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Time-averaged copper concentrations from continuous exposures predicts pulsed exposure toxicity to the marine diatom, Phaeodactylum tricornutum: importance of uptake and elimination

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Abstract

Intermittent, fluctuating and pulsed contaminant discharges result in organisms receiving highly variable contaminant exposures. Current water quality guidelines are predominantly derived using data from continuous exposure toxicity tests, and most frequently applied by regulators with the assumption that concentrations from a single sampling event will provide a meaningful approach to assessing potential effects. This study investigated the effect of single and multiple (daily) dissolved copper pulses on the marine diatom, Phaeodactylum tricornutum, including measurements of copper uptake and elimination to investigate the toxic mechanism. Copper pulses of between 0.5 and 24 h and continuous exposures with equivalent 72-h time-averaged concentrations (TACs) resulted in similar biomass inhibition of P. tricornutum, with continuous exposures often being marginally more toxic. Rates of cell division generally recovered to control levels within 24 h of the copper pulse removal. Upon resuspension in clean seawater, the extracellular copper per cell decreased rapidly, whereas the intracellular copper per cell decreased slowly. Negligible loss of copper from the total algal biomass indicated that P. tricornutum did not have an effective mechanism for eliminating copper from cells, rather the intracellular copper decreased as a result of dilution by cellular division as the algal growth rate recovered. The measurement of copper uptake after 72-h exposure and kinetics of elimination thereafter suggest that continuous exposures are marginally more toxic to P. tricornutum than pulsed copper exposures with equivalent TACs because slow internalization and saturation of algal membrane transport sites results in less copper uptake into pulse-exposed cells than continuously-exposed cells coupled with dilution of internalized copper via cellular division in the post-exposure period. In the case of P. tricornutum, the results indicate that water quality guidelines for copper based on continuous exposure will be conservative when applied to short-term discharges.

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Time-averaged copper concentrations from continuous exposures predicts pulsed exposure toxicity to the marine diatom, *Phaeodactylum tricornutum*: Importance of uptake and elimination

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ABSTRACT

Intermittent, fluctuating and pulsed contaminant discharges result in organisms receiving highly variable contaminant exposures. Current water quality guidelines are predominantly derived using data from continuous exposure toxicity tests, and most frequently applied by regulators with the assumption that concentrations from a single sampling event will provide a meaningful approach to assessing potential effects. This study investigated the effect of single and multiple (daily) dissolved copper pulses on the marine diatom, *Phaeodactylum tricornutum,* including measurements of copper uptake and elimination to investigate the toxic mechanism. Copper pulses of between 0.5 and 24 h and continuous exposures with equivalent 72-h time-averaged concentrations (TACs) resulted in similar biomass inhibition of *P. tricornutum*, with continuous exposures often being marginally more toxic. Rates of cell division generally recovered to control levels within 24 h of the copper pulse removal. Upon resuspension in clean seawater, the extracellular copper per cell decreased rapidly, whereas the intracellular copper per cell decreased slowly. Negligible loss of copper from the total algal biomass indicated that *P. tricornutum* did not have an effective mechanism for eliminating copper from cells, rather the intracellular copper decreased as a result of dilution by cellular division as the algal growth rate recovered. The measurement of copper uptake after 72-h exposure and kinetics of elimination thereafter suggest that continuous exposures are marginally more toxic to *P. tricornutum* than pulsed copper exposures with equivalent TACs because slow internalisation and saturation of algal membrane transport sites results in less copper uptake into pulse-exposed cells than continuously-exposed cells coupled with dilution of internalised copper via cellular division in the post-exposure period. In the case of *P. tricornutum*, the results indicate that water quality guidelines for copper based on continuous exposure will be conservative when applied to short-term discharges.

1. INTRODUCTION

Waterways adjacent to industrialised catchments are likely to receive intermittent toxicant inputs from urban stormwater run-off, and industrial effluent discharges resulting in significant temporal fluctuations in toxicant concentrations, referred to here as pulsed exposures. Most water quality guidelines (WQGs) for contaminants and calculations of safe dilution levels for industrial effluents are derived using no-effect concentration data (e.g. NOEC, EC10) from toxicity tests that employ continuous exposure to a range of toxicant concentrations for pre-determined test durations (ANZECC/ARMCANZ, 2000, Warne et al., 2013). The regulation of industries that undertake controlled but non-continuous discharges is likely to be improved by use of guidelines that consider the magnitude, duration and frequency of toxicant exposure and recovery post-exposure.

The effects of pulsed exposure to organic contaminants such as insecticides and pesticides have been the most studied class of contaminants historically, with fewer and more recent studies of metal contaminants (Diamond et al., 2005; Hoang et al., 2007; Holdway et al., 1994; Naddy et al., 2000; Schulz and Liess, 2000). The observed responses to and relationships between pulse concentration, duration and recovery time in toxicant-free media have been mixed, with the toxic responses varying for different types of contaminants and exposure scenarios, biological species and stage of their life cycle. A few studies have found that for different exposure scenarios with an equivalent contaminant dose, longer exposure durations resulted in greater toxicity. This was observed for survival of *Pimephales promelas* exposed to cadmium and copper (Diamond et al., 2005), survival and reproduction of *Daphnia magna* exposed to zinc (Diamond et al., 2006), reproduction of *D. magna* exposed to fenoxycarb (Hosmer et al., 1998) and immobilisation of *Chironomus riparius* exposed to carbamate toxicants (Kallander et al., 1997). In those studies the greater toxicity was attributed to either a longer duration for toxicant accumulation or the lack of an opportunity to recover in toxicant-free media. However, many studies have reported the opposite, with shorter duration pulsed exposures of higher concentrations causing greater toxicity than longer exposures of lower concentrations because the higher concentrations that achieve an equivalent dose overwhelm the test organisms for even short exposures. For exposures scenarios with equivalent doses, shorter exposures of higher concentrations were more toxic to survival of *D. magna* exposed to arsenic and copper (Hoang et al., 2007), survival of *Daphnia pulex* exposed to copper (Ingersoll and Winner, 1982), survival of *Melanotaenia fluviatilis* exposed to fenvalerate (Holdway et al., 1994), and growth (body mass) of *Limnephilus lunatus* exposed to fenvalerate (Schulz and Liess, 2000). Several studies have also reported no difference in toxicity to organisms caused by continuous and pulsed exposures when expressing effects based on time-averaged dose, such as survival of *Melita plumulosa* and *D. magna* exposed to dissolved copper (Angel et al., 2010) and selenium (Hoang et al., 2007), respectively.

Overall, the effects of fluctuating concentrations of many toxicants on biota still remains poorly quantified, and determining how to apply this information when deriving WQGs or checking compliance against WQGs requires further research. There is also a lack of research on the chronic effects of pulsed exposures, such as to microalgae, which are critical to ecosystems because they reside at the bottom of the food chain. The toxic effects of continuous copper exposures have been well documented, with many species of microalgae particularly sensitive to copper due to their large surface area and the fact that their cell membranes are in direct contact with the exposure medium (Stauber et al., 2000; Stauber and Davies, 2000). The relative sensitivity of different algal species to copper exposure depends on the interaction of copper at the surface of the cell and subsequent internalisation, interaction with specific binding sites, and intracellular detoxification or elimination from the cell (Knauer et al., 1997a; Morelli and Scarano, 2001; Stauber and Davies, 2000). However, virtually no information on the toxicity of pulsed exposures on microalgae exists, despite such exposures being expected to occur frequently in the field from anthropogenic sources such as effluent discharges, algicide and pesticide applications.

This study investigated the effects of fluctuating dissolved copper concentrations on the growth of the copper-sensitive marine alga, *Phaeodactylum tricornutum*. The effects of continuous and pulsed exposures of copper on algal growth, and recovery following pulses were quantified and contrasted. The uptake of copper into *P. tricornutum* cells following exposure and the kinetics of copper elimination from cells in the post-exposure period was measured to investigate the mechanism of continuous and pulsed copper toxicity. For this study, the implications of the results for the ability of current water quality guidelines to protect against toxicity and predict toxicity from pulsed exposures is discussed.

2. METHODS

2.1. General analytical

All glass and plasticware for chemical analyses and toxicity testing were cleaned by soaking in 10% (v/v) HNO_3 (BDH, Analytical Reagent grade) for a minimum of 24 h followed by thorough rinsing with deionised water (Milli-Q, 18 MΩ·cm; Academic Water System). Measurements of water pH were made using a Wissenschaftlich-Technische Werkstattan (WTW) meter equipped with a pH probe (Orion sure-flow combination pH 9165BN) and calibrated against pH 4.0 and 7.0 buffers (Orion Pacific, Sydney, NSW, Australia). Temperature and salinity were made using a LF 320 WTW conductivity meter (Weilheim, Germany) and electrode (TetraCon 325, WTW). The meters were calibrated and used according to manufacturer's instructions.

2.2. Algal cultures and bioassay preparation

The marine diatom, *Phaeodactylum tricornutum* (Bohlin) was originally obtained from the Collection of Living Microalgae, Hobart, Australia. It was chosen because it is sensitive to copper (72-h growth rate IC50 = 10 ± 4 µg/L), it is easy to count, and does not clump or adsorb to the walls of the test containers (Angel et al., 2013; Levy et al., 2008). All cultures were maintained in f_2 growth medium (half-strength f medium) at 21±2ºC on a 12:12 h light: dark cycle (Philips TL 40 W fluorescence daylight, 72 µmol photons/m/s) (Franklin et al., 2001). Cultures were frequently checked microscopically for the presence of bacteria after incubation of a streaked sub-sample on an agar plate (2% Bacto agar, 0.1% pepsin, and 0.1% yeast; Oxoid) in the dark. If no colonies were present and bacteria were not observed microscopically, these cultures were deemed axenic.

