to binding sites may be controlled by competition with other ions or by physical changes to the membrane of the organism. The uptake of essential metals, often involves specific pathways (e.g., ion channels) that are central for meeting metabolic requirements. Non-essential, toxicologically significant metals are not considered to have specific uptake mechanisms and appear to follow existing pathways of essential metals (Chen et al., 2010; Abboud and Wilkinson, 2013).

Over the past few years, equilibrium models such as the biotic ligand model (BLM), which aim to predict dissolved metal toxicity to aquatic organisms as a function of external metal concentration and water chemistry characteristics, have been increasingly developed. The BLM modeling approach has been proposed as a tool to quantitatively predict the manner in which water chemistry affects the speciation and biological availability of metals in aquatic systems (Di Toro et al., 2001; Santore et al., 2001; Slaveykova and Wilkinson, 2005). Due to its mechanistic facility in dealing with the interactions of components, the BLM approach has the potential to make significant advances to the risk assessment of metal mixtures. The BLM has the potential to predict both biological effects and bioaccumulation in metal mixtures. Because the BLM, assumes that the metal species in solution and those adsorbed to the biological surface are at thermodynamic equilibrium, the interactive effect of a binary metal mixture is necessarily antagonistic, i.e. in the presence of a second ion, lower (or unchanged) uptake or biological effects is always predicted (Hassler et al., 2004; Norwood et al., 2003). However, the uptake process is complex and the presence of secondary cations can also have less predictable effect on metal uptake. Increased uptake in the presence of a potentially competing ion was often observed in the literature but it is not consistent with BLM predictions (Chen et al., 2010; Hassler and Wilkinson, 2003; Worms and Wilkinson, 2007). In this paper, Ni, Pb and Cu uptake by *Chlamydomonas reinhardtii* has been quantified in single monometallic systems and binary metal mixtures. Metal ions were selected because of their contrasting toxicity and essentiality. Ni and Cu belong to the category of essential metals in metabolically available form, whereas, Pb belongs to the category of non-essential metals and is known to be toxic. The goal of this study was to determine whether metal uptake could be predicted by free ion concentrations when organisms were exposed to Ni in binary metal mixtures. Results for Ni bioaccumulation in the presence of Pb or Cu by the unicellular green alga, *C. reinhardtii*, were discussed in the context of the BLM.

2. Materials and methods

2.1. Test organism and culture conditions

C. reinhardtii (wild type 2137 *mt*+), a unicellular green alga, was employed in this study mainly due to the relative ease by which it is possible to culture and control its metabolic state, size and surface area distribution (Harris, 1989). *C. reinhardtii* is also a useful species due to an existing complete knowledge of its genetic makeup (Merchant et al., 2007). Algae were transferred from a week-old Tris–acetate–phosphate (TAP) (Harris, 1989) agar plate to a (4 ×) diluted TAP solution ($I=10^{-2}$ M) (Kola and Wilkinson, 2005). Cells were grown in an incubation chamber at 20 °C, under a 12:12 h light:dark regime using fluorescent lighting (80 µmol photons $m^{-2}\ s^{-1}$) and rotary shaking (100 rpm) until a density of $(2–3)\times 10^6$ cells mL^{-1} was achieved, generally after 4 days. Cells were then diluted in fresh media to 1.0×10^5 cells mL^{-1} and once again allowed to attain logarithmic growth. Once cells attained mid-exponential growth ($(1–3)\times 10^6$ cells mL^{-1}) generally after 2 more days, they were harvested by centrifugation (3700*g*, 4 min) into 50 mL sterile (polypropylene) centrifuge tubes, resuspended in 10^{-2} M MES (2-(*N*-morpholino)ethanesulfonate, sodium salt, Sigma) at pH 6, centrifuged and harvested a second time, then resuspended in exposure solutions. Cell density was determined using a UV–visible spectrophotometer. The optical density of a cell culture was measured at 665 nm.

2.2. Preparation of chemicals

Experimental solutions were prepared in 10^{-2} M MES media as the pH buffer. The solutions were buffered to pH 6 to minimize the potential role of carbonate complexes, which are expected to become important in the range of pH 6–8 (Hassler et al., 2004). Stocks of metal solutions were prepared using standard solutions of $Pb(NO_3)_2$, $Cu(NO_3)_2 . 3H_2O$ and $Ni(NO_3)_2$ (analytical grade, Fluka). Nitrate was chosen as the counter ion because of its low tendency to form metal complexes. The solutions were then diluted to the desired concentrations using ultra pure water. For pH adjustment, concentrated solutions of nitric acid (suprapur, Sigma) were used. Under these conditions, in the absence of added ligand, free metals represented > 96% of the total metal in solution (Chen et al., 2010). All culture media and experimental solutions were sterilized prior to use. All manipulations of algal cultures were performed under laminar flow conditions and all bottle borders were flame sterilized. All exposure solutions were equilibrated 1 day prior to use. This period was sufficient to reach the metal speciation. All polycarbonate materials were acid washed (minimum 24 h in 0.1% HNO_3), and rinsed 3 × with deionized water and 3 × in Milli-Q water ($R > 18$ MΩ cm; TOC < 2 µg L^{-1}). In order to evaluate biological and analytical variability, solutions were prepared in triplicates. The experimental results given are the average values.

2.3. Bioaccumulation measurements

In the first set of experiments, single metal biouptake experiments were performed in 150 mL of an experimental medium containing 5×10^{-7} M free metal, 10^{-5} M of Ca^{2+} added as the nitrate salt and 10^{-2} M MES as the pH buffer (pH 6.0). Low concentrations of free metal ions were selected for this study, reflecting real environmental conditions. In fact, in most aquatic ecosystems, only very small proportions of dissolved metals are present as free hydrated ions with the majority of the metal being complexed by ligands or adsorbed on the surfaces of particles and colloids (Buffle, 1988). Calcium was added in order to block metal efflux to the experimental media (Hassler et al., 2004; Worms and Wilkinson, 2007). In addition, a low amount of calcium is essential to the maintenance of cellular integrity and reactivity. Bioaccumulation was quantified in short-term experiments (< 65 min) in order to decrease the likelihood of physiological changes in the algae or physicochemical changes in the medium (e.g. decreasing