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The influence of pH on zinc lability and toxicity to a tropical freshwater microalga

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Abstract

Increased focus on the development and application of bioavailability-based metal water quality guideline values requires increased understanding of the influence of water chemistry on metal bioavailability and toxicity. Development of empirical models, such as multiple linear regression models, requires the assessment of the influence of individual water quality parameters as toxicity modifying factors. This study investigated the effect of pH on the lability and toxicity of zinc to a tropical green microalga (*Chlorella sp.*). Zinc speciation and lability were explored using the Windermere Humic Aqueous Model (WHAM7), ultrafiltration and diffusive gradients in thin-films (DGT). Zinc toxicity increased significantly with increasing pH from pH 6.7 to 8.3, with 50% growth inhibition effect concentrations (EC50) decreasing from 185 to 53 µg $Zn.L^{-1}$ across the pH range. Linear relationships between DGT-labile zinc and dissolved zinc did not vary across the tested pH range, nor did the linear relationship between dissolved (>0.45 µm) zinc and ultrafiltered (<3 kDa)

zinc. Our findings show that zinc toxicity to this freshwater alga is altered as a function of pH across environmentally realistic pH ranges and these toxicity changes could not be explained by zinc speciation and lability as measured by DGT and WHAM7.

Introduction

Metal bioavailability is influenced by many aspects of water chemistry such as major ions, pH, hardness, alkalinity and dissolved organic matter (Stumm and Morgan, 1996). Establishing robust bioavailability-based guidelines is dependent on defining relationships between toxicity and important water chemistry parameters (Adams et al., 2020). Models have been developed to explain these relationships and now form the basis of some water quality guidelines (Brix et al., 2020). Models have ranged from basic empirical models, such as hardness correction algorithms (ANZECC & ARMCANZ, 2000; USEPA, 1985) and multiple linear regressions (MLRs) (Brix et al., 2017), to quasi-mechanistic models such as the biotic ligand model (BLM) (Di Toro et al., 2001).

The BLM is a chemical equilibrium-based model that utilises the principle that metal accumulates at a biotic ligand site e.g. fish gill or algal cell membrane. The model is used to predict the extent to which metal accumulation/binding occurs at the biotic ligand site and how that accumulation relates to toxicity (Adams et al., 2020; Paquin et al., 2002). The model accounts for metal speciation and the influence of competitive ions when considering binding at the biotic ligand and potential metal accumulation. Since its development, the BLM has been incorporated into some regional risk assessment frameworks, both in Europe (Schlekat et al., 2010; Van Sprang et al., 2009) and the United States, with the USEPA developing a BLM-based criteria for copper (USEPA, 2007).

The development of the BLM and its utilisation in some regulatory frameworks has highlighted the effectiveness of understanding the role water chemistry plays on metal bioavailability and consequently, toxicity. However, the full BLM requires at least 10 input water chemistry parameters, not all of which are always available from monitoring data. Recently, there has been a renewed interest in the use of empirical models, such as MLR models, as they can be simpler to use than BLM

approaches, and often require fewer input variables (Brix et al., 2020, 2017; CCME, 2018). Several examples of water quality guidelines developed from MLR models are present in the literature. Brix *et al.* (2017) and Stauber *et al.* (2020) developed MLR-based copper and nickel guideline values, respectively, with both species-specific and pooled models, which were subsequently compared to BLM approaches and found to have similar precision in predicted toxicity for copper and nickel, respectively, under a range of typical water quality conditions. DeForest *et al.* (2020, 2018) used MLR models to develop a water quality guideline for total aluminum (USEPA, 2018), finding that the models were able to adequately predict chronic aluminum toxicity for >90% of cases for all organisms tested.

A critical step in the development of empirical models is understanding the influence of individual water chemistry parameters as toxicity modifying factors (TMFs). An important characteristic for most metals is pH, as metals will have differing speciation across a pH range which in turn can lead to differences in toxicity. Metal accumulation may also be influenced by pH through competition with H^+ at organism binding sites. In regards to the influence of pH on metal toxicity to freshwater microalgae, results have varied among studies, emphasising that the relationship between toxicity and pH is both metal- and organism-specific (Deleebeeck et al., 2009; Heijerick et al., 2002; Wilde et al., 2006).

An understanding of the importance of metal bioavailability has resulted in increased interest and the subsequent development of methods to measure different metal fractions using kinetic approaches (Davison and Zhang, 1994; Zhang and Davison, 2015). These methods include the diffusive gradients in thin films (DGT) technique, a diffusion-based sampling technology. DGT provides an *in-situ* kinetic measurement of the average labile metal concentration over the time deployed (Zhang and Davison, 2015). The method relies on a binding resin that binds cations overlaid by a diffusion layer (comprised of a diffusive gel and filter membrane) which restricts mass transport based on molecular diffusion (Davison and Zhang, 1994). The DGT technique discriminates between metal species based on size and lability, and as such, it provides metal concentrations that

are potentially bioavailable without needing to consider possible complexing ligands present in the solution (Apte et al., 2005; Macoustra et al., 2020). However, the relationship between DGT-labile metal measurements and biological response under changing water chemistry is not well established. Several studies have assessed the influence of TMFs such as dissolved organic matter (DOM) and water hardness on DGT lability for several metals and how this relates to organism toxicity, with Macoustra *et al.* (2020, 2019) assessing the effects of DOM on lability of copper and nickel and Paller *et al.* (2019) assessing the influence of DOM and water hardness on lability of copper and zinc. The findings of these studies suggest that the use of DGT in conjunction with bioavailability models may be a useful tool to assess metal toxicity over a range of water quality conditions. However, limited information is available on the influence of pH on DGT labile metals and their relationship to observed toxicity.

