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Copper and zinc tolerance of two tropical microalgae after copper acclimation

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Abstract

Current toxicity tests with microalgae are often criticized as being overly sensitive to metals because algae are cultured in metal-deficient media. If such bioassays overestimate copper toxicity in surface waters, the relevance of water quality guidelines derived from these tests is questionable. In this study, the effect of acclimation to copper at environmentally relevant concentrations, on the sensitivity of the marine diatom Nitzschia closterium and the freshwater green alga Chlorella sp. to copper and zinc was examined. N. closterium was acclimated in culture medium containing 5 or 25 g Cu L-1 for 200 days, while Chlorella sp. was acclimated in medium containing 2 g Cu L-1 for 100 days. Changes in algal growth rates and copper and zinc tolerance were monitored using standard growth inhibition toxicity tests in minimal medium over 72 h. Neither of the two acclimated N. closterium cultures had increased zinc or copper tolerance compared with that of the nonacclimated algae, nor were there any changes in control growth rates. Similarly, no changes in copper tolerance or control growth rates were observed for the acclimated Chlorella sp. culture. This was supported by measurements of intracellular and extracellular copper which confirmed that there were no differences in copper accumulation in either acclimated or nonacclimated algae. These results suggest that these algae grown in standard culture media are generally no more sensitive than algae grown in a metal-enriched medium. This supports the continued use of current laboratory bioassays with microalgae, as part of a suite of tests for assessing metal bioavailability, for use in ecological risk assessments and for providing data for the derivation of water quality guidelines. Copyright 2007 Wiley Periodicals, Inc. Environ Toxicol 22: 234-244, 2007.

Keywords

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Copper and zinc tolerance of two tropical microalgae after copper acclimation

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ABSTRACT

Current toxicity tests with microalgae are often criticised as being overly sensitive to metals because algae are cultured in metal-deficient media. If such bioassays overestimate copper toxicity in surface waters, the relevance of water quality guidelines derived from these tests is questionable. In this study, the effect of acclimation to copper at environmentally relevant concentrations, on the sensitivity of the marine diatom *Nitzschia closterium* and the freshwater green alga *Chlorella* sp. to copper and zinc was examined. *N. closterium* was acclimated in culture medium containing 5 or 25 µg Cu L^{-1} for 200 days, while *Chlorella* sp. was acclimated in medium containing 2 µg Cu L^{-1} for 100 days. Changes in algal growth rates and copper and zinc tolerance were monitored using standard growth inhibition toxicity tests in minimal medium over 72 h. Neither of the two acclimated *N. closterium* cultures had increased zinc or copper tolerance compared to the non-acclimated algae, nor were there any changes in control growth rates. Similarly, no changes in copper tolerance or control growth rates were observed for the acclimated *Chlorella* sp. culture. This was supported by measurements of intracellular and extracellular copper which confirmed that there were no differences in copper accumulation in either acclimated or non-acclimated algae. These results suggest that these algae grown in standard culture media are generally no more sensitive than algae grown in a metal-enriched medium. This supports the continued use of current laboratory bioassays with microalgae, as part of a suite of tests for assessing metal bioavailability, for use in ecological risk assessments and for providing data for the derivation of water quality guidelines.

Keywords:

Copper, zinc, algae, tolerance, acclimation, growth rate

INTRODUCTION

Algal bioassays are currently used to assess the impacts of contaminants on aquatic ecosystems as well as to assist in the development of water quality guidelines. Chronic algal toxicity tests typically measure the decrease in growth rate or cell biomass after a 72-h exposure to the contaminant. Algae have been found to be particularly sensitive to metals due to their high surface-to-volume ratio and the variety of membrane metal-ion binding sites, that differ in both affinity and specificity (Megharaj *et al,* 2003).

Both copper and zinc are essential elements required for the normal functioning of enzyme systems within algae. However, both metals are toxic when algae are exposed to concentrations exceeding those required for optimal growth. Both metals disrupt photosynthesis, respiration, ATP production and pigment synthesis, as well as inhibit cell division (Sunda and Huntsman, 1983; Cid *et al.*, 1996, Stauber and Florence, 1987, De Filippis *et al.*, 1981, Stauber and Florence, 1990).

Both biotic and abiotic factors affect the sensitivity of algae to copper and zinc (Stauber and Davies, 2000). Algal responses to metals depend on the particular species (with differing metal uptake rates and detoxification pathways), the chosen test endpoint, prior exposure, laboratory test conditions (including light, temperature, nutrient medium, cell density and exposure time), and water quality (dissolved organic matter, hardness, pH). For example, Stauber and Florence (1989) found that the use of different test media had significant impacts on the bioavailability of metals, with 72-h IC_{50} values ranging from 16 to >200 μg Cu L^{-1} for *Chlorella protothecoides* grown prior to the bioassay in different growth media. The initial algal inoculum size has also been found to affect metal toxicity, with copper toxicity decreasing as initial cell density increased (Franklin *et al*., 2002a). Decreased copper toxicity was related primarily to greater copper adsorption by algal cells, resulting in depletion of dissolved copper in solution.

Interspecies differences in sensitivities to metals can also be influenced by prior exposure to metals. Algae isolated from polluted environments typically have higher tolerance for metals than laboratory isolates, due to an induced tolerance from exposure to high metal concentrations. 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 to these high metal concentrations has also been explored in laboratory

74 environments, with increases in copper or zinc concentrations in algal growth media leading to an
75 increased tolerance towards those metals (Muyssen and Janssen, 2001; Bossuyt and Janssen
76 2004). Muyssen and Janssen (2001) studied zinc tolerance in two commonly used algae,
77 *Raphidocelis subcapitata* and *Chlorella vulgaris*, which were acclimated in medium containing
78 65 μg Zn L^{-1}. They found that zinc tolerance increased, with the 72-h EC_{50} increasing three-fold
79 compared to non-acclimated algae grown in International Organisation for Standardisation (ISO)
80 medium containing 1.4 μg Zn L^{-1}.

81 Some algae that develop a tolerance for one metal can also display an increased tolerance to
82 another metal, particularly if the route of metal uptake and mode of toxic action is similar.
83 Stokes and Drier (1981) found that a copper-tolerant isolate of *Scenedesmus* also displayed co-
84 tolerance to nickel and cobalt, despite no previous exposure to these metals. This change in metal
85 tolerance due to prior metal exposure may be a result of either physiological acclimation or
86 genetic adaptation. Loss of metal tolerance when algae are subsequently cultured in standard
87 growth medium at low metal concentrations, is generally interpreted as physiological acclimation
88 only. Stokes and Drier (1981) reported that the copper-tolerant *Scenedesmus* species, grown in
89 copper-deficient medium, lost its tolerance to copper and co-tolerance to nickel and cobalt.
90 Similarly, Muyssen and Janssen (2001) found that the zinc tolerance in *Raphidocelis subcapitata*
91 and *Chlorella vulgaris* was lost upon the algae being returned to standard growth medium,
92 suggesting that this was physiological acclimation rather than genetic adaptation.

93 Changing metal tolerance has significant implications for the applicability of current algal
94 toxicity tests for assessing metal bioavailability in natural waters. If algae are cultured for long
95 periods in metal-deficient medium in the laboratory, they could become overly-sensitive to
96 metals compared to natural algal populations, and hence bioassays could over-estimate metal
97 toxicity in natural waters. Furthermore, the relevance of water quality guidelines derived from
98 these tests could be questionable. The importance and consequences of metal acclimation of
99 algae in laboratory culture, and their subsequent sensitivity to metals in toxicity tests, have rarely
100 been investigated. The aim of this study, therefore, was to determine the acclimation/adaptation
101 response to copper of two tropical algae commonly used in toxicity testing in Australasia: the
102 marine diatom *Nitzschia closterium* and the freshwater green alga *Chlorella* sp. These two
103 species were acclimated to environmentally realistic concentrations of dissolved copper in
104 copper-supplemented culture media, and changes in tolerance to copper and zinc over several
105 months were monitored in minimal medium using standard 72-h growth rate inhibition bioassays.

