Toxicity and bioaccumulation of copper in three green microalgal species

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Abstract

The effective concentrations of copper on the inhibition of the growth of *Scenedesmus obliquus*, *Chlorella pyrenoidosa* and *Closterium lunula* at 96 h (96 h EC₅₀) were determined to be 50, 68 and 200 µg/l, respectively. The low initial bioaccumulation of Cu by *C. lunula* was found to be responsible for its tolerance to Cu. The amount of Cu accumulated by all three microalgae reached the maximum value and decreased quickly after the peak followed by a slow decrease over the next 6 d. Bioaccumulation of Cu by *C. lunula* was directly proportional to the initial Cu concentration. After reaching the first peak after 1 d, the bioconcentration factor of Cu by microalgae declined to its minimum value during the exponential growth phase but increased in the stationary growth phase again. This indicates that desorption of Cu from microalgae was higher during the exponential growth phase but lower in the stationary growth phase. Smaller microalgae with low 96 h EC₅₀ values are more efficient in removing Cu from wastewater.

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1. Introduction

Copper is one of the most important and potentially toxic metallic pollutants (Chang and Sibley, 1993). The toxicity of copper is mainly related to free ions (Knauer et al., 1997) and its sensitivity varies among microalgae (Soldo and Behra, 2000). Toxic metals may affect microalgal photosynthesis, growth, enzyme activity and respiration. The effective concentration of heavy metal that inhibits 50% microalgal growth at 96 h (96 h EC₅₀) is widely used as an index of toxicity (Hornstrom, 1990). One mechanism of tolerance by algae is physiological exclusion of metal ions due to reduced cell membrane permeability (Brown et al., 1988). Internal metal im-

mobilization by the formation of copper bodies can also occur (Nassiri et al., 1997). Binding to organic complexes, especially cell exudates, is another possible mechanism regulating the detoxification of metal ions (Xue et al., 1988; Xue and Sigg, 1990; Rijstenbil et al., 1994; Gerringa et al., 1995). It has been reported that microalgal production of glutathione, thiols or superoxide dismutase activity (SOD) can be stimulated by copper, which may also be a defense mechanism against copper toxicity (Rijstenbil et al., 1994, 1998a,b; Rijstenbil and Wijnholds, 1996; Okamoto and Colepicolo, 1998; Leal et al., 1999).

Sorption of heavy metals from aqueous solution by microalgae has attracted much attention for the wastewater treatment, which can often reduce the concentration of heavy metals greatly. Sorption capacities of marine algal biomass for cadmium, copper and lead were found to be greater than those of other types of biomass (Yu et al., 1999). Knauer et al. (1997) observed

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that bioaccumulation of copper by microalgae consisted of an initial fast passive adsorption process followed by a slow active intracellular uptake. The first fast step is reversible and contains the processes of adsorption, ion exchange, coordination, complexation, chelation and microprecipitation. The second slow uptake is often irreversible and can be due to a number of mechanisms including covalent bonding, surface precipitation, redox reactions or most often, diffusion into the cell interior and binding to proteins and other intracellular sites (Kuyucak and Volesky, 1989). Hassall (1962) reported that dead cells can uptake more metals than living cells, indicating a possible desorption ability of metals by the living cells.

Although both the toxicity and the bioaccumulation of copper in microalgae have been extensively studied, little information is available on the relationships between the two. Here, the inhibition effects of copper on the growth of three microalgal species were evaluated and the long-term bioaccumulation processes of copper in microalgae were investigated. It was found that the less initial bioaccumulation amount of Cu by *Closterium lunula* was responsible for its strong tolerance to Cu, which was important for both evaluation of water quality and treatment of heavy metal polluted wastewater.

2. Materials and methods

2.1. Chemicals and microalgae

Analytical grade of CuCl_2 was used in the experiments. *Chlorella pyrenoidosa*, *Scenedesmus obliquus* and *C. lunula* were bought from the Institute of Hydrobiology, Academia Sinica, China.