As part of a larger study to provide chronic zinc toxicity data for algae-specific MLR models, the first objective of the current study was to assess the influence of pH (6.7 – 8.3) on the toxicity of zinc to the tropical freshwater microalga, *Chlorella* sp. and to determine if any changes in the observed toxicity were due to differences in metal lability (determined using DGTs) and speciation (measured by ultrafiltration and modelled using WHAM7). As highlighted by Brix *et al.* (2020), data relating to the response of algae and aquatic plants under different water quality conditions is limited, therefore, the results of this study fill an important knowledge gap and will add to the literature on the bioavailability and toxicity of zinc to aquatic organisms under various pH conditions.

Methods

2.1 General laboratory techniques

General glassware and plasticware were cleaned in a dishwasher (Smeg GW4060, Gallay Scientific) using a detergent rinse cycle (Gallay clean A powder detergent, Gallay scientific) and acid rinse cycle (2% HNO_3, Merck), and finished with thorough rinses with ultrapure water (UPW, 18 MΩ.cm, Milli-Q®, Millipore). All glassware and 5 mL polypropylene subsample vials and lids (Technoplas) used in

testing and analysis were soaked in 10% HNO_3 (Merck) for >24 h and thoroughly rinsed with UPW before testing.

2.2 Algal culturing

All algal growth inhibition bioassays were conducted using the tropical freshwater green microalga *Chlorella* sp. (isolate 12), isolated from Papua New Guinea (Stauber and Apte, 1996). Cultures were maintained 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^{-2}.s^{-1}). Algae were transferred into new media weekly and 5 – 7 day old cultures were used for test initiation to ensure exponential growth during testing.

2.3 Toxicity testing

All bioassays were conducted using modified synthetic test water based on the standard USEPA recipe (USEPA, 2002) adjusted to a final hardness of 90 mg $CaCO_3$.L^{-1}. All test treatments were adjusted to the required pH using dilute HCl or KOH and pH was maintained using MOPS (3-N-morpholinopropanesulfonic acid) buffer (free acid form, Merck) to give a final MOPS concentration of 0.5 g.L^{-1} (2.4 mM) in each treatment. MOPS has been shown not to influence metal speciation (Kandegedara and Rorabacher, 1999). Furthermore, De Schamphelaere *et al.* (2004) demonstrated that MOPS was not toxic to *Raphidocelis subcapitata* (formerly known as *Pseudokirchneriella* subcapitata*)* and did not affect the toxicity of zinc to *R. subcapitata* over the tested concentration range of 0.5 – 1 g.L^{-1}. Preliminary tests were conducted to determine the minimum concentration of MOPS needed to reduce pH drift to ± 0.1 units and to verify that MOPS was not toxic to the *Chlorella* sp. used in this study.

Growth inhibition bioassays were conducted using silanised 250 mL Erlenmeyer flasks containing 75 mL of prepared test media. Each flask was spiked with 1.5 mg NO_3^-.L^{-1} ($NaNO_3$) and 0.15 mg PO_4^{3-}.L^{-1} (KH_2PO_4) to sustain exponential growth over the 72 h test. Stock solutions (20 and 1000 mg.L^{-1}) of zinc were prepared using analytical grade zinc chloride ($ZnCl_2$, Sigma-Aldrich) and appropriate volumes were spiked into test flasks. Zinc concentration series (of at least 10 concentrations and

controls (in triplicate) ranging from 0 - 2000 µg $Zn.L^{-1}$) were tested at five pH levels (nominal pH 6.5, 7.0, 7.5, 8.0, 8.5). An unbuffered concentration series (initially adjusted to pH 7.5) without the addition of MOPS was also tested for comparison.

Following a 24-h pre-equilibration period at test conditions and immediately prior to algal inoculation, 25 mL of media was taken from each flask for chemical analysis. To inoculate the test, *Chlorella* sp. cells were harvested, centrifuged (170 g, 7 min, 25 ± 1 °C) and washed with test media. Centrifugation and washing of the algae were repeated three times to ensure removal of culture medium. The remaining algae concentrate was spiked into each test flask at a cell density of 2 – 4 $\times 10^3$ $cells.mL^{-1}$ (Franklin et al., 2002). Tests were conducted in incubator cabinets (LABEC) under constant conditions: 27 ± 1 °C, 12:12 photoperiod, and light intensity of 140 ± 20 µmol $photons.m^{-2}.s^{-1}$ for 72 h. All tests were carried out in duplicate or triplicate to account for inter-test variability.

Algal cell densities were determined at 24, 48 and 72 h by flow cytometry (FACSVerse, BD Biosciences). Population growth rates were assessed as the slope of the linear regression of the log-transformed cell density as a function of time (Franklin et al., 2001). Growth rates were normalised as a percentage of control response to pool inter-test data and account for inter-test variability. Test acceptability criteria included a copper reference toxicant which was run concurrently with each test (72-h 50% effect concentration of 3.8 ± 3.3 µg $Cu .L^{-1}$), <20% coefficient of variation in control growth rates, and >1.2 doublings per day in controls. The pH of buffered tests was required to be maintained at ± 0.1 pH unit over the 72-h test to meet test acceptability criteria.

2.4 Chemical analyses

Metal subsamples were collected at the start (0 h) and end (72 h) of each test from each test flask. Metal subsamples were filtered through acid-rinsed 0.45 µm syringe filters (polyethersulfone membrane, Sartorius). Where total metal subsamples (unfiltered) were collected, test media was poured directly into 5 mL vials. Ultrafiltration was used to assess the colloidal fraction (operationally defined as >3 kDa) in selected tests. Ultrafiltration was performed by passing algae-inoculated test

media through a 0.45 µm filter, as described above, with filtrate placed in an acid-rinsed centrifugal filtration device with a 3 kDa membrane (modified polyethersulfone membrane, Macrosep Advanced, PALL). Devices were then centrifuged at 170 g for >30 min, and a subsample was collected. When ultrafiltration was used, total and dissolved metal subsamples were collected concurrently to provide metal fractions of <3 kDa, >3 kDa - <0.45 µm, and >0.45 µm. All metal samples collected were acidified to 0.2% HNO_3 (Tracepur, Merck) and stored below 4 °C until analysis. All metals were analysed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Agilent 720ES) with a minimum instrument detection limit of 0.16 µg Zn $.L^{-1}$. Quality assurance consisted of matrix-matched calibration standards, blanks, and drift standards.

