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Accumulation, assimilation and growth inhibition of copper on freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) in the presence of EDTA and fulvic acid

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Abstract

Accumulation and growth inhibition of Cu to fresh water alga (*Scenedesmus subspicatus* 86.81 SAG) and the influences of ethylenediaminetetraacetic acid (EDTA) and fulvic acid (FA) were examined. These results demonstrated that both EDTA and FA could reduce toxicity of Cu to alga by the way of preventing Cu from being adsorbed by cell wall of alga. When dissolved Cu ($Cu_{\text{dissolved}}$), extracellular Cu ($Cu_{\text{extracellular}}$), and intracellular Cu ($Cu_{\text{intracellular}}$) were differentiated, our results showed that the concentration level of extracellular Cu ($[Cu_{\text{extracellular}}]$) was a good indicator for measuring the toxic effects of Cu on alga growth in complex matrix. Either in the absence or in the presence of EDTA and FA, the concentration of intracellular Cu increased to $0.6\text{--}1.5 \times 10^{-8} \mu\text{M}$ per cell when the growth inhibition reached to about 50%. We found that the acute toxicity of copper on unicellular alga could be interpreted by its accumulation at a discrete site or biotic ligand at alga cell wall and critical accumulation of Cu associated with EC_{50} was determined to be $1 \times 10^{-8} \mu\text{M}$ per cell. Therefore, the Biotic Ligand Model (BLM) could be extended to predict the influence of copper on growth inhibition of alga.

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1. Introduction

Copper is ubiquitous in the environment. Its presence in aquatic systems arises from a variety of

point and non-point source discharges, of both naturally occurring and man-made origin. Although Cu is an essential element, which is required for the proper sustenance of all plants and animals, including aquatic life (Lee and Allen, 1998; Landner and Lindstrom, 1999; Sigg et al., 1999), at elevated levels Cu becomes toxic. Therefore, Cu contamination has drawn great attention in the past few decades. Regulating the discharge

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of Cu to the environment is vitally important. To develop water quality criteria (WQC) for Cu, it requires an understanding of how much Cu is discharged in the environment and how much actually causes toxic effects in the environment.

It has become apparent that the toxic effects of heavy metals towards aquatic organisms depend not only on their concentrations but also on the forms of their occurrence. Predicting metal toxicity through certain kinds of indicator is, therefore, the pursuing of researchers. In previous studies, much work indicated that the activity of free Cu ions in bulk aqueous phase is a good predictor for Cu toxicity to many biological species (Sunda and Guillard, 1976; Anderson and Morel, 1978; Brand et al., 1986; Moffett and Brand, 1996). The Cu uptake has, therefore, been described by the free-ion-activity model (FIAM; Morel and Hering, 1993), which has been interpreted to imply that a constant degree of biological effect will occur at a constant chemical activity of the free metal ion, independent of other water quality parameter. However, the free-ion activity does not appear to be a good predictor of toxicity across some water-quality conditions (Campbell, 1995). The recent development on the prediction of Cu toxicity was based on the Biotic Ligand Model (BLM; International Copper Association, 2000), in which free-ion activity is a necessary but not sufficient component to describe metal accumulation and toxicity. The toxicity of metals to organisms is assumed to occur as the result of free metal ion reaction with the physiologically active binding sites and the accumulation at the binding sites is controlled by the free Cu concentration in aqueous phase. By using the BLM, accumulation of Cu at the biotic ligand at a critical level can be used to predict Cu toxicity over a broad range of water quality conditions. BLM has been developed for fathead minnows, *Daphnia pulex* and *Ceriodaphnia dubia*, based on the assumption that acute toxicity of Cu should be interpreted by its accumulation at specific sites on the cell membrane (International Copper Association, Ltd., 2000). BLM has not been extended to predict the toxicity of Cu to unicellular alga.

An alternative in predicting toxicity of metals in complex matrix is the use of internal dose. Toxic effects are observed in aquatic organisms when the body burden of toxic metals reaches some critical level. The advantage of using internal dose is that it may avoid the interferences of matrix effects from biological accumulation. But if the chemical substance acts through a different mode of toxic action, the same level of toxicity occurs at lower internal dose exposed to biota (Jarvinen and Ankey, 1999).

