One Size Doesn't Fit All

Developing Bioavailability-based Models for Zinc Toxicity to Microalgae

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Submitted in fulfilment of the requirement for the degree of Doctor of Philosophy

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2023

CERTIFICATE OF ORIGINAL AUTHORSHIP

I, Gwilym AV Price, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Life Sciences, Faculty of Science at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

This research is supported by an Australian Government Research Training Program.

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Date: 28 November 2023

COVID-19 IMPACT STATEMENT

The research conducted within this thesis was completed between March 2019 and December 2023. The COVID-19 pandemic and subsequent Australian and NSW-specific lockdowns impacted the experimental and field components of this thesis. The 2020 NSW lockdown delayed and then slowed the rate at which experimental work (toxicity testing and chemical analysis) could be completed across a 6-month period. The 2021 NSW lockdown again delayed and slowed the rate at which experimental work could be completed, and delayed and limited field work needed to collect natural water samples from around Australia.

KEYWORDS

bioavailability, biotic ligand model, chemistry, dissolved organic carbon, ecotoxicology, environmental management, flow cytometry, freshwater, hardness, microalgae, model validation, multiple linear regression, pH, speciation, statistics, toxicity, water quality guidelines, water quality parameters, zinc

ABSTRACT

Increasing urbanisation and industrialisation are intensifying pressures on natural ecosystems. This includes metal contamination in freshwater environments. Australia is a major miner and refiner of zinc ore. These operations could lead to increased risk of zinc contamination to freshwater systems due to spills, discharge and runoff. Australia implements national default water quality guidelines to manage this risk. The current guideline values for zinc, however, do not adequately account for water chemistry parameters that are known to modify zinc toxicity to aquatic organisms.

To develop national zinc water quality guidelines that account for a range of water chemistries and are relevant to Australian freshwater conditions, a detailed understanding of how water chemistry influences zinc toxicity is needed. To date, there has been limited understanding of how water chemistry affects zinc toxicity to microalgae, a key group of organisms in freshwater ecosystems that are also used in Australian water quality guideline development. Thus, this thesis investigated the influence of a range of water chemistry conditions on the chronic toxicity of zinc to a freshwater microalga, *Chlorella* sp.

Firstly, the influences of pH, hardness, and dissolved organic carbon (DOC) on zinc toxicity to *Chlorella* sp. were investigated. The microalgae became more sensitive to zinc with increasing pH and less sensitive to zinc with increasing hardness. The influence of DOC was variable and dependent on the chemical composition of the DOC, with one DOC source increasing zinc toxicity while the other had limited effect.

Secondly, a detailed assessment of the commonly used bioavailability-based model validation method, the ‘factor-of-2 rule’, was undertaken. This rule is solely based on acute fish and daphnia toxicity data and therefore may not be appropriate to all test organisms and effect levels. The datasets investigated highlighted that larger variability exists in low effect levels and supported the use of a factor-of-3 rule.

Thirdly, multiple linear regression models to predict zinc toxicity to *Chlorella* sp. were developed using toxicity data from this thesis. Models were independently validated using zinc-spiked natural Australian waters to assess model performance under environmentally realistic conditions. The models performed poorly when predicting toxicity in the natural waters, with models consistently overpredicting toxicity. This consistent overprediction questions the underlying assumption that models developed from synthetic laboratory waters can be directly applied to natural water samples.

Approaches to bioavailability-based environmental risk management are discussed considering the experimental data and models produced in this thesis. The findings presented in this thesis contribute to a broader understanding of how water chemistry influences zinc toxicity to microalgae and assists with developing bioavailability-based zinc water quality guidelines for Australia.

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LIST OF PUBLICATIONS AND REPORTS

The contents of Chapters 2 – 6 in this thesis have been published in five papers. Collaboration and contributions from co-authors have been acknowledged at the beginning of each chapter.

The publications are:

1. **Price, G. A. V**., Stauber, J. L., Holland, A., Koppel, D. J., van Genderen, E. J., Ryan, A. C., & Jolley, D. F. (2021). The Influence of pH on Zinc Lability and Toxicity to a Tropical Freshwater Microalga. *Environmental Toxicology and Chemistry*, 40(10), 2836–2845. https://doi.org/10.1002/ETC.5177

2. **Price, G. A. V**., Stauber, J. L., Holland, A., Koppel, D. J., van Genderen, E. J., Ryan, A. C., & Jolley, D. F. (2022). The influence of hardness at varying pH on zinc toxicity and lability to a freshwater microalga, *Chlorella* sp. *Environmental Science: Processes & Impacts*. https://doi.org/10.1039/d2em00063f

3. **Price, G. A. V**., Stauber, J. L., Jolley, D. F., Koppel, D. J., van Genderen, E. J., Ryan, A. C., & Holland, A. (2023). Natural organic matter source, concentration, and pH influences the toxicity of zinc to a freshwater microalga. *Environmental Pollution*, 318, 120797. https://doi.org/10.1016/J.ENVPOL.2022.120797

4. **Price, G. A. V**., Stauber, J. L., Stone, S., Koppel, D. J., Holland, A., & Jolley, D. (2022). Does toxicity test variability support bioavailability model predictions being within a factor of 2? *Environmental Chemistry*, 19(4), 177–182. https://doi.org/10.1071/EN22050

5. **Price, G. A. V**., Stauber, J. L., Jolley, D. F., Koppel, D. J., van Genderen, E. J., Ryan, A. C., & Holland, A. (2023). Development and Validation of Multiple Linear Regression Models for Predicting Chronic Zinc Toxicity to Freshwater Microalgae. *Environmental Toxicology and Chemistry*. https://doi.org/10.1002/etc.5749

The following report was prepared for the funding body:

1. Stauber, J. L., **Price, G. A. V**., Evans, A., Gadd, J., Holland, A., Batley, G. E., Binet, M. T., Golding, L. A., Hickey, C. W., Harford, A. J., Jolley, D. F., Koppel, D. J., McKnight, K. S., Morais, L., Ryan, A. C., Thompson, K., Van Genderen, E. J., Van Dam, R. A., & Warne, M. St. J. (2022). Towards Bioavailability-Based Guideline Values for Zinc for Australian and New Zealand Freshwaters. CSIRO Report EP2022-0801. CSIRO, Australia

The following publication was prepared with contributions from my thesis:

1. Stauber, J. L., Gadd, J., **Price, G. A. V**., Evans, A., Holland, A., Albert, A., Batley, G. E., Binet, M. T., Golding, L. A., Hickey, C., Harford, A., Jolley, D., Koppel, D., McKnight, K. S., Morais, L. G., Ryan, A., Thompson, K., Van Genderen, E., Van Dam, R. A., & Warne, M. S. J. (2023). Applicability of Chronic Multiple Linear Regression Models for Predicting Zinc Toxicity in Australian and New Zealand Freshwaters. *Environmental Toxicology and Chemistry*, https://doi.org/10.1002/etc.5722

CONFERENCE PRESENTATIONS AND OUTREACH

1. 2019 SETAC Australasia, 7-10 July, Darwin: **Price, G. A. V**., Stauber, J. L., Holland, A., van Genderen, E. J., Jolley, D. F. *The Influence of Water Chemistry on Zinc Toxicity – Back to Basics*

2. 2020 SETAC North America 41st, 15-19 November, Forth Worth, Texas: **Price, G. A. V**., Stauber, J. L., Holland, A., Koppel, D. J., van Genderen, E. J., Jolley, D. F. *Understanding the Influence of pH, Hardness and Dissolved Organic Carbon on Zinc Toxicity to a Tropical Freshwater Microalga*

3. 2021 SETAC 10th Young Environmental Scientists Meeting, 22-26 Feb (Virtual Meeting): **Price, G. A. V**., Stauber, J. L., Holland, A., Koppel, D. J., van Genderen, E. J., Jolley, D. F. *Understanding the Influence of pH, Hardness and Dissolved Organic Carbon on Zinc Toxicity to a Tropical Freshwater Microalga*

4. 2021 SETAC AU 30 August - 2 September (Virtual meeting): Evans, A., **Price, G. A. V**., Stauber, J. L., Jolley, D. F., Holland, A. *The influence of toxicity modifying factors (pH, DOC and water hardness) in Australian natural waters on toxicity and bioavailability of zinc to the cladoceran Ceriodaphnia dubia*

5. 2022 SETAC Europe 32nd, 15-19 May, Copenhagen: **Price, G. A. V**., Stauber, J. L., Holland, A., Koppel, D. J., van Genderen, E. J., Ryan, A., Jolley, D. F. *Development and validation of multiple linear regression models for predicting chronic zinc toxicity to freshwater algae in Australian natural waters*

6. 2022 SETAC Europe 32nd, 15-19 May, Copenhagen: Stauber, J. L., **Price, G. A. V**., Evans, E., Gadd, J., Holland, A., et al. *Towards Bioavailability-based Guideline Values for Zinc in Australian and New Zealand Freshwaters*

7. 2022 AFSS, 28 November - 2 December, Batemans Bay: Holland, A., Stauber, J. L., **Price, G. A. V**., Macoustra, G., Koppel, D. J., Jolley, D. F. *Contrasting effects of dissolved organic matter on metal toxicity and bioavailability in freshwaters*

8. 2023 IZA Bioavailability Workshop, 27-28 April, Sydney: **Price, G. A. V**., Stauber, J. L. *Towards Bioavailability-based Guideline Values for Zinc in Australian and New Zealand Freshwaters*

9. 2023 SETAC Australasia, 7-11 August, Townsville: **Price, G. A. V**., Stauber, J. L., Jolley, D. F., Koppel, D. J., Holland, A. *DOM toxicity amelioration is a scam! Well… at least for some Australian DOM sources when microalgae are exposed to zinc*

10. 2023 SETAC Australasia, 7-11 August, Townsville: Stauber, J. L., **Price, G. A. V**., Gunkel-Gillon, P., Dominique, Y., Golding, L. *Deriving site-specific freshwater nickel, cobalt and chromium guidelines in the presence of naturally high background concentrations: A case study from New Caledonia*

ACKNOWLEDGEMENTS

I acknowledge the traditional custodians of the lands where I lived and worked during this thesis, the Dharawal and muwinina people. I pay my respects to elders past and present.

A thesis is an endeavour of knowledge, discovery, and personal growth, made possible through the selfless acts of those around you. This thesis was no different. Much of the support I received, from so many different people, cannot be measured on an ICP, counted on a flow cytometer or modelled in lines of code. The love, support, and care I received throughout this journey has often been immeasurable on the microscale, yet always clearly evident and felt on the macroscale as I sit here writing the last page of this thesis. Why was this the last page to write? Because it is the hardest. Not because of the need to wrack one's brain to find actions and people to acknowledge, it is hard because I know I am unable to appropriately put into the words the gratitude I have for my support team.

If you listed the names Jenny Stauber, Aleicia Holland, Darren Koppel, and Dianne Jolley to anyone in the field of environmental chemistry and ecotoxicology, there would be a general consensus that you would be hard pressed to find a more knowledgeable, caring, fun, and fantastic supervisory team. I feel so lucky to have been mentored and guided by you all. I can attribute much of my expertise to you all. Starting as far back as CHEM214 lectures by Di, to as recently as the review of this thesis where you all provided so many useful writing tips and skills. Thank you for helping me establish my research career - I would not be where I am today without you all. I look forward to continuing to work with you all. I would like to thank Prof. Alaina Ammit for your guidance and positivity as you helped me navigate the systems of UTS from afar.

Sharing this journey with friends turned some of the hardest times into some of the best. Sarah and Kitty – thank you for your friendship, laughter, advice, and company over coffee. You both made an empty Building 2 feel full. Thank you for teaching me so much about the world of science and the world itself – I am a better scientist and person because of you both. Thank you to the CSIRO and Jolley research groups for providing a network of friendship I will have forever, you have all helped me so much in the lab, the field, the office, the tea room, at conferences, on the touch footy field and beyond. A special thanks to Jenny Stauber, Kitty McKnight, Monique Binet, Lisa Golding, David Spadaro, Sarah Stephenson, Merrin Adams, Graeme Batley, and Stuart Simpson for welcoming me into the CSIRO Team. It has been a privilege to be part of the Lucas Heights team.

Thank you to my family, who have always been there to offer advice or a laugh, a jump start when my car has broken down, shared a drink with me at the Charles or the Patch, visited me in Hobart,

or simply provided good company when we are together. Sally, Mum, Dad, Anwen, Ceridwen, Eluned, Sam, Jake, Tom, and Adam – thank you.

Sally, your contributions to this thesis and my life are perhaps the most immeasurable. You have been the one constant across these four years, despite a large portion of this time being spent apart due to lockdowns, jobs and field work. Thank you for your love, kindness, support, and patience. I look forward to having a little more free time to spend with you.

THESIS STRUCTURE

This thesis is written and structured as a compilation of five published peer-reviewed journal articles (Chapters 2 – 6). The thesis opens with an introductory chapter (Chapter 1) discussing background context, relevant literature, and the aims and objectives of the thesis. A final chapter (Chapter 7) discusses the results of all five research chapters and places the research within the broader scientific field of environmental chemistry and ecotoxicology. The final chapter also provides commentary on the implication for and practicalities of regulatory use of the research in this thesis. Each research chapter is published and listed in the *List of Publications and Reports*. Research chapters have been reformatted to unify this thesis.

As this thesis has been prepared as a compilation of journal articles, each chapter provides its own introduction and methodology section, and with that there is some repetition of information.

Only 3% of the water on our planet is fresh. Yet these precious waters are rich with surprise. All life on land is ultimately dependent on fresh water.

- David Attenborough

Chapter 1: Introduction

1.1 CONTEXT STATEMENT

Increasing urbanisation, industrialisation, and natural resource demands associated with growing human populations are increasing pressure on natural ecosystems. Associated with these anthropogenic stressors are increasing metal concentrations in freshwaters. Zinc, in particular, is a common contaminant of concern and is listed on the United States Environmental Protection Agency (USEPA) *Priority Pollutant List*. Australia is a major miner and refiner of zinc ore, and zinc contamination poses a substantial risk to Australia's freshwater ecosystems. Australia uses water quality guidelines as part of the *Water Quality Management Framework* to manage and protect freshwater ecosystems. However, current national default water quality guidelines for zinc may not adequately account for a range of water chemistry parameters that are known to modify zinc toxicity to aquatic organisms and therefore these guidelines may be either under- or over-protective.

Water quality guidelines are developed using toxicity data from tests on a range of species, including microalgae. Microalgal toxicity data for zinc is only available for one freshwater species, *Raphidocelis subcapitata*, using water chemistries most relevant to the northern hemisphere. To date, our understanding of how the sensitivities to zinc of Australian-relevant microalgae change under Australian water chemistries is limited. As such there is uncertainty in the relevance and protectiveness of the current zinc water quality guidelines. These knowledge gaps regarding microalgal zinc toxicity limit the ability to incorporate water chemistry parameters, as toxicity modifying factors, into water quality guideline values using bioavailability-based toxicity models, which have been popularised in recent years. Of the previously developed bioavailability-based models for zinc toxicity, none have been validated in natural Australian freshwaters. The appropriateness of these models for use under Australian freshwater chemistries is unknown.

The primary aim of this thesis was to investigate if the currently established microalgal zinc toxicity paradigms under varying water chemistry conditions extended to a freshwater microalga, *Chlorella* sp., a model organism used for aquatic toxicity testing in Australia.

This aim was addressed by developing high quality, chronic zinc toxicity data for *Chlorella* sp. This toxicity data was used to develop multiple linear regression (MLR) models to predict zinc toxicity under different water chemistry conditions. Finally, MLR models were validated using a range of Australian natural waters. This work was undertaken in response to the growing interest in, and need to accurately account for, the modifying effects of water

chemistry to metal toxicity in Australia and New Zealand's national water quality guidelines (ANZG, 2018). For the effective development of these guidelines, robust zinc toxicity models require validation under Australian-relevant conditions.

This introductory chapter briefly reviews the relevant chemistry of Australian freshwater environments, the sources of zinc, the role of water chemistry in toxicity modification and the current status of water quality guidelines and bioavailability modelling approaches. The chapter closes with a summary of current knowledge gaps and the aims and specific objectives of this thesis.

