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EFFECT OF INITIAL CELL DENSITY ON THE BIOAVAILABILITY AND TOXICITY OF COPPER IN MICROALGAL BIOASSAYS

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Abstract—Algal toxicity tests based on growth inhibition over 72 h have been extensively used to assess the toxicity of contaminants in natural waters. However, these laboratory tests use high cell densities compared to those found in aquatic systems in order to obtain a measurable algal response. The high cell densities and test duration can result in changes in chemical speciation, bioavailability, and toxicity of contaminants throughout the test. With the recent application of flow cytometry to ecotoxicology, it is now possible to use lower initial cell densities to minimize chemical speciation changes. The speciation and toxicity of copper in static bioassays with the tropical freshwater alga *Chlorella* sp. and the temperate species *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*) were investigated at a range of initial cell densities (10^2–10^5 cells/ml). Copper toxicity decreased with increasing initial cell density. Copper concentrations required to inhibit growth (cell division) rate by 50% (72-h median effective concentration [EC50]) increased from 4.6 to 16 μg/L for *Chlorella* sp. and from 6.6 to 17 μg/L for *S. capricornutum* as the initial cell density increased from 10^2 to 10^5 cells/ml. Measurements of anodic stripping voltammetry–labile, extracellular, and intracellular copper confirmed that at higher initial cell densities, less copper was bound to the cells, resulting in less copper uptake and lower toxicity. Chemical measurements indicated that reduced copper toxicity was due primarily to depletion of dissolved copper in solution, with solution speciation changes due to algal exudates and pH playing a minor role. These findings suggest that standard static laboratory bioassays using 10^4 to 10^5 algal cells/ml may seriously underestimate metal toxicity in natural waters.

Keywords—Algae Copper Bioavailability Bioassay Flow cytometry

INTRODUCTION

Algal bioassays are routinely employed as part of a battery of toxicity tests to assess the environmental impacts of contaminants on aquatic ecosystems. Standard tests measure the decrease in growth rate (cell division rate) or final cell biomass (cell yield) after a 48- to 96-h exposure. Growth endpoints are the basis of most chronic algal toxicity tests and are particularly environmentally relevant because changes in population growth may influence species succession and community structure and function.

Several protocols for algal toxicity testing are currently available [1,2]. In these methods, experimental variables, including test inoculum, light intensity, temperature, duration, and media composition, are standardized to increase inter- and intralaboratory test precision. While significant attention has been given to the way in which incubation conditions [3,4], test duration [5,6], and test media [7,8] influence the sensitivity of laboratory bioassays, the effect of initial cell density on the algal response to toxicants has been less studied. Evidence exists that the sensitivity of a toxicity test increases with decreasing initial cell density [9–11]. Steemann Nielsen and Kamp-Nielsen [12] referred to this phenomenon as the biomass effect and concluded that copper toxicity decreased as the concentration of cells in suspension increased. Most test protocols recommend an initial cell density of 10^4 to 10^5 cells/ml, as it is considered a compromise between maximizing the sensitivity of the bioassay and having sufficient cells to determine cell density changes over the duration of the test [13]. Wong and Couture [14] and more recently Blaise and Ménard [15] recommended an algal inoculum of 10^5 and 10^6 cells/ml, respectively, which is several orders of magnitude higher than that found even in algal bloom conditions in natural waters. Because algal cells divide daily, final cell biomass after 2- to 4-d growth tests is even higher, and this can lead to binding of the test substance with the increasing number of cells [16]. This may be particularly important in tests with highly toxic elements at low concentrations [16]. Algal metabolism at high cell densities can also cause pH drift from CO_2 depletion and subsequent chemical alteration of the test substance [17]. Increased exudate production from an increasing number of cells may also influence chemical speciation through the formation of nontoxic Cu-exudate complexes [18].

While it is now well established that the physicochemical form of a metal (speciation) is a critical factor controlling metal bioavailability and hence toxicity in natural waters, fewer studies have investigated the effect of the algae themselves on the speciation of metals in solution. To date, this issue has not been addressed in standard algal bioassays, largely because of the difficulty of counting cells at low cell densities. The lowest cell concentration able to be counted using conventional techniques, such as the microscope and automatic particle counters (e.g., Coulter Counter, Toronto, ON, Canada), is typically 10^4 cells/ml. With the recent application of flow cytometry to algal toxicity testing [19,20], the capability now exists to carry out toxicity tests at much lower cell densities to minimize speciation changes.

Flow cytometry allows rapid, multiparameter analysis of individual cells in a moving fluid [19,20]. This technique has the ability to analyze thousands of cells per second and has sufficient sensitivity to analyze cell densities that are more typical of algal concentrations in natural systems (10^2 cells/

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ml). It also has the advantage over conventional counting techniques in that it can differentiate live from dead algal cells and other particles on the basis of chlorophyll *a* fluorescence (autofluorescence) and light scattering, thereby eliminating background particle interference.

The main objective of the present study was to investigate the effect of initial cell density (10^2–10^5 cells/ml) on the toxicity of copper to two freshwater chlorophytes. *Selenastrum capricornutum* (*Pseudokirchneriella subcapitata*) is commonly used as a toxicity test species in North America, and *Chlorella* sp. is a standard tropical test species used in Australasia that is particularly sensitive to copper [13]. Results from previous research indicate that algal bioassays carried out at higher initial algal cell densities lead to less metal bound per cell, resulting in lower toxicity [16,17]. However, this will be true only if dissolved copper in solution becomes limiting. A relationship between cellular metal content and the toxicity response at different algal cell densities has not yet been demonstrated.

For copper to exert an adverse effect on cells, it must first bind to receptors on the cell surface, followed by transport across the membrane and release into the cytoplasm [21]. A second objective was to compare extracellular (membrane-bound) and intracellular copper concentrations at each cell density (10^3–10^5 cells/ml) to determine whether toxicity was proportional to the amount of copper associated with the cell at each cell density. Copper speciation was determined by geochemical modeling and direct chemical measurement using anodic stripping voltammetry (ASV) to predict whether potentially bioavailable copper differed at the different cell densities. The overall aim was to improve current algal growth bioassays to reduce metal speciation changes associated with metal–cell interactions, so that metal bioavailability determined in algal toxicity tests more closely represents metal bioavailability in natural aquatic systems.

MATERIALS AND METHODS

Algal cultures

Selenastrum capricornutum (more recently called *P. subcapitata*) was obtained from the American Type Culture Collection (Rockville, MD, USA) and cultured axenically in U.S. Environmental Protection Agency (U.S. EPA) medium [2] on a 24-h light cycle (Philips TL 40-W cool-white fluorescent lighting, Danvers, MA, USA; 70 μmol photons/m^2/s) at 24°C. *Chlorella* sp. 12 was isolated from Lake Aesake, Strickland River, Papua New Guinea. The culture was maintained axenically in JM/5 media [22] on a 12:12-h light:dark photo cycle (Philips TL 40-W cool-white fluorescent lighting, 75 μmol photons/m^2/s) at 27°C.

