1-1-2007

Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity

Jacqueline Levy
University of Wollongong, jl53@uow.edu.au

Jenny L. Stauber
CSIRO, jenny.stauber@csiro.au

Dianne F. Jolley
University of Wollongong, djolley@uow.edu.au

Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity

Abstract

Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals. However, interspecies differences in sensitivity to metals are not well understood. The relationship between metal-algal cell binding and copper sensitivity of marine microalgae was investigated using a series of 72-h growth-rate inhibition bioassays and short-term (1-h) uptake studies. A range of marine algae from different taxonomic groups were screened to determine whether copper adsorption to the cell membrane was influenced by biotic factors, such as the ultrastructure of cell walls and cell size. Minutocellus polymorphus was the most sensitive species to copper and Dunaliella tertiolecta the least sensitive, with 72-h IC50 values (concentration to inhibit growth-rate by 50%) of 0.6 and 530 microg Cu/L, respectively. Copper solution-cell partition coefficients at equilibrium (K(d)) were calculated for six species of algae on a per cell and surface area basis. The largest and smallest cells had the lowest and highest K(d) values, respectively (on a surface area basis), with a general (non-linear) trend of decreasing K(d) with increasing cell surface area (p=0.026), however, no relationship was found between K(d) and copper sensitivity, nor cell size and copper sensitivity. Interspecies differences in copper sensitivity were not related to cell size, cell wall type, taxonomic group or K(d) values. The differences in sensitivity may be due to differences in uptake rates across the plasma membrane, in internal binding mechanisms and/or detoxification mechanisms between the different microalgal species.

Keywords

Sensitivity, marine, microalgae, copper, effect, biotic, factors, copper, adsorption, toxicity, CMMB

Disciplines

Life Sciences | Physical Sciences and Mathematics | Social and Behavioral Sciences

Publication Details

Levy, J., Stauber, J. L. & Jolley, D. F. (2007). Sensitivity of marine microalgae to copper: The effect of biotic factors on copper adsorption and toxicity. Science of the Total Environment, 387 (1-3), 141-154.

Sensitivity of marine microalgae to copper: the effect of biotic factors on copper adsorption and toxicity

Jacqueline L. Levy[*,1,2], Jennifer L. Stauber[1,3] and Dianne F. Jolley[2]
[1] Centre for Environmental Contaminants Research, CSIRO Land and Water, Private Mail Bag 7, Bangor, New South Wales 2234, Australia.
[2] GeoQuest, University of Wollongong, New South Wales 2522, Australia.
[3] Current address: Department of Biology and Chemistry, City University, Tat Chee Ave, Kowloon, Hong Kong
* Corresponding author: telephone +61297106928; fax +61297106837; email Jacqueline.Levy@csiro.au

Abstract
Microalgae are sensitive indicators of environmental change and, as the basis of most freshwater and marine ecosystems, are widely used in the assessment of risk and development of environmental regulations for metals. However, inter-species differences in sensitivity to metals are not well understood. The relationship between metal-algal cell binding and copper sensitivity of marine microalgae was investigated using a series of 72-h growth-rate inhibition bioassays and short-term (1-h) uptake studies. A range of marine algae from different taxonomic groups were screened to determine whether copper adsorption to the cell membrane was influenced by biotic factors, such as the ultrastructure of cell walls and cell size. *Minutocellus polymorphus* was the most sensitive species to copper and *Dunaliella tertiolecta* the least sensitive, with 72-h IC50 values (concentration to inhibit growth-rate by 50%) of 0.6 and 530 µg Cu/L, respectively. Copper solution-cell partition coefficients at equilibrium (K_d) were calculated for six species of algae on a per cell and surface area basis. The largest and smallest cells had the lowest and highest K_d values, respectively (on a surface area basis), with a general (non-linear) trend of decreasing K_d with increasing cell surface area ($p = 0.026$), however, no relationship was found between K_d and copper sensitivity, nor cell size and copper sensitivity. Interspecies differences in copper sensitivity were not related to cell size, cell wall type, taxonomic group or K_d values. The differences in sensitivity may be due to differences in uptake rates across the plasma membrane, in internal binding mechanisms and/or detoxification mechanisms between the different microalgal species.

Keywords
toxicity; marine; microalgae; copper; adsorption

Introduction

The biotic ligand model (BLM) is a relatively new approach in environmental regulation which should improve our ability to derive site-specific water quality criteria for metals. It takes into account metal speciation in solution and the competition for metal binding at the cell surface, both of which depend on a range of water quality parameters such as pH, hardness and dissolved organic carbon (DOC). While the BLM is capable of accurately predicting acute copper toxicity to invertebrates and fish at fairly high copper concentrations, its extension to predicting chronic toxicity at low metal concentrations in marine systems will depend on gaining a better mechanistic understanding of copper uptake, toxicity and detoxification processes in biota. Copper is an essential nutrient for aquatic life, but can be toxic at concentrations as low as 1 μg/L. Sources of copper in the environment may be natural or anthropogenic, with weathering, mining activities, industrial discharge, fertilisers, pesticides, algicides and antifouling paints all potential contributors to copper in the near-shore marine environment (Stauber and Davies, 2000).

Water quality guidelines for metals are increasingly being derived from species sensitivity distributions (ANZECC/ARMCANZ, 2000). However, currently we have a limited mechanistic understanding of why some species are sensitive to contaminants and others are tolerant. Both abiotic factors, such as pH, salinity and DOC, and biotic factors, such as route of uptake, organism size/age and internal detoxification processes, can influence metal tolerance (Buchwalter and Cain, 2005). Abiotic factors are considered in the BLM, however less emphasis has been placed on biotic factors that may also influence species sensitivity.

Because of their key position as primary producers in aquatic ecological systems, microalgae are sensitive indicators of environmental change and are therefore important test species for

the regulatory assessment of metals. However, the sensitivities of microalgae to metals such as copper vary over several orders of magnitude (Stauber and Davies, 2000). While it is known that copper toxicity in microalgae is generally manifested only after the uptake of copper into the cell (Stauber and Davies, 2000), little is known about the specific sites to which copper binds on the algal cell wall/cell membrane, the nature of the site of toxic action (biotic ligand), or the exact mode of action of copper in microalgae.

Copper adsorption to algal cells is described by equation 1:

$$Cu(II)L_{water} + X_{cell} \rightarrow Cu(II)X_{cell} + L_{water} \quad (1)$$

where L = ligand and X_{cell} = sites on the cell membrane (Sunda, 1989). Copper binds rapidly and non-specifically to many sites on the cell membrane, including carboxylic, sulfhydryl and phosphate groups (Crist et al., 1990), and specifically to copper transport sites. Generally, copper sorption to cell surfaces is considered rapid (~ 10 minutes), and is able to reach a pseudo-equilibrium with copper in solution, while internalisation of the metal across the plasma membrane is much slower (hours) (Gonzalez-Davila et al., 1995). At low copper concentrations however, copper transport may become diffusion-limited and copper uptake is under kinetic control (Stauber and Davies, 2000). Once copper is internalised, there is some evidence in marine algae that copper oxidises thiol groups in the cytoplasm, leading to a lowering of the ratio of reduced to oxidized glutathione, which in turn affects spindle formation and cell division (Stauber and Florence, 1987).

Franklin et al. (2004) showed that two freshwater green algae (*S. capricornutum* and *Chlorella* sp.) had similar copper cell partition coefficients and similar sensitivities to copper.

They suggested that species sensitivity to copper may be predicted from copper-cell partition coefficients (K_d), but that further work was required to determine these for a range of microalgae.

This study was undertaken as part of a larger study investigating the influence of copper binding, uptake rates and intracellular detoxification processes on the sensitivity of different marine microalgae to copper. The relationship between rapid copper-algal cell binding over 1 h and copper toxicity over 72 h was investigated using growth-rate inhibition bioassays with a range of marine microalgae. This study aimed to determine if the copper sensitivity of algae was dependent on the amount of copper adsorbed to the cell, as determined by the copper-cell partition coefficient (K_d). In particular the aim was to establish whether copper adsorption to the cell membrane, and subsequent toxicity, was influenced by biotic factors, such as the ultrastructure of cell walls or cell size, or whether trends could be established based on algal taxonomic groupings.

Methods

Algal cultures

All marine microalgae were originally obtained from the CSIRO Collection of Living Microalgae, Marine and Atmospheric Research, Hobart, Australia. A description of the 11 marine species and the culture conditions used are given in Table 1. The two tropical species, *Isochrysis* sp. and *Proteomonas sulcata*, were maintained in growth cabinets at 27 ± 2°C, while the remaining nine temperate species were maintained at 21 ± 2°C. All cultures were maintained on a 12:12 h light/dark cycle at 70 µmol photons/m^2/s (TL 40W cool white fluorescent lighting).

Growth-rate inhibition bioassays

The chronic toxicity of copper to 11 marine microalgae was determined using 72-h growth-rate inhibition bioassays. A batch method was used for the growth-rate inhibition bioassays using 250-mL borosilicate glass Erlenmeyer flasks, coated with Coatasil silanising solution (APS Ajax Finechem, Seven Hills, NSW, Australia) to prevent adsorption of copper to the glass. Test flasks were soaked in 10% (v/v) nitric acid (Merck, Kilsyth, Victoria, Australia) overnight and rinsed thoroughly with high-purity Milli-Q® deionised water (>18 MΩ/cm, Millipore, North Ryde, NSW, Australia). Cultures were checked regularly microscopically and streaked onto agar plates (2% bacto agar, 0.1% pepsin, and 0.1% yeast) and incubated in the dark to check for the presence of bacteria. If no colonies were present and bacteria were not observed, these cultures were deemed axenic.