1.2 WATER CHEMISTRY OF AUSTRALIAN FRESHWATER ENVIRONMENTS

Australian freshwater ecosystems are characterised by their unique hydrology, geology, climate, and diverse aquatic life. The water chemistry of these ecosystems varies spatially. For example, river systems in Kakadu National Park are often characterised by low pH, very soft waters (<5 mg $CaCO_3.L^{-1}$) and low dissolved organic carbon (DOC), while rivers in south-west Western Australia often have very hard waters (>400 mg $CaCO_3.L^{-1}$), high pH and high DOC (Price et al., 2023a; Stauber et al., 2023). In addition to the large spatial variability in water chemistry, there is also strong temporal variation driven by seasonal rains. The wet and dry seasons associated with many parts of Australia result in large changes in DOC concentration and composition, hardness and pH at locations throughout the year (Acharya et al., 2023; Holland et al., 2018).

Australia has distinct ecosystems and geologies that result in highly varied freshwater chemistries. Until recently, there had been no single dataset available for Australian water chemistry, with a recent study by Stauber et al. (2023) publishing a collation of water chemistries from around Australia (Table 1.1). The dataset was collated from a broad range of sources, including government, academic and private institutes that conduct routine water chemistry monitoring. Such institutes include state water corporations and environmental protection authorities, the Bureau of Meteorology, state and federal departments of environment and resource management, and private water utilities. Comparing this data to a similar US water chemistry dataset published in Brix et al. (2020) and a European dataset published by Merrington et al. (2020) demonstrates Australia's differences in water chemistry to North America and Europe. Low water hardness (<30 mg $CaCO_3.L^{-1}$), common to parts of the Northern Territory, is not common in the US, with a median and 10th – 90th percentile range of 136 and 31 – 324 mg $CaCO_3.L^{-1}$, respectively. The European water hardness 10th – 90th percentile range is more comparable to Australia; however, the median water hardness is substantially higher than

that of Australian waters (Table 1.1). The uniquely low hardness in Australian waters (median of 62 mg $CaCO_3.L^{-1}$) required specific consideration by Peters et al. (2018, 2021) when validating nickel toxicity models in Australian freshwaters and highlights why Australian water quality frameworks recommend local water chemistry validation for metal toxicity models derived overseas (Warne et al., 2018).

Table 1.1: Median and range (10th to 90th percentile) of key water chemistry parameters in Australian freshwaters. '–' indicates jurisdictions where no data was available. Australian data adapted from Stauber et al. (2023), United States data adapted from Brix et al. (2020) and European data adapted from Merrington et al. (2020).

State/Territory	pH	Hardness (mg $CaCO_3.L^{-1}$)	DOC ($mg.L^{-1}$)
QLD	7.1 (6.4–7.9)	78 (11–289)	-
NSW	7.4 (6.5–8.2)	43 (13–373)	4.4 (2.2–8.2)
VIC	7.1 (6.4–7.7)	38 (11–379)	4.0 (2.0–13)
TAS	7.0 (6.1–7.7)	35 (11–290)	4.4 (2.4–11)
SA	8.0 (7.1–8.7)	69 (42–109)	4.7 (3.0–8.6)
WA	7.3 (6.5–7.9)	85 (22–1108)	13 (4.0–30)
NT	7.1 (5.6–8.3)	55 (4–316)	4.0 (1.0–14)
ACT	7.7 (7.1–8.3)	-	-
Australia (overall)	7.4 (6.6–8.3)	62 (12–440)	6.7 (2.7–21)
United States	7.8 (7.0–8.3)	136 (31–324)	3.5 (1.5–7.7)
Europe	7.7 (6.4–8.3)	180 (13–555)	5.0 (0.96–17)

1.3 ZINC

1.3.1 Sources and uses of zinc

Zinc is the 24th most abundant element in the Earth's crust and primarily occurs as sphalerite (ZnS), with smaller deposits of carbonates ($ZnCO_3$) and silicates (Zn_2SiO_4) present in weathered sections of orebody (Huston, 2020; Rumble, 2023). Zinc is the 10th most extracted element by mass with approximately 13 million tonnes extracted globally in 2022. Identified global zinc resources are estimated at 1.9 billion tonnes (U.S Geological Survey, 2023). In 2022, 85% of global zinc mine production occurred in 11 countries: Australia, Bolivia, Canada, China, India, Kazakhstan, Mexico, Peru, Russia, Sweden, and the United States (Rumble, 2023; U.S Geological Survey, 2023).

As of 2018, Australia contained 29% of global economic resources of zinc, the highest of any country. Australia is the third largest zinc producer, behind China and Peru, producing 9% of global zinc (Huston, 2020). Zinc resources and mining activities are widely distributed across all Australian states and the Northern Territory (Figure 1.1).

The physical properties of zinc, including resistance to weathering, low melting point and alloying ability have led to its use across a range of industries. More than 50% of global zinc use is for galvanising products to produce protective coatings on steel. Other important uses of zinc include as diecast objects (as a zinc-aluminium alloy), low flammability and high energy density batteries, skin products as zinc-oxide, and health supplements (Huston, 2020; U.S Geological Survey, 2023).

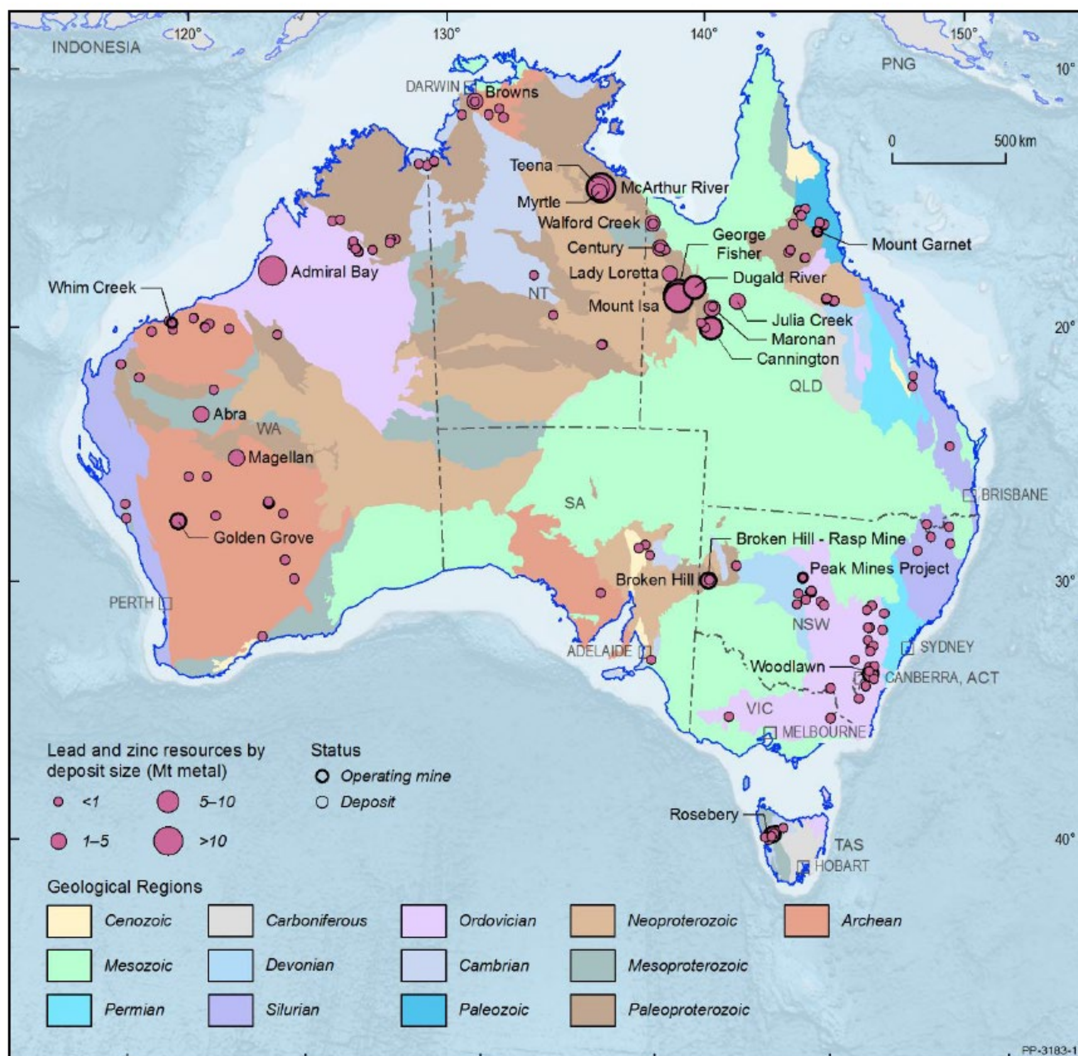

Figure 1.1: Australian lead-zinc deposits and operating mines (Huston, 2020).

1.3.2 Zinc in aquatic systems

When zinc enters an aquatic environment, it can remain in the water column, be taken up by organisms (as an essential trace element), or be deposited in sediment through complexation, adsorption, or precipitation (CCME, 2018). Zinc can enter freshwaters naturally through weathering and leaching of surrounding rock, and anthropogenically through mining and other industrial and urban discharges (McDonald et al., 2022; Tabelin et al., 2018).

Background aquatic concentrations of zinc depend on the geology of the region and can therefore vary substantially between locations. There has been limited published data on the background concentrations of zinc in unimpacted surface waters in Australia until recently with the publication of Stauber et al. (2023). The study collected unimpacted natural freshwaters from around Australia and New Zealand. All New Zealand waters had dissolved (<0.45 µm) zinc concentrations below the limit of detection (<1 $\mu g.L^{-1}$), while the Australian waters varied between 0.2 and 1.4 $\mu g.L^{-1}$ for dissolved zinc and 0.3 to 5.7 $\mu g.L^{-1}$ for total zinc. The study noted that New Zealand waters were analysed separately to Australian waters, resulting in different reported limits of detection. A review of background zinc concentrations in Canadian freshwaters found these varied by region and were higher than those reported for Australia and New Zealand (CCME, 2018). River water from the Canadian Northwest Territories had estimated natural dissolved background zinc concentrations of 5.3 $\mu g.L^{-1}$, while measurements from British Columbia ranged from 5 to 20 $\mu g.L^{-1}$ of total zinc. A collation of freshwater metals data from the United Kingdom found background dissolved zinc concentrations were typically below 5 $\mu g.L^{-1}$ (Peters et al., 2012), similar to those found in Australia (Stauber et al., 2023).

Reported concentrations of zinc contamination in Australian surface waters varies significantly as the magnitude of contamination is dependent on the polluting industry, mitigation measures and the length of the contamination event. For example, McDonald et al. (2022) studied metal contamination in urban stormwater systems in Melbourne and reported measured zinc concentrations (<0.45 µm) up to 11 $\mu g.L^{-1}$. The study then modelled predicted zinc concentrations (<0.45 µm) during and after a storm event and found modelled zinc concentrations dropped from a peak of 60 $\mu g.L^{-1}$ during the storm event down to 23 $\mu g.L^{-1}$ immediately post-storm. A study by Kavehei et al. (2021) investigated metal concentrations downstream of a New South Wales mine and found zinc concentrations (<0.45 µm) in water ranging from 34 to 286 $mg.L^{-1}$ across a 30 year period following mine closure. Comparison of these two studies highlights that the range of contamination of zinc in surface waters varies substantially both spatially and temporally. Both concentration and exposure time are important parameters when assessing environmental risk.

Zinc is an important essential element for most aquatic plants and animals and is broadly required for the activity of over 300 enzymes (McCall et al., 2000). The essentiality of zinc is well documented in all phyla (Vallee & Falchuk, 1993). For example, zinc is required in aquatic plants and organisms for electron transport, phosphorylation, gene expression and enzymatic function (El-Agawany & Kaamoush, 2023; Muyssen & Janssen, 2002). At higher concentrations zinc has been shown to be toxic to a wide range of aquatic organisms and can exert these toxic effects via several mechanisms. In fish and aquatic invertebrates acute zinc toxicity is associated with

disruption to calcium homeostasis (Muyssen et al., 2006; Spry & Wood, 1985). In fish, elevated zinc concentrations interfere with calcium uptake at the gill, resulting in calcium deficiency and hypocalcemia. For invertebrates, zinc and calcium compete for binding sites on the apical membrane of gill epithelium (Hogstrand et al., 1996; Spry & Wood, 1985).

The predominant inorganic species of zinc in natural waters (pH $\leq$ 8.5) is the +2 valence state. With increasing pH, the relative distribution of the hydrolysed species $ZnOH^+$ and $Zn(OH)_2$ increases (Stumm & Morgan, 1996). This is discussed further below (section 1.4.1, Figure 1.2). Zinc speciation is also dependent on the presence of natural dissolved organic matter, as zinc can form complexes with DOC functional groups, such as carboxylates, phenols, amines, and thiols (Aiken et al., 2011). The stability and relative presence of Zn-DOC complexes is also dependent on water pH and the presence and concentration of major ions (Florence & Batley, 1977).

1.4 TOXICITY MODIFYING FACTORS AND BIOAVAILABILITY

For many metal contaminants, toxicity to aquatic organisms is associated with disruption of essential ion balances due to the uptake of metal ions into the organism; however, toxic modes of action can vary considerably among metals and between organisms (Niyogi & Wood, 2004).

Water chemistry is known to influence metal bioavailability and subsequently metal toxicity (Di Toro et al., 2001). Cations (e.g., hydrogen, calcium, magnesium, sodium), organic ligands such as DOC, and inorganic ligands (e.g., carbonates, hydroxides and chlorides) influence an organism's response to metals (Deleebeeck et al., 2009; Franklin et al., 2000; Gensemer et al., 2018; Macoustra et al., 2019; Park et al., 2009; Wilde et al., 2006). Such water chemistry properties are commonly referred to as toxicity modifying factors (TMFs). TMFs can be grouped into two general categories: (1) those that compete for biological uptake with the metal, and (2) those that complex the metal, thus altering speciation (Di Toro et al., 2001). The magnitude and type of influence of each TMF is variable and dependent on the metal and organism. Additionally, the ability of TMFs to influence metal toxicity is dependent on the interactions between water chemistry properties (Di Toro et al., 2001).

While there are many identified water chemistry parameters that are TMFs to metals in freshwater environments, several studies have highlighted the importance of three key parameters: pH, hardness, and DOC (Brix et al., 2017, 2020; CCME, 2018; DeForest et al., 2023). These parameters have been shown to be the drivers of most toxicity modification for metals and as such, will be discussed below in greater detail and form the basis for much of the research presented in this thesis.

1.4.1 pH

The role of pH as a TMF has been shown to be highly dependent on the test organism. As pH is a function of both hydrogen and hydroxide ion concentrations, the influence of pH as a TMF is through both competition of the hydrogen ion with the metal at the biotic ligand of the organism and through complexation with hydroxide ions to form metal hydroxides (Wilde et al., 2006). A biotic ligand is a site on an organism where ionoregulatory processes can be disturbed by metal binding (Adams et al., 2020). As such, the influence of pH on the toxicity of a metal will depend upon: the relative binding affinities of hydrogen and the metal to the biotic ligands on the organism; the complexing capacity of hydroxides to the metal; and the relative toxicity of those metal hydroxides produced (François et al., 2007; Long et al., 2004). Furthermore, changes in pH will significantly influence the speciation of a dissolved metal (Figure 1.2), thus changing the toxicity of the metal in solution (Stumm & Morgan, 1996).

