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## **Uptake and internalisation of copper by three marine microalgae: comparison of copper-sensitive and copper-tolerant species**

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## Abstract

Although it has been well established that different species of marine algae have different sensitivities to metals, our understanding of the physiological and biochemical basis for these differences is limited. This study investigated copper adsorption and internalisation in three algal species with differing sensitivities to copper. The diatom *Phaeodactylum tricornutum* was particularly sensitive to copper, with a 72-h IC50 (concentration of copper to inhibit growth rate by 50%) of  $8.0 \mu\text{g Cu L}^{-1}$ , compared to the green algae *Tetraselmis* sp. (72-h IC50  $47 \mu\text{g Cu L}^{-1}$ ) and *Dunaliella tertiolecta* (72-h IC50  $530 \mu\text{g Cu L}^{-1}$ ). At these IC50 concentrations, *Tetraselmis* sp. had much higher intracellular copper ( $1.97 \pm 0.01 \times 10^{-13} \text{ g Cu cell}^{-1}$ ) than *P. tricornutum* ( $0.23 \pm 0.19 \times 10^{-13} \text{ g Cu cell}^{-1}$ ) and *D. tertiolecta* ( $0.59 \pm 0.05 \times 10^{-13} \text{ g Cu cell}^{-1}$ ), suggesting that *Tetraselmis* sp. effectively detoxifies copper within the cell. By contrast, at the same external copper concentration ( $50 \mu\text{g L}^{-1}$ ), *D. tertiolecta* appears to better exclude copper than *Tetraselmis* sp. by having a slower copper internalization rate and lower internal copper concentrations at equivalent extracellular concentrations. The results suggest that the use of internal copper concentrations and net uptake rates alone cannot explain differences in species sensitivity for different algal species. Model prediction of copper toxicity to marine biota and understanding fundamental differences in species sensitivity will require, not just an understanding of water quality parameters and copper-cell binding, but also further knowledge of cellular detoxification mechanisms.

## Keywords

Uptake, internalisation, copper, three, marine, microalgae, comparison, copper, sensitive, copper, tolerant, species, CMMB

## Disciplines

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

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**Uptake and internalisation of copper by three marine microalgae: Comparison of  
copper sensitive and copper tolerant species**

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## Abstract

Although it has been well established that different species of marine algae have different sensitivities to metals, our understanding of the physiological and biochemical basis for these differences is limited. This study investigated copper adsorption and internalisation in three algal species with differing sensitivities to copper. The diatom *Phaeodactylum tricornutum* was particularly sensitive to copper, with a 72-h IC<sub>50</sub> (concentration of copper to inhibit growth rate by 50%) of 8.0 µg Cu L<sup>-1</sup>, compared to the green algae *Tetraselmis* sp. (72-h IC<sub>50</sub> 47 µg Cu L<sup>-1</sup>) and *Dunaliella tertiolecta* (72-h IC<sub>50</sub> 530 µg Cu L<sup>-1</sup>). At these IC<sub>50</sub> concentrations, *Tetraselmis* sp. had much higher intracellular copper ( $1.97 \pm 0.01 \times 10^{-13}$  g Cu cell<sup>-1</sup>) than *P. tricornutum* ( $0.23 \pm 0.19 \times 10^{-13}$  g Cu cell<sup>-1</sup>) and *D. tertiolecta* ( $0.59 \pm 0.05 \times 10^{-13}$  g Cu cell<sup>-1</sup>), suggesting that *Tetraselmis* sp. effectively detoxifies copper within the cell. By contrast, at the same external copper concentration (50 µg L<sup>-1</sup>), *D. tertiolecta* appears to better exclude copper than *Tetraselmis* sp. by having a slower copper internalization rate and lower internal copper concentrations at equivalent extracellular concentrations. The results suggest that the use of internal copper concentrations and net uptake rates alone cannot explain differences in species sensitivity for different algal species. Model prediction of copper toxicity to marine biota and understanding fundamental differences in species-sensitivity will require, not just an understanding of water quality parameters and copper-cell binding, but also further knowledge of cellular detoxification mechanisms.

**Keywords:** Toxicity; ultrastructure; exudates; Cu; internalisation; uptake

## **Introduction**

To increase our fundamental understanding of why and how species differ in their sensitivities to contaminants, several approaches have been used, including biological monitoring, physiological comparisons of contaminant uptake, studies of sub-cellular partitioning and detoxification, and predictive and biodynamic modelling (Buchwalter and Cain, 2005). Recent studies in metals-based ecotoxicological research have focussed heavily on generating models capable of predicting toxicity based on water quality parameters such as pH, hardness, salinity and dissolved organic matter. These parameters influence both the speciation of the metal in solution and the binding of metals at the organism interface, ultimately affecting metal internalisation and toxicity. Various equilibrium-based models have been proposed to predict toxicity, including the Free Ion Activity Model (FIAM) (Morel, 1983), the extended FIAM (Brown and Markich, 2000) and the Biotic Ligand Model (BLM) (Di Toro et al., 2001; Santore et al., 2001; De Schamphelaere and Janssen, 2002; De Schamphelaere et al., 2003). The BLM has been used to successfully predict acute toxicity to freshwater fish and invertebrates at relatively high metal concentrations, however, its application to marine species such as microalgae, under low chronic exposure conditions, is still under development. Some exceptions to the BLM have been noted in the literature (Campbell, 1995; Hassler et al., 2004a). A more mechanistic-based approach, which takes into account the kinetics of metal binding and uptake, may be required to better understand metal toxicity and detoxification processes in algae.

Uptake of metal into algal cells is considered to be a two-part process when the solution in direct contact with the cell, the diffusion layer, is at equilibrium with the surrounding bulk medium. Firstly, fast metal adsorption to sites on the exterior of the cell membrane occurs, with the metal-binding sites consisting of both metabolically active sites at which copper may

enter the cell, and non-active sites. Previous research has shown that inter-species differences in the sensitivity of marine microalgae to copper were not related to the adsorption of copper to a variety of different algal cell walls and surfaces (Levy et al., 2007). The second step in metal uptake is the internalisation of metal across the cell membrane. It is hypothesised that metal internalisation occurs via ion pores, channels or transporters in the algal cell membrane (Campbell, 1995). For charged metal ions, this is generally considered to be the rate-limiting step in the uptake of metal, and thus a primary factor considered in metal exposure routes and in modelling metal uptake and toxicity. However, it has been shown that neutrally-charged lipophilic copper complexes can cross the cell membrane quickly, exerting a greater toxicity on microalgae than would otherwise be expected (Stauber and Florence, 1987).

The majority of BLM studies have focussed on the acute responses of a number of species of fish to metals, where the gill has proven to be the biotic ligand. Microalgae have also gained attention in the BLM literature (Santore et al., 2001; De Schamphelaere et al., 2003; Heijerick et al., 2002). The BLM assumes that for algae the “biotic-ligand” consists of specific sites on the plasma membrane, which do not change upon exposure to a toxicant, or under different water quality regimes, e.g. changes in pH, and that no major biological regulation is induced upon metal binding to the ligand (Campbell et al., 2002; Hassler et al., 2004a). Recent work has suggested that this assumption may not hold true for algae, as exposure to metal mixtures may change membrane permeability and thus metal uptake (Franklin et al., 2002a), or changes in pH may cause conformational changes in surface proteins responsible for metal transport, thus altering the internal flux of metals (Francois et al., 2007). Furthermore, internalisation of lipophobic metal across the lipophilic cell membrane is not always the rate-limiting step in metal uptake, e.g. Campbell et al. (2002) showed that silver uptake in algae is limited by diffusion from the bulk media to the surface of cells, while consequent adsorption

and internalisation are fast. The BLM also failed to explain the toxicity of zinc to *Chlorella kesslerii*, a green microalga, where zinc uptake could not be predicted from the solution chemistry or cell-bound zinc, possibly due to the production of membrane-bound zinc transporters (Hassler and Wilkinson, 2003). Although equilibrium models have had some success in predicting acute responses of organisms, it is clear that microorganisms have a dynamic relationship with the environment around them (Worms et al., 2006).

Algae are capable of regulating their internal cell environment and also their immediate surroundings, e.g. through the production of exudates with metal binding capacity (Megharaj et al., 2003; Worms et al., 2006). Other detoxification mechanisms employed by algae may include: (i) the exclusion of metals through changes in membrane permeability; (ii) binding or sequestration of metals at non-metabolically active sites within the cell, e.g. in the cell wall; (iii) binding to cysteine-rich phytochelatins; (iv) induction of stress-proteins or antioxidants; or (v) efflux of metal into solution. Toxicity, therefore, is not just a function of exposure to contaminants, but also of internal biological sequestration (Luoma and Rainbow, 2005).

The aim of this study was to further investigate the species-sensitivity concept - why is one species of marine microalgae more or less sensitive to copper than another species? Copper was chosen as the contaminant of concern due to its high aquatic toxicity at environmentally relevant concentrations and increasing use as a replacement for tributyltin in antifouling biocides (Stauber and Davies, 2000; Warnken et al., 2004). This paper compares copper sensitivities, uptake rates and intracellular copper concentrations in three marine microalgae: the diatom *Phaeodactylum tricornutum* (Bacillariophyceae) and the green algae *Tetraselmis* sp. (Prasinophyceae) and *Dunaliella tertiolecta* (Chlorophyceae). It also examines ultrastructural cell changes in response to copper, determines if copper inclusions are visible

in the cells using electron microscopy and investigates the production of exudates as a potential detoxification mechanism for copper in algal cells.

## Methods

### *Algal cultures*

The marine microalgae *Phaeodactylum tricornutum* Bohlin, *Tetraselmis* sp. and *Dunaliella tertiolecta* (Butcher) (strains CS-29/4, CS-87 and CS-175, respectively) were originally obtained from the Collection of Living Microalgae, CSIRO Marine and Atmospheric Research, Hobart, Australia. All cultures were maintained in f<sub>2</sub> growth medium (half-strength f medium; Guillard and Ryther, 1962) at 21 ± 2°C (12:12 h light/dark cycle, 70 µmol photons m<sup>-2</sup> s<sup>-1</sup>, Philips TL 40W cool white fluorescent lighting). Cultures were checked regularly microscopically, streaked onto agar plates (2% Bacto agar, 0.1% pepsin, and 0.1% yeast; Oxoid, Bacto Laboratories, Liverpool, NSW, Australia) 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.