In this study, unicellular freshwater alga (*Scenedesmus subspicatus* 86.81 SAG) was used to evaluate the toxicity of Cu in the presence of EDTA, a synthetic organic ligand and FA, a typical natural existing organic ligand. The aim of this study was to determine toxic predictor among different forms of Cu in aqueous phase and to differentiate whole body burden from extracellular Cu concentration to elucidate the mode of toxic action in freshwater alga. Through the results, both external and internal dose–effect relationship were compared.

2. Materials and methods

2.1. Reagents and plasticware

All plastic ware and glassware were soaked for at least 24 h in 10% HNO₃, thoroughly rinsed with ultrapure water (ELGA, UK). All the chemicals used for cultures and experiments were of analytical grade or better. The culture medium and all the other solutions were prepared with ultrapure water. Stock solution of Cu was prepared from analytical grade CuSO₄ · 5H₂O (Backer Analyzed Reagent, J.T. Baker, The Netherlands) and diluted to test solution on the day of use from the stock. Stock solution of EDTA was prepared from its sodium salt (Fluka ACS, Germany) and diluted 100-fold for preparation of test solution. The FA was extracted from the sediment collected from Guanting Reservoir in Beijing, China and was purified by following the method described previously (Wang, 1987). The test solution was prepared from stock solution of Cu, stock

solution of EDTA or FA, as well as culture medium.

2.2. Bioassays

The bioassays were carried out following the method of OECD-guideline 201 (OECD, 1984). Unicellular freshwater green alga was kindly provided by Netherlands Organization for Applied Scientific Research (TNO). Culture medium for alga was prepared according to OECD-guideline 201 (OECD, 1984) and pH was adjusted to 8.0. Alga was grown at a photon irradiance of approximately $120 \mu\text{M m}^{-2} \text{s}^{-1}$ (cool white fluorescent tubes) in a 12 h light: 12 h dark regime by constant shaking at 100 rpm and temperature of $25 \pm 2^\circ\text{C}$. The bioassays were performed in 250-ml polycarbonate flasks (Erlenmeyer flasks from NALGENE®) to minimize adsorptive losses of Cu to the wall of containers, and over as short as possible time period (72 h) to minimize possible effects of algal exudates.

To prepare the test solution, 1-ml stock solution of EDTA and 1-ml stock solution of Cu were added to a 250-ml polycarbonate flask and the final volume was adjusted to 100-ml with OECD-medium. In the experiments, two different levels of EDTA (17 and $34 \mu\text{M}$) and three levels of FA (1.0, 2.5 and 5.0 mg l^{-1}) were used, respectively. Each treatment consisted of five concentration levels of Cu and a control. Four replicates of test solution for each concentration level of Cu or control were prepared. One flask in each treatment was used for the determination of the total added Cu concentration and the remaining three flasks were added with alga and were used for bioassay and the differentiation of Cu. In the treatments with EDTA and the treatment with Cu alone, Cu concentrations ranged from 10 to $160 \mu\text{M}$ by a 0.5-fold dilution series. However, in the presence of FA, as precipitation occurred at high Cu concentration, concentrations of Cu were tested between the range from 2.5 to $40 \mu\text{M}$ by a 0.5-fold dilution series.

The test cultures were inoculated with exponential-phase algal cells from the stock culture. The initial cell concentration was approximately 2×10^4 cells per ml. The absorbance of alga suspen-

sion was measured at 625 nm on a spectrophotometer (HITACHI U-1100) at 24, 48 and 72 h after the start of the culture. The cell density was obtained using a calibration curve of absorbance versus cell number, which was counted by using a microscope. The pH of solution was checked after 72 h to ensure that it did not vary by more than 1.0 U.

The percentage of growth inhibition in relation to the control was calculated from the total amount of Cu in the culture. The concentration that leads to 50% growth reduction was calculated according to OECD-guideline 201 (OECD, 1984) and was recorded as EC_{50} .