The various bioassay protocols were based on the OECD Guideline 201 (2005) and Stauber et al*.,* (1994). Sub-cultures of *P. tricornutum* cells in the exponential phase of growth (3- to 6-d old) were washed three times in 0.45 µm filtered clean seawater (Port Hacking, Sydney, Australia) by centrifuging (Spintron GT-175BR, 7 mins × 700 g) before inoculation (Franklin et al., 2001). Cells were inoculated into 250 mL borosilicate glass Erlenmeyer flasks (silanised with Coatasil to reduce metal adsorption) containing 50 or 100 mL of clean filtered seawater supplemented with 15 mg NO_3^-/L and 1.5 mg PO_4^{3-}/L. Relatively low initial cell densities of 5 (±0.5) $\times 10^3$ cells/mL were used to better reflect those found in aquatic systems and to minimise copper losses through adsorption to algal cell surfaces during the bioassay (Franklin et al., 2002). The flasks were capped loosely with glass lids, incubated for 72 h on a 12:12 h light: dark cycle at 21±1°C, shaken twice daily to prevent CO_2 limitation, and were positioned randomly in test cabinets daily to ensure equivalent illumination. Test results were considered acceptable if control mean growth rates were 1.5±0.5 doublings per day, and the coefficient of variation in the controls (CV) was <20% (Stauber et al., 1994). The pH of test solutions was measured at the start and end (72 h) of tests to confirm increases in pH did not occur, which would indicate dissolved CO_2 had decreased and possibly become limited.

Algal cell densities were measured using a flow cytometer (FACSCalibur, Becton Dickinson, Biosciences) calibrated using TruCount fluorescent beads (BD Trucount TM tubes, BD Biosciences) and gates in chlorophyll *a* fluorescence (Franklin et al*.,* 2001). The inhibition of biomass yield for treatments was expressed as a percentage of the control biomass yield and was calculated using equation 1.

$$I = 100 - (100 \times (R_C - R_T) / R_C) \qquad \text{EQUATION 1}$$

where, I was the inhibition of biomass expressed as a percentage of the control biomass yield, R_C was the mean control biomass yield, and R_T was the biomass yield of each treatment replicate.

2.3. Pulsed exposure-effect bioassays

Dissolved copper pulses were generated by spiking seawater from stock solutions (5 and 100 mg/L) prepared from copper sulfate ($CuSO_4.5H_2O$, AR grade, APS Finechem, Sydney, Australia) and terminated by replacing 99% of copper-spiked water with clean seawater (Angel et al., 2010). Removing copper-spiked water involved centrifuging test solutions (50 ml Cellstar, Greiner; Spintron GT-175BR, 1400 g × 4 min) to create an algal pellet, followed by careful withdrawal of the supernatant so that only 0.5 mL remained above the algal pellet. Algal pellets were resuspended (vortex) in clean seawater (containing 15 mg NO_3^-/L and 1.5 mg PO_4^{3-}/L) equivalent to test starting volume and replaced into clean test flasks. Preliminary tests indicated that the centrifugation of *P. tricornutum* cells did not negatively impact the rate of growth, but some cells were lost during the removal of supernatant. Therefore, the cell densities were measured before and after replacement of test solution to account for any losses.

A range of exposure scenarios were designed to investigate the toxicity from pulsed copper exposures in chronic 72-h bioassays using inhibition of biomass yield as the effect endpoint (Table 1). The 72-h biomass yield inhibition was compared to the time averaged concentration (TAC) of dissolved copper (Angel et al., 2010), calculated according to equation 2.

$$TAC = ((P_C \times P_D) + R_C \times (72 - P_D)) / 72 \qquad \text{EQUATION 2}$$

where, P_C was the mean measured dissolved copper pulse concentration (µg/L), P_D was the pulse duration (h), and R_C was the recovery concentration (µg/L) calculated as the mean measured dissolved copper concentration between pulses (i.e. RC ~1% of pulse concentration). The P_C was calculated by assuming a linear decrease in dissolved copper between the measured concentrations at the start and end of the copper pulse and taking the average over this duration, (Angel et al., 2010). Similarly, R_C was calculated by assuming a linear change between dissolved copper, measured immediately after water replacement and immediately before the subsequent pulse or end of bioassay, and taking the average.

The experiments investigated the effects of concentration and duration of multiple pulsed exposures, the inhibition and recovery of algal growth resulting from single pulsed exposure, and comparisons of toxicity from different exposure scenarios with equivalent time-averaged concentrations (TACs) of dissolved copper (Table 1, Figures S1, S2 and S3 of the Supplementary Information). The tests employed nominal concentrations in the range 10 - 356 µg Cu/L for durations of 0.5 - 72 h (continuous exposure), where measurements of dissolved copper allowed calculation of time-averaged exposure concentrations. For tests investigating equivalent exposures, the dissolved copper TACs were designed to be approximately the 72-h growth rate IC50 (50% inhibition concentration). Biomass yield inhibition was used to assess toxicity rather than growth rate inhibition

because pulsed exposures resulted in variable growth rates over the 72-h tests. Biomass yield inhibitions were greater than growth rate inhibitions due to the exponential growth of control algae (Nyholm, 1985).

Controls and continuous exposure treatments were subjected to the same centrifugation and water replacement procedure so that all treatments received the same handling; copper was re-spiked into continuous exposures after water replacement. All exposure bioassays included reference treatments exposed continuously for 72 h to a range of dissolved copper concentrations to ensure the algal sensitivity to copper was similar between tests and for determination of the 72-h biomass yield IC50.

2.4. Measurement of intracellular and extracellular copper in Phaeodactylum tricornutum

The intra- and extra-cellular copper was measured in *P. tricornutum* after 72-h continuous or pulsed exposures and over 72 h post-exposure using the method described by Levy et al. (2008). The copper bound to the surface of the cell (extracellular copper) was operationally defined as the copper extracted by EDTA (0.01 M for 20 min). The copper internalised within algal cells (intracellular copper) was operationally defined as the copper remaining after the removal of extracellular copper. Three replicates were measured for each treatment time-point. At each time-point, cell densities were measured and test assays were centrifuged (1400 g × 4 min). The supernatant was then filtered (0.45 µm) for analysis of dissolved copper, and the cell pellet extracted for extracellular copper and the remaining pellet digested with nitric acid to obtain the intracellular copper.

For continuous exposures, *P. tricornutum* cells were exposed to 0, 2, 3, 5, 10, 20, 30 and 50 µg Cu/L for 72 h followed by measurement of intra- and extra-cellular copper. For uptake from pulsed exposures, *P. tricornutum* cells were exposed to 1, 4 and 8-h daily pulses and continuously (as per Table 1) followed by measurement of intra- and extra-cellular copper after 72 h. For copper elimination kinetics studies, *P. tricornutum* cells were continuously exposed to a nominal dissolved copper concentration of 15 µg/L (shown in preliminary tests to give reproducible uptake without residual copper after water replacement) for 72 h, followed by water replacement with clean seawater (as per pulsed exposures) and measurement of extra- and intra-cellular copper at 0, 0.5, 1, 2, 4, 10, 20, 30, 48, and 72 h.

2.5. Copper analyses

Dissolved copper (<0.45 µm) in test solutions was measured following filtration (acid-washed 0.45-µm cellulose nitrate filters, 25 mm, Sartorius Minisart), and acidification to 0.2% (v/v) (Tracepur, Merck) (Angel et al., 2013). The dissolved copper was determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES, Spectroflame EOP, Spectro Analytical Instruments) calibrated

with multi-element matrix-matched standards (QCD analysts and Plasma Chem Corp), and results were only accepted when analytical drift was less than 10%.

2.6. Statistical analyses

The dissolved copper concentrations that resulted in the IC50 were calculated using linear interpolation (ToxCalc Version 5.0.23C, Tidepool Software). To compare the effect of TACs from continuous and pulsed exposures, a log−logistic concentration−response model was applied to the inhibition data obtained from twelve continuous exposure tests run to monitor sensitivity during pulsed exposure tests. Differences in the mean percentages of 72-h biomass yield inhibition from different pulsed and continuous exposure treatments were examined using a one-factor analysis of variance for tests comparing equivalent time-averaged exposures and a two-factor analysis of variance for comparing the effects of exposure concentration and duration (ANOVA) (NCSS, Kaysville, UT). A repeated measure of means test was employed to test for significant differences in cell division rates over successive 24 h intervals in tests investigating recovery from single pulsed exposures. Kurtosis, Omnibus, and Levene tests were used to test for normality and homogeneity of variances. In cases where these assumptions were violated, appropriate transformations were performed (Sokal and Rolf, 1995). The Tukey-Kramer multiple-comparisons test was used as a post-hoc procedure to test for multiple comparisons upon means (NCSS).

3. RESULTS AND DISCUSSION

During the 72-h toxicity tests, the pH ranged from 8.0 to 8.6. The dissolved copper concentration decreased, on average, by 16% during the course of pulsed or continuous exposures, with the lowest concentration treatments decreasing by as much as 45% over 72 h. Copper losses from solution were attributed to a combination of adsorption onto the test vessel, algal surfaces, and possibly complexation with algal exudates excreted into the test solutions.

The relationship between algal biomass as a percentage of controls and the time-averaged concentration (TAC) of dissolved copper measured in twelve continuous exposure tests is shown in Figure 1 (separate tests undertaken over a 6-month period). The algal biomass as a percentage of controls decreased as the duration of continuous exposure increased from 24 to 72 h (Figure 1A). From this dataset, the dissolved copper TACs that resulted in algal biomass inhibition of 50% relative to controls (IC50) for the 24-, 48-, and 72-h durations were 6.0 (5.2-7.1), 4.8 (4.3-5.4) and 2.6 (2.2-3.2) µg/L, respectively. This compared to IC50s based on nominal concentrations of 7.5 (6.7-8.3), 6.3 (5.7-6.9) and 3.4 (2.8-4.1) µg/L for 24-, 48- and 72 h, respectively.

The relationship between 72-h algal biomass and dissolved copper (nominal and TACs) are shown for the twelve tests in Figure 1B. The biomass yield IC50 for individual tests calculated using measured dissolved copper TACs varied in the range 1.5-4.1 µg/L. When the data from the twelve tests were pooled, the 72-h mean IC50 and IC20 (20% inhibition relative to control) (95% confidence interval) values were 2.6 (2.2-3.2) and 0.7 (0.6-1.1), respectively. The biomass yield IC50 determined using TAC data was approximately 74% of the value determined using nominal concentrations and reflects the loss of dissolved copper over the exposure duration due to adsorption onto test vessels and/or algal surfaces.