### *Growth-rate inhibition bioassays*

The chronic toxicity of copper to the three algae was determined using 72-h growth-rate inhibition bioassays, following an initial screening process (Levy et al., 2007). The method used is the same as that described in Levy et al. (2007) and the bioassay conditions are summarised in Table 1. At least five different copper treatments and a control (copper-free filtered seawater plus nitrate and phosphate) were prepared for each toxicity test from a CuSO<sub>4</sub>·5H<sub>2</sub>O stock solution. Nominal concentrations of copper used in the toxicity tests were 4, 8, 12, 30 and 60 µg Cu L<sup>-1</sup> for *P. tricornutum*, 5, 10, 30, 60, 125, 250, 500 µg Cu L<sup>-1</sup> for *Tetraselmis* sp., and 10, 50, 100, 250, 500, 750 and 900 µg Cu L<sup>-1</sup> for *D. tertiolecta*. Three



replicates were tested per treatment. Cells in exponential growth phase (cultures 5-6 d old) were used to inoculate the test treatments after washing the cells three times in filtered seawater to remove residual culture medium. Due to the fragility of *Tetraselmis* sp., this culture was only rinsed once to prevent cell lysis. The test medium was then inoculated with  $2-4 \times 10^3$  cells mL<sup>-1</sup>. This low cell density was used to better simulate algal concentrations in seawater and to avoid copper limitation in solution over the duration of the bioassay (Franklin et al., 2002b). A sub-sample (5 mL) was immediately filtered through an acid-washed 0.45 µm membrane filter (MiniSart, Sartorius, Oakleigh, VIC, Australia) and dissolved copper was determined after acidification by inductively coupled plasma-atomic emission spectrometry (ICP-AES). The flasks were capped with glass lids, incubated for 72 h (12:12h light/dark photocycle, 140 µmol photons m<sup>-2</sup> s<sup>-1</sup>, 21°C). Test flasks were rotated 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 (BD-FACSCalibur, Becton Dickinson BioSciences, San Jose, CA, USA) as 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<sub>10</sub> (cell density) versus time (h). Growth rates for treatment flasks (divisions day<sup>-1</sup>) were calculated ( $\mu \times 24 \times 2.303/\ln 2$ ) and expressed as a percentage of the control growth rates.

#### *Statistical analysis of toxicity data*

The 72-h IC<sub>50</sub>, i.e. the inhibitory concentration to reduce growth rate by 50%, was calculated using linear interpolation (ToxCalc, Ver 5.0.23C, Tidepool Software, San Francisco, CA, USA). Measured copper concentrations were used in all calculations of toxicity endpoints. The data were tested for normality and homogeneity of variance, and Dunnett's multiple

comparison test was used to determine which treatments differed significantly from controls (1 tailed,  $p \leq 0.05$ ) to estimate the no-observable effect concentration (NOEC) and the lowest-observable effect concentration (LOEC). Where data were pooled to gain a single IC<sub>50</sub> value based on multiple tests, the Bonferroni t test or the Wilcoxon Rank Sum Test were used to compare which treatments differed significantly from controls, because the numbers of replicates in each treatment were unequal (e.g. more control replicates).

#### *Algal cell size*

Algal cell sizes ( $\pm$  copper) were measured over the test period using phase-contrast microscopy and an eye-piece micrometer (400 $\times$  magnification). The cell dimensions ( $\mu\text{m}$ ) of individual cells were measured ( $n \geq 30$ ), including cell length and cell width at the widest point for *P. tricornutum*, and length and width for the prolate ellipsoid-shaped *D. tertiolecta* and *Tetraselmis* sp. Surface area (SA) and volume (V) were calculated for *P. tricornutum* by visualising the cell as two cones: SA of each cone =  $\pi r \sqrt{h^2 + r^2}$ , where h = half length of cell and r = half width of cell;  $V = 1\pi r^2 h/3$ . The equation for a prolate ellipsoid was used for *D. tertiolecta* and *Tetraselmis* sp.;  $SA = 2\pi b^2 + 2\pi a^2 b^2 / \sqrt{(a^2 - b^2)} * \text{ASIN}(\sqrt{(a^2 - b^2)}/a)$ , where a = length and b = width;  $V = 4\pi ab^2/3$ . Statistically significant changes in cell surface area ( $\mu\text{m}^2$ ) or volume ( $\mu\text{m}^3$ ) were tested using the Student's t test ( $p < 0.05$ ).

#### *Determination of extra- and intracellular copper concentrations*

Algae were exposed to various concentrations of copper for up to 72 h, in a test medium of filtered seawater with minimal nutrients (15 mg  $\text{NO}_3^- \text{L}^{-1}$ , 1.5 mg  $\text{PO}_4^{3-} \text{L}^{-1}$ ) under similar conditions as the growth-rate inhibition bioassays. Algae, at initial cell densities of  $3.0 (\pm 0.3) \times 10^3 \text{ cells mL}^{-1}$ , were allowed to grow for one day in copper-free test media prior to the addition of copper. *P. tricornutum* was exposed to 10 ( $\approx$ 72-h IC<sub>50</sub>), 30 and 50  $\mu\text{g Cu L}^{-1}$ ,

*Tetraselmis* sp. to 10, 50 ( $\approx$ 72-h IC<sub>50</sub>) and 100  $\mu\text{g Cu L}^{-1}$  and *D. tertiolecta* to 50 and 500 ( $\approx$ 72-h IC<sub>50</sub>)  $\mu\text{g Cu L}^{-1}$ . This allowed direct comparison of intracellular copper concentrations at the 72-h IC<sub>50</sub> for each alga, and at a common concentration of 50  $\mu\text{g Cu L}^{-1}$ . A modified method of Franklin et al. (2002b) was used to determine copper adsorbed to the cell (extracellular fraction) and copper inside the cell (intracellular fraction) in triplicate samples collected at time-points from 0 to 72 h (0, 0.5, 1, 2, 4, 10, 20, 30, 48 and 72h). The method is summarised as a schematic diagram in Figure 1. Depending on cell density, several flasks were used per replicate (generally 1-5 flasks), with three replicates per treatment. The total volume used for each replicate was measured by mass. Blank solutions (no algae) were also prepared in each sample batch. Cell density data from the flow cytometer and solution volumes (weighed and converted using seawater density of 1.03  $\text{g mL}^{-1}$ ) were used to calculate the number of cells in all measurements of intra- and extracellular copper.

Preliminary experiments showed that a 20-min exposure to the EDTA washing solution effectively removed extracellular copper without damaging the integrity of the cells. Other researchers have successfully used EDTA for this purpose, without damaging cells (Franklin et al., 2002b; Hassler et al., 2004b) and without causing efflux of zinc from cells (Mirimanoff and Wilkinson, 2000). Preliminary work also showed that two different methods were required to collect cells. *P. tricornutum* and *D. tertiolecta* were centrifuged, however *Tetraselmis* sp. frequently lysed if centrifuged multiple times, however it was easily resuspended off filter papers, unlike the other species. Therefore, filtration through an acid-washed glass unit with a 0.45  $\mu\text{m}$  pore size GH-polypropylene membrane filter was used for collecting *Tetraselmis* sp. fractions. When the appropriate method was used, cell membranes appeared intact and cells appeared healthy when observed under phase-contrast microscopy.

### *Copper uptake rates*

Intracellular copper concentrations were plotted against exposure time (0.5 to 72 h) and the copper uptake rate was calculated as the slope (m) of the regression line with units of  $10^{-15}$  g Cu cell<sup>-1</sup> h<sup>-1</sup> or  $10^{-18}$  g Cu  $\mu\text{m}^{-3}$  h<sup>-1</sup>. Note that this is the net copper uptake/internalisation rate because we have not specifically calculated copper efflux rates from the cells. These slopes were compared statistically using t tests (comparing 2 slopes, two-tailed,  $p = 0.05$ ) and analysis of covariance tests (comparing  $> 2$  slopes, one-tailed,  $p = 0.05$ ), with Tukey's post-hoc analysis to detect differences in uptake rates (Zar, 1974).

### *Copper analyses*

A variety of methods were used to measure copper concentrations based on the detection limits required and the matrix of the sample. The higher concentrations of copper in the saline dissolved and rinse fractions were measured easily using inductively coupled plasma-atomic emission spectrometry (ICP-AES; Spectro Flame-EOP, Spectro Analytical Instruments, Kleve, Germany), however instruments with lower detection limits were required for the other fractions. The saline matrix for dissolved and extracellular fractions was incompatible with graphite furnace-atomic absorption spectroscopy (GF-AAS), and thus anodic stripping voltammetry (ASV) with a hanging mercury drop electrode (Metrohm 646 Voltammetric Analyzer; Berchem, Belgium) was used to measure copper concentrations in the extracellular fractions following sample acidification to  $\text{pH} < 3$ . The low concentrations of copper in the intracellular and flask-adsorbed fractions were best measured by GF-AAS (4100ZL Perkin-Elmer instrument, Norwalk, CT, USA). Regardless of the analytical method used, copper concentrations were calculated from matrix-matched calibration curves using serial dilution of a standard (QCD Analysts, Eaglewood, FL, USA; Australian Chemical Reagents, Cu Elemental Standard, 1000 mg Cu L<sup>-1</sup> in 2% HNO<sub>3</sub>).

### *Algal exudate analyses*

Two methods were used to detect algal exudates in copper-exposed cells. Algae ( $2-4 \times 10^3$  cells mL<sup>-1</sup>) were inoculated into filtered seawater with minimal nutrients, 15 mg NO<sub>3</sub><sup>-</sup> L<sup>-1</sup> and 1.5 mg PO<sub>4</sub><sup>3-</sup> L<sup>-1</sup>, ( $\pm 50 \mu\text{g Cu L}^{-1}$ ) for 72 h as per the standard growth inhibition tests.