The results of this study aimed to provide a better understanding of the environmental relevance of using these bioassays to assess metal bioavailability in natural waters at environmentally relevant metal concentrations.

MATERIALS AND METHODS

Algal cultures

Nitzschia closterium was originally obtained from the Microalgae Culture Collection (CSIRO Marine and Atmospheric Research, Hobart, Australia). The tropical alga, isolated from the Coral Sea, Australia in 1981, was maintained in half strength G medium (Loeblich and Smith, 1968). This medium had measured total and dissolved copper concentrations of <2 μg L^{-1}, and total and dissolved zinc concentrations of 79 ± 1 μg Zn L^{-1} and 76 ± 2 μg Zn L^{-1}, respectively.

Chlorella sp. 12 was isolated from Lake Aesake, Papua New Guinea in 1995 and maintained axenically in JM/5 media (Thompson *et al*., 1988). This medium had measured background concentrations of total and dissolved copper of 1.4 ± 0.3 μg L^{-1} and total and dissolved zinc of <1 μg L^{-1}.

Both species were incubated on a 12:12 h light:dark cycle (75 ± 5 μmol photons m^{-2} s^{-1}, Phillips TL 40 W cool white fluorescent lighting) at 27 ± 1 ^{o}C. Both cultures were renewed weekly by inoculating 0.1 mL into freshly autoclaved medium.

Acclimation of algae

N. closterium was grown in half strength G medium supplemented with either 5 or 25 μg Cu L^{-1} (added as $CuSO_4.5H_2O$). There was good agreement between nominal and measured copper concentrations, with measured total and dissolved copper concentrations of 4.3 ± 0.1 μg Cu L^{-1} and 27 ± 1 μg Cu L^{-1} for the +5 and +25 μg Cu L^{-1} supplemented media, respectively.

Chlorella sp. was cultured in JM/5 media supplemented with 2 μg Cu L^{-1}. Measured copper concentrations in the copper-supplemented medium were 3.8 ± 0.2 μg L^{-1} total copper and 3.4 ± 0.4 μg L^{-1} dissolved copper. The composition of both the copper supplemented media (G and JM/5) were identical to the non-acclimated baseline media for all other constituents, including zinc.

135 ***Growth inhibition bioassays***

136 To determine algal tolerance to copper and zinc over several months, the effect of copper and
137 zinc individually on 72-h algal growth rates in minimal medium was assessed for each of the pre-
138 acclimated algal cultures – *N. closterium* baseline and +5 µg Cu L^{-1} (+5Cu), and the *Chlorella* sp.
139 baseline and +2 µg Cu L^{-1} (+2Cu) cultures. Tests were conducted over a 100- and 200-day
140 period for *Chlorella* sp. and *N. closterium*, respectively. Range-finder and definitive toxicity
141 tests were carried out according to the method of Stauber *et al.* (1994), as summarised below.

142 The *N. closterium* toxicity tests were carried out in filtered seawater (pH 8.0 ± 0.2, salinity 34 ‰,
143 dissolved copper <0.5 µg/L, dissolved zinc <10 µg/L), which was supplemented with nitrate (15
144 mg NO_3^- L^{-1}) and phosphate (1.5 mg PO_4^{3-} L^{-1}). For *Chlorella* sp., a synthetic soft water
145 (hardness 80 - 90 mg $CaCO_3$ L^{-1}, alkalinity 54 mg $CaCO_3$ L^{-1} and pH 7.5 ± 0.2) was
146 supplemented with nitrate (15 mg NO_3^- L^{-1}) and phosphate (0.15 mg PO_4^{3-} L^{-1}). Light and
147 temperature conditions for the toxicity tests for both species were the same as those used for
148 culture maintenance. Cultures were shaken twice daily by hand.

149 Metal stock solutions were prepared from copper sulphate ($CuSO_4.5H_2O$, Ajax Chemicals) and
150 zinc chloride ($ZnCl_2$, Sigma), acidified to pH <2 using HCl (Suprapur grade, Merck), and stored
151 at 4°C. Controls, together with at least five metal concentrations, each in triplicate, were
152 prepared for toxicity testing. Copper concentrations ranged from 10 - 160 µg Cu L^{-1} for *N.*
153 *closterium* and 2 - 20 µg Cu L^{-1} for *Chlorella* sp., and zinc concentrations ranged from 50 - 600
154 µg Zn L^{-1} for *N. closterium* and 15 - 200 µg Zn L^{-1} for *Chlorella* sp. (Tables 1 and 2). Fifty
155 milliliters of toxicity test medium was dispensed into 250-mL borosilicate glass Erlenmeyer
156 flasks, pre-coated with silanizing solution (Coatsil, Ajax Chemical, Auburn, NSW, Australia) to
157 reduce adsorption of metals to the flask walls. All glassware was acid washed in 10% HNO_3
158 before use. Subsamples (5 mL) were immediately taken from each flask, acidified and analysed
159 for copper and zinc by inductively coupled plasma – atomic emission spectrometry (Spectroflame
160 EOP). Measured copper and zinc concentrations were used to calculate all toxicity test
161 endpoints.

162 Exponentially-growing algal cells of each species were centrifuged (2800 rpm x 7 min) and
163 washed three times before use in the bioassay to remove culture medium. Each flask was
164 inoculated with pre-washed cells to give an initial cell density of 2 - 4 x 10^4 cells mL^{-1}. The pH

was monitored throughout the test and cell density was measured each day for three days using a Coulter Multisizer 2Z Particle Analyser with a 70 µm aperture.

Test endpoints and statistical analysis

The algal growth rate (cell division rate) in each flask over 72 h was calculated using regression analysis. A regression line was plotted of the $\log_{10}$ cell density against time (h) to determine the slope of the line for each flask, equivalent to the cell division rate per hour (µ) and calculated as doublings/day for each treatment. Growth rates for *N. closterium* and *Chlorella* sp baseline cultures were 1.4 ± 0.1 and 1.4 ± 0.1 doublings/day respectively. Algal growth rates in each treatment were expressed as a percentage of the control growth rate. A concentration-response curve was obtained by plotting the percentage control growth rate versus the measured metal concentrations. The IC_{50},, IC_{25} and IC_{10} were calculated using Linear Interpolation in ToxCalc Version 5.0.23 (Tidepool Software). After testing the data for normality and homogeneity of variance, Dunnett's Multiple Comparison Test was used to determine which concentrations were significantly different to the controls in order to estimate LOEC and NOEC values. The Students t-test ($p \leq 0.05$) was used to determine significant difference between treatments.

Measurements of intra- and extracellular copper concentrations

Intracellular and extracellular copper concentrations were determined for algal cells exposed to copper concentrations equivalent to their 72-h IC_{50} values. Intracellular and extracellular metal was measured in the *N. closterium* non-acclimated baseline and 5 µg Cu L^{-1} acclimated cultures, and the *Chlorella* sp. non-acclimated baseline and 2 µg Cu L^{-1} cultures, using a modified method of Franklin *et al.* (2002a).