3.1. Effect of pulse duration

The effect of pulse duration and concentration was investigated by exposing the algae to daily pulsed exposures (i.e. 3 pulses during the 72-h test period) of 30, 100, 200 and 600 µg Cu/L (Table 1 and Figure 2A). Measured concentration profiles are shown in Figure S1 (Supplementary Information). There was a significant ($p<0.05$) interaction between exposure concentration and duration with the algal biomass inhibition increasing as either was increased. Algal biomass inhibition was not necessarily significantly different between successive increments in concentration or duration, but there was a significant ($p<0.05$) difference over the range tested. For the 100, 200 and 600 µg Cu/L pulse exposures there was significant residual dissolved copper between pulses that could potentially contribute towards toxicity. While this only represented approximately 1% of the copper pulse concentration, it was significant because of the high sensitivity of the algal species, i.e.

the inter-pulse copper concentrations could be expected to exert significant biomass inhibition (Franklin et al., 2001). The inclusion of this dissolved copper in the TAC calculation more accurately accounted for the toxicity. The relatively short pulsed exposures (0.5 to 4 h) coupled with residual copper between pulsed exposures consistently elicited significant biomass inhibition. The calculated TACs for pulsed exposures generally provided a good prediction of the observed algal biomass inhibition with similar inhibitions between continuous and pulsed exposures for similar TACs (Figure 2B, Supplementary Figure S4).

3.2. Growth rate recovery following single 24-h pulses

In this study, the rate of algal cell division was compared over successive 24-h periods for both pulsed and continuous exposures which had near-equivalent TACs: single 30 µg/L 24-h pulsed and 10 µg/L continuous (72-h) exposure (nominal) (Table 1 and Figure 3). A profile of each exposure scenario using measured concentrations is shown in Figure S2 (Supplementary Information). The control treatments had a mean cell division rate of 1.6±0.1 doublings/day over the duration of bioassay. All pulsed treatments had significantly ($p<0.05$) greater inhibition of cell division rates during exposure than before or after exposure. For the 30 µg/L 0-24 h pulse, the mean cell division rate during 0-24, 24-48 and 48-72 h were 0.7±0.1, 1.4±0.1 and 1.9±0.2 doublings/day, respectively, indicating recovery of *P. tricornutum* growth rate to marginally less ($p<0.05$) than control levels in the 24-48 h period and significantly ($p<0.05$) greater than control levels in the 48-72 h period. Treatments exposed to 30 µg/L copper for 24 h during the 24-48 and 48-72 h stages had significantly lower ($p<0.05$) 72-h biomass inhibition than that exposed in the 0-24 h stage, probably because they had more time to grow exponentially before being exposed to copper. The 10 µg/L continuous exposure resulted in significantly ($p<0.05$) greater 72-h biomass inhibition than any of the 24-h 30 µg/L pulse exposures despite having a marginally lower TAC.

3.3. The effect of pulse concentration and duration (with equivalent dissolved copper TACs) on biomass yield inhibition

The predictive nature of using TACs is demonstrated for a range of exposures of different copper pulse concentrations and durations with equivalent nominal TACs in Table 1 and Figure 2B. A profile of each exposure scenario using measured concentrations is shown in Figure S3 (Supplementary Information). The dissolved copper TACs were between 8.7 and 11.2 µg/L, with the lowest value determined for the continuous exposure and the highest value recorded for the 1 h pulsed exposure. There was a significant ($p<0.05$) difference between 72-h algal biomass inhibition of

treatments with continuous exposure causing the greatest inhibition (89% inhibition) despite having the lowest TAC (Table 1). The 4- and 8-h pulsed exposures resulted in similar 72-h algal biomass inhibitions (72 and 76% inhibition) that were significantly ($p<0.05$) lower than that of continuous exposure and generally similar to that of the 0.5, 1 and 2 h pulsed exposures (66-69%). The marginally greater toxicity for longer duration treatments indicated that the rates of copper internalisation during exposure and copper elimination and algal recovery during the post-exposure period were likely to be important factors and prompted their measurement in subsequent tests.

3.4. Copper uptake from exposure and elimination post-exposure

Following 72 h exposures up to 50 µg/L, the intra- and extra-cellular copper in *P. tricornutum* cells reached 490 $\times 10^{-16}$ and 1200 $\times 10^{-16}$ g/cell, respectively (Supplementary Information Figure S5A). The algal biomass was inhibited with increasing 72-h intra- and extra-cellular copper concentrations up to 130 and 380 $\times 10^{-16}$ g/cell, respectively, after which inhibition appeared to plateau as intra- and extra-cellular copper increased to 490 and 1200 $\times 10^{-16}$ g/cell, respectively (Supplementary Information Figure S5B and C). The concentrations of extra- and intra-cellular copper after 72 h did not increase significantly ($p>0.05$) for exposures above 20 and 30 µg/L, respectively.

Following 72 h exposure to 15 µg/L dissolved copper the extracellular copper concentration of algae resuspended in clean seawater decreased by 72% after 1 h and was not significantly ($p>0.05$) different to unexposed cells after 8 h (Figure 4A). For these cells, the intracellular copper per cell increased by 24% after 4 h post-exposure before decreasing at a relatively constant rate over the remainder of the post-exposure to negligible levels after 72 h (Figure 4B). The rapid decrease in extracellular copper post-exposure was probably due to copper desorption from the cell surface into clean seawater, as many of the adsorption sites on the surface of algal cells are non-specific and have weak binding affinities with metals (Campbell, 1995; Hassler et al., 2004; Knauer et al., 1997a). The small increase in intracellular copper in the first 4 h post-exposure indicated some internalisation occurred post-exposure, probably from the internalisation of the surface-bound copper that was irreversibly (non-EDTA extractable) bound to transport proteins (Slaveykova and Wilkinson, 2002). Hassler et al*.,* (2004) also found that the concentration of transporter-bound copper was only a small fraction of the total surface-bound metal in the green alga, *Chlorella kesslerii*.

The cell density of *P. tricornutum* cells in solution was inversely related to the intracellular copper ($R^2 \geq 0.98$), decreasing by 21% in the initial 4 h post-exposure (Figure S6 of the Supplementary Information), after which it increased over the remainder of the 72 h (Figure 4B). In the 0-24, 24-48 and 48-72 h post-exposure periods the *P. tricornutum* exhibited a growth rate (mean ± S.D.) of 0.75 ±

0.21, 2.88 ± 0.47 and 0.97 ± 0.21 doublings/day, respectively. This indicated that the algal cells were beginning to recover their normal growth rates in the initial 24 h post-exposure, exhibited higher-than-normal growth rates in the subsequent 24-48 h period, before attaining normal growth rates in the final 48-72 h of the recovery period. As a result of the inverse relationship between intracellular copper per cell and algal cell density, the total intracellular copper in the bioassay population did not decrease over the 72-h post-exposure period (Figure 4C).

3.5. Copper internalisation within pulse-exposed algal cells

The relationship between intracellular copper, algal biomass inhibition and the time-averaged concentration (TAC) of continuous, 1, 4 and 8 h pulsed exposures designed to have equivalent TACs is shown in Figure S7 and Table S1 of the Supplementary Information. Algal biomass inhibition increased linearly with intracellular copper, suggesting copper internalisation led to toxicity. The observed toxicity (algal biomass inhibition) increased in the order of 4-h pulsed exposure < 8-h pulsed exposure < continuous and 1-h pulsed exposure. The TACs of all pulsed exposures were similar (6.4 - 6.5 µg/L), while the continuous exposure TAC (5.5 µg/L) was marginally lower due to greater losses of dissolved copper over the longer exposure period. The residual copper remaining between pulses due to the high concentration of the 1 h pulse resulted in this treatment having higher toxicity than the other pulse exposure treatments.

3.6. Discussion of toxic mechanism

In the uptake tests, after 72-h exposure to dissolved copper concentrations up to 50 µg/L, the extra- and intra-cellular copper concentrations did not increase significantly ($p>0.05$) for dissolved copper concentrations above 20 and 30 µg/L, respectively (Figure S5 of Supplementary information), indicating saturation of copper adsorption sites and transport proteins on the algal cell surface above these concentrations (Campbell, 1995; Sunda and Huntsman, 1998). In a corresponding study by Levy et al. (2008) that investigated the kinetics of copper uptake in *P. tricornutum* over 72 h, a rapid increase in the extracellular copper concentration occurred before it reached a plateau (at each exposure concentration) as exposure duration increased, while the intracellular copper increased linearly with exposure duration but did not plateau. The saturation of extracellular sites on *P. tricornutum* occurred for copper concentrations above 30 µg/L (Figure S8 of the Supplementary Information), which was higher than in the current study. However, that study did not measure

uptake between 10 and 30 µg/L to confirm if saturation occurred in this concentration range (Levy et al., 2008).

In the study by Levy et al. (2008), copper was internalised in *P. tricornutum* much more slowly than it bound at the cell surface (extracellular Cu), which is supported by previous studies that reported metal internalisation as the rate limiting step of metal uptake into microalgae (Hassler et al., 2004; Knauer et al., 1997a; Slaveykova and Wilkinson, 2002). Based on this study, for organisms with slower copper internalisation rates, toxicity thresholds (EC10, EC50) would be predicted to occur at higher TACs for short-duration pulses than for continuous exposures because of inadequate time for copper internalisation to occur before the external exposure is removed. This explains the marginally (sometime significant and sometimes not significant, p=0.05 higher biomass inhibition (greater toxicity) for many continuous exposures relative to pulsed exposures with equivalent TACs (Figure 2B).

Following 72-h exposure to concentrations up to 50 µg/L, the algal population growth was inhibited with increasing 72-h intra- and extra-cellular copper concentrations up to 130 and 380 $\times 10^{-16}$ g/cell, respectively, after which inhibition appeared to plateau as intra- and extra-cellular copper increased to 490 and 1200 $\times 10^{-16}$ g/cell, respectively (Figure S5B and S5C of Supplementary information). These results indicate that even marginal increases in the intracellular copper can elicit severe inhibition of algal cell division. For *P. tricornutum,* the maximum inhibition occurred at ≥ 130 $\times 10^{-16}$ g/cell intracellular copper, approximately a quarter of the maximum intracellular copper measured, indicating that the intracellular uptake sites were not saturated when this response occurred. The observation of a plateau in inhibition as intracellular copper concentrations increased above 130 $\times 10^{-16}$ g/cell indicates that the intracellular uptake sites were not saturated when this response occurred. It is possible that additional binding sites were induced as a response to higher copper concentrations such as by the formation of phytochelatins, starch granules or lipid bodies (Andrade et al., 2004; Morelli and Scarano, 2001; Smith et al., 2014).

