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The use of time-averaged concentrations of metals to predict the toxicity of pulsed complex effluent exposures to a freshwater alga

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Abstract

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The use of time-averaged concentrations of metals to predict the toxicity of pulsed complex effluent exposures to a freshwater alga

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ABSTRACT

Intermittent, fluctuating and pulsed contaminant discharges may result in organisms receiving highly variable toxicant exposures. This study investigated the toxicity of continuous and pulsed exposures of a complex, neutralised drainage water (NDW) and dissolved copper-spiked dilute NDW to the green alga, *Pseudokirchneriella subcapitata*. The effects of single pulses of between 1 and 48 h duration and continuous exposures (72 h) on algal growth rate inhibition were compared on a time-averaged concentration (TAC) basis. Algal growth rates generally recovered to control levels within 24 to 48 h of the pulse removal. Continuous exposures to NDW resulted in similar or marginally higher toxicity to the algae when compared to pulsed exposures of equivalent TAC (% NDW). The toxicity of the NDW was attributed mostly to the metals, with the major cations potentially causing effects that are both additive (direct toxicity) and antagonistic (lower bioavailability of trace metals). For dissolved copper in dilute NDW, the pulsed exposures caused slightly higher toxicity than continuous exposures of equivalent dissolved copper TAC, with much of the difference explained by differences in labile copper concentrations between treatments. The results indicate that water quality guideline values for toxicants derived from continuous chronic exposures may be relaxed for pulsed exposures by a factor related to the TAC with the intent to provide an adequately protective but not overly-conservative outcome. The study highlights the influence that natural water quality parameters such as water hardness and DOC can have metal speciation and toxicity, and indicates that these parameters are particularly important for site-specific water quality guideline value derivation where, on a TAC basis, pulsed exposures may be more toxic than continuous exposures typically used in guideline value derivation.

CAPSULE ABSTRACT

This study investigated the effects of continuous and pulsed exposures of a complex effluent in natural water to a sensitive microalgal species and assessed the ability of time-averaged concentrations to predict toxicity.

1. INTRODUCTION

Waterways may experience significant temporal fluctuations in concentrations of contaminants as a result of industrial, agricultural and urban-runoff inputs (Burton et al., 2000; Simpson et al., 2014). This may be particularly pronounced in close proximity to effluent discharge points, where concentrations of chemicals in receiving waters may rapidly decrease after the discharge has ended (Angel et al., 2010a; Beck and Birch, 2014; Simpson et al., 2014). Variations in rainfall duration, frequency and intensity also have a strong influence on contaminant concentration in waters receiving stormwater inputs (Burton et al., 2000). As a consequence, organisms within the receiving environment are frequently exposed to pulses, rather than constant concentrations of contaminants, and the assessment of the risk posed by the varying water quality (i.e. contaminants and associated non-chemical stressors such as variations in water pH, salinity, dissolved oxygen, suspended solid concentrations) should be considered (Gordon et al., 2012; Hoffman et al., 2000; Hogan et al., 2013). Pulses of dissolved metals may also occur in continuous exposure toxicity tests when the solubility limit is exceeded (Angel et al., 2015; Gilmore et al., 2016). Most whole effluent toxicity (WET) / direct toxicity assessment (DTA) test methods have been developed to assess continuous point source discharges, but frequently are applied in the same manner to episodic discharges (US EPA, 2002a, b).

There is generally a poor understanding of the magnitude, duration and frequency of pulsed exposure events or the biological effects resulting from such exposures. Few studies have investigated the effects of pulsed exposures of complex effluents that are typical of many trade waste and industrial discharges (i.e. often containing multiple potential chemical toxicants and other stressors), and there is little understanding of how factors that influence the bioavailability of contaminants may modify the outcomes when assessing potential impacts from episodic discharges that result in pulsed exposures to organisms. For example, variations in the ionic compositions (e.g. water conductivity, hardness) and dissolved organic matter will influence the bioavailability of many contaminants, particularly metals (Paquin et al., 2002; Apte et al., 2005). A number of studies have investigated the effects of pulsed exposures of a few chemicals added to synthetic media (Angel et al., 2017; Diamond et al., 2005; Hoang et al., 2007), and there is a need for more information on the effects of pulsed exposures in natural waters of chemical mixtures (e.g. effluents) and contaminants whose bioavailability is strongly influenced by water composition. The regulation of effluent discharges is likely to be improved by the consideration of these factors in assessment frameworks.

In previous studies, we have observed that the time-averaged concentration (TAC, where the TAC is equivalent to the net exposure the organisms receive) of pulsed dissolved copper exposures was a good predictor of toxicity to the marine amphipod, *Melita plumulosa* (Angel el al., 2010b), the marine alga, *Phaeodactylum tricornutum* (Angel el al., 2015a), and the freshwater algae, *Psedokirchneriella subcapitata and Chlorella* sp (Angel el al., 2017). The designs of many other studies have not always enabled a direct comparison of the observed organism toxicity with the TAC of the major toxicants, however much of the literature indicates that over a range of pulse duration-magnitude exposures, the toxicity to organisms is of a similar or lesser magnitude for exposures to pulses that result in a similar or lower TAC of the major toxicants (Diamond et al., 2005; Hoang et al., 2007; Naddy et al., 2000). Beyond the different study methodologies, the comparison of toxicity and toxicant exposure on a TAC-basis may be complicated by many factors, including changes in the organism's life-stage sensitivity over the time-scale of longer toxicant exposure durations, acclimatisation of organisms following multiple exposures, and variations in recovery between pulses before test endpoints are assessed (Chen et al., 2012; Diamond et al., 2006; Hoang and Klaine, 2007; Hosmer et al., 1998; Naddy et al., 2000). When similar toxicity is observed for different types of exposure with equivalent TACs, the toxicant exposure duration and concentration may be varied in an inversely proportional manner and result in similar toxicity to an organism.

The current study investigated the toxicity of a freshwater effluent that contained known concentrations of metals and major ions that were expected to cause toxicity to aquatic organisms. The test organism studied was a freshwater microalga (*P. subcapitata*), selected as microalgae are among the most sensitive organisms to contaminants (Golding et al., 2015; Stauber and Davies, 2000). The position of microalgae at the bottom of the food chain implies that their toxicity during exposure and rate of biomass recovery post exposure affects higher trophic organisms directly through toxicant entry into the food chain and indirectly when decreased biomass leads to starvation of grazing invertebrates.

Two hypotheses were evaluated: (i) that exposures (continuous and pulsed) with an equivalent TAC (% of effluent water) result in equivalent toxic effects, and (ii) that the algal population recovers rapidly from pulsed exposures of ≤48 h in duration. Two series of waters were studied: (i) a neutralised drainage water (NDW) from a dairy farm area impacted by acidity due to oxidised acid-sulfate soils; and (ii) a less-contaminated natural water spiked with dissolved copper (CuW), for comparison to previous studies conducted in synthetic freshwater, and for assessing the influence of metal bioavailability. The algal growth rate inhibition (toxicity) resulting from continuous and pulsed exposures was compared on a TAC basis for all tests, and algal cell densities of treatments were compared to controls to measure algal toxicity and recovery during and after exposure, respectively.

Particularly for metals, the toxicity of waters is most strongly related to the concentrations of non- or weakly complexed metals (e.g. 'free' aqua ions and weak inorganic metal-ligand complexes) (Campbell, 1995; Paquin et al., 2002). An appreciable portion of filterable metals in many natural waters may be strongly complexed by organic ligands or in colloidal forms that are non-labile and exhibit low bioavailability and hence toxicity (Apte et al., 2005; Bowles et al., 2006; Eriksen et al., 2001). Only a few studies have compared organism toxicity from continuous and pulsed toxicant exposures in natural waters where metal complexation may significantly influence the observed toxicity. In the present study, we used measurements of metal lability to provide information on metal bioavailability, where greater lability is predicted to result in greater metal bioavailability. The contribution of major cations to the observed toxicity was also evaluated.

2. METHODS

2.1 Test waters

Two natural waters were collected and filtered through an acid-washed 0.45 µm cartridge filter (Sartobran P sterile midicap, Sartorius Stedium Biotech, Germany) and refrigerated in acid-washed 5-L high-density polyethylene containers in the dark below 4 ^{o}C until use. Approximately 20 L of an acidic, metal-rich, water was collected from a drainage channel of a farm at Mobilong, South Australia (Simpson et al., 2014). At this location the soils are impacted by acidity due to oxidised acid-sulfate soils that result in the farm drainage waters containing a range of metals and metalloids at concentrations that exceed Australian and New Zealand freshwater water quality guideline values (WQGVs) (ANZECC/ARMCANZ, 2000), and potentially a number of farm-derived chemicals (trace concentrations of some herbicides below WQGVs). Approximately 60 L of river water was collected from the Murray River, a few kilometres upstream of where the farm drainage waters are discharged to the river system on an intermittent basis. The farm drainage water pH was 3.2, and the Murray River pH was 7.8 and had an alkalinity of 95 mg $CaCO_3$/L which resulted in quite rapid neutralisation of the acidic drainage water inputs (Mosley et al., 2015).

The neutralised drainage water (NDW, pH 7.8) was prepared by adding small volumes of 1 M NaOH (Merck) to the Mobilong water without resulting in significant dilution of the drainage water. The NDW was allowed to stand at room temperature for 24 h before it was filtered (0.45 µm) to remove precipitates, after which it was used within 2 h to prepare test treatments. Metal and major cation concentrations at various pH values are shown in Table S1 of the Supporting Information (SI).

Two series of water treatments were used for ecotoxicity tests: NDW series and CuW series. The NDW treatments were prepared by diluting the NDW in Murray River water, where NDW-100, NDW-50, NDW-30, NDW-10, NDW-2 correspond to 100% (undiluted), 50%, 30%, 10% and 2% NDW dilutions, respectively. The NDW dilutions were selected following preliminary toxicity testing that covered a full range of toxic responses (i.e. 0 to 100% toxicity). A feature of the NDW series was the relatively high concentrations of Al, Co, Ni, Mn and Zn (Table S1 and S2), but not copper (<1.5 µg/L) compared to Australian and New Zealand water quality guideline values for 95% species protection (ANZECC/ARMCANZ, 2000).

The CuW treatments were prepared by spiking dissolved copper ($CuSO_4.5H_2O$ (AR grade, APS Finechem) in deionised water) into diluted NDW-2 (i.e. 2% NDW in Murray River water). The NDW-2 was chosen for these tests after preliminary testing of copper toxicity in NDW-2 and NDW-10, as algae were more sensitive to copper in this water. The CuW treatments were spiked with 5, 10, 20, 25,

30, 40 and 50 µg Cu/L (named CuW-5, CuW-10, CuW-20, etc.). The purpose of the CuW series was to make a comparison of continuous and pulsed copper exposure in natural waters with the findings of previous studies in synthetic waters (Angel el al., 2010b; 2015a; 2017), as the TAC may be affected by changes in copper speciation due to complexation in natural waters.