Copper complexed by algal exudates was determined as the difference between dissolved copper (measured by ICP-AES) and ASV-labile copper (measured by anodic stripping voltammetry) using the method described in Franklin et al. (2002b). Controls included: (1) seawater + 50  $\mu\text{g Cu L}^{-1}$  (no algae), (2) seawater + algae (no copper), and (3) seawater + algae, filtered after 72 h, + 50  $\mu\text{g Cu L}^{-1}$ . Copper standards were also measured just prior to and after the measurement of some samples, to ensure that algal exudates were not interacting with the electrode, causing a reduction in the ASV signal.

In a separate test, total carbohydrate concentrations and total protein concentrations in *D. tertiolecta* exudates were measured after an extracellular polymeric substances (EPS) fractionation process to separate “loose EPS” (exudates in the water column) from “capsular EPS” (exudates attached to the outside of cells) as outlined in Barranguet et al. (2004).

Control cells or Cu-exposed cells (500  $\mu\text{g Cu L}^{-1}$ ) were grown for 72 h under standard bioassay conditions and then centrifuged to pellet the replicate prior to fractionation. Total carbohydrate concentrations ( $\mu\text{g}$  of glucose equivalents mL<sup>-1</sup> or cell<sup>-1</sup>) were measured using the phenol-sulfuric acid assay (Dubois et al., 1956) and using glucose as a standard. Total protein concentrations ( $\mu\text{g}$  of albumin equivalents mL<sup>-1</sup> or cell<sup>-1</sup>) were measured using the bicinchoninic acid protein assay (BCA Protein Assay Kit, Pierce, Rockford, IL, USA) (Berges et al., 1993) and using human albumin as a standard.

### *Transmission electron microscopy (TEM)*

Control cells (no copper) and cells exposed to copper for 72 h at close to IC<sub>50</sub> concentrations (15 µg L<sup>-1</sup> for *P. tricornutum*, 50 µg L<sup>-1</sup> for *Tetraselmis* sp., 500 µg L<sup>-1</sup> for *D. tertiolecta*) were prepared for TEM using a method modified for algal cells from Au et al. (1999) (chemicals sourced from Electron Microscopy Sciences, Hatfield, PA, USA). TEM on *P. tricornutum* cells was carried out at the Australian Nuclear Science and Technology Organisation, Sydney (200 kV, JEOL 2000FXII transmission electron microscope) while TEM on the other two algae was undertaken at City University, Hong Kong (Philips Tecnai 12 BioTWIN transmission electron microscope, FEI, Netherlands).

## **Results**

### *Growth-rate inhibition tests*

Algal control growth rates were acceptable, with values of  $1.78 \pm 0.08$ ,  $1.37 \pm 0.26$  and  $1.39 \pm 0.02$  divisions day<sup>-1</sup> for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta*, respectively.

Changes in pH were minimal for test controls, with initial pH of controls (seawater + nutrients) of  $8.0 \pm 0.1$ , and changes typically less than 0.3 pH units by the end of the tests.

For copper treatments, pH increased by < 0.5 pH units.

The sensitivities of the three species to copper over 72 h are shown in Figure 2. The results for each alga are the combination of three separate bioassays, pooled together, with controls for each alga normalised to 100% for each test. As the concentration of copper in the test solutions increased, algal growth rates decreased (Figure 2). *P. tricornutum* was the most sensitive species to copper, with a 72-IC<sub>50</sub> value of 8 µg Cu L<sup>-1</sup> (Table 2). *Tetraselmis* sp., a moderately copper-tolerant species, had a 72-h IC<sub>50</sub> value of 47 µg Cu L<sup>-1</sup>, while the

extremely copper-tolerant chlorophyte *D. tertiolecta* had a 72-h IC<sub>50</sub> of 530 µg Cu L<sup>-1</sup>, well above expected environmental concentrations of copper. The NOEC values were < 1.5, 7 and 8 µg Cu L<sup>-1</sup> for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta*, respectively. The LOEC values were 1.5, 22 and 42 µg Cu L<sup>-1</sup> for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta*, respectively (Table 2).

#### *Changes in cell size upon copper exposure*

In order to normalise cellular copper concentrations to a surface area or volume basis, algal cell size in each bioassay was determined (Table 2). The cell sizes of *Tetraselmis* sp. and *D. tertiolecta* did not change over time in controls or upon copper exposure. Exposure of *P. tricornutum* to 10 µg Cu L<sup>-1</sup> caused cells to increase in size. At higher exposure concentrations there was no further increase in size. The surface area of *P. tricornutum* cells increased from 60-130 µm<sup>2</sup> in controls to a maximum of 500 µm<sup>2</sup> in copper-treated cells and cell volume increased from 50-70 µm<sup>3</sup> in controls to 430 µm<sup>3</sup> in copper-treated cells. Extracellular copper concentrations for all three algae were normalised on a surface area basis while intracellular copper concentrations and uptake rates were normalised on a volume basis. The actual size of cells at each time-point and copper exposure was used to normalise *P. tricornutum* copper concentrations.

#### *Dissolved copper concentrations in solution*

Low initial cell densities (10<sup>3</sup> cells mL<sup>-1</sup>) were used to avoid copper depletion in solution over the course of 72 h, however dissolved copper concentrations in solution were monitored over the exposure period. In tests exposing *P. tricornutum* to 10, 30 and 50 µg Cu L<sup>-1</sup>, the 72-h dissolved copper concentrations were 99-101%, 90-96% and 85-92% of concentrations measured at 1 h, respectively. For *D. tertiolecta* tests, measured final copper concentrations

were 85% and 87% of the 1-h concentrations in the 50  $\mu\text{g L}^{-1}$  and 500  $\mu\text{g Cu L}^{-1}$  treatments. However, for tests with *Tetraselmis* sp. the concentration of dissolved copper decreased over 72 h, with final concentrations only 51-60% of the 1-h concentration for all treatments. Previous work has shown that *Tetraselmis* sp. has a much higher  $K_d$  value (solution-cell metal partition coefficient) for copper than other marine microalgae, suggesting that it adsorbs relatively more copper to cell surfaces compared with other species (Levy et al., 2007).

### *Extracellular copper*

In general, extracellular copper (operationally defined as the copper removed by washing with EDTA) increased over the first 5-10 h for each alga (Figure 3), reaching a constant value, followed by a small decrease with longer exposures of 48-72 h (Figure 3a, 50  $\mu\text{g Cu L}^{-1}$ ). Increasing dissolved copper concentrations led to increased extracellular copper e.g. *D. teriolecta* (Figure 3b). However, for both *P. tricornutum* and *Tetraselmis* sp., while an initial increase in copper concentration resulted in increased extracellular copper, as the concentration of dissolved copper increased above the alga's IC50 value, extracellular copper reached a plateau. For example, for *P. tricornutum* at 72 h, extracellular copper was  $4 \pm 2$ ,  $12 \pm 5$  and  $10.2 \pm 0.4 \times 10^{-14}$  g Cu cell<sup>-1</sup> for 10, 30 and 50  $\mu\text{g Cu L}^{-1}$  exposures, respectively.

### *Algal exudates*

The decrease in extracellular copper with increasing exposure time in *Tetraselmis* sp. may have been due to a re-establishment of pseudo-equilibrium between cell surfaces and bulk solution following the observed decline in dissolved copper in solution. However, it could also be due to the production of extracellular ligands (or cell-surface ligands) by algal cells. This could change the speciation of copper in solution, altering the pseudo-equilibrium between cell surface sites and bulk solution. To help interpret the decrease in extracellular



copper for *Tetraselmis* sp., the production of algal exudates was investigated by measuring ASV-labile copper in solutions of all three algae exposed to  $50 \mu\text{g Cu L}^{-1}$  for 72 h. Control and  $50 \mu\text{g Cu L}^{-1}$  solutions were filtered ( $0.45 \mu\text{m}$ ) after 72 h to remove algal cells. ASV-labile copper measurements in two controls (seawater +  $50 \mu\text{g Cu L}^{-1}$  and algal controls spiked with  $50 \mu\text{g Cu L}^{-1}$  post-filtration) showed that ligands, if present, were not adhering to the electrode or artificially lowering the ASV-labile copper signal. After a 72-h exposure to  $50 \mu\text{g Cu L}^{-1}$ , the ASV-labile copper concentrations were  $92 \pm 9\%$ ,  $94 \pm 9\%$  and  $114 \pm 3\%$  of the dissolved copper concentrations for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta*, respectively. This suggests that the decrease in extracellular copper concentrations was not due to a decrease in labile copper in solution and that major production of exudates in response to  $50 \mu\text{g Cu L}^{-1}$  was unlikely.

Exudate production by *D. tertiolecta* exposed to  $500 \mu\text{g Cu L}^{-1}$  was also determined by measuring carbohydrate and protein in the loose EPS and capsular EPS fractions. Seawater blanks for capsular EPS had carbohydrate and protein concentrations that were below detection limits ( $< 4 \times 10^{-12}$  g glucose equivalents  $\text{mL}^{-1}$  and  $< 4 \times 10^{-12}$  g albumin equivalents  $\text{mL}^{-1}$ ). Carbohydrate and protein were not detected in the loose EPS fraction of copper-treated cells. In the capsular EPS fraction, carbohydrate concentrations were higher in copper-exposed cells ( $20 \pm 3 \times 10^{-12}$  g glucose equivalents  $\text{cell}^{-1}$ ) compared to controls ( $3.8 \pm 0.4 \times 10^{-12}$  g glucose equivalents  $\text{cell}^{-1}$ ). Protein concentrations were also higher ( $25 \pm 2 \times 10^{-12}$  g albumin equivalents  $\text{cell}^{-1}$ ) compared to controls ( $9 \pm 2 \times 10^{-12}$  g albumin equivalents  $\text{cell}^{-1}$ ). This suggests that exposure to high copper concentrations induced exudate production at the cell surface in *D. tertiolecta*.