In the copper elimination tests, the lack of a decrease in the total intracellular copper in all *P. tricornutum* cells (Figure 4C) indicates this species was not able to efflux internalised copper from cells, as measured previously in the freshwater alga, *Chlorella kesslerii* (Hassler and Wilkinson, 2003) and *Selenastrum capricornutum* (Wolterbeek et al., 1995). The results from this study suggest that the main mechanism by which *P. tricornutum* recovers from copper exposure is by diluting intracellular copper during cellular division. On the assumption that the internalised copper was evenly distributed within the cell, cellular division would be expected to result in half the cellular contents of the parent algae being passed to each daughter cell. Adequate cellular division prevents copper from rising to toxic levels in the cells, and may prevent cellular detoxification mechanisms from becoming overwhelmed. The rapid increase in biomass inhibition and corresponding decrease in

cell division with small increases in intracellular copper (Figure 4B) suggests the ability of the algal cells to dilute copper between daughter cells through cellular division decreases rapidly as exposure concentration increases within a narrow range.

The inability of *P. tricornutum* to efflux copper does not necessarily mean that it has no copper detoxification mechanisms. Detoxification mechanisms that do not involve efflux have been found in algal species including *P. tricornutum*, such as the incorporation of intracellular copper into inert bodies such as phytochelatins, starch granules, lipid droplets, and thiols, as well as the production of extracellular ligands such as exudates that complex copper (Smith et al., 2014; Amiard et al., 2006; Andrade et al., 2004; Morelli and Scarano, 2001. The freshwater alga, *Scenedesmus subspicatus*, *Chlamydomonas tremulans* and *Chlamydomonas clinobasis* were able to maintain normal cellular growth rates for intracellular copper concentrations of 6×10^{-6} g/cell, indicating some species are able to internalise very high concentrations of copper without exhibiting toxicity, probably by using intracellular detoxification (Knauer et al., 1997b). Following 72 h exposure to copper at their respective IC50 values, approximately four times more intracellular copper was measured in the marine alga, *Tetraselmis* sp. than in *P. tricornutum* or *Dunaliella tertiolecta*, suggesting *Tetraselmis* sp. employed intracellular detoxification mechanisms to ameliorate toxicity (Levy et al. 2008). Although *P. tricornutum* has been shown to produce phytochelatins in response to dissolved copper exposure (Smith et al., 2014), the sharp increase in biomass inhibition as intracellular copper increased above approximately 10×10^{-16} g/cell (Figure 4), suggests that *P. tricornutum* was not able to store significant quantities of intracellular copper without experiencing toxic effects. *P. tricornutum* is also a relatively sensitive species to copper exposure (Franklin et al., 2001) meaning detoxification mechanisms may be overwhelmed at relatively low dissolved copper concentrations. Levy et al. (2008) also measured negligible complexation of dissolved copper by algal exudates, ruling out *P. tricornutum* using this form of detoxification.

An interesting result from the single pulsed-exposure test and the copper elimination test was that the growth rate of 'previously exposed' cells suspended in clean sea-water was higher than controls in the 0-48 h post exposure period. One possible reason for this is that cellular division was inhibited whilst other cellular functions were not. The highest ratio of reduced to oxidised glutathione has been observed to occur just before cell division and growth inhibition has been shown to increase as the ratio is lowered (Hare and Schmidt, 1969, Kosower and Kosower 1978, Stauber and Florence, 1987). The marine diatoms *N. closterium* and *P. tricornutum* have also been observed to increase in size when exposed to copper (Levy et al., 2008; Stauber and Florence, 1987). Stauber and Florence (1987) proposed that increases in cell size observed in *N. closterium* occurred because cell division was negatively impacted while carbon fixation was not altered, resulting in cells swelling in size. The results of this study suggest that in addition to increases in *P. tricornutum* cell

size measured by Levy et al. (2008), energy not used during mitotic cycle inhibition is available for higher than normal cell division in the post-exposure period once the ratio of reduced to oxidised glutathione returns to normal levels.

3.7. Accuracy of TAC for predicting toxicity and assessing potential effects in relation to WQGs

The combined relationship between biomass rate inhibition and dissolved copper TACs from the pulsed and continuous exposures is shown in Figures 2B and S4 (Supplementary Information). The TAC of dissolved copper was generally good at predicting toxicity, although there was some variability in the 4-12 µg/L exposure range, which is close to the growth rate IC50 and probably reflects differences in algal sensitivity between tests. No particular pulsed exposure scenario stood out as particularly more or less toxic, however, continuous exposure was consistently marginally (sometimes significant ($p<0.05$)) more toxic than equivalent TAC pulsed exposures and was sometimes more toxic than pulsed exposures that had a higher TAC.

The copper uptake measurements for pulsed exposures (Section 3.5) showed there was little change in the TACs (between 5.5 and 6.5 µg/L) as the intracellular copper increased in the range 75 to 130 $\times 10^{-16}$ g/cell (Figure S7 and Table S1 of the Supplementary Information), indicating that longer exposures at lower concentrations (either continuous or the residual copper from the 1 h pulse) result in greater uptake than shorter exposures at higher concentrations (equivalent TAC). The marginally higher toxicity of continuous exposures than pulsed exposures for equivalent exposures is likely due to lower internalised copper (Figure S7) for pulsed exposures. This was attributed to the degree of copper internalisation for equivalent exposure TACs being higher for continuous exposure because of the longer duration and possibly the saturation of algal membrane transport proteins at the higher concentrations used for pulsed exposures (Figure 4A). For example, tripling the exposure concentration from 10 to 30 µg/L resulted in intra- or extracellular copper increasing by less than 3-fold, so a pulse with an equivalent TAC as continuous exposure would not result in proportional uptake.

The combined results shown in Figure 2B indicate that TACs are generally predictive of toxicity, but there are small differences between different exposure scenarios (pulsed vs continuous) of equivalent exposure TAC. Effect thresholds based on TACs from continuous exposure were conservatively predictive of toxicity from pulsed exposures with equivalent TACs because there was less than proportional internalisation of copper from pulsed exposures. Considering the natural variability that can occur in biological tests, the use of TACs was quite a good predictor of toxicity and the comparison of TACs to WQGs should result in an adequate, but not overly conservative measure of dissolved copper exposure, when applied to short-term discharges.

In applying WQGs to assess the risk of toxicity posed by fluctuating concentrations of metals, the comparison of the TAC with the WQG may be appropriate for many contaminants. However, there remains considerable uncertainty about the application of this approach to some contaminants (e.g. pesticides), and whether is it applicable to all organisms. Furthermore, while better characterisation of concentration-time parameters may improve our ability to predict effects occurring during laboratory based toxicity tests, these tests may not always adequately represent exposure or how organisms respond in the field to fluctuating contaminant concentrations caused by pulsed discharges (Burton et al., 2000; Liber et al., 2007; Ward et al., 2013). Better consideration of toxicokinetics/ toxicodynamics of contaminant exposure and effects through the use of modelling approaches is likely to further improve our understanding of the impact of fluctuating contaminant exposure on aquatic organisms (Ashauer et al., 2006; Ashauer and Brown, 2008; Vogs et al., 2013). Modelling also provides the ability to extrapolate to a wide-range of exposure scenarios that may occur in the field rather than having to test each exposure scenario in the laboratory.

4. CONCLUSIONS

This study indicated that the effects of fluctuating copper concentrations on the response of the copper-sensitive marine alga, *Phaeodactylum tricornutum*, can be interpreted (conservatively) by comparing the mean copper concentrations in solution to effects thresholds. Continuous and 0.5-, 1-, 2-, 4-, and 8-h pulsed copper exposures that were predicted to result in an equivalent copper dose (mean copper concentration) had similar effects on the growth of *P. tricornutum*. Biomass inhibition increased rapidly as the extra- and intra-cellular copper increased. Upon resuspension in clean seawater, the extracellular copper per cell decreased rapidly, whereas the intracellular copper per cell decreased slowly. Negligible loss of copper from the total algal biomass indicated that *P. tricornutum* did not have an effective mechanism for eliminating copper from cells, rather the intracellular copper decreased as a result of dilution by cellular division as the algal growth rate recovered. The results suggest continuous exposures are marginally more toxic to *P. tricornutum* than pulsed copper exposures at equivalent TACs due to the slow copper internalisation rates and possible saturation of membrane transport proteins that occurs for pulsed exposures at high exposure concentrations. In the case of algae, the results indicate that WQGs based on continuous exposure will be conservative when applied to short-term discharges.

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References

Amiard, J., Amiard-Triquet, C., Barka, S., Pellerin, J., Rainbow, P. 2006. Metallothioneins in aquatic invertebrates: Their role in metal detoxification and their use as biomarkers. Aquat. Toxicol. 76, 160-202.

Andrade, L. Farina, M., Filho, G. 2004. Effects of copper on *Enteromorpha flexuosa* (Chlorophyta) in vitro. Ecotox. Environ. Saf. 58, 117-125.

Angel, B., Simpson, S., Jolley, D. 2010. Toxicity to *Melita plumulo*sa from intermittent and continuous exposures to dissolved copper. Environ. Toxicol. Chem. 29, 2823-2830.

Angel, B., Batley, G., Jarolimek, C., Rogers, N. 2013. The impact of size on the fate and toxicity of nanoparticulate silver in aquatic systems. Chemosphere 93, 359-365.

ANZECC/ARMCANZ, 2000. Australian and New Zealand guidelines for fresh and marine water quality. Australia and New Zealand Environment and Conservation Council/Agricultural and Resource Management Council of Australia and New Zealand, Canberra, Australia.

Ashauer, R., Boxall, A., Brown, C. 2006. Predicting effects on aquatic organisms from fluctuating or pulsed exposure to pesticides. Environ. Toxicol. Chem. 25, 1899-1912.

Ashauer, R., Brown, C. 2008. Toxicodynamic assumptions in ecotoxicological hazard models. Environ. Toxicol. Chem. 27, 1817-1821.