Samples taken for dissolved organic carbon (DOC) analysis were collected before the addition of MOPS and passed through 0.45 µm membrane filters (polyethersulfone, Sartorius) and acidified with concentrated sulphuric acid (H_2SO_4) in glass amber vials. DOC samples were stored below 4°C until analysis by the non-purgeable organic carbon method (TOC-L series, Shimadzu).

Subsamples for physicochemical analysis, including conductivity (model 30/10 FT, YSI) and dissolved oxygen (Oximeter 330, WTW) were collected from each treatment at the start and end of each test, with subsamples for pH (probe ROSS 815600, Thermo Fischer) measurements being collected every 24 h throughout the test.

2.5 Zinc speciation and lability

Concentrations of zinc species in each test solution were modelled using the equilibrium metal speciation model, Windermere Humic Acid Model (WHAM7). Input parameters consisted of pH, temperature, major ions (Mg^{2+}, Ca^{2+}, K^{+}, Na^{+}, Cl^{-}, SO_4^{2-} and CO_3^{2-}), and an open atmosphere assumption was applied to all speciation calculations.

DGT-labile zinc was measured in at least six zinc concentrations at pH 6.7 and 8.3. A Chelex-100-based binding resin (Na form, 100 – 200 wet mesh) and polyacrylamide diffusive gel were synthesized and assembled into DGT pistons in accordance with procedures outlined by Zhang and

Davison (1995). DGT pistons were deployed in acid-washed polycarbonate vials in 100 mL of test media and inoculated with algal cell densities equivalent to toxicity test flasks. DGT samplers deployed in test vessels were placed on an orbital shaker (90 – 100 rpm) to ensure that the diffusive boundary layer was negligible. Following a 72-h deployment, binding gels were eluted in 1 M HNO_3 for >24 h, and then diluted 10-fold prior to ICP-AES analysis. DGT-labile zinc concentrations were calculated using equations detailed in Zhang and Davison (1995).

2.6 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), cowplot (Wilke, 2019) and ggpubr (Kassambara, 2018). Growth rate inhibition normalised to a percent of the respective control growth rate of that treatment was used as the biological response to derive all toxicity values. Effect concentrations for 10, 20 and 50 percent effect relative to controls (EC10, EC20, and EC50) were calculated using 2-parameter or 3-parameter models. Model selection was based on Akaike's information criterion (AIC) and residual standard error of the model using the mselect function within drc (model parameters listed in Table S1). For all data, a Weibull model with the model upper limit parameter fixed to 100 was the best model. When full effect responses (i.e. EC100) were observed, the lower limit parameter was fixed to 0. The EDcomp and comped functions within drc were used for significance testing of EC values among pH treatments, and the significance of pH as a toxicity modifying factor was determined using ANOVA and F-test as described in Ritz et al., (2015). Relationships between ultrafiltered zinc and total zinc, and DGT-labile zinc and dissolved zinc were determined using linear regressions. Algal growth was compared at varying concentrations of MOPS using ANOVA and a post-hoc Tukey multiple pairwise-comparison to determine if MOPS had any effect on algal growth. All metal concentrations in models and results were measured concentrations.

Results

3.1 Test acceptability and QA/QC

Test acceptability criteria were achieved for all tests. The pH variability was no greater than ± 0.1 units of the average pH in each test treatment (Table 1). Dissolved organic carbon (DOC) concentrations were low, less than 1 mg C.L^{-1} and hardness values did not vary significantly across tests. Control growth rates were acceptable in all pH tests (Figure 1). Tests at pH 8.3 had slightly higher control growth rates compared to the tests at lower pH; however, mean growth rates were within typical growth rates for *Chlorella sp*. Dissolved metal subsamples collected at test initiation (day 0) and completion (day 3) had an average loss of zinc across the test duration of <10%, with the exception of very low zinc treatments (< 10 µg Zn.L^{-1}), where losses were between 0.03 to 3.7 µg Zn.L^{-1}. The mean of day 0 and day 3 metal concentrations was used to model toxicity.

Table 1: The physicochemical characteristics of the test media. Data is pooled across the number of repeated tests (n). Hardness was calculated using measured Ca and Mg concentrations. pH values are the average flask value across the pooled tests. [a] The unbuffered control test pH range represents the start (day 0) and end (day 3) pH values.

Average pH	n	Hardness (mg $CaCO_3$ .L^{-1})	DOC (mg C.L^{-1})	Major ions (mg.L^{-1})		
				Ca	Mg	Na
6.7	3	93	0.66	15.1	13.8	30
7.1	2	93	0.44	15.2	13.5	30
7.7	2	94	0.60	15.3	13.5	30
8.0	2	94	0.60	15.3	13.5	30
8.3	3	93	0.69	15.2	13.3	30
7.5 – 8.3 [a]	5	93	0.54	15.2	13.3	30