2.2. Medium

The culture medium consisted of 0.200 g of $(\text{NH}_4)_2\text{SO}_4$, 0.030 g of superphosphate, 0.080 g of MgSO_4 , 0.100 g of NaHCO_3 , 0.025 g of KCl, 0.15 ml of 1% (w/v) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ solution and 1000 ml of distilled water. Initial pH of the medium was adjusted to 7.0 using 1 N HCl solution. The medium and all experimental utensils used were sterilized at 124 °C for 20 min.

2.3. Culture condition

For studies of both toxicity and bioaccumulation, microalgae were grown in a 100 ml flask containing 30 ml inoculums at 24 °C with a light intensity of $40 \mu\text{mol m}^{-2} \text{s}^{-1}$ in a 12–12 h light–dark circle. A spectrophotometer was used to measure the optical density of culture solution at 650 nm (OD650 nm) to express microalgal biomass and the initial OD650 nm was pre-

pared as 0.010. Cu stock solution of 500 mg/l was prepared using sterilized distilled water, which was further diluted to different initial levels of Cu using the medium. The experiment was conducted in triplicate. The data presented here were the average values of three parallel samples and their relative standard deviations were less than 10%.

2.4. Calculation of 96 h EC50

Cu toxicity expressed as the 96 h EC50 was calculated by a linear regression between probability unit (PU) and log (initial Cu concentration, $\mu\text{g/l}$). PU was obtained using a relationship table between PU and the reaction ratio between the OD650 nm when Cu is added and the OD650 nm when Cu is not added at 96 h (Zhou and Zhang, 1989). Ninety hour EC50 is calculated by a linear regression dose reaction equation when PU is set to 5. The dose response equation was χ^2 tested with 95% confidence.

2.5. Measurement of dry weight concentration of microalgae

Dry weight of microalgae was determined with a digital balance after the cells filtered on a 0.22 μm membrane were dried at 103 °C for 2 h. Good linear relationships between dry weight concentration (DWC) and OD650 nm were obtained. For *C. pyrenoidosa*, $\text{DWC (mg/l)} = -1.94 + 343.46 \times \text{OD650 nm}$ ($r = 0.9990$). For *S. obliquus*, $\text{DWC (mg/l)} = -3.88 + 617.97 \times \text{OD650 nm}$ ($r = 0.9920$). For *C. lunula*, $\text{DWC (mg/l)} = 0.75 + 495.67 \times \text{OD650 nm}$ ($r = 0.9997$). These linear regression equations were used to estimate the DWC in culture solution.

2.6. Measurement of Cu, bioaccumulation and bioconcentration factor

The standard analytical method recommended by the Chinese EPA (Chinese EPA, 1989) was used to measure the total ambient Cu concentration in the medium. Nitric acid of 5 ml was added to a filtered medium of 30 ml (0.22 μm membrane), which was heated to 100 °C for about 5 h, and then distilled water was added to keep the volume of 30 ml constant. Five milliliter of 0.2% (w/v) sodium diethyldithiocarbamate solution was added to the solution and the pH of medium was adjusted to 8.5 using $\text{NH}_3\text{H}_2\text{O}$. The purple complex of Cu was formed in the presence of cresyl red, which was extracted by 5 ml of tetrachlorated carbon. The absorbance of extracting solution was measured at the wavelength of 440 nm with a spectrophotometer and the limit of detection was 2 $\mu\text{g/l}$ of Cu.

Accumulation amount of Cu was calculated as the difference between the initial and final Cu concentrations at different time intervals in the solution, which included Cu bound in cell-wall adhering precipitate plus accumulated Cu in the cells. The bioconcentration factor (BCF) was calculated as the ratio between the bioaccumulation amount of Cu ($\mu\text{g}/\text{mg}$ -dry wet) and the Cu concentration in solution.

2.7. Controls

Controls consisted of 100 $\mu\text{g}/\text{l}$ Cu in the medium without algae. All the other experimental conditions are the same as those of algae. The change of total Cu concentration was less than 3% in the period of 6 d.