Growth inhibition bioassays

For *S. capricornutum*, the test medium (used for diluent water and controls) consisted of the standard U.S. EPA media without ethylenediaminetetracetic acid (EDTA), with an alkalinity of 9 mg $CaCO_3$/L and water hardness of 15 mg $CaCO_3$/L. Temperature and light conditions for the toxicity tests were identical to those used for culture maintenance. For *Chlorella* sp., a synthetic soft water [2] having a water hardness of 80 to 90 mg $CaCO_3$/L and an alkalinity of 54 mg $CaCO_3$/L was supplemented with nitrate (15 mg NO_3^-/L) and phosphate (0.15 mg PO_4^{3-}/L). Toxicity tests for *Chlorella* sp. were conducted at a higher light intensity of 140 μmol photons/m^2/s. For both species, the pH of the test medium was 7.5 ± 0.2. Cultures were shaken twice daily by hand.

Copper stock solutions (5 and 100 mg/L) were prepared from copper sulfate ($CuSO_4 \cdot 5H_2O$) and acidified to pH <2 by the addition of HCl. Controls, together with at least five metal concentrations (each in triplicate), were prepared. Copper concentrations ranged from 1.25 to 15 μg Cu/L for bioassays at 10^2 cells/ml, from 1.25 to 20 μg Cu/L for bioassays at 10^3 cells/ml, and from 2.5 to 30 μg Cu/L for bioassays at 10^4 and 10^5 cells/ml. Fifty milliliters of toxicity test medium was dispensed into 250-ml borosilicate glass conical flasks, precoated with a silanizing solution (Coatasil, Ajax Chemicals, Auburn, NSW, Australia) to reduce adsorption of metals to the flask walls. All glassware was acid washed in 10% concentrated HNO_3 before use. Subsamples (5 ml) were immediately taken from each flask and acidified prior to determination of total dissolved copper by graphite furnace atomic absorption spectrometry (GFAAS) using a 4100ZL Perkin-Elmer (Norwalk, CT, USA) instrument. Measured copper concentrations were used to calculate toxicity endpoints.

To determine the influence of cell density on the toxicity of copper to *Chlorella* sp., bioassays were conducted at initial cell densities of 4.5 ± 0.8 × 10^2, 2.1 ± 1.4 × 10^3, 3.8 ± 0.4 × 10^4, and 2.6 ± 1.0 × 10^5 cells/ml. For *S. capricornutum*, initial cell densities of 4.0 ± 0.6 × 10^2, 3.9 ± 0.6 × 10^3, 3.7 ± 0.3 × 10^4, and 3.2 ± 0.4 × 10^5 cells/ml were used. Exponentially growing cells of each species were centrifuged and washed three times before use in the bioassay to remove culture medium. The centrifugation speed was 2,500 rpm (Jouan CR4.11) for a duration of 7 min each spin. Each flask was inoculated with a known concentration of prewashed cells to give the initial cell density required. Temperature and pH were monitored throughout the test. Definitive bioassays using the same nominal copper concentrations at each cell density were conducted a minimum of three times. Subsamples (3 ml) from each flask were taken daily and algal cell densities determined by flow cytometry.

Flow cytometric analysis (cell counts)

Algal cell density was determined daily using a Bryte HS Flow Cytometer (Bio-Rad, Richmond, CA, USA) equipped with a xenon ion excitation lamp (488 nm). The flow cytometer measures optical signals such as light scattering and fluorescence of individual cells in a fluid stream. Two light-scatter detectors identify the morphology of the cell. The forward-angle light-scatter (LS1 = <15°) detector provided data on cell size, while the side-angle light-scatter (LS2 = 15–85°) detector provided information on cell size/shape. Chlorophyll *a* or autofluorescence was detected as red fluorescence in FL3 (660–700 nm). Nonalgal particles and dead cells were excluded from the analysis by setting an acquisition threshold on FL3 (positioned to the left of the distribution of healthy control cells). Data were collected in one-dimensional histograms comprising 256 channels. The flow cytometer was operated using a constant flow rate of 20 μl/min and a pressure setting of 0.7 bar. Samples were accumulated for a preset time of 130 s.

The Bio-Rad Bryte flow cytometer is unique in that the cells are counted directly as the area under the curve from a plot of cell number versus chlorophyll *a* fluorescence, without the need for internal calibration with beads. This instrument has shown good agreement (r^2 values > 0.97) with microscope and Coulter counts at cell densities of 10^4 to 10^5 cells/ml and

with theoretical counts (obtained by dividing the highest microscope count by the appropriate dilution factor) at low cell densities of 10^2 to 10^3 cells/ml (data not shown). The algal cell density was determined daily during the 72-h copper exposure. Growth (cell division) rate was determined from the regression slope and expressed in doublings per day.

Cell size measurements

In each toxicity test, changes in cell size were detected in LS1 and measured after a 72-h copper exposure. The mean diameters of *Chlorella* sp. and *S. capricornutum* cells were determined from a flow cytometric calibration curve using spherical latex beads of known diameter (1.5–13.5 μm). For *Chlorella* sp., surface area and volume were then calculated from the measured diameter using the equation of a sphere. Visual measurements of *Chlorella* sp. diameter using phase contrast microscopy confirmed those obtained by flow cytometry.

Estimates of cell surface area and volume for *S. capricornutum* were problematic using flow cytometry because of the arcuate shape of the cells. Confocal microscopy was therefore used to accurately determine the surface area and volume of *S. capricornutum* and a correction factor applied to all flow-cytometric determinations of cell diameter.

Measurement of intra- and extracellular copper concentrations

To investigate the mechanism by which cell density may influence copper toxicity, intra- and extracellular copper concentrations were also determined in at least two definitive experiments at each cell density. Initial cell densities used were 4.0×10^3, 3.8×10^4, and 2.0×10^5 cells/ml for *Chlorella* sp. and 3.4×10^3, 3.9×10^4, and 3.5×10^5 cells/ml for *S. capricornutum*. At the end of each bioassay (72 h), triplicate flasks from each copper concentration, plus controls, were subsampled.

In a Class-100 clean room, a 40-g subsample from each flask was weighed into an acid-washed (50% concentrated HNO_3) Oak Ridge polytetrafluoroethylene (PTFE) centrifuge tube (Nalgene, UK) and centrifuged for 20 min at 3,500 rpm (~2,000 *g*) in a Jouan CR4.11 centrifuge. The supernatant solutions (20 ml) were pipetted into clean, acid-washed (10% concentrated HNO_3) polycarbonate vials and acidified with 40 μl of concentrated HNO_3 (Merck Suprapur, Whitehouse Station, NJ, USA). These samples were analyzed for dissolved copper by GFAAS. The remaining supernatant solution was discarded and the algal pellet resuspended in 20 ml of 0.02-M EDTA and shaken for 30 s to remove any copper bound to the external cell surface [23]. Preliminary experiments confirmed that cells did not rupture during this treatment. The samples were centrifuged for 20 min at 3,500 rpm, and the supernatant was retained for copper analysis by GFAAS. Copper in this fraction was referred to as surface-bound (extracellular) copper. Carryover of copper from the supernatant was typically <5% of the extracellular copper.