2.2. Algal cultures and bioassay preparation

Toxicity tests were performed with the single-celled freshwater green microalga, *P. subcapitata,* using bioassay protocols based on the OECD Guideline 201 (2005). Toxicity test treatments were inoculated with relatively low initial algal cell densities of 5 ($\pm$0.5) $\times 10^3$ cells/mL so that they were representative of those typically found in aquatic systems, and to ensure algal growth was not limited by a lack of nutrients or CO_2 during testing (Angel et al., 2015b). Algal cell densities were counted using a 4-color BD-FACSCalibur™ (Becton Dickinson BioSciences, San Jose, CA, USA) flow cytometer, and the measurement of a dilution series of each algal species was used to validate the accuracy of algal cell density measurements in the 10^3 cells/mL range (Angel et al., 2017).

The algal cell densities in toxicity test treatments were counted daily and the algal growth rates were determined using linear regression analysis to fit log_{10} cell density versus time (h) for each sample. The growth rate (i.e. cell division rate) was determined from the regression slope and expressed in terms of doublings/d and expressed as % toxicity (inhibition of cell division rate). Effect concentrations (EC) were calculated using measured concentrations of the treatment (% NDW, µg Cu/L), to determine the concentrations resulting in 10, 20 and 50% toxicity after 72 h tests compared to controls (i.e. EC10, 20 or 50). The EC values were calculated using linear interpolation of the logistic sigmoidal model described by Barnes et al. (2003). Additional details on the culturing of the algae, preparation of culture media, the bioassay procedures and quality assurance are shown in Section S1.1 of the SI.

2.3. Pulsed-exposure bioassays

To simulate exposures after discharges enter a river (treatments representative of what organisms are exposed to in the field), the *P. subcapitata* was inoculated into water treatments (NDW and CuW series) within 20 min of their preparation. Pulsed exposures were ended by replacing approximately 99% of treatment water with control water using centrifugation as described previously (Angel et al., 2015a; 2017). This approach does not cause toxicity in control treatments, and further details are provided in Section S1.2 of the SI. The control waters (exposure between pulses) were Murray River water for the NDW series and Murray River water plus NDW-2 for the CuW series.

Preliminary continuous exposure tests were performed for both the NDW and CuW treatments to determine effect thresholds in order to assist with pulse test design (i.e. choice of concentrations and durations). These continuous exposure test series were repeated at the time of each pulse exposure experiment, to check the repeatability of the algal sensitivity (72-h growth rate IC) test results. The control and continuous exposure treatments were subjected to the same centrifugation and water renewal procedure as the pulse exposures so that the algae within all treatments received the same disturbance due to handling.

The pulsed-exposure experiments investigated the effects of pulse duration: 24, 48, and 72 h for NDW-100 (undiluted); 1, 5, 24 and 48 and 72 h for CuW40 (40 µg/L) treatments (Table 1). Pulsed exposure durations of ≤48 h were tested because the total test duration was 72 h and pulses up to 48 h in duration were considered appropriate durations to mimic those that occur in the field. For example, rainfall events of hours to days in duration may result in overflow of waters from drainage channels into the Murray River, and controlled pumping to discharge usually occurs for less than 1 day. The NDW was tested undiluted and with duration ≥24 h to achieve a toxic response, as minimal toxicity was elicited at lower concentrations and/or durations.

2.4. Water composition influencing toxicity

The NDW contained a range of metals (Al, Co, Mn, Ni, and Zn) and major cations (Ca, K, Mg and Na) that were expected to contribute to the observed toxicity due to their high concentrations (Tables S1 and S2 of the SI). To assess the influence of these elements on the toxicity, continuous exposure tests were undertaken on treatments that were prepared by spiking Murray River water with: (i) a metal mixture (Al, Co, Ni, Zn), (ii) manganese alone, and (iii) a major cation mixture (Ca, K, Mg,

Na), in a similar manner to the testing of natural and synthetic mine water reported by van Dam et al. (2014a). Treatments were prepared in triplicate, in which the concentrations of the elements were intended to be the same as those in the NDW-2, -30 or -100 treatments. The toxicities of these waters were compared to the toxicity of the NDW.

2.5. Calculation of time-averaged concentrations

For each exposure treatment (continuous and pulsed), the concentration of metals and major cations of interest was measured immediately after spiking, before and after water renewal, and at the end of the 72-h tests. Linear changes between each measured dissolved metal concentration were assumed to allow exposure models to be constructed that had 0.25 h increments. These exposure models / profiles represented the increases (pulse) and decreases (pulse removal or losses due to adsorption, etc.) in dissolved metal concentration that occurred over the 72-h test periods (e.g. Figure S1 of the SI). The TAC was calculated as the averaged of each 0.25 h increment over the 72-h test and is equivalent to the average area under the exposure curve (Angel et al., 2015a; 2017).

For the NDW treatments and the test investigating the toxicity of different components (metal mixture, manganese, and major cation mixture) of the NDW (Section 2.4), the TACs of individual metals and major cations were converted to percentages based on their proportion of the initial (t=0 h) concentration in undiluted NDW. The average of all individual metal or major cation % TACs in each of these treatments was calculated to determine the mean % TAC so that a comparison of these treatments with the NDW treatments could be made on the same scale. The 72-h algal growth rate inhibition (toxicity) was compared on the basis of the calculated %TAC for NDW treatments and the TAC of dissolved copper (the main toxicant) for CuW treatments (Angel et al., 2015a; 2017).

2.6. Measurement of Chelex-labile metals and copper complexing capacity

For selected water treatments, the proportion of the dissolved metals present in labile forms was determined using measurements of Chelex-labile metals, and for copper, the copper complexing capacity of the water was also determined, as described by Bowles et al. (2006). A detailed description of the procedure and quality control results is shown in Section S1.3.

2.7. General analytical methods

All glass and plasticware was cleaned by soaking in 10% (v/v) HNO_3 (BDH, Analytical Reagent grade) for a minimum of 24 h followed by thorough rinsing with ultrapure water before use in chemical analyses and toxicity testing (Milli-Q, 18 MΩ·cm; Academic Water System). Measurements of water pH were made using a meter (Wissenschaftlich-Technische Werkstattan) and probe (Orion sure-flow combination pH 9165BN) following calibration using pH 4.0 and 7.0 buffers (Orion Pacific, Sydney, NSW, Australia).

Test solutions for dissolved metal analysis were filtered (acid-washed 0.45-µm cellulose nitrate filters, 25 mm, Sartorius Minisart) through plastic syringes (Terumo), and acidified (0.2% v/v, Tracepur, Merck) before analysis by inductively coupled plasma atomic emission spectrometry (ICPAES) (Varian) (Angel et al., 2015b). The ICPAES was calibrated with matrix-matched calibration standards and operated using conditions recommended by the manufacturer. The analytical quality control for Al, Cd, Co, Cr, Cu, Fe, Mn, Ni and Zn included analysis of three matrix-matched blank solutions for calculation of the limit of detection (3σ, 0.06-0.5 µg/L), spike recoveries (92-103%) to ensure there were no matrix artefacts, and measurement of the certified reference water TM28.4 (National Research Centre, Canada) to validate the accuracy of the analysis (88-105%).

Table 1. The tested treatment conditions, time-averaged concentrations (TACs), and toxicity of pulsed NDW or CuW exposures to *Pseudokirchneriella subcapitata*

Treatment	Duration (h)		Concentration (% NDW)		72-h TAC (% NDW)	72-h toxicity (%)
	exposure	Recovery	Nominal [a]	Measured [b]		
Control (Murray River water, NDW-0 continuous)	72	-	-	0 ± 0.6	0 ± 0.6	0 ± 2.2
NDW-2 continuous	72	-	2	2.2 ± 0.1	2.2 ± 0.1	0 ± 2.6
NDW-10 continuous	72	-	10	10.3 ± 1.6	10.3 ± 1.6	0 ± 0.8
NDW-20 continuous	72	-	20	19.7 ± 0.3	19.7 ± 0.3	9 ± 1
NDW-33 continuous	72	-	33	31.4 ± 0.2	31.4 ± 0.2	28 ± 5
NDW-50 continuous	72	-	50	47.3 ± 2.1	47.3 ± 2.1	63 ± 9.1
NDW-67 continuous	72	-	67	66.0 ± 0.2	66.0 ± 0.2	97 ± 1
NDW-100 continuous	72	-	100	99.9 ± 0.3	99.9 ± 0.3	100 ± 0.4
NDW-100 24-h pulsed	24	48	100	98	36 ± 2.5	35 ± 3
NDW-100 48-h pulsed	48	24	100	97	67 ± 3.6	71 ± 4

	Duration (h)		Concentration (µg Cu/L)		72-h TAC (µg/L)	72-h toxicity (%)
	exposure	Recovery	Nominal [a]	Measured [b]		
Control (Murray River water)	72	-	-	0.7 ± 0.2	0.7 ± 0.2	0 ± 2.6
Control (CuW-0 continuous)	72	-	-	0.9 ± 0	0.9 ± 0	0 ± 0.4
CuW-5 continuous	72	-	5	4.6 ± 0.3	4.6 ± 0.3	0 ± 1.1
CuW-10 continuous	72	-	10	7.7 ± 0.9	7.7 ± 0.9	0.9 ± 1.1
CuW-20 continuous	72	-	20	15.4 ± 1.1	15.4 ± 1.1	25 ± 2.5
CuW-25 continuous	72	-	25	19.5 ± 1.3	19.5 ± 1.3	39 ± 2.1
CuW-30 continuous	72	-	30	23.6 ± 1.2	23.6 ± 1.2	58 ± 1.9
CuW-50 continuous	72	-	50	39.9 ± 1.5	39.9 ± 1.5	101 ± 2.2
CuW-40 1-h pulsed	1	71	40	34.1	2.3 ± 0.1	2 ± 3
CuW-40 5-h pulsed	5	67	40	32.9	4.5 ± 0.3	9 ± 4
CuW-40 24-h pulsed	24	48	40	32.0	11.9 ± 0.3	30 ± 4
CuW-40 48-h pulsed	48	24	40	31.8	21.5 ± 0.3	75 ± 8
CuW-40 continuous	72	-	40	-	33.4 ± 0.5	81 ± 6

[a] Nominal NDW or CuW (in NDW-2) concentration spiked into treatments at start of tests
[b] Measured pulse concentrations are the means of measured values at the beginning and end of each pulse duration. Measured continuous exposure concentrations are the 72-h TAC (% NDW) or 72-h TAC
Measured pulse concentration, 72-h TAC (% NDW) and TACs, and inhibition are the mean of three replicate measurements, of which the errors shown represent one standard deviation of the mean

2.8. Statistical analyses

The logistic sigmoidal model of Barnes et al. (2003) was used to calculate the 10, 20 and 50% effect concentrations (EC10, EC20 and EC50), and had its constants derived by minimising the residuals between the measured data and the model. The model calculations were applied to pooled data from each type of test, with three continuous and two pulsed exposure test replicates performed for the NDW and CuW series.