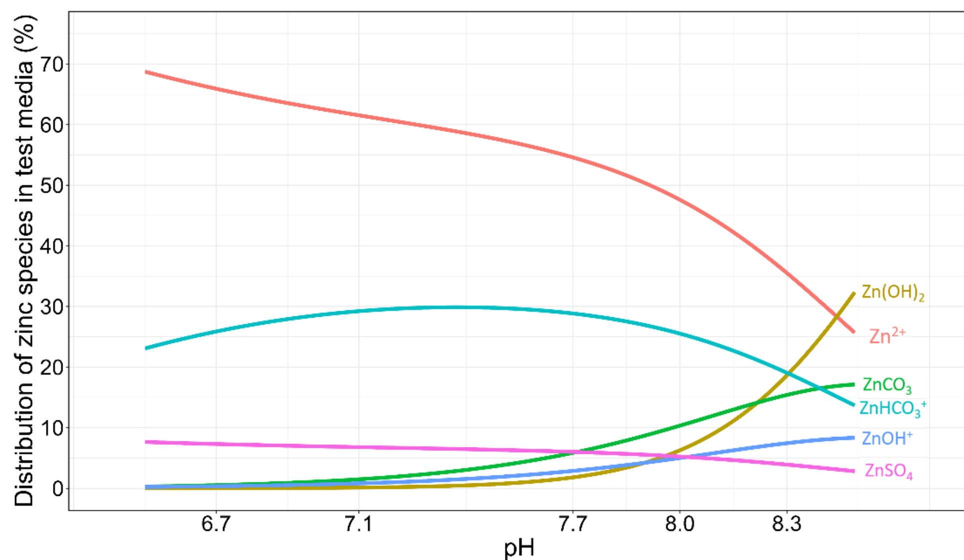

Figure 1.2: Zinc speciation at varying pH ranging from pH 4 to 10. Speciation was modelled using major ion concentrations consistent with a modified synthetic test water USEPA recipe (2002). Major ion concentrations were - Na: 30 mg.L^{-1}, Mg: 13.8 mg.L^{-1}, Ca: 15.1 mg.L^{-1}, K: 2.1 mg.L^{-1}, Cl: 1.9 mg.L^{-1}, SO_4: 84.1 mg.L^{-1}, CO_3: 68.6 mg.L^{-1}.

For many aquatic organisms, decreasing water pH increases the overall toxicity of metals (Erickson et al., 1996; Heijerick et al., 2003; Mager et al., 2011; Park et al., 2009). Such trends are generally explained by the increase in the proportion of the metal concentration existing as free metal ions (M^{n+}), where the free metal ion concentration is commonly related to metal bioavailability (Campbell, 1995). However, this is not always the case, with several studies, particularly those using microalgae, finding that decreased pH decreases metal toxicity (De Schamphelaere et al., 2005; Deleebeeck et al., 2009; Franklin et al., 2000).

Few studies have investigated the toxicity of zinc to freshwater microalgae, with even fewer examining the influence of pH on zinc toxicity. Wilde et al. (2006) assessed the toxicity of zinc

to the microalgae *Chlorella* sp. at varying pH. The study found that increasing the pH from 6.0 to 8.0 resulted in a 30-fold increase in toxicity. The researchers suggest the increased metal toxicity is likely due to the reduced competition between hydrogen ions (H^+) and zinc at the algal cell surface, resulting in more zinc binding to the algal cell. Studies by Heijerick et al. (2002a) and De Schamphelaere et al. (2004) observed similar trends, finding zinc toxicity to the microalgae *R. subcapitata* increased with increasing pH.

These three studies showed similar zinc toxicity trends with increasing pH; however, the magnitude of change varied between the microalgae species, as did the median effect concentrations (i.e., EC50 values, the concentration where 50% of test population is affected). For the *R. subcapitata* studies the EC50 values ranged from 95 to 11 $\mu g.L^{-1}$ across a pH range of 6.8 to 7.8 (Heijerick et al., 2002a) and 200 to 74 $\mu g.L^{-1}$ across a pH range of 6 to 8 (De Schamphelaere et al., 2004). These relative changes and absolute concentration in EC50 values for *R. subcapitata* were substantially less than the results found by Wilde et al. (2006) for *Chlorella* sp. across a similar pH range, with EC50 values decreasing from 1680 to 52 $\mu g.L^{-1}$ across a pH range of 6 to 8. The differences in the influence of pH on modifying toxicity and the absolute concentrations of the EC50 values between the studies may be explained by species-specific sensitivities to both zinc and hydrogen ions; however, the method of buffering used to control pH across tests may have confounded results (De Schamphelaere et al., 2004; Esbaugh et al., 2013). The Wilde et al. (2006) study used a 3-N-morpholinopropanesulfonic acid (MOPS) sodium salt, whereas Heijerick et al. (2002a) and De Schamphelaere et al. (2004) used a free-acid form of MOPS. The added sodium, which may compete with zinc at the algal cell surface, may have contributed to the differences in observed toxicity between the two species. This is further discussed and tested in Chapter 2.

The results of the above-mentioned studies indicate that a more comprehensive understanding of the species-specific influence of water chemistry is needed to elucidate the effects of pH on the toxicity of zinc to microalgae.

1.4.2 Hardness

Water hardness is also known to modify toxicity of a variety of metal contaminants to a range of freshwater organisms. The influence and degree at which hardness acts as a TMF is variable among different test organisms and metals, with some studies showing significant protective effects while others show no effect (Charles et al., 2002; Hyne et al., 2005; Markich et al., 2005; Naddy et al., 2003). Given hardness is typically measured as calcium and magnesium ions, the influence of hardness as a TMF is attributed to increased competition from calcium and magnesium with the metal contaminant at the biotic ligand binding sites (Paquin et al., 2002).

Relatively few studies have focused on the ameliorative effect of hardness on metal toxicity to microalgae. Charles et al. (2002) investigated the effect of hardness on uranium toxicity to *Chlorella* sp. and found a 50-fold increase in water hardness (8 – 400 mg $CaCO_3.L^{-1}$) resulted in a 5-fold decrease in toxicity, with 72-h growth rate EC50 values increasing from 56 to 270 µg $U.L^{-1}$. Speciation modelling revealed changes in uranium species over the hardness test range were not significant and therefore the hardness ameliorative effect was likely due to ion competition at the algal cell surface.

The number of studies investigating the effects of hardness on zinc toxicity to freshwater microalgae is limited, with most freshwater zinc toxicity research focusing on cladoceran and fish species. Of the few studies involving microalgae, all used the species, *R. subcapitata*. Heijerick et al. (2002a) studied the individual effects of calcium and magnesium ion concentrations on the toxicity of zinc to *R. subcapitata*. Both cations had a protective effect on zinc toxicity, with magnesium having a greater protective effect compared to calcium. A 10-fold change in calcium concentrations resulted in a 1.7-fold decrease in zinc toxicity, whereas for magnesium, a 6.5-fold decrease in zinc toxicity was observed across a 10-fold concentration change in magnesium.

1.4.3 Dissolved organic carbon

Dissolved organic carbon acts an important TMF in freshwater ecosystems through its ability to complex metals, resulting in a reduction in metal bioavailability (Wood et al., 2011). DOC is typically a complex mixture of organic molecules derived from the decomposition of lignin-rich materials, the decay of animals and microbes, and microbial exudates (Al-Reasi et al., 2011). DOC reduces metal bioavailability and toxicity through chelation of metals via the numerous functional groups on the main forms of DOC, humic and fulvic acids. Such functional groups include carboxyl and hydroxyl groups (Figure 1.3). Such structures vary significantly depending on the source of the DOC.

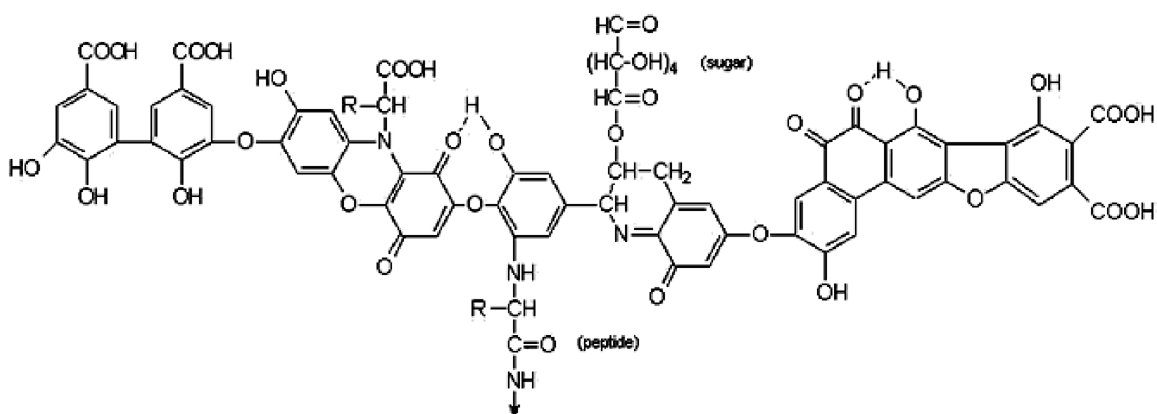

Figure 1.3: Generalised structure of a humic acid molecule (Stevenson, 1982).

Several studies have investigated the ameliorative effect of DOC on metal toxicity to microalgae. Macoustra et al. (2019) used DOC concentrates extracted from Australian freshwaters to investigate the influence of DOC concentration and source on copper toxicity to *Chlorella* sp. This study found significant reductions in toxicity (up to 22-fold) when comparing the 10 mg $C.L^{-1}$ treatments to controls. Similar observations have been made in numerous studies investigating a range of different test species, DOC sources and metals (Gensemer et al., 2018; Park et al., 2009; Ryan et al., 2004; Trenfield et al., 2011).

As with both pH and hardness, the number of studies investigating the effect of DOC on zinc toxicity to microalgae is limited. A study by Koukal et al. (2003) observed a significant decrease in zinc toxicity to the green microalgae *R. subcapitata* in the presence of two commercially available humic acids, but not in the presence of a fulvic acid. The magnitude of toxicity reduction in the presence of the humic acid was dependent on the source, with the peat-derived humic acid being more protective than the soil-derived humic acid. To date, no studies have investigated the effects of DOC on zinc toxicity to the microalgae *Chlorella* sp. Furthermore, no studies, prior to the work presented in this thesis, have investigated the independent influence of natural DOC on zinc toxicity to any freshwater microalgae, nor have any studies assessed this influence under varying pH conditions.

1.5 WATER QUALITY GUIDELINES

The effective protection of the environment requires scientifically robust management frameworks. For the management of contaminants entering aquatic environments in Australia and New Zealand, water quality guideline values are used as part of the Australian and New Zealand Guidelines for Fresh and Marine Water Quality (ANZG, 2018). The water quality guidelines provide industry, environmental regulators, and governments with a management tool to manage and protect aquatic ecosystems.

1.5.1 Aquatic ecotoxicology

Large amounts of ecotoxicity data are required to develop water quality guidelines. Aquatic ecotoxicology is the study of how contaminants, such as metals and organic chemicals, impact aquatic organisms. The discipline aims to quantify these contaminant effects on individual species and aquatic ecosystems. It is a multidisciplinary science that applies aspects of chemistry, toxicology, biology, ecology, and statistics. Toxic effects of contaminants can be observed and quantified at different levels, from effects at the molecular level such as changes in proteins and metabolites, through to entire ecosystem effects, such as changes in biodiversity. Toxicity is defined by the observed lethal or sub-lethal effects following contaminant exposure that is

typically benchmarked to a control group or population. Toxicity data used in guideline development is classed into two categories: 1) Acute toxicity, which refers to observed effects following an exposure duration that is over a short period of the organism's life; and 2) Chronic toxicity, which is observed effects following a longer period of exposure that is typically a substantial portion of the organism's life or a sensitive early life stage (Warne et al., 2018). Chronic toxicity normally occurs at lower contaminant concentrations relative to acute toxicity and the Australian and New Zealand guidelines are predominantly based on chronic toxicity data, where available (ANZG, 2018).

Toxicity data is mostly generated using standardised laboratory-based experiments, where an organism is exposed to a contaminant, and effects are observed over a fixed exposure duration. These experiments are known as bioassays or toxicity tests. Typically, an organism or group of organisms of the same species are exposed under laboratory-controlled conditions to a concentration series of a contaminant until a defined response (known as an endpoint) occurs or the predetermined exposure duration finishes (OECD, 2011a, 2011b). The selection of a test organism is based on the goals of the experiment, with organism sensitivity to the contaminant and its relevance to the geographical region of interest being key considerations. Given it is impractical to test all organisms in an ecosystem, it is important to select species that are representative of a range of sensitivities that may be present in that ecosystem (Jin et al., 2015). In this thesis a microalga, *Chlorella* sp., is exposed for 72 h to a concentration series of zinc, and growth rates/cell division rates (the endpoint) of the microalgal population are observed and quantified.

Toxicity tests are standardised and endorsed by regulatory bodies such as the Australian and New Zealand Governments (ANZG), the United States Environmental Protection Agency (USEPA) and the Organisation of Economic Co-operation and Development (OECD). These standardised methods create a framework for toxicity testing parameters and provide guidance on what is required from toxicity testing for data to be used in water quality guidelines. Such parameters include test organisms, culturing protocol, quality assurance, test reporting minimum standards and exposure conditions. Standardisation of toxicity testing is vital for interlaboratory comparison and test reproducibility, both key requirements for developing robust water quality guidelines (ANZG, 2018).

1.5.2 Current status on guideline development

The current methodology for the derivation of water quality guidelines in Australia requires a multidisciplinary approach using multiple lines of evidence such as ecotoxicology, chemistry, ecology, and statistics, and is described in detail by Warne et al. (2018) and Batley et al. (2018). Two different methods can be used to derive guidelines: the species sensitivity distribution (SSD)

and assessment factor (AF) methods. The SSD method is the preferred and most common method used in Australia and is the focus of this section (Warne et al., 2018).

Toxicity and associated physicochemical data are first collated and undergo a screening process to ensure all data meets the minimum requirements outlined in Warne et al. (2018). Such requirements include the use of appropriate control treatments, whether concentrations were analytically verified, and the appropriate measurement of physicochemical parameters, among others. Following this screening, where multiple toxicity values exist for a species, a single toxicity value per species is needed prior to use in an SSD. This is achieved by taking geometric means for each given species, endpoint and test duration combination. Once all data is deemed suitable there is a minimum number of species required for SSD development. In Australia, at least eight species from across four taxonomic groups is preferred (Warne et al., 2018); however, requirements differ across countries/jurisdictions. For example, 10-15 species from 8-9 different taxonomic groups is recommended in Europe (OECD, 2011b), while the United States recommend at least 15 species of invertebrate and fish (USEPA, 2005). In general, across all jurisdictions the consensus is that the more data from a wide range of taxonomic groups, the greater the likelihood that the SSD will be protective across a broad range of species. Once an SSD is derived, a protection concentration (PCx) can be estimated, where x represents the percentage of species protected. For example, typically a PC95 is calculated, which represents a water quality guideline value that is protective of 95% of species (Figure 1.4).

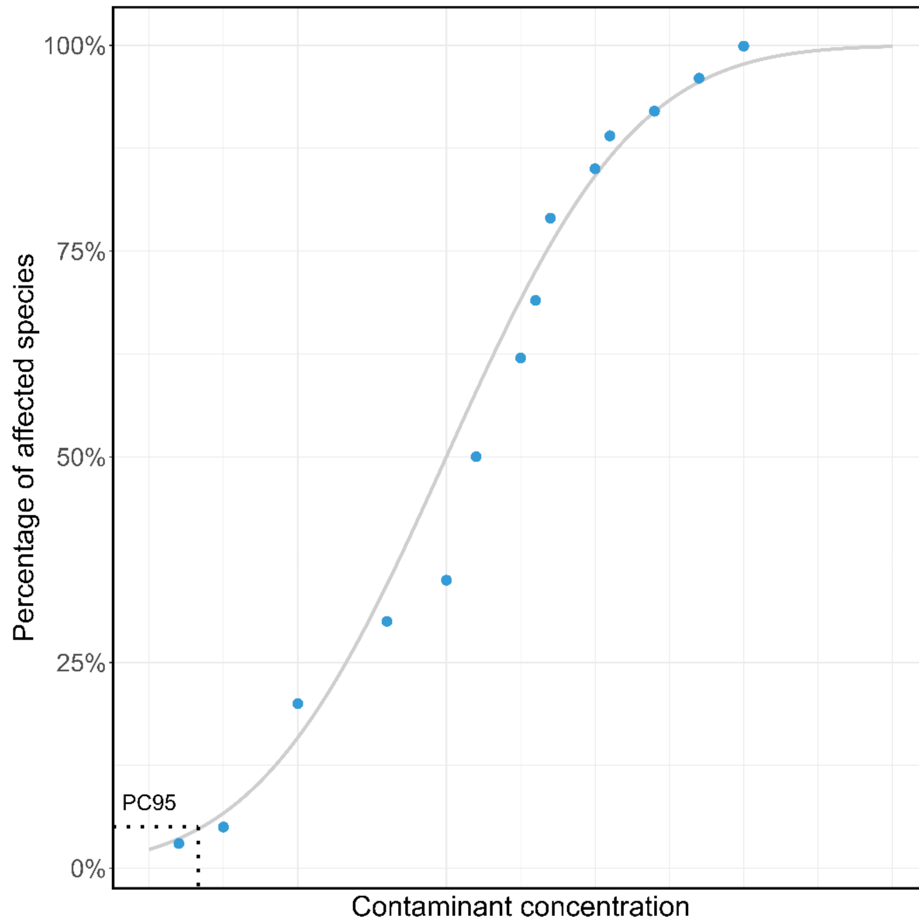

Figure 1.4: Generic species sensitivity distribution. Blue circles indicate individual species sensitivity data, grey curve indicates cumulative distribution model used to calculate protection concentrations. PC95 indicates the protection concentration that protects 95% of species.