2.3. Chemical measurements and differentiating forms of Cu

The method we used to distinguish the extracellular Cu and intracellular Cu was followed the procedure described by Franklin et al. (2000). They used 0.02 M EDTA to remove the Cu bound to *Chlorella* sp. and found EDTA treatment did not cause cell lysis. Shortly, at the end of the bioassay, 40.0 ± 0.1 g of suspension from each flask was centrifuged for 20 min at 3500 rpm and 10-ml of supernatant was transferred into a polycarbonate tube. Concentrated HNO_3 was added to the solution to a final concentration of 3%, followed by HR-ICP-MS (Finnigan MAT Element, Germany) measurement to measure the dissolved Cu concentration. The suspension from each flask was centrifuged for 5 min at 3500 rpm in order to obtain a distinct algal pellet. The supernatant was discarded and the algal pellet was re-suspended in 15-ml of 0.02 M EDTA. The suspension was shaken for approximately 30 s to remove Cu adsorbed on the cell walls then centrifuged for another 20 min at 4000 rpm. The supernatant was used to measure the extracellular Cu concentration by HR-ICP-MS. The algal pellets were left to dry and then acid-digested with 2-ml of concentrated HNO_3 at 90°C over night. The samples were diluted by ultrapure water until the HNO_3 is approximately 3% and then analyzed for intracellular Cu concentration by HR-ICP-MS.

2.4. Quality control

In the HR–ICP–MS measurement, calibration was made from standard solution containing 1000 mg l⁻¹ of Cu stock solution (Perkin–Elmer Co., the Netherlands). ¹¹⁵In was used as an internal standard which was for optimizing the operating parameters and ion lens voltages. Ultrahigh sensitivity element CD was used to achieve the highest sensitivity for the determinations at ng l⁻¹ range in complicated sample matrices. In order to reduce the risk of contamination, sample manipulation was carried out in a clean bench. Before each measurement, all container and chemical were checked for background contamination. Nebulizer, spray chamber, torch, Teflon tubes etc. were cleaned with HNO₃ (7 M, 24 h) and rinsed with ultrapure water (18.2 MΩ cm⁻¹). Recoveries were checked with standard addition of Cu. A 3% v/v HNO₃ (65% Merck, suprapur) solution was used for washing the tubes in order to remove any contamination and ‘memory effect’ prior to sample and standard introduction to the HR–ICP–MS. Typical instrument conditions for the HR–ICP–MS and sample introduction system were listed in Table 1.

3. Results and discussion

Percentages of growth inhibition and concentrations of different forms of Cu with and without EDTA were shown in Table 2. From the results,

Table 1
Instrument condition for the HR–ICP–MS and sample introduction system

Sample (Nebulizer) gas flow rate	1.05 l min ⁻¹
Auxiliary gas flow rate	1 l min ⁻¹
Plasma gas flow rate	16.9 l min ⁻¹
RF power	1240
Sample liquid flow rate	50 μl min ⁻¹
Resolution mode	Low and middle
Resolution	300 and 3000
Scanning mode	E-scan
Measurement time	0.10
Measurements per peak	20
Numbers of scans × passes	5 × 5

algal growth was significantly inhibited when total concentration of Cu in the culture was increased. The correlation coefficients between the percentage of inhibition and total Cu concentrations were 0.937, 0.865 and 0.960 corresponding to EDTA levels of 0, 17 and 34-μM. Significant correlations could also be found between the percentage of inhibition and dissolved Cu concentration or extracellular Cu concentration. In fact, both dissolved and extracellular Cu concentrations increased significantly with the increase of total Cu concentration at different concentration levels of EDTA ($r > 0.97$). In the treatments containing 17 and 34 μM of EDTA, more Cu remained in aqueous phase and the concentration of extracellular Cu was significantly reduced. As EDTA and divalent metal forms 1:1 complex in aqueous phase and the complex is non-bioavailable for alga adsorption or absorption (Allison and Brown, 1991) it was reasonable that the increase of extracellular Cu occurred at higher total Cu concentration when concentration of EDTA was higher.

