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Abstract

The concentrations of metal contaminants often fluctuate in estuarine waters; yet we have limited knowledge about the effects of intermittent exposures on estuarine organisms. Using 10-d lethality bioassays with the epibenthic amphipod *Melita plumulosa*, different combinations of intermittent (pulsed) dissolved Cu exposure were investigated, varying Cu concentration, pulse duration, and time between pulses. Negligible organism mortality was observed immediately after single 12- to 62-h duration pulsed exposures of 100 to 900 mg/L dissolved Cu. However, delayed mortality was observed in the subsequent 96-h nonexposure period, after which negligible additional mortality occurred during the remainder of the 240-h tests. For multiple pulsed exposures, increasing the time between pulses from 0 to 144 h did not result in significantly different mortality rates for 300 and 400 mg/L dissolved copper, indicating that the organisms did not recover between pulses. Organism mortality exhibited a strong relationship with the time-averaged concentration (TAC) resulting from the combination of exposure concentration and duration. The lethal concentration to 50 (LC50), 20 (LC20), and 10% (LC10) (95% confidence interval) of the test population for the combined TAC exposure–survival data were 86 (71–103), 44 (30–64), and 30 (18–49) mg Cu/L, respectively, which were similar to the respective values of 100 (87–114), 55 (43–70), and 39 (28–54) mg Cu/L determined for continuous exposure. The results from the current study support the use of analytical techniques capable of determining the time-averaged concentration of metals, because they will more accurately predict the effects of toxicants on organisms than single time-point measurements.

Keywords

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Toxicity to *Melita plumulosa* from intermittent and continuous
exposures to dissolved copper

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concentration

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20

Abstract

The concentrations of metal contaminants often fluctuate in estuarine waters, yet there is limited knowledge on the effects of intermittent exposures on estuarine organisms. Using 10-d lethality bioassays with the epibenthic amphipod, *Melita plumulosa*, different combinations of intermittent (pulsed) dissolved copper exposure were investigated: varying copper concentration; pulse duration; and time between pulses. Negligible organism mortality was observed immediately after single 12- to 62-h duration pulsed exposures of 100-900 $\mu\text{g L}^{-1}$ dissolved copper. However, delayed mortality was observed in the subsequent 96-h non-exposure period, after which, negligible additional mortality occurred during the remainder of the 240-h tests. For multiple pulsed exposures, increasing the time between pulses from 0 to 144 h did not result in significantly different mortality for 300 and 400 $\mu\text{g L}^{-1}$ dissolved copper, indicating that the organisms did not recover between pulses. Organism mortality exhibited a strong relationship with the time-averaged concentration (TAC) resulting from the combination of exposure concentration and duration. The LC_{50} , LC_{20} , and LC_{10} (95% confidence interval) of the combined TAC exposure-survival data were 86 (71-103), 44 (30-64), and 30 (18-49) $\mu\text{g Cu L}^{-1}$, respectively, which were similar to the respective values of 100 (87-114), 55 (43-70), and 39 (28-54) $\mu\text{g Cu L}^{-1}$ determined for continuous exposure. The results from this study support the use of analytical techniques capable of determining the time-averaged concentration of metals, as they will more accurately predict the effects of toxicants on organisms than single time-point measurements.

46 **Introduction**

47 Traditional toxicity tests examine the effects of contaminant concentration on biological
48 systems using a range of continuous contaminant exposures over a given time [1, 2].

49 However, *in-situ* contaminant concentrations are affected by a multitude of events such
50 as stormwater run-off, industrial discharges, and variable water flows [3]. These events
51 frequently cause temporal fluctuations of contaminant concentrations and lead to
52 intermittent/pulsed exposure of organisms inhabiting such systems.

53 Bioassays that consider fluctuations in contaminant exposures will better represent the
54 biological effects that may occur in the environment. However, information on the
55 effects of intermittent contaminant exposure is sparse and mostly limited to a few
56 freshwater organisms, such as the water flea, *Daphnia magna*, and the fathead minnow,
57 *Pimephales promelas* [4, 5, 6, 7]. For example, laboratory bioassays showed that the
58 interval between pulsed copper exposures of the same overall duration resulted in
59 significantly different toxicity to *Pimephales promelas* [4].

60 Field-based *in-situ* exposures are more environmentally realistic than static laboratory
61 bioassays, but are often difficult to undertake [3, 8, 9]. Therefore, most previous studies
62 have employed carefully designed laboratory-based studies to investigate various
63 aspects of pulsed contaminant exposure including:

- 64 (i) the potential for organism recovery or acclimation between exposures [5, 10];
- 65 (ii) tolerance [11];
- 66 (iii) delayed (latent) effects that occur after the exposure period [5, 12]; and,
- 67 (iv) the effects of exposure duration and concentration [5, 10, 12].

68 Greater understanding of these processes will improve our ability to predict the risk to
69 organism health in the field [3, 6, 9, 13].

The complexity of predicting toxicity from pulsed exposures is highlighted by previous studies that have shown that changing the concentration and duration, or the interval between pulsed exposures (to achieve equivalent time-averaged concentrations (TAC) of contaminants) may result in either greater or lesser toxic effects, depending on the species of organism and contaminant. A 20-min exposure to fenvalerate caused greater acute toxicity in rainbow fish (*M. fluviatilis*) than an equivalent exposure to TACs over 40, 60, and 120 min [12]. Greater contaminant uptake and toxicity was reported for *Salmo gairdneri* exposed to pulsed copper ($21\text{--}465\ \mu\text{g L}^{-1}$ for 4-5 h), than those exposed continuously to equivalent TACs [14]. Significant mortality was not observed in *D. magna* exposed to zinc pulses ($250\text{--}2500\ \mu\text{g L}^{-1}$) for 3-6 h, but was significant for 12-h pulses [6]. Greater toxic effects were observed in rainbow fish (*M. fluviatilis*) exposed continuously to cadmium ($0.033\text{--}3.3\ \text{mg L}^{-1}$) and zinc ($0.33\text{--}33.33\ \text{mg L}^{-1}$) than those exposed to equivalent TACs in 2-h pulses [15]. Survival of the fathead minnow *P. promelas* exposed to two copper pulses ($5\text{--}40\ \mu\text{g L}^{-1}$, 3-24 h) increased as the recovery period between pulses increased from 24 to 48 h, but then decreased as the recovery time further increased up to 144 h [4].