Differences in the mean percentages of 72-h toxicity (growth rate inhibition) from continuous and pulsed exposure test treatments were examined using a one-factor analysis of variance for tests comparing equivalent TACs (ANOVA) (NCSS statistical software, Kaysville, UT). A repeated measure of means test was employed to test for significant (p<0.05) differences in cell division rates over successive 24-h intervals in tests investigating recovery (from toxicity, i.e. return of growth rate to control level) from single copper pulsed exposures. Kurtosis, Omnibus, and Levene tests were used to test for normality and homogeneity of variances. No transformations were required as these assumptions were not violated. The Tukey-Kramer multiple-comparisons test was used as a post-hoc procedure to test for multiple comparisons upon means (NCSS statistical software).

3. RESULTS AND DISCUSSION

The concentrations of dissolved metals and major cations measured in a preliminary test of the drainage water before neutralisation and 24 h after neutralisation to pH values up to 7.8 are shown in Table S1 of the SI. The NDW had 101, 348, 8280, 381 and 86 µg/L of Al, Co, Mn, Ni and Zn, respectively, and 707, 81, 750 and 7710 mg/L of Ca, K, Mg and Na, respectively (Table S2 of the SI). The CuW (i.e. NDW-2) water had 3, 6, 177, 9 and 1 µg/L of Al, Co, Mn, Ni and Zn, respectively, <0.5 µg/L Cu, and 21, 4, 20 and 220 mg/L of Ca, K, Mg and Na, respectively (Table S2 of the SI). The dissolved organic carbon (DOC) concentrations of the NDW-100, NDW-10, NDW-2 and Murray River water were 16.7, 4.74, 3.80 and 3.61 mg/L, respectively (Table S5 of the SI), which was proportional to the dilution factor. The dissolved inorganic carbon concentrations of the NDW-100, NDW-10, NDW-2 and Murray River water were 0.58, 10.7, 11.4 and 11.6 mg/L, respectively (Table S5 of the SI),. The hardness of the NDW-100, NDW-10, NDW-2 and Murray River water were 4840, 507, 101 and 6.6 mg $CaCO_3$/L, respectively.

3.1. Comparison of the toxicity of continuous and pulsed exposures of the NDW

The quality control / quality assurance criteria were met in all toxicity tests; the growth rates of controls were >2.4 doublings/day, replicate means had relative standard deviations <5%, and the pH did not change by more than 0.3 over the duration of tests.

The relationship between algal toxicity (growth-rate inhibition) and continuous and pulsed exposures to NDW, represented as time-averaged concentrations (TAC, % NDW) for dissolved metals is shown in Figure 1A. For the continuous NDW exposure series, there was no significant (p>0.05) algal toxicity for dilutions up to NDW-10, above which toxicity was significant (p<0.05) and increased up to 100% toxicity for the NDW-100 (i.e. undiluted NDW). The EC10, 20 and 50 (and 95% confidence intervals) values calculated for the NDW series (on a TAC basis) were 27 (22-33), 32 (27-38) and 44 (40-48) % NDW for the continuous exposures, and 22 (19-26), 29 (26-33) and 46 (43-49) for the pulsed exposures, respectively. The overlap of confidence intervals for the corresponding ECx values of the continuous and pulsed exposures indicates that the NDW resulted in a similar (predictable) level of toxicity independent of the exposure scenario when compared on a TAC basis for toxicities ≤50%. There was, however, somewhat of a difference between continuous and pulsed NDW exposure for treatments with equivalent TACs at higher effects levels, with the greatest difference occurring for an equivalent TAC of NDW-67, where continuous exposure resulted in 26.4% higher toxicity than the pulsed exposure. This is not particularly concerning from a regulatory point of view as water quality guideline values are usually derived using low effect data such as EC10s (Warne et al., 2014).

The TAC-toxicity comparison was influenced by the procedure used to calculate the TAC for the NDW (i.e. due to the multiple metals and cations considered in its calculation). As the metals Al, Co, Ni and Zn exerted the greatest toxicity (see Section 3.2) the relationships between algal toxicity and the TAC were calculated using only the mean concentration of these metals for comparison (i.e. excluding the major cations and manganese) (Figure S2 of the SI) and found to be similar to that shown in Figure 1A.
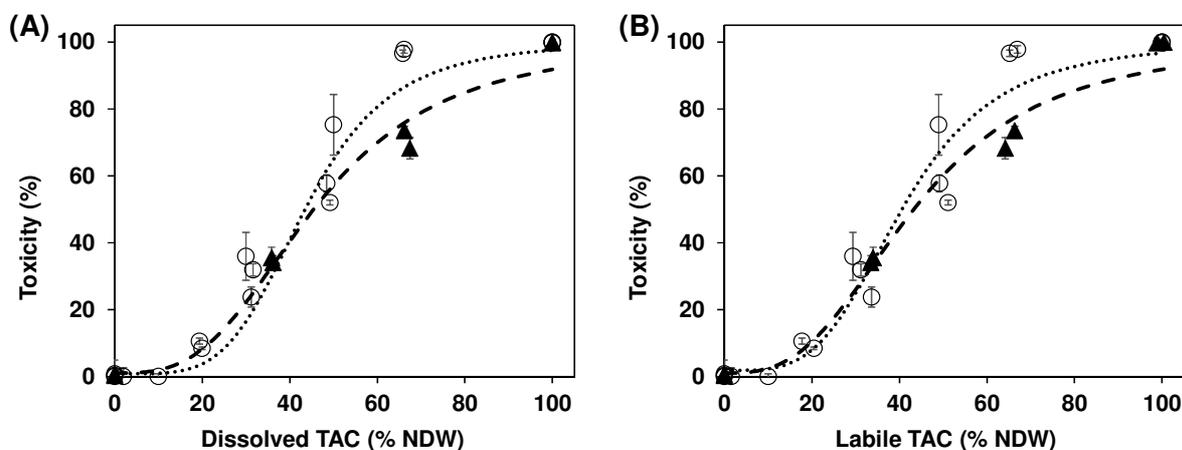

Fig. 1. The relationship between toxicity to *P. subcapitata* from continuous (○) and pulsed (▲) exposures and (A) the dissolved or (B) the labile time-averaged concentration (TAC) of Al, Co, Mn, Ni, Zn, Ca, K, Mg and Na in the neutralised drainage water (% NDW). The dotted and dashed model lines were used to calculate EC10, 20 and 50 values for continuous and pulsed exposure, respectively.

3.2. Influence of NDW composition on toxicity

The NDW contained a range of metals that were expected to contribute to the observed toxicity, however, changes in the ionic composition (e.g. concentration of Na, K, Ca, Mg, chloride, sulfate) may also contribute to toxicity (Simmons, 2012; van Dam et al., 2014a) and may also modify the toxicity of many of these metals through calcium and magnesium cations competing for binding sites at the algal surface (Campbell, 1995; Paquin et al., 2002). The elements in the NDW with the greatest dissolved concentrations (Al, Co, Mn, Ni and Zn, and Ca, K, Mg and Na) were considered to be the most likely to contribute to the observed toxicity, and TACs of these elements in the continuous NDW exposures are provided in Table S2 of the SI. Other elements or chemicals present in the NDW at lower concentrations that were considered unlikely to be responsible for the algal toxicity are shown in Table S3 of the SI.

A comparison between the toxicity of the NDW series and the Murray River water spiked with the elements (i) Al, Co, Ni, and Zn, (ii) manganese (alone), or (iii) Na, K, Mg, and Ca, at concentrations proportional to those elements in the NDW-2, -30, and -100 is shown in Figure 2 and Table S4 of the SI. For each of these element treatments, the toxicity increased with increasing TAC. For the element treatments nominally equivalent to NDW-100, the metal mixture resulted in comparable toxicity (94% toxicity), while lower toxicity than the NDW was measured for the manganese (6%) and major cation mixture (35%) treatments. For the element treatments nominally equivalent to NDW-30, the toxicity from the metal mixture (61%) was significantly (p<0.05) higher than that from that of NDW-30 (36%), while manganese (1%) and the major cations (10%) caused significantly (p<0.05) lower toxicity than NDW-30. Consequently, the toxicity of the NDW was attributed mostly to the metals, with the major cations potentially causing effects that are both additive (direct toxicity as shown by the toxicity of the major cation mixture treatments) and antagonistic (lower bioavailability of trace metals in presence of major cations, as shown by the metal mixture treatment causing greater toxicity than the equivalent NDW-30 treatment), and only a very small toxic effect from the manganese up to 8.3 mg/L. These findings indicate that *P. subcapitata* is relatively tolerant of waters containing high hardness, for example, despite a water hardness of 507 mg CaCO$_3$/L for the 10% NDW treatment the algae did not experience toxicity
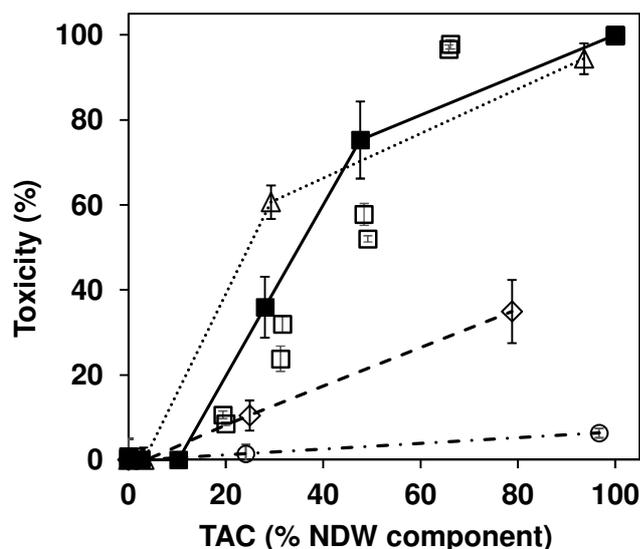
Fig 2. The toxicity of *P. subcapitata* for exposures to dilutions of NDW (■, □), and three element-treatments (in Murray River water) proportional to element concentration in the NDW: metal-mixture (Al, Co, Ni, Zn) (△), major cations (Na, K, Mg, Ca) (◇), and manganese (○). The ■, △, ◇, and ○ treatments were performed using the same batch of algae (i.e. same test) to compare the different element treatments. The □ continuous NDW treatment values are from pulsed NDW exposure tests where continuous exposure was also tested for comparison of toxicity.