1.5.3 Bioavailability-based guidelines

It is well known that metal toxicity is dependent on its bioavailability, where metal bioavailability is a measure of the metal's ability to interact with an organism's biological receptors, such as gills or cellular membranes (Adams et al., 2020). There has been a strong interest and focus over the last 20 years to incorporate concepts of bioavailability into water quality guidelines (Brix et al., 2020; Campbell, 1995). The earliest incorporation of empirical bioavailability models into water quality guidelines was the introduction of the hardness-based guidelines for metals by the USEPA in 1985, where a metal-specific equation was used to modify the water quality guideline value depending on the hardness of the water being tested. These hardness equations were incorporated into the Australian and New Zealand water quality guidelines in 2000 (ANZECC & ARMCANZ, 2000).

Since the adoption of hardness equations, the influence of other water chemistry parameters on metal bioavailability and toxicity has been further investigated, finding that parameters such as pH and DOC also play important roles (Adams et al., 2020). Interestingly, in the years following

the adoption of hardness equations, research found that these equations were inappropriate in protecting aquatic organisms to chronic copper exposures. The covariation of hardness with alkalinity and pH was blamed for equation inaccuracies, with the experiments used to develop the equations not controlling for alkalinity and pH change with changing hardness (Markich et al., 2005). This finding led to the removal of the hardness equation for copper guidelines in Australia; however, as there was insufficient research on other metals, such as zinc, the hardness equations still remain in the current guidelines for other metals.

To better represent the role of water chemistry in metal bioavailability, a mechanistic model called the biotic ligand model (BLM) was developed (Di Toro et al., 2001; Santore et al., 2001). The BLM is underpinned by the concept that metal toxicity is a function of metal accumulation to a biotic ligand site located on the organism. The affinity between the metal of interest and cations in solution with various ligands are modelled and equilibrium constants are derived. For model implementation, 10 water chemistry parameters are inputted, and concentrations of different complexes (including a metal-biotic ligand complex) are calculated and used to predict toxicity. Initial models developed by Di Toro et al. (2001) and Santore et al. (2001) were for acute copper toxicity but have since expanded to include a range of metals, organisms and chronic toxicity endpoints (De Schamphelaere & Janssen, 2002; Heijerick et al., 2002a, 2005; Nys et al., 2014). The copper BLM was first introduced into water quality criteria by the USEPA in 2007, however its uptake by regulators and end users was limited (Brix et al., 2017). It is suggested the lack of uptake was likely due to perceptions that the BLM is too complicated and insufficiently transparent for implementation by regulators. This led to the repopularising of empirical models and the subsequent development of multiple linear regression (MLR) models for predicting metal toxicity (Brix et al., 2017).

Multiple linear regression model approaches provide an empirical, statistical means to include key water chemistry parameters into a model to predict toxicity to aquatic organisms, which can then be incorporated into a water quality guideline. This approach is similar in concept to the original hardness-based equations. While the use of empirical models such as MLRs appear less complex, their derivation is built on significant mechanistic understanding of metal and water chemistry interactions established during the development of BLMs, providing confidence in the robustness of the method (Adams et al., 2020). The approaches to MLR derivation and validation are discussed in detail by Brix et al. (2020) and Garman et al. (2020) as well as in Chapter 5, 6 and 7 of this thesis (Price et al., 2022b, 2023a). In brief, toxicity data for a specific metal and species is collected via literature or experimentation. The species and range of chemistries included will depend on the water chemistry of the region for which the MLR is developed. The selection of water chemistry parameters to include in the model is typically determined by prior information or data availability. In

general, the three main parameters used in MLR models have been pH, hardness and DOC, with these parameters consistently shown to be most important for divalent metals (Brix et al., 2017, 2020, 2023; DeForest et al., 2018, 2023; Niyogi & Wood, 2004; Price et al., 2023a). Following data collection and quality checks, MLR models are developed using step-wise regression analysis (Brix et al., 2017). Model validation methods have evolved continuously since the original Brix et al. (2017) models, with three core types of validation methods: autovalidation, independent validation and cross species validation (Garman et al., 2020).

As is the case for SSD development, it is important to develop bioavailability models for a range of taxonomic groups and species where possible in order to better understand the range of influences that water chemistry has on different species. Several approaches have looked at combining datasets from different species of the same taxonomic group to produce a trophic-level model (Brix et al., 2021; Croteau et al., 2021; DeForest et al., 2023), while others have focused on species-specific models (Brix et al., 2023; DeForest et al., 2018, 2020; Price et al., 2023a). In the case of microalgae, there has been insufficient data available for multiple species to produce a trophic-level model, with all microalgal bioavailability models (prior to the work in this thesis) based on *R. subcapitata*. Expanding the understanding of water chemistry on zinc toxicity to microalgae and the subsequent development of new models was a key aim of this thesis.

1.6 THESIS OUTLINE

This thesis investigated the role of key water chemistry parameters on modifying the toxicity of zinc using the well-studied tropical freshwater green microalga, *Chlorella* sp. as a model organism.

Toxicity tests were conducted, and bioavailability models were developed and validated specifically under Australian water chemistry conditions to investigate the direction and magnitude of zinc toxicity modification by key water chemistry parameters under environmentally relevant conditions. Different measurements of zinc speciation and lability were also investigated to understand the mechanisms by which toxicity modifying factors influenced zinc toxicity to *Chlorella* sp.

The overarching aim was to improve risk assessment and management of zinc in Australian freshwater ecosystems by investigating if the currently established microalgal zinc toxicity paradigms under varying water chemistry extend to the tropical freshwater microalga, *Chlorella* sp. This was investigated using three main objectives:

> *Objective 1: To assess the influence of key water chemistry parameters on zinc toxicity to a tropical freshwater microalga.*

The influence of toxicity modifying factors on zinc toxicity was investigated in three chapters. **Chapter 2** explored the effect of pH on zinc toxicity to *Chlorella* sp. specifically for Australian conditions by determining the toxicity of zinc across the median pH range representative of Australia's freshwater ecosystems. **Chapter 3** built on this work by investigating the interactive effects of pH and hardness on zinc toxicity and compared these effects to the hardness-based algorithms currently applied under Australian and New Zealand freshwater guidelines. **Chapter 4** elucidated the influence of DOC on zinc toxicity, considering the role of DOC concentration, DOC quality characteristics, and the pH of the exposure solution. Across the three chapters, zinc speciation measurements and modelling were utilised to understand mechanisms of toxicity modification. The diffusive gradients in thin-films (DGT) technique and ultrafiltration were used to consider changes in zinc lability and complexation, while the Windermere Humic Aqueous Model (WHAM7) was used to assess expected changes in chemical speciation.

> *Objective 2: To determine the relevance of current validation methods for bioavailability modelling.*

The applicability of the commonly applied 'factor-of-2 rule' as a primary validation method for bioavailability models was assessed under this objective (**Chapter 5)**. This work analysed an extensive toxicity dataset obtained from multiple repeated tests encompassing a wide range of contaminants, species, and effect levels. An analysis was conducted on the suitability of the current validation technique to determine the appropriateness of its use at lower effect levels (i.e., EC10 and EC20 values) and across different species and metals.

> *Objective 3: To develop and validate empirical toxicity models to underpin bioavailability-based water quality guidelines for zinc.*

Multiple linear regression models for a range of effect levels were developed for *Chlorella* sp. using datasets generated under Objective 1. Models were independently validated using toxicity data generated from six zinc-spiked natural Australian freshwaters with a range of water chemistries. Alternative models in the literature developed with a different microalga were also independently validated to assess cross-species validation and applicability of those models to Australian waters. Model development and validation results are presented in **Chapter 6**.

The conclusions from these studies are summarised in **Chapter 7**, and the implications for a more comprehensive incorporation of bioavailability into aquatic environmental risk assessment and use in water quality guidelines are discussed. Recommendations for future work are also outlined.

Chapter 2: The influence of pH on zinc toxicity to *Chlorella* sp.

In Chapter 2 the chronic toxicity of zinc with varying exposure water pH was assessed for the freshwater microalgae, *Chlorella* sp. A pH range relevant to Australian natural waters was tested, with zinc toxicity increasing with increasing pH. Diffusive gradients in thin-films and speciation modelling were also investigated to understand the role of zinc speciation change and its relation to zinc toxicity. The work presented in this chapter has been published in the below cited publication.

Highlights

- Zinc toxicity increased linearly as test water pH was increased.
- Changes in zinc toxicity with increasing pH cannot be explained by changes in speciation.
- DGT-labile zinc concentrations were unchanged around the tested pH range.

Price, G. A. V., Stauber, J. L., Holland, A., Koppel, D. J., Genderen, E. J. Van, Ryan, A. C., & Jolley, D. F. (2021). The Influence of pH on Zinc Lability and Toxicity to a Tropical Freshwater Microalga. Environmental Toxicology and Chemistry, 40(10), 2836–2845. https://doi.org/10.1002/ETC.5177

I developed the experimental design and conducted all toxicity tests. I completed all chemical and statistical analyses, data visualisation and interpretation, and I prepared the manuscript for publication. All authors contributed to study conceptualisation and editing of the manuscript before submission.

2.1 INTRODUCTION

Metal bioavailability is influenced by many aspects of water chemistry such as major ions, pH, hardness, alkalinity and dissolved organic matter. 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 influence of water chemistry 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. 2017; CCME 2018; Brix et al. 2020). Several examples of water quality guidelines developed from MLR models are present in the literature. Brix et al. (2017) and Stauber et al. (2021) 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 chemistry conditions. DeForest et al. (2018, 2020) 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 quantifying the influence of individual water chemistry parameters as toxicity modifying factors (TMFs). An important characteristic for

most metals is pH, as many 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., 2002a; Wilde et al., 2006).

An understanding of the importance of metal bioavailability has resulted in increased interest in and the subsequent development of methods to measure different metal fractions using kinetic approaches (Davison & Zhang, 1994; Zhang & 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 & 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 & Zhang, 1994). The DGT technique discriminates among 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., 2021). 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 carbon (DOC) and water hardness on DGT lability for several metals and how this relates to organism toxicity, with Macoustra et al. (2019; 2021a) assessing the effects of DOC on lability of copper and nickel and Paller et al. (2019) assessing the influence of DOC and water hardness on the 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 chemistry conditions. However, limited information is available on the influence of pH on DGT labile metals and their relationship to observed toxicity.

The first objective of this study was to assess the influence of pH (6.7 – 8.3) on the toxicity of zinc to a 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 (estimated with the Windermere Humic Acid Model and ultrafiltered zinc concentrations). As highlighted by Brix et al. (2020), data relating to the response of algae and aquatic plants under different water chemistry conditions are limited, therefore, the results of the present 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.

2.2 METHODS

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% (v/v) 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% (v/v) HNO_3 (Merck) for >24 h and thoroughly rinsed with UPW before testing.

2.2.2 Algal culturing

All algal growth inhibition bioassays were conducted using the tropical freshwater green microalga *Chlorella* sp. (Stauber & Apte, 1996). Cultures were maintained in Jaworksi's medium 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 to 7 day old cultures were used for test initiation to ensure exponential growth during testing.

2.2.3 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} and the test protocol is based on Organisation for Economic Co-operation and Development test guideline 201 (2011a) with modifications described in Franklin et al. (2005). All test treatments were adjusted to the required pH using dilute HCl or KOH (not NaOH) to maintain a constant sodium concentration across all tests. The 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 & Rorabacher, 1999). Furthermore, De Schamphelaere et al. (2004) demonstrated that MOPS was not toxic to *R. subcapitata* and did not affect the toxicity of zinc to *R. subcapitata* over the tested concentration range of 0.5 to 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 the present study. Experimental media did not contain any metal buffering (e.g., ethylenediaminetetraacetic acid (EDTA)) nor trace metals as micronutrients.

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 g NO_3^-.L^{-1} ($NaNO_3$) and 0.15 g PO_4^{3-}.L^{-1} (KH_2PO_4) stocks giving final concentrations of 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 to 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 (1048 g, 7 min, rotor radius 15 cm; Spintron GT-175BR, 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 to account for inter-test variability, except tests at pH 6.7 and 8.3, which were carried out in triplicate, with the additional test used for ultrafiltration and DGT experiments.

Algal cell densities were determined at 0, 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). Cell densities were obtained from plots of side angle light scatter (SSC) and chlorophyll *a* fluorescence intensity (FLB3) with manual gating. A threshold of 200 (arbitrary units) was set to exclude background noise from non-algal particles and gating allowed for the exclusion of dead cells as described in detail by Stone et al. (2019). Growth rates were normalised as a percentage of control response to pool inter-test data and account for inter-test variability.

Copper reference toxicant tests were concurrently run with each toxicity test. Tests were considered acceptable if reference test control growth rates 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 ± 1.1µg $Cu.L^{-1}$ (mean ± SD, n = 20). Toxicity tests also required <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.

2.2.4 Chemical analyses

Metal subsamples were collected at the start (0 h) and end (72 h) of each test from each test flask and filtered through acid-rinsed rinsed (flushed with 30 mL of both 10% HNO_3 and ultrapure water) 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, 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 1048 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% (v/v) 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 multi-element calibration standards, blanks, and drift standards.

Samples taken for dissolved organic carbon (DOC) analysis were collected before the addition of MOPS and passed through acid-rinsed 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.

2.2.5 Zinc speciation and lability

Two approaches to investigate zinc speciation and lability were used: the metal speciation model, Windermere Humic Acid Model (WHAM, version 7) and the diffusive gradients in thin-films (DGT) technique.

WHAM estimated zinc speciation under each test condition with input parameters consisting of dissolved zinc, pH, temperature, major ions (Mg^{2+}, Ca^{2+}, K^+, Na^+, Cl^-, SO_4^{2-}, CO_3^{2-}, NO_3^- and PO_4^{3-}), and DOC. An open atmosphere assumption (pCO_2 = 0.00038 atm) was applied to all speciation calculations as per DeForest and Van Genderen (2012).

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, 200 – 400 wet mesh) and polyacrylamide diffusive gel were synthesised and assembled into DGT pistons in accordance with procedures outlined by Amato et al. (2019). 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. The orbital shaker was placed in an incubator cabinet (LABEC) under conditions matching the toxicity tests. 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).

2.2.6 Statistical analysis and modelling

Statistical analyses were performed using the R statistical software (v4.3.0; R Core Team, 2023) 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, 2020).

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 4-parameter models. 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 limit parameter was fixed to 0, as was the case for all models in the present study, meaning only two parameters were estimated (the slope and inflection). Model selection was based on Akaike's information criterion (AIC) and residual standard error of the model using the *mselect* function within *drc*. For all toxicity datasets, a Weibull model was the best fitting model (model parameters are provided in Table A-1).

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). 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 between 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. Assumptions of homogeneity of variances and normality of residuals were tested using Levene's test and Shapiro-Wilk tests, respectively.

All metal concentrations in concentration-response models and results were measured concentrations. Standard deviation (SD) was used to specify uncertainty throughout the present study, and all significance testing was conducted at $\alpha = 0.05$.

2.3 RESULTS

2.3.1 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 2.1). Dissolved organic carbon (DOC) concentrations were low, less than 1 mg $C.L^{-1}$ (t = day 0, prior to the addition of algae) and hardness values did not vary significantly across tests. Control growth rates were acceptable in all pH tests (Figure 2.1). Tests at pH 8.3 had significantly higher control growth rates ($p = 0.0002$) 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 2.1: The physicochemical characteristics of the test media. Data are 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. DOC = dissolved organic carbon.

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

[a] The unbuffered control test pH range represents the start (day 0) and end (day 3) pH values.