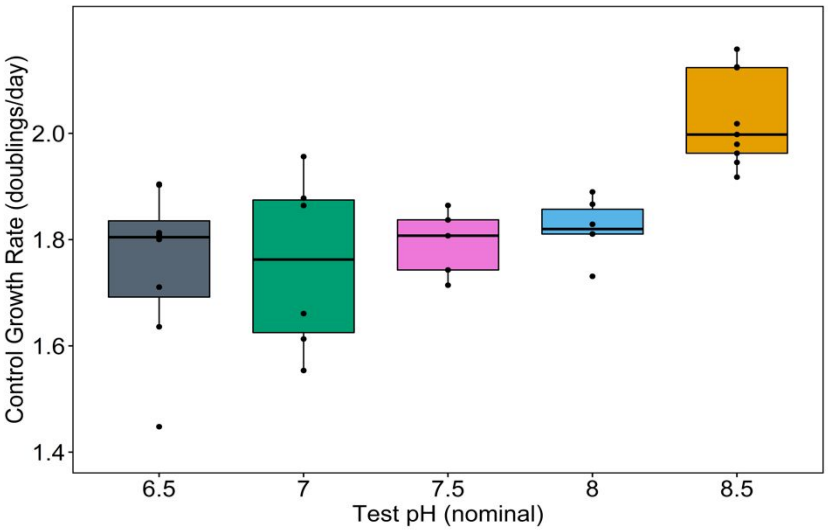

Figure 1: Comparison of growth rates as doublings per day in control treatments (no added zinc) at each pH. Graphed pH values are nominal. Individual control replicate growth rates are plotted as black points overlaid on boxplots. Boxplots show, median, first and third quartile boundaries.

3.2 Toxicity of MOPS buffer to *Chlorella sp.*

Without the addition of MOPS buffer, pH control was poor, increasing over the 72-h test duration by up to 0.8 pH units (Table 1). There was no change to *Chlorella sp*. growth rate in the presence of the MOPS buffer over the concentration range of 0 – 2.0 g MOPS.L^{-1} (Figure 2). No significant difference ($p = 0.58$) in growth rates relative to controls (no added MOPS) was observed in any treatment, with each treatment recording average growth rates within the standard control growth rates of 1.8 ± 0.5 doublings per day. These results are similar to De Schamphelaere *et al.* (2004) who found no observed toxicity to *R. subcapitata* when exposed to concentrations of MOPS up to 1 g.L^{-1}. Concentrations above 0.5 g MOPS.L^{-1} were found to be sufficient to maintain a pH value ± 0.1 pH-units across the 72-h period (Table 1). Based on these results 0.5 g MOPS.L^{-1} was used for buffering all test treatments.

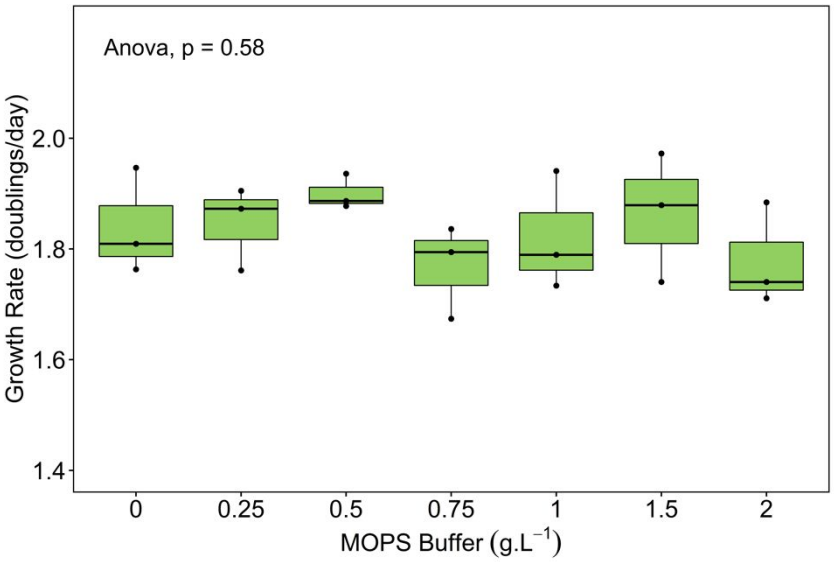

Figure 2: Comparison of growth rates as doublings per day with increasing concentrations of 3-N-morpholinopropanesulfonic acid (MOPS) buffer. MOPS concentrations are reported as nominal. Individual treatment growth rates are plotted as black points overlaid on boxplots.

3.3 The effect of pH on zinc toxicity on *Chlorella* sp.

Algal growth rates decreased with increasing zinc concentrations across all pH treatments (Table 2, Figure 3). *Chlorella sp*. sensitivity to zinc increased linearly with increasing pH from 6.7 to 8.3. The 72-h EC50 values decreased approximately 4-fold from 185 to 53 µg $Zn.L^{-1}$ (Figure 3 and 4) across the pH range (Table 2). All 72-h EC50 values were significantly different except for pH 6.7 and 7.1, pH 7.1 and 7.7, and pH 7.7 and 8.0. Similar toxicity trends were not present at lower effect concentrations; 72-h EC10 values showed no clear trend with increasing pH, with values varying from 0.79 µg $Zn.L^{-1}$ at pH 7.1 to 4.5 µg $Zn.L^{-1}$ at pH 6.7 (Table 2). There was a linear relationship between the 72-h EC50 values and pH for both measured dissolved zinc (Figure 4A) and modelled free zinc ion measurements (Figure 4C), with R^2 values of 0.89 and 0.96, respectively. Relationships between 72-h EC10 and EC20 are provided in supplementary information in Figures S1 and S2, respectively.