3. Results

3.1. Toxicity of Cu

In the absence of added Cu, microalgal biomass expressed as OD650 nm at 96 h was in the order of *C. pyrenoidosa* > *C. lunula* > *S. obliquus* (Fig. 1). The biomass decreased as the initial Cu concentration increased (Fig. 1). For *C. lunula*, OD650 nm decreased less than the other two algae (Fig. 1). The corresponding linear dose response equations between PU and log (Cu concentration, $\mu\text{g}/\text{l}$) were established in Fig. 2. Based on the regression equations, the 96 h EC50 for the inhibition of the growth of *S. obliquus*, *C. pyrenoidosa* and *C. lunula*

Fig. 1. Toxic effect of copper on the inhibition of microalgal growth at 96 h. OD650 nm was used to express microalgal biomass and initial OD650 nm was set to 0.010.

Fig. 2. Linear regression of dose response equation between PU and Log (initial Cu concentration, $\mu\text{g}/\text{l}$) at 96 h. PU was obtained using a relationship table between PU and reaction ratio that is the ratio between OD650 nm at different initial Cu concentrations and OD650 nm without added Cu.

were calculated to be 50, 68 and 200 $\mu\text{g}/\text{l}$, respectively (Fig. 2). *C. lunula* was far more tolerant to Cu than the other two algae. The 96 h EC50 values obtained were tested for significance with the X^2 test ($\alpha = 0.05$).

3.2. Growth process of microalgae

The growth process of microalgae during the 6-d period underwent a lag, exponential and stationary growth phases (Fig. 3). Because of the greatest growth rate of *C. pyrenoidosa* and the greatest sensitivity of *S. obliquus* to Cu, the order of growth was also *C. pyrenoidosa* > *C. lunula* > *S. obliquus* with an initial Cu concentration of 50 $\mu\text{g}/\text{l}$ (Fig. 3). Growth of *S. obliquus* was most inhibited and its exponential growth phase was postponed (Fig. 3). When Cu added was increased from 50 to 200 $\mu\text{g}/\text{l}$, the growth of *C. lunula* was also inhibited and the lag growth phase was prolonged (Fig. 3).

3.3. Removal of Cu from medium by microalgae

Concentrations of Cu remained in the medium were first rapidly and then slowly reduced during the 6-d experimental period (Fig. 4). When initial Cu concentration was 50 $\mu\text{g}/\text{l}$, 95%, 79% and 67% Cu were removed by *C. pyrenoidosa*, *C. lunula* and *S. obliquus*, respectively, after 6 d of exposure. Removal of Cu by *C. lunula* from the medium was proportional to the initial Cu concentration (Fig. 4).

Fig. 3. The growth process of microalgae at different initial levels of Cu.

Fig. 4. The removal of Cu from the medium with the growth of microalgae.

3.4. Accumulation amount of Cu

The amount of Cu accumulated by microalgae reached a maximum after 1 d but quickly decreased after 2 d followed by a slow decrease at the next 6 d (Fig. 5). Accumulation of Cu by *C. lunula* was much less than those of the other two species. The amount of Cu accumulated by *C. lunula* was proportional to Cu concentration in the medium (Fig. 5).

Fig. 5. The change of accumulation amount of Cu by microalgae. The accumulation amount refers to the Cu bound in cell-wall adhering precipitate plus accumulated Cu in the cells.

3.5. Bioconcentration factor of Cu

BCF reached its first peak after 1 d and then decreased at the beginning of exponential growth phase (Fig. 6). After 3 d, BCF was almost constant except when the growth of *C. pyrenoidosa* and *C. lunula* approached the stationary growth stages, where an increase in BCF occurred (Figs. 3 and 6).

Fig. 6. The change of BCF of Cu. BCF was the ratio between the accumulation amount of copper by microalgae and the level of Cu in the medium at different time intervals.