The current study was undertaken to assess the effects of various pulsed exposure parameters on the toxicity of copper to the epibenthic amphipod *Melita plumulosa*, one of the more copper-sensitive amphipod species indigenous to the eastern coast of Australia [9, 16, 17]. The effects of dissolved copper pulse concentration and duration, and the interval between multiple pulses were investigated using mortality as the test end-point. The relationship between mortality and TAC of dissolved copper was determined for each exposure to determine the potential for using TAC to predict mortality.

95 **Methods**

96 *Test media*

97 The clean seawater used in laboratory bioassays was collected from Port Hacking,
98 Sydney, Australia. The seawater was filtered through a 0.45 μm membrane filter within
99 two hours of collection, adjusted to 30 PSU with deionised water (Milli-Q, 18 $\text{M}\Omega\text{ cm}^{-1}$,
100 Millipore, Sydney, NSW, Australia), and allowed to stand at $21 \pm 1^\circ\text{C}$. Clean sediment
101 used to culture test species in the laboratory was collected at low tide from Bonnet Bay,
102 in the lower Woronora River, Sydney, Australia. Sediment from the surface layer (top
103 2-4 cm) was scooped into two clean Zip-lock bags using a clean stainless steel shovel,
104 the bags were sealed, and the sediment was stored in a cool room for a maximum of 2
105 months before use. This sediment has been characterised in the past and found to
106 contain relatively low sediment-bound and porewater contaminant concentrations and
107 has been deemed suitable for culturing *M. plumulosa* [18, 19].

108 *Collection and handling of Melita plumulosa*

109 *M. plumulosa* (Zeidler) were cultured in the laboratory as described previously [16, 18].
110 Individuals were isolated from the cultures by transferring Nylon mesh patches from the
111 stock cultures, and rinsing individuals off the patch with clean seawater into a sieve
112 (180 μm). Gravid females were identified under a dissecting microscope and pipetted
113 into a fresh culture. Juvenile *M. plumulosa* hatched 2 ± 1 d after gravid female
114 isolation, and after 16 days, juveniles of approximately equal body lengths were
115 selected for tests. Juveniles at this stage of development were able to tolerate test
116 manipulations whilst still being relatively sensitive to dissolved copper [17]. Twenty
117 organisms (16 ± 1 day old) were transferred into 250 mL polycarbonate vials containing
118 200 mL of clean seawater at the start of each test.

119 *General analytical*

Deionised water (Milli-Q) or filtered seawater was used to prepare all solutions. All plastic- or glass-ware used in laboratory experiments to measure trace metal concentrations (e.g. sampling containers and equipment, and organism culturing containers) were acid-washed before use. Measurements of pH were made according to manufacturer's instructions using a Wissenschaftlich-Technische Werkstattan (WTW, Weilheim, Germany) meter equipped with a pH probe (Orion sure-flow combination pH 9165BN). The pH probe was calibrated against pH 4.0 and 7.0 buffers (Orion Pacific, Sydney, New South Wales, Australia). Measurements of salinity and temperature were measured using a LF 320 WTW conductivity meter (Weilheim, Germany) and electrode (TetraCon 325, WTW), whilst dissolved oxygen was measured using an Oxi 196 WTW meter (Weilheim, Germany) with an oxygen electrode (EO96, WTW) following manufacturers instructions.

Dissolved metals ($<0.45\ \mu\text{m}$) were measured following filtration (acid-washed $0.45\text{-}\mu\text{m}$ cellulose nitrate filters, 25 mm, Sartorius Minisart, Goettingen, Germany), and acidifying to 0.2% (v/v) (Tracepur, Merck, Darmstadt, Germany) [19]. The dissolved metals were determined by inductively coupled plasma - atomic emission spectrometry (ICP-AES, Spectroflame EOP, Spectro Analytical Instruments, Kleve, Germany) calibrated with multi-element matrix-matched standards (QCD analysts and Plasma Chem Corp, Eaglewood, FL, USA), and results were only accepted when analytical drift was less than 10%.

Toxicity test procedures

The toxicity tests were conducted in environmental cabinets (Labec Refrigerated Cycling Incubator, Sydney, Australia) at a temperature of $21 \pm 1^\circ\text{C}$ on a 12:12 h light:dark photoperiod (light intensity of $3.5\ \mu\text{mol photons s}^{-1}\text{ m}^{-2}$) for 10 d [16]. The lids of test vessels were loosely capped and aerated by continuously bubbling air (a tube

inserted through the lid into the test solution). The amphipods were fed small amounts of fish food (0.03 mg per amphipod, Sera MicronTM, Heinsberg, Germany) at the start of the test and following each water change to prevent organism death resulting from starvation or stress [16, 18]. The pH, salinity, temperature, and dissolved oxygen were measured at the start and end of each test and during each water change (approximately 72 h).