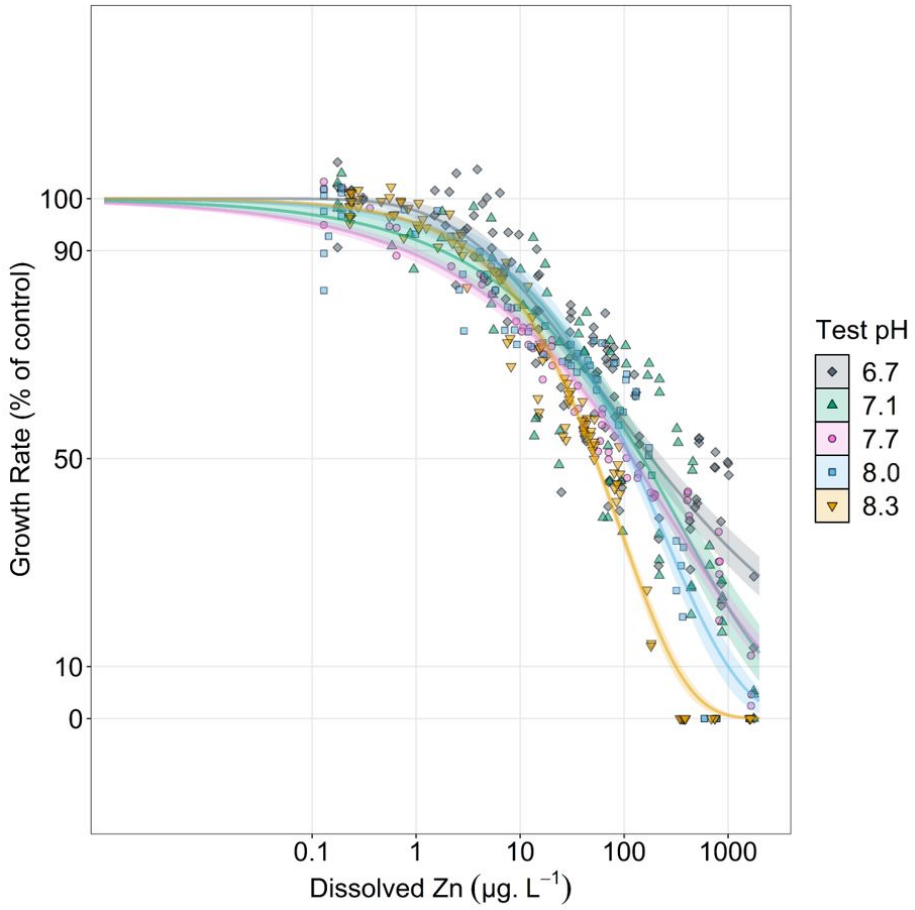

Figure 3: 72-h growth rate inhibition of Chlorella *sp. (% of control) exposed to zinc concentrations at five different pH values. Shaded ribbons represent the 95% confidence intervals. Each data point represents one individual replicate response and a corresponding measured zinc concentration. Data was pooled from separate experiments. Replicate responses were normalised to their respective controls for inter-test pooling. Individual model figures are provided in Figure S3, supplementary information.*

Table 2: 72-h effect concentrations (EC10/EC50) for population growth inhibition of Chlorella *sp. exposed to zinc under different pH conditions. Effect concentrations were calculated using pooled test data. 95% confidence intervals are shown in parentheses. Free ion EC values represent the WHAM7 calculated free ion concentration at the dissolved EC values. Control growth rate is shown as doublings per day.*

Test	Control growth rate	Dissolved (µg Zn.L^{-1})			Free ion Zn^{2+} (µg.L^{-1})		
		EC10	EC20	EC50	EC10	EC20	EC50
pH 6.7	1.8	4.5 (2.8 - 6.3)	13.7 (9.9 - 17)	185 (139 - 231)	3.0 (1.8 - 4.2)	9.1 (6.6 - 12)	122 (92 - 153)
pH 7.1	1.8	1.8 (0.33 - 3.2)	10 (4.8 - 16)	151 (112 - 191)	1.1 (0.2 - 2.0)	6.4 (3.0 - 9.9)	93 (69 - 118)
pH 7.7	1.8	0.79 (0.49 - 1.1)	5.8 (4.4 - 7.3)	120 (104 - 135)	0.45 (0.3 - 0.6)	3.3 (2.5 - 4.1)	68 (60 - 77)
pH 8.0	1.8	4.1	15.6	118	2.0	7.5	57

		(2.1 - 6.1)	(10 - 21)	(100 - 136)	(1.0 - 2.9)	(5.1 - 10)	(48 - 66)
pH 8.3	2.0	3.2	9.8	53	1.1	3.4	18
		(2.6 - 3.8)	(8.5 - 11)	(49 - 56)	(0.9 - 1.3)	(3.0 – 3.8)	(17 - 19)
Unbuffered	1.4	2.7	7.7	45	-	-	-
		(1.4 - 4.1)	(5.2 - 11)	(35 - 55)			