#### *Intracellular copper concentrations*

Despite pooling of cells, concentrations of intracellular copper in control cells not exposed to copper were close to the GF-AAS detection limit ( $0.3 \mu\text{g Cu L}^{-1}$ ). Average background concentrations of copper inside cells not exposed to copper were  $7 \pm 5$ ,  $16 \pm 8$  and  $59 \pm 45 \times 10^{-16} \text{ g Cu cell}^{-1}$  for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta* respectively on a per cell basis, and  $0.7 \pm 0.5$ ,  $0.5 \pm 0.2$  and  $1.8 \pm 1.3 \times 10^{-17} \text{ g Cu } \mu\text{m}^{-3}$ , respectively when corrected for cell volumes.

Upon exposure to copper, intracellular copper in each species increased linearly over the 72-h exposure period (Figure 4, Table 3). For some species at low copper concentrations, e.g. *D. tertiolecta* at  $50 \mu\text{g Cu L}^{-1}$ , there was a lag period of up to 20 h before intracellular copper increased. Another exception was *Tetraselmis* sp. at  $10 \mu\text{g Cu L}^{-1}$  where intracellular copper concentrations increased between 20 and 30 h, but then remained relatively constant for the rest of the 72-h period.

At the nominal concentration of  $10 \mu\text{g Cu L}^{-1}$  *Tetraselmis* sp. had similar average ( $\pm$  standard error) 72-h intracellular copper concentrations to *P. tricornutum* ( $0.22 \pm 0.02 \times 10^{-13} \text{ g cell}^{-1}$  and  $0.23 \pm 0.06 \times 10^{-13} \text{ g cell}^{-1}$ , respectively) (Table 3). At the common nominal concentration of  $50 \mu\text{g Cu L}^{-1}$  *Tetraselmis* sp. had higher 20-, 48- and 72-h intracellular copper concentrations than either *P. tricornutum* or *D. tertiolecta*, on a per cell basis and when normalised to cell volume (72-h;  $1.97 \pm 0.01$ ,  $0.62 \pm 0.02$  and  $0.33 \pm 0.01 \times 10^{-13} \text{ g cell}^{-1}$ , respectively) (Table 3).

At copper exposures similar to their respective IC50 values, the average ( $\pm$  SD) 72-h intracellular copper concentrations were  $0.23 \pm 0.06$ ,  $1.97 \pm 0.01$  and  $0.59 \pm 0.03 \times 10^{-13} \text{ g Cu cell}^{-1}$  for *P. tricornutum* ( $10 \mu\text{g L}^{-1}$  exposure), *Tetraselmis* sp. ( $50 \mu\text{g Cu L}^{-1}$  exposure) and *D.*

*tertiolecta* (500  $\mu\text{g Cu L}^{-1}$ ), respectively (Table 2). This relationship was similar on a per volume basis, with values of  $10 \pm 2$ ,  $57 \pm 0.1$  and  $27 \pm 1 \times 10^{-17} \text{ g Cu } \mu\text{m}^{-3}$  for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta*, respectively.

#### *Copper uptake rates*

Copper uptake rates for the algae were calculated using intracellular copper concentrations from 0.5 to 72 h (Figure 4). When expressed on a per cell basis all regressions were significant with  $p < 0.05$  (Figure 4). For *P. tricornutum* the uptake rates were 0.3, 0.7 and  $0.8 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$  for exposures of 10, 30 and 50  $\mu\text{g Cu L}^{-1}$ . The uptake rates at 30 and 50  $\mu\text{g Cu L}^{-1}$  were significantly higher than the uptake rate at 10  $\mu\text{g Cu L}^{-1}$  ( $p < 0.05$ ) (Figure 4). The uptake rate at 10  $\mu\text{g Cu L}^{-1}$  for *Tetraselmis* sp. was  $0.3 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ , similar to that for *P. tricornutum*, however uptake rates at 50  $\mu\text{g Cu L}^{-1}$  were much higher in *Tetraselmis* sp. ( $2.6 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ). The uptake rate for *Tetraselmis* sp. decreased at the highest copper exposure (100  $\mu\text{g Cu L}^{-1}$ ;  $1.4 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ). For *D. tertiolecta*, when the exposure concentration was increased from 50  $\mu\text{g Cu L}^{-1}$  to 500  $\mu\text{g Cu L}^{-1}$  the uptake rate increased marginally (but significantly,  $p < 0.05$ ) from 0.4 to  $0.5 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$  (Figure 4). Similar results were obtained when uptake rates were calculated on a volume basis. Poor regression coefficients were obtained when the linear uptake rates for *P. tricornutum* were expressed on a volume basis (Table 4). When the slopes were tested for significance (i.e. that there was a change in concentration over time and the slope  $\neq 0$ ) it was found that the uptake rates were not significantly different to zero for both the 10 and 50  $\mu\text{g Cu L}^{-1}$  treatments. This is likely to be related to the increase in cell volume upon copper exposure that occurs in *P. tricornutum* cells, but not *Tetraselmis* sp. or *D. tertiolecta*.

When the uptake rates at each alga's respective IC50 value were compared (on a per cell basis), they were all significantly different. *Tetraselmis* sp. (IC50 50  $\mu\text{g Cu L}^{-1}$ ) had the highest uptake rate of  $2.6 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ , when compared to *P. tricornutum* ( $0.3 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ; IC50 10  $\mu\text{g Cu L}^{-1}$ ) and *D. tertiolecta* ( $0.5 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ; IC50 500  $\mu\text{g Cu L}^{-1}$ ) (Figure 4). Similarly, when uptake rates were compared on a volume basis *Tetraselmis* sp. had a higher uptake rate ( $7.4 \times 10^{-18} \text{ g Cu } \mu\text{m}^{-3} \text{ h}^{-1}$ ) than *D. tertiolecta* ( $2.8 \times 10^{-18} \text{ g Cu } \mu\text{m}^{-3} \text{ h}^{-1}$ ) but that of *P. tricornutum* at 10  $\mu\text{g Cu L}^{-1}$  could not be compared (because the regression was not significant).

The uptake rates of the three algae, at the common copper exposure concentration of 50  $\mu\text{g L}^{-1}$ , were significantly different (on a per cell basis), with *D. tertiolecta* having the lowest rate of uptake ( $0.4 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ), compared to *P. tricornutum* ( $0.8 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ) and *Tetraselmis* sp. ( $2.6 \times 10^{-15} \text{ g Cu cell}^{-1} \text{ h}^{-1}$ ) (Figure 4). On a volume basis, *Tetraselmis* sp. had a higher rate of uptake ( $7.4 \times 10^{-18} \text{ g Cu } \mu\text{m}^{-3} \text{ h}^{-1}$ ) than *D. tertiolecta* ( $1.9 \times 10^{-18} \text{ g Cu } \mu\text{m}^{-3} \text{ h}^{-1}$ ) but that of *P. tricornutum* at 50  $\mu\text{g Cu L}^{-1}$  could not be compared (because the regression was not significant).

#### *Changes in cell ultrastructure*

The cell ultrastructure of the three algal species was examined in control cells and cells exposed to copper concentrations similar to the IC50 value for each species, i.e. 15  $\mu\text{g Cu L}^{-1}$  (*P. tricornutum*), 50  $\mu\text{g Cu L}^{-1}$  (*Tetraselmis* sp.) and 500  $\mu\text{g Cu L}^{-1}$  (*D. tertiolecta*). TEM images of control and copper-treated algal cells of each species are shown in Figure 5.

The most obvious change upon copper exposure was the cell swelling and clumping observed for *P. tricornutum*. Changes in cell shape, from the typical elongate fusiform shape of control

cells (Figure 5a) to the shorter, swollen shape (shown for the copper-treated cells in Figure 5d) were observed. This accounts for the large increase in cell volume previously noted. *Tetraselmis* sp. and *D. tertiolecta* cells did not increase in size or change shape upon copper exposure (Figures 5b, c, e and f). The main changes in ultrastructure were an increase in the number and size of vacuoles in the cell. Under higher magnification (data and images not shown), neither visible deterioration nor damage to the outer plasma-membrane of treated cells, nor damage to thylakoid membranes of the chloroplasts, was observed.

## Discussion

Previous studies in our laboratory showed that inter-species differences in copper sensitivity of algae were not due to biotic factors such as cell wall type and cell size, nor were they due to taxonomic class or equilibrium partitioning of copper to the cell surface (Levy et al., 2007). The present study has shown that the sensitivity of algal species to copper was not related to external copper binding, or exclusively to intracellular copper concentrations and uptake rates.

### *Extracellular copper and exudate production*

Despite the use of low initial cell densities, copper in solution decreased by 40-50% over 72 h for *Tetraselmis* sp. However, the decrease in dissolved copper was < 11% for *P. tricornutum* and *D. tertiolecta*. This was not surprising as previous studies showed that *Tetraselmis* sp. had a higher 1-h copper-cell partition coefficient ( $K_d$  of  $32 \pm 1 \times 10^{-10} \text{ L cell}^{-1}$ ) compared to *P. tricornutum* ( $11 \pm 1 \times 10^{-10} \text{ L cell}^{-1}$ ) and *D. tertiolecta* ( $7.6 \pm 0.6 \times 10^{-10} \text{ L cell}^{-1}$ ) (Levy et al., 2007). Copper bound externally to *Tetraselmis* sp. also decreased over 20-72 h. As dissolved copper decreased in the treatment where *Tetraselmis* sp. was exposed to  $10 \mu\text{g Cu L}^{-1}$ , this decrease in extracellular copper could be indicative of a shift in solution-cell equilibrium, i.e. a re-establishment of pseudo-equilibrium between the bulk solution and the cell interface.

Measurements of ASV-labile copper showed that for *Tetraselmis sp.* exposed to  $50 \mu\text{g Cu L}^{-1}$ , dissolved and labile copper did not differ at 72 h. Thus the decrease in extracellular copper was unlikely to be due to copper complexation by cell exudates. Interestingly, the uptake for this species at 50 and  $100 \mu\text{g Cu L}^{-1}$  remained linear over 72 h despite the decrease in extracellular copper. This may be because specific binding sites for copper on the cell membrane remained saturated, with the decrease in extracellular copper due only to reduced binding at non-active cell membrane or cell wall sites.