3.3. Influence of metal lability

For the NDW, the Chelex-labile metal measurements in dilutions ≥10% determined that labile forms were a relatively high and constant portion of the total dissolved concentrations of Co (92-97%), Ni (78-88%) and Zn (94-98%), and relatively constant but lower for Al (52-66%). Chelex-labile Mn decreased with increasing total dissolved concentration (from 95% in NDW-10 to 26% in NDW-100) (Table S5 of the SI). For the NDW-2 treatment the percentages of Chelex-labile aluminium and nickel were lower than those measured for the lesser dilutions (≥10%), but these observation were probably influenced by the concentrations being close the analytical limit of detection and thus less accurate. For aluminium and manganese a significant portion of the non-labile fraction may have been in colloidal forms (Gundersen and Steinnes, 2003; Schemel et al., 2007; Simpson et al., 2014). The increasing Chelex-labile manganese concentration with increasing NDW dilution may indicate that the NDW contained higher concentrations of manganese complexing agents than the Murray River diluent water, or that the NDW contained a higher proportion of colloidal manganese (predominantly Mn (IV oxidation state)) which partially dissociated with increasing dilution (Laxen et al., 1984).

The relationship between algal toxicity and continuous and pulsed exposures to NDW, represented as time-averaged concentration (TAC, % NDW) for Chelex-labile metals is shown in Figure 1B. The relationships between toxicity of continuous and pulsed exposure Chelex-labile metal TAC were similar to those based on dissolved metal TAC (Figure 1A). Chelex-labile metal TACs resulted in a marginally more sensitive measure of toxicity as indicated by both the continuous and pulsed exposure curves (Figure 1B) being shifted slightly to the left of those for dissolved metal TAC (Figure 1A). A plausible reason for the similarity in trends between Figures 1A and B is that a similar proportion of each dissolved metal was in Chelex-labile forms over the dilution series (i.e. similar to NDW-100, Table S3 of the SI). In other words, the algae were exposed to similar labile metal TACs in continuous and pulsed exposures of equivalent dissolved TAC (% NDW). Manganese was an exception with a variable proportion of its dissolved concentration that was Chelex-labile over the dilution range, and was not included in the Chelex-labile TAC (% NDW) calculation because it had negligible effect on toxicity (Section 3.2).

3.4. Comparison of the toxicity of continuous and pulsed exposures of the copper-spiked water

The CuW series (2% NDW spiked with copper) were prepared and studied to provide comparison to past studies in our laboratory (Angel et al., 2010b; 2015a; 2017), including an assessment of toxicity trends in more complex waters where the copper speciation and bioavailability is strongly influenced by complexation with organic ligands (Apte et al., 2005; De Schamphelaere et al., 2004; Eriksen et al., 2001). The toxicity of continuous exposure to dissolved copper spiked into NDW-2 resulted in EC10, EC20, EC50 values (µg/L TAC-Cu, 95% confidence intervals), of 11.4 (10.5-12.3), 15.9 (14.2-17.8) and 21.4 (20.7-22.1) respectively (Figure 3).

The EC10, EC20 and EC50 values determined for pulsed CuW exposures were 6.6 (5.3-8.2), 9.1 (7.8-10.7), and 15.8 (14.7-17.1) µg/L, respectively (Figure 3A). Consequently, based on the TACs for dissolved copper, the pulsed exposure was more toxic than the continuous exposure. The complexation of dissolved copper by organic ligands was expected to influence the bioavailability of copper, so the comparison of the toxicity from continuous and pulsed copper exposures was made using Chelex-labile copper concentrations (Figure 3B) and the copper complexing capacity (CuCC) of the CuW treatment water was measured. The continuous and pulsed exposure dose-response relationships were more similar when the TAC was based on Chelex-labile copper, with EC50 values of 9.9 (9.4-10.4) and 8.0 (7.3-8.7) µg/L measured, respectively. The concentration-toxicity relationship was steeper for the relationship with Chelex-labile copper than dissolved copper, indicating this fraction is more important for toxicity.
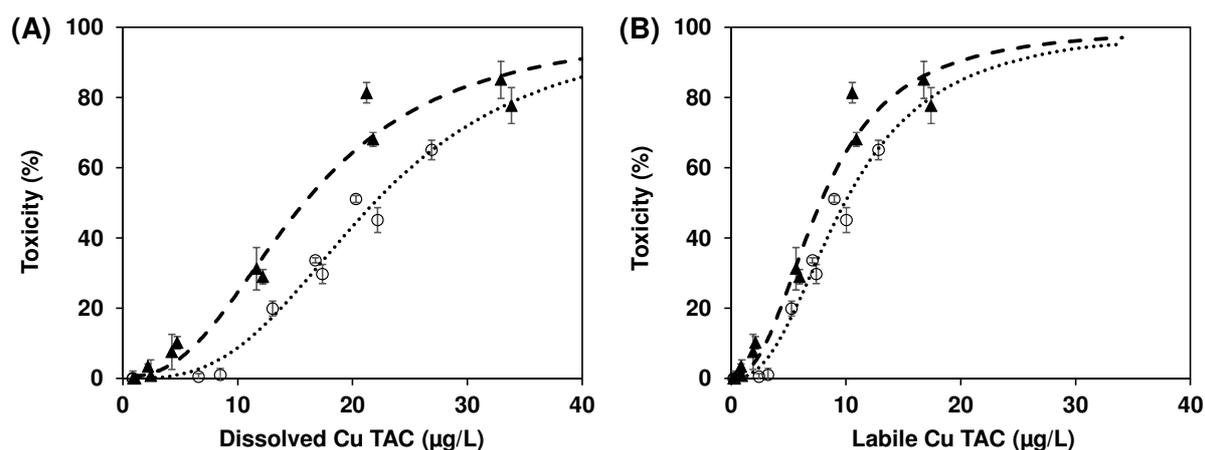

Fig. 3. The relationship between the toxicity of *P. subcapitata* from continuous (○) and pulsed (▲) exposures and the (A) dissolved or (B) labile copper TAC in 2% NDW. The dotted and dashed lines show the models used to calculate IC10, 20 and 50 values for continuous and pulsed exposure, respectively. Error bars are one standard deviation of three replicates for each treatment.

The Chelex-labile copper concentrations measured in tests investigating the effect of copper concentration and duration after spiking into CuW are shown in Table S6 of the SI. The percentage of dissolved copper in Chelex-labile forms increased as the spiked-copper concentration increased (Figure 4A), and the slope of the trend line for the relationship between Chelex-labile concentration and spiked concentration changed at copper-spike concentrations greater than approximately 15 µg/L (indicated by the two trend lines). The change in the slope of the line of best fit at 15 µg/L of dissolved copper corresponded with the exceedance of the copper complexation capacity CuCC of the water (14.6 µg/L) measured for CuW (i.e. unspiked NDW-2). For CuW treatments that had lower spiked-copper concentrations less than the CuCC there was still between 1.8 and 7.3 µg/L of labile copper, which indicates that the complexation is predominantly due to weak organic ligands (Apte et al., 1990).

The measurement of Chelex-labile copper over time after spiking CuW with 40 µg/L copper is shown in Figure 4B. The percentage of dissolved copper that was Chelex-labile had a marginal, but significant (p<0.05) decrease after 1 h, after which it decreased very slowly until 72-h post-spiking, indicating only small decreases in the labile concentration due to copper complexation occurred during pulsed exposures, the majority of which occurred in the first hour.
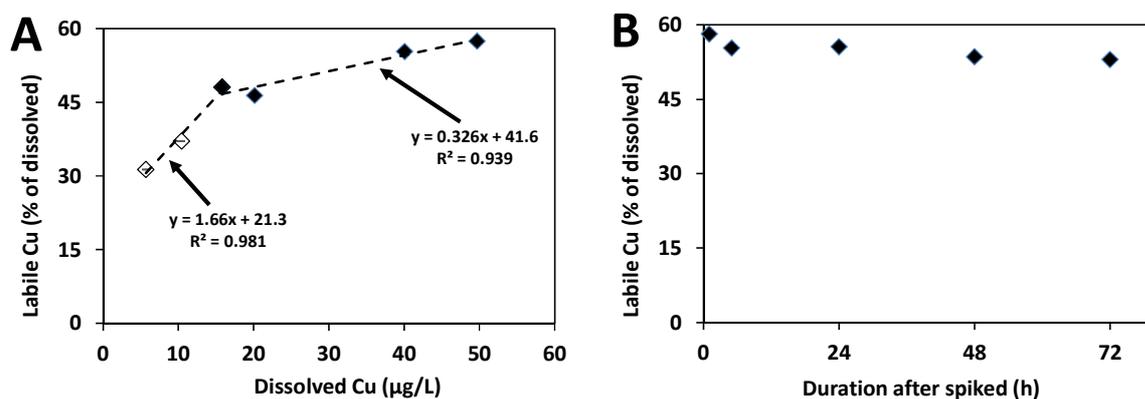
Fig 4. The relationship between the percentage of Chelex-labile copper and (A) dissolved copper concentration after 24 h equilibration in CuW water or (B) the duration after copper (40 µg/L) spiking into CuW water. Note, means (n=3) are shown for each treatment and error bars are present, but are too small to see.

3.5. Algal recovery following exposures

The toxicity to the algae for each of the 24-h time periods (0-24, 24-48, 48-72-h) during and after continuous and pulsed exposure to NDW-100 was assessed by comparing the cell division rates over each of these periods (Figure 5A). Significant ($p<0.05$) toxicity was observed during all 24-h periods in which the algae were exposed to the NDW-100. The algae growth rate (cell division) recovered rapidly after the pulsed exposures had ended, increasing significantly ($p<0.05$) in the 24-h period post-exposure (24-48 h period), and being similar (48-h pulse) or higher (24-h pulse) than the control during the 48-72 h period (i.e. 24-48-h after the pulse ended).