A control flask and three replicates at the 72-h IC_{50} copper concentration (40 µg Cu L^{-1} for *N. closterium* and 8.5 µg Cu L^{-1} for *Chlorella*), each containing 60 mL, were prepared. Two 5 mL subsamples were taken from each flask for pH and metal analyses (acidified to 0.2% (v/v) HNO_3 (Tracepur)) and replicates combined. All flasks were incubated under the same conditions as that used in the toxicity tests. At the completion of the 72-h test, 2 mL sub-samples were taken from each of the flasks and the cell density and cell size determined using a flow cytometer, as described in Stauber *et al*. (2005).

Algal cell size has previously been shown to increase during copper exposure (Franklin *et al.,* 2002a, Franklin *et al*., 2002b). To account for any changes in cell size in the presence of metal, extracellular and intracellular metal concentrations were expressed both on a per cell basis and

196 also on the basis of calculated surface area and volume, respectively. A flow cytometer was used
197 to measure cell size after a 72-h metal exposure for each treatment used in the intra/extracellular
198 experiments. The mean diameter of *Chlorella* was determined from the mean peak channel of
199 forward angle light scatter histograms (which indicate particle size) and compared to a calibration
200 curve of mean peak channel values of spherical latex beads of known diameter. The measured
201 cell diameter was used to determine the surface area and volume of *Chlorella* using the equations
202 for a sphere. *N. closterium* cell sizes were measured using a micrometer and phase-contrast
203 microscopy. *N. closterium* has a fusiform shape and two cones joined at the base were used as an
204 estimate of the surface area and volume.

205 The solution from each flask (pre-weighed) from each replicate set was filtered through an acid-
206 washed 25 mm GH Polypro (GHP) 0.45 μm membrane filter (Pall Life Sciences). A 10 mL
207 sample of filtrate was collected from each flask for *N. closterium*, while a 5 mL sample from each
208 of the two flasks in a replicate set was collected and combined (giving a total of 10 mL) for
209 *Chlorella* sp. The collected filtrate was acidified to 0.2% (v/v) HNO_3 (Dissolved Metal
210 Fraction). Filter papers (with collected algal cells) were rinsed with 5 mL of seawater and 10 mL
211 of synthetic softwater for *N. closterium* and *Chlorella* sp., respectively. This rinsate was also
212 acidified (Dissolved Rinse Fraction). *Chlorella* sp. cells on the filter paper were carefully rinsed
213 with a 0.02 M ethylenediaminetetracetic acid (EDTA) solution into an acid-washed (50%
214 concentrated HNO_3) 50-mL Oak Ridge Teflon centrifuge tube. For *N. closterium*, a phosphate
215 buffered 0.01 M EDTA in NaCl (3.4%) solution was used to rinse the algal cells. The EDTA-
216 rinsed cells were made up to 15 mL by weight, shaken for 30 s and left for 20 min. Using a
217 membrane filter, the EDTA rinsed cells were filtered and the filtrate retained for analysis of
218 extracellular bound copper (Extracellular Fraction). Algal cells collected on the filter paper were
219 again rinsed into a Teflon tube using approximately 7 mL of a 25% (v/v) concentrated HNO_3
220 solution. The volume was made to 8 mL of acid solution by weight, retaining the filter paper in
221 the solution. The solution was allowed to sit for 30 min and then microwave digested (10%
222 power of 1100W, 5 min). When cool, solutions were diluted to 10% acid with Milli-Q water, and
223 retained for analysis of intracellular copper (Intracellular Metal Fraction). Filter papers were also
224 acid-digested as a blank.

225 For mass balance calculations of copper, 50 mL of a 0.2% (v/v) HNO_3 solution was added to the
226 flasks and left overnight to remove any metal adsorbed to the flask walls. A 5-mL sub-sample
227 was taken from each flask (Flask Adsorbed Fraction).

Graphite furnace atomic absorption spectrometry (GF-AAS, Model 4100ZL) was used to measure copper in all cellular fractions for the *Chlorella* intracellular/extracellular studies. However, due to the presence of NaCl in the *N. closterium* fractions, the extracellular metal fraction was measured by anodic stripping voltammetry (ASV). Dissolved, dissolved rinse, flask adsorbed and Day 0 metal fractions were measured by inductively coupled plasma atomic emission spectrometry (ICP-AES).

RESULTS

Effect of copper pre-acclimation on copper tolerance in growth rate inhibition tests

The results of the copper growth-inhibition tests for *N. closterium* are shown in Figure 1 and Table 1. For *N. closterium* there was no significant difference between the control growth rates of the +5 μg Cu L^{-1} pre-acclimated culture (1.5 doublings/day) and the non-acclimated baseline culture (1.4 doublings/day) ($p > 0.05$). Increasing copper concentrations in the bioassay caused a decrease in algal growth rates over 72 h, with no significant difference between the mean 72-h IC_{50} for the pre-acclimated +5 μg Cu L^{-1} culture (43 ± 13 μg Cu L^{-1}, n = 6) and the non-acclimated (baseline) culture (40 ± 4 μg CuL^{-1}, n = 4) (Table 1). Copper concentration-response curves for the pre-acclimated and non-acclimated baseline cultures were similar (Figure 1a) over the 196-day acclimation period. IC_{25}, IC_{10}, NOEC and LOEC values were also similar between pre-acclimated and non-acclimated cultures (Table 1).

For the +25 μg Cu L^{-1} pre-acclimated culture, the control growth rate after a 35-day acclimation was only 0.76 doublings per day, much lower than the non-acclimated baseline control growth rate of 1.4 doublings per day. However, after a 168 day pre-acclimation, control growth rates in the +25 μg Cu L^{-1} pre-acclimated culture reached 1.5 doublings per day, similar to the baseline culture. This suggests that the algae had acclimated to the high copper concentrations in the culture medium over 168 days, prior to the bioassay.

The sensitivity of the +25 μg Cu L^{-1} culture to copper after a 35-day pre-acclimation was further demonstrated by a clear shift in the copper concentration-response curve to the left (Figure 1b), and by the lower 72-h IC_{50} of 12 (3-36) μg Cu L^{-1}, in comparison to the baseline culture. However, after 168 days, algal copper tolerance was similar to the baseline non-acclimated culture, with similar NOEC and LOEC values, concentration-response curves and 72-h IC_{50} values of 27 (14-58) μg Cu L^{-1} and 40 ± 4 μg Cu L^{-1} for the pre-acclimated and non-acclimated cultures, respectively.

The responses of the copper pre-acclimated and non-acclimated *Chlorella* cultures to copper are shown in Figure 2 and Table 2. For *Chlorella* sp. the concentration-response curves for each toxicity test conducted with the +2 μg Cu L^{-1} pre-acclimated and non-acclimated baseline cultures showed the same pattern of decreased growth with increasing copper concentration. Algal growth inhibition for the acclimated culture initially showed an increased tolerance to copper after 19 days, compared to the non-acclimated baseline culture, shown as a clear shift to the right of the concentration-response curve. However, algae in all subsequent tests (day 54 onwards) did not show any increased copper tolerance. There were no significant differences ($p > 0.05$) between the control growth rates (1.4 ± 0.1 doublings/day for both the non-acclimated and acclimated cultures) or the mean copper 72-h IC_{50} values for the pre-acclimated *Chlorella* culture (7.9 ± 1.8 μg Cu L^{-1}) and the non-acclimated baseline culture (7.3 ± 1.5 μg Cu L^{-1}), suggesting that overall, there was little change in sensitivity over the 104-day acclimation period. ICx, NOEC and LOEC values were also similar in both the pre-acclimated and non-acclimated cultures (Table 2).