The remaining cell pellet was air-dried for 2 d, and 2 ml of concentrated (15 M) HNO_3 were added. After 30 min, cells were acid digested in a microwave oven for 5 min on power setting 1 (90 W). After cooling, the samples were made up to 20 ml with Milli-Q® water (Millipore, Bedford, MA, USA) and analyzed for copper by GFAAS. Copper in this fraction was referred to as intracellular copper. Blank solutions (no algae) were also prepared in each sample batch. To calculate mass balances, copper adsorbed to the walls of the glass bioassay flasks was determined after copper was leached from each flask with 50 ml of 0.03-M HNO_3 overnight. The detection limit for copper by GFAAS was 0.5 μg/L.

It was difficult to obtain data for initial cell densities of 10^2 and 10^3 cells/ml, as insufficient cells were present to form a distinct algal pellet during the algal centrifugation procedure. Eighteen flasks (six per replicate) were used in experiments with 10^3 cells/ml to increase the volume and hence the number of cells analyzed.

Using dissolved copper and surface-bound (extracellular) copper, the copper cell distribution coefficient (K_d) was determined at initial cell densities of 10^3, 10^4, and 10^5 cells/ml as follows:

$$K_d \text{ (L/cell)} = \frac{\text{[extracellular Cu] (μg/cell)}}{\text{[equilibrium dissolved Cu] (μg/L)}}$$

To compare K_d values at the three cell densities, it was necessary to correct for changes in cell size. Extracellular copper was therefore expressed in units of μg/μm² and the resulting units of L/μm². Extracellular copper was plotted versus dissolved copper, and the K_d was determined as the slope of the linear plot (calculated by linear regression).

Measurement of labile copper

In one definitive test at each cell density, labile copper was determined in several copper treatments (10–17.5 μg Cu/L) by ASV. After 72 h, cells were centrifuged, and the supernatant solution was used to determine ASV-labile copper. The PTFE polarographic cells were filled with sample for 30 min to precondition the cell, the solution was discarded, and then 20 ml sample added. To stabilize the pH, 100 μl of 0.5-M *N*-2 hydroxyethylpiperazine-*N*-2-ethanesulfonic acid (HEPES) buffer were added followed by 40 μl of 5-M sodium nitrate. Each sample was spiked with additions of copper so as to cover a range of added copper concentrations from 0 to typically 30 μg/L. Differential pulse anodic stripping voltammetry measurements were made using a Metrohm 646 Voltammetric Analyzer (Berchem, Belgium) with a hanging mercury drop electrode. Samples were stirred and deaerated with nitrogen for 300 s before deposition for 300 s at −0.6 V versus a saturated calomel electrode. A potential scan was initiated (scan rate 3.3 mVs^{-1}, pulse height 50 mV, pulse step 2 mV) and the copper oxidation peak recorded between −0.2 and 0.2 V. The resulting titration plots were analyzed using the Van den Berg/Ruzic graphical method [24]. The amount of copper complexed by algal exudates was determined from the difference between dissolved copper and labile copper [18].

Statistical analysis

Algal growth at each copper concentration was expressed as a percentage of the control for each definitive bioassay (≥ three tests). These data at each cell density were pooled, and the concentration–response curve was plotted using weighted linear regression analysis on probit-transformed data. The EC50 values (effective concentration giving 50% reduction in algal growth rate after 72 h compared to the controls) were calculated from the pooled data using a computer program, NYHOLM-3, which was modified by Yuri Tsvetnenko from Nyholm et al. [25]. For each cell density, EC50 values were also calculated for each definitive test (nonpooled data) and were not significantly ($p > 0.05$) different.

After the data had been tested for normality and homo-

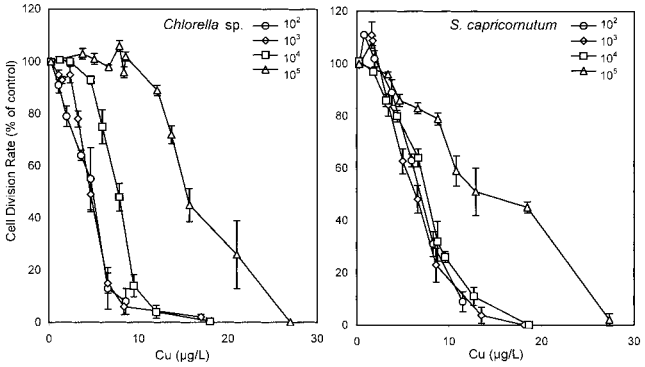

Fig. 1. Growth rate inhibition of *Chlorella* sp. and *Selenastrum capricornutum* exposed to copper for 72 h at initial cell densities of 10^2, 10^3, 10^4, and 10^5 cells/ml. Data points represent the mean ± standard error of mean. Measured copper concentrations were used.

geneity of variance, Dunnett's multiple comparison test was used to determine which concentrations were significantly different to the controls. This enabled estimation of the LOEC (lowest concentration to cause a significant effect on algal growth) and NOEC (highest concentration to cause no significant effect on algal growth) compared with that of the controls. Tests for significance between the EC50 values for each species at the different cell densities were determined using the method described in Sprague and Fogels [26]. Significance levels were tested at the $p = 0.05$ level.

RESULTS

The concentration–response curves for *Chlorella* sp. and *S. capricornutum* to copper at initial cell densities ranging from 10^2 to 10^5 cells/ml are shown in Figure 1. In the absence of copper, control growth rates ranged from 1.2 to 2.1 doublings/d for *Chlorella* sp. and from 1.2 to 1.7 doublings/d for *S. capricornutum* (Table 1). Higher control growth rates were observed at lower initial cell densities. The coefficient of variation (CV%) in controls was <10%, indicating test acceptability [2]. For all *S. capricornutum* bioassays, the pH drift was less than 0.5 pH unit over 72 h. The pH control in the unbuffered soft water was reasonable for *Chlorella* sp. (<0.5-pH-unit increase from an initial pH of 7.5) over 72 h at initial cell densities of 10^2 and 10^3 cells/ml; however, an increase of 0.3 to 1.0 pH unit was observed over 72 h at 10^4 and 10^5 cells/ml as a result of high final algal cell densities.

Copper had an inhibitory effect on the cell division rate of both species after a 72-h exposure, although minor stimulation of growth was observed at low copper concentrations, particularly for *S. capricornutum*. For the tropical alga *Chlorella* sp., as the initial cell density increased, the toxicity of copper decreased (the curve shifts to the right; Fig. 1). A significant decrease ($p < 0.05$) was observed in the 72-h EC50 value for copper at initial cell densities of 10^4 and 10^5 cells/ml, compared to those of 10^2 and 10^3 cells/ml (Table 1). At higher initial cell densities (10^4 and 10^5 cells/ml), the NOEC values (4.7 and 9.0 µg Cu/L, respectively) were higher than the 72-h EC50 values obtained at the lower initial cell densities (4.4 and 4.6 µg Cu/L for 10^2 and 10^3 cells/ml, respectively).

