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TOXICITY OF METAL MIXTURES TO A TROPICAL FRESHWATER ALGA (*CHLORELLA* SP.): THE EFFECT OF INTERACTIONS BETWEEN COPPER, CADMIUM, AND ZINC ON METAL CELL BINDING AND UPTAKE

NATASHA M. FRANKLIN,*†‡ JENNIFER L. STAUBER,† RICHARD P. LIM,‡ and PETER PETOCZ§
†Centre for Advanced Analytical Chemistry, CSIRO Energy Technology, Private Mail Bag 7, Bangor, New South Wales 2234, Australia
‡Department of Environmental Sciences, §Department of Mathematical Sciences, University of Technology, Sydney, P.O. Box 123, Broadway, New South Wales 2007, Australia

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Abstract—The individual and combined effects of copper, cadmium, and zinc on the cell division rate of the tropical freshwater alga *Chlorella* sp. were determined over 48 to 72 h. Metal mixtures were prepared based on multiples of their single-metal median effective concentration (EC50) values, i.e., toxic units (TU) using a triangular mixture design with five toxicant levels (0, 0.75, 1.0, 1.25, and 1.5 TU). Single-metal EC50 values after a 72-h exposure were 0.11, 0.85, and 1.4 μM for copper, cadmium, and zinc, respectively. Significant interactions were observed for all metal combinations after 48 and 72 h. An equitoxic mixture of Cu + Cd was more than concentration additive (synergistic) to the growth of *Chlorella* sp., while combinations of Cu + Zn, Cd + Zn, and Cu + Cd + Zn were all less than concentration additive or were antagonistic. To determine the effect of each metal on the uptake of the other, extracellular (membrane-bound) and intracellular metal concentrations, both alone and in mixtures, were compared. The increased growth inhibition observed for mixtures of Cu + Cd was due to higher concentrations of cell-bound and intracellular copper in the presence of cadmium compared with copper alone (i.e., cadmium-enhanced copper uptake). In contrast, both extra- and intracellular cadmium concentrations were reduced in the presence of copper. In mixtures of Cu + Zn, copper also inhibited the binding and cellular uptake of zinc, which resulted in decreased toxicity. Zinc had no appreciable effect on the uptake of copper by *Chlorella* sp. Our results suggest that all three metals share some common uptake and transport sites on *Chlorella* cells and that copper out competes both cadmium and zinc for cell binding. Determination of metal cell distribution coefficients (K_d) confirmed that K_d values for cadmium and zinc in single-metal exposures decreased in the presence of copper.

Keywords—Algae Metal Toxicity Synergism Antagonism

INTRODUCTION

The individual effects of copper, cadmium, and zinc on microalgae have been well documented. In trace amounts, copper and zinc are essential micronutrients that play an important role in many enzyme systems in both algae and higher plants [1,2]. At concentrations above those required for optimal growth, copper and zinc have been shown to inhibit algal growth [3–5] and to interfere with important processes such as photosynthesis, respiration, adenosine triphosphate (ATP) production, and pigment synthesis [2–4,6]. In contrast, cadmium has no known biological function, although recently, Lane and Morel [7] showed the nutritional importance of cadmium to the marine diatom *Thalassiosira weissflogii* under conditions of low zinc, typical of the marine environment. Like copper and zinc, cadmium has been shown to inhibit cell division and to alter a variety of metabolic processes in microalgae at low concentrations [5,8]. Few detailed studies have examined the combined effects of copper, cadmium, and zinc on microalgae despite the fact that these elements are commonly present together in mining, industrial, and domestic effluents.

Various models have been used to evaluate the effects of mixtures on aquatic organisms. For toxicants with similar modes of action that do not interact, concentration-additive effects can be expected [9]. In this case, mixture toxicity equals the sum of the component concentrations, expressed in toxic units (TUs). Deviations from concentration addition may occur when toxicants interact and can result in more-than-additive or less-than-additive toxicity than predicted [9]. Throughout the literature, the terms synergism and antagonism have more often been used (sometimes incorrectly) to describe more-than-additive or less-than-additive interactions, respectively. Because it is difficult to determine whether interactions are true antagonisms according to the definition of Sprague [9] or are simply less than additive, these terms will be considered synonymous in this article.

Studies on mixture toxicity involving the metals copper, cadmium, and zinc have shown a variety of joint effects ranging from less than additive to more than additive [10–13]. Often, the interaction found depends on factors such as the species of algae and the biological response measured in addition to metal combinations and their concentrations, so that no firm conclusions can be drawn on mixture toxicity. Bræk et al. [11] demonstrated that combinations of cadmium and zinc acted either synergistically or antagonistically depending on the algal species, with differences between strains of the same species. Prevot and Soyer-Gobillard [14] found that the interaction between cadmium and selenium was largely concentration dependent, i.e., less concentrated combinations produced antagonistic effects, but as soon as one of the mixture components exceeded a critical level of toxicity, synergism was observed. Starodub et al. [6] demonstrated that the type of interaction between mixtures of copper, cadmium, and lead was dependent on the biological response measured, as mixtures had an antagonistic effect on photosynthesis of *Scene-*

* To whom correspondence may be addressed (natasha.franklin@csiro.au).

desmus quadricauda but a synergistic effect on growth. Furthermore, the synergistic interaction between copper, cadmium, and lead was shown to increase at low pH [15], demonstrating that environmental factors may also influence the toxicity of metal mixtures, not just the toxicity of the individual metals.

The physiological basis for interactive effects among metals to microalgae is not well known, as few studies have measured the effect of the interaction in terms of cell binding, metal uptake, and toxicity [16]. No studies have investigated the combined effects of copper, cadmium, and zinc on metal uptake and toxicity in microalgae.

The objective of this study was to characterize the interactive effects of copper, cadmium, and zinc in various binary and ternary mixtures on the cell-division rate of the tropical freshwater alga *Chlorella* sp. To cover a wide range of combinations of metal concentrations, a triangular mixture design was used [17]. For mixture combinations resulting in more-than-additive or less-than-additive interactions, a second objective was to compare extracellular (membrane-bound) and intracellular metal concentrations, both alone and in mixtures, to determine the effect of each metal on the uptake of the other. Understanding such metal–cell interactions should enable better assessment of contaminant mixtures in aquatic systems.

MATERIALS AND METHODS

Algal cultures

The unicellular freshwater green alga *Chlorella* sp. was isolated from Lake Aesake, Strickland River, Papua New Guinea. The culture was maintained axenically in JM/5 media [18] on a 12:12-h light:dark cycle (Philips TL 40-W cool-white fluorescent lighting, Danvers, MA, USA; 75 μmol photons/m^2/s) at 27°C.

Growth-inhibition bioassays with single metals

Toxicity tests were conducted in a synthetic softwater [19] (hardness 80–90 mg $CaCO_3$/L, alkalinity 54 mg $CaCO_3$/L) supplemented with nitrate (15 mg NO_3^-/L) and phosphate (0.15 mg PO_4^{3-}/L). The pH of this minimal medium was 7.5 ± 0.1. Toxicity tests were conducted at a light intensity of 140 μmol photons/m^2/s at 27°C. Cultures were shaken twice daily by hand.