Copper stock solutions (5 and 100 mg/L) were prepared from copper sulfate ($CuSO_4.5H_2O$, AR grade, APS Ajax Finechem) and acidified with HCl (0.2% v/v, pH < 2). The medium used to prepare test treatments was filtered seawater (0.45 μm pore size, cartridge filter, Millipore) supplemented with 15 mg NO_3^-/L ($NaNO_3$, AR grade, APS Ajax Finechem) and 1.5 mg PO_4^{3-}/L (KH_2PO_4, AR grade, APS Ajax Finechem). At least five different copper treatments and a control (copper-free filtered seawater plus nitrate and phosphate) were prepared from the stock solutions in triplicate and 50 mL dispensed into 250-mL Erlenmeyer flasks.

Cells in exponential growth phase (5-6 d old) were used to inoculate the test treatments after centrifugation (2500 rpm, 7 min, rotor radius 17 cm, Spintron GT-175BR, Spintron, Melbourne, VIC, Australia) and three washes in filtered seawater to remove residual culture medium. Due to their larger size and fragility, *H. niei* and *Tetraselmis* sp. were only rinsed

once and were centrifuged at lower speed for less time (1000 rpm, 4 min) to prevent cell lysis. The test medium was then inoculated with 2-4 × 10^3 cells/mL. A sub-sample (5 mL) was immediately filtered through an acid-washed 0.45 µm membrane filter and dissolved copper was determined after acidification by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The flasks were incubated for 72 h in 12:12 h light/dark conditions at 140 µmol photons/m^2/s at 27°C (for tropical species) or 21°C (temperate species). Test flasks were rotated within the light cabinet and shaken twice daily by hand to ensure sufficient gas exchange. The pH was recorded initially and after 72 h.

The cell density in each treatment was measured daily using flow cytometry (Bio-Rad Bryte HS Flow Cytometer, Richmond, CA, USA or BD-FACSCalibur, Becton Dickinson BioSciences, San Jose, CS, USA). Cells were excited with blue light (488 nm) and chlorophyll *a* autofluorescence was measured in the 660-700 nm band (FL3) (Biorad Bryte) or > 600 nm long pass filter (BD FACSCalibur). When the FACSCalibur flow cytometer was used, TruCount fluorescent beads (BD Trucount™ Tubes, BD Biosciences) were added to each sample as an internal counting standard. Further details on the flow cytometric method are detailed in Franklin et al. (2004).

The growth rate (cell division; µ), was calculated as the slope of the regression line from a plot of $\log_{10}$ (cell density) versus time (h). Growth rates for treatment flasks (doublings/day) were expressed as a percentage of the control growth rates.

The 72-h IC50, i.e. the inhibitory concentration to reduce the growth rate by 50%, was calculated using linear interpolation (ToxCalc, Ver 5.0.23C, Tidepool Software, San Francisco, CA, USA). The data were tested for normality and homogenous variance, and

Dunnett's multiple comparison test was used to determine which treatments differed significantly from controls (1 tailed, $p \leq 0.05$) in order to estimate the no-observable effect concentration (NOEC) and the lowest-observable-effect concentration (LOEC). Toxicity end-points for *D. tertiolecta*, *Tetraselmis* sp., *P. tricornutum* and *N. closterium* were derived from multiple tests (n =3, 3, 2 and 32, respectively), while end-points for the other algae were derived from one definitive test each as part of the screening process.

Copper analyses

The concentration of dissolved copper in samples was measured by inductively coupled plasma-atomic emission spectroscopy (ICP-AES) (Spectro Flame-EOP, Spectro Analytical Instruments, Kleve, Germany). Copper concentrations were calculated from a matrix-matched calibration curve (clean seawater, acidified with 0.2% HNO_3) using serial dilution of a mixed metal standard (QCD Analysts, Eaglewood, FL, USA) and a drift standard incorporated into the analysis procedure. The detection limit for copper was typically 1 µg/L. Measured copper concentrations were used in all calculations of toxicity endpoints and K_d determinations, except where algal species were very sensitive (*M. polymorphus* and *M. pusilla*). For these two algae, the copper concentrations used to calculate toxicity endpoints were the best estimations possible based on the serial dilution factor applied to the first measurable copper concentration using ICP-AES.

Determination of the partition coefficient (K_d)

Six algal species (*D. tertiolecta*, *Tetraselmis* sp., *E. huxleyi* (with and without coccoliths), *N. closterium*, *P. tricornutum* and *H. niei*), with a broad range of copper sensitivities, were selected from a preliminary survey to determine copper adsorption to the algal cells. Species that were very sensitive to copper were not included, as the exposure concentrations were

below the detection limits of copper in seawater by ICP-AES or inductively coupled plasma-mass spectrometry (ICP-MS).

The partitioning of copper between solution and cells at equilibrium (K_d), was calculated as:

$$K_d \text{ (L/cell)} = \frac{\text{cellular Cu (µmol/cell)}}{\text{dissolved Cu (µmol/L)}} \qquad (2)$$

Cellular copper was determined by difference (Figure 1). K_d on a surface area basis was calculated by dividing K_d (L/cell) by the surface area of an algal cell (µm^2/cell). Six replicates of treatment solutions (control and five copper treatments) were prepared in seawater using the same method as the growth-rate inhibition bioassays (but without nutrients added). Thirty millilitres of each was dispensed into Teflon centrifuge tubes that had been pre-soaked in 10% HNO_3 and rinsed thoroughly with MilliQ® water. Three of six replicates (per copper concentration) were inoculated with algal cells (2-3 × 10^5 cells/mL prepared as above), while three replicates (without algae) were used to measure any copper losses from solution due to adsorption to the Teflon tubes. The appropriate time of exposure (60 min) was determined from the adsorption plateaus of plots of adsorbed copper versus time. Preliminary experiments showed that cells exposed to copper for 60 min had not internalised the copper and the copper had had no effect on algal cell size. After 60 min the tubes were centrifuged. Five mL sub-samples of supernatant were removed, acidified to pH < 3 using concentrated Tracepur HNO_3 (2 µL/mL of solution) and dissolved copper measured by ICP-AES.

Copper adsorbed to the algal cells was calculated as the difference between the dissolved copper in the treatments with algae and matching algae-free solutions. The amount of copper per cell (µmol/cell) was calculated by dividing the concentration of cellular copper (µmol/L) by the cell density (cells/L). The dissolved copper (µmol/L) was plotted against cellular

copper (μmol/cell), and the K_d (L/cell) was determined as the slope of the regression line. An example of this calculation is shown in Figure 2. The K_d was then normalised for each alga based on surface area, due to the different cell sizes of each algal species.

Determination of algal cell sizes

Algal cell size was calculated by flow cytometry for spherical cells (*D. tertiolecta*, *E. huxleyi*) using forward-angle light scatter (LS1= < 15%) (Franklin et al., 2004). Fluorescent beads of nominal diameter 2, 5, 10 or 15 μm (Coulter CC Size Standard L2, L5, L10 and L15 Latex beads, Beckman Coulter, Fullerton, CA, USA) or 3.6 μm (Flow-Set Fluorospheres, Beckman Coulter) were used to create a calibration curve for cell size. An equation was fitted to the curve, and the diameter of spherical algal cells determined. The surface area of the cell was then calculated using the equation for surface area of a sphere ($4\pi r^2$, where r = the radius of the cell). These cell sizes were also confirmed using the microscopy technique described below (n=30).

For cells that were not spherical in shape, cell size was determined on at least 30 individual cells per treatment using a micrometer and phase-contrast microscopy. The equation for the surface area (SA) of a prolate ellipsoid (where length > width = depth) was used for *Tetraselmis* sp. and *H. niei* ($SA = 2\pi b^2 + 2\pi a^2 b^2/\sqrt{(a^2-b^2)} * ASIN (\sqrt{(a^2-b^2)}/a)$, where a = length and b = width (He and Tebo, 1998). If the length equalled the width for an individual cell, the SA equation for a sphere was used instead. The cell size of *P. tricornutum* was approximated by visualising the cells as two cones joined at their base; SA of each cone = $\pi r\sqrt{(h^2+r^2)}$, where h = half length of cell and r = half width of cell. The cell size for *N. closterium* was calculated by visualising the cells as three cylinders; two small cylinders attached to each

end of a wider cylinder; SA of each cylinder = $2\pi r^2 + 2\pi rh$ where r = radius of cell and h = height of cell. The surface area of cells is reported as μm^2.

Statistical analyses

Linear and non-linear regression analysis was used to compare species sensitivity, cell surface area and partition coefficients (SPSS for Windows, version 14.0).