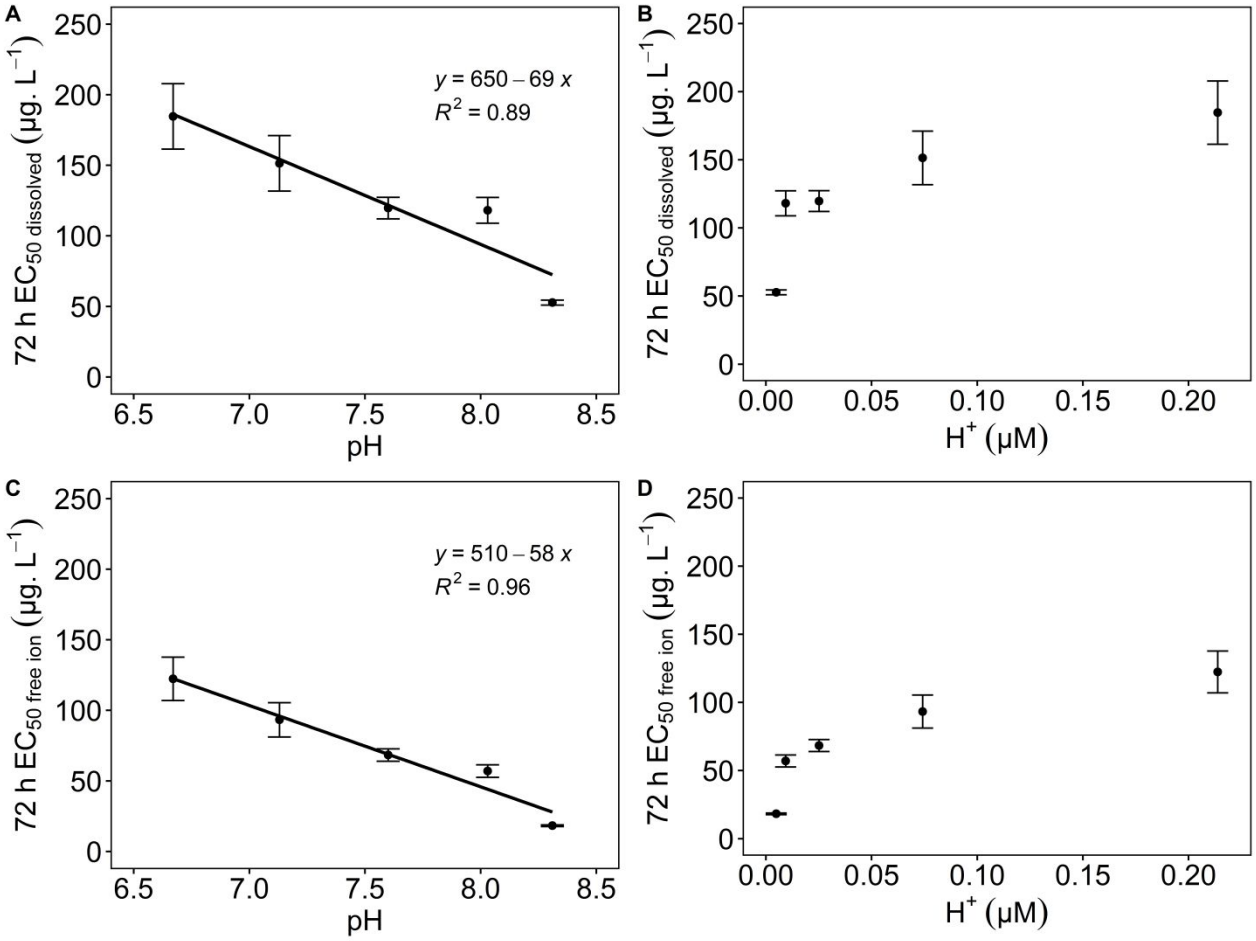

Figure 4: The effect of pH and H^+ concentrations on zinc toxicity to Chlorella sp. *across a pH range of 6.7 – 8.3. A) and B) show results using dissolved metal concentrations and C) and D) show results using WHAM7 modelled free zinc ion.*

3.4 Zinc speciation and lability

Speciation calculations (WHAM7) demonstrated that the free ion zinc (Zn^{2+}) was the major species present across the pH range tested (6.7 – 8.3). Zinc species distribution changed with changing pH, with Zn^{2+} gradually decreasing from 61% at pH 6.7 to 30% at pH 8.3. $ZnHCO_3^+$ increased from 23% at pH 6.7 to 26% at pH 7.7 before decreasing to 16%. $ZnCO_3$ increased with pH from 0.44% at pH 6.7 to 13% at pH 8.3. $Zn(OH)^-$ and $Zn(OH)_2$ increased with pH from 0.30% to 6.5% and 0.02% to 17%, respectively from pH 6.7 to 8.3. A full summary of the calculated zinc species distribution across the pH range is provided in supplementary information in Table S3 and Figure S4.

Comparison of ultrafiltered (<3 kDa) zinc concentrations and total (unfiltered) zinc concentrations at pH 6.7 and 8.3 showed that they had close to a 1:1 relationship with an average of 99.8% and 92.4% of measured total zinc concentrations present as the 'truly dissolved' or ultrafiltered fraction for pH 6.7 and 8.3, respectively (Figure 5A). This small difference, which was not significant (p=0.56), may be due to analytical variability at the low zinc concentrations close to the ICP-AES limit of reporting (0.12 – 0.31 µg $Zn.L^{-1}$). Exclusion of these low zinc concentration treatments gave a value of 99.3% truly dissolved zinc at pH 8.3 also confirming that there is likely to be no difference in truly dissolved zinc across the tested pH range.