Stock solutions (5 and 100 mg/L) of each metal were prepared from analytical reagent-grade copper sulfate ($CuSO_4.5H_2O$), cadmium sulfate ($CdSO_4.8H_2O$), and zinc sulfate ($ZnSO_4.7H_2O$) and acidified to pH <2 by the addition of hydrochloric acid (HCl). For single-metal toxicity tests, controls together with test samples of at least five metal concentrations (each in triplicate) were prepared. Fifty-five milliliters of toxicity-test medium was dispensed into 250-ml borosilicate glass Erlenmeyer® flasks (Bellco Glass, Vineland, NJ, USA), precoated with a silanizing solution (Coatasil, Ajax Chemicals, Auburn, NSW, Australia) to reduce adsorption of metals to the flask walls. Preliminary experiments showed that Coatasil had no effect on the growth of *Chlorella* sp. over 72 h. All glassware was acid washed in 10% concentrated HNO_3 before use. Subsamples (5 ml) were immediately taken from each flask at the beginning of each toxicity test and acidified (pH < 2). Total dissolved cadmium and zinc were determined by inductively coupled plasma atomic emission spectrometry (Spectroflame EOP, Littleton, MA, USA). The detection limit was 1 μg/L for zinc and 2 μg/L for cadmium. Total dissolved copper was determined by graphite furnace atomic absorption spectrometry (GFAAS) using a 4100ZL Perkin-Elmer (Norwalk, CT, USA) instrument. The detection limit for copper was 0.5 μg/L. For quality assurance purposes, all metal analyses included multipoint calibrations, spike and recovery, and matrix-matched blanks and standards. Measured concentrations of each metal were used to calculate toxicity endpoints.

Exponentially growing algal cells were centrifuged and washed three times before use in the bioassay to remove the culture medium. The centrifugation speed was 2,500 rpm (Jouan CR4.11, Winchester, MA, USA) for a duration of 7 min each spin. Each flask was inoculated with 2×10^4 to 4×10^4 cells/ml. Temperature and pH were monitored throughout the tests.

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). Two light-scatter detectors were used to identify the morphology of the cell. The forward-angle light scatter ([LS]1 = <15°) detector collected 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 (FL) 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.

Linear regression analysis was used to fit $\log_{10}$ cell density versus time (h) for each sample. The growth (i.e., cell division) rate was determined from the regression slope and expressed in doublings per day.

In each toxicity test, changes in cell size caused by the metal toxicant were detected in LS1 and measured after a 48- and 72-h exposure. The mean diameters of *Chlorella* sp. cells were determined from a flow cytometric calibration curve using spherical latex beads of known diameter (1.5–13.5 μm). The surface area and volume were then calculated from the measured diameter using the equation for a sphere. Visual measurements of cell diameters using phase-contrast microscopy confirmed those obtained by flow cytometry.

Effects on growth inhibition were expressed as the effective concentration giving 50% reduction (EC50) in algal growth rate over 72 h compared with the controls. The EC50 values in each bioassay were calculated using the computer program NYHOLM-3 (modified by Yuri Tsvetnenko from Nyholm et al. [20]). In addition, data from single-metal exposure bioassays were pooled and the combined statistical endpoints, including EC50s, calculated for each metal. Calculations were carried out assuming that the concentration–response can be described by the probit function and were performed using weighted linear regression analysis on probit-transformed data. After testing the combined data for normality and homogeneity of variance, Dunnett's multiple comparison test (ToxCalc Ver 5.0.23, Tidepool Software, San Francisco, CA, USA) was used to determine which treatments were significantly different from the controls. The no-observable-effect concentration was the concentration at which no statistically significant effect was observed compared with the control. The lowest-observable-effect concentration was the lowest concentration of the

Table 1. Triangular design template for the three-component mixture of Cu, Cd, and Zn

Total toxic units (TUs)	Mixture components (TUs)[a]		
	Cu	Zn	Cd
Control	—	—	—
0.75	—	0.38	0.38
	0.50	0.13	0.13
	0.38	—	0.38
	—	0.75	—
	0.38	0.38	—
	0.75	—	—
	0.25	0.25	0.25
	0.13	0.50	0.13
	—	—	0.75
	0.13	0.13	0.50
1.00	0.50	0.50	—
	—	1.00	—
	—	0.50	0.50
	0.17	0.67	0.17
	0.33	0.33	0.33
	1.00	—	—
	0.18	0.17	0.67
	—	—	1.00
	0.67	0.17	0.17
	0.50	—	0.50
1.25	1.25	—	—
	0.21	0.83	0.21
	—	0.63	0.63
	0.21	0.21	0.83
	—	—	1.25
	0.63	0.63	—
	0.42	0.42	0.42
	0.63	—	0.63
	—	1.25	—
	0.83	0.21	0.21
1.5	—	0.75	0.75
	1.00	0.25	0.25
	0.75	—	0.75
	—	1.50	—
	0.75	0.75	—
	1.50	—	—
	0.50	0.50	0.50
	0.25	1.00	0.25
	—	—	1.50
	0.25	0.25	1.00

[a] Nominal TU only. All TU values adjusted prior to analysis using actual EC50 (effective concentration giving 50% reduction in algal growth rate compared with the control) values (48 and 72 h) and measured metal concentrations.

metal to cause a statistically significant effect compared with the control. A significance level of 0.05 was used for all tests.

Growth-inhibition bioassays with metal mixtures

The toxicity of copper, cadmium, and zinc in binary and ternary mixtures was investigated using a triangular mixture design [19] in which the percentages of the mixture components sum to 100%. The experimental design template is shown in Table 1, with the following metal combinations tested: Cu + Cd, Cu + Zn, Zn + Cd, and Cu + Cd + Zn. Metal concentrations used in the mixture experiments were based on individual metal toxicities (EC50 values) using the TU concept proposed by Sprague and Ramsay [21], i.e., 1 TU = EC50 value. The concentration of each metal in the mixture was represented as a fraction of their EC50 values. The total TU of the mixture was the sum of their individual fractions. All TUs were adjusted prior to analysis using actual EC50 values after 48 and 72 h and measured metal concentrations. To cover a wide range of combinations of metal concentrations, four TU levels were tested (0.75, 1.0, 1.25, and 1.5 TU). All binary-mixture combinations were equitoxic, which was achieved by adding equal fractions of the EC50 value of each metal. For example, one equitoxic TU of Cu + Zn consists of 0.5 TU Cu and 0.5 TU Zn, which is 0.5 × EC50 Cu = 0.11 μmol/L (72 h) and 0.5 × EC50 Zn = 1.4 μmol/L (72 h). Because of the steep concentration-response curves for the individual metals, 0.5 TU was equivalent to the EC25 for each metal. For ternary mixtures, both equitoxic and nonequitoxic combinations were tested (Table 1). Due to the size of the experimental design (41 treatments, each in triplicate), toxicity tests were conducted over a four-week period. To minimize the effect of variation between weeks, controls, together with all treatments at the 1.0 TU level, were tested each week (i.e., repeated four times). In addition, the level of 1.25 TU was tested twice, resulting in a test size of $n = 251$. Growth-inhibition bioassays were performed using the test procedure outlined above for single-metal exposures.

Two different analytical approaches were used to determine the type of interaction between metals in the mixtures. Both methods were based on the concept of TUs. In the first approach, EC50 values (48 and 72 h) were calculated using total TUs for each single-, binary-, and ternary-metal combination (i.e., Cu, Cd, Zn, Cu + Cd, Cu + Zn, Zn + Cd, Cu + Cd + Zn). If 1 TU was bounded by the 95% confidence limits, the metals were considered to be concentration additive (i.e., no interaction). If the EC50 of the mixture was significantly lower than 1 TU, then the metals were more than concentration additive (synergistic); if the EC50 value was significantly higher than 1 TU, the metals were less than concentration additive or antagonistic.