Microorganisms are capable of altering their local environment, and one biotic process that can modify metal speciation is the production of biogenic exudates that adsorb or complex copper, effectively excluding it from the cell (Brown et al., 1988; Megharaj et al., 2003; Worms et al., 2006). Xue and Sigg (1990) found that binding of Cu(II) to exudates from the freshwater green alga *Chlamydomonas reinhardtii* was more significant in altering copper speciation than binding to algal cell surfaces. Similarly, the freshwater gram-positive bacterium, *Rhodococcus opacus*, was found to produce small ( $< 3 \text{ kDa}$ ) complexing ligands which resulted in a decrease in free  $\text{Zn}^{2+}$  in solution (Mirimanoff and Wilkinson, 2000). Research on marine algal species has found that some species are capable of producing ligands with large binding constants for copper with  $\log K$  values of 12-14 (Sunda and Huntsman, 1998; Moffett and Brand, 1996; Croot et al., 2000). A second class of ligands that are generally weaker may also be produced, with  $\log K$  values of 8-10 (Croot et al., 2000; Town and Filella, 2000). Gerringa et al. (1995) found that exudates from the diatom *Ditylum brightwellii* adsorbed copper up to  $7 \mu\text{g Cu L}^{-1}$ , with toxicity only manifesting after this dissolved copper concentration was exceeded.

In the current work ASV-labile copper was  $92 \pm 9\%$ ,  $94 \pm 9\%$  and  $114 \pm 3\%$  of dissolved copper concentrations for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta* exposed to  $50 \mu\text{g Cu L}^{-1}$  for 72 h, respectively. This suggests that cell exudates were not responsible for the observed decrease in extracellular copper. However, measurement of cell-associated carbohydrate and proteins for *D. tertiolecta* at higher copper concentrations ( $500 \mu\text{g L}^{-1}$ ), showed an increase in these moieties upon copper treatment. Stress-induced cell surface proteins have been noted for the diatom *Thalassiosira pseudonana* upon copper exposure and iron and silicon limitation (Davis et al., 2005). It is possible that exudates would have been detected by ASV if higher copper exposure concentrations were used. Previous work with both *P. tricornutum* and *D. tertiolecta* has found variable results. Croot et al. (2000) did not find ligand production by either species, however González-Davilá et al. (1995) found weakly complexing ligands produced by *D. tertiolecta* ( $\log K' 9.3$ ). Exudate production by *P. tricornutum* was found to increase with cell growth rate and during daylight hours of the diurnal cycle (Zhou and Wangersky, 1985, 1989) but decrease under conditions of nitrogen limitation (Zhou and Wagersky, 1989). In contrast, Obernosterer and Herndl (1995) found that *Chaetoceros affinis* had 30% and 100% higher exudate release when grown under nitrogen and/or phosphorus limitation, which supports the general belief that when nutrient-limited algae produce excess carbon through photosynthesis that is unable to be utilised in growth, the excess carbon is released into solution as dissolved organic matter. The test protocol used in the current study relies on minimal nutrient addition, and this could potentially explain the lack of exudates in solution for all three algal species.

#### *Intracellular copper and copper uptake rates*

Species-sensitivity could not be easily predicted on the basis of intracellular copper loadings or uptake rates. The moderately copper-tolerant *Tetraselmis* sp. (72-h  $\text{IC}_{50}$  of  $47 \mu\text{g Cu L}^{-1}$ ),

had much higher internal copper concentrations than *P. tricornutum* (the most sensitive species with a 72-h IC50 of 8.0  $\mu\text{g Cu L}^{-1}$ ) and *D. tertiolecta* (the most tolerant species with a 72-h IC50 of 530  $\mu\text{g Cu L}^{-1}$ ), at comparable exposures of 50  $\mu\text{g Cu L}^{-1}$  and at IC50 values. Uptake rates were also higher for *Tetraselmis* sp, whereas the most sensitive species, *P. tricornutum* had the lowest uptake rate. *D. tertiolecta* was very tolerant to copper and had low intracellular copper concentrations, presumably due to its ability to effectively exclude copper from the cell. Similar trends were observed when data were corrected for cell size.

In *P. tricornutum*, *Tetraselmis* sp., and *D. tertiolecta* the concentrations of copper inside the cell at the 72-h IC50 concentrations of 10, 50 and 500  $\mu\text{g Cu L}^{-1}$ , respectively, were  $23 \pm 19$ ,  $197 \pm 1$  and  $59 \pm 5 \times 10^{-15} \text{ g Cu cell}^{-1}$ . These values are of a similar magnitude to those found in other studies (see Table 5). The very tolerant *Scenedesmus subspicatus* (IC50 340  $\mu\text{g Cu L}^{-1}$ ) accumulated the most copper inside the cell ( $380\text{-}950 \times 10^{-15} \text{ g Cu cell}^{-1}$ ; Ma et al., 2003), followed by *Tetraselmis* sp. in the current study (IC50 50  $\mu\text{g Cu L}^{-1}$ ;  $197 \times 10^{-15} \text{ g Cu cell}^{-1}$ ), while the most tolerant species, *D. tertiolecta*, accumulated  $59 \times 10^{-15} \text{ g Cu cell}^{-1}$  at its IC50 of 500  $\mu\text{g Cu L}^{-1}$ . However, *Tetraselmis* sp. (current study), *Chlorella* sp. (Johnson et al., 2007; Franklin et al., 2002a, b) and *Pseudokirchneriella subcapitata* (Franklin et al., 2002a) had similar intracellular copper concentrations on a volume basis despite different sensitivities to copper. Bossuyt and Janssen (2004) found that inhibition of biomass for *P. subcapitata* did not occur at concentrations of up to  $2.9 \times 10^{-15} \text{ g Cu cell}^{-1}$  within the cell. Intracellular copper increased from 0.099 to  $21 \times 10^{-15} \text{ g Cu cell}^{-1}$  for algae acclimated to 0.5 and 100  $\mu\text{g Cu L}^{-1}$ , respectively, for 12 weeks, however a poor positive correlation ( $R^2 = 0.1$ ,  $N = 42$ ,  $P > 0.05$ ) between internal copper concentrations of acclimated algae and their chronic copper sensitivity was observed.



This research suggests that species with different sensitivities to copper do vary in the amount of intracellular copper required to give a particular effect, e.g. 50% growth-rate inhibition. Literature on various organisms, including algae and invertebrates (Luoma and Rainbow, 2005; Amiard-Triquet et al., 2006; Campbell et al., 2006) suggests that information on the form and localisation of copper inside the cell is more useful for predicting toxicity than total intracellular copper concentrations. Fractionation using ultra-centrifugation can determine the partitioning of metals within the cell (Campbell et al., 2005). Metals can be stored in biologically detoxified forms by binding to phytochelatins or precipitating in granules. Alternatively, metals may be located in fractions that are more likely to cause toxicity, e.g. bound to heat-sensitive proteins or to organelles (Campbell et al., 2006). The drawback of this method is that it is operationally defined, and must be adapted for each species used, not dissimilar to the EDTA washing procedure used in the current work.

#### *Changes in cell ultrastructure due to copper exposure*

Some changes in cell ultrastructure were observed upon exposure to copper concentrations equivalent to algal 72-h IC<sub>50</sub> values. *P. tricornutum* cells were larger in size and clumped together, while *Tetraselmis* sp. and *D. tertiolecta* cells did not change in size, but had larger-sized and an increased number of vacuoles. It is not clear if these vacuoles contained starch granules or were accumulating copper. Further ultrastructural changes may have been observed at higher copper exposure concentrations. The clumping of *P. tricornutum* cells could be due to copper oxidation of intracellular thiols, leading to a lowering of the ratio of reduced to oxidised glutathione (GSH:GSSG ratio), which has been hypothesised to inhibit mitotic spindle formation and consequently inhibit cell division in the marine diatom *Nitzschia closterium* (Stauber and Florence, 1987). Thus algae continue to photosynthesise and fix carbon but cell division is impaired, leading to swollen and enlarged cells that clump

together. Recent research by Stoiber et al. (2007) supports this hypothesis. They have shown a decrease in the ratio of GSH:GSSG, in GSH levels and growth-rate inhibition in the freshwater green alga *Chlamydomonas reinhardtii* after a 24-h copper exposure, with EC50 values of 3.1, 3.2 and 2.4  $\mu\text{g Cu L}^{-1}$ , respectively. They also found growth-rate inhibition to be highly correlated with the ratio of GSH:GSSG, suggesting that this may be a general mechanism of toxicity in microalgae under copper stress.

Previous work on the copper tolerant *Tetraselmis suecica* (96-h IC50 for growth inhibition of 170  $\mu\text{g Cu L}^{-1}$ ), found ultrastructural changes that included increased vacuolisation of the cytoplasm, the appearance of cells within multilayered cell walls, and the excretion of organic matter (Nassiri et al., 1996). X-ray microanalysis of the exudates showed a high concentration of copper in the organic matter and in the vesicles. Copper was found to be associated with sulfur and phosphorus within the vesicles. At concentrations greater than 500  $\mu\text{g L}^{-1}$ , *T. suecica* cells lost their flagella, became spherical and intra-cytoplasmic granules appeared. The increase in organic matter in the medium led to greater aggregation of cells. Previous ultrastructural research on *D. tertiolecta* exposed to 500  $\mu\text{g Pb L}^{-1}$  resulted in loosely packed, disrupted thylakoid membranes, thickening of the cell membrane (possibly due to Pb-precipitates), an increase in vacuolisation and polyphosphate bodies, and an over-accumulation of starch (Saçan et al., 2007). Simultaneous exposure to lead and aluminium (500  $\mu\text{g L}^{-1}$  each) resulted in deterioration of the cell membrane (Saçan et al., 2007). Electron dense deposits observed in the vacuoles in the Saçan et al., (2007) study were not visible in the current study. Effects on cells are metal-specific, but it appears that vacuoles may store the breakdown products, or act as a store for metals (Worms et al., 2006). Since greater vacuolisation occurred for *Tetraselmis* sp. and *D. tertiolecta* in the current study, compartmentalisation of copper may be a copper detoxification mechanism in these cells.