In Table 2, total Cu concentrations increased from about 10–160 μM and a gradual increase in algal growth inhibition was observed. The calculated 72-h EC₅₀ in the absence of EDTA was 5.4 μM. The inhibition effect was less pronounced in the presence of EDTA. Increasing EDTA concentration from 17 to 34 μM resulted in a substantial increase in the 72-h EC₅₀ from 31 to 60 μM. Our results jointed the vast evidences that the presence of EDTA reduced the toxicity of Cu (Wong et al., 1995; Hansten et al., 1996; Campbell et al. 2000).

It could be quite interesting to see from Table 2 that the concentration of intracellular Cu increased from about $1.0\text{--}1.8 \times 10^{-8}$ μM per cell when the inhibition reached to about 50% either in the absence or in the presence of different concentrations of EDTA. The result clearly indicated that the growth inhibition of alga was closely related to the binding of Cu on alga cell wall and the translocation of Cu crossing cell membrane was retarded when concentrations of intracellular Cu reached about 1.0×10^{-8} μM per cell.

Growth inhibition and concentrations of different forms of Cu in the presence of FA were shown in Table 3. The natural derived FA had similar

Table 2

Concentrations of different Cu forms and growth inhibition in the presence of EDTA

[EDTA] (μM)	[Cu] _{total} (μM)	[Cu] _{dissolved} (μM)	[Cu] _{extracellular} ($\times 10^{-8}$ μM per cell)	[Cu] _{intracellular} ($\times 10^{-8}$ μM per cell)	Growth inhibition (%)	ΔpH
0	10.5	4.0 $\pm$ 0.5	1.6 $\pm$ 0.1	1.4 $\pm$ 0.1	57	0.57
	20.8	7.4 $\pm$ 0.6	5.1 $\pm$ 2.9	1.8 $\pm$ 0.1	63	0.61
	40.6	8.6 $\pm$ 0.9	17.3 $\pm$ 0.6	1.6 $\pm$ 0.2	72	0.58
	80.7	11.3 $\pm$ 0.5	62.2 $\pm$ 4.4	1.0 $\pm$ 0.3	92	0.63
	156.1	17.8 $\pm$ 0.1	148.1 $\pm$ 9.7	1.5 $\pm$ 0.5	99	0.73
17	11.5	9.7 $\pm$ 0.1	0.3 $\pm$ 0.0	0.1 $\pm$ 0.1	1	0.09
	24.6	18.0 $\pm$ 0.9	0.8 $\pm$ 2.9	0.7 $\pm$ 0.1	34	0.14
	46.0	26.3 $\pm$ 1.5	4.2 $\pm$ 0.1	2.0 $\pm$ 0.2	60	0.20
	90.2	26.3 $\pm$ 0.2	33.3 $\pm$ 3.2	0.7 $\pm$ 0.2	85	0.20
	176.3	37.4 $\pm$ 1.5	94.4 $\pm$ 4.0	1.1 $\pm$ 0.4	95	0.35
34	9.0	9.3 $\pm$ 0.0	0.2 $\pm$ 0.0	0.1 $\pm$ 0.0	–1	0.20
	18.8	19.7 $\pm$ 0.3	0.4 $\pm$ 0.0	0.1 $\pm$ 0.0	–5	–0.30
	36.9	36.8 $\pm$ 0.9	1.2 $\pm$ 0.0	0.8 $\pm$ 0.0	33	0.94
	73.2	44.5 $\pm$ 1.5	17.6 $\pm$ 1.0	1.5 $\pm$ 0.4	57	0.56
	146.9	53.0 $\pm$ 3.0	65.0 $\pm$ 12.3	1.6 $\pm$ 0.6	91	0.86

effects of Cu adsorption and growth inhibition on alga as those of EDTA. When FA concentration increased, the growth inhibition and extracellular Cu concentration were reduced, whereas the dissolved Cu concentration increased. For example, at about 20 μM of total Cu, the percentage of inhibitions were 67, 53 and 48% in the treatments containing 1, 2.5 and 5 mg l^{-1} of FA, respectively. At the same time, concentrations of dissolved Cu

were 9.6, 15.0 and 15.2 μM , respectively, in the treatments containing 1, 2.5 and 5 mg l^{-1} of FA. The result supports the finding that the toxicity of Cu, when regarding to its total concentration, was conditional and varied with concentration of dissolved organic matter in aquatic environment (Allen and Hansen, 1996). From Table 3, one can also noted that intracellular Cu concentration increased when concentration of FA increased