The second approach used regression analysis (Minitab Ver 12, Minitab, State College, PA, USA). The input data for Minitab was algal cell division rates, expressed in doublings/day, versus the concentration of each metal, expressed in TUs. The data were analyzed using three models (linear, quadratic, and three-factor interaction models). Model effectiveness was assessed using calculated S values (S = [mean-square error]$^{1/2}$) and goodness-of-fit (r^2) values. The S value indicates the remaining variability in the data that is not described by the model. The type of interaction between copper, cadmium, and zinc was assessed such that, if the difference was positive and significant ($p < 0.05$), the interaction was less than concentration additive; if the difference was negative and significant ($p < 0.05$), the interaction was more than concentration additive; if the difference was not significant ($p > 0.05$), irrespective of sign, the interaction was concentration additive.

When interactions were found, intra- and extracellular metal concentrations were determined to investigate the effect of each metal on the uptake of the other.

Measurement of intra- and extracellular metal concentrations

Additional growth-inhibition bioassays were conducted to measure intra- and extracellular metal concentrations after 48- and 72-h exposures. Synergism between two metals was tested at the levels of 0.50, 0.75, and 1.0 TUs, while antagonism was tested at the levels 0.75, 1.0, and 1.5 TUs. In addition, a 1-h exposure of *Chlorella* sp. to copper alone (at 1 TU) and Cu + Cd (1.0 TU Cu + 1.0 TU Cd) was carried out to determine whether solution speciation changes affected metal uptake.

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 centrifuge tube 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, NJ, USA). These samples were analyzed for dissolved copper by GFAAS and dissolved cadmium and zinc by inductively coupled plasma atomic emission spectrometry. The remaining supernatant solution was discarded and the algal pellet was resuspended in 20 ml of 0.02 M ethylenediaminetetracetic acid (EDTA) and shaken for 30 s to remove any metal bound to the external cell surface [22]. Preliminary experiments confirmed that cells did not rupture during this treatment and that 20 min was the optimal time for the EDTA washing. The samples were centrifuged for 20 min at 3,500 rpm and the supernatant retained for copper, cadmium, and zinc analysis by GFAAS. This fraction was referred to as surface-bound (extracellular) metal. Analysis by GFAAS rather than inductively coupled plasma atomic emission spectrometry was more appropriate for determining this extracellular cadmium and zinc fraction due to the low extracellular metal concentrations expected in these samples. Carryover of copper from the dissolved copper in the supernatant into the extracellular fraction was typically <5% of the extracellular copper. For cadmium and zinc, however, carryover from the dissolved fraction was considerable due to the higher metal concentrations in solution needed to elicit a toxic response. Metal concentrations in this carryover fraction were subtracted from all measured extracellular metal concentrations.

The remaining cell pellet was air dried for 2 d and 2 ml of concentrated (15 M) HNO_3 added. After 30 min, cells were acid digested in a microwave oven for 5 min at low power (90 W). After cooling, the samples were made up to 20 ml with Milli-Q® water (Millipore, Bedford, MA, USA) and analyzed for copper, cadmium, and zinc by GFAAS. This fraction was referred to as intracellular metal. Carryover of copper, cadmium, or zinc from the EDTA supernatant into the intracellular fraction was typically <5% of the intracellular metal concentrations. Blank solutions (no algae) were also prepared in each sample batch. To calculate mass balances, any metal adsorbed to the walls of the glass bioassay flasks was determined after leaching each flask with 50 ml of 0.03 M HNO_3 overnight.

Using dissolved and surface-bound (extracellular) metal concentrations, the metal cell distribution coefficient (K_d) was determined for single-metal and binary combinations as follows:

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

To compare K_d values for each metal, it was necessary to correct for changes in cell size. Extracellular metal (μmol/μm^2) was plotted against dissolved metal (μmol/L), and the K_d based on cell size (L/μm^2) was determined as the slope of the linear plot (calculated by linear regression).

RESULTS

Toxicity of individual metals to Chlorella *sp.*

Control growth rates of *Chlorella* sp. (in the absence of added metals) (n = 8 tests) were 1.9 ± 0.2 and 1.6 ± 0.1 doublings/day after 48 and 72 h, respectively. The coefficient of variation ranged from 1 to 8%, which was within test acceptability limits (i.e., <20%). For each toxicity test, the pH drift was typically less than 0.5 pH units over 48 and 72 h. An increase of up to 0.8 pH units was sometimes observed in the controls by 72 h as a result of the high final algal cell densities.

Table 2. Effect of single-metal exposures of Cu, Cd, and Zn (μM) on the growth rate of *Chlorella* sp. after 48- and 72-h exposures (values in parentheses are 95% confidence limits)[a]

	48 h			72 h		
	NOEC	LOEC	EC50	NOEC	LOEC	EC50
Cu	0.05	0.07	0.09 (0.08–0.11)	0.07	0.09	0.11 (0.11–0.13)
Cd	0.06	0.19	0.85 (0.81–1.5)	<0.06	0.06	0.85 (0.81–1.5)
Zn	0.31	0.57	1.3 (1.2–1.4)	0.31	0.57	1.4 (1.2–1.5)

[a] NOEC = no-observable-effect concentration; LOEC = lowest-observable-effect concentration; EC50 = effective concentration giving 50% reduction in algal growth rate compared with the control.

The no-observable-effect concentration, lowest-observable-effect concentration, and EC50 values for copper, cadmium, and zinc in single-metal exposures are shown in Table 2. On a molar basis, copper was most toxic to *Chlorella* sp. The 72-h EC50 value for copper (0.11 μM) was approximately eight times lower than the value for cadmium (0.85 μM) and 12 times lower than the value for zinc (1.4 μM). Shorter exposure times did not significantly ($p > 0.05$) alter the toxicity of each metal (Table 2).

Toxicity of metal mixtures to Chlorella *sp.*

Figures 1 and 2 show the concentration–response curves after a 72-h exposure for single-metal and binary- and ternary-

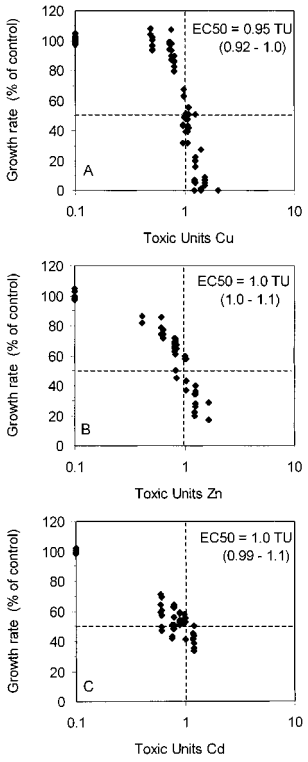

Fig. 1. Growth rate of *Chlorella* sp. (% of control) after a 72-h exposure plotted against actual toxic units of (**A**) copper, (**B**) zinc, and (**C**) cadmium tested individually. Median effective concentration (EC50); toxic unit (TU).

