



# Natural organic matter source, concentration, and pH influences the toxicity of zinc to a freshwater microalga

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## ABSTRACT

Zinc is a contaminant of concern in aquatic environments and is a known toxicant to many aquatic organisms. Dissolved organic matter (DOM) is a toxicity modifying factor for zinc and is an important water chemistry parameter. This study investigated the influence of DOM concentration, source, and water pH on the chronic toxicity of zinc to a freshwater microalga, *Chlorella* sp. The influence of DOM on zinc toxicity was dependent on both concentration and source. In the absence of DOM, the 72-h EC50 was 112  $\mu\text{g Zn.L}^{-1}$ . In the presence of a DOM high in fulvic-like components, zinc toxicity was either slightly decreased (<4-fold increase in EC10s across 15 mg C.L<sup>-1</sup> range) or unchanged (minimal difference in EC50s). In the presence of a DOM high in humic-like (aromatic and high molecular weight) components, zinc toxicity was slightly decreased at the EC10 level and strongly increased at the EC50 level. The influence of pH on zinc toxicity was dependent on the source of DOM present in the water. In the presence of DOM high in humic-like components pH did not influence toxicity. In the presence of DOM high in fulvic-like components, pH had a significant effect on EC50 values. Labile zinc (measured by diffusive gradients in thin-films) followed linear relationships with dissolved zinc but could not explain the changes in observed toxicity, with similar DGT-labile zinc relationships shown for the two DOMs despite each DOM influencing toxicity differently. This indicates changes in toxicity may be unrelated to changes in zinc lability. The results suggest that increased toxicity of zinc in the presence of DOM may be due to direct uptake of Zn-DOM complexes. This study highlights the importance of considering DOM source and characteristics when incorporating DOM into water quality guidelines through bioavailability models.

## 1. Introduction

Zinc is an essential element for aquatic organisms but can be toxic at elevated concentrations, as is observed in freshwaters following increasing urbanisation, industrialisation, and resource extraction. Water chemistry parameters, such as dissolved organic matter (DOM), pH, and hardness are known to influence metal toxicity by modifying bioavailability through changes to metal speciation and competition at cellular surfaces (Adams et al., 2020). DOM is of particular importance as a toxicity modifying factor due to its ability to influence metal bioavailability through complexation. The source and quality

characteristics of DOM are location-specific and unique to different aquatic systems, meaning that the ameliorative capacity of DOM can vary for different DOM sources and seasons and different metals (Macoustra et al., 2019; Macoustra et al., 2020; ; Macoustra et al., 2021a, Macoustra et al., 2021b). The consequence of these interactions on zinc toxicity is not yet well understood. So, an improved understanding of how water chemistry influences zinc toxicity is required to better manage and protect aquatic ecosystems (Price et al., 2021; Price et al., 2022).

There is a general acceptance that DOM influences zinc toxicity; however, this is based on relatively few studies, mostly focused on

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freshwater invertebrates. Hejjerick et al. (2003) investigated the influence of DOM on the chronic toxicity of zinc to *Daphnia magna* and found a negative linear relationship between DOM concentration and chronic (reproduction) zinc toxicity. Hyne et al. (2005) found that increasing DOM resulted in a small decrease in the acute toxicity of zinc to *Ceriodaphnia dubia*, with results only becoming significant in the presence of 10 mg C.L<sup>-1</sup>.

There are currently **no** studies that have investigated the **independent** influence of natural DOM on chronic zinc toxicity to freshwater microalgae. Recent multiple linear regression models developed for application in the Canadian freshwater zinc guidelines did not include a dissolved organic carbon (DOC) or DOM parameter for microalgae (Canadian Council of Ministers of the Environment, 2018). There are several studies that have validated bioavailability models that include a DOM component via speciation modelling tools, but it is difficult to elucidate the specific influence of DOM in these studies as other water chemistry parameters (e.g. pH and major ions) covaried with DOM concentration (De Schampheleere et al., 2005).

Metal uptake in organisms is dependent on both the free metal ion and other inorganic and organic labile metal complexes. Measuring metal speciation and lability provides insight into how much of the total metal concentration is likely to be bioavailable, which can aid in the prediction of toxicity and risk (Batley et al., 2004). The extent to which a metal in solution is bioavailable is influenced by both thermodynamic and kinetic factors. Under thermodynamic equilibrium conditions the rate limiting step of metal uptake is the assimilation of metal into the organism. The rate of metal diffusion to the cell surface is much faster, thereby establishing a pseudoequilibrium between cell surface-bound metal and metal in solution. Speciation models such as the Windermere Humic Aqueous Model VII (WHAM) can be used to estimate metal speciation at equilibrium under a set of water quality parameters. WHAM also considers the binding of metals to fulvic and humic acids, fractions of DOM, in waters (Tipping et al., 2011). However, limited studies have assessed the use of WHAM in the presence of natural Australian DOM (Macoustra et al., 2019; Macoustra et al., 2021), with no studies having assessed the use of WHAM for zinc speciation in the presence of Australian DOM.

Under kinetic conditions, metal assimilation into the organism is much faster than metal diffusion to the cell surface (Sunda and Huntsman, 1998). As a result, a concentration gradient at the cell surface is established, perturbing the local metal ion equilibria. Metal complexes may dissociate and become labile when this equilibria is perturbed (Stumm and Morgan, 1996). The diffusive gradients in thin-films (DGT) technique is a useful tool for *in situ* kinetic measurements of the average labile metal fraction over time and is increasingly being used in environmental monitoring (Davison and Zhang, 1994; Zhang and Davison, 2015). DGT-labile metal is a surrogate for potentially bioavailable metal in solution without the need to directly measure metal complexing ligands (Apte et al., 2005).

This study aimed to assess the influence of DOM characteristics from Australian sources and DOM concentration on the toxicity of zinc to an Australian freshwater microalga under different pH conditions. Different measurements of zinc in solution including total, dissolved and colloidal zinc, as well as DGT lability and WHAM speciation modelling were used to understand how zinc speciation in the presence of DOM correlated with observed toxicity. This will help inform environmental assessment practices into the suitability of using labile zinc measurements rather than dissolved metal concentrations. This study also aimed to provide important data on the independent influence of natural DOM on zinc toxicity to a freshwater microalga, which is needed to develop bioavailability-based guidelines for zinc in freshwaters.

## 2. Methods

### 2.1. Natural DOM collection

Natural organic matter was isolated from two pristine freshwater systems using *in situ* reverse osmosis following methods outlined by Serkiz and Perdue (1990). A custom-built reverse osmosis unit (Compact L series Rowater, Australia) was used for both collections, operated at 200 psi (1400 kPa) and consisted of a 20 and 5 µm polyspun filter and a thin-film composite membrane (polyamide layered with polysulfone porous support). DOM concentrates were collected from Appletree Creek (Darumbal Country, QLD Australia) and Manton Dam (Larrakia Country, NT Australia). Concentrates were treated with cation exchange resin to remove ions concentrated during the reverse osmosis and stored as detailed in Macoustra et al. (2021a,b).

Manton Dam and Appletree Creek DOMs have been optically characterised and were described by Holland et al. (2018). Manton Dam DOM is a circumneutral DOM composed of mainly humic-like (35%) and fulvic-like (46%) substances, with a minor component of protein-like (19%) substances. In contrast, Appletree Creek DOM is naturally acidic, aromatic, of high molecular weight and composed of predominantly humic-like (56%) substances with less amounts of fulvic-like (36%) and protein-like (8%) substances. Analysis of the DOM composition showed minor differences in composition between the time of collection and the time of toxicity testing and these are shown in the supplementary information. Detailed DOM characterizations are reported in Tables S1–S3 and fluorescence excitation emission scans are provided in Fig. S1.

### 2.2. General laboratory techniques

General glassware and plasticware were cleaned in a dishwasher using a detergent rinse, (Gallay clean A powder detergent, Gallay scientific), acid rinse (2% HNO<sub>3</sub>, Merck) and ultrapure water rinse cycles (UPW, 18 MΩ cm, Milli-Q®, Millipore). Glassware and polypropylene sample vials (Technoplas) used in testing and analysis were soaked (>24 h) in 10% (v/v) HNO<sub>3</sub> (Merck) and thoroughly rinsed with UPW before use.