Previous studies investigating the effects of contaminant pulses on macro-organisms have often simulated contaminant pulses by spiking the solution with the desired contaminant and transferring the organism (with tweezers or by pipette) into clean water to terminate the pulse [6, 20, 21]. In our studies, these methods resulted in unacceptably high mortalities in juvenile amphipod controls (no copper). An alternative technique was adopted, in which approximately 95% of copper-spiked water was carefully replaced with clean seawater at pulse termination. To terminate copper pulses or make water changes two acid-washed 100 mL syringes were used: one to remove copper-spiked water, and another to slowly add clean seawater to minimise organism stress. Metal analyses showed that $\leq 5\%$ of the copper concentration remained after each clean water change. A small number of organisms accidentally removed with the syringe were immediately replaced into bioassays. This approach resulted in acceptable survival of juvenile controls in preliminary tests with ≥ 16 of the initial 20 individuals ($\geq 80\%$) surviving to the end of the 10-d tests. Continuous exposure bioassays were tested before pulsed exposure bioassays to investigate the sensitivity of *M. plumulosa* and determine the lethal concentration to 20% of the population (LC20). The concentrations of dissolved copper employed in the pulsed exposure bioassays were selected to ensure that the 95% water replacements would result in residual dissolved copper concentrations (between pulses) that were below the LC20. This ensured that mortality

in the bioassays could only be attributed to the pulsed exposure and not the residual copper between pulsed exposures.

Copper pulses were generated by spiking seawater from stock solutions (5 and 100 mg L⁻¹) prepared from copper sulfate (CuSO₄.5H₂O, AR grade, APS Finechem, Sydney, Australia). For the longer exposure durations, water changes were generally performed every 72 h, or at the end of the specified pulse period.

Lethality of copper to *M. plumulosa* was preceded by organism immobility, as exposed organisms exhibited very slow movement in the time between exposure and mortality. Generally, organisms that suffered mortality either decomposed rapidly or were preyed upon by the remaining organisms and were no longer present in solution. The number of live amphipods was counted immediately before each water change. Amphipods were deemed to be alive if they swam when the solutions were agitated, and were counted regardless of any small behavioural impairments brought on by copper toxicity.

Continuous and pulse exposure bioassay procedures

A range of exposure scenarios were designed to investigate the toxicity from pulsed copper exposure in acute 10-d bioassays using mortality as the effect endpoint. The mortality after each treatment was compared to the time averaged concentration (TAC) of dissolved copper, which was calculated according to equation 1.

$$\text{TAC} = ((\text{PC} \times t_p) + \text{RC} \times (240 - t_p)) / 240 \quad \text{Equation 1}$$

where PC is the mean measured dissolved copper pulse concentration (µg L⁻¹), *t_p* is the pulse duration (h), and RC is the mean measured dissolved copper concentration between pulses (i.e. recovery concentration ~5% of pulse concentration (µg L⁻¹)) and 240 represents 10 d (in hours). PC was calculated by averaging the copper concentrations measured at the start and end of each pulsed exposure, assuming a linear decrease in metal concentration over the pulse duration [22]. RC was calculated by

averaging the copper concentrations measured immediately after water replacement and immediately before the subsequent pulse or water replacement.

This study investigated the effects of concentration and duration in single pulses, the number of pulses, the concentration in multiple pulsed exposures, the interval between multiple pulsed exposures of equivalent TAC (equal concentration and overall exposure duration), and pulsed exposures with equivalent TACs, but different concentrations and durations (Table 1 and Figures S1, S2, S3, S4 and S5 of the Supporting Information).

Continuous exposure scenarios were also investigated. The tests employed concentrations in the range 30 – 900 $\mu\text{g Cu L}^{-1}$ for durations of 4 h to continuous exposure, and exposures were initiated 24 h after bioassays were started.

The survival of 16-d old *M. plumulosa* not exposed to copper over the course of the 10-d bioassays was unaffected by three to five water changes occurring between 24 and 96 h apart. Consequently, all survival data is presented as percentage of control, tested for significance at the $p < 0.05$ level.

Statistical analyses

The dissolved copper concentrations that resulted in 10, 20 and 50% lethality (LC_{10} , LC_{20} and LC_{50}) were calculated using a Microsoft Excel® based program [23].

Differences in the mean percentages of survival from different pulse and continuous exposure treatments were examined using a one-way analysis of variance (ANOVA) (NCSS, Kaysville, UT). 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 [24]. The Tukey-Kramer multiple-comparisons test was used as a post-hoc procedure to test for multiple comparisons upon means (NCSS).

Results

During the 10-d water-only tests, the pH ranged from 7.9 to 8.2, the salinity was in the range of 29-31 PSU, and the dissolved oxygen was >94% saturation for all tests. The dissolved copper concentration decreased, on average, by 20% during the course of exposures, with the lower concentrations treatments decreasing by as much as 46% during a 48-h period. Metal losses from solution occur through a combination of adsorption onto the test vessel and the added food, and organism exoskeleton, and through active uptake into organisms.

Continuous exposure to copper resulted in 10-day LC₅₀ (50% lethality, 95% confidence interval), LC₂₀ and LC₁₀ values of 100 (87-114), 55 (43-70), and 39 (28-54) $\mu\text{g Cu L}^{-1}$, respectively.

When exposed to single pulses of 100, 200, 300, 400, 600 and 900 $\mu\text{g Cu L}^{-1}$, the 10-d survival of *M. plumulosa* was not significantly different to controls for exposure durations \leq 96, 35, 19, 10, 8, and 4 h, respectively, but significant lethality occurred at durations \geq 160, 62, 29, 18, 12, and 7 h, respectively (Figures S6 A and B of the Supporting Information for 300 and 400 $\mu\text{g Cu L}^{-1}$ pulsed exposure treatments). In general, single pulsed exposure treatments with similar TACs of dissolved copper showed similar lethality to *M. plumulosa*. For the 100, 200, 300, 400, 600, and 900 $\mu\text{g Cu L}^{-1}$ exposures, the LOECs occurred at TACs of 62 ± 6 , 66 ± 9 , 47 ± 10 , 54 ± 5 , 54 ± 7 , and 51 ± 10 $\mu\text{g Cu L}^{-1}$, respectively, which were not significantly different from each other. The 96-h 100 $\mu\text{g Cu L}^{-1}$ and 10-h 900 $\mu\text{g Cu L}^{-1}$ single pulsed exposures resulted in TACs of 56 ± 5 and 65 ± 6 $\mu\text{g Cu L}^{-1}$, respectively, and did not cause significantly ($p < 0.05$) different organism lethality. This was also the case for the 20-h 200 $\mu\text{g Cu L}^{-1}$ and 10-h 400 $\mu\text{g Cu L}^{-1}$ single pulsed exposures, which resulted in TACs of 35 ± 4 and 40 ± 4 $\mu\text{g Cu L}^{-1}$, respectively, and the 35-h 200 $\mu\text{g Cu L}^{-1}$ and 18-h 400 $\mu\text{g Cu L}^{-1}$