Burton, G., Pitt, R., Clark, S. 2000. The role of traditional and novel. toxicity test methods in assessing stormwater and sediment contamination. Crit.l Rev. Environ. Sci. Technol. 30, 413-447.

Campbell, P. 1995. Interactions between trace metals and aquatic organisms: a critique of the free ion activity model. In: Metal Speciation and Bioavailability in Aquatic Systems. John Wiley: Chichester. Eds: A. Tessier and D.R. Turner. pp. 45–102.

Diamond, J., Bowersox, M., Latimer, H., Barbour, C., Bearr, J., Butcher, J. 2005. Effects of pulsed contaminant exposures on early life stages of the fathead minnow. Arch. Environ. Con. Toxicol. 49, 511-519.

Diamond, J.M., Klaine, S.J., Butcher, J.B. 2006. Implications of pulsed chemical exposures for aquatic life criteria and wastewater permit limits. Environ. Sci. Technol. 40, 5132-5138.

Franklin, N., Stauber, J., Lim, R. 2001. Development of flow cytometry based algal bioassays for assessing the toxicity of metals in natural waters. Environ. Toxicol. Chem. 20, 160-170.

Franklin, N., Stauber, J., Apte, S., Lim, R. 2002. Effect of initial cell density of copper in microalgae bioassays. Environ. Toxicol. Chem. 21, 742-751.

Hare, T., Schmidt, R., 1969. Synthesis of reduced glutathione from an endogenous source of sulfur immediately prior to nuclear division in synchronized *Chlorella*. Biochim. Biophys. Acta 192, 537-&.

Hassler, C., Wilkinson, K. 2003. Failure of the biotic ligand and free-ion activity models to explain zinc bioaccumulation by *Chlorella kessleri*. Environ. Toxicol. Chem. 22, 620-626.

Hassler, C., Slaveykova, V., Wilkinson, K. 2004. Discriminating between intra- and extra-cellular metals using chemical extractions. Limnol. Ocean.: Meth. 2, 237-247.

Hoang, T.C., Gallagher, J.S., Tomasso, J.R., Klaine, S.J. 2007. Toxicity of two pulsed metal exposures to Daphnia magna: Relative effects of pulsed duration-concentration and influence of interpulse period. Arch. Environ. Con. Toxicol. 53, 579-589.

Holdway, D., Barry, M., Logan, D., Robertson, D., Young, V., Ahokas, J. 1994. Toxicity of pulse-exposed fenvalerate and esfenvalerate to larval Australian crimson-spotted fish (*Melanotaenia fluviatilis*). Aquat. Toxicol. 28, 169-187.

Hosmer, A., Warren, L., Ward, T. 1998. Chronic toxicity of pulse-dosed fenoxycarb to *Daphnia magna* exposed to environmentally realistic concentrations. Environ. Toxicol. Chem. 17,1860–1866.

Ingersoll, C., Winner, R. 1982. Effect on *Daphnia pu*lex (deGeer) of daily pulse exposures to copper or cadmium. Environ. Toxicol. Chem. 1, 321-327.

Kallander, D., Fisher, S., Lydy, M. 1997. Recovery following pulsed exposure to organophosphorus and carbamate insecticides in the midge, *Chironomus riparius*. Arch. Environ. Contam. Toxicol. 33:29–33.

Kosower, N. Kosower, E. 1978. The glutathione status of cells. Int. Rev. Cytol. 54, 109-160.

Knauer, K., Behra, R., Sigg, L. 1997a. Adsorption and uptake of copper by the green alga Scenedesmus subspicatus (Chlorophyta). J. Phycol. 33, 596-601.

Knauer, K., Behra, R., Sigg, L. 1997b. Effects of free Cu^{2+} and Zn^{2+} ions on growth and metal accumulation in freshwater algae. Environ. Toxicol. Chem. 16, 220-229.

Levy, J., Angel, B., Stauber, J., Poon, W., Simpson, S., Cheng, S., Jolley, D. 2008. Uptake and internalisation of copper by three marine microalgae: Comparison of copper-sensitive and copper-tolerant species. Aquat. Toxicol. 89, 82-93.

Liber, K., Goodfellow, W., Green, A., Clements, W., den Besten, P., Galloway, T., Gerhardt, A., Simpson, S. 2007. In situ-based effects measures: considerations for improving methods and approaches. Integr. Environ. Assess. Manag. 3, 246-257.

Morelli, E., Scarano, G. 2001. Synthesis and stability of phytochelatins induced by cadmium and lead in the marine diatom *Phaeodactylum tricornutum*. Mar. Environ. Res. 52, 383-395.

Naddy, R., Johnson, K., Klaine, S. 2000. Response of *Daphnia magna* to pulsed exposures of chlorpyrifos. Environ. Toxicol. Chem. 19:423–431.

Nyholm, N. (1985). Response variable in algal growth inhibition tests – Biomass or growth rate. *Water Res.* 19, 273-279.

OECD (2005) Test Guideline 201: Freshwater alga and cyanobacteria growth inhibition test, revised version organisation for economic co-operation and development (OECD). Organisation for Economic Cooperation and Development, Paris, France.

Schulz, R., Liess, M. 2000. Toxicity of fenvalerate to caddisfly larvae: Chronic effects of 1- vs 10-h pulse exposure with constant doses. Chemosphere. 41:1511–1517

Smith, C., Steele, J., Stauber, J., Jolley, D. 2014. Copper-induced changes in intracellular thiols in two marinediatoms: *Phaeodactylum tricornutum* and *Ceratoneis closteri*um. *Aquat. Toxicol.* 156, 211-220.

Slaveykova, W., Wilkinson, K. 2002. Physicochemical aspects of lead bioaccumulation by Chlorella vulgaris. Environ. Sci. Technol. 36, 969-975.

Sokal, R., Rohlf, F. 1995. Biometry: the principles and practices in biological research. Freeman, New York.

Stauber, J,, Tsai, J., Vaughan, G., Peterson, S., Brockbank, C. 1994. Algae as indicators of toxicity of the effluent from bleached eucalypt kraft pulp mills. National Pulp Mills Research Program Technical Report No 3, CSIRO, Canberra, Australia.

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

Stauber, J., Benning, R., Hales, L., Eriksen R., Novak, B. 2000. Copper bioavailability and amelioration of toxicity in Macquarie Harbour, Tasmania, Australia. Mar. Freshwater Res. 51, 1-10.

Stauber, J., Florence, T. 1987. The mechanism of toxicity of ionic copper and copper-complexes to algae. Mar. Biol. 94, 511-519.

Sunda, W., Huntsman, S. 1998. Processes regulating cellular metal accumulation and physiological effects: Phytoplankton as model systems. Sci. Tot. Environ. 219, 165-181.

ToxCalc (1994). ToxCalc users guide. Comprehensive toxicity data analysis and database software, version 5.0.23C, Tidepool Scientific Software, McKinleyville, CA, USA.

Vogs, C., Bandow, N., Altenburger, R. 2013. Effect propagation in a toxicokinetic/toxicodynamic model explains delayed effects on the growth of unicellular green algae scenedesmus vacuolatus. Environ. Toxicol. Chem. 32, 1161-1172.

Ward, D., Simpson, S., Jolley, D. 2013. Slow avoidance response to contaminated sediments elicits sublethal toxicity to benthic invertebrates. Environ. Sci. Technol. 47, 5947-5953.

Warne, M., Batley, G., Braga, O,, Chapman, J., Fox, D., Hickey, C., Stauber, J., Van Dam, R. 2014. Revisions to the derivation of the Australian and New Zealand guidelines for toxicants in fresh and marine waters. Environ. Sci. Poll. Res. 21, 51-60.

Wolterbeek, H., Viragh, A., Sloof, J., Bolier, G., Vanderveer, B., Dekok, J. 1995. On the uptake and release of zinc (Zn-65) in the growing alga *Selenastrum capricornutum* Printz. Environ. Pollut. 88, 85-90.

Table 1. Copper exposure conditions, time-averaged concentrations (TACs) and 72-h biomass yield inhibition in pulsed-exposure bioassays

Number of pulses	Pulsed exposure period			Recovery between pulses			72-h TAC (µg/L)	72-h inhibition[a,b] (%)
	Duration (h)	Pulse concentration (µg Cu/L)		Duration (h)	Recovery concentration (µg Cu/L)			
		Nominal	72 h measured		Nominal	72 h measured		
Effect of multiple pulse concentration and duration								
3	0.5, 1, 2, 4	30	21 – 23	23.5, 23, 22, 20	0.3	0.5 - 1.2	15, 27, 40, 51	16, 27, 39, 55
3	0.5, 1, 2, 4	100	83 – 87	23.5, 23, 22, 20	1	1.2 - 2.2	3.7, 7.1, 15, 27	60, 68, 76, 75
3	0.5, 1, 2, 4	200	163 – 166	23.5, 23, 22, 20	2	1.4 - 2.9	3.7, 4.9, 8.4, 14	65, 63, 79, 87
3	0.5, 1, 2, 4	600	541 – 543	23.5, 23, 22, 20	6	4.7 - 6.3	0.8, 1.7, 2.8, 5.0	92, 94, 95, 96
Growth rate recovery following single pulses[a]								
1	24 (0-24 h)	30	21	48	0.3	0.5	8.7	59±6
1	24 (24-48 h)	30	20	48	0.3	0.3	9.1	49±6
1	24 (48-72 h)	30	18	48	0.3	0.8	8.6	40±5
Continuous	72	10	5.7	-	-	-	7.5	83±4
Effect of varying pulse concentration and duration								
3	0.5	326	243	23.5	3.3	4.0	10.6	68±7
3	1	195	151	23	2.0	4.1	11.2	66±7
3	2	108	79	22	1.1	2.6	10.7	69±5
3	4	57	38	20	0.6	2.0	10.3	72±4
3	8	29	22	16	0.3	1.5	10.1	76±7
Continuous	Continuous	10	7.5	-	-	-	8.7	89±3

[a] Measurement of biomass yield inhibition (mean ± standard deviation, n=4)

[b] Where standard deviation not reported due to space constraints, the RSDs were in the range of 0.7-15%