### *Other detoxification mechanisms*

Binding of metals to cysteine-rich phytochelatins is also a potential copper detoxification pathway but was not explored in this work. In a natural freshwater environment, phytochelatin concentrations increased in periphyton when copper and zinc increased in the biomass as a result of sediment resuspension (Le Faucheur et al., 2005). Induction of phytochelatins occurred in the green alga *Scenedesmus vacuolatus* at free copper concentrations of  $8 \times 10^{-11}$  M (Le Faucheur et al., 2006). Copper-phytochelatin complexes have been found in *P. tricornutum* after 1-h exposures, with increases in the Cu-phytochelatin complex with time (Morelli and Scarano, 2004). Despite this ability to bind copper to phytochelatin, this alga is still very sensitive to copper. Glutathione reductase activity was also found to increase in copper-exposed *P. tricornutum* cells, suggesting that the cells were trying to restore the concentration of reduced glutathione in the cell which is the precursor for phytochelatin (Morelli and Scarano, 2004). As metal increases in the cell, binding to phytochelatin may be overwhelmed, thus leading to toxicity. *D. tertiolecta* exposed to zinc and cadmium resulted in induction of phytochelatin synthesis at metal concentrations below those impacting on growth (Hirata et al., 2001). Other marine microalgae have also been shown to produce phytochelatins upon metal exposure, including *Emiliania huxleyi* and *Thalassiosira weissflogii* (Kawakami et al., 2006).

### **Conclusion**

Inter-species sensitivity to copper in marine microalgae, as measured by growth rate inhibition, did not relate solely to internal cellular copper concentrations nor to copper uptake rates. It is likely that species sensitivity also depends heavily on cell detoxification mechanisms. The most sensitive algal species (*P. tricornutum*, 72-h IC<sub>50</sub> 8 µg Cu L<sup>-1</sup>) had

the lowest copper uptake rate when exposed to concentrations equivalent to its IC<sub>50</sub> value. Cells were shown to swell and clump upon copper exposure. Intracellular copper concentrations in *P. tricornutum* were lower than in *Tetraselmis* sp. and yet *P. tricornutum* was five times more sensitive to copper, suggesting that potential detoxification mechanisms in *P. tricornutum* are overwhelmed and effects are observed at relatively low doses. The moderately-tolerant *Tetraselmis* sp. (72-h IC<sub>50</sub> of 47 µg Cu L<sup>-1</sup>) had the highest copper uptake rate of all three species and had the highest ratio of intracellular to extracellular copper. This alga may be able to detoxify copper within the cell, thus showing only moderate sensitivity despite the higher internal copper concentrations. The most tolerant species of algae, *D. tertiolecta* (72-h IC<sub>50</sub>, 530 µg Cu L<sup>-1</sup>), had the highest concentrations of copper in the extracellular fraction, one of the lowest internal concentrations of copper at its IC<sub>50</sub> and a very low ratio of intracellular to extracellular copper at its IC<sub>50</sub>. Concentrations of both carbohydrate and protein material associated with the surface of *D. tertiolecta* cells increased upon exposure to 500 µg Cu L<sup>-1</sup> suggesting that *D. tertiolecta* may gain its tolerance to copper through effective exclusion of copper from the cell.

Understanding differences in species-sensitivity and, by extension, predicting toxicity of copper to algal species, will require knowledge not only of water quality parameters, copper-cell binding and internalisation rates, but also of intracellular processes and detoxification mechanisms. This will require further research to resolve the exact nature of the biotic ligand in algae and to determine the biological and chemical fate of copper within these cells.

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Table 1. Summary of growth-rate inhibition bioassay protocol and test conditions for algal cells used in intracellular copper determinations.

Test type	Static
Temperature	21 ± 2°C
Light quality	Cool white fluorescent lighting
Light intensity	100 µmol photons m <sup>2</sup> s <sup>-1</sup>
Photoperiod	12 h light: 12 h dark
Test chamber size	250 mL
Test volume	
<i>Toxicity bioassay</i>	50 mL
<i>Intracellular copper determinations</i>	50 to 5 × 50 mL
Renewal of test solution	None
Age of test organism	4-5 days
Initial cell density in test chambers	3 × 10 <sup>3</sup> cells mL <sup>-1</sup>
Number of replicates	3
Shaking rate	Twice daily by hand
Test medium	0.45 µm filtered seawater + 15 mg NO <sub>3</sub> <sup>-</sup> L <sup>-1</sup> and 1.5 mg PO <sub>4</sub> <sup>3-</sup> L <sup>-1</sup>
Copper concentrations	CuSO <sub>4</sub> ·7H <sub>2</sub> O
<i>Toxicity bioassay</i>	Minimum of 5 and a control
<i>Intracellular copper determinations</i>	2-3, including IC50 [Cu] and 50 µg Cu L <sup>-1</sup> for each alga
Test duration	72 h
Test end-point	Growth-rate inhibition

Table 2. Sensitivity of *Phaeodactylum tricornutum*, *Tetraselmis* sp. and *Dunaliella tertiolecta* to copper (72-h).

Alga	72-h Growth-Rate Inhibition ( $\mu\text{g L}^{-1}$ )			Cell Dimensions (Control Cells) <sup>a</sup>			
	IC50 (95% CL)	LOEC	NOEC	Length ( $\mu\text{m}$ )	Width ( $\mu\text{m}$ )	SA ( $\mu\text{m}^2$ )	Vol ( $\mu\text{m}^3$ )
<i>P. tricornutum</i>	8.0 (4.7- 8.3)	1.5	<1.5	24-30	2.5-3.5	60-130	50-70
<i>Tetraselmis</i> sp.	47 (46-49)	22	7	8-11	5-9	240 $\pm$ 60	330 $\pm$ 140
<i>D. tertiolecta</i>	530 (450-600)	42	8	7-10	6-8	220 $\pm$ 50	310 $\pm$ 110

<sup>a</sup> Cell size was determined from analysis of at least 30 random cells using a phase-contrast microscope at 400  $\times$  magnification and an eye-piece micrometer. Cell dimensions are cell length and cell width at the widest section for the pennate diatom (*P. tricornutum*); and length and width (width = depth) for prolate ellipsoid cells (*D. tertiolecta* and *Tetraselmis* sp.). Surface area and volume were determined using equations for a prolate ellipsoid for *Tetraselmis* sp. and *D. tertiolecta* and the surface area and volume are given as mean  $\pm$  standard deviation. Surface area and volume were calculated for *P. tricornutum* by visualising the cell as two cones.

1 Table 3. Extracellular and intracellular copper in three algae after 72-h exposure to various dissolved copper concentrations <sup>a</sup>.  
 2

Alga	[Cu] ( $\mu\text{g L}^{-1}$ )	Per cell (72 h)			Normalised to Surface Area and Volume of Cells	
		Intracellular $10^{-13} \text{ g cell}^{-1}$	Extracellular $10^{-13} \text{ g cell}^{-1}$	Intra:Extra	Intracellular $10^{-17} \text{ g Cu } \mu\text{m}^{-3}$	Extracellular $10^{-17} \text{ g Cu } \mu\text{m}^{-2}$
<i>P. tricornutum</i>	10	$0.23 \pm 0.06$	$0.4 \pm 0.1$	0.5	$10 \pm 2$	$9 \pm 1$
	30	$0.48 \pm 0.05$	$0.9 \pm 0.1$	0.5	$11 \pm 1$	$21 \pm 2$
	50	$0.62 \pm 0.02$	$1.0 \pm 0.03$	0.6	$14 \pm 1$	$23 \pm 1$
<i>Tetraselmis</i> sp.	10	$0.22 \pm 0.02$	n.d. <sup>b</sup>	- <sup>c</sup>	$6.7 \pm 0.7$	n.d.
	50	$1.97 \pm 0.01$	$1.1 \pm 0.1$	2	$57 \pm 0.1$	$45 \pm 3$
	100	$1.17 \pm 0.20$	$1.6 \pm 0.3$	0.7	$19 \pm 4$	$39 \pm 12$
<i>D. tertiolecta</i>	50	$0.33 \pm 0.01$	$1.1 \pm 0.3$	0.3	$14 \pm 1$	$60 \pm 15$
	500	$0.59 \pm 0.03$	$5.7 \pm 0.9$	0.1	$27 \pm 1$	$320 \pm 40$

3  
 4 <sup>a</sup> Results are given as mean  $\pm$  standard error of three to nine replicates.

5 <sup>b</sup> n.d. = not detected

6 <sup>c</sup> Ratio could not be calculated at 72-h because extracellular copper was too low.

Table 4. The uptake rate of copper into algal cells over the period of a chronic toxicity test (72 h) expressed on a volume basis (to account for different cell sizes).

Alga and Copper Treatment	Uptake Rate ( $10^{-18}$ g Cu $\mu\text{m}^{-3}$ h $^{-1}$ ) <sup>a</sup>	Equation of Line $y = mx + b$	R <sup>2</sup>	p Value <sup>b</sup>	
<i>P. tricornutum</i>					
10 $\mu\text{g}$ Cu L $^{-1}$	0.47	$y = 0.047x + 5.4$	0.09	0.058	
30 $\mu\text{g}$ Cu L $^{-1}$	0.80	$y = 0.080x + 5.4$	0.17	<b>0.009</b>	
50 $\mu\text{g}$ Cu L $^{-1}$	0.65	$y = 0.065x + 8.8$	0.17	0.067	
<i>Tetraselmis</i> sp.					
10 $\mu\text{g}$ Cu L $^{-1}$	0.99	$y = 0.099x + 2.0$	0.61	<b>&lt;0.005</b>	a
50 $\mu\text{g}$ Cu L $^{-1}$	7.4	$y = 0.74x - 2.3$	0.93	<b>&lt;0.005</b>	b
100 $\mu\text{g}$ Cu L $^{-1}$	1.8	$y = 0.18x + 7.4$	0.86	0.058	
<i>D. tertiolecta</i>					
50 $\mu\text{g}$ Cu L $^{-1}$	1.9	$y = 0.19x + 0.88$	0.94	<b>&lt;0.005</b>	a
500 $\mu\text{g}$ Cu L $^{-1}$	2.8	$y = 0.28x + 5.55$	0.95	<b>&lt;0.005</b>	b

<sup>a</sup> Uptake rates were calculated by plotting the mean ( $\pm$  SE) value for replicates at each time-point and finding the slope of the straight line, with R<sup>2</sup> values indicative of goodness of fit. Note that Pearson's R<sup>2</sup> values were not high for *P. tricornutum* on a volume basis. Significance of regression was tested in SPSS Ver 14.0 for windows using all replicates plotted individually, with bold face type of p values indicative of a significant regression (for a line  $y = \alpha + \beta x$  where  $\beta$  = true population slope, is  $\beta \neq 0$  at greater than 95% confidence, i.e. we are confident there is an actual slope to the line).