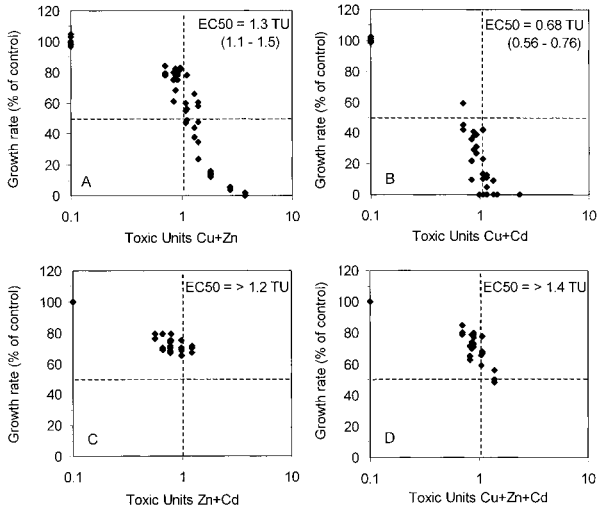

Fig. 2. Growth rate of *Chlorella* sp. (% of control) after a 72-h exposure plotted against actual toxic units (TU) of mixtures (**A**) Cu + Zn, (**B**) Cu + Cd, (**C**) Zn + Cd, and (**D**) Cu + Zn + Cd. Mixtures with EC50 (effective concentration giving 50% reduction in algal growth rate compared with the control) values significantly ($p < 0.05$) >1 TU are less than concentration additive, while EC50 values significantly ($p < 0.05$) <1 TU are more than concentration additive.

metal combinations expressed as TU. The toxicity of the individual metals (in the mixture experiments) was the same as that determined in the preliminary bioassays with each metal (Table 2). The concentration–response curves for copper, cadmium, and zinc alone intersected the line of 50% growth inhibition at the 1 TU level (Fig. 1A through C). The corresponding single-metal EC50 values were all within the 95% confidence limits of 1 TU, determined in preliminary tests.

For an equitoxic mixture of Cu + Zn (Fig. 2A), there was a shift in the concentration–response curve to the right, resulting in an increase in the 48-h EC50 (not shown) and 72-h EC50 (Fig. 2A) values to 1.1 and 1.3 TUs, respectively. Because these values were significantly ($p < 0.05$) higher than 1 TU, Cu + Zn combinations were considered to be less than concentration additive in their effect on growth. In contrast, an equitoxic mixture of Cu + Cd caused a shift in the concentration–response curve to the left (Fig. 2B), resulting in 48- and 72-h EC50 values that were significantly ($p < 0.05$) less than 1 TU (0.78 and 0.68 TU after 48 and 72 h, respectively). Therefore, the interaction between Cu + Cd was considered to be more than concentration additive. For combinations of Zn + Cd and Cu + Zn + Cd (Fig. 2C and D), a 50% effect was not observed after both 48 and 72 h; therefore, EC50 values were reported as greater than the highest TU tested. These mixture combinations were less than concentration additive in their effect on the growth rate of *Chlorella* sp.

Using regression analysis, several models were used to describe the toxicity of copper, cadmium, and zinc to *Chlorella* sp. The cell-division rate equations based on the linear, quadratic, and three-factor interaction models after a 72-h exposure are given in Table 3. A comparison of the *S* and r^2 values in Table 3 indicate that the data are better represented by either the quadratic model or the three-factor interaction model than by the linear model. For example, up to 80% of the variability in the data is explained by the three-factor interaction model, which includes interactive terms for binary and ternary mixtures, in contrast with 54% for linear terms only. The similarity in the *S* and r^2 values obtained for the quadratic and three-factor interaction models showed that the additional three-factor interaction between the metals does not contribute largely to the overall response. All models showed a significant negative effect (i.e., toxicity) of each metal alone on the cell division rate of *Chlorella* sp. Significant interactions were detected for all metal combinations, with the type of interaction depending on the sign (i.e., negative = more than concentration additive, positive = less than concentration additive) and the strength of the interaction depending on the numerical value. In agreement with the previous method of identifying mixture interactions, equitoxic mixtures of Cu + Cd were more than concentration additive in their effect on the cell division rate of *Chlorella* sp. (i.e., negative interaction), while mixtures of Cu + Zn, Cd + Zn, and Cu + Cd + Zn were less than concentration additive (i.e., positive interaction) (Table 3). The same type of interactions between copper, cadmium, and zinc were observed after a 48-h exposure (data not shown). The strength of the interactions between the metal combinations could also be assessed by comparing like terms (i.e., binary combinations with binary combinations). For example, using the three-factor interaction model, the less-than-concentration-additive interaction between Cu + Zn and Cd + Zn mixtures were the same (i.e., +1.3 CuZn and +1.3 CdZn). A comparison of 48- and 72-h data showed that the strength of the mixture interactions was greatest after a 72-h exposure (e.g., +0.64 CuZn at 48 h and +1.3 CuZn at 72 h) for all metal combinations, with the exception of Cd + Zn mixtures. The effect of variation between weeks was also tested in the model and was shown to be statistically significant but very small, explaining a further 3% of the variability in the data.

In addition to effects on cell division, each metal caused a change in cell size of *Chlorella* sp. after a 48- and 72-h exposure, as indicated by shifts in LS1. The mean surface area of control cells was 20 ± 2 μm^2. Cell size increased with increasing concentrations of copper, cadmium, and zinc in the medium, with maximum increases observed after 48 h. In single-metal experiments, copper caused the largest increases in cell size (i.e., up to 51% after 72 h), followed by cadmium (up to 27% after 72 h) and zinc (up to 18% after 72 h). How-

Table 3. Cell division rate equations for *Chlorella* sp. after 72 h based on linear and quadratic regression models[a]

Statistical model	*S*	r^2 (%)
Linear model		
Growth rate = 1.7 − 1.1Cu* − 0.75Cd* − 0.47Zn*	0.28	54
Quadratic model		
Growth rate = 1.8 − 1.2Cu* − 0.80Cd* − 0.86Zn* − 0.99CuCd* + 1.6CuZn* + 1.6CdZn*	0.19	79
Three-factor interaction model		
Growth rate = 1.8 − 1.2Cu* − 0.81Cd* − 0.87Zn* − 1.3CuCd* + 1.3CuZn* + 1.3CdZn* + 4.2CuCdZn*	0.19	80

[a] $S = \sqrt{\text{Mean-square error}}$; r^2 = goodness-of-fit; * = statistically significant variable at $p = 0.05$.

ever, all binary-metal combinations caused similar cell size increases, with up to 50% of cells being enlarged compared with control cells.

To further investigate mixture interactions, intra- and extracellular metal concentrations were determined for the two types of interactions identified, i.e., more than concentration additive (Cu + Cd) and less than concentration additive (Cu + Zn). Although less-than-concentration-additive interactions were observed for other metal combinations, only the Cu + Zn combination was used. Due to the differences in the size of *Chlorella* sp. as a result of metal exposures, it was necessary to correct the extra- and intracellular metal concentrations for surface area and volume, respectively. Molar concentration units were used for stoichiometric comparison of the uptake of each metal into the cell.

Synergism between Cu and Cd

Interactions between copper and cadmium at the cell surface were investigated at three TU levels (0.5, 0.75, and 1.0 TUs) corresponding to copper concentrations of 0.06, 0.09, and 0.13 μM and cadmium concentrations of 0.44, 0.67, and 0.89 μM. Uptake of each metal was determined alone and in the presence of an equitoxic concentration of the second metal.