Effect of copper pre-acclimation on intacellular and extracellular copper

Intra- and extracellular copper concentrations of *N. closterium* were measured in the +5 μg Cu L^{-1} pre-acclimated and the non-acclimated baseline cultures (Table 3). The copper mass balance (i.e. copper in the cells, copper on the cells, copper in solution and copper on the flask) was generally good, with 87-105% copper recovery in the replicates. For the control (no added copper), intracellular and extracellular copper concentrations were below detection limits ($< 0.5 \times 10^{-15}$ g/cell) for both the pre-acclimated and non-acclimated *N. closterium* cultures. For the 40 μg Cu L^{-1} treatment, 51% and 54% of added copper was associated with the cells (intra- and extracellular copper) for the non-acclimated and the pre-acclimated cultures, respectively. There was higher extracellular copper (on a per cell basis) in the algae grown in the baseline medium compared to the algae grown in the +5 μg Cu L^{-1} medium. However, due to the large variation in the baseline extracellular copper concentrations, this difference was not statistically significant ($p > 0.05$). There was also no significant difference in intracellular copper (expressed either on a per cell basis or a cell volume basis) between the two cultures. This supports the results of the growth rate inhibition bioassays, and previous studies, which have shown that growth inhibition is related to intracellular copper concentrations (Franklin *et al.*, 2002).

For *Chlorella* cultures, the intra- and extracellular copper concentrations (on a per cell basis) were not significantly different between pre-acclimated and non-acclimated algal cells (Table 3). The intra- and extracellular copper concentrations for the control (no added copper) were again below detection limits for both the acclimated and non-acclimated cultures (3). For both non-acclimated and pre-acclimated cultures, 57% and 66% of added copper was associated with the algal cells, respectively. The copper mass balance was good, with 95-120% recovery for all of the replicates. There was no difference in the ratio of extra- to intracellular copper between the pre-acclimated and non-acclimated cultures for copper when expressed on a per cell, per volume or per surface area basis. These findings further support the *Chlorella* growth-inhibition results, revealing no difference in sensitivity to copper between copper pre-acclimated and non-acclimated cultures.

Effect of pre-acclimation to copper on zinc co-tolerance

The effect of prior exposure to copper on algal co-tolerance to zinc was determined for both the copper pre-acclimated and non-acclimated *N. closterium* and *Chlorella* cultures. Control growth rates between copper pre-acclimated and non-acclimated cultures of *N. closterium* were similar in all zinc toxicity tests (>1 doubling/day). Algae grown prior to the bioassays in the medium supplemented with 5 µg Cu L^{-1} had similar sensitivity to zinc as the non-acclimated baseline culture (Figure 3). The mean 72-h IC_{50} for the 5 µg Cu L^{-1} pre-acclimated culture was 273 ± 58 µg Zn L^{-1}, which was not significantly different to the mean zinc 72-h IC_{50} for the non-acclimated baseline culture of 226 ± 105 µg Zn L^{-1}. This indicates that acclimation to 5 µg Cu L^{-1} had no effect on zinc tolerance in tropical *N. closterium*.

The 72-h IC_{50} of 186 µg Zn L^{-1} for the +25 µg Cu L^{-1} copper-acclimated algae was within one standard deviation of the corresponding mean value for the non-acclimated culture (226 µg Zn L^{-1}) (Table 4). After a 168-day acclimation, there was no significant difference between the 72-h IC_{50} values of 194 µg Zn L^{-1} for the copper-pre-acclimated culture and 226 µg Zn L^{-1} for the non-acclimated culture, which was also supported by the similar concentration response curves (Figure 4).

The effect of zinc on the growth rate of *Chlorella* sp. from both baseline and +2 µg Cu L^{-1} pre-acclimated cultures is shown in Figure 5. The 72-h IC_{50} value for zinc for the pre-acclimated culture (115 ± 18 µg Zn L^{-1}) was not significantly different to the non-acclimated culture (110 ± 41 µg Zn L^{-1}), suggesting that pre-acclimation to copper had no effect on co-tolerance to zinc.

DISCUSSION

Sensitivity of N. closterium and Chlorella sp. to copper and zinc

The freshwater green alga *Chlorella* sp. was more sensitive to both copper and zinc than the marine diatom *N. closterium*. Both algal species were also found to have a greater sensitivity to copper than zinc, in agreement with other reported studies with microalgae using similar test protocols (Franklin *et al*., 2001, Wilde *et al*., 2005).

Comparison of IC_{50} values found in this study with literature data is difficult, as differences in test procedures and test conditions affect algal sensitivity to metals (Stauber and Davies, 2000). While the temperate clone of *N .closterium* has been widely used throughout Australasia, few studies have examined metal sensitivity of the tropical strain used in this study. Earlier unpublished data from our laboratory suggests that the 72-h IC_{50} values for zinc and copper for the tropical clone reported here are similar to that found previously (197 µg Zn L^{-1} and 33 µg Cu L^{-1}) (J. Stauber, unpublished data).

The toxicity of zinc and copper to *Chlorella* sp. reported in this study was also similar to that found previously by Franklin and coworkers, who reported 72-h IC_{50} values for *Chlorella* sp. of 7.3 and 7.9 µg Cu L^{-1}, and 92 µg Zn L^{-1} (Franklin *et al*. 2002a,b). Intracellular and extracellular copper concentrations for *Chlorella* sp. were also similar and dependent on the external dissolved copper concentrations. Intracellular ($66 \pm 17 \times 10^{-8}$ ng/µm^3) and extracellular ($21 \pm 7 \times 10^{-8}$ ng/µm^2) copper concentrations in baseline *Chlorella* sp. after a 72 exposure to 8.5 µg Cu L^{-1} in this study were similar to that found by Franklin *et al*. (2002b), who reported intra- and extracellular copper concentrations of 68×10^{-8} ng/µm^3 and 25×10^{-8} ng/µm^2, respectively for the same *Chlorella* sp. exposed to 8.2 µg Cu L^{-1} for 72 h.

Acclimation of N. closterium and Chlorella sp.

This study demonstrated that both the copper pre-acclimated tropical *N. closterium* and *Chlorella* sp. cultures showed no increase in copper tolerance in comparison with the non-acclimated algal cultures. This suggests that the copper concentration in the culture medium does not influence algal copper tolerance under the test conditions and low copper concentrations used in this study. This agrees with Bossuyt and Janssen (2004), who also found no increased copper tolerance for the freshwater green alga *Pseudokirchneriella subcapitata* acclimated to 1-35 µg Cu L^{-1}, compared to algae grown in the control (no added copper) medium. However, Bossuyt and Janssen (2004) found differences in tolerance in cultures acclimated to higher copper

concentrations (60-100 μg Cu L^{-1}). It is possible that the copper concentrations to which *Chlorella* sp. was acclimated in our study were too low to cause increased copper tolerance. However, because our *Chlorella* sp. was much more sensitive to copper than the freshwater algae used by Bossuyt and co-workers, the copper concentration in the medium could not be further increased, without causing copper-stress, indicated by increased sensitivity and poor control growth rates. The environmental relevance of acclimating algae to such high copper concentrations is also questionable. In pristine freshwaters, typical dissolved copper concentrations are 0.3-3 μg/L., with concentrations up to 40 μg/L reported for mine-impacted rivers (Stauber and Davies, 2000). These concentrations are substantially lower than those used by Bossuyt and co-workers. In surface open ocean seawater, dissolved copper ranges from 0.03-0.15 μg/L, while in nearshore waters concentrations are typically 0.09-0.3 μg/L, although concentrations of up to 14 μg/L have been reported in some highly contaminated estuaries around the world. (Stauber and Davies, 2000).