For *S. capricornutum*, copper toxicity was also cell density dependent. Copper was at least two times less toxic to *S. capricornutum* at 10^5 cells/ml (EC50 values of 17 µg Cu/L) than at 10^2 to 10^4 cells/ml (EC50 values ≤ 7.5 µg Cu/L) (Table 1). A significant decrease ($p < 0.05$) was observed in the 72-h EC50 value for copper at initial cell densities of 10^4 cells/ml compared to that of 10^3 cells/ml (7.5 and 6.2 µg Cu/L, respectively). No significant difference ($p > 0.05$) was observed between copper toxicity at 10^2 and 10^3 cells/ml.

Copper also caused a change in cell size of both species after a 72-h exposure, as indicated by shifts in LS1. For *Chlorella* sp., the mean surface area of control cells without copper was 33 ± 11 µm² ($n = 11$ tests). Maximum increases in cell size were observed in bioassays at initial cell densities of 10^3 and 10^4 cells/ml, with greater than 50% of cells being enlarged compared with control cells at 10 µg Cu/L (Fig. 2). At 10^5 cells/ml, increases in cell size were less than 20% at all copper concentrations.

Copper caused a small concentration-dependent increase in the size of *S. capricornutum* cells relative to the control cells after a 72-h exposure; however, increases of less than 20% were typically observed at all cell densities (data not shown). Control cells, without copper, had a mean cell surface area of 53 ± 7 µm² ($n = 4$ tests) as determined by flow cytometry and 53 ± 13 µm² ($n = 10$ cells) as determined by confocal microscopy. Occasionally, two peaks in the LS1 distribution were observed for *S. capricornutum*, making estimates of the mean cell size less reliable.

Table 1. Effect of initial cell density on the toxicity of copper to *Chlorella* sp. and *Selenastrum capricornutum* after a 72-h exposure

Initial cell density (cells/ml)	*Chlorella* sp.				*S. capricornutum*			
	Growth rate[a]	NOEC[b] (µg/L)	LOEC[c] (µg/L)	EC50[d] (µg/L)	Growth rate	NOEC (µg/L)	LOEC (µg/L)	EC50 (µg/L)
10^2	2.0 ± 0.2	1.1	2.0	4.6 E[e] (3.5–6.0)	1.7 ± 0.2	1.9	3.8	6.6 HI (5.9–7.3)
10^3	2.1 ± 0.1	2.4	3.3	4.4 E (3.9–5.0)	1.6 ± 0.1	3.4	4.8	6.2 H (5.5–6.9)
10^4	1.7 ± 0.1	4.7	6.0	7.3 F (6.7–8.0)	1.3 ± 0.2	1.8	3.2	7.5 I (6.8–8.2)
10^5	1.2 ± 0.1	9.0	12	16 G (14–18)	1.2 ± 0.1	4.6	6.6	17 J (14–20)

[a] Doublings per day.
[b] NOEC = no-observable-effect concentration.
[c] LOEC = lowest-observable-effect concentration.
[d] EC50 = effective concentration giving 50% reduction in algal growth rate compared to the control ($n = 3$–8 tests).
[e] E to J denote whether effective concentrations of 50% (EC50) values are significantly ($p \leq 0.05$) different from each other (same letter means no significant [$p > 0.05$] difference).

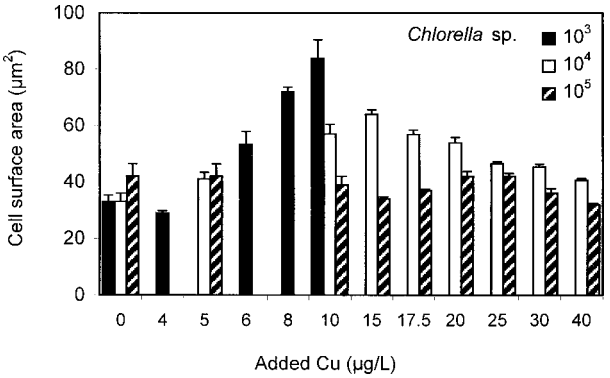

Fig. 2. The effect of copper on the mean cell surface area of *Chlorella* sp. after a 72-h exposure (n = 2–5 tests). Selected nominal copper concentrations are shown.

To investigate why copper was less toxic to *Chlorella* sp. and *S. capricornutum* at higher initial cell densities, dissolved copper in solution was measured at the beginning and end of the bioassays. In addition, membrane-bound (extracellular) and intracellular copper concentrations were determined at initial cell densities of 10^3, 10^4, and 10^5 cells/ml after a 72-h exposure (Fig. 3A and B). Determination of extra- and intracellular copper at 10^2 cells/ml was not possible because of the low cell numbers and subsequent low cellular copper concentrations (less than the GFAAS detection limit of 0.5 μg/L). Furthermore, no significant difference ($p > 0.05$) was observed in copper toxicity between bioassays at initial cell densities of 10^2 and 10^3 cells/ml for both species.

A mass balance for copper after 72 h was determined with recovered copper >82% for both *Chlorella* sp. and *S. capricornutum* (Fig. 3A and B). At 10^3 cells/ml, approximately 50 to 60% of the total copper at the end of the bioassay was still present in solution (dissolved Cu), with the intra- and extracellular copper fractions representing 4 to 17%, depending on the copper concentration used. In contrast, at 10^5 cells/ml, dissolved copper represented only 20% of the total copper added, with most of the copper at the end of the bioassay associated with the cells (>60%). Dissolved copper in solution

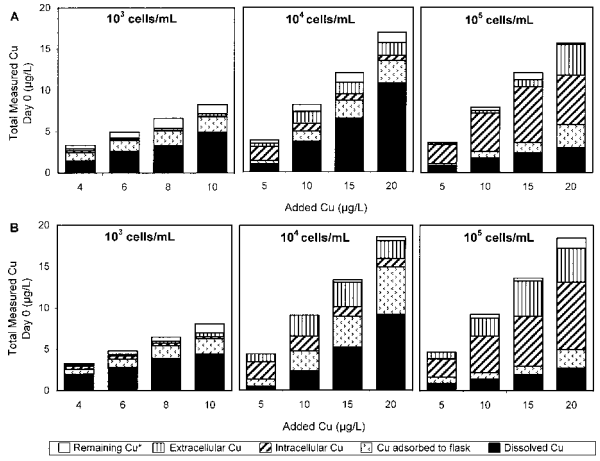

Fig. 3. A mass balance for copper (μg/L) after a 72-h exposure as a fraction of total measured copper on day 0 for (**A**) *Chlorella* sp. and (**B**) *Selenastrum capricornutum* at initial cell densities of 10^3, 10^4, and 10^5 cells/ml (remaining Cu* = amount of Cu not accounted for by the mass balance as a fraction of the total measured Cu on day 0).