A mass balance (i.e., total of all metal fractions = dissolved + extracellular + intracellular + flask-bound metal) for copper and cadmium after 48 and 72 h confirmed that recovered copper was greater than 86% and recovered cadmium was greater than 97%. In experiments with copper alone, dissolved copper was 15 to 35% of the total copper added, with most of the copper at the end of the bioassay associated with the cells (40–60%). A considerable portion of copper (13–30%) adsorbed to the walls of the flasks throughout the test despite silanization of the glass prior to the bioassay. In contrast, in experiments with cadmium alone, percentage adsorption losses of cadmium to the glass flasks were minimal (4–7%) due to the higher concentrations of cadmium used. Most of the cadmium at the end of the bioassay was dissolved in solution (73–92%), with typically less than 15% of the total cadmium associated with the cells.

Figure 3A through D shows surface-bound (extracellular) and intracellular concentrations of copper and cadmium alone and in the presence of the second metal after a 72-h exposure. For each metal alone, the cellular concentration (intracellular + extracellular) of copper and cadmium increased with increasing metal concentration in the medium. This effect was much more pronounced for copper than for cadmium, with a greater than 20-fold increase in extracellular copper over the concentration range tested compared with a 1.5-fold increase in extracellular cadmium. The presence of the second metal significantly ($p < 0.05$) altered the cellular metal uptake of copper and cadmium compared with the single-metal exposures. There was a significant ($p < 0.05$) increase in the amount of copper bound to the cells (extracellular copper) in the presence of cadmium at all concentrations tested (Fig. 3A). For example, at 0.09 μM copper, there was a 20-fold increase in extracellular copper in the presence of cadmium compared with copper alone. Consequently, intracellular copper also increased in the presence of cadmium, with up to two times more copper located intracellularly when cells were exposed in a mixture of the two metals (Fig. 3B) compared with copper alone. In contrast, both extracellular and intracellular cadmium concentrations were significantly ($p < 0.05$) reduced in the presence of copper compared with a cadmium-only exposure

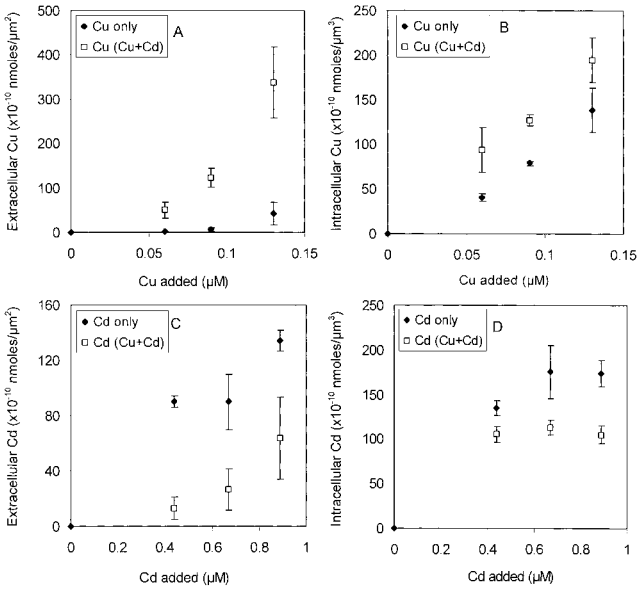

Fig. 3. Surface-bound (extracellular) and intracellular concentrations of Cu and Cd in a single and equitoxic binary mixture after a 72-h exposure. (**A**) Extracellular Cu, (**B**) intracellular Cu, (**C**) extracellular Cd, and (**D**) intracellular Cd. (**A**) and (**B**) show Cu alone and Cu in the presence of Cd, respectively. (**C**) and (**D**) show Cd alone and in the presence of Cu, respectively. Data points represent the mean ± standard deviation of the mean.

(Fig. 3C and D). Unlike copper, intracellular cadmium concentrations were regulated, with little change with increasing cadmium concentration in solution. Extracellular cadmium increased up to sevenfold in a mixture with copper, with a less than twofold increase in intracellular cadmium at all concentrations tested. Results obtained after a 48-h exposure (data not shown) also showed significantly ($p < 0.05$) more copper and less cadmium associated with the cells (i.e., extra- and intracellular) in the Cu + Cd mixture compared with each metal alone.

Although an equitoxic mixture of copper and cadmium had up to 25 times more dissolved cadmium than dissolved copper in solution after 72 h, much more copper was bound to the cell than cadmium on a molar basis. This suggests that *Chlorella* sp. may have a stronger affinity for binding copper than cadmium. However, intracellular concentrations of the two metals were similar except at the 1 TU level, where intracellular copper concentrations were higher than those of cadmium.

To determine whether increased binding of copper in the presence of cadmium was related to solution speciation changes, a short-term (1-h) uptake experiment was carried out at the 1.0 TU level for copper alone and Cu + Cd (1.0 TU Cu + 1.0 TU Cd). Over this time, effects on cell division were negligible, thereby allowing a direct comparison of copper uptake between treatments. One assumption of the free-ion activity model is that copper-cell binding is rapid compared with the slower uptake of copper through the cell membrane [23]; therefore, only total cellular copper (i.e., intracellular + extracellular copper) was determined. It was assumed that this fraction was predominately extracellular copper. It was hypothesized that, in the copper-only exposure, copper may bind/complex a component of the test medium (e.g., phosphate, algal exudates), reducing copper bioavailability. However, in an equitoxic mixture of copper (0.13 μM) and cadmium (0.89 μM),

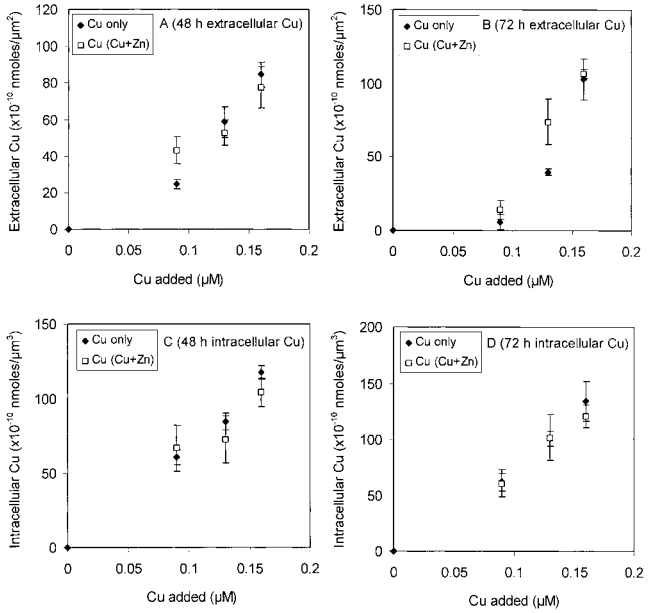

Fig. 4. Surface-bound (extracellular) and intracellular concentrations of Cu in a single and equitoxic binary mixture of Cu and Zn after 48- and 72-h exposures. All figures show Cu alone and Cu in the presence of Zn. (**A**) and (**B**) are extracellular Cu after 48- and 72-h exposures, respectively, and (**C**) and (**D**) are intracellular Cu after 48- and 72-h exposures, respectively. Data points represent the mean ± standard deviation of the mean.