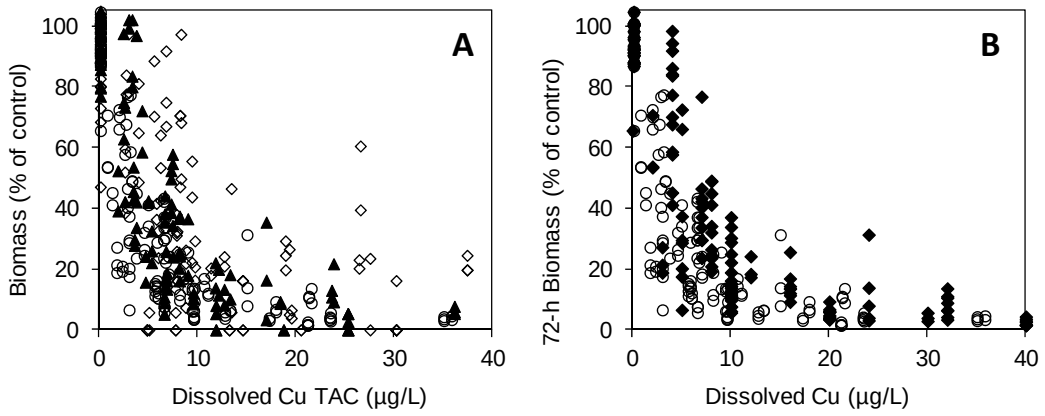


Figure 1. The relationship between biomass inhibition of *P. tricornutum* and dissolved copper in twelve continuous exposure bioassays; A) comparison between 24 h (◇), 48 h (▲), and 72 h (○) exposure to dissolved copper TACs, and B) comparison between 72-h nominal (◆) and TAC (○) dissolved copper.

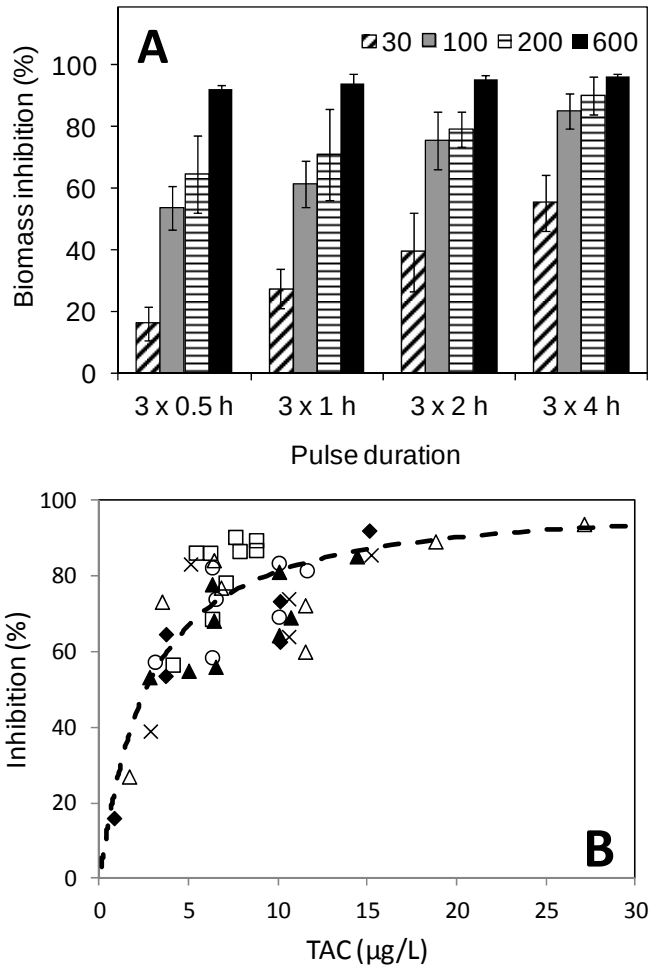


Figure 2. A) The 72-h biomass inhibition (mean ± standard deviation, n=4) for pulsed exposure treatments of 30, 100, 200 and 600 µg Cu/L, and B) The relationship between 72-h biomass inhibition of *P. tricornutum* and dissolved copper TAC in all bioassays tested for exposure durations of 0.5 (◆), 1 (△), 2 (×), 4 (▲), 8 (○) h and equivalent continuous exposures (□). The broken line is the model fit of the data obtained from twelve tests run to monitor species sensitivity during pulsed exposures. Error bars represent the standard deviation of four replicates.

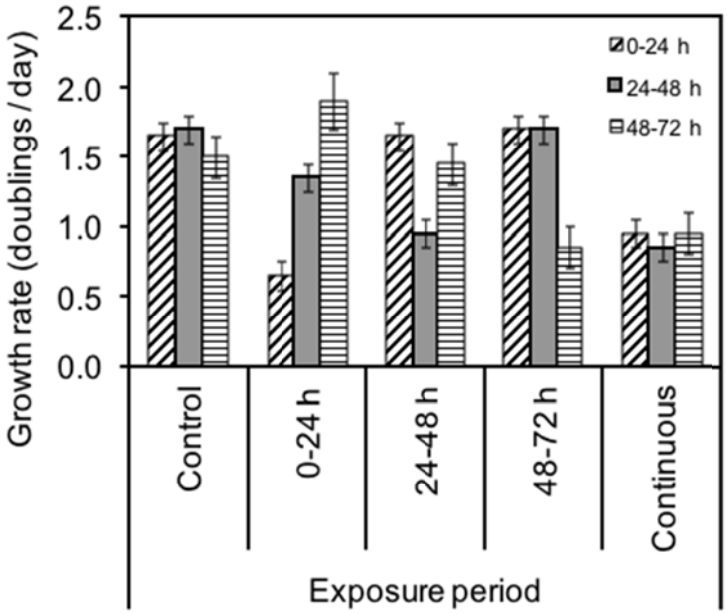


Figure 3. The growth rates (doublings/day) of *P. tricornutum* over 0-24 h, 24-48 h and 48-72 h periods in tests comparing the effect of single 24-h pulsed copper exposures of 30 µg/L delivered in either the 0-24 h, 24-48 h or 48-72 h periods of the 72-h bioassay compared to continuous exposure to 10 µg/L. Error bars represent the standard deviation of four replicates.