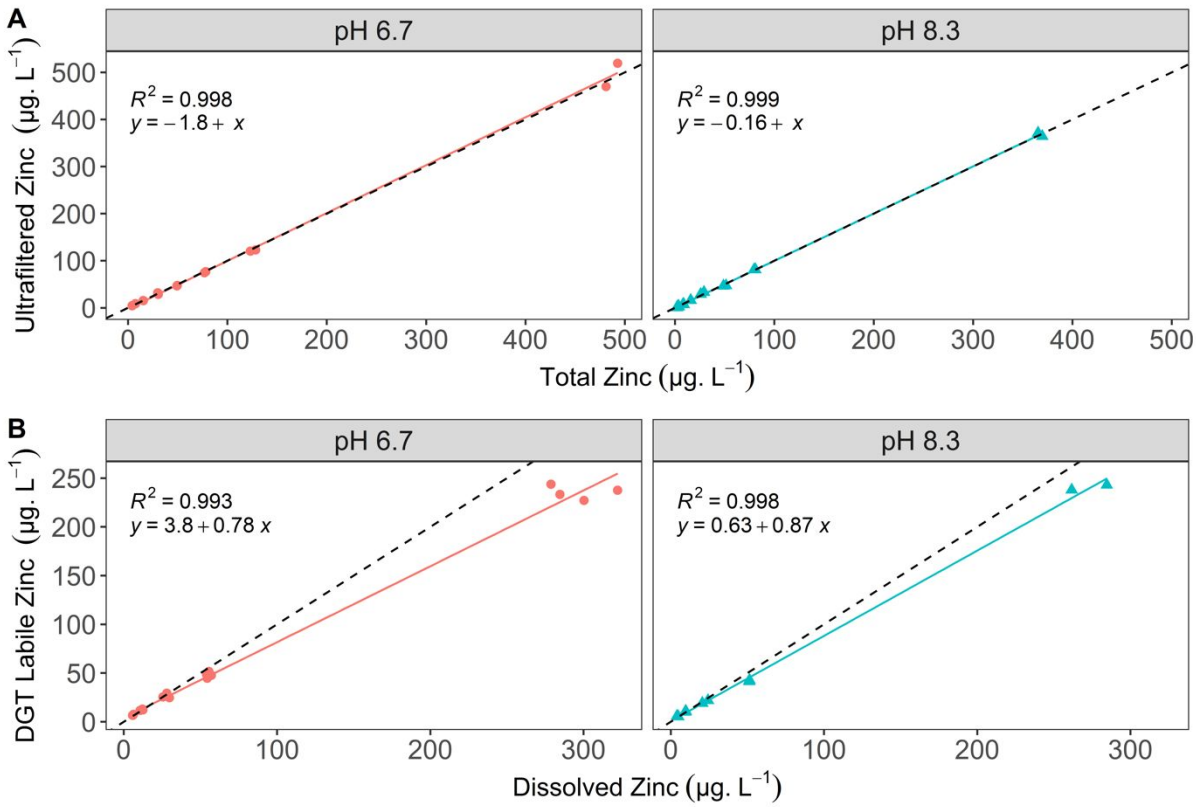

Figure 5: Comparison of A) ultrafiltered (<3 kDa) zinc concentrations compared to total zinc (unfiltered) concentrations; and B) DGT-labile zinc concentrations to dissolved (day 3) zinc concentrations. Dashed black line indicates the 1:1 ratio and correlation coefficients are calculated using the Pearson method.

Due to the size of the DGTs, they were deployed into 120 mL polycarbonate vials rather than the silanised glass toxicity test flasks and large losses of zinc over time were observed. These decreases are likely due to insufficient pre-equilibration times (24 h), with average dissolved zinc losses of 45%

across the test duration. Mean dissolved zinc concentrations assume an even weighting for both day 0 and day 3, which implies losses of zinc to the vessel are linear across the exposure time. This is unlikely the case and rates of losses may be exponential rather than linear (Simpson et al., 2003). Therefore DGT-labile zinc (DGT_{Zn}) was compared to day 3 dissolved zinc.

DGT_{Zn} was 95% and 99% of day 3 dissolved zinc at pH 6.7 and 8.3, respectively, with no apparent zinc concentration-dependent effects observed for either pH. Linear regression indicates that the relationship between DGT_{Zn} and dissolved zinc was linear for both pH 6.7 and pH 8.3, with R^2 values of 0.993 and 0.998, respectively (Figure 5B). Ratios of DGT_{Zn} and dissolved zinc were not significantly different ($p=0.80$) between the two pH values, suggesting that the pH range tested did not significantly affect the lability of zinc as measured by DGT. When comparing DGT_{Zn} to mean dissolved metals, DGT_{Zn} was 68% and 64% of the dissolved zinc at pH 6.7 and 8.3, respectively. There was no significant difference ($p=0.108$) between the two pH values, and as such does not alter the finding that DGT-lability was unaffected across the pH range.

Discussion

4.1 Relationship between pH and zinc toxicity

Based on 72-h EC50 values, there was an approximately 4-fold increase in zinc toxicity as the pH increased from pH 6.7 to 8.3. This increase was significantly less than the 20-fold increase in zinc toxicity found by Wilde et al. (2006) for the same algal species across a similar pH range of 6.5 to 8.0, with EC50 values decreasing from 970 to 52 µg $Zn.L^{-1}$. These findings are also less than reported by Heijerick et al. (2002) who found an 11-fold increase in zinc toxicity from pH 6.8 to 7.8 for the alga *R. subcapitata*, with EC50 values decreasing from 95 to 11 µg $Zn.L^{-1}$. Similar toxicity trends have been reported for other metals for microalgae (Franklin et al., 2000; Heijerick et al., 2002; Wilde et al., 2006). Franklin et al. (2000) reported a 23-fold and 1.7-fold increase in copper and uranium toxicity, respectively, to a Northern Territory (Australia) *Chlorella* species isolate across a narrower pH range

of 5.7 to 6.5. Deleebeeck et al. (2009) observed a 1.8-fold increase in nickel toxicity across a pH range of 6.45 to 7.92 for *R. subcapitata.* Such differences in magnitude of metal toxicity are likely explained by multiple factors including biological differences across species, different initial cell densities in exposure bioassays and the various buffering techniques used (De Schamphelaere et al., 2004; Esbaugh et al., 2013; Franklin et al., 2002). For example, Franklin et al. (2002) found that increasing the initial cell density of *Chlorella* sp. from 10^2 to 10^5 cells.mL^{-1} resulted in a 3.5-fold decrease in copper toxicity with EC50 values ranging from 4.6 to 16 µg.L^{-1}. The study found increased algal cells resulted in a decrease in extracellular copper binding, thus decreasing toxicity.

The influence of buffers can be seen when comparing this study to Wilde et al. (2006). Zinc toxicity to the same culture of *Chlorella sp*. deviated significantly between the two studies when different buffers were used. In the study by Wilde et al. (2006), 2 mM MES (2-[N-morpholino]ethanesulfonic acid sodium salt) was used for pH 6.5 and 2 mM PIPES (piperazine-N,N'-bis[2-ethanesulfonic acid] disodium salt) was used for pH 7.0. The Wilde et al. (2006) results represent a 5-fold and 4-fold decrease, relative to the current study, in zinc toxicity at pH 6.5 and 7.0, respectively, when using these buffers. This reduction in toxicity may be explained by increased sodium concentrations from sodium-salt buffers, compared to no increase in sodium from the free-acid form of buffer used in the current study. Heijerick et al. (2002) has demonstrated the ameliorative effect of sodium to *R. subcapitata*, where an increase in sodium from 2.7 to 7.2 mM resulted in a 2.1-fold reduction in zinc toxicity.