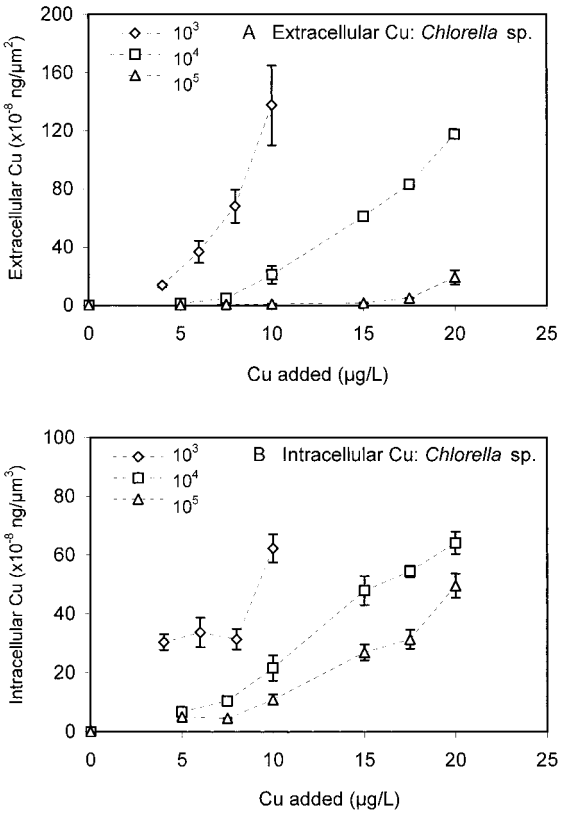

Fig. 4. Surface-bound (extracellular) and intracellular copper for *Chlorella* sp. at initial cell densities of 10^3, 10^4, and 10^5 cells/ml. Data points represent the mean ± standard error of mean (n = 3).

by 72 h decreased markedly with increasing initial cell density. At all cell densities, a considerable portion of copper (7–30%) adsorbed to the walls of the flasks throughout the test, despite silanization of the glass flasks prior to the bioassay. This fraction was overestimated, however, as it included the carryover from the dissolved copper solution that remained in the flask but was not adsorbed. Later experiments in which the flasks were rinsed with Milli-Q water prior to adding the acid showed that this carryover represented approximately 20% of the flask-adsorbed fraction. The concentration of flask-bound copper decreased with increasing initial cell density in bioassays with both species (Fig. 3A and B).

Because of the differences in the size of *Chlorella* sp. at each cell density, it was necessary to correct the extra- and intracellular copper concentrations on the basis of surface area and volume, respectively. Although cell size increases were small for *S. capricornutum*, extra- and intracellular copper concentrations were also corrected for cell surface area and volume, respectively, so that results were comparable between species. Similar trends were observed for *S. capricornutum* with or without the cell size correction.

Uptake of copper by *Chlorella* sp. at initial cell densities of 10^3, 10^4, and 10^5 cells/ml is shown in Figure 4. The cellular copper content (intracellular + extracellular copper) increased with increasing copper concentration in the media at all cell densities. Measured concentrations of extracellular copper showed that as the cell density increased from 10^3 to 10^5 cells/ml, significantly ($p < 0.05$) less copper was bound at the cell surface. Consequently, intracellular copper also decreased with increasing initial cell density (Fig. 4). For example, at 10 μg Cu/L, a threefold decrease was observed in intracellular copper

Table 2. Ratio of surface-bound (extracellular) to intracellular copper for *Chlorella* sp. and *Selenastrum capricornutum* after a 72-h exposure at initial cell densities of 10^3 to 10^5 cells/ml

Initial cell density (cells/ml)[a]	Added Cu (μg/L)	*Chlorella* sp. Extra/intra Cu	*S. capricornutum* Extra/intra Cu
10^3	0	—	—
	2	—	0.13 ± 0.02
	4	0.45 ± 0.01	0.17 ± 0.11
	6	1.08 ± 0.09	0.30 ± 0.06
	8	2.16 ± 0.17	0.36 ± 0.03
	10	2.19 ± 0.39	0.96 ± 0.12
10^4	0	—	—
	2.5	—	0.26 ± 0.02
	5	0.20 ± 0.06	0.18 ± 0.02
	7.5	0.49 ± 0.16	0.27 ± 0.05
	10	0.87 ± 0.29	0.59 ± 0.07
	12.5	—	0.76 ± 0.11
	15	1.30 ± 0.22	1.27 ± 0.26
	17.5	1.53 ± 0.15	—
	20	1.85 ± 0.22	1.29 ± 0.50
10^5	0	—	—
	2.5	—	0.08 ± 0.01
	5	0.10 ± 0.07	0.11 ± 0.01
	7.5	0.19 ± 0.00	0.15 ± 0.03
	10	0.11 ± 0.09	0.16 ± 0.01
	12.5	—	0.16 ± 0.02
	15	0.07 ± 0.03	0.26 ± 0.12
	17.5	0.16 ± 0.03	—
	20	0.40 ± 0.19	0.17 ± 0.00

[a] Exact cell densities used were 4.0×10^3, 3.8×10^4, and 2.6×10^5 cells/ml for *Chlorella* sp. and 3.4×10^3, 3.9×10^4, and 3.5×10^5 cells/ml for *S. capricornutum*.

as the cell density increased from 10^3 to 10^4 cells/ml and a further twofold decrease with increasing cell density from 10^4 to 10^5 cells/ml was found.

The ratio of extracellular to intracellular copper also differed between the different cell densities for *Chlorella* sp. (Table 2). For example, at 10 μg Cu/L and 10^3 cells/ml, 30% of the total cellular copper was located intracellularly, and 70% was bound to the cell surface (extra:intra Cu = 2.19 ± 0.39). In contrast, at 10^5 cells/ml, the majority of cellular copper (92%) was located intracellularly with only 8% bound to the cell surface (extra:intra Cu = 0.11 ± 0.09). The ratio of extracellular to intracellular copper typically increased with increasing copper concentration in the media at all cell densities, although this trend was less evident at 10^5 cells/ml.

Uptake of copper by *S. capricornutum* is shown in Figure 5. Similar results were obtained as for *Chlorella* sp., reflecting the similarity in the 72-h EC50 values at each cell density (Table 1). The cellular copper content (intracellular + extracellular Cu) increased with increasing copper concentration in the media. *Selenastrum capricornutum* cells at 10^3 cells/ml accumulated significantly ($p < 0.05$) more extra- and intracellular copper for the same added copper concentration in the media compared to 10^4 and 10^5 cells/ml. For example, at 10 μg Cu/L, intracellular copper increased 14-fold as the cell density decreased from 10^5 to 10^3 cells/ml. On a cell surface area/volume basis, the ratio of extracellular to intracellular copper differed at the different cell densities. At 10 μg Cu/L and 10^3 cells/ml, 50% of the total cellular copper was located extracellularly, with the fraction of membrane-bound copper decreasing to 37% at 10^4 cells/ml and 14% at 10^5 cells/ml (Table 2).