### 2.3. Organism culturing and toxicity testing

Growth inhibition toxicity tests were conducted using a tropical freshwater green microalga *Chlorella* sp. Algae were cultured in JM media at 2/5 strength (Thompson et al., 1988) at 27 ± 1 °C on a 12: 12 light/dark cycle (75 µmol photons.m<sup>-2</sup>.s<sup>-1</sup>). Algal cultures were transferred weekly into fresh media, and 5 to 7-day old cultures were used for toxicity testing to ensure algae were in an exponential growth phase throughout the testing period.

All toxicity tests were conducted using modified synthetic test water based on the standard USEPA recipe (USEPA, 2002) adjusted to a final hardness of 90 mg CaCO<sub>3</sub>.L<sup>-1</sup>. The test protocol followed the Organisation for Economic Co-operation and Development test guideline 201 (OECD, 2011) with modifications as described by Franklin et al. (2005). Both natural DOM concentrates (Manton Dam stock: 138 mg C.L<sup>-1</sup>; Appletree Creek stock: 148 mg C.L<sup>-1</sup>) were diluted with the synthetic test waters to produce a series of DOM concentrations (nominally 0, 2, 5, 10 and 15 mg C.L<sup>-1</sup>, Table 1). All test waters were adjusted to the required pH using dilute HCl or KOH (not NaOH) to maintain a constant sodium concentration across all tests.

The 72-h growth inhibition bioassays were conducted in 75 mL of test solution in 250 mL borosilicate conical flasks coated in a silanising solution (Coatasil, Thermofisher Scientific) to reduce zinc adsorption to the glass. Each test flask was supplemented with 75 µL of 1.5 g NO<sub>3</sub>.L<sup>-1</sup> (NaNO<sub>3</sub>) and 0.15 g PO<sub>4</sub><sup>3-</sup>.L<sup>-1</sup> (KH<sub>2</sub>PO<sub>4</sub>) stocks giving final concentrations of 1.5 mg NO<sub>3</sub>.L<sup>-1</sup> (NaNO<sub>3</sub>) and 0.15 mg PO<sub>4</sub><sup>3-</sup>.L<sup>-1</sup> (KH<sub>2</sub>PO<sub>4</sub>) to sustain exponential growth over the 72-h test. Each DOM treatment

**Table 1**

The 72-h effect concentrations (EC10/EC50) for growth rate inhibition of *Chlorella* sp. exposed to zinc under different dissolved organic matter (DOM) concentrations, source, and pH conditions. EC values were calculated using pooled test data ( $n = 2$ ). 95% confidence intervals are shown in parentheses. EC20 data are provided in [Supplementary Table S7](#).

DOM Source	[DOC] (mg C.L <sup>-1</sup> )	pH	Mean control growth rate (doublings/day)	Dissolved (µg.L <sup>-1</sup> )	
				EC10	EC50
Control (no added DOM, buffered)	<1	7.7	1.63	1.6 (1.0–2.1)	112 (96–127)
Manton Dam	2.5	7.6	2.40	2.0 (1.2–2.9)	71 (58–83)
	5.4	7.6	2.32	3.5 (2.4–4.7)	86 (76–97)
	10.1	7.6	2.32	4.5 (2.9–6.1)	107 (92–122)
	15.1	7.6	2.32	6.1 (3.6–8.6)	126 (103–149)
	5.5	6.7	2.33	2.7 (1.7–3.6)	34 (25–42)
	5.5	8.3	2.25	2.8 (2.1–3.5)	37 (33–40)
Appletree Creek	2.0	7.6	2.17	1.8 (1.0–2.6)	17 (11–23)
	4.6	7.6	2.17	2.3 (0.64–3.9)	18 (10–26)
	8.8	7.6	2.21	2.9 (1.1–4.6)	20 (9–30)
	13.0	7.6	2.11	3.4 (1.5–5.4)	25 (12–37)
	4.9	6.7	2.19	2.2 (1.7–2.6)	19 (16–22)
	4.9	8.3	2.14	2.0 (1.4–2.5)	19 (16–21)
	Control (no added DOM, unbuffered)	<1	7.5–8.2 <sup>a</sup>	1.88	1.3 (0.61–2.1)
Suwannee River Reference NOM	2.5	7.5–8.2 <sup>a</sup>	2.10	6.8 (5.3–8.2)	72 (64–81)
	4.4	7.5–8.2 <sup>a</sup>	2.10	15 (12–19)	80 (72–89)
	8.7	7.5–8.2 <sup>a</sup>	2.14	20 (14–26)	127 (107–146)

<sup>a</sup> pH range of unbuffered tests represents the start and end pH values.

consisted of a zinc concentration series from 0 (Control) to 5000 µg Zn. L<sup>-1</sup> using a stock solution of ZnCl<sub>2</sub> (analytical grade, Sigma-Aldrich). The pH was maintained throughout the tests using 2-N-morpholinopropanesulfonic acid (MOPS) buffer (free acid form; Merck) to give a final MOPS concentration of 0.5 g.L<sup>-1</sup> (2.4 mM), shown previously to have no effect on this microalga (Price et al., 2021). Test solutions were equilibrated for >24 h before addition of the algae (Van Genderen et al., 2020).

A 25 mL subsample of test solution was taken from each flask for chemical analysis immediately prior to algal inoculation. *Chlorella* sp. in exponential growth phase (5–7 days old) were centrifuged (1048 g, 7 min, rotor radius 15 cm; Spintron GT-175BR, 25 ± 1 °C) and washed with test solution. Centrifugation and washing were repeated three times to ensure removal of nutrient-rich culture medium. The algal concentrate was spiked into each test flask to give a final cell density of 2–4 × 10<sup>3</sup> cell.mL<sup>-1</sup> (Franklin et al., 2002). Test flasks were kept in incubator cabinets (LABEC) under constant 27 ± 1 °C, 12:12 photoperiod, and light intensity of 140 ± 20 µmol photons.m<sup>-2</sup>.s<sup>-1</sup> for 72 h.

Algal cell densities in each test flask were determined at 0, 24, 48 and 72 h using flow cytometry (FACSVerse, BD Biosciences). Side angle light scatter (SSC) and chlorophyll *a* fluorescence intensity (FLB3) were plotted with manual gating to obtain cell densities. A threshold of 200 (arbitrary units) was set to exclude background noise from non-algal particles and gating was used to exclude dead cells as detailed in Stone et al. (2019). Population growth rates, as doublings per day, were calculated from cell densities and used as the response variable in the toxicity tests (Franklin et al., 2001). Growth rates (i.e., cell division rates) were expressed as a percentage of the control growth rate in each test to account for inter-test variability and to allow data to be pooled.

Copper reference toxicant tests were run concurrently with each toxicity test. The test was considered acceptable if control growth rates in the reference tests were 1.7 ± 0.4 doublings per day (mean ± standard deviation [SD],  $n = 20$ ) and the median effect concentration (EC50) was within internal database limits of 2.6 ± 1.1 µg Cu.L<sup>-1</sup> (mean ± SD,  $n = 20$ ). Controls in DOM tests needed to have a growth rate of >1.2 doublings per day within the 72-h test period. Test pH variability was required to be less than ±0.1 of the average test pH for the 72-h test.

An additional set of algal toxicity tests was conducted with the well-characterised commercial standard Suwannee River natural organic matter (NOM) (IHSS) to provide a reference with a standard DOM source (Mager et al., 2011; Trenfield et al., 2011). A 100 mg.L<sup>-1</sup> stock of Suwannee River NOM was diluted with synthetic test waters to produce

a series of DOM concentrations (2.5, 4.4 and 8.7 mg C.L<sup>-1</sup>), as described previously for the Appletree Creek and Manton Dam DOM tests. Test pH was adjusted to 7.5 with KOH and equilibrated for >24 h before addition of the algae. Suwannee River reference tests were not buffered.