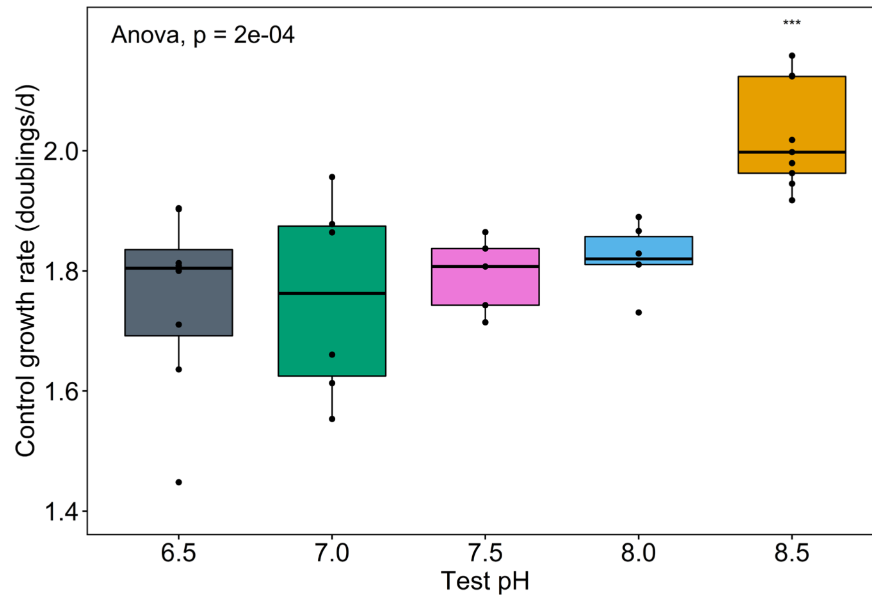

Figure 2.1: Comparison of growth rates as doublings per day in control treatments (no added zinc) at each pH. Graphed pH values are nominal. Individual control replicate growth rates are plotted as black points overlaid on boxplots. Boxplots show, median, first and third quartile boundaries.

2.3.2 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 2.1). There was no change to *Chlorella* sp. growth rate in the presence of the MOPS buffer over the concentration range of 0 to 2.0 g MOPS.L^{-1} (Figure 2.2). 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. (2004b) who found no observed toxicity to *R. subcapitata* 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 2.1). Based on these results 0.5 g MOPS.L^{-1} was used for buffering all test treatments.

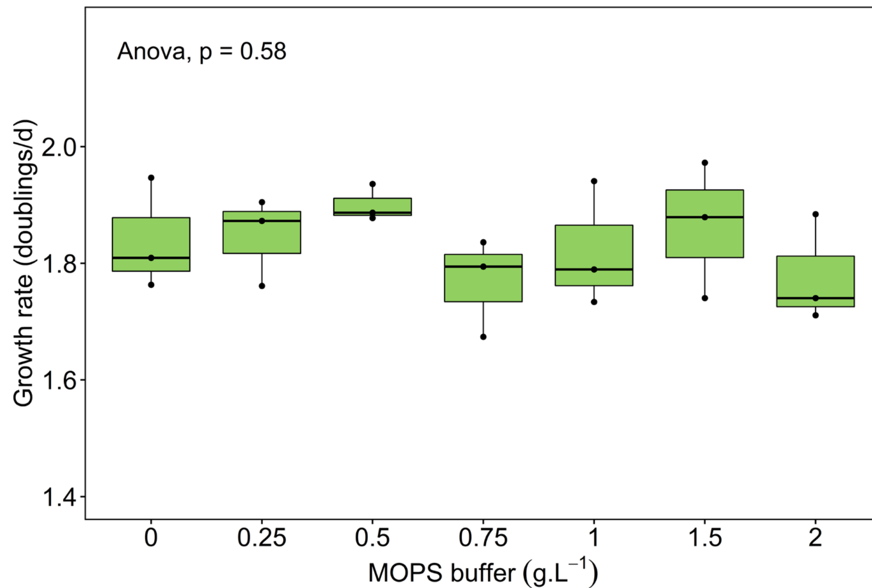

Figure 2.2: Comparison of growth rates as doublings per day with increasing concentrations MOPS buffer. MOPS concentrations are reported as nominal. Individual treatment growth rates are plotted as black points overlaid on boxplots. MOPS = 3-N-morpholinopropanesulfonic acid

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

Algal growth rates decreased with increasing zinc concentrations across all pH treatments (Table 2.2, Figure 2.3). *Chlorella* sp. sensitivity to zinc increased linearly with increasing pH from 6.7 to 8.3 (Table 2.2 and Figure 2.4). The 72-h EC50 values decreased approximately 4-fold from 185 to 53 µg Zn.L^{-1} (Figures 2.3 and 2.4) across the pH range (Table 2.2). 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 2.2). There was a linear relationship between the 72-h EC50 values and pH for both measured dissolved zinc (Figure 2.4A) and modelled free zinc ion measurements (Figure 2.4C), with R^2 values of 0.89 and 0.96, respectively. Relationships between 72-h EC10 and EC20 are provided in Figures A-1 and A-2, respectively.

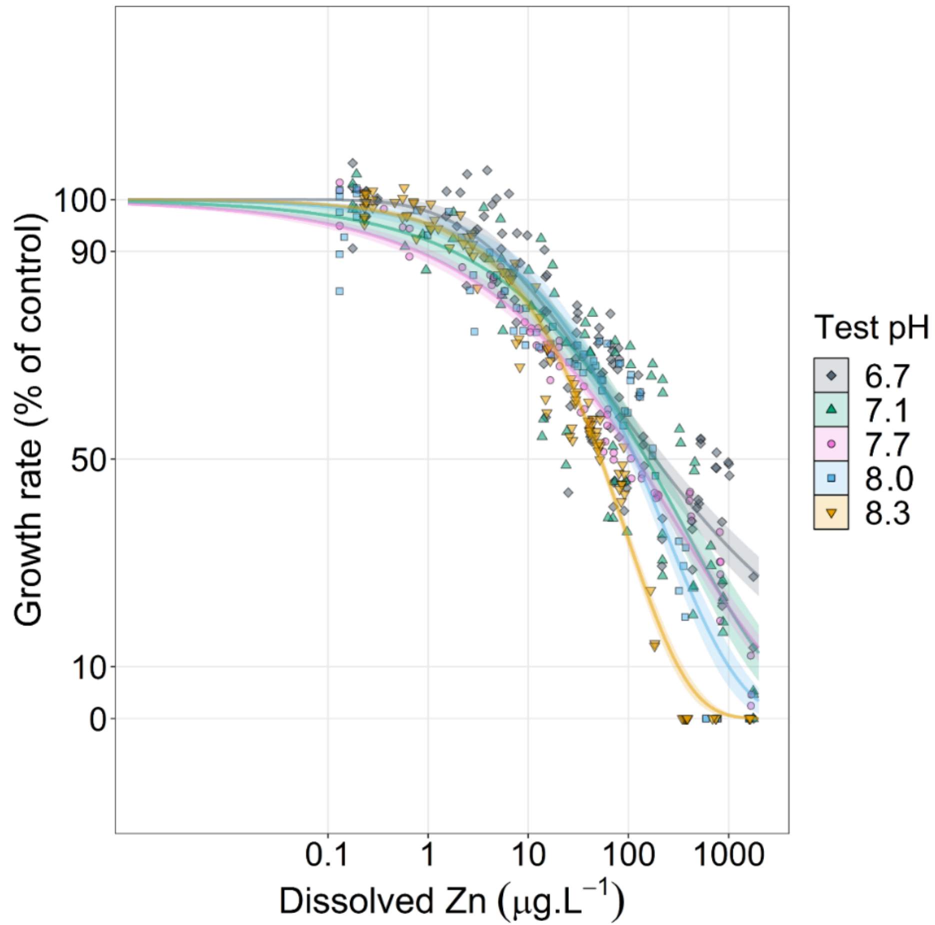

Figure 2.3: 72-h growth rate inhibition of *Chlorella* sp. (% of control) exposed to zinc concentrations at five different pH values. Shaded ribbons represent the 95% confidence intervals. Each data point represents one individual replicate response and a corresponding measured zinc concentration. Data are pooled from separate experiments. Replicate responses were normalised to their respective controls for inter-test pooling. Individual model figures are provided in Figure *A*-3.

Table 2.2: 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 WHAM 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	13.7	185	3.0	9.1	122
		(2.8 - 6.3)	(9.9 - 17)	(139 - 231)	(1.8 - 4.2)	(6.6 - 12)	(92 - 153)
pH 7.1	1.8	1.8	10	151	1.1	6.4	93
		(0.33 - 3.2)	(4.8 - 16)	(112 - 191)	(0.2 - 2.0)	(3.0 - 9.9)	(69 - 118)
pH 7.7	1.8	0.79	5.8	120	0.45	3.3	68
		(0.49 - 1.1)	(4.4 - 7.3)	(104 - 135)	(0.3 - 0.6)	(2.5 - 4.1)	(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)			

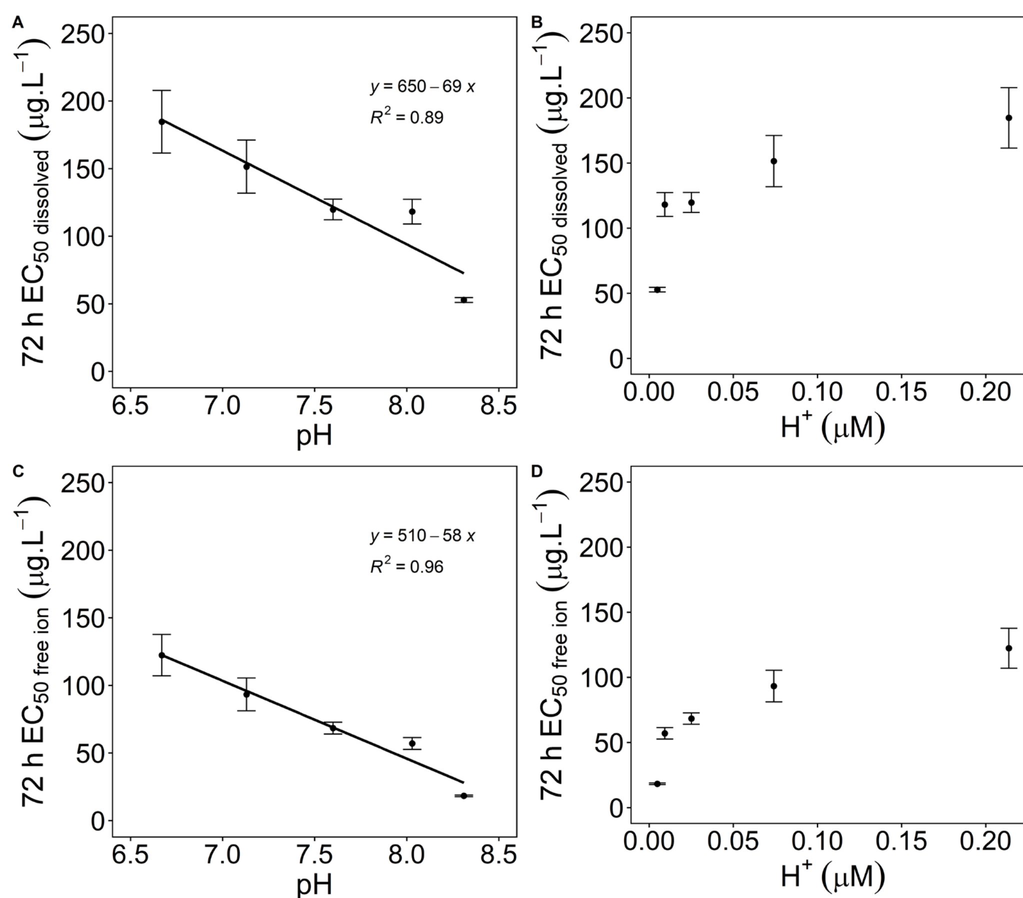

Figure 2.4: The effect of pH and H^+ concentrations on zinc toxicity to *Chlorella* sp. across a pH range of 6.7 – 8.3. A) and B) show results using dissolved metal concentrations and C) and D) show results using WHAM modelled free zinc ion. Error bars = standard error, ECx = x% effect concentration.

2.3.4 Zinc speciation and lability

Speciation calculations (WHAM) 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 62% at pH 6.7 to 31% at pH 8.3. $ZnHCO_3^+$ increased from 24% at pH 6.7 to 26% at pH 7.7 before decreasing to 17%. $ZnCO_3$ increased with pH from 0.44% at pH 6.7 to 14% at pH 8.3. $Zn(OH)^+$ and $Zn(OH)_2$ increased with pH from 0.30% to 6.7% 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 Table A-3 and Figure A-4.

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% (SD = 0.1, n = 16) and 92.4% (SD = 0.2, n = 16) of measured total zinc concentrations present as the 'truly dissolved' or ultrafiltered fraction for pH 6.7 and 8.3, respectively (Figure 2.5). 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.

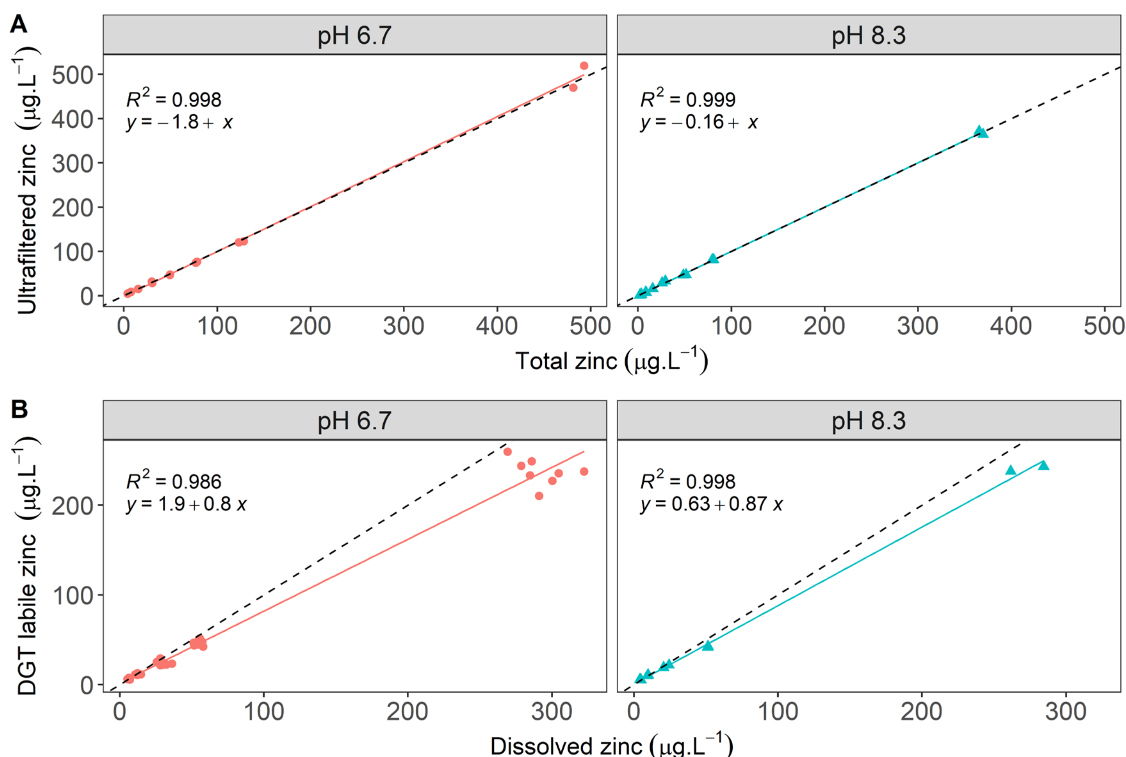

Figure 2.5: Comparison of A) ultrafiltered (<3 kDa) zinc concentrations compared to total zinc (unfiltered) concentrations; and B) DGT-labile zinc concentrations to dissolved (day 3) zinc concentrations. Dashed black line indicates the 1:1 ratio and correlation coefficients are calculated using the Pearson method. DGT = diffusive gradient in thin-films.

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 zinc binding to the test vessel as well as losses due to uptake by the DGT device, with average dissolved zinc losses of 45% (SD = 7, n = 19) 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 and DGT device 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 was compared to day 3 dissolved zinc as it is likely more representative of the mean concentration across the exposure period.