The K_d values obtained after a 72-h copper exposure are

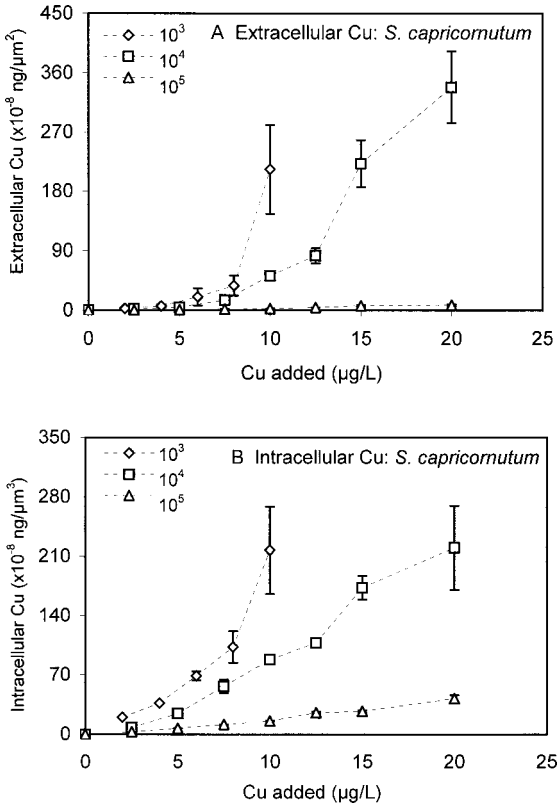

Fig. 5. Surface-bound (extracellular) and intracellular copper for *Selenastrum capricornutum* at initial cell densities of 10^3, 10^4, and 10^5 cells/ml. Data points represent the mean ± standard error of mean (n = 3).

shown in Table 3 and ranged from 11×10^{-11} to 25×10^{-11} L/μm² in bioassays with *Chlorella* sp. and from 10×10^{-11} to 30×10^{-11} L/μm² in bioassays with *S. capricornutum*.

For *Chlorella* sp. and *S. capricornutum*, a clear concentration–response relationship was observed between extracellular copper (and intracellular copper) and growth inhibition; that is, the more copper bound at the cell surface, the greater the uptake of copper through the cell membrane and the greater the effect on cell division.

Figures 6 and 7 show plots of extra- and intracellular copper versus growth inhibition at each cell density for *Chlorella* sp. and *S. capricornutum*, respectively. For both species, toxicity was dependent on the amount of extracellular copper (Figs. 6A and 7A) and consequently on the amount of intracellular copper (Figs. 6B and 7B), and this relationship was similar at each cell density. For example, 50% growth inhibition of *Chlorella* sp. occurred at approximately 15×10^{-8} ng/μm² of extracellular copper and 30×10^{-8} ng/μm³ of intracellular copper,

Table 3. Copper cell distribution coefficient (K_d) values for *Chlorella* sp. and *Selenastrum capricornutum* after a 72-h exposure

Initial cell density (cells/ml)[a]	K_d ($\times 10^{-11}$ L/μm²)	
	Chlorella sp.	*S. capricornutum*
10^3	25	30
10^4	11	30
10^5	17	10

[a] Exact cell densities used were 4.0×10^3, 3.8×10^4, and 2.6×10^5 cells/ml for *Chlorella* sp. and 3.4×10^3, 3.9×10^4, and 3.5×10^5 cells/ml for *S. capricornutum*.

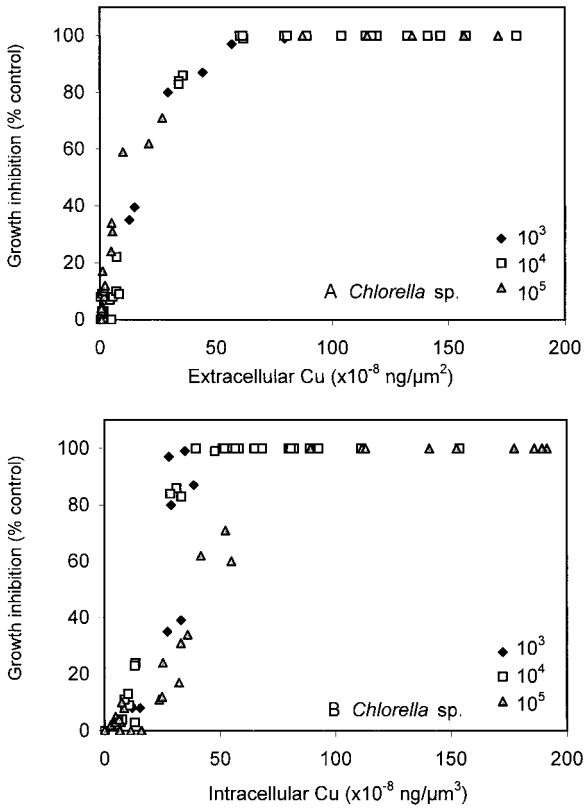

Fig. 6. Relationship between (**A**) extracellular copper and growth inhibition and (**B**) intracellular copper and growth inhibition for *Chlorella* sp. after 72 h at initial cell densities of 10^3, 10^4, and 10^5 cells/ml.

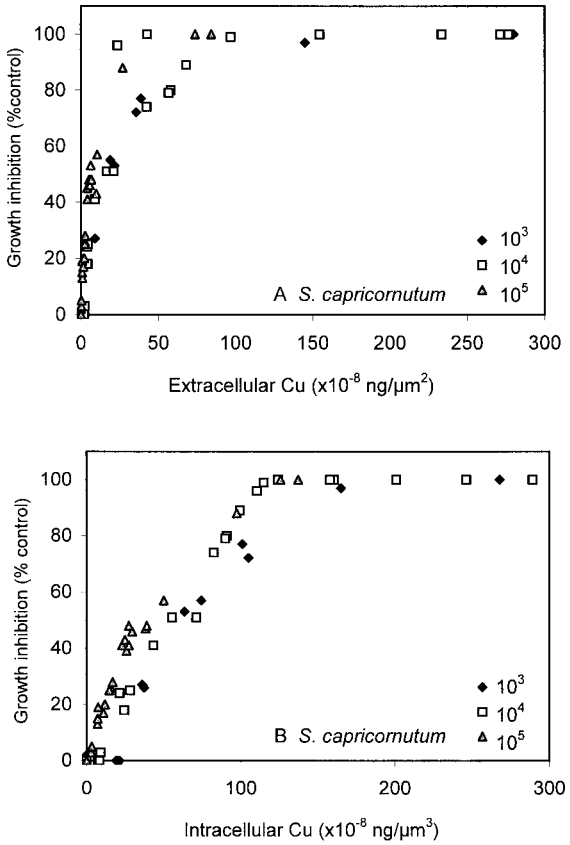

Fig. 7. Relationship between (**A**) extracellular copper and growth inhibition and (**B**) intracellular copper and growth inhibition for *Selenastrum capricornutum* after 72 h at initial cell densities of 10^3, 10^4, and 10^5 cells/ml.

regardless of the initial number of cells. Similarly for *S. capricornutum*, 50% inhibition occurred at 15×10^{-8} ng/μm^2 of extracellular copper and 60×10^{-8} ng/μm^3 of intracellular copper. This confirmed the results of the toxicity bioassays, which showed greater toxicity at 10^3 cells/ml because of greater copper penetration into the cells.