For CuW water spiked with 40 µg/L dissolved copper, significant ($p<0.05$) toxicity was observed in the initial 24-h period in which the copper exposure occurred only for treatments with copper pulse durations ≥5 h (Figure 5B). In the 24-h period(s) after the dissolved copper exposures had ended, the growth rates increased for most treatments. For the 5- and 24-h pulsed copper exposures, the growth rate increased significantly ($p<0.05$) in the 24-h period after the pulse ended. No toxicity was observed for the 5-h pulsed copper exposures in the monitoring period beyond 24 h. For the 24-h pulsed copper exposures, the toxicity in the 24-48-h period after the pulse had ended was also significantly lower ($p<0.05$) than in the 0-24 h period. No toxicity was observed for the 24-h pulsed copper exposures in the monitoring period beyond 48 h. The results indicated that toxicity to the algae decreases rapidly following both the pulsed mixed metal exposures (NDW, Figure 5a) and the dissolved copper pulsed exposures (CuW with 40 µg/L Cu) (i.e. growth rates rapidly recover).

The 24-48-h recovery timeframe is the same as that measured for pulsed dissolved copper exposures to the marine alga, *Phaeodactylum tricornutum* in natural seawater, and the freshwater algae, *P. subcapitata* and *Chlorella* sp. in synthetic freshwater (Angel et al., 2015a; 2017). For many microalgal species, a 24-h period allows for approximately two cellular divisions / doublings, so the 24-48-h timeframe for recovery is relatively long compared to the algal lifecycle. Other higher trophic species may recover (from toxic exposures to certain contaminants) over timeframes equivalent to a fraction of their lifecycle so it is not surprising that algae can recover over such short periods after exposure has ended (Angel et al., 2010b; Brent and Herricks, 1998). The rapid decrease in toxicity to the algae once the exposure ceased has importance for assessing long-term impacts of short-term contaminant exposures in natural aquatic systems. The direct effects on algal populations and indirect effects to organisms that feed on them may be negligible when there is a sufficient duration between contaminant pulsed exposures to allow algal recovery to occur. Rapid recoveries of algal growth rates after termination of a contaminant pulsed exposure will also result in rapid dilution of any contaminant internalised during the exposure, thus reducing the transfer to higher trophic species via dietary exposure (Angel et al., 2015a; Cain et al., 2011).
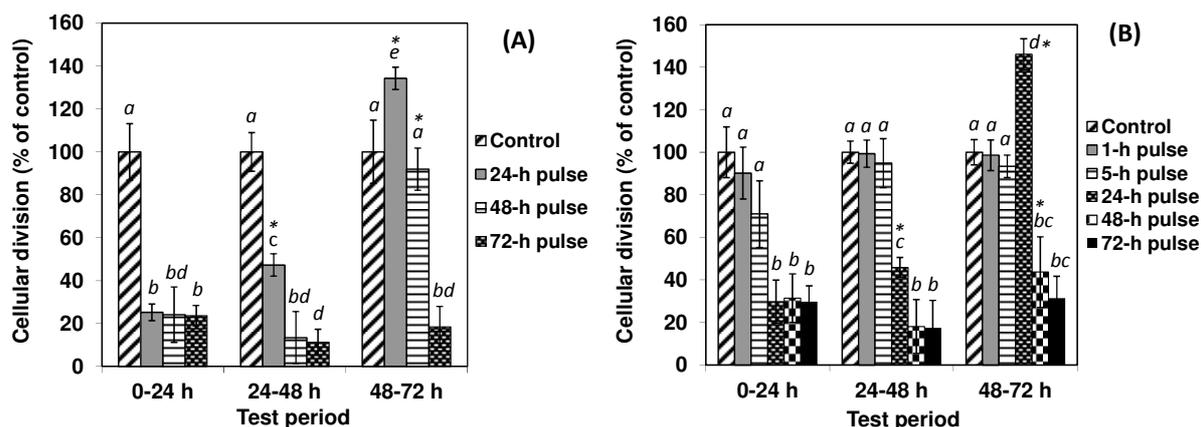
Fig. 5. Comparison of algal cell division rates over the three successive 24-h periods of the 72-h tests of continuous (72-h) and pulsed exposure to (A) NDW-100 and (B) CuW spiked with 40 µg/L dissolved copper. All pulsed exposures commenced at the start of the test (first 24-h period). Within the figures, the italic *a, b, c, d* and *e*, denote significant (p<0.05) differences between treatments relative to the controls (all '*a*'). The asterisks denote significant (p<0.05) increases in the rate of cell division in the two 24-h post-exposure periods compared to the initial 24-h period for any given exposure duration. Error bars are one standard error (n=3).

3.6. Factors affecting the toxicity of P. subcapitata in the tested natural waters

This study supports previous findings that major cations and/or organic ligands in natural waters can cause toxicity themselves, or have additive / antagonistic interactions with other toxicants (Apte et al., 2005; De Schamphelaere et al., 2003; Peters et al., 2011). Several studies have shown that water hardness has no effect on the toxicity of copper to the freshwater algae *P. subcapitata* and *Chlorella* sp. (De Schamphelaere et al., 2003; Markich et al., 2005). However, other studies have found the presence of major cations reduced the toxicity of metals such as Al (Parent and Campbell, 1994), Ni (Deleebeeck et al., 2009) and Zn (Heijerick et al., 2002) to *Chlorella pyrenoidosa* (Al) and *P. subcapitata* (Ni and Zn), supporting the lower toxicity of the NDW than the equivalent trace metal component treatment shown in Figure 2. The protective effects of major cations is interpreted as increased competition between ions such as calcium and magnesium for metal binding sites at a gill or algal cell membrane, as conceptualised in the biotic ligand model (Paquin et al., 2002).

The major cation element treatment was less toxic than the trace metal element treatment, but still elicited significant (p<0.05) toxicity for treatments equivalent to higher percentage NDW treatments (Figure 2). Major cations may directly affect freshwater organisms via osmotic stress related to the combined major ion concentrations (i.e. a general salinity effect), or toxicity of specific major ions. Simmons (2012) investigated the toxicity of Ca, K and Na on *P. subcapitata* growth inhibition and reported EC10s and EC50s in the range 80-690 and 470-1560 mg/L, respectively. Comparisons of major cation concentrations in the current study with those that caused toxicity in studies by Simmons (2012) and van Dam et al. (2014a) suggest that calcium, sodium and magnesium, but not potassium, are likely to have contributed to the toxicity of *P. subcapitata*.

Dissolved organic matter (DOM, as characterised by increased concentrations of DOC) is widely recognised as ameliorating the toxicity of some metals in natural water, particularly for algae (Apte et al. 2005; De Schamphelaere et al., 2003; 2004; Heijerick et al., 2005). There are several mechanisms by which the DOM could have ameliorated the copper toxicity to *P. subcapitata* in the current study. The first is that the ligands associated with DOM could complex dissolved copper and prevent or slow availability at the algal cell surface uptake sites due to negligible (strong binding) or slow (weak binding) metal dissociation (Apte et al. 2005; De Schamphelaere et al., 2004). The second potential factor is that the DOM may bind to the surface of the algal cells and block metal receptor sites due to steric hindrance by the bound DOM such as observed for fulvic acid binding to the green algae, *Chlorella pyrenoidosa* (Campbell et al., 1997). A third factor is potential ternary complex formation between algal cell surface binding sites, DOM, and metals (Lamelas et al., 2009).

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3.7. The use of TACs to predict the toxicity of pulsed exposures

There are several factors reported in the scientific literature that may lead to continuous and pulsed exposures of equivalent TACs of toxicants causing different toxicity in exposed organisms. Changes in the organism metabolism and the rates of uptake and depuration of toxicants, such as during moulting, may influence the degree of toxicity during different life-stages of the test organisms (Chen et al., 2012; Hoang and Klaine, 2007; Hosmer et al., 1998; Naddy et al., 2000). Younger daphnids (<24-h old) experienced greater toxicity from pulsed fenoxycarb exposure (Hosmer et al. 1998), with the authors suggesting that younger daphnids had less resistance and a higher metabolism than older organisms that led to greater uptake and toxicity of fenoxycarb. However, Hoang and Klaine (2007) and Naddy et al. (2000) found younger organisms are not always the most sensitive. Naddy et al. (2000) reported older *D. magna* (3, 7 and 14 d old) were more sensitive to pulsed chlorpyrifos exposure than neonates, and Hoang and Klaine (2007) reported that 2 to 4-d old *D. magna* experienced greater 21-d mortality from pulsed arsenic, copper, selenium and zinc exposure than younger or older organisms. Incomplete organism recovery and/or acclimatisation are two additional factors that can lead to pulsed exposures being more or less toxic than continuous exposures of equivalent TACs of toxicants. Several of the above factors may lead to an underestimation of organism toxicity and should be considered when assessing whether a TAC-approach is appropriate for regulating pulsed exposures.

The current study found that for NDW exposure dilutions that induced <50% toxicity, pulsed exposures caused similar or marginally lower toxicity to *P. subcapitata* than continuous exposures (Figure 1A). The finding of similar TACs on a Chelex-labile metal basis for equivalent exposures is likely to be the reason for this, as higher Chelex-labile metal concentrations would be expected to be more toxic (Apte et al. 2005; Eriksen et al., 2001). For NDW exposure dilutions that induced >50% toxicity, the marginally higher toxicity of the continuous NDW exposure compared to the pulsed exposure may be attributed to either saturation of algal uptake sites at the higher pulsed exposure concentration required to achieve an equivalent TAC, resulting in greater metal uptake in the equivalent continuous exposure (i.e. Michaelis-Menten saturation), and/or exposure to higher major cation concentrations in the equivalent pulsed exposure leading to greater competition for algal uptake sites (Levy et al., 2008; Peters et al., 2011).

For the CuW tests, the pulsed exposures of dissolved copper caused similar or marginally higher toxicity to the algae than continuous exposure of equivalent TAC (Table 1). The similarity in toxicity profiles between pulse and continuous Chelex-labile copper measurements indicated that the complexation of copper in CuW water explained most of the difference in toxicity. The results showing labile metal TACs were good predictors of toxicity are supportive of measurement techniques such as diffusive gradients in thin films (Li et al., 2005) that can measure time-integrated labile metal concentrations.

The finding of pulsed dissolved copper exposures being marginally more toxic than continuous exposures when compared on a TAC-basis is contrary to our previous findings of similar toxicity between the two exposure scenarios in synthetic freshwater (Angel et al., 2017). This is an example of another factor that may lead to differences in toxicity between different contaminant exposure scenarios that contain an equivalent TAC. Therefore, for different exposures with equivalent dissolved TACs of metals, the ability of ligands within natural waters to complex a greater proportion of metals in continuous (lower concentration, longer duration) than pulsed exposures (higher concentration, shorter duration) can lead to higher TACs based on labile metal concentrations for the pulsed exposure (conceptual diagram shown in Figure S3 of the SI) and should be considered if the TAC approach of assessing pulsed contaminant risk is applied.