Increases in zinc toxicity with increasing pH are typically not observed in many other organisms, such as *Daphnia magna* (Heijerick et al., 2003) and rainbow trout (De Schamphelaere and Janssen, 2004), where in both cases a linear decrease in toxicity with increasing pH is observed. This relationship is often explained by the changes in metal speciation, with the free metal ion becoming less dominant as pH increases, due to the increased availability of hydroxide ions (OH^-) to form metal hydroxide complexes. This difference seen between microalgae and other organisms highlights the importance of considering microalgae when developing bioavailability-based water quality criteria.

4.2 Zinc speciation

The changes in zinc toxicity to *Chlorella* sp. with increasing pH cannot be explained by zinc speciation changes in solution. Speciation modelling using WHAM7 showed that over the pH range tested, as the pH increased, the percentage of total zinc as the free Zn^{2+} ion decreased by 30%, and the percentage of zinc as $ZnCO_3$, $Zn(OH)^+$ and $Zn(OH)_2$ increased. Such changes in metal speciation do not explain the apparent increase in zinc toxicity with pH, given that Zn^{2+} is generally considered to be the most bioavailable and thus most toxic form of zinc (Morel, 1983). Although the optimization of biotic ligand binding constants for $Zn(OH)^+$ has been shown to improve zinc BLM models and therefore is likely to contribute to toxicity to some species (Deforest and Van Genderen, 2012), it is unknown whether zinc hydroxides or carbonates are directly toxic to microalgae. Rather, the increase in toxicity with increasing pH has been widely attributed to reduced proton competition with the free metal ion at the algal cell surface (Mebane et al., 2020; Parent and Campbell, 1994; Worms et al., 2007) and this is supported by our results. Nonlinearity observed between zinc toxicity (as dissolved zinc or free ion activity, Figure 4B and 4D, respectively) and proton concentrations observed in this study is consistent with previous studies (Heijerick et al., 2002; Wilde et al., 2006). The relationship between toxicity and proton concentration appears to be metal-specific, with copper toxicity showing a linear relationship with proton concentration for several different organisms (Brix et al., 2017). Given the differences between metals, the way Zn^{2+} binds to the algal cell likely explains the nonlinearity observed - zinc may bind to multiple binding sites, not only sites involved in proton competition (Deleebeeck et al., 2009). Additionally, it has been suggested that the number of zinc/H^+ competitive binding sites may change as the pH changes (De Schamphelaere et al., 2005; Heijerick et al., 2002). Others have suggested that toxicity changes arise as a result of conformational changes in transport proteins, which may lead to increasing metal-binding affinity at the algal cell wall (François et al., 2007; Parent and Campbell, 1994). Changes in algal cellular zinc concentrations (intracellular and extracellular) across a pH range of 6.5 to 8.0 were examined for *Chlorella sp*. by Wilde et al (2006). The study found extracellular zinc concentrations increased 3-fold

from pH 6.5 to 8.0, while intracellular zinc did not change with added dissolved zinc concentrations as pH changed. Increased extracellular zinc with increased pH may provide further evidence to proton competition being the driver of toxicity changes seen in the current study.

Ultrafiltration measurements found that there was no difference between ultrafiltered zinc concentrations at the two pH treatments, suggesting that there were no significant changes in colloidal or truly dissolved zinc across the tested pH range. Results of the DGT measurements also found no significant difference in DGT-labile zinc concentrations relative to dissolved zinc concentrations across the pH range tested. This suggests that zinc lability is unchanged across the test pH range, while the organism response at different pH values suggests that zinc bioavailability has changed. Such results provide more evidence that proton competition rather than metal speciation changes is primarily responsible for changes in the observed toxicity to this alga.

In recent years there has been increased research into linking DGT-labile metal measurements to metal bioavailability in order to predict metal toxicity to test organisms (Koppel et al., 2019; Philipps et al., 2018). The DGT technique has previously been shown to be subject to uptake effects with changing pH, where Zhang and Davison (1995) demonstrated that above pH 5 the DGT-labile cadmium in pH-adjusted ultrapure water was unaffected by proton competition, with uptake effects from elevated proton concentrations being present only at lower pH (2.3 to 5). The results of the current study agree with those findings and highlight that algal sensitivity to metal/proton competition is outside the range of DGT measurements affected by cationic competition, and therefore DGT measurements do not reflect the effects of pH on zinc toxicity.

DGT as a tool to predict bioavailability under varying water qualities has recently been studied. For example, Macoustra et al (2019) found that ratios of DGT-labile copper to dissolved copper concentrations were affected similarly by DOC source to the same species of *Chlorella* used in our study, suggesting that the DGT-labile fraction may be a good predictor of protective effects of DOC. However, similar to results of the current study, Paller et al (2019) found DGT-labile zinc did not

change greatly with varied water hardness, while zinc toxicity to *Ceriodaphnia dubia* varied significantly. It is widely considered that both pH and hardness act to modulate metal bioavailability through cationic competition with pH also affecting speciation, whereas DOC ameliorates toxicity through complexation and reducing bioavailability (Di Toro et al., 2001; Paquin et al., 2002). The studies of Macoustra et al (2019) and Paller et al (2019) along with the current study highlight DGT measurement's usefulness and limitations in predicting changes in metal bioavailability under varying water quality parameters.

Conclusions

This study showed that zinc toxicity to a tropical freshwater alga varied as a function of pH, with a linear relationship between EC50 values and pH. Increases in pH, across a pH range of 6.7 to 8.3, resulted in a 4-fold increase in zinc toxicity. Measurements of DGT-labile zinc and ultrafiltered zinc were unaffected by pH across the tested range, although WHAM predicted a decrease in Zn^{2+} concentrations and an increase in $ZnCO_3$, $Zn(OH)^+$ and $Zn(OH)_2$ species. These results highlight that zinc speciation and lability does not solely explain zinc toxicity across varying pH values in freshwater. The toxicity results of this study will add to the limited data on algal response to zinc under different water quality conditions. The findings of this study provide further evidence that microalgae respond to metal toxicity in a converse manner to animals under varying pH and highlights the importance of considering algae/plant specific modelling for bioavailability-based guideline derivation.

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