Results

Algal species sensitivities to copper

The effects of copper on the growth rates of the eleven marine microalgae are shown in Table 2. Growth rates in the controls were greater than or equal to 1.1 doublings/day, except for the large dinoflagellate *H. niei* and *E. huxleyi* with coccoliths, which grew more slowly (0.9 and 1.0 doublings/day, respectively). Algal growth rates for each species decreased with increasing concentrations of copper. The 72-h IC50 values ranged from 0.6 µg Cu/L for the small centric diatom *Minutocellus polymorphus* to 530 µg Cu/L for the tolerant chlorophyte *Dunaliella tertiolecta.*

Low cell densities were used in the bioassays to avoid copper depletion in solution over the test duration which may otherwise lead to an underestimation of copper toxicity in the bioassays (Franklin et al., 2002). To confirm this, copper was measured daily in test solutions for several copper-tolerant species. For *Tetraselmis* sp., copper in the highest test concentration decreased slightly from 112 µg/L to 101 µg/L over 72-h, indicating that there was little depletion of copper in solution over the test. For *D. tertiolecta* the measured copper decreased from 938 µg/L (Day 0) to 559 µg/L (Day 3) at the highest copper concentration tested (nominally 1000 µg Cu/L). To determine whether this copper loss was due to

adsorption to algal cells and test containers, or due to precipitation of copper in seawater, copper was determined in filtered and unfiltered (algal-free) copper test solutions. Dissolved and total copper were not significantly different (paired t tests, $p > 0.05$ at all test concentrations), suggesting that copper losses were due to adsorption to flask and algal cell surfaces rather than to precipitation in seawater. Further confirmation was obtained using dialysis (1000 kDa molecular weight cut-off, ~1 nm; Spectra/Por 7 dialysis membrane, Extech Supplies – Cole Palmer product). Free copper, $Cu(H_2O)_6^{2+}$, and simple inorganic species, e.g. $Cu(H_2O)_4Cl_2$, with approximate diameters of 1 nm, would be able to cross the dialysis membrane whereas inorganic and organic colloids with diameters from 10-200 nm would not be dialysable (Apte and Batley, 1995). If the solubility limit for copper in seawater had been exceeded then copper could precipitate as colloidal material. However, this copper fraction would not be able to cross the dialysis membrane, leading to lower total copper concentrations on the inside of the dialysis membrane compared to on the outside of the membrane. Dialysis tubing was filled with 10 mL of seawater and left to equilibrate in seawater before transfer to a test medium containing 495 µg total Cu/L (3 replicates) with nutrient concentrations matched to the growth bioassays. After 24-h, the concentration of copper in dialysis bags (440 ± 3 µg/L), i.e. truly dissolved copper, was not significantly different ($p > 0.05$) to the concentration of dissolved copper in the external medium (447 ± 6 µg/L), showing that the solubility limit for copper in seawater had not been exceeded.

The toxicity of copper to two different clones of two species of coccolithophorids was determined to establish the effect of the presence of coccoliths (calcium carbonate scales) on copper sensitivity. For *E. huxleyi*, the clone with coccoliths present was significantly ($p > 0.05$) more sensitive to copper than the clone without coccoliths, with 72-h IC50 values of 15 and 20 µg Cu/L, respectively (significance tested using Sprague and Fogels, 1977). Similarly,

the *Gephyrocapsa oceanica* clone with coccoliths was more sensitive to copper than the clone without coccoliths, with 72-h IC50 values of 17 µg Cu/L and > 25 µg Cu/L, respectively. However, microscopic examination of the *G. oceanica* clone without coccoliths showed that when treated with copper, cells with coccoliths were produced.

Regression analysis of cell surface area (μm^2) with toxicity of copper to the alga (72-h IC50) revealed no significant linear relationship ($R^2 = 0.02$; $p = 0.67$, data not shown). No general trend appeared to link the toxicity of copper to the size of the cell.

Copper partition coefficients (K_d)

Preliminary experiments showed that higher cell densities (10^5 cells/mL) than those used in the bioassays were required to determine cellular copper by difference. Adsorption of copper to algae was rapid, reaching a plateau after approximately 40 min. A 1-h exposure time was chosen, as this was sufficient to ensure that equilibrium between copper in solution and copper on the cells had been reached, and short enough to ensure that copper internalisation was minimal.

Copper adsorption to algal cells increased linearly with copper concentration, and the lines of best fit used to predict K_d had R^2 values ranging from 0.84 to 1.0 (e.g. Figure 2). Partition coefficients (K_d) ranged from 7.6 to 32×10^{-10} L/cell, with *D. tertiolecta* having the lowest partition coefficient on a per cell basis, and *Tetraselmis* sp. having the highest partition coefficient on a per cell basis, i.e. *Tetraselmis* sp. adsorbs relatively more copper per cell than any other alga (Table 3). On a surface area basis, K_d values varied from 2.0 to 24×10^{-12} L/μm^2, with the largest cell *H. niei* having the lowest K_d and the smallest cell, the *E. huxleyi* clone without coccoliths, the highest K_d. Regression analysis showed that there was no

significant linear relationship ($p = 0.11$) between cell surface area and K_d (on a surface area basis) but a significant nonlinear exponential relationship was found ($p = 0.026$). The cell surface area explains 66% of the variation in K_d ($L/\mu m^2$) (Figure 3a). However, the relationship was not significant when K_d was expressed as L/cell ($p = 0.95$). No significant linear relationship between algal sensitivity to copper (72-h IC50) and copper-cell partitioning, either on a surface area basis (Figure 3b; $p = 0.82$) or on a per cell basis ($p = 0.96$) was found. This relationship was also tested for significance with toxicity on a normal (non-log) basis, and also with the exclusion of *Dunaliella tertiolecta*, and in no test was a significant linear relationship between partition coefficient and toxicity, expressed as 72-h IC50 values, found ($p > 0.05$ in all cases).

Discussion

Species-sensitivity to copper

The sensitivity of the 11 marine species to copper varied by three orders of magnitude, with IC50 values ranging from 0.6-530 µg Cu/L. It is difficult to compare the sensitivity of these species with literature data due to the different test conditions, cell densities and test media used (Stauber and Davies, 2000). However, the results are in accordance with previous studies in our laboratory that also used a minimal nutrient medium to avoid complexation of metals and consequent under-estimation of copper toxicity (Franklin et al., 2004, Franklin et al., 2001a, Franklin et al., 2001b, Stauber and Davies, 2000). Of the species tested, the most sensitive species was the small centric diatom *M. polymorphus*. The naked chlorophyte (no cell wall) *Dunaliella tertiolecta* was the most tolerant species to copper, with a 72-h IC50 of 530 µg Cu/L. This was similar to reported 72-h IC50 values of 576 µg Cu/L (Peterson and Stauber, 1996) and > 600 µg Cu/L (Franklin et al., 2000) for this alga. The 72-h IC50 values reported in the current study are in agreement with 72-h IC50 values reported for *N.*

closterium (18 µg/L this study, 14 µg/L in Stauber and Davies, 2000), *P. tricornutum* (8 µg/L this study, 10-20 µg/L in Franklin et al, 2001a, 2001b, 2004), *M. pusilla* (1.2 µg/L this study, 3.1 µg/L in Franklin et al., 2004) and *H. niei* (4.8 µg/L this study, 16 µg/L in Franklin et al., 2004). Four of eleven algal species (or thirteen strains) tested had LOEC values less than the current Australasian marine water quality guideline for copper of 1.4 µg Cu/L (ANZECC/ARNCANZ, 2000), and *P. tricornutum* had a LOEC of 1.5 µg Cu/L. This suggests that the guideline may be under-protective for many sensitive marine microalgae.

Factors affecting species sensitivity to copper

Cell size

Small cells have large surface area to volume ratios and have been reported to be more sensitive to copper than larger species (Quigg et al., 2006). In the current study, the three most sensitive species *M. polymorphus*, *M. pusilla* and *Isochrysis* sp. were amongst the four smallest species in our study. *M. pusilla* had the smallest surface area, yet was about twice as tolerant as *M. polymorphus*. However, the dinoflagellate *H. niei* was the largest species tested and was one of the most sensitive algal species to copper, with a 72-h IC50 of 4.8 µg/L. Regression analysis showed no significant relationship between cell surface area and sensitivity to copper. Also, the clones of *E. huxleyi* and *G. oceanica* that did not produce coccoliths were smaller than the clones with coccoliths, yet were less sensitive.

There is limited data on the effect of cell size on copper sensitivity in microalgae. The effect of cell size on copper sensitivity in the pennate diatom *Haslea ostrearia* (Simonsen) was studied by Joux-Arab et al. (2000). With apical axes of 40, 65 and 85 µm in length, sensitivity was highest for the 85 µm strain and lowest for the 65 µm strain, i.e. copper sensitivity was not related to cell size. In a study of freshwater phytoplankton in mesocosms

(Le Jeune et al., 2006), smaller phytoplankton (3 to 20 µm) dominated communities (> 90% of total biomass) over larger phytoplankton (20-250 µm) after 27-day exposures to copper concentrations of 160 µg/L (complexing capacity of the waters was 100 ± 10 and 110 ± 10 µg/L in the spring and summer samplings, respectively). Although it was suggested that smaller organisms should be more sensitive to copper because of the higher cell surface to volume ratio, small cells may also recover faster due to their rapid growth rates.

From the current study and limited literature, it appears that there is no clear evidence that small microalgae are more sensitive to copper than larger species.