DGT-labile zinc was 95% (SD = 14, n = 9) and 99% (SD = 19, n = 10) 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-labile zinc 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 2.5B). Ratios of DGT-labile zinc 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-labile zinc to mean dissolved metals,

DGT-labile zinc was 68% (SD = 8, n = 9) and 64% (SD = 6, n = 10) of the mean 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.

2.4 DISCUSSION

2.4.1 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. (2002a) 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., 2002a; 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 and 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 present 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 present study. Heijerick et al. (2002a) 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 metal toxicity with increasing pH are not always observed, as explored in a meta-analysis by Wang et al. (2016). The meta-analysis found that, when comparing 110 datasets representing a variety of taxa from the USEPA ECOTOX database (http://www.epa.gov/ecotox/), 42% of datasets followed a decreasing toxicity with increasing pH pattern (positive correlation), while an increasing toxicity with increasing pH (negative correlation) accounted for 18% of datasets. This positive correlation, not observed in the present study, 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. These differences across species highlight the importance of considering numerous taxa when developing bioavailability-based water quality criteria.

2.4.2 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 WHAM showed that over the pH range tested, as the pH increased, the proportion of 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, especially given bioavailability can be related to free metal ion concentration (Campbell, 1995). Although the optimisation 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 & 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 & 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 2.4B and 2.4D, respectively) and proton concentrations observed in the present study is consistent with previous studies (Heijerick et al., 2002a; 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 among 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., 2002b). 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 membrane (François et al., 2007; Parent & Campbell, 1994). Protons have also been suggested to alter permeability of the plasma membrane on the algal cell, thereby influencing metal binding and uptake (Macfie et al., 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 in contrast, intracellular zinc did not increase as the added zinc concentration increased or as the pH increased.

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., 2018a). 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 present study agree with those findings and highlight that algal sensitivity to metal/proton competition is not reflected in DGT measurements, 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, suggesting that the DGT-labile fraction may be a good predictor of protective effects of DOC. However, similar to results of the present 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 by increasing the competition between the free metal ion and other cations, 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 present study highlight DGT

measurement's limitations in predicting changes in metal bioavailability under varying water chemistry parameters.

2.5 CONCLUSIONS

The present 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 do not solely explain zinc toxicity across varying pH values in freshwater. The toxicity results of the present study will add to the limited data on algal response to zinc under different water chemistry conditions. The findings of the present study provide further evidence that different organisms respond to metal toxicity under varying pH in different ways and highlights the importance of considering numerous taxa when modelling for bioavailability-based guideline derivation.

Chapter 3: The influence of hardness at varying pH on zinc toxicity to *Chlorella* sp.

The current Australian freshwater guideline values for zinc incorporate a hardness correction algorithm to modify the guideline depending on the water hardness of the ecosystem being assessed. This algorithm was derived predominately from North American fish toxicity data using experimental designs not controlled for alkalinity and pH. As such, this algorithm may not be truly representative of the effects of changing hardness on zinc toxicity to microalgae. Chapter 3 assessed the influence of varying hardness and pH on the chronic toxicity of zinc to the freshwater microalgae, *Chlorella* sp., and investigated the effectiveness of the current hardness algorithm. Environmentally relevant water hardness and pH ranges were tested, with increased hardness generally being protective to zinc toxicity. The diffusive gradients in thin-films technique and speciation modelling were used to understand if the protective nature of hardness was correlated to the lability and speciation of zinc. The work presented in this chapter has been published in the below cited publication.

Highlights

- Increasing hardness had a protective effect on zinc toxicity up to 93 mg $CaCO_3.L^{-1}$.
- Increasing protection to zinc toxicity plateaued above hardness 93 mg $CaCO_3.L^{-1}$.
- DGT-labile zinc does not correlate with organism toxicity response as hardness increases.
- Current zinc hardness-algorithms used in water quality guidelines may not be appropriate.

Price, G. A. V., Stauber, J. L., Holland, A., Koppel, D. J., Van Genderen, E. J., Ryan, A. C., & Jolley, D. F. (2022). The influence of hardness at varying pH on zinc toxicity and lability to a freshwater microalga, *Chlorella* sp. Environmental Science: Processes & Impacts. https://doi.org/10.1039/d2em00063f

I developed the experimental design and conducted all bioassays. I completed all chemical and statistical analyses, data visualisation and interpretation, and I prepared the manuscript for publication. All authors contributed to study conceptualisation and editing of the manuscript before submission.

3.1 INTRODUCTION

Metal toxicity to an organism is directly related to the bioavailability of that metal to the organism. Bioavailability is significantly influenced by *in situ* water chemistry, such as pH, hardness (measured as Ca and Mg), alkalinity, and dissolved organic carbon (DOC) (Di Toro et al., 2001). This occurs via several mechanisms including: metal speciation changes (e.g., pH and alkalinity), cation competition for binding sites at the biotic ligand (e.g., pH and hardness) and metal complexation to organic ligands (e.g., DOC) (Adams et al., 2020).

Numerous empirical and mechanistic models have been developed to explain relationships between toxicity and water chemistry, including hardness algorithms (USEPA, 1985), multiple linear regressions (MLRs) (Brix et al. 2017; Stauber et al. 2021) and biotic ligand models (BLMs) (Santore et al. 2001; De Schamphelaere and Janssen 2002). These have subsequently been used to develop water quality guidelines. The USEPA (1985) first incorporated a bioavailability term through a hardness-dependent algorithm. This algorithm was then incorporated into other water quality regulatory frameworks, such as the Australian and New Zealand water quality guidelines (ANZECC and ARMCANZ 2000). Since the development of these hardness algorithms, the role of other toxicity modifying factors (TMF) (pH, major ions, dissolved organic carbon (DOC)) have been further investigated (see review by Adams et al. (2020)). In the case of copper, rigorous testing of the dependence of copper toxicity on hardness highlighted that the hardness algorithms were not appropriate, resulting in removal of the hardness algorithm for copper guideline values in Australia and New Zealand (Markich et al., 2005, 2006). Markich et al. (2005) explained that a key reason for the discrepancies between the original results used to derive the copper hardness-algorithms and the more recent studies disputing the algorithm, was the covariation of hardness with alkalinity and pH. Hardness and dissolved inorganic carbon (DIC, as measured by alkalinity and pH) influence metal bioavailability in different ways. Hardness affects bioavailability through calcium and magnesium competition at the biotic ligand, while DIC affects metal speciation in solution through complexation with carbonates, so it is important to separate these effects and assess them individually (Markich et al., 2005).

Such rigorous assessment of the hardness algorithms has not occurred for other metals, such as zinc. The current Australian and New Zealand water quality guideline values (ANZG, 2018) for zinc use the original 1985 hardness-dependent algorithm, as shown in Equation 3.1.

$$Hardness\ corrected\ guideline = GV(\frac{H}{30})^{0.85} \qquad (3.1)$$

where GV is the guideline value in $\mu g.L^{-1}$ normalised at a hardness of 30 mg $CaCO_3.L^{-1}$, and H is the measured hardness (mg $CaCO_3.L^{-1}$) (Warne et al., 2018).

This zinc hardness algorithm was derived largely from North American fish acute toxicity data and was not verified for freshwater microalgae due to the lack of high-quality data. Consequently, it is uncertain if the algorithm is protective of zinc toxicity to microalgae. Despite hardness being one of the most widely studied TMFs, there are limited studies on the effect of true water hardness (decoupled from alkalinity and pH) on zinc toxicity to freshwater microalgae and of those studies, most have been done on the green freshwater microalga *R. subcapitata* (De Schamphelaere et al., 2005; Heijerick et al., 2002a; Van Regenmortel et al., 2017). The increased understanding of the importance of metal bioavailability in aquatic environments has led to the development of methods that measure different metal fractions using kinetic approaches (Davison and Zhang 1994). The diffusive gradients in thin films (DGT) sampling technique uses diffusion-based sampling and provides *in situ* kinetic measurements of the average labile metal concentration across the time of deployment (Zhang & Davison, 2015). A chelating resin that selectively binds cations is overlaid by a diffusive gel and a 0.45 µm filter membrane that restrict mass transport based on molecular diffusion (Davison & Zhang, 1994). In contrast, bioavailability models (such as the biotic ligand model) assume a thermodynamic equilibrium exists in bulk solution, which is not always the case (Di Toro et al., 2001). Here, kinetic approaches (as measured by DGT) may be more useful. DGT measurements can be used to check if all dissolved metal is labile; this can be of particular importance in toxicity tests using algae, which, when present at high cell density, can release exudates that complex metals and greatly reduce lability and hence bioavailability (Franklin et al., 2002). DGT measurements can be compared to speciation estimates (determined with the Windermere Humic Aqueous Model (WHAM)), to help explain changes in toxicity under various water chemistry conditions.

The concepts of bioavailability and lability are similar, so it can be hypothesised that measures of lability will be correlated to observable changes in bioavailability and toxicity over varying water chemistry conditions. However, the relationship between DGT lability and metal toxicity under varying water chemistry is not well established. Macoustra et al. (2019; 2021b) found that DGT-labile copper concentrations were correlated to microalgae toxicity with changing DOC concentration. Chapter 2 (Price et al. 2021) assessed the influence of pH on the relationship between DGT-labile zinc and microalgal toxicity finding lability was not correlated to toxicity with changing pH. Paller et al. (2019) found hardness had a small influence on copper and zinc lability, relative to the influence of DOC. These studies suggested that DGT measurements combined with bioavailability models may be useful as a tool for assessing metal toxicity over a range of water chemistries. However, there is still limited information on how DGT measurements can be directly linked to observed toxicity.

This study had three main objectives: 1) to assess the influence of increasing hardness, at three different pH values, on the toxicity of zinc to a tropical freshwater microalga *Chlorella* sp. using

environmentally relevant hardness values (5 to 400 mg $CaCO_3$.L^{-1}) at an environmentally relevant pH range (6.7 to 8.3); 2) to determine whether DGT-labile zinc measurements correlate with zinc toxicity at different water hardness levels; and 3) to compare the influence of hardness on zinc toxicity against the current hardness algorithms applied to the US and Australian and New Zealand freshwater guidelines to assess the protectiveness of the algorithm to microalgae.

3.2 METHODS

3.2.1 General laboratory techniques and reagents

All 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), acid rinse cycle (2% (v/v) HNO_3, Merck), and finished with an ultrapure water (UPW) rinse cycle (18 MΩ.cm, Milli-Q®, Millipore). All glassware and polypropylene sample vials and lids (Technoplas) used in testing and analysis were soaked (>24 h) in 10% (v/v) HNO_3 (Merck) and thoroughly rinsed with UPW before testing.

3.2.2 Organism cultures

Algal growth inhibition bioassays were conducted using the tropical freshwater green microalga, *Chlorella* sp. (Stauber & Apte, 1996). Algae were cultured in Jaworski's medium 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 weekly into new media, and 5 to 7-day old cultures were used for test initiation to ensure continual exponential growth throughout the testing period.

3.2.3 Toxicity testing

All toxicity tests were conducted using modified synthetic water based on the USEPA recipe (USEPA, 2002) and the test protocol is based on Organisation for Economic Co-operation and Development test guideline 201 (2011b) with modifications described in Franklin et al. (2005). To ensure that only true water hardness was varied and carbonate/bicarbonate alkalinity remained constant across all test solutions, $NaHCO_3$ and KCl salts were kept constant at 96 mg.L^{-1} and 4 mg.L^{-1}, respectively, based on the 'moderately hard' (90 mg $CaCO_3$.L^{-1}) recipe (USEPA, 2002). Concentrations of $CaSO_4.2H_2O$ and $MgSO_4.7H_2O$ were modified to prepare water of varying hardness (nominally 5 – 400 mg $CaCO_3$.L^{-1}) and kept at a constant ratio (2:1) for all treatments. Concentrations of salts used to prepare test solutions are presented in Table B-1. All test solutions were adjusted to the required pH with dilute HCl or KOH, with pH being 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 previously been shown to be

non-toxic to microalgae and to not influence toxicity at 0.5 $g.L^{-1}$ (De Schamphelaere et al., 2004; Price et al., 2021).

The 72-h growth inhibition bioassays were conducted in 250 mL conical flasks coated in a silanizing solution (Coatasil, Thermofisher Scientific) with 75 mL of test solution. Each flask was spiked with 75 µL of 1.5 g $NO_3^-.L^{-1}$ ($NaNO_3$) and 0.15 g $PO_4^{3-}.L^{-1}$ (KH_2PO_4) stocks giving final concentrations of 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 (5, 20 and 1000 $mg.L^{-1}$) of zinc were prepared using analytical grade zinc chloride (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 – 5000 µg $Zn.L^{-1}$) were tested at four different hardness concentrations (5, 31, 93 and 402 mg $CaCO_3.L^{-1}$) and at three different pH values (pH 6.7, 7.6 and 8.3) at each hardness. These pH and hardness ranges were chosen as it covers the 10th – 90th percentile range of Australian and New Zealand natural water values (Stauber et al. 2023) and allows all tests to be conducted using a single buffering agent. EC50 values at 93 mg $CaCO_3.L^{-1}$ hardness were taken from Chapter 2 (Price et al. 2021) and included in the analysis of the present study.

A 25 mL aliquot of test solution was taken from each flask for chemical analyses prior to algal inoculation but after a 24-h pre-equilibration period at the test conditions. Algal cells in culture media 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 culture medium. Algal concentrate was spiked into test flasks to give a final cell density of 2 – 4 x 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. Tests of all pH and hardness treatments were repeated at least once to account for inter-test variability.

Algal cell densities in test solutions were determined using flow cytometry (FACSVerse, BD Biosciences) at 0, 24, 48 and 72 h. Cell densities were obtained by plotting side angle light scatter (SSC) versus chlorophyll *a* fluorescence intensity (FLB3) with manual gating. A threshold of 200 (arbitrary units) was set to exclude background noise from non-algal particles and gating allowed for the exclusion of dead cells as described in detail by Stone et al. (2019). Cell densities were used to calculate population growth rates (Franklin et al., 2001). Population growth rates were normalised as a percentage of the test's control response to account for inter-test variability and allow data to be pooled.

A copper reference toxicant test was run concurrently with each hardness series test. Tests were conducted in 'moderately hard' modified synthetic water (90 mg $CaCO_3.L^{-1}$, pH adjusted to 7.5) (USEPA, 2002). The test was considered acceptable if control algal growth rates in reference

toxicant tests were 1.7 ± 0.4 doublings per day (mean ± SD, n = 20) and the copper reference median effect concentration (EC50) was within internal database limits of 2.6 ± 1.1 µg $Cu.L^{-1}$ (mean ± SD, n = 20). Additionally, toxicity tests required <20% coefficient of variation in control growth rates, and >1.2 doublings per day in controls. Test pH variability was required to be within ± 0.1 pH unit of the average test pH for the 72-h test.

3.2.4 Chemical analyses

Subsamples (<0.45 µm) were collected from each test flask at the start (0 h) and end (72 h) of each test for dissolved metal analysis. Subsamples were filtered through acid-rinsed (flushed with 30 mL of both 10% HNO_3 and ultrapure water) 0.45 µm syringe filters (polyethersulfone membrane, Sartorius). All metal samples were acidified to 0.2% (v/v) HNO_3 (Tracepur, Merck) and stored below 4°C until analysis. Metals samples were analysed by inductively coupled plasma-atomic emission spectrometry (ICP-AES, Agilent 730ES) with a minimum instrument detection limit of 0.16 µg $Zn.L^{-1}$. Matrix-matched multi-element calibration standards, blanks and drift-standards were used for quality assurance.

Samples were collected from the bulk test solution for DOC and alkalinity analysis prior to the addition of MOPS. Test solutions were filtered through acid-rinsed 0.45 µm filters (polyethersulfone, Sartorius), DOC samples were acidified with concentrated sulfuric acid (H_2SO_4) in glass amber vials and alkalinity samples were collected in high-density polyethylene containers and stored below 4°C until analysis. DOC was determined by the non-purgeable organic carbon method (TOC-L series, Shimadzu) and alkalinity was determined using automated titration.

Physicochemical measurements including conductivity (model 30/10 FT, YSI) and dissolved oxygen (Oximeter 330, WTW) were made from each treatment at the start and end of each test, and measurements for pH (probe ROSS 815600, Thermo Fischer) were collected every 24 h throughout the test. All instruments were calibrated before use in accordance with manufacturer instructions.