#### 2.4. Measured zinc lability and ultrafiltration

Zinc lability was measured using the diffusive gradients in thin-films (DGT) technique (Davison and Zhang, 1994). The DGT devices accumulate metals that are labile to iminodiacetic acid functional groups on the Chelex binding resin. As such, DGT-labile metal concentrations include free metal ions (M<sup>2+</sup>), simple inorganic metal complexes and any readily labile organic metal complexes (Van Leeuwen et al., 2005; Koppel et al., 2021). Chelex-100-based binding resins (Na form, 100–200 wet mesh) and polyacrylamide diffusive gel were synthesized and assembled into DGT pistons according to procedures outlined by Amato et al. (2019). DGT pistons were deployed in polycarbonate containers with test solutions and kept on an orbital shaker (90–100 rpm) to ensure the diffusive boundary layer was negligible. The orbital shaker was placed in the incubator cabinet with the toxicity tests. DGT test solutions matched the toxicity test solutions in DOM concentration and pH, zinc concentration and initial algal cell densities. DGT-labile zinc was measured in four to five zinc concentrations between 0 and 400 µg.L<sup>-1</sup>. DGT pistons were deployed for 72 h. Once retrieved, the Chelex binding gel layer was placed in 1 M HNO<sub>3</sub> and eluted for >24 h. Gel elutions were then diluted 10-fold with ultrapure water prior to analysis. DGT-labile zinc concentrations were calculated as detailed by Koppel et al. (2019). Throughout the present study, DGT measured zinc is referred to as DGT-labile zinc. DGT-labile zinc measurements are provided in [Table S8](#).

Ultrafiltration was used to assess the colloidal fraction of zinc (operationally defined as >3 kDa). Ultrafiltration was performed by filtering algal-inoculated test subsamples through a 0.45 µm filter, with filtrate being placed directly into acid-rinsed centrifugal filtration devices fitted with 3 kDa membranes (modified polyethersulfone membrane, Macrosep Advanced; PALL). Devices were then centrifuged at 1048 g for >30 min, and filtrate was subsampled. Ultrafiltration samples were taken at the start and end of each test from 4 to 8 zinc concentrations in each definitive test. Where ultrafiltration was used, total (no filtration) and dissolved metal (<0.45 µm) subsamples were collected concurrently. All samples were acidified to 0.2% (v/v) HNO<sub>3</sub> (Tracepur, Merck) and stored below 4 °C until analysis.

## 2.5. Chemical analyses

Dissolved metal subsamples (<0.45 µm) were collected from all test flasks at the start (0 h) and end (72 h) of each test. Samples were filtered through acid-rinsed (flushed with 30 mL of both 10% HNO<sub>3</sub> and ultrapure water) 0.45 µm syringe filters (polyethersulfone membrane, Sartorius). All metal samples were acidified to 0.2% (v/v) HNO<sub>3</sub> (Tracepur, Merck) and stored below 4 °C until analysis. Throughout the present study, dissolved zinc is defined as measured and filtered (<0.45 µm) zinc unless otherwise stated. All ultrafiltered, dissolved, and total metal samples were analysed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Agilent 730 ES) that had a minimum instrument detection limit of 0.16 µg Zn.L<sup>-1</sup> calculated as three times the standard deviations of the acidified blank solutions. Matrix-matched calibration standards, blanks and drift-standards were used for quality assurance.

Samples for DOC analysis were collected from bulk test solutions prior to the addition of MOPS. Samples were filtered through acid-rinsed (flushed with 60 mL of both 10% HNO<sub>3</sub> and ultrapure water) 0.45 µm filters (polyethersulfone, Sartorius), and acidified with concentrated sulfuric acid (H<sub>2</sub>SO<sub>4</sub>) in glass amber vials. Samples were stored below 4 °C until analysis by the non-purgeable organic carbon method (TOC-L series, Shimadzu).

## 2.6. WHAM estimated metal speciation

WHAM Version VII was used to estimate zinc speciation for all zinc exposures under all test conditions. Input parameters included pH, temperature, major ions (Mg<sup>2+</sup>, Ca<sup>2+</sup>, K<sup>+</sup>, Na<sup>+</sup>, Cl<sup>-</sup>, SO<sub>4</sub><sup>2-</sup>, CO<sub>3</sub><sup>2-</sup>, NO<sub>3</sub><sup>-</sup> and PO<sub>4</sub><sup>3-</sup>), and concentrations of colloidal fulvic and humic acid (mg C.L<sup>-1</sup>) based on measured proportions of fulvic and humic acid in each DOM. Humic- and fulvic-like proportions in both DOM (Table S1) were determined using fluorescence excitation emission scans followed by parallel factor (PARAFAC) analysis (Holland et al., 2018). An open atmosphere assumption (pCO<sub>2</sub> = 0.00038 atm) was applied to all speciation calculations as per DeForest and Van Genderen (2012).

Speciation modelling was calculated to test a secondary stressor hypothesis (Tables S5 and S6, discussed section 3.2), specifically at the EC10 and EC50 concentrations (Tables S9 and S10, discussed section 3.4) and for all experimental replicates (Tables S11 and S12). DOM-bound zinc is discussed in section 3.4. DOM-bound zinc is defined as the concentration of zinc bound to fulvic and humic acids as calculated in the speciation modelling (Table S10).

## 2.7. Statistical analysis and modelling

Statistical analyses were performed using the R studio environment (version 4.0.2, R Core Team, 2016) with the extension package *drc* (Ritz et al., 2015). Figures were produced using the extension packages *ggplot2* (Wickham, 2016) and *ggpubr* (Kassambara, 2020).

Population growth rate inhibition normalised to a percent of the respective control population growth rate of that test was used as the response variable to derive all toxicity estimates. Normalising population growth rates to the test's control response allowed for data across repeat (n = 2) tests to be pooled for analysis. Effect concentrations for 10, 20 and 50 percent growth rate inhibition relative to controls (EC10, EC20 and EC50) were calculated using 4-parameter Weibull or log-logistic models. Akaike's information criterion (AIC) and model residual standard error were used for model selection via the *mselect* function within *drc*, as well as visual assessments of model fit. The models used and their parameters are listed in Table S4. As response data were normalised to a percentage of control, all models had upper limit parameters fixed to 100. When full effect responses (i.e., EC100) were observed, the lower asymptote parameter was fixed to 0, meaning only the midpoint and slope parameters were estimated. When this was not the case (i.e., lower asymptote >0), the lower asymptote parameter was

not fixed.

Visual assessments of fit are an important component of concentration-response model selection as the *mselect* function chooses the best fitting model for the entire dataset, occasionally to the detriment of choosing the best fitting model for the upper 50% of the data, where EC10, EC20 and EC50 values are derived. This was of particular importance in several datasets used within the current study where a plateau in response was observed across given zinc concentrations not representing the traditional sigmoidal-like concentration response shape. In these situations, model lower asymptotes were set at the plateau (rather than at 0% growth rate), which provided a better fit of the models to data in the upper 50% of the curve, thus providing better estimates for the EC10, EC20 and EC50 values. Note in these scenarios the *ED* function in *drc* will calculate effect concentrations between the upper and lower limits of the model (i.e., an EC50 will represent the midpoint between upper and lower asymptotes rather than the 50% response relative to controls at 100% response). To ensure that EC values were based on response relative to controls, re-scaling, using interpolation, of the input values in the *ED* function was required.

The *comped* function within the *drc* package was used to test for differences between EC values in different experiments by applying a ratio test (Wheeler et al., 2006). Differences between ultrafiltered and DGT-labile zinc at different DOM concentrations were assessed by ANOVA and Tukey post-hoc analysis. Assumptions of homogeneity of variances and normality of residuals were tested using Levene's test and Shapiro-Wilks tests, respectively. All metal concentrations in models and results were measured concentrations. Standard deviation (SD) was used to specify variability throughout, and all significance testing was conducted at  $\alpha = 0.05$ .

## 3. Results and discussion

### 3.1. Quality assurance and control

All tests met the acceptability criteria. Control growth rates in DOM tests were acceptable in all tests (>1.2 doublings per day) (Table 1). It was noted that all tests with added DOM had increased control growth rates relative to the no added DOM controls. This was likely due to *Chlorella* sp. using the added carbon as an additional energy source and the potential additional micronutrients included with the DOM concentrate. Copper reference test control growth rates were within 1.7 ± 0.4 doublings per day. All copper reference toxicant tests' EC50 values were within the acceptability limits of 2.6 ± 1.1 µg Cu.L<sup>-1</sup>, indicating that the microalgal cultures had repeatable and comparable sensitivity across tests.