single pulsed exposures that resulted in TACs of 47 ± 6 and $54 \pm 4 \mu\text{g Cu L}^{-1}$, respectively.

The shortest pulsed exposure duration required to elicit lethality for each of the single pulsed exposure concentrations was best represented by a hyperbolic relationship (Figure 1). The area under the curve is equivalent to the combinations of dissolved copper exposure concentration and the duration that the 16-d old *M. plumulosa* can tolerate without suffering lethal effects. As the exposure concentration was decreased, the duration required to cause lethality increased and approached the LC_{20} value observed for continuous exposures. As the exposure concentration increased, the results indicated that there may be a minimum exposure duration before lethality can occur. This could reflect the time required for sufficient uptake of metabolically available copper, or the time required for the stress caused by the exposure to result in lethality. No mortality was observed during the first 96 hours of tests that involved single 12- to 62-h copper pulse exposures (initiated 24 h after the start of each 10-day bioassay), but delayed/latent mortality was observed in the proceeding 96-h post-exposure period (Figure 2). Additional mortality after this period until test completion was generally not significant. There was no evident trend between the percentage of latent mortality and the single pulse concentration or duration.

Multiple-pulsed exposures generally resulted in greater lethality than single-pulsed exposures, e.g. survival was 72 ± 18 , 40 ± 10 , 26 ± 5 , and $21 \pm 10\%$ for single, double, and triple 32-h pulsed and continuous exposures of $400 \mu\text{g Cu L}^{-1}$, respectively (Table 1, Figure 3A). A similar relationship was observed for single, double, and triple 48-h pulsed and continuous exposures of $300 \mu\text{g Cu L}^{-1}$, although the single-pulse treatment had high variability and was not significantly different (Table 1, Figure 3B). Likewise,

as the pulse duration increased, so did the likelihood of toxicity, with a similar TAC threshold for copper exposure where toxicity occurred once it was exceeded (Figure 4).

The influence of intervals between multiple pulses of equivalent TAC

Changing the interval (0-144 h) between the pulses for multiple-pulse exposures with varying time profiles (Table 1, Figure 5A) had negligible effect ($p < 0.05$) on mortality after 240 h. The survival was not significantly different for a single 48-h $300 \mu\text{g L}^{-1}$ pulse and two 24-h $300 \mu\text{g L}^{-1}$ pulses separated by 48, 96 and 144-h intervals, respectively. For these four tests the TACs were 55 ± 5 , 54 ± 7 , 57 ± 3 , $57 \pm 5 \mu\text{g Cu L}^{-1}$, and the final survival was 59 ± 9 , 59 ± 10 , 71 ± 10 , and $71 \pm 10\%$, respectively. *M. plumulosa* survival was also not significantly different for a single $400 \mu\text{g Cu L}^{-1}$ 32-h pulse and two 16-h pulses separated by 30, 56 and 120-h intervals, respectively (56 ± 10 , 54 ± 10 , 41 ± 10 , and $41 \pm 8\%$ survival), which had TACs of 73 ± 6 , 73 ± 5 , 78 ± 8 , $81 \pm 11 \mu\text{g Cu L}^{-1}$, respectively. Survival in controls experiencing the same frequency of water changes but with no added copper was $>85\%$ for all of these tests.

The effect of concentration and duration on effects of exposures with equivalent TAC

Varying the copper pulse concentration and duration (Table 1, Figure 5B) in different copper exposures with equivalent TAC did not result in significantly different survival between exposure treatments. For example, the survival was 43 ± 10 , 28 ± 10 , 41 ± 13 , and $39 \pm 10\%$ for triple 6-, 24-, and 48-h pulsed and continuous exposures, respectively, which had TACs of 113 ± 14 , 123 ± 9 , 126 ± 8 , and $122 \pm 10 \mu\text{g Cu L}^{-1}$, respectively.

290 Discussion

291 *Effect of time TACs on toxicity*

292 The relationship between 16-d old *M. plumulosa* survival and the dissolved copper TAC
 293 was compared for all exposure treatments, i.e. eighteen experiments with varying
 294 exposure profiles (Figure 6). Similar survival of *M. plumulosa* was observed for single,
 295 double, and triple pulsed and continuous exposures with similar dissolved copper TACs.
 296 The survival exhibited a clear trend and decreased from negligible to severe toxicity
 297 over the range of TACs tested. The low variability of this trend indicated that different
 298 pulsed exposure scenarios of equivalent TACs resulted in similar mortality, and
 299 suggested that organisms exposed to different exposure scenarios of equivalent TACs
 300 received equivalent copper doses.