Differences in tolerance to zinc were also reported by Muyssen and Janssen (2001) who found *Raphidocelis subcapitata* (now named *Pseudokirchneriella subcapitata*) and *Chlorella vulgaris* acclimated to 65 μg Zn L^{-1} were up to 3 times more tolerant to zinc compared to the non-acclimated algae.

For tropical *N. closterium*, additions of higher copper concentrations in the growth medium are unlikely to induce an increased copper tolerance. Based on this study, acclimating tropical *N. closterium* to higher copper concentrations is more likely to have the reverse effect of decreasing tolerance, at least during the initial acclimation period. The initial stress experienced by the 25 μg Cu L^{-1} acclimated culture, as indicated by the decreased control growth rate and lowered IC_{50} value in comparison to the baseline culture, was also reported by Bossuyt and Janssen (2004). They found that the acclimated *P. subcapitata* cultures all had significantly reduced growth rates and biomass in comparison with the control culture after one week of acclimation, particularly for those cultures acclimated to high copper concentrations (60-100 μg Cu L^{-1}). Cultures acclimated to copper concentrations at or above 35 μg Cu L^{-1} had lower growth rates compared to non-acclimated algae over the entire experimental period (12 weeks), while cultures at lower copper concentrations recovered to some extent. In contrast, the +25 μg Cu L^{-1} pre-acclimated culture in this study had decreased growth rates and copper tolerance compared to the non-acclimated culture after 35 days of acclimation, with recovery to baseline levels after 168 days. It appears that tropical *N. closterium* requires longer to acclimate to copper than *P. subcapitata*. Short

384 acclimation periods have also been found by Kuwabara and Leland (1986) who reported copper
385 acclimation occurring within days for *P. subcapitata*.

386 The intra- and extracellular copper concentrations for the pre-acclimated cultures of both tropical
387 *N. closterium* and *Chlorella* sp., when exposed to copper concentrations equivalent to their
388 copper IC_{50} values for 72 h, were not significantly different to the non-acclimated baseline
389 cultures. This supports the toxicity test findings, which showed no difference in copper tolerance
390 between the acclimated and non-acclimated cultures. Intracellular and extracellular copper
391 concentrations in *P. subcapitata* acclimated to copper concentrations ranging from 0.5 (control)
392 to 100 μg Cu L^{-1} were also reported by Bossuyt and Janssen (2005). Internal copper
393 concentrations were found to increase with copper concentration in the culture medium, however
394 no change was found between acclimation concentrations of 1 and 5 μg Cu L^{-1}. The control
395 culture (no added copper) had lower cellular copper concentrations than those acclimated to low
396 non-toxic concentrations of copper, which Bossuyt and Janssen interpreted as copper deficiency.
397 The intracellular copper concentrations in Bossuyt and Janssen's study were measured
398 immediately after algae were transferred from stock cultures into fresh sterile media, as opposed
399 to our study where measurements were taken after algae were grown in minimal bioassay test
400 solutions (± copper) for 72 h.

401 Growth rates were also compared between the pre-acclimated and non-acclimated cultures to
402 determine whether algae were potentially copper deficient in baseline medium. Control growth
403 rates for both the acclimated *Chlorella* sp. and 5 μg Cu L^{-1} acclimated *N. closterium* cultures
404 were not significantly different to non-acclimated baseline cultures. This indicates that based on
405 growth rates, the growth media for both *Chlorella* sp. 12 and *N. closterium* are unlikely to be
406 copper deficient. Bossuyt and Janssen (2004) also reported no differences in growth rates in *P.*
407 *subcapitata* cultures acclimated to copper concentrations of 0.5 - 12 μg Cu L^{-1}. However, cultures
408 acclimated to higher copper concentrations had reduced growth rates. For zinc, Muyssen and
409 Janssen (2001) found control growth rates in non-acclimated cultures to be higher than in zinc-
410 acclimated *C. vulgaris* cultures. In contrast, zinc acclimated *R. subcapitata*, had higher growth
411 rates than the non-acclimated algae at all test concentrations (Muyssen and Janssen, 2001),
412 indicating potential zinc deficiency.

413 Co-tolerance typically occurs when the binding sites and uptake pathways of different metals at
414 the cell-water interface are similar. As metal coordination sites on the cell surface are never
415 entirely specific for a single metal or nutrient, competition for membrane transport sites and

intracellular binding sites can occur for metals with similar ionic radii and coordination geometry (Sunda and Huntsman, 1998). Copper and zinc have similar ionic radii and both bind strongly to oxygen- and nitrogen-containing ligands. Therefore, it is possible that the uptake and bioavailability of copper could also influence zinc uptake and toxicity in algal cells. This study found that tropical *Nitzschia closterium,* pre-acclimated to medium with added copper, did not have increased co-tolerance to zinc when compared to algae cultured in medium with no added copper. Similarly, *Chlorella* sp. 12 did not show increased zinc tolerance in the copper pre-acclimated culture compared to the non-acclimated baseline culture.

CONCLUSIONS

The findings that the tolerance of tropical *Nitzschia closterium* and *Chlorella* sp. to copper and zinc generally did not depend on concentrations of copper in the pre-exposure culture medium, suggests that continued culturing of these species in low metal medium does not influence their response to copper or zinc in algal growth inhibition bioassays in minimal media. Such culture media, used as standard test media in Australasia, does not appear to be metal deficient, as growth rates of the copper pre-acclimated and non-acclimated cultures in 72-h bioassays were similar. Together, this suggests that standard bioassays with these two species do not over-estimate copper or zinc bioavailability and toxicity in natural waters compared to bioassays undertaken with algae pre-cultured in metal-replete media. This study supports the continued use of algal bioassays for determining bioavailability, toxicity and hazard/risk of metals in aquatic environments at environmentally realistic metal concentrations.

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498

Table 1. Effect of copper on growth rate over 72 h of tropical *Nitzschia closterium* grown prior to the bioassay in baseline (no added copper) and copper-supplemented media (Parenthesis are 95% confidence intervals).

Culture	Acclimation Period (days)	IC_x (µg L^{-1})			NOEC (µg L^{-1})	LOEC (µg L^{-1})
		IC_{50}	IC_{25}	IC_{10}		
Baseline Mean ± 1 SD[a]	0 - 154	40 ± 4	21 ± 3	11 ± 4	10[c]	29[c]
+5Cu	47	43 (39 – 48)	15 (10 – 24)	5 (1 – 12)	5	9
	62	51 (20 – 71)	17 (10 – 23)	9 (0 – 16)	5	11
	88	23 (17 – 33)	10 (7 – 18)	6 (2 – 9)	6	12
	143	44 (27 – 66)	25 (18 – 57)	14 (10 – 20)	16	39
	154	62 (-)	16 (0 – 49)	10 (0 – 20)	6	17
	196	33 (0 – 37)	11 (9 – 18)	8 (7 – 10)	7	14
	Mean ± 1 SD[b]	43 ± 13	16 ± 5	9 ± 3	7[c]	17[c]
+25Cu	35	12 (3 – 36)	3 (0 – 30)	1 (0 – 4)	3	16
	168	27 (14 – 58)	13 (9 – 16)	8 (1 – 12)	7	19

[a] Mean ± one standard deviation of four IC_x values from four baseline toxicity tests
[b] Mean ± one standard deviation of six IC_x values from six toxicity tests over 196 days
[c] Geometric mean