4. Discussion and conclusion

4.1. Relationship between toxicity and accumulation amount

C. lunula was the most tolerant to Cu (Figs. 1 and 2), which accumulated the least Cu among the three microalgae studied especially in 1 d (Fig. 5). The great initial adsorption of Cu may cause serious damage to the algal cells, which may be responsible for the lower values of 96 h EC50 and greater sensitivities of *C. pyrenoidosa* and *S. obliquus*. However less accumulated Cu at similar initial Cu levels did not damage *C. lunula* because of its ability to exclude Cu. It was reported that the tolerance of *Dunaliella* to heavy metals was due to its lesser uptake of heavy metals (Gimmler et al., 1991). *Enteromorpha*, which is frequently used as an indicator species for heavy metal contamination, contained relatively great concentration of Cu (Rijstenbil et al., 1998a,b). The chelation removal of heavy metals from the cell surface after the binding of Cu with organic complexes (including cell exudates) is another possible mechanism for the detoxification of metal ions (Xue et al., 1988; Xue and Sigg, 1990; Rijstenbil et al., 1994; Gerringa et al., 1995).

The tolerance of *C. lunula* to Cu may be due to physiology. When the initial Cu level was 198.7 µg/l, the amount of Cu accumulated by *C. lunula* was greater than that of *C. pyrenoidosa* or *S. obliquus* exposed to 50 µg Cu/l (Fig. 5). However, its growth was not significantly inhibited (Fig. 3), indicating that *C. lunula* may have other mechanisms to detoxify Cu. It has been reported that production of glutathione and other thiols as the copper-complexing ligands were promoted by algae under the stress of accumulated Cu (Rijstenbil and Wijnholds, 1996; Leal et al., 1999). SOD, providing antioxidant protection in algae, increased during the first day of exposure to Cu (Okamoto and Colepicolo, 1998).

4.2. Accumulation amount of Cu

Smaller cells have relatively greater surface area and more sites for binding metals than those of larger cells, so accumulation amount of Cu in smaller cells are generally greater than those in larger cells (Khoshmanesh et al., 1997). Our results supported this conclusion where accumulation of Cu by algae was less in larger cells (*C. pyrenoidosa* > *S. obliquus* > *C. lunula*; Figs. 4–6).

4.3. Accumulation kinetics

The long-term accumulation of Cu by microalgae depends on the processes of the fast passive adsorption, slow active uptake and desorption (Kuyucak and Volesky, 1989; Knauer et al., 1997). The fast passive adsorption was responsible for the first peaks of both

accumulation amount and BCF of Cu by microalgae at day 1 (Figs. 5 and 6). Accumulation of Cu by microalgae was diluted by the rapid growth of microalgae. The stress of Cu absorbed by microalgae can stimulate the secretion of organic ligands that can bind and exclude heavy metal ions from cells, which may contribute to the decrease of BCF during the exponential growth phase (Fig. 6). The increase of BCF during the stationary growth phase may be due to the death of algal cells, which were losing active desorption abilities. Dead algal cells can uptake more metals than living cells because of the losing the desorption ability of metals (Hassall, 1962). Since the growth of *S. obliquus* was more sensitive to Cu (Figs. 1 and 2), the time for reaching exponential growth phase and the minimum BCF was delayed (Figs. 3 and 6). Desorption ability of microalgae was tightly related to the growth phase of microalgae. pH of medium slightly increased from initial 7.0 to about 8.0 during the 6-d period, which caused the change of Cu speciation.

Growth rate is a dominant factor to determine the biodegradation of organic compound by *C. pyrenoidosa* (Yan et al., 1995). Our finding indicated that the growth phase also played an important role in determining active desorption ability of Cu by microalgae. The results presented here can explain why Cu accumulation was less during the spring or summer but greater in the autumn or winter (Leivuori and Vallius, 1998). The exponential growth of microalgae generally appeared in the spring or summer. In short-term, the adsorption equilibrium of metals by microalgae followed the Freundlich or Langmuir isotherm. But for long-term exposure, a different model needs to be studied to fit the accumulation data.

4.4. Conclusion

(1) *C. lunula* was the most tolerant while *S. obliquus* most sensitive to Cu. Lesser initial accumulation of Cu might be responsible for a greater tolerance of microalgae to Cu. (2) Three microalgal species tested were able to desorb more Cu during the exponential growth phase but less in the stationary growth phase. (3) Microalgae with smaller cell volume but greater sensitivity to Cu are recommended for the efficient removal of Cu from wastewater.

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