### 3.2. Influence of DOM concentration and source on zinc toxicity

In the absence of DOM, *Chlorella* sp. was sensitive to zinc exposure, with an EC10 and EC50 of 1.3 ± 0.3 and 48 ± 4 µg.L<sup>-1</sup>, respectively. In the presence of Suwannee River DOM, EC10 and EC50 values were significantly (EC10: p < 0.0001, z = 28.1, EC50: p < 0.0001, z = 5.29) increased relative to tests with no added DOM, with EC10 and EC50 values of 6.8 ± 0.9 and 72 ± 5 µg.L<sup>-1</sup>, respectively (Table 1). Increasing Suwannee River DOM concentration led to further significant (EC10: p < 0.0001, z = 18.1, EC50: p < 0.0001, z = 10.3) increases in both the EC10 and EC50 values, with an approximate 15- and 2.6-fold change across the <1–8.7 mg C.L<sup>-1</sup> range, respectively (Figs. S2 and S3). These results are as expected, as DOM presence is typically associated with a decrease in bioavailable metal through complexation (Wood et al., 2011). Suwannee River DOM has also been shown to have similar ameliorative effects on other metals and organisms, with Mager et al. (2011) reporting reduced lead toxicity to *Ceriodaphnia dubia* in the presence of 4 mg C.L<sup>-1</sup> Suwannee River DOM and Kozlova et al. (2009) who found small reductions (3-fold) of nickel toxicity to *Daphnia pulex* across a large concentration range of Suwannee River DOM from 0.5 to

41 mg C.L<sup>-1</sup>.

The influence of natural Australian DOM on the toxicity of zinc to *Chlorella* sp. differed from the results of the Suwannee River DOM reference tests and were dependent on the source of the DOM (Fig. 1, Table 1). However, it is important to note that the Suwannee River reference tests were not chemically buffered by MOPS.

In the presence of Manton Dam DOM, DOM concentration had a small yet significant ( $p < 0.05$ ) influence on zinc toxicity relative to results in the absence of DOM, with the trend of this influence differing between EC10 and EC50 values. The EC10 results showed a significant increase ( $p = < 0.0001$ ,  $z = 20.7$ ) from  $1.6 \pm 0.3$  to  $6 \pm 1 \mu\text{g.L}^{-1}$  as DOM concentration increased from no added DOM ( $< 1 \text{ mg C.L}^{-1}$ ) to  $15.1 \text{ mg C.L}^{-1}$ , respectively (Fig. 2, Table 1). The EC50 results showed a small ameliorative capacity of the Manton Dam DOM across the tested range, with no significant difference ( $p = 0.312$ ,  $z = 1.01$ ) between EC50 values in the no added DOM test ( $\text{EC}_{50} = 112 \pm 8 \mu\text{g.L}^{-1}$ ) and the highest DOM concentration test at  $15.1 \text{ mg C.L}^{-1}$  ( $\text{EC}_{50} = 126 \pm 11 \mu\text{g.L}^{-1}$ ). Interestingly, there were small but significant decreases in EC50 values between the no added DOM treatment and the low concentration Manton Dam DOM treatments, with EC50 values of  $71 \pm 6$  ( $p = 0.012$ ,  $z = 2.50$ ) and  $86 \pm 5$  ( $p = 0.033$ ,  $z = 2.13$ )  $\mu\text{g.L}^{-1}$  in the 2.5 and 5.4 mg C.L<sup>-1</sup> treatments, respectively. There were also significant increases ( $p < 0.0001$ ,  $z = -6.09$ ) in EC50 values between low (2.5 mg C.L<sup>-1</sup>) and high (15.1 mg C.L<sup>-1</sup>) concentration Manton Dam DOM treatments suggesting that there may be some trend of protective effects with increasing DOM concentration.

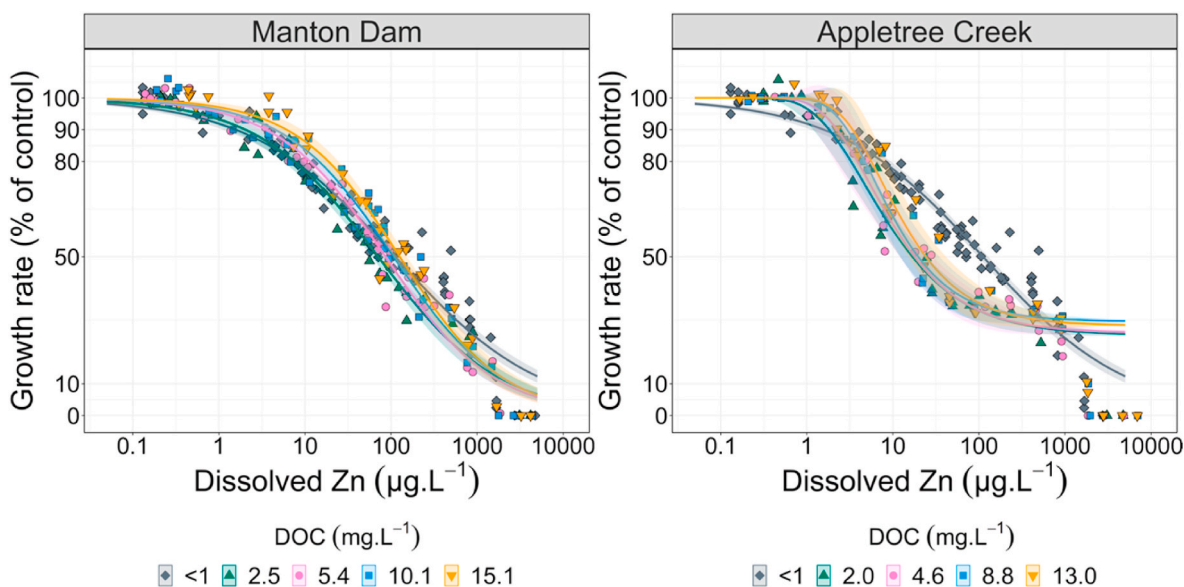
Despite these small observed increases in toxicity in low Manton Dam DOM treatments, overall, there appears to be limited influence of Manton Dam DOM on zinc toxicity at the EC50 level, and only a small influence at the EC10 level, with EC10 values increasing by  $< 4$ -fold across a 15 mg C.L<sup>-1</sup> range. This Manton Dam DOM has previously been shown to have an influence on copper toxicity using the same *Chlorella* sp. strain (Macoustra et al., 2019). Macoustra et al. (2019) reported a 17- and 13-fold increase in copper EC10 and EC50 values, respectively, across a DOM concentration range of  $< 1$ –8.4 mg C.L<sup>-1</sup>. This greater ameliorative capacity is consistent with copper having a larger binding affinity to DOM compared to zinc (Tipping et al., 2011).