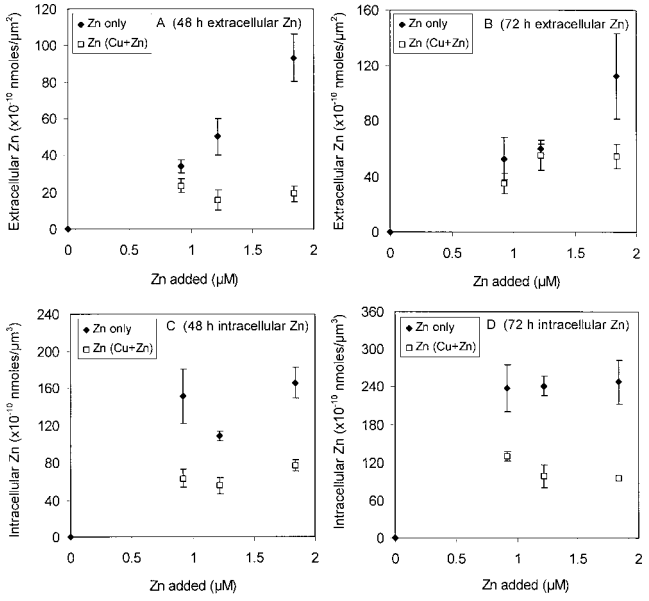

Fig. 5. Surface-bound (extracellular) and intracellular concentrations of Zn in a single and equitoxic binary mixture of Cu and Zn after 48- and 72-h exposures. All figures show Zn alone and Zn in the presence of Cu. (**A**) and (**B**) are extracellular Zn after 48- and 72-h exposures, respectively, and (**C**) and (**D**) are intracellular Zn after 48- and 72-h exposures, respectively. Data points represent the mean ± standard deviation of the mean.

cadmium may out compete copper for binding this component, displacing copper and leading to more copper in solution available for uptake. A measurable increase in the concentration of cellular copper was therefore expected in the Cu + Cd mixture after 1 h compared with copper alone.

Copper had no effect on the size of *Chlorella* sp. after a 1-h exposure, and therefore cellular copper was expressed on a per cell basis. Identical values of 4.8×10^{-7} nmoles Cu/cell were obtained in the presence and absence of cadmium after a 1-h exposure. This suggested that initial changes in solution speciation were not responsible for the increased binding of copper in the presence of cadmium observed after 48 and 72 h.

Antagonism between Cu and Zn

Interactions between copper and zinc at the cell surface were investigated at three TU levels (0.75, 1.0, and 1.5 TUs) corresponding to copper concentrations of 0.09, 0.13, and 0.19 μM and zinc concentrations of 0.92, 1.22, and 1.84 μM.

A mass balance for copper and zinc showed that, after both 48 and 72 h, recovered copper was greater than 83% and recovered zinc ranged from 92 to 103%. Dissolved copper increased with increasing copper concentrations in the medium, and ranged from 25 to 67% of the total copper added. The percentage of copper associated with the cells decreased with increasing copper concentration but was typically 20 to 40% of the total copper added. Most of the zinc at the end of the bioassay was still dissolved in solution (58–83%). Zinc also adsorbed to the glass flasks (13–28%) despite silanization of the glass prior to the bioassay. Typically, less than 10% of the total zinc was bound to the algal cells after 48 or 72 h.

Extra- and intracellular metal concentrations after 48- and 72-h exposures are shown in Figures 4 and 5 for copper and zinc, respectively. For each individual metal alone, the amount of copper and zinc bound to the cells increased with increasing metal concentration in the medium (Figs. 4A and B and 5A and B). Intracellular copper concentrations also increased with increasing copper in solution (4C and D). However, intracellular zinc remained fairly constant as the zinc concentration in solution increased from 0.92 to 1.84 μM (Fig. 5C and D), suggesting that the cells may be capable of regulating internal concentrations of zinc, similar to that found for cadmium. In a mixture of Cu + Zn, zinc did not inhibit the cellular uptake of copper. Extracellular copper concentrations were similar or slightly higher in the presence of zinc compared with copper alone (Fig. 4A and B). Similarly, the intracellular copper was not affected by the presence of zinc (Fig. 4C and D). This is also evident from a plot of extracellular copper versus intracellular copper in the presence and absence of zinc (not shown). The similar slopes obtained for copper alone (0.91, $r^2 = 0.92$) and Cu + Zn (1.0, $r^2 = 0.67$) indicate that zinc had no appreciable affect on copper uptake by *Chlorella* sp. after both 48 and 72 h. Therefore, it appears that the less-than-concentration-additive response observed for a mixture of Cu + Zn could not be attributed to a decrease in the amount of copper associated with the algal cells.

In contrast, the copresence of copper in the medium significantly ($p < 0.05$) altered the cellular metal uptake of zinc compared with that of zinc alone. Extracellular zinc was significantly ($p < 0.05$) reduced (1.5–2-fold) in a mixture with copper after a 48-h exposure at all zinc concentrations (Fig. 5A). After 72 h, this effect was only significant ($p < 0.05$) at the highest zinc concentration tested (Fig. 5B). Intracellular zinc was consistently lower (about half) in the presence of copper after both a 48- and 72-h exposure (Fig. 5C and D).

Although an equitoxic mixture of copper and zinc had at least 20 times more dissolved zinc in solution than dissolved copper after both 48 and 72 h, similar amounts of copper and

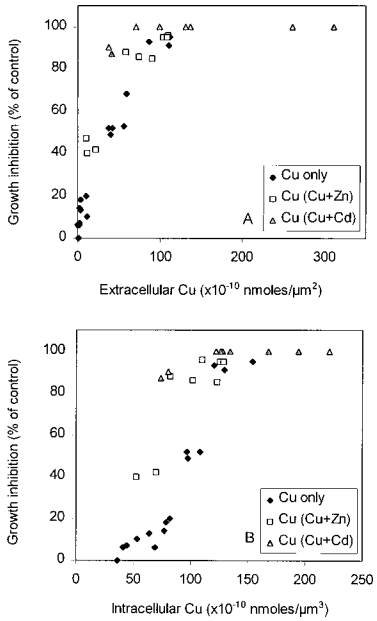

Fig. 6. Relationship between (**A**) extracellular copper and growth inhibition and (**B**) intracellular copper and growth inhibition for *Chlorella* sp. after 72 h in the presence and absence of equitoxic concentrations of cadmium and zinc.

zinc were bound to the cells on a molar basis. Intracellular concentrations of the two metals were also similar.

A common similarity between mixture combinations of Cu + Zn and Cu + Cd was that the presence of copper in the medium significantly reduced the cellular uptake of the second metal. This suggests that copper may be the dominant metal in determining toxicity. Figure 6A and B shows plots of extra- and intracellular copper versus growth inhibition for copper alone and in the presence of equitoxic concentrations of cadmium and zinc. Toxicity (i.e., growth inhibition) was related to extracellular copper concentrations, as indicated by the similar curves obtained in the presence and absence of the other metals (Fig. 6A). The severe growth inhibition observed for mixtures of Cu + Cd meant that most of the data points were at 100% inhibition, thereby making it difficult to compare with copper alone. Growth inhibition was also strongly related to intracellular copper concentrations despite the addition of zinc at concentrations that severely inhibited growth in single-metal exposures. A slight shift in the curve to the left was observed for copper in the presence of zinc compared with copper alone, indicating that the intracellular concentration of the second metal partially influenced the toxicity of the mixture.