506 **Table 2. Effect of copper on the growth rate over 72 h of *Chlorella* sp. grown prior to the**
507 **bioassay in baseline (no added copper) and copper-supplemented media (Parentheses are**
508 **95% confidence intervals).**

Test	Acclimation Period (days)	IC_x (µg L^{-1})			NOEC (µg L^{-1})	LOEC (µg L^{-1})
		IC_{50}	IC_{25}	IC_{10}		
Baseline Mean ± 1 SD[a]	0 - 104	7.3 ± 1.5	5.6 ± 0.9	4.7 ± 0.6	4.2[c]	7.9[c]
+2Cu	12	6.8 (5.8 – 8.0)	5.0 (4.0 – 6.0)	2.5 (0.7 – 6.6)	4.3	8.7
	19	11.1 (10.3 – 12.6)	9.8 (9.0 – 10.7)	9.1 (0 – 9.6)	8.8	12.4
	54	7.4 (5.7 – 9.2)	5.6 (4.9 – 7.2)	4.7 (4.4 – 5.7)	4.3	6.7
	82	6.8 (6.2 – 7.7)	5.4 (4.8 – 5.9)	4.6 (4.0 – 4.9)	4.1	7.8
	104	7.2 (4.0 – 11.7)	5.2 (3.6 – 11.9)	4.0 (3.3 – 11.9)	3.4	7.4
	Mean ± 1 SD[b]	7.9 ± 1.8	6.2 ± 2.0	5.0 ± 2.5	5.1[c]	8.7[c]

509 [a] Mean ± one standard deviation of three IC_x values from three baseline toxicity tests
510 [b] Mean ± one standard deviation of five IC_x values from five toxicity tests over 104 days
511 [c] Geometric mean

512 **Table 3. Intracellular and extracellular copper concentrations for baseline and copper pre-acclimated *N. closterium*[a] and**
513 ***Chlorella* sp.[b] cultures.**

Culture	Copper	Intracellular copper		Extracellular copper		Extra : Intra Cu ratio	Growth inhibition
	($\mu g\ L^{-1}$)	per cell ($\times 10^{-6}$ ng/cell)	per cell volume ($\times 10^{-8}$ ng/μm^3)	per cell ($\times 10^{-6}$ ng/cell)	per cell surface area ($\times 10^{-8}$ ng/μm^2)	per cell/ per surface area-vol	(%)
N. closterium							
Baseline	Control	$\leq$ 0.5[c]	$\leq$ 0.04	$\leq$ 0.4	$\leq$ 0.05	0.8/1.25	-
+5Cu	Control	$\leq$ 0.5	$\leq$ 0.04	$\leq$ 0.4	$\leq$ 0.05	0.8/1.25	-
Baseline	40	80 ± 36	10 ± 4	160 ± 100	28 ± 17	2.0/3.6	76
+5Cu	40	52 ± 9	6.7 ± 1.2	56 ± 2	9.6 ± 3.5	1.2/1.9	81
***Chlorella* sp.**							
Baseline	Control	$\leq$ 0.5[a]	$\leq$ 1.0	$\leq$ 0.5	$\leq$ 0.7	1/1.4	0
+2Cu	Control	$\leq$ 1.0	$\leq$ 3.0	$\leq$ 0.8	$\leq$ 1.5	0.8/0.5	0
Baseline	8.5	37 ± 10	66 ± 17	15 ± 4	21 ± 7	0.40/0.32	87
+2Cu	8.5	26 ± 4	47 ± 8	9.6 ± 2.9	14 ± 4	0.38/0.29	80

514 [a] Measurements were made after 156 days copper acclimation, followed by a 72-h exposure to 40 µg Cu L^{-1} in standard growth inhibition tests. (Parenthesis
515 represent ± one SD)

516 [b] Measurements were made after an 82-day acclimation, followed by a 72-h exposure to 8.5 µg Cu L^{-1} in standard growth inhibition tests. Each value represents
517 mean ± one SD (n= 3 for baseline, n = 2 for +2Cu).

518 [c] Detection limits based on instrument detection limitations and measured cell density.
519

520 **Table 4. Effect of zinc on the growth rate of tropical *Nitzschia closterium* (non-acclimated**
521 **and copper pre-acclimated cultures) over 72 h. (Parentheses are 95% confidence intervals)**

Culture	Acclimation Period (days)	IC_x (µg L^{-1})			NOEC (µg L^{-1})	LOEC (µg L^{-1})
		IC_{50}	IC_{25}	IC_{10}		
Baseline Mean[a]	0 - 196	226 ± 105	142 ± 79	84 ± 64	97[c]	152[c]
+5Cu	47	364 (285 – 599)	176 (137 – 214)	31 (21 – 47)	120	265
	62	275 (204 – 324)	146 (22 – 244)	42 (15 – 102)	66	156
	88	203 (163 – 339)	105 (36 – 123)	48 (14 – 114)	77	164
	143	269 (153 – 331)	93 (48 – 306)	37 (19 – 35)	<99	99
	196	254 (242 – 271)	191 (172 – 217)	102 (35 – 141)	<89	89
	Mean[b]	273 ± 58	142 ± 43	52 ± 29	85[c]	143[c]
+25Cu	35	186 (126 – 232)	116 (0 – 199)	67 (0 – 142)	62	145
	168	194 (112 – 312)	121 (87 – 160)	87 (28 – 119)	77	172

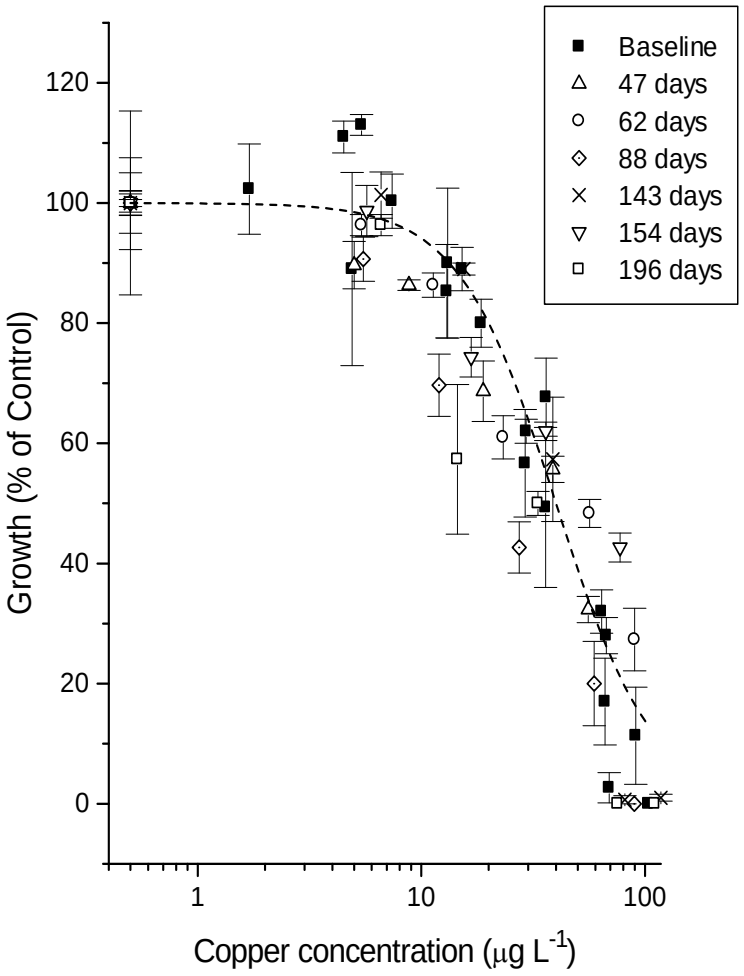
522 [a] Mean ± one standard deviation of four IC_x values from four baseline toxicity tests
523 [b] Mean ± one standard deviation of five IC_x values from five toxicity tests over 196 days
524 [c] Geometric mean
525