Copper speciation in solution was estimated by direct chemical measurement using ASV to determine labile or weakly complexed copper and by geochemical modeling. The difference between dissolved copper and labile copper in solution was assumed to be copper complexed by algal exudates (Table 4). As the initial cell density increased from 10^2 to 10^5 cells/ml, dissolved copper in solution decreased markedly in bioassays with both species. For *Chlorella* sp. at 10 μg Cu/L, the percentage of copper still in solution (dissolved Cu) after a 72-h exposure decreased from 71 to 69, to 57, and to 29% as the initial cell density increased from 10^2 to 10^5 cells/ml. The ASV-labile copper also decreased with increasing initial cell density for *Chlorella* sp.; however, complexed copper was similar, suggesting that some background complexing capacity may exist in solution regardless of the number of cells. However, at higher copper concentrations (e.g., 15–17.5 μg Cu/L), an increase was observed in complexed copper in solution, suggesting that algal exudates may play a role in complexing copper and reducing copper–cell binding at higher initial cell densities (10^5 cells/ml) (Table 4).

For *S. capricornutum*, dissolved copper in solution rep-

Table 4. Copper speciation measurements for *Chlorella* sp. after a 72-h exposure. Values represent a single measurement from the labile copper sample at each cell density

Initial cell density (cells/ml)[a]	Added Cu, day 0 (μg/L)	Total measured Cu, day 0 (μg/L)	Dissolved Cu (μg/L)	Labile Cu (μg/L)	Complexed Cu (μg/L)
10^2	10	7.6	5.4	ND[b]	ND
10^3	10	8.9	6.1	4.3	1.8
10^4	10	8.2	4.7	3.0	1.7
10^5	10	7.9	2.3	<0.5	1.8–2.3
10^4	15	12.0	7.2	3.0	1.7
10^5	15	12.1	3.2	<0.5	2.7–3.2
10^4	17.5	13.3	7.4	6.4	1.0
10^5	17.5	13.7	4.7	<0.5	4.2–4.7

[a] Exact cell densities used were 4.0×10^2, 3.3×10^3, 3.7×10^4, and 2.1×10^5 cells/ml.
[b] ND = not determined.

resented 67% of the added copper after 72 h at 10^2 cells/ml, with as little as 15% still present in solution at 10^5 cells/ml (data not shown). The ASV-labile copper was below detection limits at initial cell densities of 10^3, 10^4, and 10^5 cells/ml, so complexed copper could only be estimated and was similar regardless of the initial number of cells. Labile copper was not measured at 10^2 cells/ml.

Because of increases in pH over the 72-h bioassay, particularly for *Chlorella* sp. at initial cell densities of 10^4 and 10^5 cells/ml, the speciation of copper in the test water at pH 7.5, 8.0, and 8.5 was estimated using the Harwell pH Redox Equilibrium speciation code (HARPHRQ, S. Markich, ANSTO, Sydney, NSW, Australia, personal communication). At pH 7.5, 51% of copper would be present as the free hydrated metal ion (Cu^{2+}) with 41% as $CuOH^+$. With an increase in the pH to 8.0, the estimated percentage of Cu^{2+} decreased to 23%, while the proportion of $CuOH^+$ increased to 60%. At pH 8.5, Cu^{2+} represented only 6.6%, while the proportion of $CuOH^+$ was 53%.

DISCUSSION

The results obtained in the cell density experiments with *Chlorella* sp. and *S. capricornutum* showed that copper toxicity decreased when the initial cell density increased. Similar effects of reduced toxicity with increased algal inoculum have been reported for a number of contaminants, including Cu, Cd, and Zn [9,11]. It has been suggested that decreased toxicity with increasing initial cell density was due in part to adsorption onto the algal biomass [9]. However, these authors did not investigate chemical speciation changes in solution or metal concentrations on and in the cell.

In this study, measurements of extra- and intracellular copper for *Chlorella* sp. and *S. capricornutum* confirmed that less copper was bound to the cells, resulting in less copper uptake and lower toxicity at higher initial cell densities. Toxicity was proportional to the amount of copper associated with the cell at each cell density.

In ideal toxicity test systems, where dissolved copper in solution is constant over time, cell density should have no influence on copper toxicity because copper losses to algal cells are only a small proportion of the total dissolved copper in solution. If copper concentrations in solution are depleted over the course of the bioassay, because of binding to an increased number of algal surfaces at high initial cell densities, then copper toxicity in solution may be underestimated. Decreased bioavailability of copper may therefore be due to greater adsorption of copper by an increased number of algal cell surfaces, leading to a depletion of dissolved copper in solution and therefore reduced copper binding per cell.

The surfaces of algae contain a number of functional groups with high affinity for metal ions and carry a net negative charge, due mainly to carboxylic, sulfhydryl, and phosphatic groups [27,28]. These groups are binding sites that transport metal ions across the cell membrane and into the cell. Several studies have shown that two distinct phases occur in the uptake of metals by algal cells: a rapid adsorption that is complete within 10 min, followed by a slower, facilitated transport into the cytoplasm of the cell [18,29]. Adsorption of metal ions to the cell surface determines the initial toxicant loading of the cells, and this is likely to be dependent on the algal cell surface area and also the nature of the binding sites [30]. Higher cell concentrations provide a greater surface area with more cellular binding sites. For highly toxic elements, such as copper, used at low concentrations (<20 μg Cu/L in this study), a depletion of the toxicant concentration in solution may result [16]. Indeed, in bioassays with *Chlorella* sp. and *S. capricornutum*, dissolved copper in solution decreased markedly as the initial cell density increased from 10^2 to 10^5 cells/ml.

The release of algal exudates into the medium may also be responsible for reducing copper bioavailability in solution at higher cell densities. Complexation of copper by organic exudates, including glycolic acid, polysaccharides, and carbohydrates, has been reported for a number of algae, including *Dunaliella tertiolecta* [18], *Chlorella* spp. [31], *Phaeodactylum tricornutum*, and *Thalassiosira weissflogii* [32]. Xue et al. [29] showed that copper was complexed by exudates from *Chlamydomonas reinhardii* and therefore was not directly available for cellular uptake [21]. It is possible that at higher cell densities, exudate concentrations in solution are higher, causing a measurable increase in the complexation of copper in solution. By measuring ASV-labile copper, we were able to separate the primary effect of adsorption of copper to algal surfaces with that of complexation/adsorption of copper by algal exudates. The results obtained suggest that complexation of copper by algal exudates was not responsible for the differences in toxicity observed for *Chlorella* sp., at least for bioassays at initial cell densities of 10^3 and 10^4 cells/ml. At these cell densities, complexed copper did not increase with increasing numbers of cells. At 10^5 cells/ml, complexed copper increased with increasing copper concentration in the medium, suggesting that algal exudates may play a role in reducing copper–cell binding at higher cell densities. An increase in the amount of exudates in response to an increase in copper concentration has also been observed for *Emiliania huxleyi* [33], *Dunaliella tertiolecta* [18], and a number of microalgae from the Adriatic Sea [34].