Comparison of K_d values for single- and binary-metal mixtures

The K_d values were calculated after a 48- and 72-h exposure for each metal alone and in the presence of a second metal (Table 4). In a single-metal exposure, K_d values were highest for copper (84 and 139 $\times$ 10^{-12} L/μm^2), followed by cadmium (11 and 16 $\times$ 10^{-12} L/μm^2) and zinc (6.5 and 8.1 $\times$ 10^{-12} L/μm^2). The K_d value for copper alone appeared to increase with increasing exposure time from 48 to 72 h (Table 4). Measurement of dissolved copper in solution and extracellular copper showed that both these fractions decreased with increasing exposure time. For the K_d value to increase, dissolved copper would therefore have to decrease more than extracellular copper, suggesting that dissolved copper in solution may be depleted. The K_d value for cadmium in a single-metal exposure also increased from 48 to 72 h; however, this was due to an increase in extracellular cadmium from 48 to 72 h while dissolved cadmium in solution remained similar (i.e., not depleted). For zinc alone, there was only a small increase in the K_d value from 48 to 72 h. Both extracellular and dissolved concentrations of zinc remained similar over 48 to 72 h.

Table 4. K_d and r^2 values obtained in single and binary combinations of Cu, Cd, and Zn

Metal ion	System	Molar ratio	48 h K_d ($\times$ 10^{-12} L/μm^2)	48 h r^2	72 h K_d ($\times$ 10^{-12} L/μm^2)	72 h r^2
Cu	Cu	—	84	0.70	139	0.94
Cd	Cd	—	11	0.82	16	0.83
Zn	Zn	—	6.5	0.94	8.1	0.82
Cu	Cu + Cd	1:7	232	0.89	706	0.78
Cu	Cu + Zn	1:10	85	0.76	204	0.92
Cd	Cu + Cd	1:7	2.3	0.61	5.2	0.74
Zn	Cu + Zn	1:10	1.2	0.53	4.2	0.71

In the Cu + Cd mixture, the K_d value for copper increased to 232 and 706 $\times$ 10^{-12} L/μm^2 after 48- and 72-h exposures, respectively, compared with bioassays with copper alone. In contrast, the partitioning of cadmium between the cells and solution was reduced in the presence of copper compared with cadmium alone. These K_d values reflect the changes in extracellular metal concentrations for a Cu + Cd mixture. In the mixture of Cu + Zn, copper K_d values were similar to copper alone (Table 4). However, the K_d value for zinc was reduced in the presence of copper compared with zinc alone. These K_d values for copper and zinc also reflect the changes in the measured concentrations of these metals at the cell surface in single-metal and binary-metal exposures.

DISCUSSION

Mixture combinations of copper, cadmium, and zinc had different effects on the growth of *Chlorella* sp. and these could not be predicted from the effects of the individual metals. An equitoxic mixture of Cu + Cd was more than concentration additive to the growth of *Chlorella* sp., while combinations of Cu + Zn, Cd + Zn, and Cu + Cd + Zn were all less than concentration additive or antagonistic. It has been suggested that whether mixture combinations are synergistic or antagonistic depends on whether one metal facilitates the uptake of the other or whether they compete for the same transport sites on the cell membrane [24].

The surfaces of algae contain a number of functional groups with high affinity for metal ions and carry a net negative charge mainly due to carboxylic, sulfhydryl, and phosphatic groups [25,26]. These groups are binding sites that transport metal ions across the cell membrane and into the cell. Several studies have shown that there are two distinct phases in the uptake of metals by algal cells, i.e., a rapid adsorption (passive uptake) that is complete within 10 min, followed by a slower, facilitated transport into the cytoplasm of the cell [27,28]. Metal coordination sites on the cell surface are never entirely specific for a single metal or nutrient, and competition for membrane transport sites and intracellular binding sites can occur for metals with similar ionic radii and coordination geometry [29]. Copper and zinc bind strongly to oxygen- and nitrogen-containing

ligands. In contrast, cadmium is known to bind more strongly with sulfur-containing ligands [30].

Competitive interactions between nutrient and inhibitory metals have been identified in a variety of microalgae. The manganese uptake system has been shown to bind and transport a number of chemically similar metals, including cadmium and possibly zinc and copper [31,32]. Evidence of competitive binding between cadmium and manganese was shown in *Chlorella pyrenoidosa* [32] and *Thalassiosira pseudonana* [31] and between zinc and manganese in *Chlamydomonas* sp. [33].

In the present study, the less-than-concentration-additive response observed for mixture combinations of Cu + Zn, Cd + Zn, and Cu + Cd + Zn after a 48- and 72-h exposure is probably due to competitive binding of these metals at the cell surface. Results showed that, for an equitoxic mixture of copper and zinc, the presence of copper in the medium significantly reduced the amount of zinc bound to the cell (extracellular zinc) and intracellular zinc concentrations compared with zinc alone. Determination of metal cell distribution coefficients (K_d) confirmed that K_d values for zinc decreased in the presence of copper. Although zinc concentrations in the mixture were many-fold higher than copper concentrations, zinc had no appreciable effect on the uptake of copper and copper K_d values were similar in the absence and presence of zinc after a 48-h exposure. This suggests that copper and zinc share common uptake and transport sites on *Chlorella* cells and that copper out competes zinc for cell binding. This results in the inhibition of zinc uptake in the presence of copper, with subsequent reduction in growth inhibition.

In agreement with our results, Zhang and Majidi [34] showed that, when copper was added to a solution containing zinc, the amount of zinc adsorbed by the green alga *Stichococcus bacillaris* decreased due to competitive binding, and the transport of zinc into the algae was also inhibited. Bræk et al. [10] reported that competition between copper and zinc ions was the likely reason for the antagonistic effects on growth of the marine diatom *Phaeodactylum tricornutum*. These authors concluded that all divalent cations act on the same site in *P. tricornutum*. Using radioisotope tracer studies, they later showed that zinc inhibited the uptake of cadmium in the same alga [11].

Although extra- and intracellular metal concentrations were not measured, mixture combinations of Cd + Zn and Cu + Cd + Zn were also less than concentration additive. Similar effects between cadmium and zinc on cell division have been previously reported for *Kirchneriella subcapitata*, *Monoraphidium contorium* [35], and *Chlorella pyrenoidosa* [12]. Ting et al. [16] showed that, for equimolar concentrations of cadmium and zinc, the presence of the second metal did not affect the instantaneous uptake (i.e., binding) of the other metal by *Chlorella vulgaris*. However, the long-term uptake of zinc into the cell was inhibited by the presence of cadmium. In this case, it was suggested that competition between these metal ions did not occur, with cadmium and zinc possibly binding to different surface components of the cell wall, which have different affinities for these metals.

Mixture combinations of Cu + Cd used in this study were more than concentration additive toward the growth of *Chlorella* sp. This effect between Cu + Cd has also been reported for *Dunaliella salina*, *Chlamydomonas bullosa* [13], and the bacterium *Pseudomonas fluorescens* [36]. Synergism between two metals has generally been interpreted as one cation facilitating the uptake and subsequent toxic effect of the other, and it is assumed that competition for common uptake, transport, or toxicity sites is not involved [13]. In the present study, the reduction in the amount of extra- and intracellular cadmium in the presence of copper suggests that copper and cadmium do compete for binding sites on *Chlorella* sp. to some extent, with copper being preferentially bound. Competition alone, however, does not explain the increased amount of extra- and intracellular copper detected in the presence of cadmium compared with copper alone. Increased growth inhibition in response to increased intracellular copper was found.