Cell wall type

The naked chlorophyte *D. tertiolecta* was the most tolerant species to copper. It appears that the absence of a cell wall did not render it more sensitive to copper than other green algae with cell walls, such as the prasinophytes *M. pusilla* and *Tetraselmis* sp., which had much lower IC50 values. In contrast, a wall-less clone of the freshwater unicellular green alga *C. reinhardtii* was reported to be more sensitive to cadmium, copper and nickel than a strain with a cell wall (Macfie et al, 1994). Yet neither total copper, weakly-bound nor strongly-bound copper (operationally defined as externally-bound and internally-bound copper, respectively) differed significantly between the two strains (Macfie and Welbourne, 2000), suggesting that the cell wall itself did not modify copper toxicity in this alga. However, in exposures to cadmium, the walled strain accumulated more metal than the wall-less strain (Macfie and Welbourne, 2000), while in a study by Kola and Wilkinson (2005), the maximum internal flux of cadmium was five fold higher for wild type *C. reinhardtii*, when compared to a wall-less strain.

The sensitivity of two coccolithophorids, *E. huxleyi* and *G. oceanica*, to copper was examined for two clones, a normal strain which produces coccoliths and a strain which does not produce coccoliths, to determine whether a difference in this external structural feature would be reflected in differences in sensitivity to copper. The normal strains of both *E. huxleyi* and *G. oceanica* were slightly, but significantly, more sensitive to copper than the non-coccolith producing strains. This was contrary to expectation. The coccolith producing cells have a larger diameter and the presence of many small coccoliths increased the surface area of these cells, thus prior literature would suggest a greater number of inert or non-biologically active sites for copper to bind to, reducing the concentration of copper in solution with less copper available for uptake into cells. Alternatively it has been proposed that smaller cells, with a larger surface area to volume ratio, should be more sensitive to toxicants, yet this is also not the case. The experimental examination of adsorption revealed that the *E. huxleyi* copper seawater-cell partition coefficients (K_d) (on a per cell basis) were not significantly different between the two strains. So why are the coccolith-absent strains more sensitive to copper if it is not related to adsorption sites? It is possible that the mechanism by which calcium is taken up to produce coccoliths, also enhances copper uptake, thereby increasing the sensitivity of the coccolith-bearing clones. Coccolithophorids require calcium for the production of coccoliths (Rost and Riebesell, 2004) and have been shown to have much higher uptake rates of calcium when compared to non-coccolith forming haptophytes, e.g. *E. huxleyi* compared to *Isochrysis galbana* (Sorrosa et al., 2005). However, this explanation is unlikely because (1) the closely related but non-coccolith forming Prymnesiophyte, *Isochrysis* sp., was much more sensitive than any of the coccolithophorid clones in this study, and (2) there is no evidence from freshwater studies with other organisms that copper uptake occurs via calcium channels (Niyogi and Wood, 2004).

Taxonomic grouping

Of the 11 algal species investigated, there was no indication that any one taxonomic class of algae was more sensitive to copper than another. The five most sensitive species to copper (with 72-h IC50 values of < 5 µg/L) included five different classes of algae (a diatom, a prasinophyte, a cryptomonad, a prymnesiophyte and a dinoflagellate). Most of the literature data on the toxicity of copper to microalgae in laboratory growth inhibition bioassays have focused on diatoms, so it is difficult to determine if one specific class of algae is more sensitive than another. Stauber and Davies (2000) summarised the copper sensitivities of marine phytoplankton, including 16 diatoms, one chlorophyte, one dinoflagellate and one cyanophyte, and found similar sensitivities of all algae except the chlorophyte *Dunaliella tertiolecta,* which was tolerant to copper. Algae from the genera *Dunaliella* and *Tetraselmis* have been identified as highly tolerant species, e.g. the highly saline tolerant *Dunaliella salina* is also tolerant to metals, and has been reported to effectively outcompete other algal species in highly metal-stressed conditions (Moreno-Garrido et al., 2005; DeKuhn et al., 2006). Similarly, *Tetraselmis chui* was also reported as one of the most tolerant algal species to metal stressors (Moreno-Garrido et al., 2005).

While the green algae *Dunaliella* spp. and *Tetraselmis* spp., are often found to be tolerant, considerable variation in toxicity among green algae occurs. *D. tertiolecta* was found to have higher tolerance to copper than *D. salina* (Nikookar et al., 2005) which may have been related to lower concentrations of copper in the cell and an increase in ascorbate peroxidase activity. Ismail et al. (2002) found that *Tetraselmis* sp. was three times less sensitive than *T. tetrahele* from the same genera, with 96-h IC50 values of 130 and 370 µg/L, respectively, showing variation in toxicity within closely related algal species. Moreno-Garrido et al. (2000) found sensitivity to copper (72-h IC50 µg/L) to decrease in the order *Isochrysis galbana*

(prymnesiophyte; 0.4 µg/L) >> *Chlorella autotrophyca* (green alga; 9.6 µg/L) ≈ *P. tricornutum* (diatom; 9.8 µg/L) > *Nannochloris atomus* (green alga; 16.7 µg/L), showing that green algae can be just as sensitive as other taxonomic groups. In a study of toxicity of copper to marine algae from a variety of taxonomic groups (using chlorophyll *a* measurements in micro-well plates to assess growth inhibition), Satoh et al. (2005) found that *Isochrysis galbana* and the cyanophyte *Synechoccus* sp. were of similar tolerance, with 72-h IC50 values of 4.2 mg Cu/L and 5.3 mg Cu/L, respectively. Two green algae *Prasinococcus* sp. and *Tetraselmis tetrahele* fell in the same range of sensitivity (IC50 values of 5.4 and 7.4 mg Cu/L) while two more green algae (*Chlorococcum littorale* and *Chlorococcum* sp.) were more tolerant with 72-h IC50s of 10.2 and 11.7 mg Cu/L, respectively. The diatom (*Cylindrotheca* sp.) fell within the sensitivities of the green algae, with a 72-h IC50 of 7.7 mg Cu/L, while the dinoflagellate *Heterocapsa* sp. was most tolerant to copper with an IC50 of 11.6 mg/L, in contrast to the current study where *H. niei* was one of the most sensitive species. The high (mg/L) 72-h IC50 values reported in Satoh et al. (2005) compared to those in the present study are likely to be due to the use of a standard culture medium as the test medium, and the cell densities were presumably higher than in our study in order to obtain sufficient chlorophyll *a* to use as a surrogate for biomass. Despite the wide range of species studied from different groups in Satoh et al. (2005) there was less than a three fold difference in sensitivity, while in the current study sensitivity ranged over four orders of magnitude.

In this study the diatoms *M. polymorphus* and *P. tricornutum* were two of the more sensitive species to copper, with IC50 values of 0.6 and 8.0 µg Cu/l, respectively. However, the other diatom tested, *N. closterium*, was more tolerant, with an IC50 of 18 µg Cu/L. The relative sensitivity of diatoms to copper compared to other algal classes is unclear from the literature. Sunda and Guillard (1976) found that the lowest observed free ion concentration to affect

growth was 1.9 ng Cu^{2+}/L for the diatom *Thalassiosira pseudonana* and 2.5 ng Cu^{2+}/L for the green alga *Nannochloris atomus*, suggesting greater tolerance of the green alga. However, in a study by Hawkins and Griffiths (1982), a dinoflagellate (*Amphidinium carterae*) and a diatom (*P. tricornutum*) were found to be the most tolerant species to copper with IC50 values of 7.0 pCu (6.4 µg Cu^{2+}/L) while two species of green algae, *Stichococcus baciallaris* and *D. tertiolecta*, were most sensitive with IC50 values of 10.1 and 9.1 pCu (5 and 50 ng Cu^{2+}/L), respectively. Another marine diatom, *Amphora coffeaeformis*, was noted to be very copper tolerant, even growing on copper-based antifouling paints (Brown et al., 1988). Tolerance in *A. coffeaeformis* was found to be related to the production of soluble exudates and mucilage which presumably decreased copper uptake into the cell. Mucilage layers may play a very important role in copper adsorption with Tien et al. (2005) finding that copper Freundlich adsorption capacity constants (K_f) were higher in four mucilaginous freshwater species including three cyanophytes and a chlorophyte (3.96-12.62 mg Cu per g) when compared to the non-mucilaginous algal species including a chlorophyte, two diatoms and a dinoflagellate (0.36 to 3.63 mg Cu per g). In addition, cells with mucilage required more time to reach adsorption maxima and copper adsorption per unit area was larger. Exudate production and cell surface changes in response to metal stress have been suggested as possible detoxification mechanisms for many algae, and may help alter metal speciation in the immediate local environment around cells (Gonzalez-Davila et al., 1995; Croot et al., 2000).

In a study of succession in a marine phytoplankton community following copper exposure, Gustavson et al. (1999) found that while a pollution-induced community tolerance response was observed, with short-term tolerance that increased concurrently with a decrease in total number of species, diversity and richness (at 15 µg Cu/L), they could not make any general assumptions regarding particular copper-sensitive or copper-tolerant groups of algae. Similar

conclusions regarding species specificity have been reached with respect to freshwater algal communities, e.g. Genter and Lehman (2000) who emphasised that copper and metal sensitivity was very species-specific.

It is clear from the current study and the variety of responses to copper by different algal taxonomic groups observed in the literature that the relationship between copper and species-sensitivity is complex, and is unlikely to be related to taxonomic groupings.