For *S. capricornutum*, it is unlikely that algal exudates played a major role in the amelioration of copper toxicity observed at high algal cell densities because complexed copper was similar regardless of the initial number of cells. Surprisingly, the background complexing capacity of the *S. capricornutum* medium was higher than that for *Chlorella* sp., even with the omission of EDTA, which would otherwise complex copper [2]. For *S. capricornutum*, it is suggested that depletion of dissolved copper in solution with increasing initial cell density was due primarily to the increased algal surface area available for copper binding.

Further evidence of limitation of dissolved copper concentrations in solution was obtained by comparing the amount of copper in solution at equilibrium with the amount of copper on the cells (extracellular copper). This copper cell distribution coefficient (K_d) reflects the partitioning of copper between the cells and the solution. By measuring dissolved copper in solution after 72 h and extracellular copper, we confirmed that both these fractions decreased with increasing initial cell density. For the K_d value to be a constant, dissolved and extracellular copper concentrations would therefore have to decrease proportionally. Within the error of the experimental procedure, the K_d values for *Chlorella* sp. were similar at all cell densities, suggesting that surface-binding characteristics do not alter with cell density or cell size. An exception was for *S. capricornutum* at 10^5 cells/ml, where the K_d value was one-third of the K_d values at 10^3 and 10^4 cells/ml. A similar decrease in the partition coefficient with increasing concentration of particles in suspended sediments (not algae) was observed by Apte et al. [35]. This inverse relationship between

K_d and the concentration of particles is a commonly observed but poorly understood phenomenon in partitioning studies [36].

A possible limitation of the K_d approach used in this study was that sorption equilibrium was characterized after a 72-h exposure while some cells were still dividing rather than after short exposures of several hours [37]. However, preliminary experiments showed that copper is rapidly adsorbed to algal cells, attaining equilibria within minutes, compared to slower uptake and effects on cell division. The experimental conditions under which the K_d values were determined were the same for both species at all cell densities. The K_d values after a 48-h exposure to copper (not shown) were also similar to those after a 72-h exposure. In addition, the K_d values for *Chlorella* sp. and *S. capricornutum* were very similar, in agreement with the similar 72-h EC50 values for copper for both species. Future work may involve determining K_d values for a variety of algal species with a range of sensitivities to copper to determine whether species sensitivity to copper can be predicted from copper–cell binding.

The increase in pH observed in the *Chlorella* sp. bioassays may also explain the decreased bioavailability of copper due to a change in copper speciation in solution throughout the bioassay. This increase was largest at the higher initial cell densities (10^4 and 10^5 cells/ml) because of an increase in photosynthetic activity and depletion of CO_2. This resulted in a lower concentration of the free hydrated metal ion (Cu^{2+}) in solution, offset by an increase in the proportion of $CuOH^+$. Experimental evidence indicates that copper present as the free metal ion or as weak or labile complexes that are able to dissociate at the cell membrane is more bioavailable than copper in strong or inert complexes [38]. It is not known whether $CuOH^+$ is of similar toxicity as Cu^{2+}; therefore, small changes in pH and consequent changes in copper speciation may possibly contribute to the decreased toxicity observed at high initial cell densities. Vasseur et al. [9] concluded that increases in pH of the test medium containing a high number of cells was the likely mechanism responsible for the decreased toxicity of copper, cadmium, and zinc to *S. capricornutum* with increasing algal inoculum from 10^4 to 10^6 cells/ml. The 72-h EC50 values obtained with copper were 10, 65, 105, and 280 μg/L for initial cell densities of 2×10^4, 2×10^5, 6×10^5, and 2×10^6 cells/ml, respectively. These authors, however, observed much greater increases in the pH of the test media (>3-pH-unit increase) than that observed in this study with *Chlorella* sp. and *S. capricornutum*. In highly productive algal biofilms, it was also suggested that pH may play a role in lowering copper toxicity through the formation of insoluble species [39] at high copper concentrations (up to 2 mg/L). The authors concluded that the packing of cells in the biofilm, resulting in a larger surface area exposed to copper, was also a determining factor for the overall tolerance of the biofilm to copper [39].

The decrease in copper toxicity with increasing initial cell density has important ramifications for static laboratory bioassays with algae. Our results suggest that in standard algal bioassays using 10^4 to 10^5 cells/ml, the algae themselves may alter the solution concentration and speciation of copper, and therefore these bioassays may potentially underestimate metal toxicity.

Several options are available to overcome depletion of dissolved copper in solution throughout the bioassay. The use of continuous or flow-through systems, such as turbidostats and chemostats, involve the continuous supply of fresh medium and toxicant to the cells. However, the high cost and practical difficulties have restricted the application of these techniques to routine algal bioassays. Static renewal, used in higher-organism tests, is difficult with microalgal tests because concentrating the cells by centrifugation each day leads to reduced growth rates and subsequent failure of the test to meet acceptability criteria (J.L. Stauber, unpublished data). Alternatively, metal ion buffers may be used to keep the free metal ion concentration constant; however, this technique is not appropriate for determining the toxicity of copper in natural waters. Use of low initial cell densities is therefore critical to limit depletion of dissolved copper in solution throughout the bioassay and to reduce copper speciation changes due to complexation to algal exudates. With the recent application of flow cytometry to algal toxicity testing [19,20], the capability now exists to conduct bioassays at cell densities that are more typical (10^2–10^3 cells/ml) of natural aquatic systems. In bioassays with *Chlorella* sp. and *S. capricornutum*, an initial cell density of between 2 and 4×10^3 cells/ml is recommended since copper bioavailability, and hence toxicity, was similar to bioassays at 10^2 cells/ml. However, even at low algal cell densities, adsorptive losses to the test containers were still considerable, despite presilanization of the glass flasks. The issue of alternative test containers in microbial bioassays is worthy of more attention and has been recently reviewed by Stauber and Davies [40]. Use of low algal cell densities will help reduce metal speciation changes associated with metal–cell interactions, so that metal bioavailability determined in algal toxicity tests more closely estimates metal bioavailability in natural aquatic systems.

CONCLUSIONS

The aim of this study was to use flow cytometry to develop more environmentally relevant toxicity tests using lower initial cell densities, which more closely reflect cell densities found in aquatic systems. Results obtained for *Chlorella* sp. and *S. capricornutum* showed that copper toxicity decreased with increasing initial cell density from 10^2 to 10^5 cells/ml. Measurement of extra- and intracellular copper confirmed that less copper was bound to the cells, resulting in less copper uptake and lower toxicity at higher initial cell densities. Decreased copper toxicity at higher cell densities was due primarily to greater copper adsorption by algal cells, resulting in depletion of the equilibrium concentration of dissolved copper in solution. Algal exudates may also play a role in the depletion of dissolved copper at higher cell densities.

Standard algal bioassays that use 10^4 to 10^5 cells/ml may potentially underestimate metal toxicity. It is recommended that initial cell densities do not exceed 10^3 cells/ml to avoid depletion of dissolved copper in solution. In this way, metal bioavailability determined in laboratory toxicity tests should more closely estimate metal bioavailability in natural aquatic systems.

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