The ECx values determined for both continuous and pulsed exposures of copper in the current study were substantially higher (less toxicity) than those determined in the synthetic freshwater for this algal species; depending on the ECx were between a factor of 7 to 32 times higher. These factors are much greater than those caused by the difference in toxicity of continuous and pulsed exposures with equivalent dissolved copper TACs. In other words, tests conducted in synthetic waters are inherently conservative relative to these natural waters due to the latter having greater dissolved organic matter and higher hardness that generally result in lower metal bioavailability. These findings have significance to the application of site-specific WQGVs to assessing risk posed by pulsed exposures to natural contaminated waters when the WQGVs being applied were originally derived from continuous exposures of organisms to the natural contaminated waters (van Dam et al., 2014b; 2017). In some such cases, the TAC derived

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from continuous exposures may not always be sufficiently conservative for assessing potential effects from pulsed exposures. As suggested in Angel et al. (2017), in such circumstances it may be applicable to apply a safety factor when deriving a TAC-based WQGV to ensure the 'threshold' remains suitably protective but not overly conservative. Such a safety factor should also consider other factors such as changes in life stage that may influence the toxicity of pulsed contaminant exposures.

4. CONCLUSIONS

Following pulsed exposures to either NDW or CuW water series, the green alga, *P. subcapitata* recovered its growth rate rapidly (i.e. no toxicity within 24-48-h post exposure). Continuous exposures to NDW resulted in similar or only marginally higher toxicity to the algae when compared to pulsed exposures of equivalent TAC (% NDW). The toxicity of the NDW was attributed mostly to the metals (Al, Co, Ni and Zn), with the major cations (Na, K, Mg, Ca) potentially causing effects that are both additive (direct toxicity, but lower than metals) and antagonistic (lowering the bioavailability of metals). For dissolved copper, the pulsed exposures caused greater toxicity than continuous exposures of equivalent TAC of copper, and much of the difference could be attributed to differences in the labile copper concentrations between treatments. The study highlights the influence of metal speciation in natural waters on the toxicity of pulsed exposures, and shows this factor should be considered when considering the toxicity of dissolved contaminants on a TAC-basis for different exposure scenarios. The results indicate that the consideration of metal lability may improve the application of WQGVs. While the study is generally supportive of considering TACs when applying guidelines, the results indicate that an appropriate safety factor may be necessary to ensure adequate conservatism to the range of organisms targeted for protection. Tests with sensitive organisms from other trophic levels such as invertebrates are required to inform the degree of conservatism required.

References

Angel, B.M., Hales, L.T., Simpson, S.L., Apte, S.C., Chariton, A.A., Shearer, D.A., Jolley, D.F., 2010a. Spatial variability of cadmium, copper, manganese, nickel and zinc in the Port Curtis Estuary, Queensland, Australia. Mar. Freshwater Res. 61, 170–183.

Angel, B.M., Simpson, S.L., Jolley, D.F. 2010b. Toxicity to *Melita plumulosa* from intermittent and continuous exposures to dissolved copper. Environ. Toxicol. Chem. 29, 2823–2830.

Angel, B.M., Simpson, S.L., Chariton, A.A., Stauber, J.L., Jolley, D.F., 2015a. Time-averaged copper concentrations from continuous exposures predicts pulsed exposure toxicity to the marine diatom, *Phaeodactylum tricornutum*: Importance of uptake and elimination. Aquat. Toxicol. 164, 1–9.

Angel, B.M., Vallotton, P., Apte, S.C., 2015b. On the mechanism of nanoparticulate CeO_2 toxicity to freshwater algae. Aquat. Toxicol. 168, 90-97.

Angel, B.M., Apte, S.C., Batley, G.E., Golding, L.A., 2016. Geochemical controls on aluminium concentrations in coastal waters. Environ Chem 13, 111-118.

Angel, B.M., Simpson, S.L., Granger, E., Goodwyn, K., Jolley, D.F., 2017. Time-averaged concentrations are effective for predicting chronic toxicity of varying copper pulse exposures for two freshwater green algae species. Environ. Pollut. 230, 787-797.

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.

Apte, S.C., Gardner, M.J., Ravenscroft, J.E., Turrell, J.A., 1990. Examination of the range of copper complexing ligands in natural-waters using a combination of cathodic stripping voltametry and computer-simulation. Anal. Chim. Acta 235, 287-297.

Apte, S.C., Batley, G.E., Bowles, K.C., Brown, P.L., Creighton, N., Hales, L.T., Hyne, R.V., Julli, M., Markich, S.I., Pablo, F., Rogers, N.J., Stauber, J.L., Wilde, K., 2005. A comparison of copper speciation measurements with the toxic responses of three sensitive freshwater organisms. Environ. Chem. 2, 320-330.

Barnes, M.B., Correll, R.L., Stevens, D., 2003. A simple spreadsheet for estimating low-effect concentrations and associated confidence intervals with logistic dose response curves. CSIRO Mathematical and Information Sciences Report, Canberra, Australia. http://www.cmis.csiro.au/Mary.Barnes/EC50_20050905.xls.

Beck, H.J., Birch, G.F., 2014. The nature and source of irregular discharges to stormwater entering Sydney estuary, Australia, Environ. Pollut. 188, 172-176.

Bowles, K.C., Apte, S.C., Batley, G.E., Hales, L.T., Rogers, N.J., 2006. A rapid Chelex column method for the determination of metal speciation in natural waters. Anal. Chim. Acta 558, 237-245.

Brent, R.N., Herricks, E.E., 1998. Postexposure effects of brief cadmium, zinc, and phenol exposures on freshwater organisms. Environ. Toxicol. Chem. 17, 2091–2099.

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

Cain, D., Croteau, M.N., Luoma, S., 2011. Bioaccumulation dynamics and exposure routes of Cd and Cu among species of aquatic mayflies. Environ. Toxicol. Chem. 30, 2532-2541.

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.

Campbell, P.G.C., Twiss, M.R., Wilkinson, K.J., 1997. Accumulation of natural organic matter on the surfaces of living cells: Implications for the interaction of toxic solutes with aquatic biota. Can. J. Fish. Aquat. Sci. 54, 2543-2554.

Chen, W.-Y., Lin, C.-J., Ju, Y.-R., Tsai, J.-W., Liao, C.-M., 2012. Assessing the effects of pulsed waterborne copper toxicity on life-stage tilapia populations. Sci. Total Environ. 417, 129-137.

Deleebeeck, N.M.E., De Schamphelaere, K.A.C., Janssen, C.R., 2009. Effects of Mg2+ and H+ on the toxicity of Ni2+ to the unicellular green alga *Pseudokirchneriella subcapitata*: Model development and validation with surface waters. Sci. Total Environ. 407, 1901-1914.

De Schamphelaere, K.A.C., Vasconcelos, F.M., Heijerick, D.G., Tack, F.M.G., Delbeke, K., Allen, H.E., Janssen, C.R., 2003. Development and field validation of a predictive copper toxicity model for the green alga Pseudokirchneriella subcapitata. Environ. Toxicol. Chem. 22, 2454-2465.

De Schamphelaere, K.A.C., Vasconcelos, F.M., Tack, F.M.G., Allen, H.E., Janssen, C.R., 2004. Effect of dissolved organic matter source on acute copper toxicity to *Daphnia magna*. Environ. Toxicol. Chem. 23, 1248-1255.

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. Contam. 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.

Eriksen, R. Mackey, D. van Dam, R. Nowak, B. 2001. Copper speciation and toxicity in Macquarie Harbour, Tasmania: an investigation using a copper ion selective electrode. Mar. Chem. 74, 99-113.

Gillmore, M.L., Golding, L.A., Angel, B.M., Adams, M.S., Jolley, D.F., 2016. Toxicity of dissolved and precipitated aluminium to marine diatoms. Aquat Toxicol 174, 82-91.

Golding, L.A., Angel, B.M., Batley, G.E., Apte, S.C., Krassoi, R., Doyle, C.J., 2015. Derivation of a Water Quality Guideline for Aluminium in Marine Waters. Environ. Toxicol. Chem. 34, 141-151.

Gordon, A.K., Mantel, S.K., Muller, N.W.J., 2012. Review of toxicological effects caused by episodic stressor exposure. Environ. Toxicol. Chem. 31, 1169-1174.

Gundersen, P., Steinnes, E., 2003. Influence of pH and TOC concentration on Cu, Zn, Cd and Al speciation in rivers. Water Res. 37, 307–318.

Heijerick, D.G., De Schamphelaere, K.A.C., Janssen, C.R., 2002. Biotic ligand model development predicting Zn toxicity to the alga *Pseudokirchneriella subcapitata*: possibilities and limitations. Comp. Biochem. Phys. C 133, 207-218.

Heijerick, D.G., Bossuyt, B.T.A., De Schamphelaere, K.A.C., Indeherberg, M., Mingazzini, M., Janssen, C.R., 2005. Effect of varying physicochemistry of European surface waters on the copper toxicity to the green alga *Pseudokirchneriella subcapitata*. Ecotoxicology 14, 661-670.

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

Hoang, T.C., Klaine, S.J., 2007. Influence of organism age on metal toxicity to Daphnia magna. Environ. Toxicol. Chem. 26, 1198-1204.

Hoffman, R.S., Capel, P.D., Larson, S.J., 2000. Comparison of pesticides in eight U.S. urban streams. Environ. Toxicol. Chem. 19, 2249–2258.

Hogan, A.C., Trenfield, M.A., Harford, A.J., van Dam, R.A., 2013. Toxicity of magnesium pulses to tropical freshwater species and the development of a duration-based water quality guideline. Environ. Toxicol. Chem. 32, 1969–1980.

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.

Lamelas, C., Pinheiro, J.P., Slaveykova, V.I., 2009. Effect of humic acid on Cd(II), Cu(II), and Pb(II) uptake by freshwater algae: kinetic and cell wall speciation considerations. Environ. Sci. Technol. 43, 730-735.

Laxen, D, Davison, W, Woof, C. 1984. Manganese chemistry in rivers and streams. Geochim. Cosmochim. Acta 48:2107–2111.

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

Li, W., Zhao, H., Teasdale, P.R., John, R., Wang, F., 2005. Metal speciation measurement by diffusive gradients in thin films technique with different binding phases. Anal. Chim. Acta 533, 193-202.

Markich, S.J., Batley, G.E., Stauber, J.L., Rogers, N.J., Apte, S.C., Hyne, R.V., Bowles, K.C., Wilde, K.L., Creighton, N.M., 2005. Hardness corrections for copper are inappropriate for protecting sensitive freshwater biota. Chemosphere 60, 1-8.