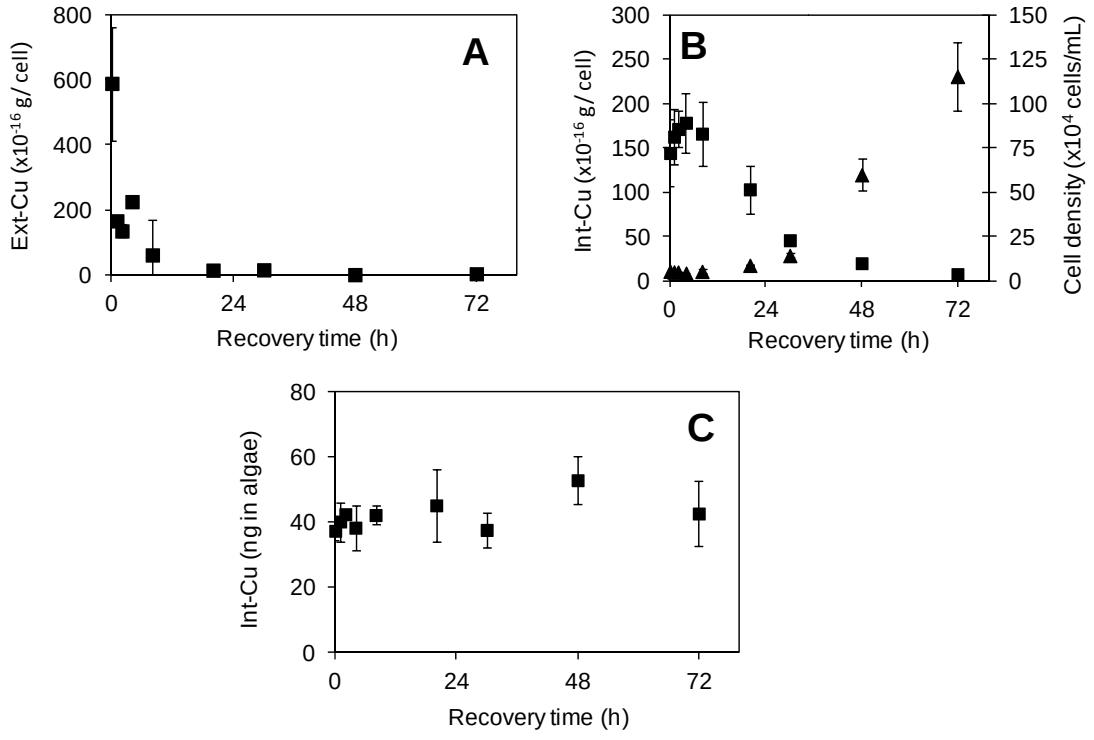


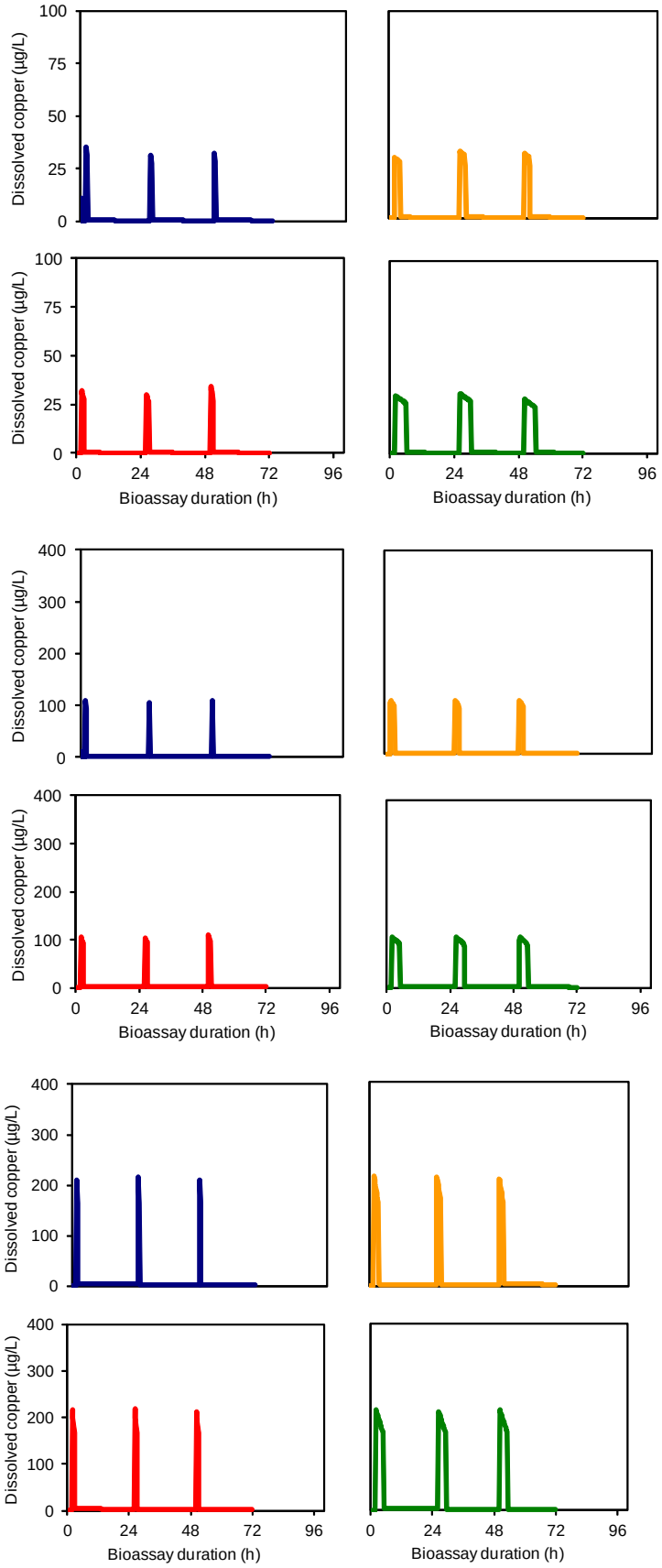
Figure 4. Elimination rates of (A) extracellular copper; (B) intracellular copper (■) and cell density (▲); and (C) total intracellular copper in all bioassay cells (accounting for dilution due to increased cell density). Error bars represent the standard deviation of four replicates.

Supplementary Information

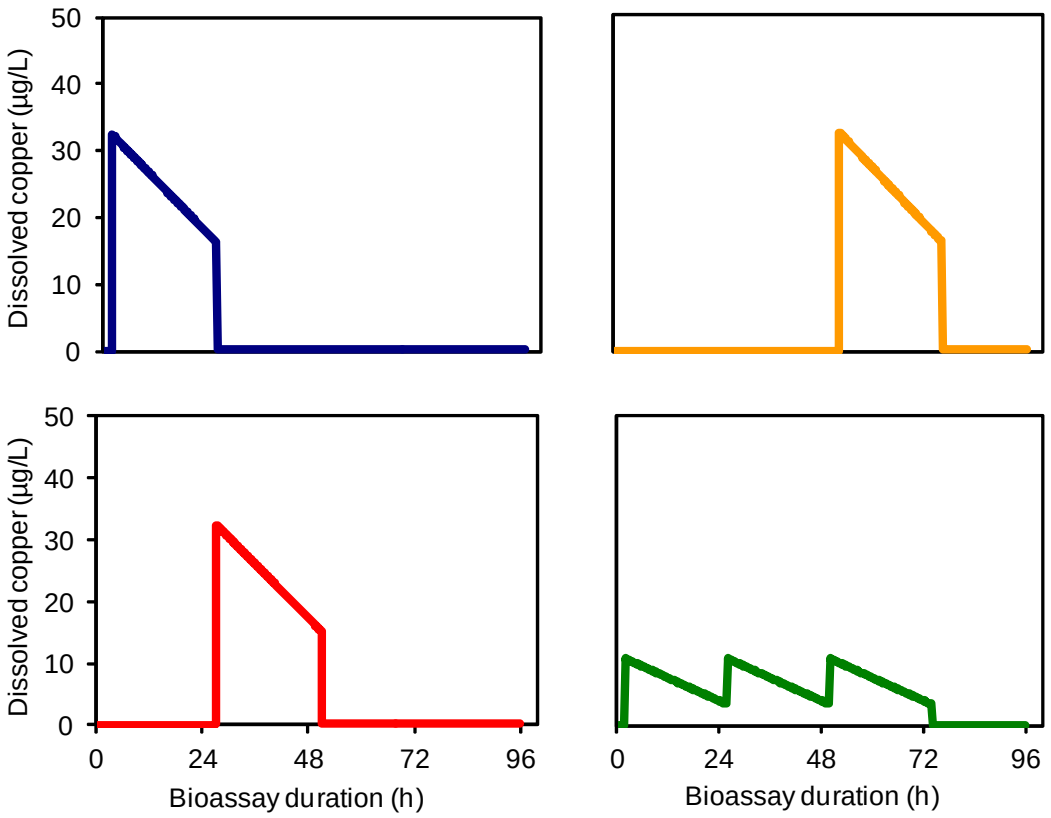
For manuscript titled “Time-averaged copper concentrations from continuous exposures predicts pulsed exposure toxicity to the marine diatom, *Phaeodactylum tricornutum*: Importance of uptake and elimination” by Brad M. Angel, Stuart L. Simpson, Anthony A. Chariton, Jenny L. Stauber, Dianne F. Jolley

Supplementary Table S1. Dissolved copper TACs, extra- and intracellular copper concentrations, and 72-h biomass yield inhibitions determined for 1, 4 and 8 h pulsed and continuous exposure scenarios

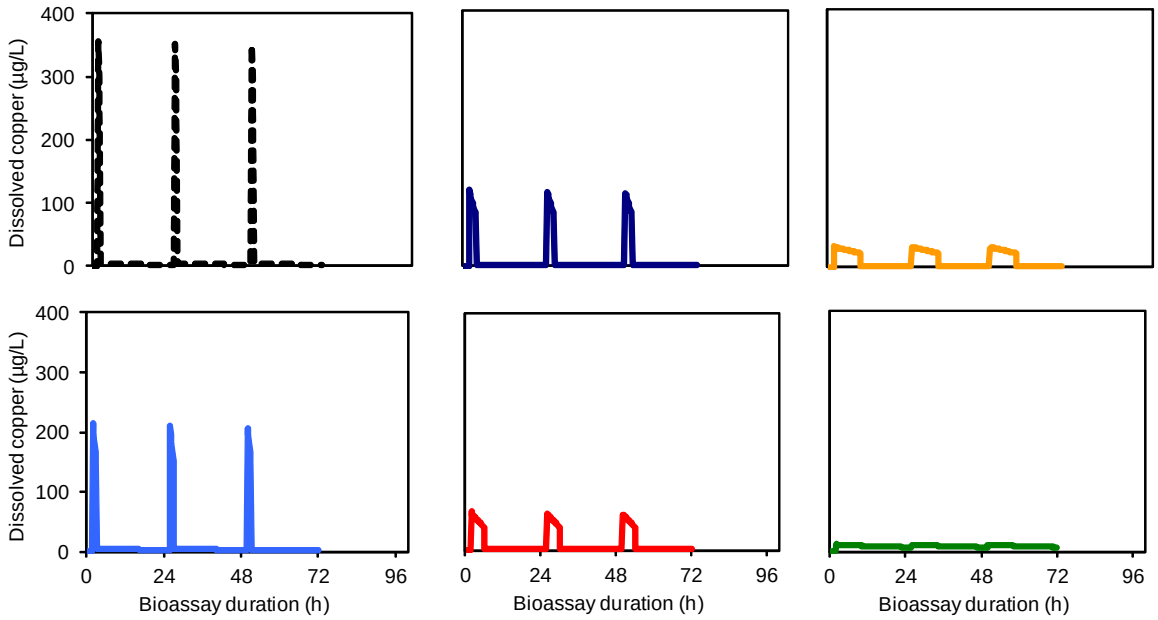
Exposure scenario	Dissolved Cu TAC	Ext-Cu	Int-Cu	Biomass yield inhibition
	(µg/L)	($x10^{-16}$ g Cu/cell)		(%)
Control	<0.3	30	3	0
1-h pulse	6.5	300	130	82±3
4-h pulse	6.4	91	75	65±3
8-h pulse	6.5	59	92	70±2
Continuous	5.5	290	120	83±2



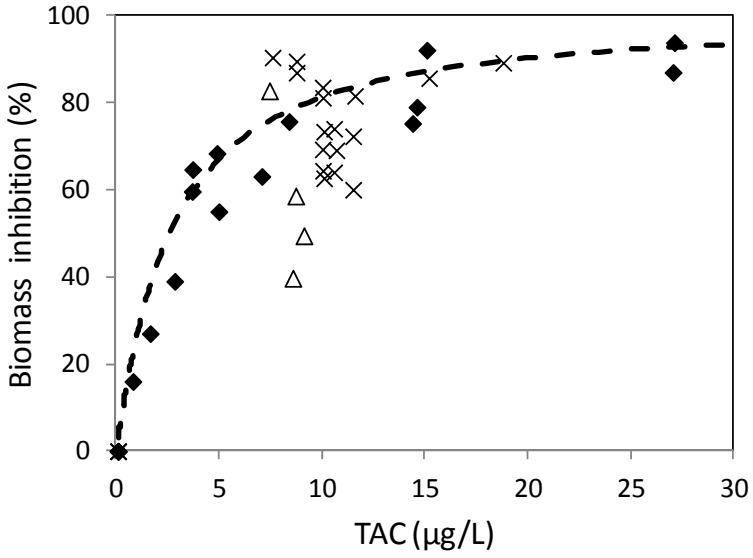
Supplementary Figure S1. Measured dissolved copper for exposure scenarios investigating the effect of pulse duration for nominal daily dissolved copper pulses of 30, 100, 200 and 600 µg/L.