The EC10 results in the presence of Appletree Creek DOM followed a

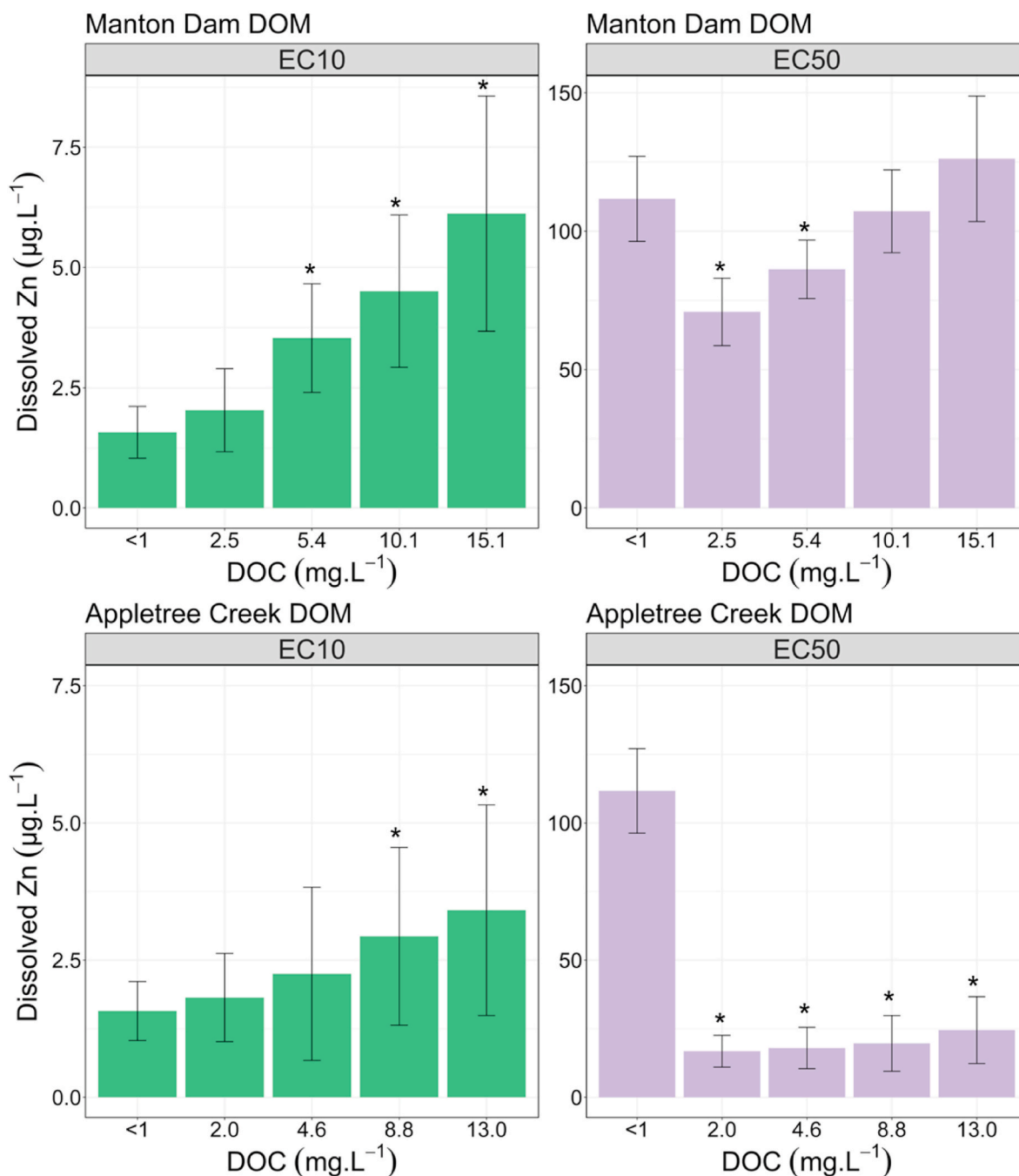
similar trend to the ones observed for Suwannee River and Manton Dam DOM. The EC10 values showed a small but significant increase ( $p < 0.0001$ ,  $z = 5.09$ ) from  $1.6 \pm 0.3$  to  $3 \pm 1 \mu\text{g.L}^{-1}$  as DOM concentrations increased from  $< 1$  (no added DOM) to  $13.0 \text{ mg C.L}^{-1}$ , respectively (Fig. 2, Table 1). Unexpectedly, the EC50 results in the presence of Appletree Creek DOM contrasted with the overall trends observed for the Manton Dam and Suwannee River DOM (Figs. 1 and 2). The EC50 values in the presence of all Appletree Creek DOM treatments were significantly ( $p < 0.05$ ) lower, relative to the no added DOM results, suggesting that the presence of Appletree Creek DOM increased zinc toxicity. The concentration of Appletree Creek DOM did not influence the magnitude of the increase in toxicity, with EC50 values ranging from  $17 \pm 3$  to  $25 \pm 6 \mu\text{g.L}^{-1}$  (which were not significant  $p = 0.070$ ,  $z = 1.81$ ), across a DOM concentration range of 2.0–13.0 mg C.L<sup>-1</sup>.

In the presence of Appletree Creek DOM, concentration-response model slope parameters were significantly influenced relative to the slope parameter of the no added DOM concentration-response models (Fig. 1). Significant steepening ( $p < 0.0001$ ,  $z = 13.5$ ) of the slope parameter (Table S4) highlights that rate of inhibition in algal growth relative to zinc concentration increased and explains why the trends between EC10 and EC50 values are not consistent. Such changes in slope parameters were not observed in the presence of Manton Dam DOM (Fig. 1) and may suggest that an alternative mechanism for toxicity is occurring in the presence of Appletree Creek (Ritz et al., 2006). These increases in toxicity are unlikely to be explained by the Appletree Creek DOM having a mode of toxicity independent of zinc as the control growth rates for all Appletree Creek treatments were high and met all test acceptability criteria (Table 1).

Our results suggest that the increased toxicity of zinc in the presence of Appletree Creek DOM may be due to direct uptake of the Zn-DOM complexes, particularly any lipid-soluble complexes, or due to the formation of a ternary (DOM-Zn) complex at the cell surface (Lamelas et al., 2005). In the literature, there are several examples of organically-bound metals having increased bioavailability and thus increased toxicity or metal uptake (Errecalde et al., 1998; Lamelas et al., 2005; Aristilde et al., 2012). Errecalde et al. (1998) investigated the influence of citrate on the toxicity and uptake of zinc and cadmium in *Selenastrum capricornutum* (now named *R. subcapitata*). The presence of citrate ( $100 \mu\text{mol.L}^{-1}$ )



**Fig. 1.** Concentration-response curves indicating the effect of Manton Dam dissolved organic matter (DOM) (LHS panel) and Appletree Creek DOM (RHS panel) at increasing concentrations of DOM ( $< 1$ –15 mg.L<sup>-1</sup>) on the growth rate of *Chlorella* sp. when exposed to dissolved zinc. All tests were conducted at a constant pH of 7.6. Shaded ribbons represent the 95% confidence intervals. Each datapoint represents an individual replicate response and a corresponding measured zinc concentration. Data are pooled from separate experiments. Replicate responses were normalised to their respective controls for inter-test pooling. Note that the exposures with Appletree Creek DOM have biphasic response curves and the models are fitted to the first lower asymptote.



**Fig. 2.** Comparison of EC10 and EC50 values for zinc toxicity on the growth rate of *Chlorella* sp. when exposed to dissolved zinc as a function of dissolved organic carbon (DOC) concentration for Manton Dam DOM (upper panel) and Appletree Creek DOM (lower panel) at a fixed pH of 7.6. Error bars indicate the calculated lower and upper 95% confidence intervals. Note variable y-axis scales. \* Indicates significant difference ( $p < 0.05$ ) from the no added DOM treatment ( $<1 \text{ mg.L}^{-1}$ ).

caused an increase in toxicity (compared to no added citrate) for both metals, with EC50 values for the metal ions  $\text{Cd}^{2+}$  and  $\text{Zn}^{2+}$  decreasing by 7- and 6-fold, respectively. The uptake of  $\text{Cd}^{2+}$  was also increased in the presence of citrate (uptake experiments were not conducted for  $\text{Zn}^{2+}$ ). Aristilde et al. (2012) found similar results for zinc uptake in the marine phytoplankton *Emiliania huxleyi* and *Thalassiosira weissflogii* in the presence of cysteine (nM concentrations), operationally defined by the authors as a “weak organic ligand”. Interestingly, the increased zinc uptake due to cysteine only occurred in the presence of  $\mu\text{M}$  concentrations of EDTA, a strong binding ligand, at concentrations an order of magnitude greater than cysteine. The authors also repeated the experiments using other “weak organic ligands” and found similar enhanced zinc uptake in the presence of glutathione, phytochelatin and histidine.

The presence of similarly acting ligands in Appletree Creek DOM may be causing effects similar to those described by Errecalde et al. (1998) and Aristilde et al. (2012). However, as highlighted by Zhao et al. (2016), care is needed when extrapolating results from these studies using isolated organic ligands to results using the complex matrix of naturally-sourced DOM.

Experiments to measure zinc uptake, extracellular or intracellular zinc in the presence of DOM would help provide more evidence as to the cause of the enhanced toxicity in the presence of Appletree Creek DOM. A recent study by Hourtané et al. (2022) investigated the influence of Suwannee River humic acid on platinum toxicity to *Chlorella fusca* and *Chlamydomonas reinhardtii*. The study found that the presence of the humic acid increased platinum toxicity and used intracellular metal

concentrations to further evaluate this increased toxicity. Intracellular platinum increased in the presence of the humic acid. The researchers suggested that humic acid can absorb to algal cell walls (Campbell et al., 1997), and being amphiphilic can modify membrane permeability thereby increasing the uptake of platinum. Given that Appletree Creek DOM has a high humic fraction this may provide a plausible hypothesis to the observed increases in toxicity in the present study.