526 **Table 5. The effect of zinc on the growth rate of *Chlorella* sp. cultures (non-acclimated and**
527 **copper pre-acclimated cultures) over 72 h. (Parentheses are 95% confidence intervals)**

Test	Acclimation Period (days)	IC_x (µg L^{-1})			LOEC (µg L^{-1})	NOEC (µg L^{-1})
		IC_{50}	IC_{25}	IC_{10}		
Baseline	Mean ± 1 SD [a]	110 ± 41	61 ± 35	28 ± 21	20[c]	44[c]
+2Cu	12	110 (86 – 144)	21 (1 – 84)	6 (3 – 12)	<14	14
	19	115 (98 – 127)	77 (41 – 109)	39 (3 – 63)	27	86
	54	140 (91 – 182)	85 (57 – 109)	22 (1 – 35)	32	84
	82	98 (77 – 111)	63 (37 - 1100	31 (15 – 51)	32	80
	Mean ± 1 SD [b]	115 ± 18	62 ± 29	24 ± 14	30[c]	53[c]

528 [a] Mean ± one standard deviation of IC_x values (n=5 for IC50, n=4 for IC25 and IC10)
529 [b] Mean ± one standard deviation of four IC_x values from four toxicity tests over 82 days
530 [c] Geometric mean
531
532

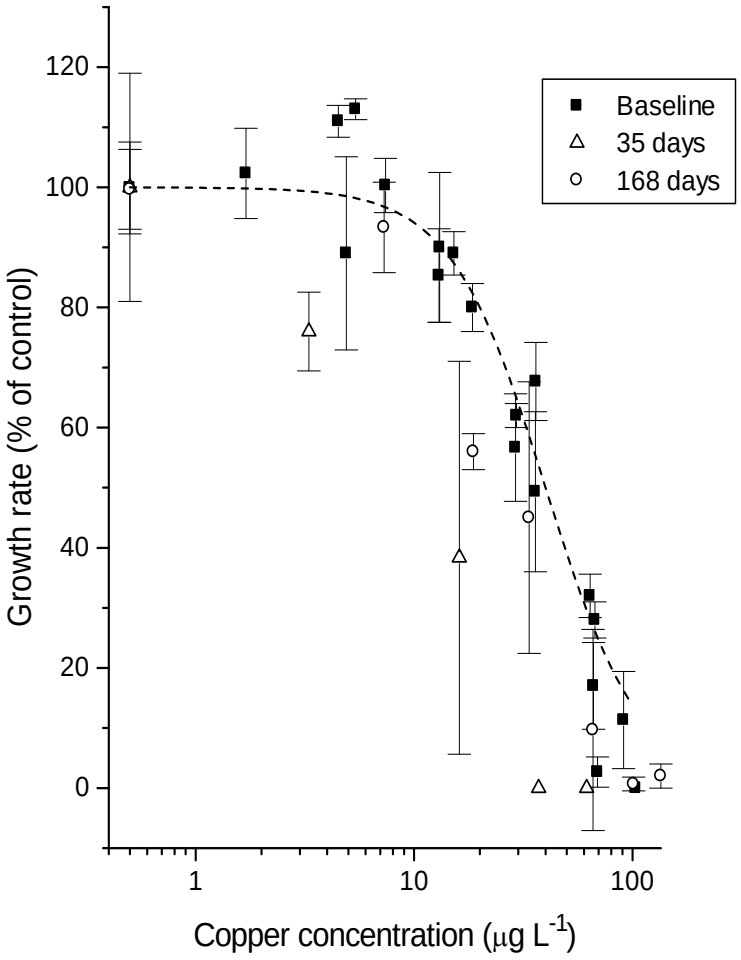
533



534

535 Figure 1a: The effect of 72-h copper exposure on the growth rate of *Nitzschia closterium* pre-
536 cultured in a +5 μg L^{-1} copper-supplemented medium for up to 196 days, compared to a standard
537 baseline culture (no copper added).

538



539
540 Figure 1b. The effect of 72-h copper exposure on the growth rate of *Nitzschia closterium* pre-
541 cultured in a +25 μg L^{-1} copper-supplemented medium for up to 168 days, compared to standard
542 baseline culture (no copper added)
543

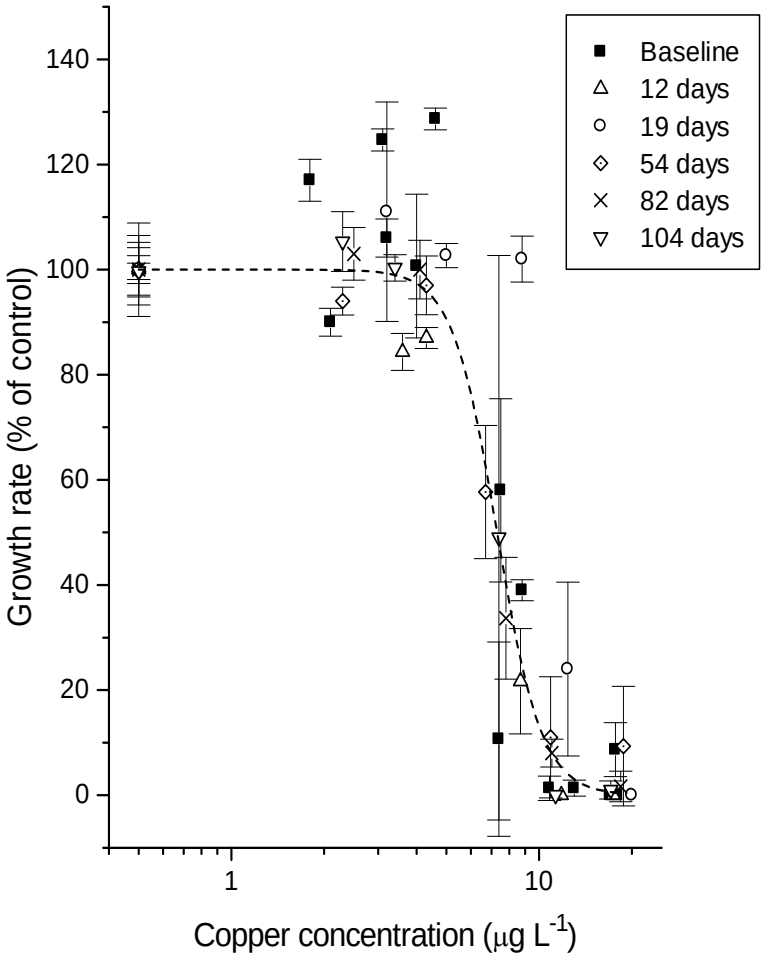


Figure 2. The effect of 72-h copper exposure on the growth rate of *Chlorella sp.* pre-cultured in a +2 μg L^{-1} copper-supplemented medium for up to 104 days, in comparison to a standard baseline culture (no copper added).