3.2.5 Zinc speciation and lability

Two methods were used to investigate zinc speciation and lability: the Windermere Humic Acid Model (WHAM, version 7) and the diffusive gradients in thin-films (DGT) technique.

WHAM was used to estimate zinc speciation in each test solution. Input parameters included dissolved zinc, pH, temperature, major ions (Mg^{2+}, Ca^{2+}, K^+, Na^+, Cl^-, SO_4^{2-}, CO_3^{2-}, NO_3^- and PO_4^{3-}), and DOC. An open atmosphere assumption (pCO_2 = 0.00038 atm) was applied to all

speciation calculations as per DeForest and Van Genderen (2012). Nominal concentrations for major anions and cations (except Mg^{2+} and Ca^{2+}) were used (input data provided Table B-2).

DGT-labile zinc was measured in six zinc concentrations between 0 and 400 µg.L^{-1} at hardness concentrations of 31 and 402 mg $CaCO_3$.L^{-1}, with DGT-labile data for 93 mg $CaCO_3$.L^{-1} taken from Chapter 2 (Price et al. 2021). The Chelex-100-based chelating resin (Na form, 200 – 400 wet mesh) and polyacrylamide diffusive gel were synthesised and assembled into DGT pistons following procedures by Amato et al. (2019). DGT pistons were deployed in acid-washed polycarbonate containers with test solution and inoculated with algal cell densities equivalent to initial densities used in the toxicity tests. DGT samplers were deployed in separate vessels to the toxicity tests as they did not fit inside a 250 mL conical flask. Deployed DGT samplers were placed on an orbital shaker (90 – 100 rpm) to ensure that the diffusive boundary layer was negligible. The orbital shaker was placed in an incubator cabinet (LABEC) under the same conditions as the toxicity tests. DGT samplers were retrieved following 72-h deployment, binding gels were eluted in 1 M HNO_3 for >24 h, and then diluted 10-fold for ICP-AES analysis. DGT-labile zinc concentrations were calculated in accordance with equations detailed by Zhang and Davison (1995). DGT experiments were only conducted at pH 6.7, as findings in Chapter 2 (Price et al. 2021) had shown that pH within the tested range (6.7 to 8.3) did not significantly influence DGT-labile zinc concentrations.

3.2.6 Statistical analysis and modelling

Statistical analyses were performed using the R statistical software (v4.3.0; R Core Team, 2023) with the extension package *drc* (Ritz et al., 2015). Figures were produced using the extension packages *ggplot2* (Wickham, 2016) and *ggpubr* (Kassambara, 2020).

Growth rate inhibition normalised to a percent of the respective control growth rate of that test was used as the response variable to derive all toxicity estimates. Effect concentrations for 10, 20 and 50 percent growth inhibition relative to controls (EC10, EC20 and EC50) were calculated using 4-parameter log-logistic or Weibull models. Akaike's information criterion (AIC) and model residual standard error were used for model selection via the *mselect* function within *drc* (model parameters are provided in Table B-3). 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 limit parameter was fixed to 0, meaning only two parameters were estimated (the slope and inflection). When this was not the case (i.e., lower asymptote >0), the lower asymptote was not fixed (Ritz, 2010). Note in scenarios where the lower asymptote was >0, 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 mid-point 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.

Ratio tests (Wheeler et al., 2006) via the *comped* function within *drc* were used for significance testing of EC values among tests. Stepwise linear regression was used to test for significant interaction between hardness and pH as TMFs. The *stepAIC* function from the *MASS* package was used following approaches used by Brix et al. (2017). DGT-labile zinc results were compared using ANOVA and post-hoc Tukey multiple pairwise-comparisons. Assumptions of homogeneity of variances and normality of residuals were tested using Levene's test and Shapiro-Wilk tests, respectively.

All metal concentrations in concentration-response models and results were measured concentrations. Standard deviation (SD) was used to specify variability, and all significance testing was conducted at $\alpha = 0.05$.

3.3 RESULTS AND DISCUSSION

3.3.1 Quality assurance and control

All tests met the test acceptability criteria. The pH variability within tests was no greater than ± 0.1 units of the average pH in each test treatment. Dissolved organic carbon concentrations were kept low, with less than 1 mg C.L^{-1} reported in test solutions for all tests on day 0 in the absence of MOPS. Hardness concentrations (measured as Ca and Mg) in each test remained consistent, with no differences between tests of the same hardness concentration (Table B-4). Alkalinity varied slightly across all hardness concentrations (Table B-4); however, relative to the large concentration range of the hardness treatments, the changes in alkalinity were small.

Control growth rates were acceptable in all tests (>1.2 doublings per day) (Table 3.1). Copper reference toxicant tests run concurrently had a mean EC50 of 2.0 (± 0.4) µg.L^{-1}, indicating that the microalgal cultures had repeatable and comparable sensitivity across tests. Dissolved metal subsamples collected on day 0 and day 3 in toxicity tests had average losses of measured zinc across the test duration of <10%, except for very low zinc treatments (<10 µg Zn.L^{-1}), where losses were between 0 and 7.6 µg Zn.L^{-1}. The mean of day 0 and day 3 metal concentrations were used in all toxicity modelling.

3.3.2 The effect of hardness at different pH values on zinc toxicity to *Chlorella* sp.

Test hardness had an inverse correlation with zinc toxicity at all pH values tested (Figure 3.1, Table 3.1), with decreasing toxicity corresponding with increasing hardness. Exceptions to these trends included a significant ($p < 0.0001$) decrease in EC50 values in the pH 6.7 test series from

184 µg.L^{-1} at 93 mg $CaCO_3$.L^{-1} to 96 µg.L^{-1} at 402 mg $CaCO_3$.L^{-1} and a significant ($p < 0.0001$) decrease in EC10 in the pH 7.6 test series from 2.1 µg.L^{-1} at 31 mg $CaCO_3$.L^{-1} to 0.8 µg.L^{-1} at 93 mg $CaCO_3$.L^{-1} (Table 3.1).

Increasing water hardness is often found to reduce zinc toxicity (CCME, 2018). Hardness (and/or individual Ca^{2+} and Mg^{2+} concentrations) is a widely studied TMF for zinc; however, there are limited studies on direct effects of total hardness on algal species with most studies focusing on invertebrates and fish (Meyer et al., 2007). One study using microalgae by Heijerick et al. (2002a) investigated the individual influence of Ca^{2+} and Mg^{2+} rather than total hardness on zinc toxicity to *R. subcapitata*. Similar to the present study, the researchers reported significant decreases in toxicity with increasing concentrations for both calcium and magnesium, with a 1.7-fold increase in zinc EC50 values when calcium increased from 10 to 80 mg.L^{-1} and a 6.5-fold increase when magnesium increased from 6 to 60 mg.L^{-1}. Similar ameliorative effects of increasing hardness on zinc toxicity have been shown for other freshwater organisms including acute and chronic toxicity to cladocerans (Heijerick et al., 2002b, 2005; Paller et al., 2019) and acute toxicity to fish (Barron & Albeke, 2000). These trends are also apparent among other metals including nickel (Deleebeeck et al., 2009) and uranium (Charles et al., 2002), but with a notable exception of copper, for which chronic toxicity has been shown to be minimally influenced by hardness (Hyne et al., 2005; Markich et al., 2005, 2006). However, there are conflicting results around the influence of hardness on copper toxicity with recent toxicity models developed by Brix et al. (2021) showing hardness had a significant influence on both acute and chronic copper toxicity to a range of freshwater species. The authors also noted that hardness had a larger effect on acute toxicity models compared to chronic toxicity models.

Several mechanisms have been proposed to explain the observed ameliorative influence of hardness on zinc toxicity. Decreases in toxicity are generally attributed to competition between cations for binding sites at the biotic ligand on the organism. For rainbow trout, calcium has been shown to have a much greater ability to inhibit zinc uptake compared to magnesium, likely due to Ca^{2+} competing with Zn^{2+} for gill surface adsorption and uptake into the cell. In comparison, Mg^{2+} likely only competes with Zn^{2+} for cellular adsorption sites (Alsop & Wood, 1999). This has been attributed to Ca^{2+} and Zn^{2+} sharing the same apical transport channel (Hogstrand et al., 1996). These observations in fish may not directly translate to microalgae, with Heijerick et al. (2002) finding the opposite trend was true for *R. subcapitata,* where Mg^{2+} had a greater toxicity-reducing capacity compared to Ca^{2+}. The calculated biotic ligand stability constants for Ca^{2+} were similar between microalgae and daphnids, but Mg^{2+} stability constants were more than 0.5 log units greater for microalgae. This suggests that magnesium has a higher binding affinity to the microalgal biotic ligand than for the daphnid biotic ligand, therefore providing greater toxicity amelioration. Additionally, zinc uptake mechanism studies by Reid et al. (1996) using the alga

Chara corallina demonstrated that zinc influx into the algal cell was unlikely to be via the Ca^{2+} channels as zinc influx was unaffected by blockage of these channels by lanthanum (La^{3+}).

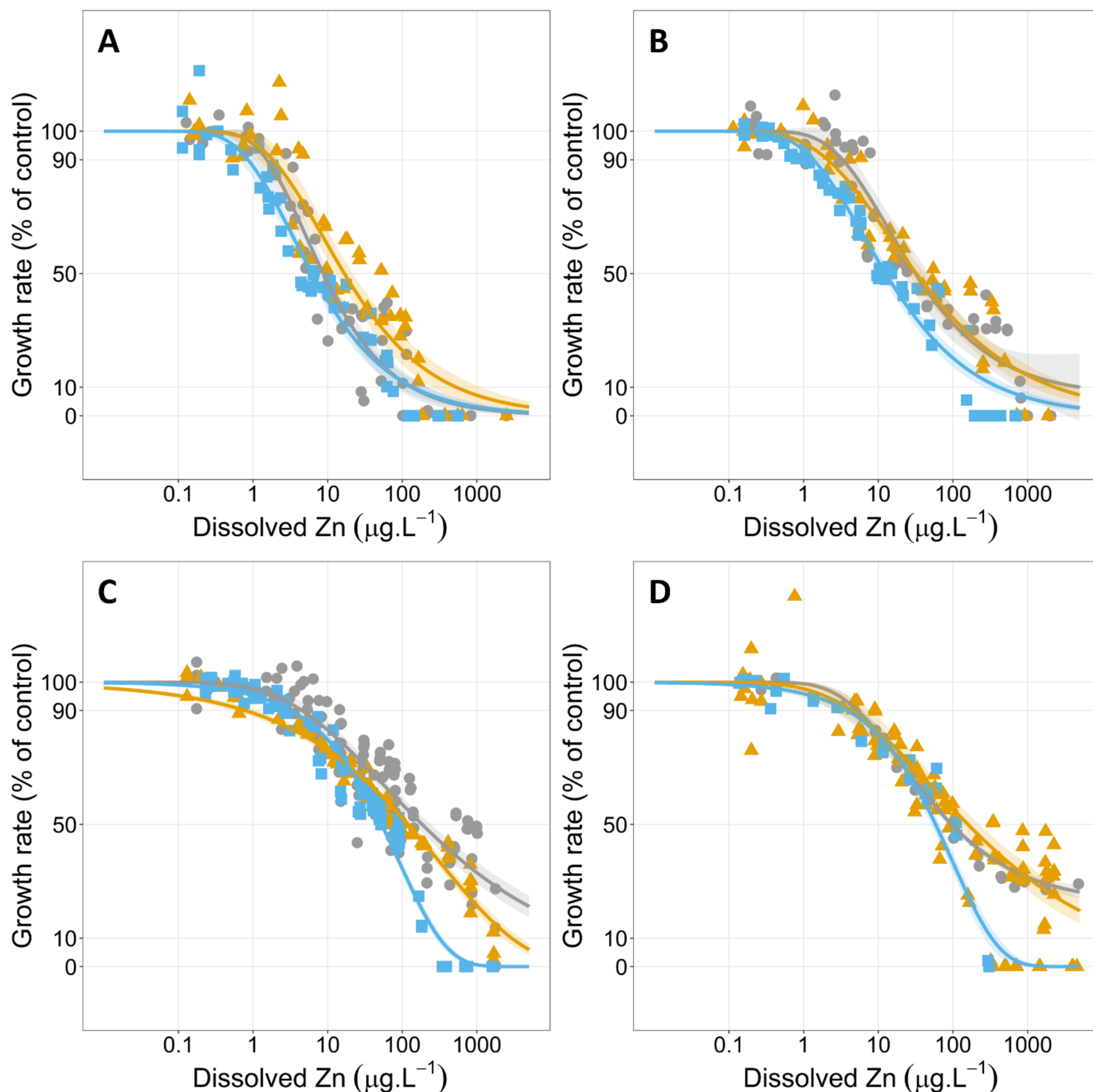

Figure 3.1: Concentration-response curves indicating the effect of pH and hardness on the growth rate of *Chlorella* sp. when exposed to dissolved zinc. Hardness concentrations tested were 5 (A), 31 (B), 93 (C) and 402 (D) mg $CaCO_3.L^{-1}$. Each hardness concentration was tested at pH 6.7 (grey circles), 7.6 (yellow triangles) and 8.3 (blue squares). 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.

Table 3.1: The 72-h effect concentrations (EC10/EC50) for population growth inhibition of *Chlorella* sp. exposed to zinc under different hardness treatments at 3 different pH conditions. Effect concentrations were calculated using pooled test data (n = 2). 95% confidence intervals are shown in parentheses. Free ion EC values represent the WHAM calculated free ion concentration at the dissolved EC values. Control growth rate is shown as doublings per day. EC20 data is provided in Table B-5.

Hardness ($mg\ CaCO_3.L^{-1}$)	**pH**	**Control growth rate**	**Dissolved zinc ($\mu g.L^{-1}$)**		**Zn^{2+} ($\mu g.L^{-1}$)**	
			EC10	**EC50**	**EC10**	**EC50**
5	6.7	1.7	1.5 (1.0 – 2.1)	8.7 (7.1 – 10)	1.0 (0.68 – 1.4)	5.9 (4.9 – 7.0)
	7.6	1.2	1.8 (1.0 – 2.7)	17 (12 – 21)	1.0 (0.57 – 1.5)	9.5 (7.0 – 12)
	8.3	1.7	0.85 (0.62 – 1.1)	6.2 (5.3 – 7.2)	0.29 (0.21 – 0.37)	2.1 (1.8 – 2.4)
31	6.7	1.5	3.3 (2.1 – 4.5)	31 (22 – 40)	2.2 (1.4 – 3.0)	20 (15 – 27)
	7.6	1.4	2.1 (1.3 – 2.9)	32 (24 – 40)	1.2 (0.74 – 1.6)	18 (14 – 23)
	8.3	1.9	1.3 (1.0 – 1.7)	13 (11 – 15)	0.47 (0.34 – 0.59)	4.3 (3.6 – 5.1)
93	6.7	1.8	4.5 (2.8 – 6.3)	184 (138 – 231)	3.0 (1.8 – 4.2)	122 (92 – 153)
	7.6	1.8	0.79 (0.49 – 1.1)	120 (104 – 135)	0.45 (0.3 – 0.6)	68 (60 – 77)
	8.3	2.0	3.2 (2.6 – 3.8)	53 (49 – 56)	1.1 (0.9 – 1.3)	18 (17 – 19)
402	6.7	1.8	5.3 (4.3 – 6.3)	96 (66 – 125)	3.2 (2.6 – 3.8)	59 (41 – 77)
	7.6	1.2	4.4 (1.8 – 7.0)	159 (107 – 211)	2.4 (0.96 – 3.8)	6.9 (3.8 – 10)
	8.3	1.7	3.9 (2.4 – 5.3)	59 (51 – 66)	1.4 (0.87 – 2.0)	22 (19 – 25)