301 The LC₅₀, LC₂₀, and LC₁₀ (95% confidence interval) of the combined TAC exposure-
 302 survival data were 86 (71-103), 44 (30-64), and 30 (18-49) µg Cu L⁻¹, respectively
 303 (Figure 6), which were similar to the values of 100 (87-114), 55 (43-70), and 39 (28-54)
 304 µg Cu L⁻¹ determined for continuous exposure, respectively. This further supported the
 305 finding that different exposures with similar TACs resulted in similar toxicity. This
 306 trend is supported by studies in which similar toxicity was the result of different
 307 exposure scenarios with equivalent metal TACs for *D. magna* exposure to selenium [20]
 308 and *P. promelas* exposure to cadmium [25]. The results of our study indicated that
 309 continuous exposure bioassays with *M. plumulosa* are likely to accurately predict the
 310 acute-lethality effects of pulsed exposures with similar TACs, and supports the use of
 311 TAC-effect data to predict the risk of acute toxicity [2, 6].

312 Our results suggest that in cases where single time-point sampling is used to assess
 313 water quality, overestimations of the risk of toxicity may occur if the high concentration
 314 is not sustained over a significant duration of the life cycle of the organism. These

results suggest that TACs calculated from temporal data on metal concentrations in the field would be a better predictor of potential toxicity than single time-point concentration measurements. This study supports the use of techniques that integrate dissolved metal concentrations over time, such as diffusive gradients in thin films (DGT) samplers [26, 27] and peepers [28].

The current study has shown that mortality of *M. plumulosa* exposed to dissolved copper was related to the TAC and not the type of exposure (pulsed or continuous). However, many studies have reported variations in toxic responses from different contaminant exposures with equivalent TACs for other biological species or contaminants. Longer exposures to lower concentrations of cadmium and zinc with the rainbow fish *M. fluvalilis*, and cadmium and copper with the rainbow trout *S. gairdnerii* and water flea *D. magna* showed greater toxicity than those of equivalent TACs with shorter exposures to higher concentrations [15, 25, 29]. In these cases, the exposure duration was shown to have a greater influence on toxicity than the exposure concentration, possibly due to relatively low contaminant uptake by the organism in shorter exposures.

The opposite response was reported when the midge *C. riparius* was exposed to cadmium, and the water flea *D. magna* exposed to copper, with greater toxicity observed for equivalent TAC exposures of high concentration and short duration than for low concentration long duration [20, 30]. These results indicated the pulse concentration had a greater influence on toxicity than pulse duration. This may occur because pulses with higher concentrations facilitate metal transport to the target sites within organisms, as reported for the green mussel *Perna viridis* exposed to cadmium, *S. gairdnerii* exposed to copper, and *D. magna* exposed to arsenic and copper [14, 20, 31].

Latent toxicity

The latent (delayed) mortality following single copper pulsed exposure (in the 24-96 h period) generally occurred during the 96-h post-exposure period (96-192 h bioassay duration) (Figure 2). Latent mortality during this post-exposure period accounted for an average of 79% of the total mortality that occurred by the end of the 240-h bioassays. The high percentage of mortality that occurred directly after the pulse ended highlights the need to observe bioassay populations after the exposure period to account for the full toxic effect of contaminants. Our results indicated that shorter duration bioassays (e.g. <96 h) may underestimate toxicity as individuals may be counted before they succumb to mortality. However, in the absence of ongoing exposure, a long post-exposure observation is not necessary. The results from the current study suggest a post-exposure observation period of 96 h for amphipods such as *M. plumulosa* should be adequate for contaminants to give rise to most or all of the acute toxic effects.

Latent toxicity has been reported for many other species and contaminants, including *D. magna* exposed to pulses of zinc, *D. Pulex*, macroinvertebrates exposed to pulses of diflubenzuron, and *C. dubia*, *H. azteca*, and *P. promelas* exposed to pulses of cadmium and zinc [5, 6, 32, 33]. However, latent mortality was not observed in *P. promelas* exposed to copper and cadmium, *D. magna* exposed to pulses of copper or ammonia, and for *C. dubia*, *H. azteca*, and *P. promelas* exposed to pulses of phenol [4, 5, 6, 25]. The degree of latent toxicity that occurs for different toxicants and organisms may be attributed to different mechanisms of action [10].

The delayed mortality may be due to slow copper depuration rates after the pulsed exposures ended [20]. The rate of depuration reported for adult *M. plumulosa* (40-d old) following 24-h exposure to radiolabelled dissolved copper was much lower than the rate of uptake [22]. This meant that copper remained within the organism for a period well

after the exposure duration. Similarly slow copper depuration rates were observed in the amphipod, *Chaetogammarus marinus* [34]. The long residence time of metals in these amphipods allows a longer duration for the metal to exert toxic effects, and is likely to indicate poor regulation of copper by these species [31]. The study by King *et al.* [22] of uptake and depuration of copper in *M. plumulosa* used adult organisms (rather than the juveniles in the current study) and copper exposure concentrations of 0.4-50 $\mu\text{g L}^{-1}$. These concentrations were below or similar to the LC20 (55 $\mu\text{g/L}$) and it is possible that physiological responses would be different for the younger *M. plumulosa* organisms in the present study that were exposed to the copper concentrations higher than 55 $\mu\text{g/L}$.

Many studies have reported a net accumulation of copper in amphipods during exposure, because the depuration rates were lower than the accumulation rates [34, 35, 36, 37, 38]. This was observed in *C. marinus* during dissolved copper exposure and in *Orchestia gammarellus* during dissolved cadmium, silver, and zinc exposure [34, 39]. The net accumulation of metals may lead to the amount of metal increasing above the critical body threshold (CBT) of the organism [22, 40], which may be the cause of mortality occurring in the post-exposure period in the present study. It is possible that the mortality of *M. plumulosa* in the current study was the result of immobilisation leading to starvation in combination with direct toxic effects, as proposed for a previous study by Van der Hoeven and Gerritsen [33].

The effect of intervals between pulsed exposures

The inclusion of various intervals between pulsed copper exposures did not have a significant effect on the survival of *M. plumulosa*. This, coupled with observations of organism immobility, indicates that *M. plumulosa* was not able to recover from the dissolved copper pulsed exposures. Organism recovery has been reported in many

pulsed exposure studies, including *D. magna* exposure to chlorpyrifos [21, 40], copper and cadmium [20], *P. promelas* exposure to copper [4], and *C. riparius* exposure to carbofuran and propoxur [41].