Mosley, L.M., Daly, R., Palmer, D., Yeates, P., Dallimore, C., Biswas, T., Simpson, S.L., 2015. Predictive modelling of pH and dissolved metal concentrations and speciation following mixing of acid drainage with river water. Appl. Geochem. 59, 1-10.

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

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.

Paquin, P. Gorsuch, J. Apte, S. Batley, G. Bowles, K. Campbell, P. Delos, C. Di Toro, D. Dwyer, R. Galvez, F. Gensemer, R. Goss, G. Hogstrand, C. Janssen, C. McGeer, J. Naddy, R. Playle, R. Santore, R. Schneider, U. Stubblefield, W. Wood, C. Wu, K. The biotic ligand model: a historical overview. Comp. Biochem. Physiol. C 2002, 133, 3-35.

Parent, L., Campbell, P.G.C., 1994. Aluminum bioavailability to the green alga *Chlorella pyrenoidosa* in acidified synthetic soft water. Environ. Toxicol. Chem. 13, 587-598.

Peters, A., Lofts, S., Merrington, G., Brown, B., Stubblefield, W., Harlow, K., 2011. Development of biotic ligand models for chronic manganese toxicity to fish, invertebrates, and algae. Environ. Toxicol. Chem. 30, 2407-2415.

Schemel, L.E., Kimball, B.A., Bencala, K.E., Runkel, R.L., Cox, M.H., 2007. Colloid Formation of mixed Al–Fe colloidal sorbent and dissolved-colloidal partitioning of Cu and Zn in the Cement Creek – Animas River Confluence, Silverton. Colorado. Appl. Geochem. 22, 1467–1484.

Simmons, J.A., 2012. Toxicity of major cations and anions (Na^+, K^+, Ca^{2+}, Cl^-, and SO_4^{2-}) to a macrophyte and an alga. Environ. Toxicol. Chem. 31, 1370-1374.

Simpson, S.L., Vardanega, C.R., Jarolimek, C., Jolley, D.F., Angel, B.A., Mosley, L.M. (2014). Metal speciation and potential bioavailability changes during discharge and neutralisation of acidic drainage water. Chemosphere, 103, 172–180.

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

US EPA. 2002a. Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms. Fifth Edition. United States Environmental Protection Agency Office of Water, Washington D.C. (EPA-821-R-02-012).

US EPA. 2002b. Short-term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms. Fourth Edition. United States Environmental Protection Agency Office of Water, Washington D.C. (EPA-821-R-02-013).

van Dam, R.A., Harford, A.J., Lunn, S.A., Gagnon, M.M., 2014a. Identifying the cause of toxicity of a saline mine water. Plos One 9, 1-13.

van Dam, R.A., Humphrey, C.L., Harford, A.J., Sinclair, A., Jones, D.R., Davies, S., Storey, A.W., 2014b. Site-specific water quality guidelines: 1. Derivation approaches based on physicochemical, ecotoxicological and ecological data. Environ. Sci. Pollut. Res. 21, 118-130.

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

SUPPLEMENTARY INFORMATION (SI)

SI Methods

S1.1 Algal cultures and bioassay preparation

The *P. subcapitata* cells (Strain 22662) were originally obtained from the American Type Culture Collection (ATCC), Maryland, USA, and were cultured axenically in synthetic freshwater (SFW) under continuous light (Philips TL 40 W cool white fluorescent lighting, Danvers, MA, USA, 70 µmol photons/m^2/s) at 24±1°C. Sub-cultures of *P. subcapitata* cells in the exponential phase of growth (3 to 6 d old) were washed three times with filtered (0.45 µm) River Murray water by centrifuging (Spintron GT-175BR, 7 mins × 700 g) before inoculation into 50 mL of treatment solutions contained within 250 mL borosilicate glass flasks that had previously been silanised with the reagent Coatasil to reduce metal adsorption (Angel et al., 2015b).

The flasks were capped loosely with clear plastic lids, and incubated in the same way as each respective culture, shaken twice daily to prevent CO_2 limitation, and were repositioned randomly in test cabinets daily to ensure equivalent illumination. Test results were considered acceptable if control mean growth rates were greater than 1 doubling per day, and the coefficient of variation in the controls (CV) was <20% (Stauber and Davies, 2000). 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.

S1.2. Copper pulse exposure bioassays

The renewal of test solutions to remove pulses involved the removal of NDW- or copper-spiked water by transferring treatment solutions into 50 mL centrifuge tubes (Cellstar, Greiner) and centrifuging (Spintron GT-175BR, 1400 g × 4 min) to create an algal pellet, followed by careful withdrawal of the supernatant so that approximately 0.5 mL remained above the algal pellet. Algal pellets were resuspended (vortex) in a volume of fresh control water equal to that before water replacement and transferred into clean glass flasks. The water replacements were designed so that the dissolved metal concentrations after water replacement were below the concentrations that elicited 10% growth rate inhibition (IC10) in continuous exposures. Preliminary tests indicated that the centrifugation of *P. subcapitata* did not negatively impact the rate of growth, but approximately 10% of the algal cells were lost during the removal of supernatant from the algal pellet. Therefore, the cell densities were measured before and after replacement of test solution and a correction applied to account for any losses (Angel et al., 2015a; 2017).

S1.3. Measurement of Chelex-labile copper and copper complexing capacity

Briefly, the waters were passed through a column containing Chelex-100 resin at a precise and fast flow rate (48 ± 4 mL/min), such that only free metal and rapidly dissociable complexes were measured as a Chelex-labile fraction (i.e. available for uptake onto the Chelex resin). The Chelex-labile metal fractions were calculated as the difference between the column influent and effluent concentrations. Chelex-labile copper measurements of NDW-2 water spiked with 40 µg/L dissolved copper were made 1, 5, 24, 48, 72 h after spiking to assess changes in labile copper over the exposure duration of this pulse concentration. The Chelex method was also used to determine the copper complexation capacity of the Murray River water, NWD-2 and NDW-10, using treatments spiked with 0, 50, 100 and 150 µg/L dissolved copper and equilibrated for 24 h at 24°C. The copper complexing capacity was calculated by determining the x-intercept of the relationship between Chelex-labile copper and dissolved copper for the copper-spiked treatments (Bowles et al., 2006). Method QA/QC consisted of running a copper nitrilotriacetic acid (NTA) solution that contained an in-house (n=89) average (±2 x S.D.) of 12 (±4)% Chelex-labile copper before sample measurement and an inorganic copper solution that contained an in-house (n=145) average (±2 x S.D.) of 97 (±4)% Chelex-labile copper at the end of each sample batch to validate that the resin capacity had not been exceeded. All analysis batches during Chelex-labile metal and CuCC measurements met these criteria.

Supplementary Information (SI)
SI Figures and Tables
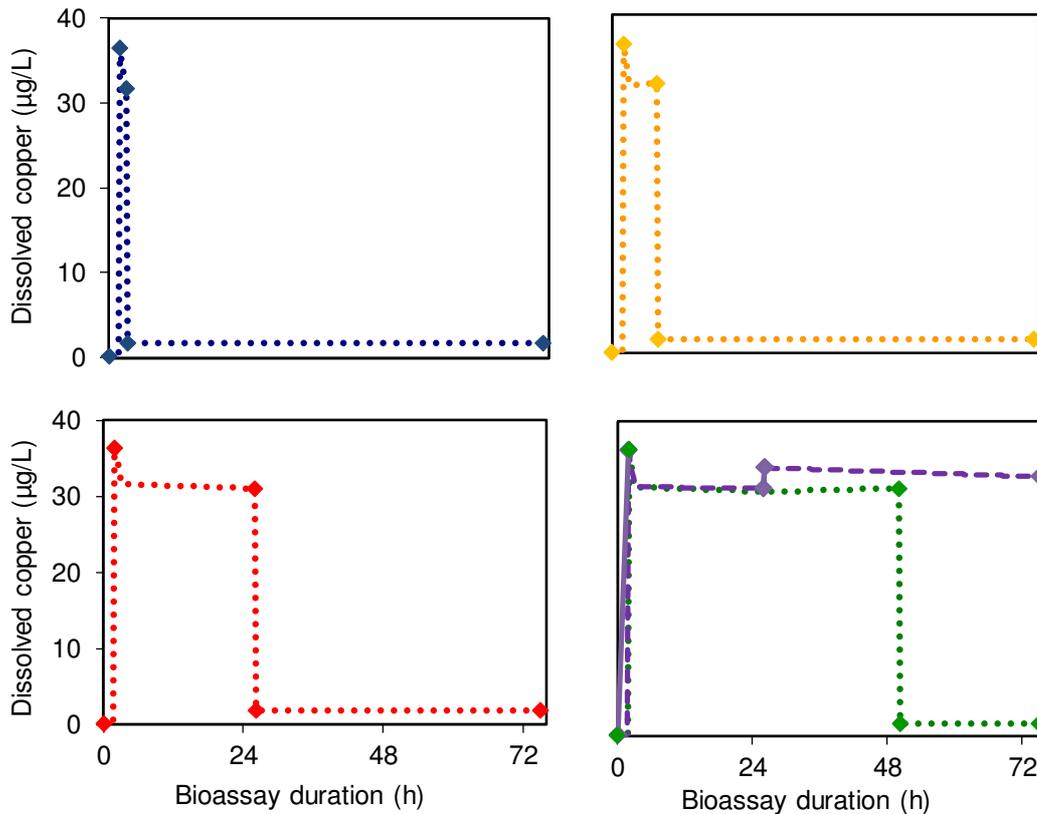
Fig S1. Example of dissolved metal exposure profiles (dotted lines) constructed from measured concentrations (icons) and used to calculate TACs. This example is for *P. subcapitata* exposed to 40 µg/L dissolved copper for 1, 5, 24, 48 and 72-h exposure durations.
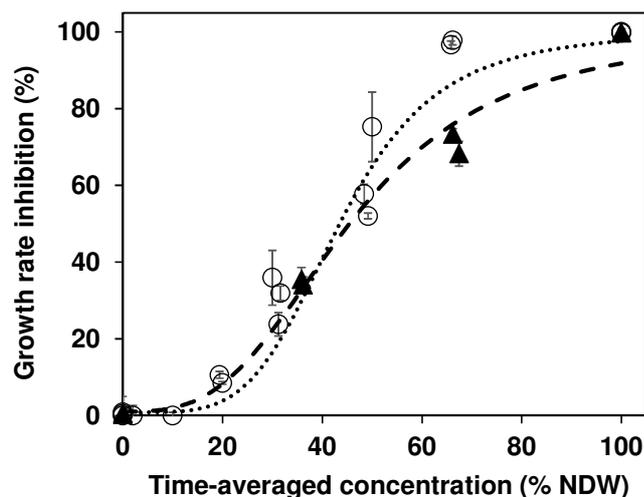
Fig S2. The relationship between growth rate inhibition of *P. subcapitata* for continuous (○) and pulsed (▲) exposures and the time-averaged concentration (TAC) of the neutralised drainage water (% NDW). The TAC calculations used the measured concentrations of the significant metals (Al, Co, Ni and Zn). The dotted and dashed model lines for continuous and pulsed exposure, respectively, show the IC10, 20 and 50 values calculated using only Al, Co, Ni and Zn are essentially the same as those calculated using TACs derived from Al, Co, Mn, Ni, Zn, Ca, K, Mg, and Na.
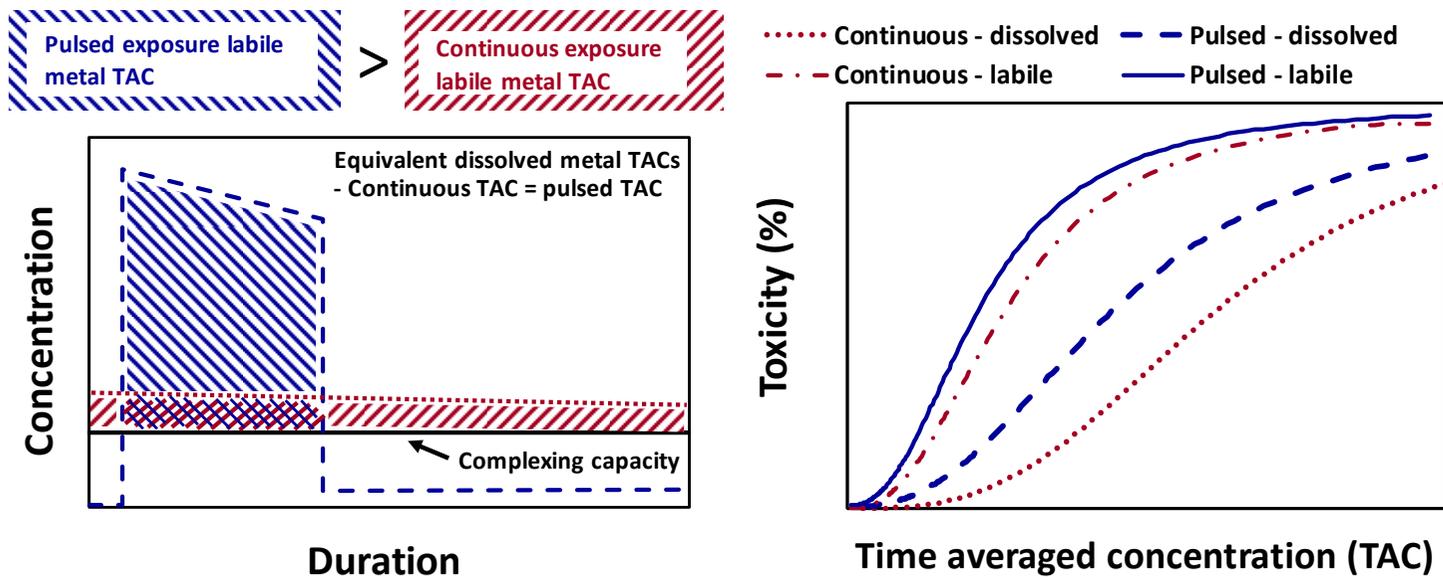
Fig S3. Conceptual diagram of equivalent continuous and pulsed dissolved TAC exposures resulting in a higher labile TAC for the pulsed exposure due to the complexation capacity of the water, and therefore higher toxicity of the pulsed exposure on a dissolved TAC basis.