It was hypothesized that cadmium may be altering copper speciation in solution by displacing copper from copper-phosphate complexes or algal exudates. This would result in more bioavailable copper in solution in the presence of cadmium and consequently increased copper-cell binding. Results obtained after a 1-h exposure, however, showed that there were similar concentrations of cellular copper in the presence and absence of cadmium, suggesting that cadmium did not appreciably affect copper speciation in solution. In fact, the K_d value for copper decreased in the presence of cadmium compared with copper alone after a 1-h exposure, indicating that less copper was partitioning onto cells, possibly due to direct competition for cell-binding sites. This is in contrast with 48- and 72-h exposures, in which Cu K_d values and extracellular copper increased in the Cu–Cd mixture compared with copper alone. These differences suggest that cadmium may be directly facilitating the uptake of copper by *Chlorella* sp. and that this effect is related to the time of exposure.

It is possible that cadmium caused conformational changes to membrane proteins or increased membrane permeability, resulting in the greater influx of copper into the cell. These effects may not have been evident after only a 1-h exposure. Copper and cadmium have been previously shown to cause damage to the membranes of microbial cells [3,37]. Further uptake experiments over the course of the bioassay (i.e., 6, 12, and 24 h) would be needed to determine at what point cadmium enhances the uptake of copper, resulting in the synergistic effect observed.

In both the Cu + Cd and Cu + Zn experiments, the amount of copper bound to the cell was similar or greater than that of either cadmium or zinc despite dissolved concentrations of these metals being far in excess of dissolved copper. There are numerous studies on the metal adsorption capacities of algal cells and other microorganisms, primarily investigating their use in water treatment. In short-term biosorption studies with a variety of microorganisms, it has been shown that the uptake of copper in a single- and multimetal mixture was considerably greater than that of cadmium and/or zinc [38,39]. Furthermore, in binary and ternary mixtures of these metals, the presence of copper decreased the biosorption of cadmium and zinc [38]. It is difficult to compare our results with these studies because they were designed to obtain maximum metal biosorption and therefore used high cell biomass and typically acidic to neutral conditions. Nevertheless, there is good agreement with our results, which indicate increased binding of copper to *Chlorella* sp. compared with cadmium and zinc.

Differences in the binding of copper, cadmium, and zinc may be related to ionic properties of the metals, such as electronegativity, hardness, and ionic radius [40,41]. Pearson's theory of hard and soft acids and bases states that hard acids prefer to coordinate to hard bases and soft acids to soft bases [42]. The values of increasing absolute hardness (η) for Cu^{2+}, Cd^{2+}, and Zn^{2+} ions are 8.29, 10.29, and 10.88, respectively [42]. The

single-metal K_d values obtained in the present study also follow this trend, with the highest K_d associated with the softest metal, copper, and the lowest K_d with the hardest metal, zinc.

The distribution coefficient, K_d, was also used to characterize the binding of copper, cadmium, and zinc to *Chlorella* sp. The much higher K_d value obtained for copper in single-metal exposures compared with cadmium and zinc further suggests that copper preferentially binds to algal cells. In addition, there was a relationship between K_d values and the order of toxicity of copper, cadmium, and zinc to *Chlorella* sp. Copper, with a higher K_d, was the most toxic metal, followed by cadmium and then zinc in single-metal exposures. This suggests that species sensitivity to metals under identical experimental conditions may be predicted from calculated K_d values.

The use of 10^4 cells/ml as the initial inoculum was necessary to have sufficient cells to measure intracellular copper after 48- and 72-h exposures. However, recent work [43] has shown that, at this cell density, dissolved copper in solution is depleted over the course of the bioassay, resulting in decreased extra- and intracellular copper concentrations in *Chlorella* sp. compared with bioassays carried out at lower cell densities (10^3 cells/ml). In contrast, cadmium and zinc were not depleted in solution. If dissolved copper had not been depleted in solution, it is possible that even greater effects of copper on cadmium and zinc binding would have been found. Cu + Cd synergism and Cu + Zn antagonism may have been even greater if lower cell densities had been used.

Metal concentrations used in this study were related to the relative toxicity of each metal based on the toxic unit concept, and mixture combinations were equitoxic. It has been demonstrated that, at different concentrations, metals can interact to produce contrasting effects (e.g., synergism vs antagonism or vice versa). Lam et al. [44] showed that the combined effect of copper and cadmium on the growth of *Chlorella vulgaris* decreased with increasing metal concentration, i.e., less concentrated combinations were strongly synergistic but higher concentrations resulted in antagonistic effects. The concentrations used by these authors were substantially higher (i.e., up to 5 and 10 mg/L of cadmium and copper, respectively) than those used in the present study. Concentration-dependent mixture toxicity has also been observed in higher plants. Sharma et al. [45] found that the toxicity of binary mixtures of copper, cadmium, and zinc to *Silene vulgaris* were antagonistic or nonadditive at less concentrated combinations, but as soon as one of the mixture components exceeded a critical level of toxicity, synergism was the predominant effect. Further work to examine the effect of metal concentrations on the synergistic interactions would be useful to identify if there is a threshold concentration at which cadmium enhances copper uptake in *Chlorella* sp.

The differing responses of both algae and higher plants to combinations of copper, cadmium, and zinc indicate that the interactive effects of metal mixtures is extremely complex. The final result of the interaction depends on the species, metal combinations, metal concentrations, and the biological response measured. In addition, environmental factors, such as pH and water hardness, that strongly influence single-metal toxicity will also affect the toxicity of metal mixtures. Consequently, the ability to satisfactorily predict the way in which interactions between metals may affect aquatic organisms has been limited.

To provide a more mechanistic basis for predicting the toxicity of metals to aquatic biota, the biotic ligand model has recently been developed and tested with fish and invertebrates [46]. According to this model, the bioavailability of metals (i.e., their uptake into the organism) can be decreased by decreasing the concentration of the free metal ion and thereby decreasing the potential for metal-cell binding or by increasing the concentrations of competing cations, thus decreasing the amount of metal bound to receptor sites on the cells.

Competing cations may include Ca^{2+}, H^+, or other metal ions. The antagonistic responses observed in the present study support the biotic ligand model. The competition between copper and zinc ions for common cellular binding sites resulted in a decrease in the cellular content of zinc, accompanied by no change in copper binding, leading to lower intracellular zinc and reduced growth inhibition. Further work to measure metal-cell binding constants may help extend the biotic ligand model to other organisms such as algae.

One key assumption of the biotic ligand model is that metal-cell binding does not alter the characteristics of the biotic ligand itself (in this case the cell). The synergistic effect observed for Cu + Cd may have been due in part to alteration in the cell membrane or its permeability. If this is the case, the current biotic ligand model may not be predictive of chronic toxicity of copper and cadmium mixtures to microalgae.

At present, interactions between combinations of metals are not considered in water quality guidelines despite the fact that metal contaminants rarely occur alone. Our results showed that the toxicity of binary and ternary mixtures of copper, cadmium, and zinc could not be predicted on the basis of the toxicity of the individual metals. Of major environmental concern are those interactions resulting in synergism, as observed in this study for mixtures of copper and cadmium.

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