In many invertebrates and fish, metal exposure induces the production of metallothioneins, which may be used to regulate and detoxify metals such as cadmium, copper, and zinc by binding to them in forms which are non-toxic [31, 42, 43]. The production and role of metallothioneins varies widely among different species of amphipods exposed to metals [37, 44, 45, 46]. The lack of organism recovery in the period between pulsed exposures in the current study may indicate that the *M. plumulosa* species have inadequate detoxification mechanisms, including metallothionein production [4, 40, 47].

Additive mortality was not observed for successive pulses of 32 and 48 h. This was likely due to the severe mortality from the initial pulse, resulting in fewer of the most copper-sensitive organisms remaining during subsequent exposures. It is possible that mortality is additive for multiple pulse exposures which result in mid-range mortalities (i.e. 30-70%), but becomes less than additive for exposures which show severe mortality, due to more tolerant organisms remaining which are not affected by copper to the same degree as the more sensitive individuals in the population.

Conclusions

The relationship observed between *M. plumulosa* mortality and the time-averaged copper concentration (TAC) of dissolved copper in each test indicated that similar toxicity occurred for similar TACs, regardless of the pulse exposure regime. A clear transition from negligible to severe mortality was shown as the TAC increased in the range 30-120 $\mu\text{g Cu L}^{-1}$. The TAC of single pulses that gave rise to significant mortality was in the range 47-66 $\mu\text{g Cu L}^{-1}$ and was similar for the different single pulse

concentrations tested. The LOEC and LC₅₀ of the combined TAC exposure-survival data were 44 (30-64) and 86 (71-103) $\mu\text{g Cu L}^{-1}$, respectively, and similar to those determined from continuous exposure. The results of this study support the use of TAC exposure-effects data from a particular exposure scenario being used to predict toxicity, including the use of continuous exposure in standard toxicity tests to derive contaminant guidelines and evaluate sample toxicity. This study also supports the use of techniques that measure metal concentrations over time, rather than single time-point measurements to evaluate contaminant risk in the field.

A large proportion of the mortality occurred during a 96-h period (96-192 h bioassay duration) that proceeded the initial 96-h period in which the pulse exposures occurred. This highlights the need to perform short-term monitoring of organisms in the post-exposure period to account for the majority of the exposure toxicity. The mortality of organisms was not significantly different when they were exposed to two pulses of copper separated by various durations, indicating that *M. plumulosa* was not able to recover from the first pulse, irrespective of the recovery duration (to a maximum of 144 h) in clean seawater before the second copper pulse. Surviving exposure to the first copper pulse did not prevent mortality from the second pulse, and may indicate that the initial exposure did not induce detoxification mechanisms such as the production of metallothioneins.

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Figure Captions

Figure 1. Mortality of 16-d old *M. plumulosa* following 10-d bioassays (20 individuals, 3 replicates per treatment, mean \pm standard deviation): the relationship between the duration of a single pulsed exposure and the pulse concentration

Figure 2. Latent mortality (mean \pm standard deviation, n=3) of 16-d old *M. plumulosa* following single exposures within the 24-86-h period (dashed lines): dissolved copper pulses of 200 $\mu\text{g L}^{-1}$, 62 h (\square), 300 $\mu\text{g L}^{-1}$, 48 h (\blacklozenge), 400 $\mu\text{g L}^{-1}$, 24 h (\triangle) or 600 $\mu\text{g L}^{-1}$, 12 h (\bullet)

Figure 3. The survival (mean \pm standard deviation, n=3) of 16-d old *M. plumulosa* over the duration of the 10-d bioassays investigating the effects of the number of pulses of (A) 300 $\mu\text{g L}^{-1}$, 48 h and (B) 400 $\mu\text{g L}^{-1}$, 32 h

Figure 4. Three pulses between 4- and 48-h duration plus a continuous exposure of 300 $\mu\text{g L}^{-1}$ copper to *M. plumulosa* over a 10-d bioassay: (A) measured concentrations of dissolved copper and (B) survival (mean \pm standard deviation, n=3). Bars with symbols indicate treatments were significantly different from controls, treatments with the same symbol were not significantly different, and treatments with different symbols were significantly different.

Figure 5. The measured concentrations of dissolved copper in tests employing equivalent exposure TACs to investigate (A) the effect of the interval between multiple exposures of 400 $\mu\text{g Cu L}^{-1}$, and (B) the effect of varying the concentration and duration

Figure 6. The relationship between the mean 10-d survival of *M. plumulosa* and dissolved copper TAC for single, double, triple, and continuous exposures

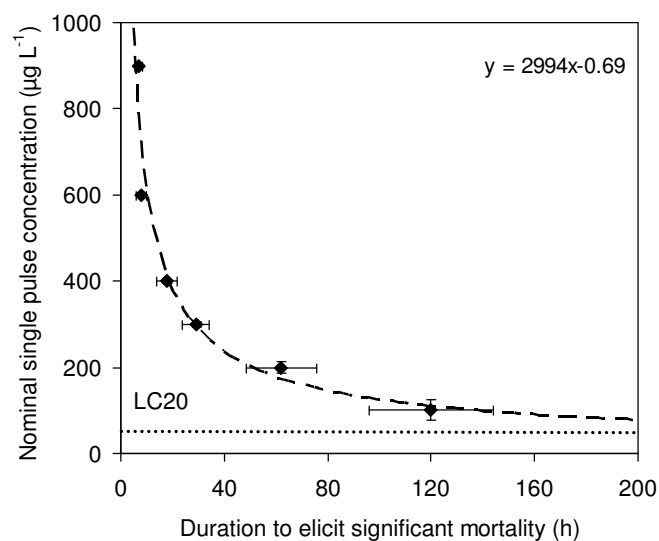


Figure 1. Mortality of 16-d old *M. plumulosa* following 10-d bioassays (20 individuals, 3 replicates per treatment, mean \pm standard deviation): the relationship between the duration of a single pulsed exposure and the pulse concentration