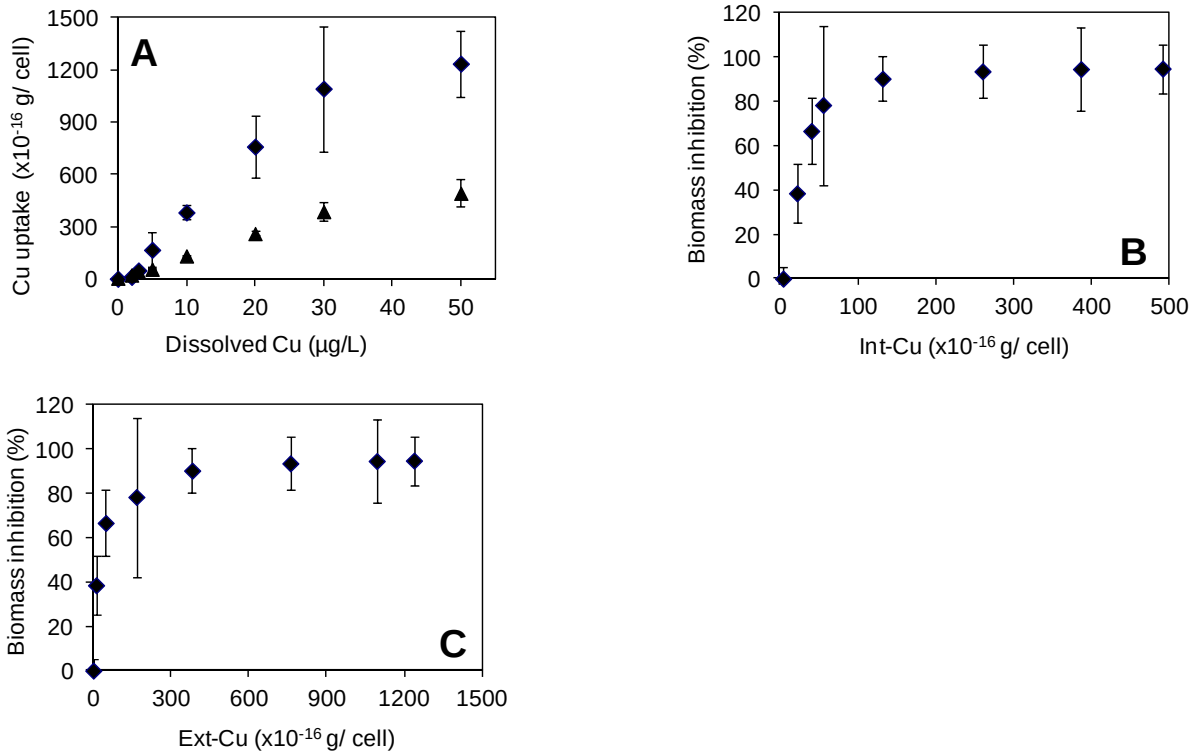
Supplementary Figure S2. Measured dissolved copper for exposure scenarios investigating the recovery of algal growth rate following single 30 µg/L 24 h pulsed exposures in the 0-24, 24-48 and 48-72 h stages of the bioassay compared to an equivalent dose of 10 µg/L dissolved copper in a continuous exposure.



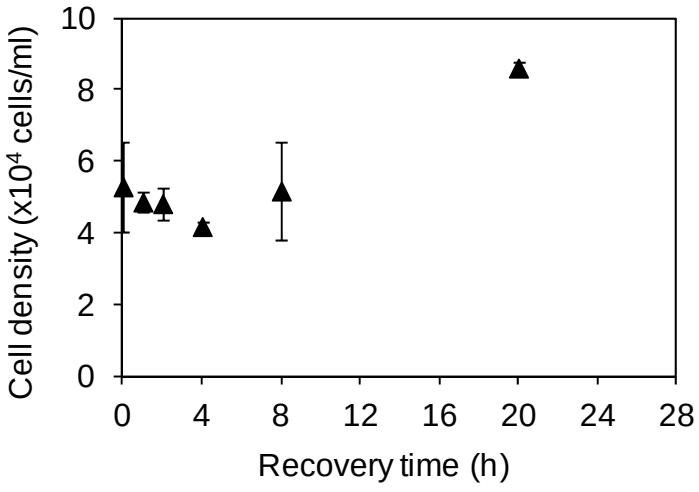
Supplementary Figure S3. Exposure scenarios investigating the effect of equivalent doses of dissolved copper delivered in daily pulsed exposures with different concentrations and durations.



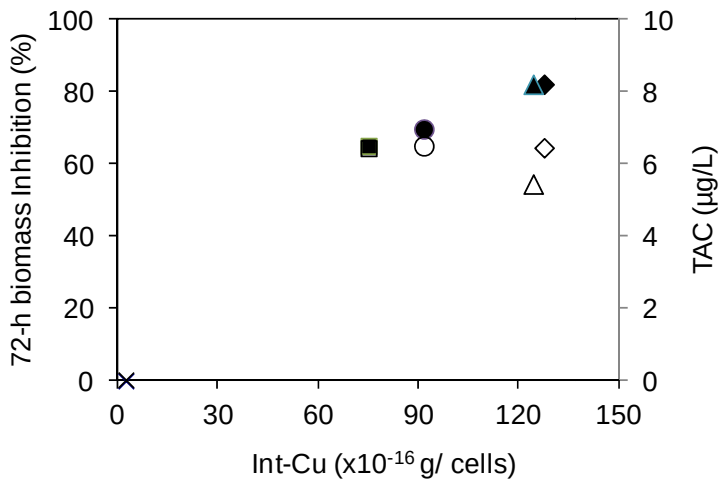
Supplementary Figure S4. A comparison of the relationship between biomass inhibition of *P. tricornutum* and dissolved copper TAC over 72 h for tests investigating the effect of pulse duration (□) on growth rate recovery following single pulses (□), and the effect of concentration and duration on toxicity elicited by exposures with equivalent dissolved copper TAC (□).The broken line is the model fit through the data obtained from twelve tests run to monitor species sensitivity during pulsed exposures.



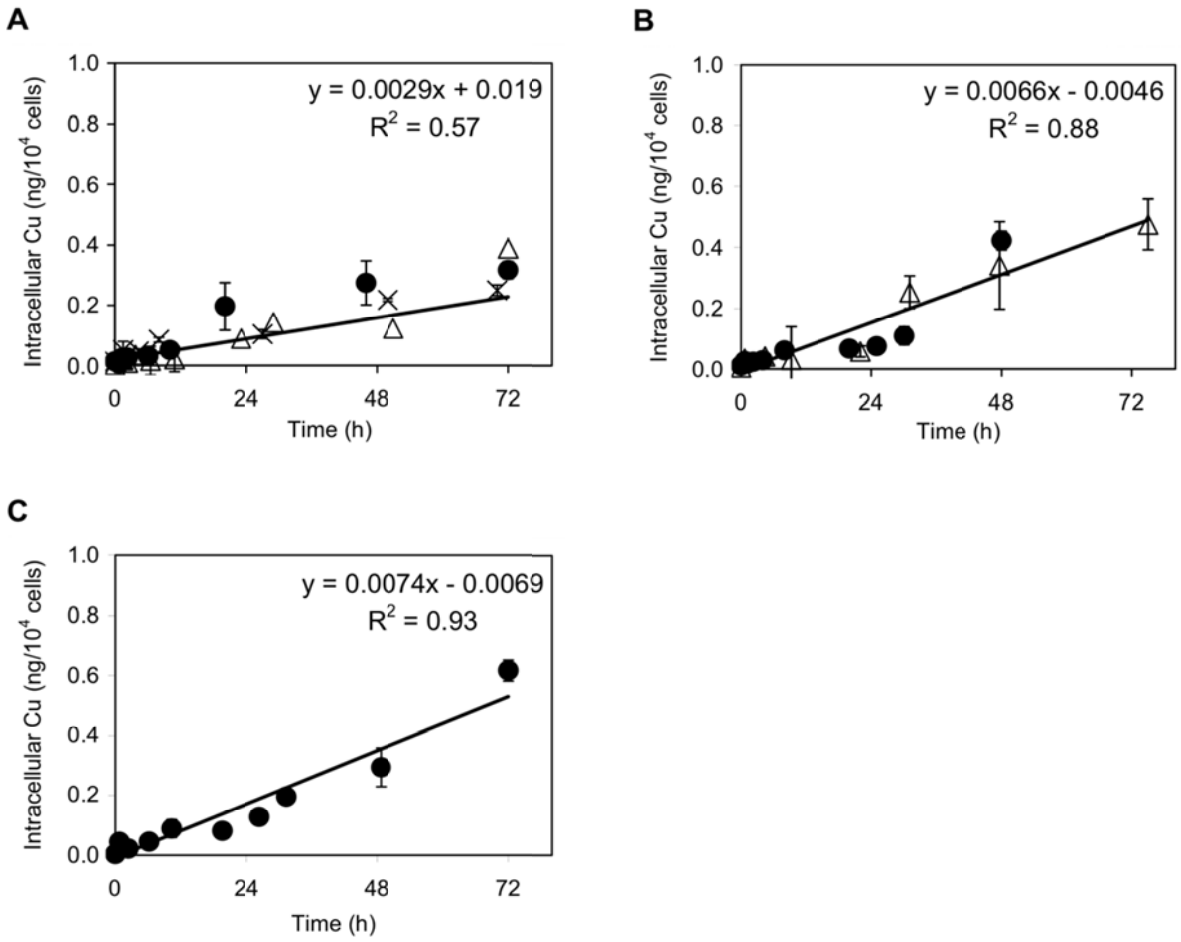
Supplementary Figure S5. A) The effect of dissolved copper on 72-h extracellular (◆) and intracellular (▲) copper, B) relationship between 72-h biomass yield inhibition and intracellular copper, and C) relationship between 72-h biomass yield inhibition and extracellular copper. Error bars represent the standard deviation of three replicates.



Supplementary Figure S6. The *P. tricornutum* cell density in the initial 28 h of the recovery period (resuspension in clean seawater) following 72-h exposure to 15 µg Cu/L.



Supplementary Figure S7. The relationship between inhibition (closed symbols) and the dissolved copper TAC (open symbols), and intracellular copper for control (×), 1 (◆), 4 (■), 8 (●) h pulsed and continuous (▲) exposures.



Supplementary Figure S8. The intracellular copper per cell within *P. tricornutum* during continuous exposure to A) 10 µg Cu/L, B) 30 µg Cu/L, and C) 50 µg Cu/L, where different symbols represent replicate tests at each exposure concentration (Levy et al., 2008).