Metal-cell partitioning

In the current study, copper water-cell partition coefficients (K_d) varied four-fold on a per cell basis and 12-fold when corrected for cell surface area. The prasinophyte *Tetraselmis* sp. (K_d $32 \pm 1 \times 10^{-10}$ L/cell) adsorbed the most copper per cell, relative to the concentration of dissolved copper in solution. Relative adsorption declined in the order *Tetraselmis* sp. > diatom *Nitzschia* sp. (K_d 24×10^{-10} L/cell) > dinoflagellate *H. niei* ($12 \pm 7 \times 10^{-10}$ L/cell) = diatom *Phaeodactylum* sp. ($11 \pm 1 \times 10^{-10}$ L/cell) > green alga *D. tertiolecta* (K_d $7.6 \pm 0.6 \times 10^{-10}$ L/cell) = coccolithophorid *E. huxleyi* regardless of the presence of coccoliths (with coccoliths, K_d $8 \pm 1 \times 10^{-10}$ L/cell; without coccoliths $8 \pm 9 \times 10^{-10}$ L/cell). Where more than one test for K_d values was carried out ($n \geq 3$), e.g. *D. tertiolecta*, *Tetraselmis* sp. and *P. tricornutum*, the results agreed closely and the standard deviation was small giving confidence that the K_d measurements for all species were sufficiently precise. The one exception was *E. huxleyi* (without coccoliths) where the standard deviation in K_d was unexplainably large A study by Quigg et al (2006) found that of seven phytoplankton tested, the diatom *Thalassiosira weissflogii*, accumulated the most copper per cell over one hour, while the two chlorophytes (*Pyramimonas parkeae* and *Tetraselmis levis*) also accumulated high concentrations of copper. This contrasts with the current work, where the chlorophyte

accumulated the least copper relative to the concentration of copper in solution. These differences suggest that adsorption of metals to cell surfaces appears to be non-discriminatory for cell wall type.

Adsorption of metals to cell surfaces was not related to the sensitivity of algal species after 72-h exposures to copper (Figure 3b). The hypothesis under investigation was derived from the work of Franklin et al. (2002) who showed that two freshwater green algae, *Selenastrum capricornutum* (now *Pseudokirchneriella subcapitata*) and *Chlorella* sp. had similar K_d values (25 and 30 × 10^{-11} L/μm^2) and similar sensitivities to copper (72-h IC50s of 6.6 and 4.6 μg Cu/L, respectively). These authors hypothesised that copper toxicity in freshwater algae may be predicted by copper-cell binding. The present study, however, using a wider range of species derived from the marine environment, does not support these findings. The K_d values calculated in the current study were approximately one order of magnitude lower than those found for the two freshwater algae in the Franklin et al. (2002) study, in which K_d values were calculated after 72 h (i.e. after cells were growing and dividing). These differences may be because the current study calculated K_d values after only one hour, which was sufficient time for a pseudo-equilibrium between copper in solution and copper cell-binding to be established, and was without the confounding factors of cell growth (Gowrinthinan and Rao, 1991; Gonzalez-Davila et al, 1995; Quigg et al., 2006).

While there was no relationship between copper sensitivity and partition coefficient (K_d), there was a relationship between cell size and partition coefficient on a surface area basis. The copper partition co-efficient (K_d in L/μm^2) decreased with increasing cell surface area in an exponential relationship ($R^2 = 0.66$, $p = 0.026$) (Figure 3a). However, this relationship was not significant when the K_d was expressed on a per cell basis. This is similar to that reported

by Quigg et al. (2006), who found that, despite the copper accumulation rate being the highest for the smallest cell, copper accumulation rates were not linearly related to cell surface area for seven phytoplankton species (Quigg et al., 2006).

Other factors

Algal sensitivity to copper is more likely to be related to copper internalisation than to adsorption to non-specific surface binding sites. Binding of copper to the biotic ligand, as yet unknown in algae but assumed to be on the plasma membrane (DeSchamphelare et al., 2005), is the critical step before internalisation of copper and subsequent toxicity (Kola and Wilkinson, 2005). Different uptake rates for algal species could lead to differences in metal-sensitivity. Quigg et al. (2006) found that the most copper-sensitive species, a cyanobacterium (*Synechococcus* sp.), had a 2-3 fold greater copper uptake rate on a surface area basis, than eukaryotic algae such as *Tetraselmis levis* and *E. huxleyi*.

Growth inhibition in microalgae has also been related to intracellular copper concentrations (Stauber and Florence, 1987, Franklin et al., 2002). However, biota may bioaccumulate metals in non-metabolically active forms, so internal metal loadings do not always reflect differences in sensitivity (Buchwalter and Cain, 2005, Luoma and Rainbow, 2005). Detoxification mechanisms include exclusion, internal sequestration and active efflux mechanisms. For example, copper may be prevented from entering algal cells by the release of exudates that bind copper in solution, reducing the bioavailable fraction of metal, and thereby reducing toxicity. Alternative physical exclusion mechanisms may include reduced membrane permeability (Megharaj et al., 2003) or alteration of the metal species at the cell surface. Once copper is internalised, the production of cysteine-rich phytochelatins can bind excess copper, rendering it less toxic through subcellular partitioning of metals to inactive

sites. Phytochelatins were induced by free copper concentrations of 8×10^{-11} M for the freshwater green alga *Scenedesmus vacuolatus* (LeFaucher et al., 2006) and in the marine diatom *P. tricornutum* (Morelli and Scarano, 2004) but not in *Thalassiosira weissflogii* (Miao and Wang, 2007). Similarly, sequestration of copper in the cell wall of the marine diatom *Skeletonema costatum* (Nassiri et al., 1997) and accumulation of copper in thykaloid membranes of the freshwater green alga *Oocystis nephrocytioides* (Soldo and Behra, 2005), away from internal metabolic systems, were suggested to be the major detoxification mechanisms for these algae. The induction of proteins due to stress (toxicant-induced or via nutrient deficiency) has been noted in some algal cells (Davis et al., 2005) and the induction of the antioxidant superoxide dismutase has been noted for the marine prasinophyte *Tetraselmis gracilis* when exposed to cadmium. Finally, efflux mechanisms may be used to pump metal back into solution, potentially as a different, less toxic metal species. Population dynamics and growth rates can also play a role, as an increase in cell density will provide a greater surface area, effectively diluting the concentration of toxicant per cell (Franklin et al., 2002, Megharaj et al., 2003, Luoma and Rainbow, 2005).

Interspecies differences in sensitivity of microalgae to copper may also be related to their habitat (estuarine versus coastal versus oceanic environments) and their prior exposure to copper. Sunda (1989) showed that small changes in metal bioavailability in the open ocean affected the type of algal species that occurred. In contrast, Quigg et al. (2006) showed that copper accumulation rates in algae were not related to geographic position, e.g. coastal versus oceanic environments.

Some algae have been shown to adapt, either physiologically or genetically, to high metal concentrations, resulting in decreased sensitivity to metals. Twiss (1990) reported

Chlamydomonas acidophila isolated from acidic, copper-contaminated soils, had algistatic copper concentrations 20-125 times higher than laboratory strains. Acclimation or adaptation of freshwater algae to these high metal concentrations has also been explored in laboratory environments, with increases in copper or zinc concentrations in algal growth media leading to an increased tolerance towards those metals (Muyssen and Janssen, 2001; Bossuyt and Janssen 2004). In contrast, Johnson et al. (2007) found no increased tolerance to copper or zinc in the marine alga *N. closterium* or the freshwater alga *Chlorella sp* after acclimation to copper in culture medium over 100 days.

Conclusions

Inter-species differences in copper sensitivity of marine microalgae were not related to biotic factors, such as cell wall type and cell size, nor to taxonomic class, nor to equilibrium partitioning of copper to the cell. Adsorption of copper to cells was rapid and it was not possible to discriminate between non-specific copper binding sites and high affinity active sites on the cell wall/cell membrane. It is possible that copper sensitivity is better related to copper uptake rates (internalisation) and intracellular detoxification processes. These factors are currently being investigated in our laboratory.

Acknowledgments

The authors wish to acknowledge Merrin Adams, Monique Binet, Sarah Stephenson, Janine Wech and Jenny Boge at CSIRO Land and Water for their invaluable assistance in the laboratory. This work was supported by an Australian Postgraduate Award and a CSIRO postgraduate scholarship.

References

Apte SC, Batley GE. Trace metal speciation of labile chemical species in natural waters and sediments: Non-electrochemical approaches. In: Tessier A, Turner DR, editors. Metal Speciation and Bioavailability in Aquatic Systems, IUPAC, John Wiley and Sons, 1995, pp. 259-306.

Australian and New Zealand Environment and Conservation Council; Agricultural and Resource Management Council of Australia and New Zealand (ANZECC/ARMCANZ). Australian and New Zealand Guidelines for Fresh and Marine Water Quality, Paper 4, Volume 1. ANZECC/ARMCANZ; 2000.

Amiard-Triquet C, Berthet B, Joux L, Perrein-Ettajani H. Significance of physicochemical forms of storage in microalgae in predicting copper transfer to filter-feeding oysters (*Crassostrea gigas*). Environ Toxicol 2006;21:1-7.