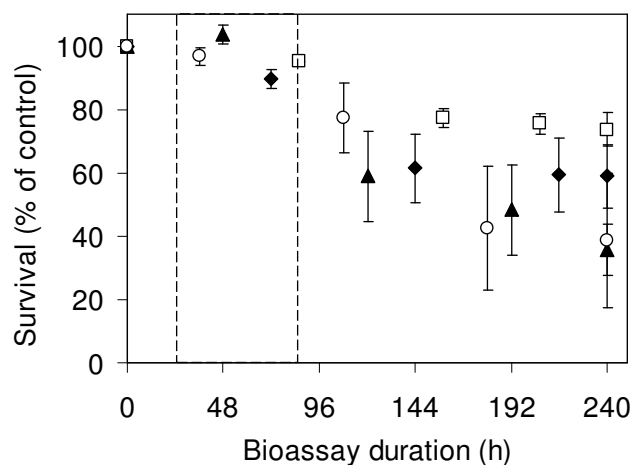


Figure 2. Latent mortality (mean \pm standard deviation, $n=3$) of 16-d old *M. plumulosa* following single exposures within the 24-86-h period (dashed lines): dissolved copper pulses of 200 $\mu\text{g L}^{-1}$, 62 h (□), 300 $\mu\text{g L}^{-1}$, 48 h (◆), 400 $\mu\text{g L}^{-1}$, 24 h (△) or 600 $\mu\text{g L}^{-1}$, 12 h (●)

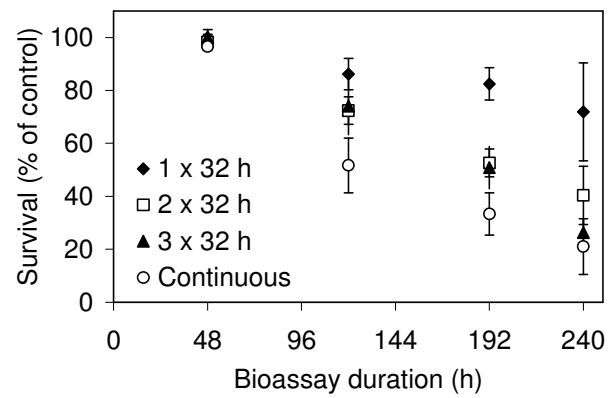
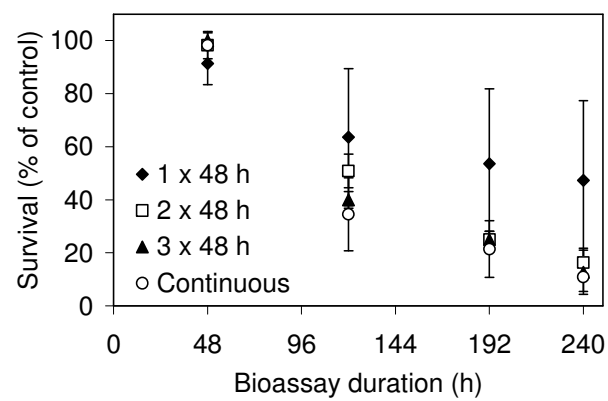
A**B**

Figure 3. The survival (mean \pm standard deviation, $n=3$) of 16-d old *M. plumulosa* over the duration of the 10-d bioassays investigating the effects of the number of pulses of (A) 300 $\mu\text{g L}^{-1}$, 48 h and (B) 400 $\mu\text{g L}^{-1}$, 32 h

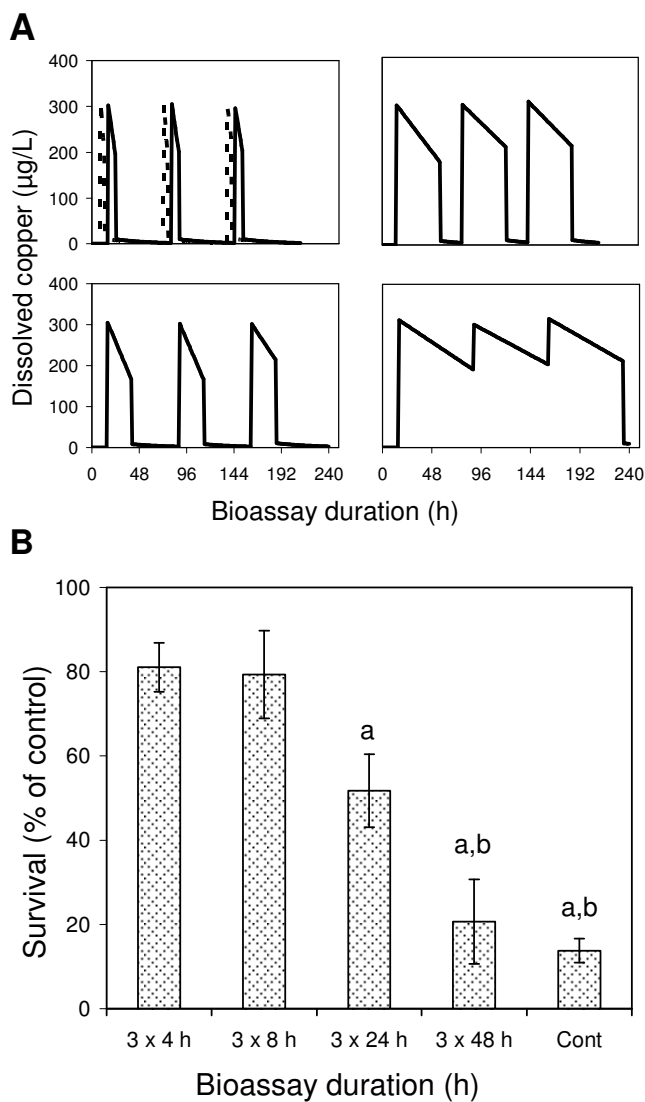


Figure 4. Three pulses between 4- and 48-h duration plus a continuous exposure of $300 \mu\text{g L}^{-1}$ copper to *M. plumulosa* over a 10-d bioassay: (A) measured concentrations of dissolved copper and (B) survival (mean \pm standard deviation, $n=3$). Bars with symbols indicate treatments were significantly different from controls, treatments with the same symbol were not significantly different, and treatments with different symbols were significantly different.