<sup>b</sup> The lowercase letters (a,b,c) are used to denote slopes (uptake rates) for each individual alga that are significantly different using analysis of covariance and Tukey's post-hoc analysis (*Tetraselmis* sp.) or t-tests (*D. tertiolecta*) (as described in Zar, 1974). Where slopes were not significantly to zero they have been excluded from the analysis.



Table 5. Comparison of intracellular copper concentrations at or near the IC50 for a number of marine and freshwater algae.

Alga	Exposure concentration (µg Cu L <sup>-1</sup> )	IC50 concentration (µg Cu L <sup>-1</sup> )	Exposure time (h)	Intracellular copper concentration at endpoint		Reference
				(× 10 <sup>-15</sup> g Cu cell <sup>-1</sup> )	(× 10 <sup>-17</sup> g Cu µm <sup>-3</sup> )	
<b>Marine</b>						
<i>Phaeodactylum tricornutum</i>	10	8	72	23 ± 19	9.9 ± 6.9	This study
<i>Tetraselmis</i> sp.	50	47	72	197 ± 1	57 ± 0.2	This study
<i>Dunaliella tertiolecta</i>	500	530	72	59 ± 5	27 ± 2	This study
<i>Nitzschia</i> sp.	40	40	72	80 ± 36	10 ± 4	Johnson et al. (2007)
<b>Freshwater</b>						
<i>Chlorella</i> sp.	1.1-30	1.1-30 <sup>a</sup>	48	29		De Schamphelaere et al. (2005)
	8.5	7.3	72	37 ± 10	66 ± 17	Johnson et al. (2007)
	8.3	7	72		~80-100	Franklin et al. (2002a)
	4.4	4.4 <sup>b</sup>	72	6	30 <sup>b</sup>	Franklin et al. (2002b)
		1.5-35 (pH 6.5-5.7) <sup>a</sup>	72	14 (9-23)		Franklin et al. (2000)
<i>Pseudokirchneriella subcapitata</i>	18-46	18-46 <sup>a</sup>	72	42-71 <sup>a</sup>		Franklin et al. (2000)
		37	72	13.7		De Schamphelaere et al. (2005)
	6.2	6.2 <sup>b</sup>	72		60 <sup>b</sup>	Bossuyt and Janssen (2004)
<i>Scenedesmus subspicatus</i>		340 <sup>c</sup>	72	380-950		Franklin et al. (2002b)
<i>Chlamydomonas reinhardtii</i>	1.3-5.1	2.4	24	13- 14 <sup>d</sup>		Ma et al. (2003)
						Stoiber et al. (2007)

<sup>a</sup> Varies with pH; toxicity increases with increasing pH.

<sup>b</sup> IC50 depends on cell density, but internal concentration of copper to cause 50% effect varied little with cell density.

<sup>c</sup> IC50 when no EDTA or fulvic acid present.

<sup>d</sup> Total cellular copper not specifically intracellular copper.

Figure 1. Schematic diagram for isolating extracellular and intracellular copper fractions for *P. tricornutum* and *D. tertiolecta*. For *Tetraselmis* sp., centrifugation was replaced with filtration.

Figure 2. Concentration-response curves for *P. tricornutum*, *Tetraselmis* sp. and *D. tertiolecta* exposed to copper for 72 h. Results are pooled from three tests, with mean control growth rates normalised to 100% in each bioassay and the growth rate for each replicate plotted as a percentage of mean control growth rate.

Figure 3. Concentration of extracellular (EDTA extractable) copper per cell over a 72-h exposure (a) to 50  $\mu\text{g Cu L}^{-1}$  for the three algae, *P. tricornutum* (■), *Tetraselmis* sp. (◇) and *D. tertiolecta* (▲) and (b) to 50 (▲) and 500  $\mu\text{g Cu L}^{-1}$  (△) for *D. tertiolecta*. Three replicates taken at each time-point for each exposure concentration, with error bars an indication of standard error.

Figure 4. Concentration of intracellular copper per cell for each algal species following exposure to copper from 0 to 72 h. Control cells at 0-h (■) (n = 12-15). (a) *P. tricornutum* exposed to 10 (×---×), 30 (◆--◆) or 50  $\mu\text{g Cu L}^{-1}$  (△—△). (b) *Tetraselmis* sp. exposed to 10 (×---×), 50 (△—△) or 100  $\mu\text{g Cu L}^{-1}$  (●—●) (no time points prior to 24-h for 100  $\mu\text{g L}^{-1}$  tests). (c) *D. tertiolecta* exposed to 50 (△—△) or 500  $\mu\text{g Cu L}^{-1}$  (▲—▲). Each point represents the mean and standard error at any one time-point (with n = 3-6).

Figure 5. Transmission electron microscope (TEM) images under low magnification. (a), (b) and (c) are control cells for *P. tricornutum*, *Tetraselmis* sp., and *D. tertiolecta*, respectively. (d), (e) and (f) are cells exposed to their IC50 Cu concentration after 72-h and are *P.*

*tricornutum* ( $15 \mu\text{g Cu L}^{-1}$ ), *Tetraselmis* sp. ( $50 \mu\text{g Cu L}^{-1}$ ), and *D. tertiolecta* ( $500 \mu\text{g Cu L}^{-1}$ ), respectively. The scale bars indicates  $2 \mu\text{m}$ . Cell swelling and clumping observed for *P. tricornutum* copper-exposed cells. Increased numbers and size of vacuoles in *Tetraselmis* sp. and *D. tertiolecta* copper-exposed cells.