Table 3

Concentrations of different Cu forms and growth inhibition in the presence of FA

[FA] mg l^{-1}	[Cu] _{total} (μM)	[Cu] _{dissolved} (μM)	[Cu] _{extracellular} ($\times 10^{-8}$ μM per cell)	[Cu] _{intracellular} ($\times 10^{-8}$ μM per cell)	Growth inhibition (%)	ΔpH
1	2.5	0.7 $\pm$ 0.1	0.5 $\pm$ 0.1	0.2 $\pm$ 0.1	30	–0.07
	4.8	1.72 $\pm$ 0.34	1.1 $\pm$ 0.1	0.4 $\pm$ 0.1	37	0.23
	8.8	4.45 $\pm$ 0.1	2.4 $\pm$ 0.1	0.6 $\pm$ 0.1	56	0.37
	19.0	9.58 $\pm$ 0.9	5.8 $\pm$ 0.9	0.7 $\pm$ 0.0	67	0.25
	37.0	12.37 $\pm$ 1.9	26.4 $\pm$ 1.8	0.9 $\pm$ 0.1	82	0.47
2.5	2.4	1.02 $\pm$ 0.0	0.4 $\pm$ 0.0	0.3 $\pm$ 0.1	8	0.22
	4.9	1.73 $\pm$ 0.1	0.9 $\pm$ 0.1	0.6 $\pm$ 0.2	28	0.41
	10.5	5.39 $\pm$ 0.2	1.6 $\pm$ 0.1	1.1 $\pm$ 0.0	40	0.46
	21.1	15.04 $\pm$ 0.9	2.8 $\pm$ 0.3	1.2 $\pm$ 0.2	53	0.58
	41.7	20.36 $\pm$ 0.9	12.3 $\pm$ 1.6	1.1 $\pm$ 0.2	63	0.85
5.0	2.3	1.4 $\pm$ 0.7	0.35 $\pm$ 0.3	0.11 $\pm$ 0.1	–2	–0.14
	4.8	4.3 $\pm$ 0.1	0.67 $\pm$ 0.4	0.32 $\pm$ 0.1	0	0.29
	9.6	6.2 $\pm$ 1.9	1.46 $\pm$ 0.7	0.70 $\pm$ 0.1	30	–0.23
	20.0	15.2 $\pm$ 2.2	3.77 $\pm$ 1.5	1.24 $\pm$ 0.2	48	–0.05
	38.5	17.6 $\pm$ 0.3	17.51 $\pm$ 1.8	1.88 $\pm$ 0.4	60	0.07

and it increased to about $0.6\text{--}1.2 \times 10^{-8}$ μmol per cell with a mean value of 0.9×10^{-8} μmol per cell when the inhibition reached to about 50% in the presence of different levels of FA. This value is quite close to that in EDTA treatments, where the increase of intracellular Cu was retarded at 1.0×10^{-8} μM per cell.

In our experiments, both intracellular and extracellular Cu concentrations increased obviously with increase of added Cu before intracellular Cu reached 1×10^{-8} μM per cell and Cu concentration reached EC_{50} . However, intracellular Cu no longer increased when extracellular Cu increased very rapidly at EC_{50} . Therefore, the inhibition of algal growth or acute toxicity was rather caused by binding of Cu on algal cell wall than by binding at intracellular active sites. This phenomenon was quite similar to that observed for fish, where the site of action of acute toxicity of Cu is the gill cell (McDonald et al., 1989).