Bossuyt BTA, Janssen CR. Long-term acclimation of *Pseudokirchneriella subcapitata* (Korshikov) Hindak to different copper concentrations: changes in tolerance and physiology. Aquat Toxicol 2004;68:61-74.

Brown LN, Robinson, MG, Hall BD. Mechanisms for copper tolerance in *Amphora coffeaeformis*- internal and external binding. Mar Biol 1988;97:581-586.

Buchwalter B, Cain D. Species-specific responses to environmental contaminants: A case for comparative physiology. SETAC Globe 2005;May-Jun:31.

Crist RH, Martin JR, Guptill PW, Eslinger JM, Crist DR. Interaction of metals and protons with algae. 2. Ion-exchange in adsorption and metal displacement by protons. Environ Sci Technol 1990;24: 337-342.

Croot PL, Moffett JW, Brand LE. Production of extracellular Cu complexing ligands by eucaryotic phytoplankton in response to Cu stress. Limnol Oceanogr 2000;45:619-627.

Davis AK, Hillebrand M, Palenik B. A stress-induced protein associated with the girdle band region of the diatom *Thalassiosira pseudonana* (Bacillariophyta). J Phycol 2005;41:577-589.

DeKuhn RM, Streb C, Breiter R, Richter P, Neeße T, Häder DP. Screening for unicellular algae as possible bioassay organisms for monitoring marine water samples. Water Res 2006;40:2695-2703.

Franklin NM. The application of flow cytometry to the development of algal toxicity tests for assessing metal bioavailability. PhD Thesis, Environmental Biology, University of Technology, Sydney, 2003.

Franklin NM, Adams MS, Stauber JL, Lim RP. Development of an improved rapid enzyme inhibition bioassay with marine and freshwater microalgae using flow cytometry. Arch Environ Contam Toxicol 2001a;40:469-480.

Franklin NM, Stauber JL, Lim RP. Development of flow-cytometry-based algal bioassays for assessing toxicity of copper in natural waters. Environ Toxicol Chem 2001b;20:160-170.

Franklin NM, Stauber JL, Apte SC, Lim RP. Effect of initial cell density on the bioavailability and toxicity of copper in microalgal bioassays. Environ Toxicol Chem 2002;21:742-751.

Franklin NM, Stauber JL, Lim RP. Development of multispecies algal bioassays using flow cytometry. Environ Toxicol Chem 2004;23:1452-1462.

Genter RB, Lehman, RM. Metal toxicity inferred from algal population density, heterotrophic substrate use, and fatty acid profile in a small stream. Environ Toxicol Chem 2000;19:869-878.

Gonzalez-Davila M, Santana-Casiano JM, Perez-Pena J, Millero FJ. Binding of Cu(II) to the surface and exudates of the alga *Dunaliella tertiolecta* in seawater. Environ Sci Technol 1995;29:289-301.

Gowrinathan KP, Rao VNR. Uptake and accumulation of copper by two microalgae. Phykos 1991;30:13-18.

Guillard RRL, Ryther JH. Studies of marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervaceae* Cleve. Canad J Microbiol 1962;8:229-239.

Gustavson K, Petersen S, Pedersen B, Stuer-Lauridsen F, Pedersen S, Wängberg SA. Pollution-induced community tolerance (PICT) in coastal phytoplankton communities exposed to copper. Hydrobiologia 1999;416: 125-138.

Hawkins PR, Griffiths, DJ. Cupric ion tolerance in four species of marine phytoplankton. Bot Mar 1982;XXV:31-33.

He LM, Tebo BM. Surface charge properties of and Cu(II) adsorption by spores of the marine *Bacillus* sp. strain SG-1. Appl Environ Microb 1998;64:1123-1129.

Ismail M, Phang SM, Tong SL, Brown MT. A modified toxicity testing method using tropical marine microalgae. Environ Monit Assess 2002;75:145-154.

Johnson H, Adams MS, Stauber JL, Jolley DF. Copper and zinc tolerance of two tropical microalgae after copper acclimation. Environ Toxicol 2007;22:234-244.

Joux-Arab L, Berthet B, Robert JM. Do toxicity and accumulation of copper change during size reduction in the marine pennate diatom *Haslea ostrearia*? Mar Biol 2000;136:323-330.

Keller MD, Selvin RC, Claus W, Guillard RRL. Media for the culture of oceanic ultraplankton. J Phycol 1987;23:633-638.

Kola H, Wilkinson KJ. Cadmium uptake by a green alga can be predicted by equilibrium modelling. Environ Sci Technol 2005;39:3040-3047.

Le Faucher S, Schildknecht F, Behra R, Sigg L. Thiols in *Scenedesmus vacuolatus* upon exposure to metals and metalloids. Aquat Tox 2006;80:355-361.

Le Jeune AHJ, Charpin M, Deluchat V, Briand JF, Lenain JF, Baudu M, Amblard C. Effect of copper sulphate treatment on natural phytoplanktonic communities. Aquat Tox 2006;80:267-280.

Loeblich AR, Smith VE. Chloroplast pigments of the marine dinoflagellate *Gyrodinium resplendens*. Lipids 1968;3:5-13.

Luoma SN, Rainbow PS. Why is metal bioaccumulation so variable? Biodynamics as a unifying concept. Environ Sci Technol 2005;39:1921-1931.

Macfie SM, Tarmohamed Y, Welbourn PM. Effects of cadmium, cobalt, copper and nickel on growth of the green alga *Chlamydomonas reinhardtii.* The influences of the cell wall and pH. Arch Environ Contam Toxicol 1994;27:454-458.

Macfie SM, Welbourn PM. The cell wall as a barrier to uptake of metal ions in the unicellular green alga *Chlamydomonas reinhardtii* (Chlorophyceae). Arch Environ Contam Toxicol 2000;39:413-419.

Megharaj M, Ragusa SR, Naidu R. Metal-algae interactions: implications of bioavailability. In: Naidu R, Gupta VVSR, Rogers S, Kookana RS, Bolan NS, Adriano DC, editors Bioavailability, Toxicity and Risk Relationships in Ecosystems. Science Publishers, Enfield, USA, 2003, pp. 109-144.

Miao AJ, Wang WX. Predicting copper toxicity with its intracellular or subcellular concentration and the thiol synthesis in a marine diatom. Environ Sci Technol 2007;41:1777-1782.

Morelli E, Scarano G. Copper-induced changes of non-protein thiols and anti-oxidant enzymes in the marine microalgae *Phaeodactylum tricornutum*. Plant Sci 2004;167:289-296.

Moreno-Garrido I, Lubián LM, Soares AMVM. Influence of cellular density on determination of EC50 in microalgal growth inhibition tests. Ecotox Environ Safety 2000;47:112-116.

Moreno-Garrido I, Campana O, Lubián LM, Blasco J. Calcium alginate immobilized marine microalgae: Experiments on growth and short-term heavy metal accumulation. Mar Poll Bull 2005;51:823-829.

Muyssen BTA, Janssen CR. Zinc acclimation and its effect on the zinc tolerance of *Raphidocelis subcapitata* and *Chlorella vulgaris* in laboratory experiments. Chemosphere, 2001;45:507-514.

Nassiri Y, Mansot JL, Wéry J, Ginsburger-Vogel T, Amiard JC. Ultrastructural and electron energy loss spectroscopy studies of sequestration mechanisms of Cd and Cu in the marine diatom *Skeletonema costatum*. Arch Environ Contam Toxicol 1997;33:147-155.

Nikookar K, Moradshahi A, Hosseini L. Physiological responses of *Dunaliella salina* and *Dunaliella tertiolecta* to copper toxicity. Biomol Eng 2005;22:141-146.

Niyogi S, Wood CM. Biotic ligand model, a flexible tool for developing site-specific water quality guidelines for metals. Environ Sci Technol 2004;38:6177-6192.

Peterson SM and Stauber, JL. New algal enzyme bioassay for the rapid assessment of aquatic toxicity. Bull Environ Contam Toxicol 1996; 56: 750-757.

Quigg A, Reinfelder JR, Fisher N. Copper uptake kinetics in diverse marine phytoplankton. Limnol Oceanogr 2006;51:893-899.

Rost B, Riebesell U. Coccolithophores and the biological pump: responses to environmental changes. In: Thierstein HR, Young JS, editors. Coccolithophores: from molecular processes to global impact. Berlin: Springer-Verlag; 2004, pp. 99-125.

Sprague JB, Fogels A. Watch the Y in bioassay. In: Parker WR, Pessah E, Wells PG, Westlake GF, editors. Proceedings of the 3rd Aquatic Toxicity Workshop. Halifax, NS, Canada, 2-3 November 1976, Halifax, NS, Canada, 1977, pp. 107-118.

Satoh A, Vudikaria LQ, Kurano N, Miyachi S. Evaluation of the sensitivity of marine microalgal strains to the heavy metals, Cu, As, Sb, Pb and Cd. Environ Int 2005; 31: 713-722.

Soldo D, Hari R, Sigg L, Behra R. Tolerance of *Oocystis nephrocytioides* to copper: intracellular distribution and extracellular complexation of copper. Aquat Toxicol 2005;71: 307-317.

Sorrosa JM, Satoh M, Shiraiwa Y. Low temperature stimulates cell enlargement and intracellular calcification of coccolithophores. Mar Biotech 2005;7:128-133.