Figure 1

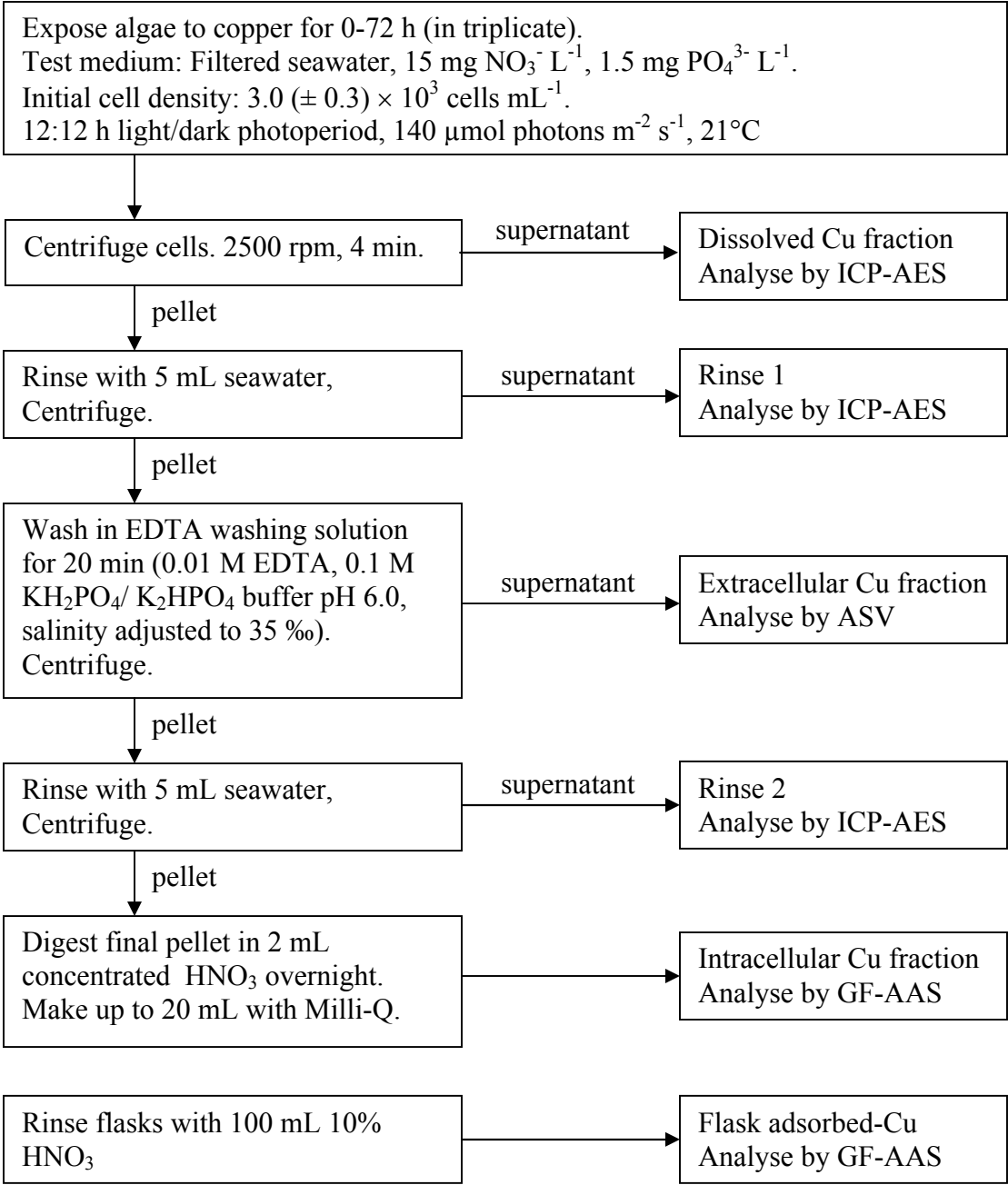


Figure 2

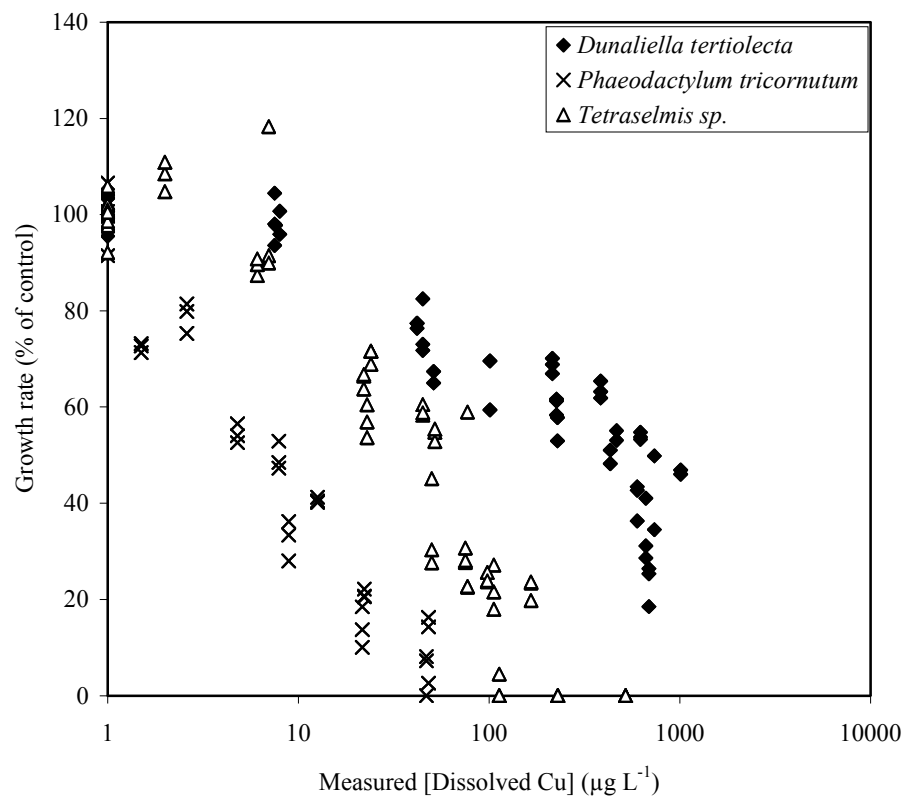


Figure 3

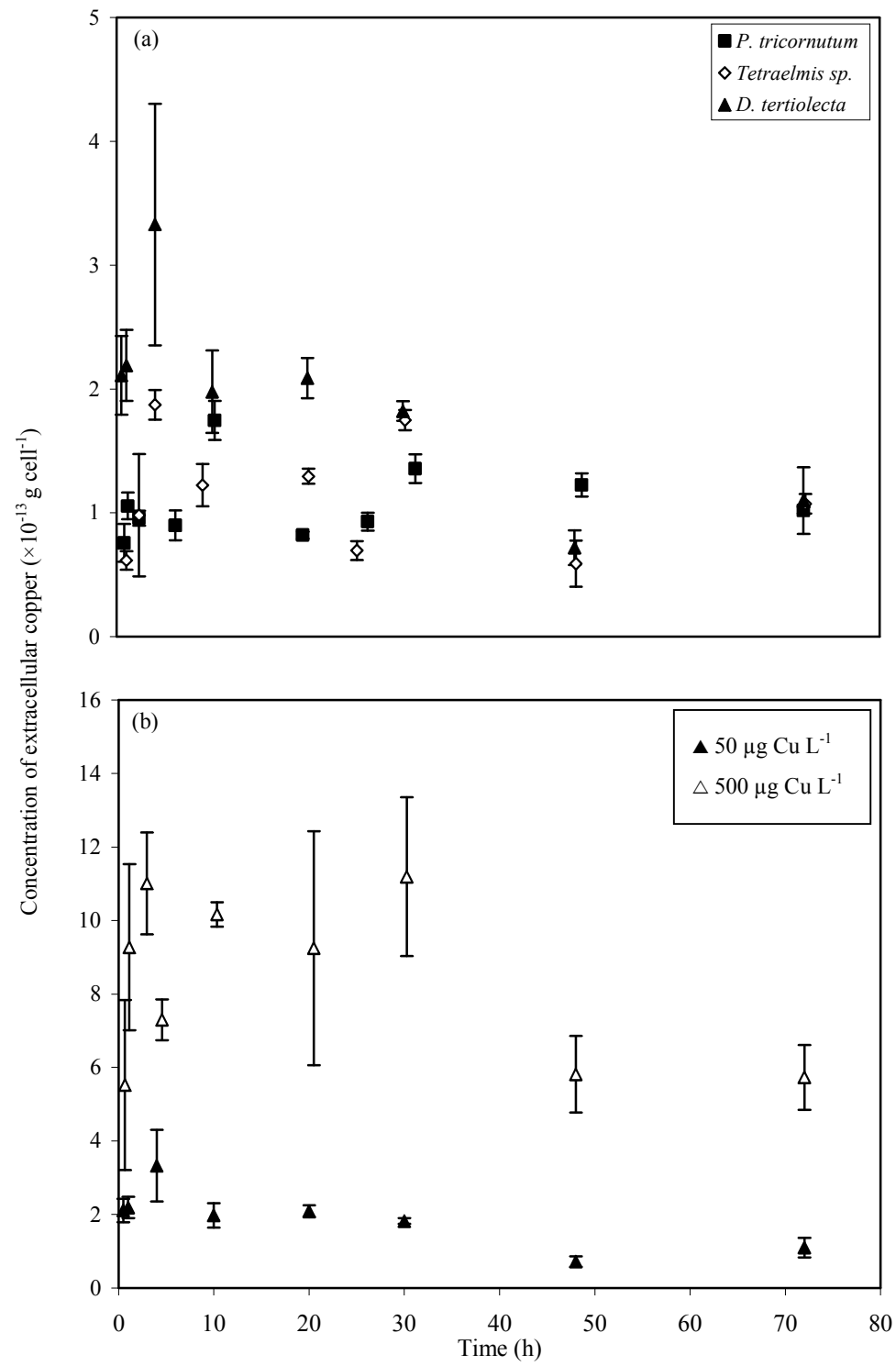


Figure 4

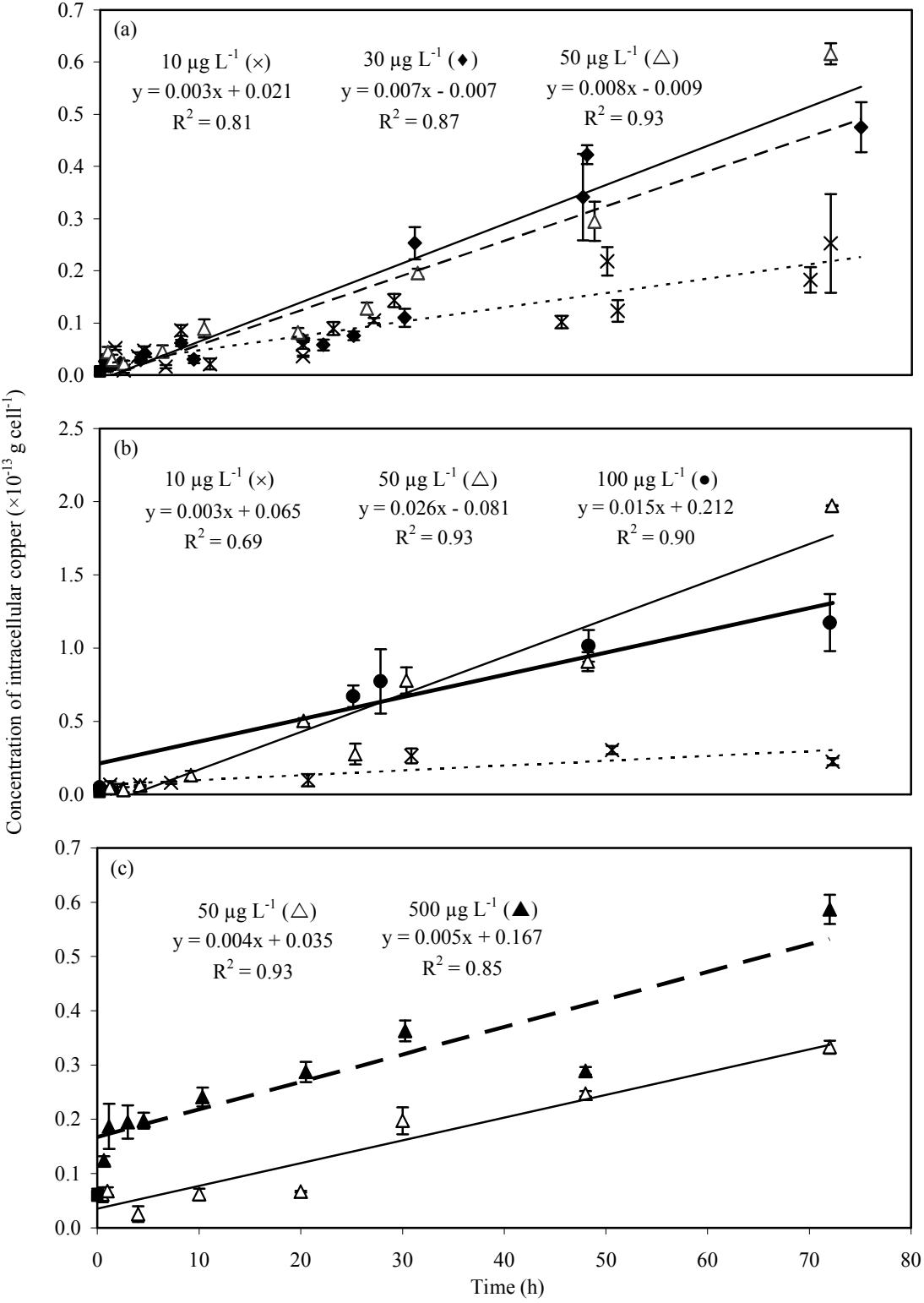


Figure 5