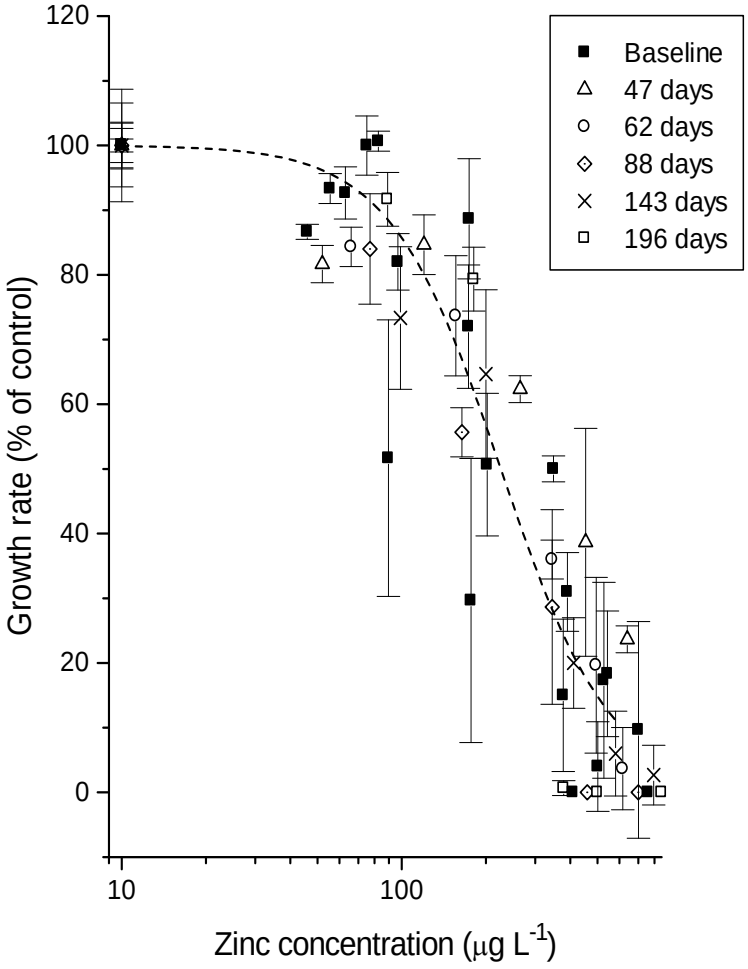
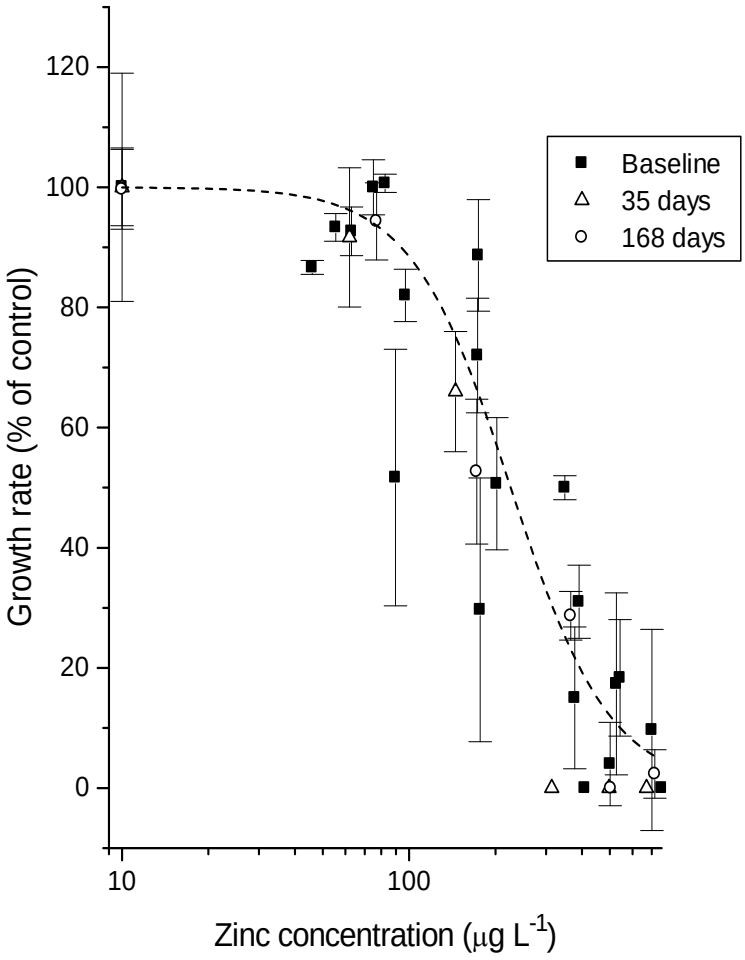


Figure 3. The effect of 72-h zinc exposure on the growth rate of *Nitzschia closterium* pre-cultured in a +5 μg L^{-1} copper-supplemented medium for up to 196 days, in comparison to the standard baseline culture (no copper added).



556
557 Figure 4. The effect of 72-h zinc exposure on the growth rate of *Nitzschia closterium* pre-
558 cultured in a +25 μg L^{-1} copper-supplemented medium for up to 168 days, in comparison to the
559 standard baseline culture (no copper added).
560

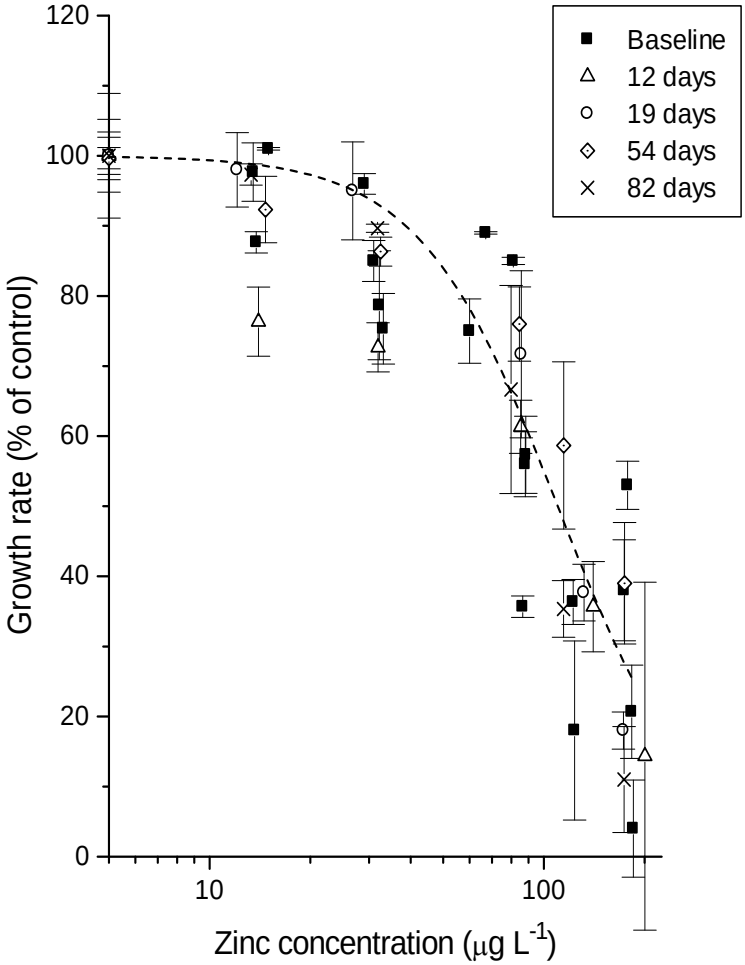


Figure 5. The effect of 72-h zinc exposure on the growth rate of *Chlorella sp.* pre-cultured in a +2 μg L^{-1} copper-supplemented medium for up to 82 days, in comparison to a standard baseline culture (no copper added).