Increased toxicity in the presence of added humic-dominant natural DOM has also been shown for nickel. Holland et al. (2017) found that in the presence of natural DOM from the Amazon Basin in Brazil, nickel toxicity to Cardinal tetras (*Paracheirodon axelrodi*) was increased, relative to tests without DOM and this was dependant on pH. Of the natural DOMs tested, nickel was most toxic in black waters characterised as having high amounts of aromatic and higher molecular weight humic-like components of allochthonous origins. Interestingly, the DOM characteristics of the Brazilian black water are very similar to that of Appletree Creek DOM and these aromatic high molecular weight

humic-like components may also be linked to the increased zinc toxicity in the current study.

An alternative to the Zn-DOM complex hypothesis discussed above, is that secondary stressors or metal mixture effects may be influencing toxicity. For example, at higher zinc concentrations, zinc may displace another metal or toxicant from the DOM. This can be tested with WHAM speciation modelling by comparing the percentage of DOM-bound copper at low concentrations ( $1 \mu\text{g Cu.L}^{-1}$ , representing the maximum measured concentration of copper in Appletree Creek DOM) in the presence of low and high zinc concentrations ( $10$  and  $5000 \mu\text{g Zn.L}^{-1}$ ). Speciation modelling shows that only at low DOM concentrations ( $2 \text{ mg C.L}^{-1}$ ) does the percentage of DOM-bound copper change greatly, decreasing from 99% to 42% DOM-bound in the presence of  $10$  and  $5000 \mu\text{g Zn.L}^{-1}$ , respectively. Modelled percentages of DOM-bound copper at higher DOM concentrations varied between 71 and 100% (Tables S5 and S6). The decrease to 42% DOM-bound copper in the low DOM concentration cannot explain how increased toxicity was seen

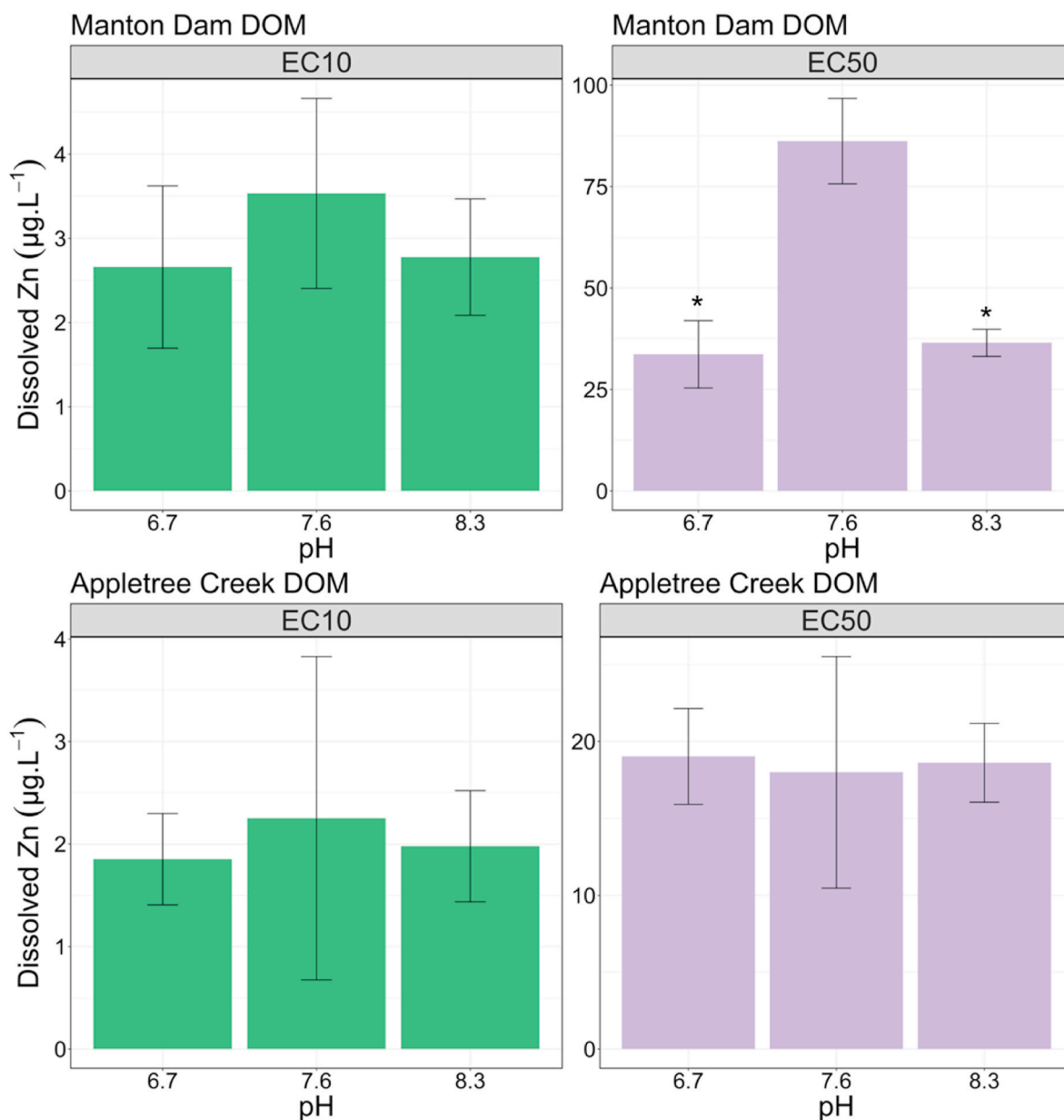


Fig. 3. Comparison of EC10 and EC50 values for zinc toxicity on the growth rate of *Chlorella* sp. when exposed to dissolved zinc as a function of pH in the presence of  $5.5 \text{ mg C.L}^{-1}$  Manton Dam DOM (upper panel) and  $4.9 \text{ mg C.L}^{-1}$  Appletree Creek DOM (lower panel). Error bars indicate the calculated lower and upper 95% confidence intervals. Note variable y-axis scales. \* Indicates significant difference ( $p < 0.05$ ) from the pH 7.5 treatment.

uniformly across all Appletree Creek DOM concentrations (Table 1) and the presence of such low concentrations of freely available copper is also unlikely to cause such a large increase in toxicity. Macoustra et al. (2019) reported an EC10 value of  $7.1 \mu\text{g Cu.L}^{-1}$  in the presence of  $2.1 \text{ mg C.L}^{-1}$  of Appletree Creek DOM for the same algae species, suggesting the displacement of low concentrations of copper from Appletree Creek DOM is unlikely to explain the greatly increased toxicity observed in the present study. Furthermore, previous studies using microalgal toxicity tests with ternary Cu–Ni–Zn mixtures found no toxic interactivity between the three metals (Van Regenmortel and De Schamphelaere, 2018). For a metal mixture to be causing the increased toxicity in the presence of Appletree Creek DOM, large synergistic interactions would be needed.

No matter the cause, the increased toxicity of zinc in the presence of DOM is contrary to expectations that DOM will ameliorate toxicity to algae and highlights the importance of assessing the influence of different natural DOM sources, especially with the renewed focus on developing empirically derived bioavailability-based water quality guidelines (Mebane et al., 2020).

### 3.3. Influence of pH on zinc toxicity in the presence of DOM

The influence of pH on zinc toxicity in the presence of DOM was dependent on the DOM source. In the presence of Appletree Creek DOM, changes in pH did not influence either the EC10 or EC50 values (Table 1, Fig. 3). Toxicity increased at the EC50 level relative to no added DOM regardless of the test pH. While measured toxicity was unaffected by pH change, concentration response curve shape was slightly influenced in the pH 8.3 tests. In all Appletree Creek treatments at pH 6.7 and 7.6, a plateauing of organism response occurred at approximately 30% growth rate (Figs. 1 and 4) from 100 to  $1000 \mu\text{g Zn.L}^{-1}$ ; this was not observed in the pH 8.3 treatment. The lack of change in EC10 and EC50 values across this pH range may indicate that an alternative mode of action for toxicity in the presence of Appletree Creek DOM (as hypothesised in section 3.2) is unaffected by proton competition at the algal binding site. This is in contrast to results previously reported for zinc with the same *Chlorella* sp. strain in the absence of DOM (Price et al., 2021).