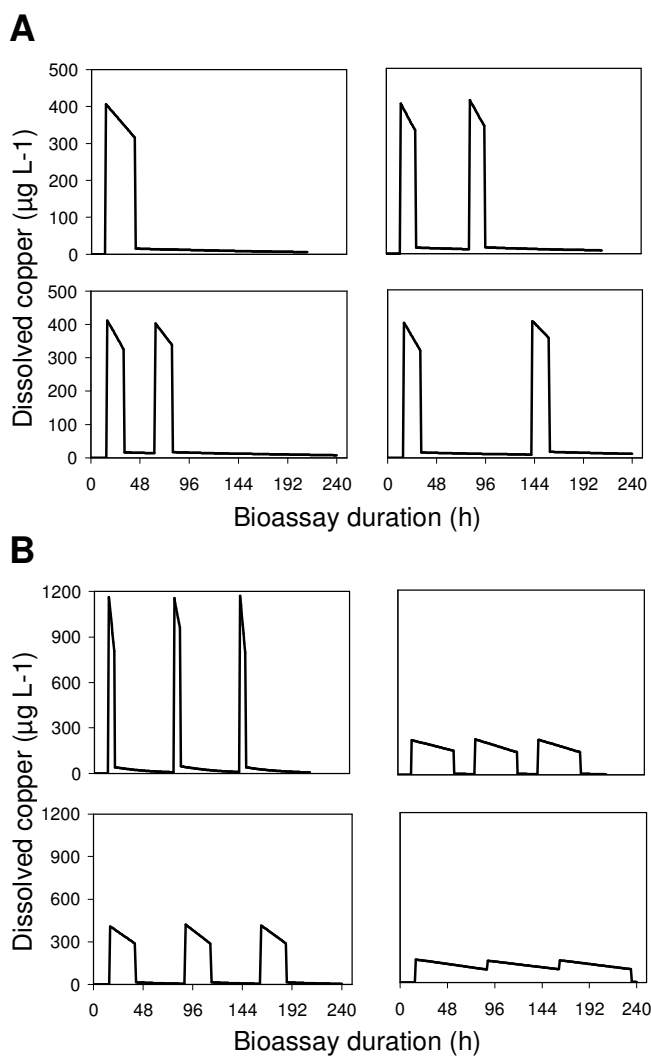


Figure 5. The measured concentrations of dissolved copper in tests employing equivalent exposure TACs to investigate (A) the effect of the interval between multiple exposures of $400 \mu\text{g Cu L}^{-1}$, and (B) the effect of varying the concentration and duration

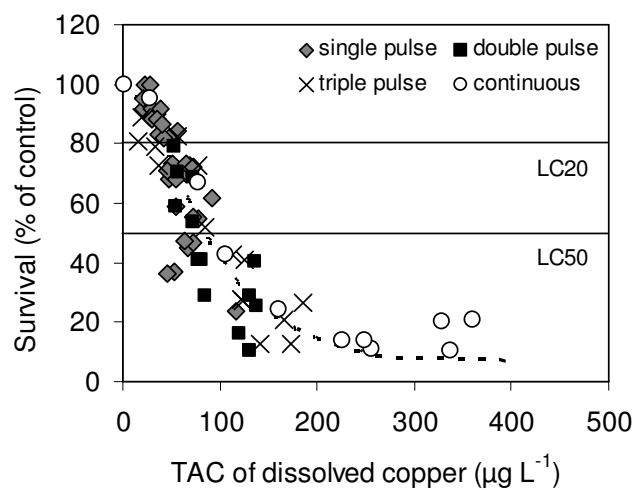


Figure 6. The relationship between the mean 10-d survival of *M. plumulosa* and dissolved copper TAC for single, double, triple, and continuous exposures

Table 1. The parameters of pulsed exposure bioassays

Number of pulses	Pulsed exposure period		Recovery between pulses	
	Duration(s) compared (h)	Nominal concentration (µg Cu/L)	Duration (h)	Nominal concentration (µg Cu/L)
Effect of concentration and duration				
1	24, 48, 96, 160	100	NA	5
1	10, 20, 35, 62	200	NA	10
1	6, 12, 19, 29	300	NA	15
1	4, 10, 18, 30	400	NA	20
1	4, 8, 12, 19	600	NA	30
1	4, 7, 10, 18	900	NA	45
Effect of single and multiple pulses				
1, 2, and 3	32	400	72	20
1, 2, and 3	48	300	72	15
Effect of multiple pulse duration ^a				
3	4, 8, 24, 48, continuous	300	68, 64, 48, 24, 0	15
Effect of interval between pulses ^a				
1	48	300, 400	0	15, 20
2	24	300	48, 96, and 144	15
2	16	400	30, 56, and 120	20
Effect of varying pulse concentration and duration ^a				
3	6	1160	66	58
3	24	410	48	21
3	48	220	24	11
Continuous	Continuous	150	-	-

NA = not applicable as only single pulse.

^a Comparisons made for equivalent time average concentrations (TAC).