4 **Table S1. The concentration of the most significant dissolved metals and major cations in NDW-100 after neutralising and allowing waters to stand for 24**
5 **h, followed by filtering, in a preliminary test. Metals with lower concentrations are shown in Table S3**

pH	Al (µg/L)	Co (µg/L)	Mn (µg/L)	Ni (µg/L)	Zn (µg/L)	Ca (mg/L)	K (mg/L)	Mg (mg/L)	Na (mg/L)
7.8	98	337	8130	396	82	722	78	765	7690
7.0	176	371	8990	424	196	744	65	798	7690
6.0	168	441	9430	487	791	751	71	794	7710
5.0	6540	458	9560	486	859	744	71	791	7680
4.0	15700	455	9730	480	868	742	67	787	7680
3.2	17100	470	10200	507	882	772	66	814	7660

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Table S2. The 72-h algal growth rate inhibition, time-averaged metal concentrations and dilutions (in brackets) measured in 72-h continuous exposure tests of NDW and potentially important components (trace metals, major cations and manganese) of the NDW

Treatment component	Dilution (%) Nominal	TAC	72-h inhibition (%)	72-h time-averaged concentrations (equivalent % NDW) Trace metals (µg/L) Al	Co	Ni	Zn	Major cations (mg/L) Ca	K	Mg	Na	Manganese (µg/L) Mn
Control [a]	0	0 ± 4.9	2 (0)	0 (0)	1 (0)	2 (0)	1 (0)	1 (0)	1 (0)	34 (0)	0 (0)
NDW	2	2.6	0 ± 2.6	3 (3)	6 (2)	9 (2)	1 (2)	21 (3)	4 (4)	20 (3)	220 (3)	177 (2)
	10	10	0 ± 0.8	10 (9)	35 (10)	42 (11)	10 (11)	67 (9)	9 (11)	70 (9)	742 (10)	918 (11)
	30	28	36 ± 7.1	21 (21)	105 (30)	117 (31)	27 (31)	182 (26)	24 (29)	194 (26)	2280 (29)	2500 (30)
	50	48	75 ± 9.1	40 (38)	171 (49)	192 (50)	44 (51)	327 (46)	39 (48)	356 (47)	4070 (53)	4140 (50)
	100	100	100 ± 0.8	101 (100)	348 (100)	381 (100)	86 (100)	707 (100)	81 (100)	750 (100)	7710 (100)	8280 (100)
Trace metals	2	3.3	0 ± 3.1	2 (2)	7 (2)	8 (2)	1 (1)	-	-	-	-	-
	30	29	61 ± 3.5	20 (21)	104 (30)	107 (28)	22 (26)	-	-	-	-	-
	100	94	94 ± 7.4	99 (102)	336 (97)	356 (93)	72 (84)	-	-	-	-	-
Major cations	2	3.1	0 ± 1.2	-	-	-	-	24 (3)	4 (5)	17 (2)	123 (2)	-
	30	25	10 ± 3.9	-	-	-	-	160 (23)	22 (27)	174 (23)	2050 (27)	-
	100	79	35 ± 3.6	-	-	-	-	522 (74)	74 (92)	600 (80)	5300 (69)	-
Manganese	2	2	0 ± 1.5	-	-	-	-	-	-	-	-	197 (2)
	30	24	1 ± 2.2	-	-	-	-	-	-	-	-	2000 (24)
	100	97	6 ± 1.1	-	-	-	-	-	-	-	-	8010 (97)

[a] Murray River water

Table S3. The concentration of selected metals and metalloids in the NDW-100 other than those listed in Table S1

Ag (µg/L)	Ba (µg/L)	Cd (µg/L)	Cr (µg/L)	Cu (µg/L)	Fe (µg/L)	Mo (µg/L)	Pb (µg/L)	Sn (µg/L)	V (µg/L)
2.2	24	0.4	0.9	1.0	62	<0.13	3.2	<0.94	27

Table S4. The 72-h pH and TACs measured for the NDW dilutions and its different components in continuous exposure toxicity tests

NDW equivalent treatment[a]	pH	NDW		Trace metals		Major cations		Manganese	
		TAC (%)	Inhibition (%)	TAC (%)	Inhibition (%)	TAC (%)	Inhibition (%)	TAC (%)	Inhibition (%)
NDW-0	7.97	0	0 ± 5	0	0 ± 5	0	0 ± 5	0	0 ± 5
NDW-2	7.94	3	0 ± 3	3	0 ± 3	3	0 ± 1	2	0 ± 1
NDW-10	7.92	10	0 ± 1						
NDW-30	7.95	28	36 ± 7	29	61 ± 4	25	10 ± 4	24	1 ± 2
NDW-50	7.92	48	75 ± 9						
NDW-100	7.86	100	100 ± 1	94	94 ± 7	79	35 ± 4	97	6 ± 1

[a]Nominal percentage dilution e.g. NDW-0 = control River Murray water, NDW-50 = 50% NDW diluted in Murray River water, NDW-100 = 100% (undiluted) neutralised drainage water

Table S5. The concentrations of dissolved organic carbon (DOC), dissolved inorganic carbon (DIC), Chelex-labile metals, and copper complexation capacity (CuCC) in NDW dilutions in Murray River Water and the proportions as a percentage of the total dissolved metal concentration

NDW (%)	DOC (mg/L)	DIC	CuCC (µg/L)	Labile Al (µg/L)	(%)	Labile Co (µg/L)	(%)	Labile Mn (µg/L)	(%)	Labile Ni (µg/L)	(%)	Labile Zn (µg/L)	(%)
0	3.61	11.6	22.0	-	-	-	-	-	-	-	-	-	-
2	3.80	11.4	14.6	0.9	25	5.9	95	181	98	4.5	61	1.5	87
10	4.74	10.7	12.4	5.6	52	31.2	95	907	95	31.5	84	8.2	94
20	-	-	-	11.8	59	58.1	96	1570	87	62.4	87	15.9	97
33	-	-	-	19.3	62	98.8	97	2290	75	103	88	26.8	97
50	-	-	-	28.2	64	140	96	2500	58	147	86	40.9	98
67	-	-	-	47.9	65	193	95	2380	40	198	83	54.7	98
100	16.7	0.58	-	55.5	66	281	92	2200	26	280	78	79.3	97

24 **Table S6. The concentrations of dissolved and Chelex-labile copper in tests investigating the**
25 **effect of copper concentration and duration after spiking into CuW (i.e. 2% NDW)**

Treatment	Dissolved copper (µg/L)	Chelex-labile copper (µg/L)
Effect of concentration in CuW		
CuW, 5 µg/L Cu	1.8	1.8
CuW, 10 µg/L Cu	3.9	3.9
CuW, 15 µg/L Cu	7.6	7.6
CuW, 20 µg/L Cu	9.3	9.3
CuW, 40 µg/L Cu	22.2	22.2
CuW, 50 µg/L Cu	28.6	28.6
Effect of duration after spiking CuW with 40 µg Cu/L		
CuW, 1 h	23.1	23.1
CuW, 5 h	21.6	21.6
CuW, 24 h	21.8	21.8
CuW, 48 h	20.8	20.8
CuW, 72 h	21.1	21.1

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