In the presence of Manton Dam DOM, pH did not have a significant influence on EC10 values but did have a significant effect ( $p < 0.05$ )

between the pH 6.7 and 8.3 EC50 values and the pH 7.6 EC50 value (Fig. 3). The decrease in EC50 value with increased pH (i.e., the pH 8.3 test compared with the pH 7.6 test) is similar in magnitude to previously reported results for *Chlorella* sp. In the absence of DOM (Price et al., 2021). Given, the general minimal influence of Manton Dam DOM concentration on zinc toxicity (Fig. 2), the decreased EC50 value may be solely a product of changed pH with little interaction from the added DOM. However, this hypothesis cannot explain the decrease in EC50 value with decreased pH (pH 6.7), as one would expect to see reduced toxicity as pH decreased, caused by the increase in proton competition. It is important to consider in the presence of DOM, proton competition is likely to occur at both algal cell binding sites and at the DOM binding ligand functional groups. As such, pH may not influence zinc toxicity in the presence of DOM in the same manner as it would in the absence of DOM. Additionally, the decrease in EC50 value at pH 6.7 may be caused by an alternative mechanism of toxicity, as concentration response curve shape changed to have a plateauing of organism response similar to the results reported for Appletree Creek (Fig. 4).

### 3.4. Zinc lability in freshwaters in the presence of organic matter

DGT-labile zinc concentrations were measured in four to five zinc treatments ( $0\text{--}400 \mu\text{g.L}^{-1}$  dissolved zinc) in the pH 7.6 tests after a 72-h deployment. DGT-labile zinc was less than dissolved zinc for all treatments. There was a strong linear relationship between DGT-labile zinc and dissolved zinc in the presence of both DOM sources and at each DOM concentration (Fig. 5). A summary of all DGT-labile and ultra-filtered zinc concentrations is provided in Table S8. Concentrations provided in Table S8 were used to calculate percentages of DGT-lability. Speciation modelling is used in toxicity models, like the biotic ligand model, to predict toxicity of a contaminant under particular water chemistry conditions (Di Toro et al., 2001). WHAM speciation modelling estimates were calculated for all treatments to assess changes in DOM-bound zinc and free ion zinc ( $\text{Zn}^{2+}$ ) for comparison to DGT-lability and toxicity results to determine if changes in speciation can explain changes in observed toxicity. Model estimates indicated a decrease in  $\text{Zn}^{2+}$  and an increase in DOM-bound zinc with increasing concentrations of DOM for both Manton Dam and Appletree Creek. WHAM input and output data is provided in Tables S9–12.

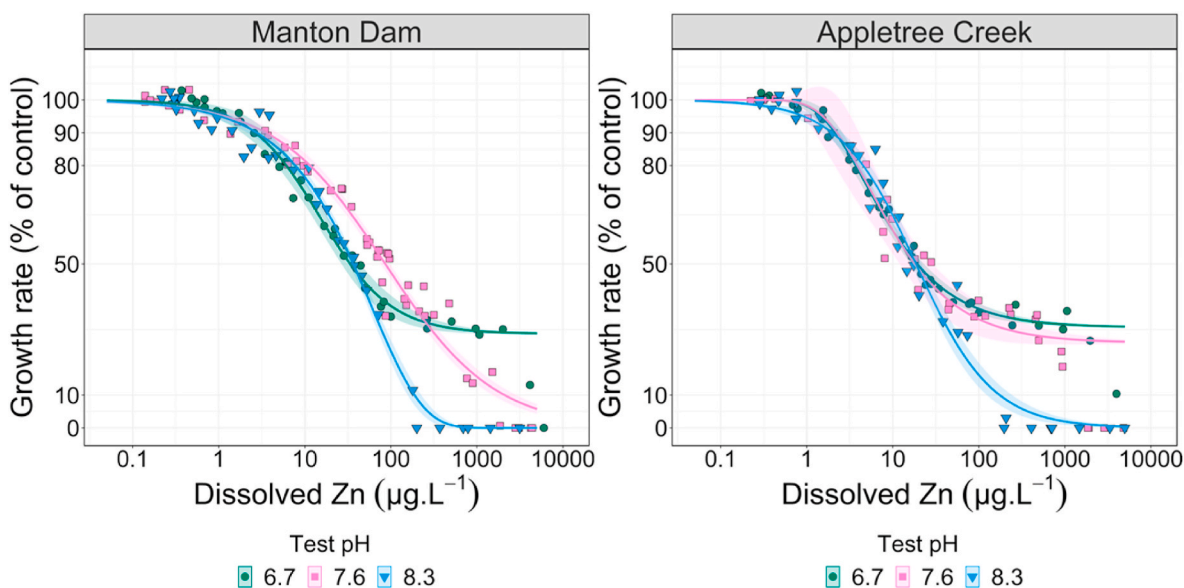
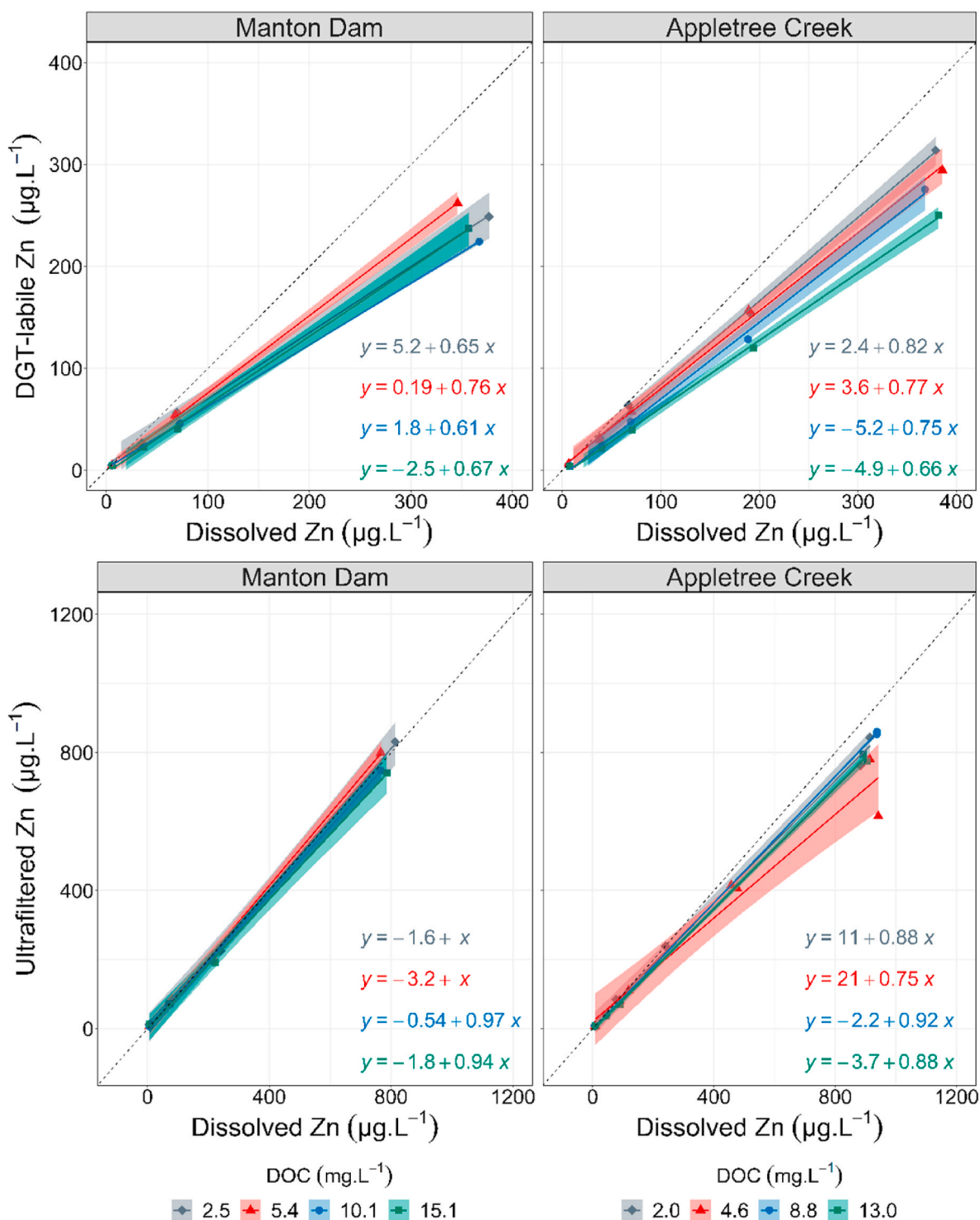


Fig. 4. Concentration-response curves indicating the effect of three pH values (6.7, 7.6 and 8.3) on the growth rate of *Chlorella* sp. when exposed to dissolved zinc in the presence of  $5.5 \text{ mg.L}^{-1}$  of Manton Dam DOM (LHS panel) and  $4.9 \text{ mg.L}^{-1}$  Appletree Creek DOM (RHS panel). Shaded ribbons represent the 95% confidence intervals. Each datapoint represents an individual replicate response and a corresponding measured zinc concentration. Data are pooled from separate experiments. Replicate responses were normalised to their respective controls for inter-test pooling.