Stauber JL, Davies CM. Use and limitations of microbial bioassays for assessing copper bioavailability in the aquatic environment. Environ Rev 2000;8:255-301.

Stauber JL, Florence TM. Mechanism of toxicity of ionic copper and copper complexes to algae. Mar Biol 1987;94:511-519.

Sunda W. Trace metal interactions with marine phytoplankton. Biol Oceanogr 1989; 6: 411-442.

Sunda W, Guillard RRL. The relationship between cupric ion activity and the toxicity of copper to phytoplankton. J Mar Res 1976;34:511-529.

Tien CJ, Sigee DC, White KN. Copper adsorption kinetics of cultured algal cells and freshwater phytoplankton with emphasis on cell surface characteristics. J Appl Phycol 2005;17:379-389.

Twiss MR. Copper tolerance of *Chlamydomonas-acidophila* (chlorophyceae) isolated from acidic, copper-contaminated soils. J Phycol 1990;26:655-659.

Table 1: Algal species investigated in this study: description, source and culture medium used

Alga	Features	Culture Number[a]	Place isolated	Culture Medium	Cell shape	Cell size (μm)[h]	Axenic
Bacillariophyceae (diatoms)							
Minutocellus polymorphus (Hargraves and Guillard) Hasle, Von Stosch and Syvertsen	Centric diatom. Siliceous cell wall	CS-3	Port Hacking, NSW, Australia	f/2[b]	Elliptical prism	4.0 × 3.7 × 3.1	N
Phaeodactylum tricornutum Bohlin	Pennate diatom. Weakly siliceous cell wall	CS-29/4	Unknown	f/2[b]		24-30 × 2.5-3.5	Y
Nitzschia closterium (Ehrenb.) W. Smith	Pennate diatom. Siliceous cell wall	CS-5	Port Hacking, NSW, Australia	f[c]		24-32 × 1-3	N
Chlorophyceae (green algae)							
Dunaliella tertiolecta (Butcher)	Naked membrane (no cell wall, but mucilage layer)	CS-175	Unknown	f/2[b]	Prolate ellipsoid	7-10 × 6-8	Y
Prasinophyceae (green flagellates)							
Micromonas pusilla (Butcher) Manton and Parke	Prasinophyceae. Small green flagellate with organic scales	CS-222	Corio Bay, VIC, Australia	f/2 + Se[d]	Sphere	2 ± 0.3[i]	N
Tetraselmis sp.	Prasinophyceae. Green flagellate	CS-87	Port Hacking, NSW, Australia	f/2[b]	Prolate ellipsoid	8-11 × 5-9	Y
Dinophyceae (Dinoflagellate)							
Heterocapsa niei (Loeblich) Morrill and Loeblich	Large, motile, fragile cell	CS-89	Port Hacking, NSW, Australia	G + Se[e]	Prolate ellipsoid	18 × 12[i]	N
Prymnesiophyceae							
Isochrysis sp.	Tropical species (27°C). Golden-brown flagellate.	CS-177	Mataira, Society Islands, Tahiti	f/2[b]	Prolate ellipsoid	4-7 × 3.5	Y
Gephyrocapsa oceanica Kamptner	Coccolithophorid. Coccoliths present ($CaCO_3$ plates). Non-motile.	CS-335	Jervis Bay, NSW, Australia	G/2 + Se[f]	Sphere	6-9	N
Emiliania huxleyi (Lohmann) Hay and Mohler	Coccolithophorid. Non-motile cell, coccoliths present, no organic scales	CS-275/01	North Atlantic, Iceland Basin	K[g]	Sphere	4-6	N
Emiliania huxleyi (Lohmann) Hay and Mohler	Coccolithophorid. Coccoliths absent	(coccolith absent clone)	Cloned from above strain	K[g]	Sphere	2-4	N
Cryptophyceae							
Proteomonas sulcata (Hill et Wetherbee)	Tropical species (27°C). Flagellate. Periplast plates outside plasma membrane.	CS-412	Fitzroy Island, QLD, Australia	f[c]	Prolate ellipsoid	5-9 × 2.5-6.5	N

All cultures are maintained and tested at 21°C unless otherwise noted.
[a] CS= CSIRO Collection of Living Microalgae, Marine and Atmospheric Research, Hobart, Australia.
[b] Half strength f medium, Guillard and Ryther (1962).

[c] f medium, Guillard and Ryther (1962).
[d] Half strength f medium, Guillard and Ryther (1962) + 1.27 mg SeO_3/L.
[e] G medium, Loeblich and Smith (1968) + 0.0013 mg/L SeO_3.
[f] half-strength G medium, Loeblich and Smith (1968) + 1.27 mg SeO_3/L.
[g] K medium, Keller et al. (1987).
[h] Unless otherwise stated, cell size was determined from analysis of 30 random cells on phase-contrast microscope at 400× magnification using an eye-piece micrometer. Cell dimensions are reported in μm and are length, width and depth for elliptical prism (*M. polymorphus*); entire cell length and width at widest section for pennate diatoms (*P. tricornutum* and *N. closterium*); length and width (width = depth) for prolate ellipsoid cells and diameter for spherical cells.
[i] Analysis of same strain in Franklin (2003) by confocal microscopy.

Table 2: The response of marine microalgae to copper in 72-h growth rate inhibition bioassays in increasing order of sensitivity

Alga	72-h IC50[e] (µg Cu/L)	95% C.L. (µg Cu/L)	LOEC[g] (µg Cu/L)	NOEC[j] (µg Cu/L)	Control Cell Division Rate (doublings/day)
D. tertiolecta[a]	530	450-600	42	8	1.39 ± 0.02
Tetraselmis sp.[a]	47	46-49	22	7	1.37 ± 0.26
G. oceanica – coccoliths[b]	>25	-	2.6	1.3	1.07
E. huxleyi – coccoliths[b]	20	16-26	-[h]	9	1.21
N. closterium[c]	18	6-30[f]	5.8	4.4	1.53 ± 0.16
G. oceanica + coccoliths[b]	17	17-18	1	<1	1.21
E. huxleyi + coccoliths[b]	15	12-18	-[h]	8	0.92
P. tricornutum[d]	8.0	4.7- 8.3	1.5	<1.5	1.78 ± 0.08
H. niei[b]	4.8	3.5-7.2	nd[i]	nd[i]	0.96
P. sulcata [b]	4.2	2.4-7.5	-[h]	<5	1.54
Isochrysis sp.[b]	4.0	3.8-4.2	1.1	<1.1	1.85
M. pusilla[b]	1.2	1.1-1.4	0.6	0.3	1.45
M. polymorphus[b]	0.6	0.5-0.8	0.2	<0.2	1.77

[a] Three separate bioassays conducted, results pooled and IC50 calculated with 95% confidence limits.
[b] One screening bioassay conducted and IC50 calculated with 95% confidence limits.
[c] n = 32, geometric mean for LOEC and NOEC.
[d] n = 2.
[e] Concentration of copper to cause a 50% inhibition of the algal cell division rate over 72 h.
[f] ± 2 SD.
[g] Lowest concentration of copper tested to cause a significant effect on the cell division rate.
[h] Value calculated for LOEC was > IC50, therefore not reported.
[i] Not determined.
[j] The highest concentration of copper tested at which there was no significant effect on cell division rate. Where results are presented as "less than", no NOEC was found using the copper concentrations tested.

Table 3: Copper partition coefficients (K_d) for algal species of varying sensitivity

Alga	IC50 (µg Cu/L)	Surface Area (µm^2)	K_d (± 95% CL) ($\times 10^{-10}$ L/cell)	K_d (± 95% CL) ($\times 10^{-12}$ L/µm^2)[d]
D. tertiolecta	530	220	7.6 (± 0.6) [a]	3.5 (± 0.7)
Tetraselmis sp.	47	220	32 (± 1) [b]	14.5 (± 0.4)
E. huxleyi – coccoliths	20	34	8 (± 9) [c]	24 (± 26)
N. closterium	18	103	24 (± 1) [c]	23 (± 1)
E. huxleyi + coccoliths	15	90	8 (± 1) [c]	8.9 (± 0.5)
P. tricornutum	8.0	126	11 (± 1) [a]	9 (± 1)
H. niei	4.8	610	12 (± 7) [c]	2 (± 1)

[a] Three separate K_d measurements.
[b] Four separate K_d measurements.
[c] One K_d measurement. Standard deviation of the slope of the line (K_d) calculated and converted to 95% confidence interval.
[d] K_d ($\times 10^{-12}$ L/µm^2) calculated by dividing K_d ($\times 10^{-10}$ L/cell) by the surface area for each alga (µm^2/cell).