It is well known that Cu toxicity to freshwater biota is markedly affected by water quality variables, such as pH, water hardness and dissolved organic carbon concentration including biologically produced organic ligands (Xue et al., 1996). These factors modify toxicity by either increasing or decreasing the bioavailable concentration required to produce a particular biological response (Chapman, 1995). Factors other than those in aquatic environment may also affect the acute toxicity. For example, Cu toxicity may also be reduced due to detoxification pathway of Cu by the algal cell. Certain algal species, including *Scenedesmus acutiformis*, *Chlorella fusca* and *Fucus vesiculosus*, have been shown to release strong ligands that reduce the bioavailability of Cu in aqueous phase if they are released in sufficient amounts (Robinson, 1989; Gledhill et al., 1999; Rijstenbil and Gerringa, 2002). Cell wall exclusion of Cu and intracellular detoxification by binding to phytochelators or polyphosphate have also been demonstrated in a variety of freshwater algal species in response to elevated Cu concentrations (Twiss et al., 1993; Knauer et al., 1997). However, exudation and detoxification effects were not considered in our experiment.

It was shown in Fig. 1a that when the growth inhibition was plotted versus the $[\text{Cu}_{\text{dissolved}}]$, the

Fig. 1. (a) Influence of EDTA on the growth inhibition of *S. Subspicatus* with the increasing of dissolved Cu concentration (b) Influence of EDTA on the growth inhibition of *S. Subspicatus* with the increasing of extracellular Cu concentration.

dose–effect relationships representing three concentration levels of EDTA shifted rightward with the increase of EDTA concentration. However, in Fig. 1b when the curves were plotted as a function of $[\text{Cu}_{\text{extracellular}}]$, these three curves gave almost constant prediction of toxicity. A similar observation was found in the case of FA (Fig. 2a, b), which the dose–effect relationships representing four levels of FA could be constant when plotted as a function of $[\text{Cu}_{\text{extracellular}}]$.

EC_{50} s calculated by extracellular Cu concentrations were within the range of $1.2\text{--}5.4 \times 10^{-8}$ μM per cell no matter what the EDTA and FA concentrations were. However, at the three different EDTA levels tested, when EC_{50} s were calculated by total added Cu concentrations, they were

Fig. 2. (a) Influence of FA on the growth inhibition of *S. Subspicatus* with the increasing of dissolved Cu concentration (b) Influence of FA on the growth inhibition of *S. Subspicatus* with the increasing of extracellular Cu concentration.

5.4, 31 and 60 μM, respectively, while, when EC₅₀s were calculated by the dissolved Cu concentrations, they were 4.1, 23 and 42 μM, respectively. Large variations in EC₅₀s based on total Cu concentrations and dissolved Cu concentrations were obtained from the tests with addition of FA as well. Therefore, it can be concluded that EC₅₀s calculated by extracellular Cu concentrations were approximately constant in comparing with the EC₅₀s calculated by total added Cu concentrations and EC₅₀s calculated by dissolved Cu concentrations. Thus, [Cu_{extracellular}] is probably a good toxicity predictor in varies concentrations of organic ligands.

All the results of this study demonstrated that both EDTA and FA could reduce toxicity of Cu to freshwater alga *S. subspicatus* 86.81 SAG by the way of preventing Cu from being adsorbed by algal cell. The results also support the applicability of BLM in predicting Cu toxicity because the binding sites on the algal cell (walls and/or

membranes) are most likely similar to those on fish gill (Pagenkopf, 1983). According to the BLM, acute toxicity of Cu on alga can be described as Cu accumulation at a discrete site of alga cell wall or biotic ligand. Concentration of extracellular Cu is probably a better indicator because it does not need to consider the matrix effects in aqueous phase. Meyer et al. (1999) used fathead minnows (*Pimephales promelas*) to evaluate Cu toxicity and found that concentration of Cu bounded on fish gill ([Cu_{gill}]) was constant predictors of toxicity when the Ca concentration increases, whereas the free-ion activities of Cu are not constant predictors of toxicity. Di Toro (2001) used larval fathead minnow to observe the effect of variations in DOC concentration on LC₅₀ of Cu and found that the gill Cu concentration can be used to predict acute mortality. In our study, it was found that extracellular Cu, other than total Cu concentration and dissolved Cu concentration is of close relationship with Cu toxicity to alga and is approximately a good predictor of Cu toxicity to freshwater alga in the presence of organic ligand. The critical accumulation of Cu associated with EC₅₀ was determined to be 1×10^{-8} μM per cell.

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