**Fig. 5.** Upper panels show the comparison of DGT-labile zinc to dissolved zinc concentrations in the presence of increasing concentrations of Manton Dam DOM (LHS panel) and Appletree Creek DOM (RHS panel). Lower panels show the comparison of ultrafiltered (<3 kDa) zinc concentrations to dissolved zinc concentrations in the presence of increasing concentrations of Manton Dam DOM (LHS panel) and Appletree Creek DOM (RHS panel). Shaded ribbons represent the 95% confidence intervals. Dashed line represents the 1:1 relationship.

Previous work with Manton Dam DOM and copper (Macoustra et al., 2019) found that DOM concentration strongly decreased copper toxicity to *Chlorella* sp. and was explained by corresponding decreases in DGT-labile copper concentrations. In the current study, DGT-labile zinc as a proportion of measured dissolved zinc decreased slightly as Manton Dam DOM concentration increased from an average of 78% of measured dissolved zinc being DGT-labile at 2.5 mg C.L<sup>-1</sup> down to 67% at 15.1 mg

C.L<sup>-1</sup>, however, this difference was not significant ( $p = 0.195$ ,  $F = 1.832$ ) (Table S8). Similarly, small decreases in ultrafiltered zinc as a proportion of measured dissolved zinc occurred as Manton Dam DOM concentration increased. Percentages of ultrafiltered zinc as a proportion of measured dissolved zinc decreased from 101% to 92% across the DOM concentration range of 2.5–15.1 mg C.L<sup>-1</sup> but again, this difference was not significant ( $p = 0.304$ ,  $F = 1.315$ ) (Table S8). The limited

influence of Manton Dam DOM on the labile and ultrafiltered zinc proportions agrees with the general limited effect on EC50 values across the tested DOM concentration range but contrasts with the small ameliorative effect on the EC10 values.

Results of the speciation modelling for Manton Dam at the EC10 and EC50 concentrations found  $Zn^{2+}$  decreased as DOM concentration was increased from 2.5 to 15.1 mg C.L<sup>-1</sup>. At the EC10 concentration a decrease in  $Zn^{2+}$  from 0.4 to 0.2  $\mu g Zn^{2+}.L^{-1}$  (a change from 18 to 3% of dissolved zinc) was found. At the EC50 concentration a decrease in  $Zn^{2+}$  from 24.7 to 11.9  $\mu g Zn^{2+}.L^{-1}$  (a change from 35 to 9% of dissolved zinc) was found. Conversely, DOM-bound zinc (as calculated by WHAM, Table S10) at the EC10 and EC50 concentrations increased as DOM concentration was increased from 2.5 to 15.1 mg C.L<sup>-1</sup>. At the EC10 concentration an increase in DOM-bound zinc from 1.4 to 5.7  $\mu g.L^{-1}$  (a change from 69 to 94% of dissolved zinc) was found, whereas at the EC50 concentration DOM-bound zinc increased from 27.4 to 105  $\mu g.L^{-1}$  (39–83% of dissolved zinc).

These decreases in  $Zn^{2+}$  concentrations and increases in DOM-bound zinc are consistent with the slight decreases in DGT-labile zinc concentrations. This is consistent with the premise that the presence of DOM-bound metals will reduce DGT-lability as not all metals complexed to organic matter are labile (Macoustra et al., 2019). Comparing the speciation estimates of DOM-bound zinc to DGT-labile zinc suggests that at least a portion of DOM-bound zinc is DGT-labile. This is particularly evident at higher DOM concentrations. For example, at 15.1 mg C.L<sup>-1</sup> of Manton Dam DOM, 67% of dissolved zinc was DGT-labile, while speciation modelling suggests that 83–94% of zinc should be DOM-bound across the EC10 to EC50 range.

In the presence of Appletree Creek DOM, DGT-labile zinc concentrations decreased with increasing DOM concentration (Fig. 5 and Table S8). These results agree with those of other studies using Appletree Creek DOM with copper and nickel, where the DGT-lability of both metals decreased in the presence of increasing DOM concentration (Macoustra et al., 2019; Macoustra, Jolley, et al., 2021). However, the decreases in DGT-labile zinc in the current study do not explain the increased toxicity (based on EC50 values) in the presence of Appletree Creek DOM (Fig. 2), with DGT-labile zinc as a proportion of measured dissolved zinc decreasing with increasing Appletree Creek DOM (87% DGT-labile zinc at 2.0 mg C.L<sup>-1</sup> to 58% DGT-labile zinc at 13.0 mg C.L<sup>-1</sup>).

Results for Appletree Creek speciation modelling had similar trends to Manton Dam. At the EC10 concentration a decrease in  $Zn^{2+}$  from 0.4 to 0.1  $\mu g Zn^{2+}.L^{-1}$  (a change from 20 to 3% of dissolved zinc) was found. At the EC50 concentration a decrease in  $Zn^{2+}$  from 5.2 to 1.5  $\mu g Zn^{2+}.L^{-1}$  (a change from 31 to 5% of dissolved zinc) was found. DOM-bound zinc at the EC10 concentration increased from 1.2 to 3.2  $\mu g.L^{-1}$  (a change from 64 to 94% of dissolved zinc) and increased at the EC50 concentration from 7.8 to 22.3  $\mu g.L^{-1}$  (a change from 46 to 89%). These trends are similar to the DGT-labile zinc results for Appletree Creek, and again suggest that a large portion of DOM-bound zinc (as estimated with WHAM) is DGT-labile.

Neither the DGT-lability results nor the speciation modelling explains the observed increased toxicity in the presence of Appletree Creek. Such discrepancies between toxicity and lability and speciation further suggests that the increased toxicity observed in the presence of Appletree Creek DOM may be occurring via a mechanism (such as increased uptake/bioavailability of Zn-DOM complexes) that is not directly related to changes in speciation in solution as measured by DGT or speciation modelling, as discussed previously in section 3.2.

#### 4. Conclusion

DOM source was important in determining the influence of DOM concentration and pH on zinc toxicity to *Chlorella* sp. In the presence of DOM with high amounts of aromatic and higher molecular weight humic-like components zinc toxicity was increased. These increases in

zinc toxicity in the presence of DOM could not be explained by changes in zinc speciation as determined by WHAM, DGT-labile zinc or ultrafiltration measurements across the DOM concentration range. Increased toxicity may be due to the formation of Zn-DOM complexes that are more readily taken up by the microalgae. Future investigations, such as the measurement of intracellular zinc, are needed to elucidate the mechanisms behind the observed toxicity. This study is the first to investigate the independent influence of natural DOM on chronic zinc toxicity to a freshwater microalga and provides high quality data useful for incorporating DOM source and concentration into bioavailability-based water quality guidelines.

#### Credit author statement

**G. Price:** methodology, software, investigation, formal analysis, data curation, writing—original draft, and visualization. **J. Stauber:** conceptualization, methodology, resources, funding acquisition, project administration, writing—review and editing, and supervision. **D. Jolley:** conceptualization, methodology, resources, writing—reviewing and editing. **D. Koppel:** software, writing—review and editing, and supervision. **E. Van Genderen:** conceptualization, and writing—review and editing. **A. Ryan:** software and writing—review and editing. **A. Holland:** methodology, investigation, software, writing—review and editing, and supervision.

#### Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

#### Data availability

Data will be made available on request.

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#### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2022.120797>.

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