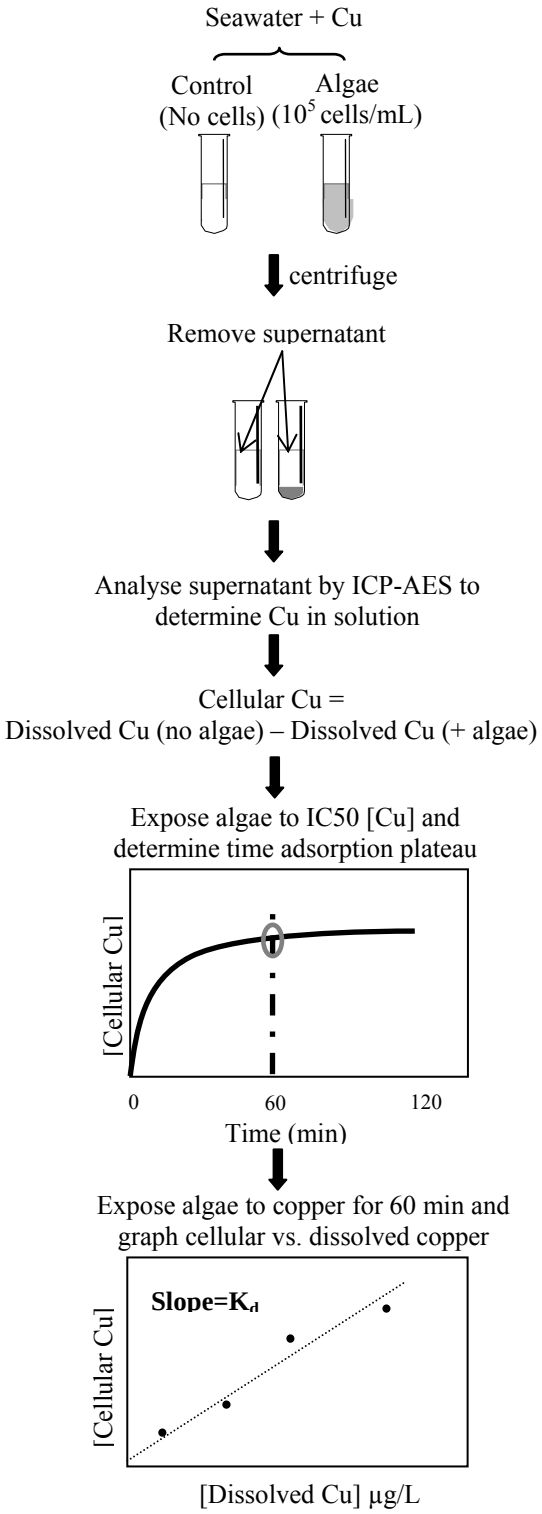

Figure 1: Outline of the method for determining the partition coefficient (K_d) of marine microalgal cells exposed to copper.

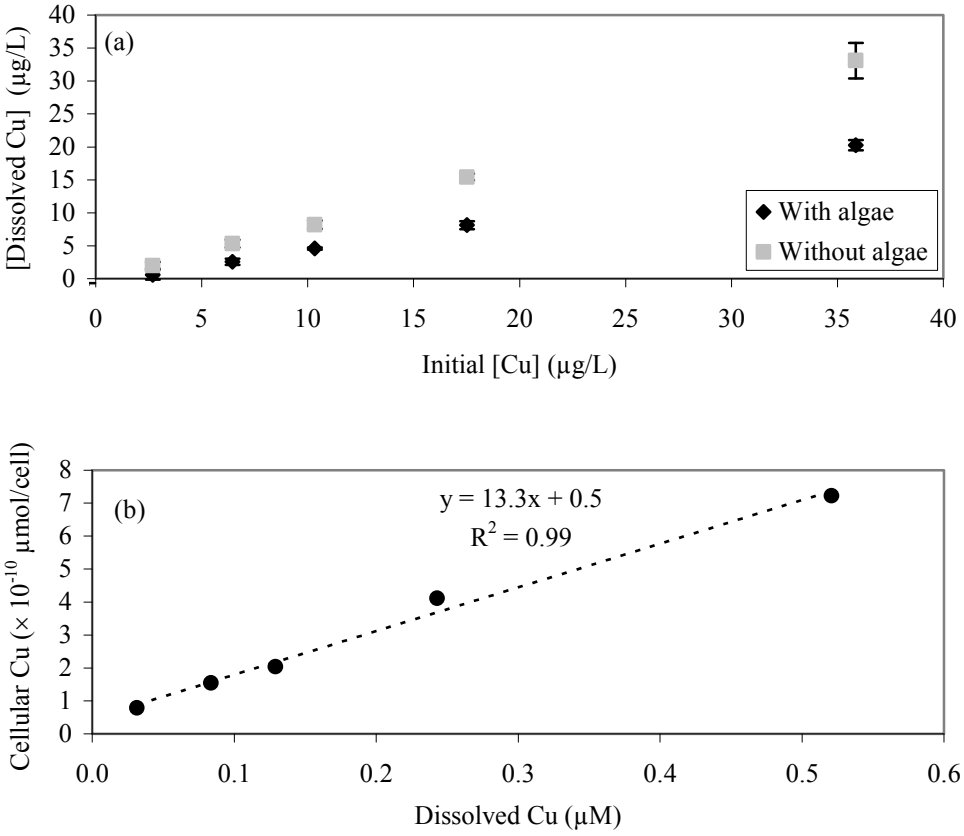

Figure 2: Example of K_d determination (one of three *P. tricornutum* tests); (a) The average concentration of copper in replicates following a 1-h exposure to copper, in the presence or absence of cells. The error bars indicate 1SD. The difference was calculated as cellular copper; (b) Cellular copper (µmol/cell) plotted against dissolved copper (µmol/L), the slope of the line is the K_d (L/cell).

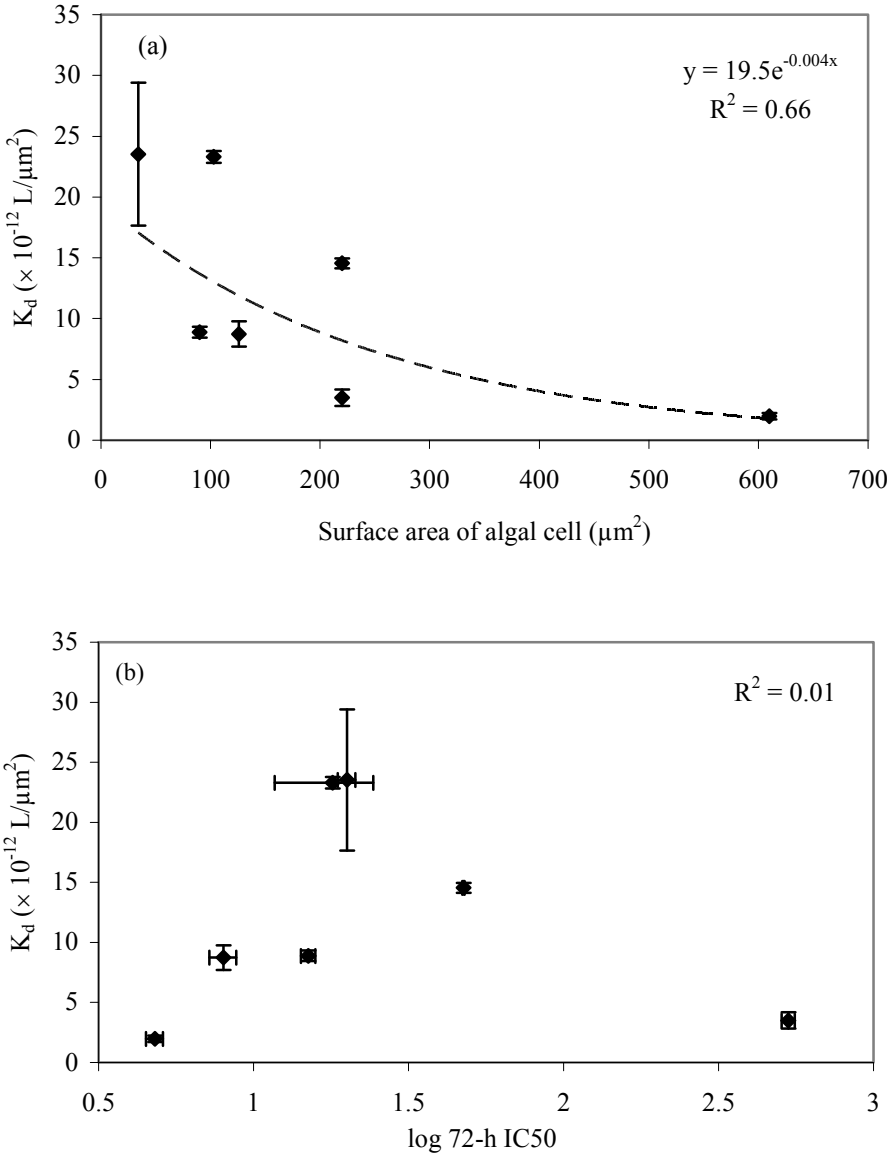

Figure 3: (a) Plot of cell surface area against partition coefficient (K_d) normalised on a surface area basis for each alga tested. There is a significant non-linear exponential relationship between K_d and surface area ($p = 0.026$) that is plotted as the dashed line. (b) Plot of species sensitivity for each alga (log 72-h IC50) against partition coefficient K_d on a surface area basis. Error bars for both the x and y axes are one standard deviation. Regression analysis found no significant linear relationship between K_d and log 72-h IC50 ($p = 0.81$). Het, Phaeo, Nitz, Ehux(+C), Ehux(-C), Tet and Dun are *Heterocapsa niei*, *Phaeodactylum tricornutum*, *Nitzschia closterium*, *Emiliania huxleyi* (plus coccoliths), *Emiliania huxleyi* (without coccoliths), *Tetraselmis* sp. and *Dunaliella tertiolecta*, respectively.