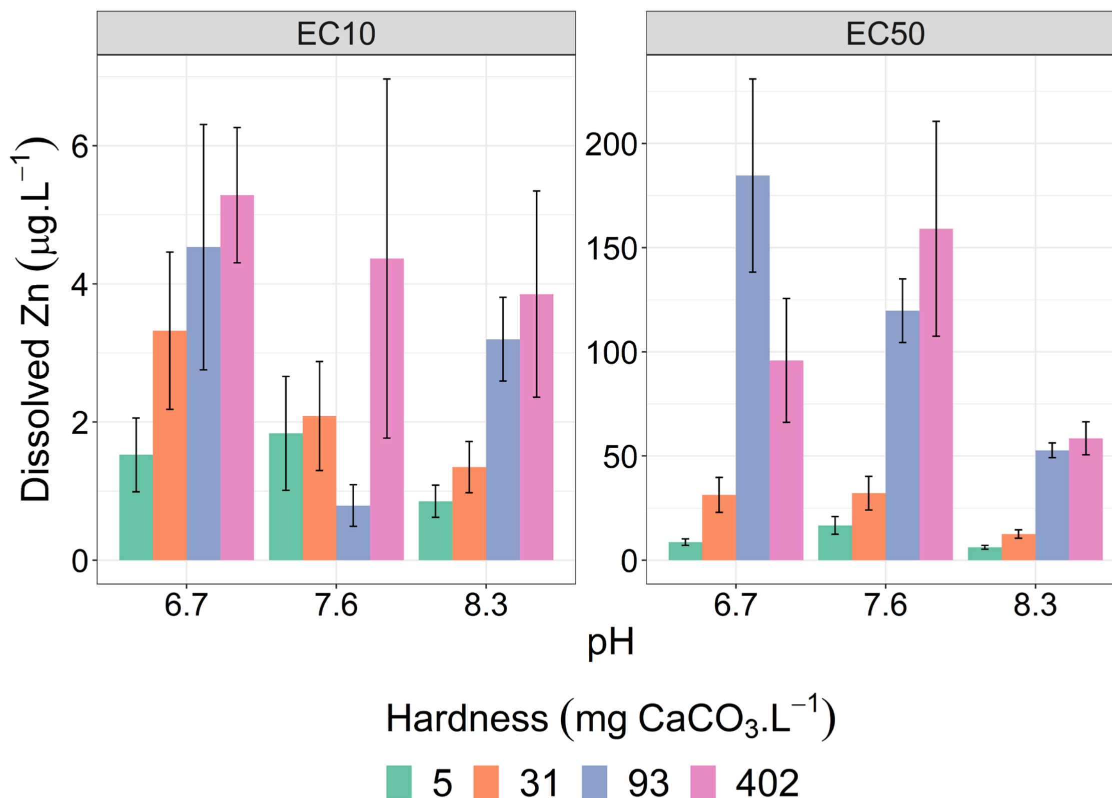

Figure 3.2: Comparison of EC10 and EC50 values for zinc as a function of hardness at 5 (green), 31 (orange), 93 (purple) and 402 (pink) mg $CaCO_3.L^{-1}$ at three different pH values. Effect concentrations at 10% (LHS panel) and 50% (RHS panel) growth rate inhibition after 72-h exposure. Error bars indicate the calculated lower and upper 95% confidences intervals. Note variable y-axis.

Increases in hardness from 5 to 93 mg $CaCO_3.L^{-1}$ resulted in a linear decrease in toxicity at all pH values and effect levels with strong linear correlation between EC50 values and hardness (R^2 = 0.99 (pH 6.7), = 0.98 (pH 7.6), = 0.97 (pH 8.3)). Despite a 4.3-fold increase in hardness from 93 to 402 mg $CaCO_3.L^{-1}$, above 93 mg $CaCO_3.L^{-1}$ there was no continued significant decrease ($p>0.05$) in toxicity at all pH values and effects levels, except for the EC10 ($p = 0.005$) at pH 7.6. Other studies have found similar plateauing in the protective effects at high hardness (and/or Ca^{2+} and Mg^{2+} ion concentrations). Rai et al. (1981) found that calcium concentrations above 20 mg.L^{-1} resulted in reduced protective effects against zinc toxicity to *Chlorella vulgaris*. Similarly, in the current study the plateauing of protective effects occurred between 15 and 71 mg Ca.L^{-1}. Heijerick et al. (2002a) also found higher concentrations of calcium and magnesium resulted in a plateau in the cations' ameliorative effect, with the authors noting that the biotic ligand model developed within that study would not be suitable for predicting zinc toxicity in higher hardness waters. It is important to note that, in natural waters, increased hardness is strongly correlated with increased pH and, these water chemistry conditions of 402 mg $CaCO_3.L^{-1}$ and pH 6.7 are unlikely. Analysis of water chemistry data in the United States by Brix et al. (2020) found that this combination of water chemistry fell outside 99% of the data collected. However, Brix et al.

(2020) also highlighted the importance of assessing toxicity in unlikely water chemistry conditions as metal-impacted sites may have unnatural associated water chemistries.

3.3.3 Interactions of hardness and pH

Changes in pH do not appear to influence the protective effects of hardness, with relative changes in toxicity with changes in hardness remaining consistent between the pH treatments (i.e., compare the hardness treatment at different pH values in Figure 3.2). At pH 6.7, an increase in hardness from 5 to 402 mg $CaCO_3.L^{-1}$ resulted in a 3.5-fold increase in EC10 values (1.5 to 5.3 $\mu g.L^{-1}$) and an 11-fold increase in EC50 values (8.7 to 96 $\mu g.L^{-1}$). Similar increases occurred at higher pH. At pH 7.6, a 2.4- and 9.4-fold increase in EC10 and EC50 values, respectively, were observed. At pH 8.3, a 4.5- and 9.5-fold increase in EC10 and EC50 values, respectively, were observed (Table 3.1). Use of stepwise linear regression confirmed this observed lack of interaction, which found interactive terms were not retained between hardness and pH for EC10 values ($p = 0.829$) and EC50 values ($p = 0.511$). Interestingly, the analysis also did not retain a pH term, with hardness being the only retained parameter. Limited interactions between the two water chemistry parameters was also found by Hyne et al. (2005), who reported a 2.5 and 2.3-fold decrease in acute zinc toxicity for *C. dubia* when hardness was increased from 44 to 374 mg $CaCO_3.L^{-1}$ at pH 7.5 and 8.4, respectively. Similar absences of interactions have also been observed for chronic nickel toxicity to *R. subcapitata* (Deleebeeck et al., 2009) and acute copper toxicity to *D. magna* (Long et al., 2004).

3.3.4 Zinc lability and speciation

DGT-labile zinc concentrations were measured in six dissolved zinc treatments (nominally 0 – 400 $\mu g.L^{-1}$), using the same test media as the 72-h algal growth-inhibition tests. DGT-labile zinc was less than dissolved zinc for all water conditions tested. The relationship between dissolved zinc and DGT-labile zinc followed strong linear relationships at each hardness tested (Figure 3.3). For greater environmental relevance, the relationship between DGT-labile and dissolved zinc between 0 and 100 $\mu g.L^{-1}$, will be discussed. The linear relationship for all dissolved zinc concentrations tested are displayed in the inset plots in Figure 3.3. DGT lability did not follow the same inverse relationship between hardness and zinc toxicity; no significant difference was detected ($p = 0.0766$) in DGT-labile zinc concentrations relative to dissolved zinc concentrations (Figure 3.3). This suggests zinc lability is unchanged across the hardness range tested (31 – 402 mg $CaCO_3.L^{-1}$) at pH 6.7. The lack of significant difference in DGT-labile zinc between hardness treatments suggests that the changes in toxicity are due to competition effects specific to the biotic ligand of the microalgae, rather than changes to zinc speciation or lability. Results of WHAM speciation modelling supports the DGT results that changes in zinc speciation

and lability are not the cause of the changes in toxicity with changes in hardness. Free zinc ion (Zn^{2+}) was the major species present in solution under all test conditions. Zn^{2+} concentrations primarily varied as a function of pH (average change across tested pH range = 31%, SD = 5.4%), with only small changes due to hardness (average change across tested pH range = 4.1%, SD = 2.4%). Increased pH (6.7 to 8.3) resulted in a decrease in the Zn^{2+} species at all hardness concentrations, decreasing from an average of 69 to 34% and 61 to 37% for 5 and 402 mg $CaCO_3.L^{-1}$ hardness, respectively. Although Zn^{2+} decreased with increasing pH, toxicity increased with increasing pH for all hardness concentrations, suggesting that Zn^{2+} concentration is not the main factor influencing toxicity change in these conditions, i.e., that the decrease in Zn^{2+} is more than compensated for by the increased pH and the 40-fold decrease in proton concentration. A full summary of zinc speciation across all test conditions is provided in Table B-6.

DGT results for the current study are similar to those of Paller et al. (2019), who measured DGT-labile zinc in low (13 mg $CaCO_3.L^{-1}$) and moderate (80 – 100 mg $CaCO_3.L^{-1}$) hardness waters in the absence of DOC. The study reported that differences in DGT-labile zinc did not correspond with the observed changes in acute zinc toxicity to *C. dubia* as hardness was varied. Philipps et al. (2018b) assessed the influence of hardness on copper DGT lability and reported no significant changes in labile copper when hardness varied from 40 – 48 to 160 – 180 mg $CaCO_3.L^{-1}$. The study also found that while DGT lability remained unchanged with hardness, DGT-labile copper correlated with copper bioaccumulation in *P. promelas*, but not for copper bioaccumulation in the freshwater mussel, *L. cariosa*.

Several studies have compared biological responses to DGT lability changes under varying water chemistries including pH (Price et al., 2021), hardness (Paller et al., 2019; Philipps et al., 2018b), and DOC (Macoustra et al., 2019; 2021b; Philipps et al., 2018b). Consensus from these studies highlights that DGT lability is not a good predictor of bioavailability change when the primary cause of bioavailability change is via cation competition (H^{+}, Ca^{2+}, Mg^{2+}). However, based on the studies with DOC, DGT may provide useful information on bioavailability in the presence of DOC, as changes in bioavailability and toxicity are associated with DOC-metal complexation.

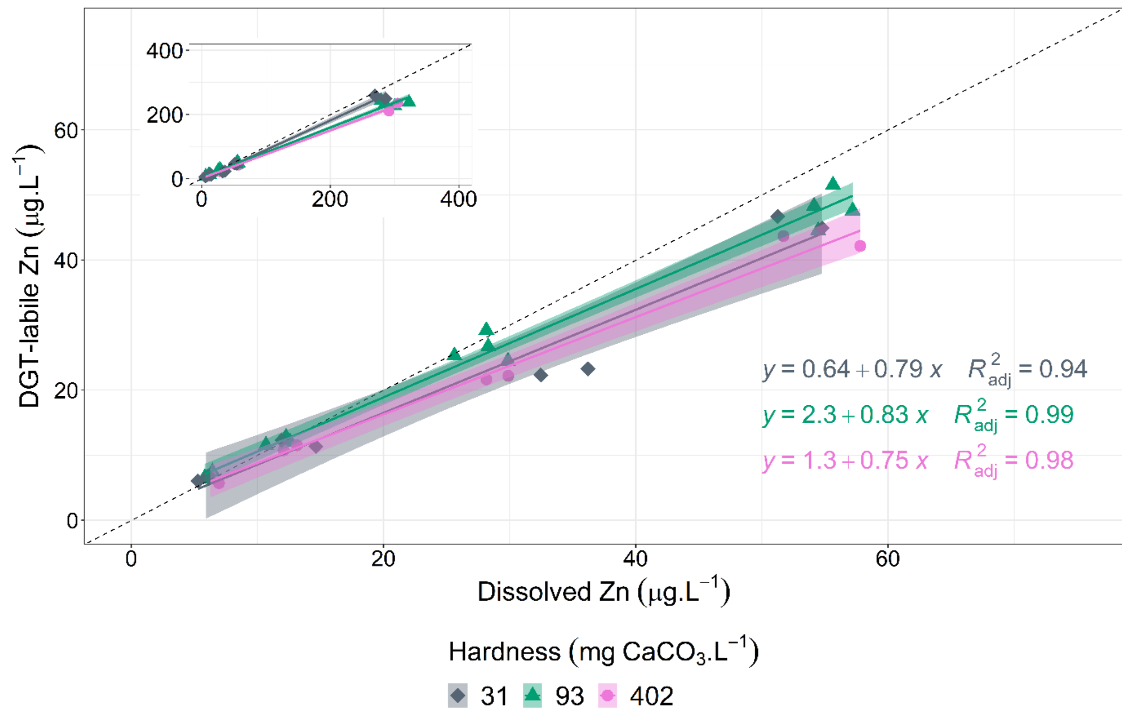

Figure 3.3: Dissolved zinc compared to DGT-labile zinc concentrations measured in 31 (grey diamonds), 93 (green triangles) and 402 (pink circles) mg $CaCO_3.L^{-1}$ at pH 6.7. Main plot displays data for zinc concentrations between 0 and 100 µg.L^{-1}. Inset plot displays all zinc concentrations determined for DGT-labile zinc (0 – 400 µg.L^{-1}). Dashed line represents a 1:1 relationship.

3.3.5 Implications for water quality guideline values

Hardness was the first TMF investigated for metals and as such hardness-dependent algorithms are incorporated into the water quality guidelines of several freshwater regulatory frameworks (ANZG, 2018; USEPA, 1995). The findings of this study along with others (Heijerick et al., 2002a; Rai et al., 1981) have important implications for hardness-dependent guideline values for zinc. The collective results indicate that environmentally relevant high hardness conditions may not provide protective effects to all freshwater organisms from zinc toxicity.

Using Equation 3.1 (Section 3.1) there is a 9-fold difference in the zinc 95% species protection guideline value over the hardness range of 31 – 402 mg $CaCO_3.L^{-1}$ from 8 to 72 µg.L^{-1}, and a 3.6-fold difference over a range of 93 – 402 mg $CaCO_3.L^{-1}$ from 21 to 72 µg.L^{-1}. The use of this algorithm may provide appropriate increases in guideline values up until 93 mg $CaCO_3.L^{-1}$ but based on the results of the current study, precaution is needed at higher hardness. Moreover, the use of the algorithm with the ANZG (2018) framework does not allow for modifying the guideline value for hardness concentrations below 30 mg $CaCO_3.L^{-1}$, potentially providing insufficient protection in very soft waters. Australia has both very soft (<10 mg $CaCO_3.L^{-1}$) (Peters, et al., 2021) and hard waters (>200 mg $CaCO_3.L^{-1}$) (Batley et al., 2018) and therefore may be subject to under protection using the current hardness-correction algorithms.

While use of BLMs and MLRs is not endorsed by the current Australian and New Zealand framework, there is scope within the framework to justify the use of such models where appropriate. Based on the results of the current study, it may be most appropriate to use established bioavailability models (such as BLMs and MLRs) to adjust default zinc guideline values where hardness is very soft or hard. However, models developed with non-algal species (such as fish and daphnids) may not be appropriate and algal specific models may be required. Such species specific models are currently being developed and applied in order to revise zinc guideline values in Australia and New Zealand.

3.4 CONCLUSIONS

This study showed that increasing hardness has a protective effect on zinc toxicity to *Chlorella* sp. up to 93 mg $CaCO_3.L^{-1}$ across an environmentally relevant pH range of 6.7 to 8.3, with further increases in hardness not offering greater protection, with no difference in zinc toxicity from 93 to 402 mg $CaCO_3.L^{-1}$. It was also demonstrated that DGT-labile zinc did not change over the hardness range of 31 to 402 mg $CaCO_3.L^{-1}$ and did not correspond to observed changes in *Chlorella* sp. response across the same hardness range, this likely means that hardness affects *Chlorella* sp. through competition rather than speciation. DGT results were supported by WHAM speciation modelling, where only small changes in zinc speciation were due to changes in hardness. The results of this study also showed that current zinc hardness-algorithms used in water quality guidelines may not be appropriate to use for high hardness waters. More flexible and robust approaches, such as MLR models and/or BLMs are currently being considered in future revisions of Australian and New Zealand water quality guidelines, which could greatly improve the ability of regulators and industry to derive site-specific zinc guideline values for protecting sensitive aquatic biota, such as *Chlorella* sp.

Chapter 4: The influence of DOC on zinc toxicity to *Chlorella* sp.

This chapter is the first study to assess the influence of natural Australian dissolved organic carbon (DOC) on the toxicity of zinc to a freshwater microalga under varying pH conditions. Chapter 4 investigated the toxicity modifying capacity of two distinct Australian DOC sources across four environmentally relevant concentrations. The modification of zinc toxicity to *Chlorella* sp. by DOC across a pH range was also assessed. Speciation modelling and measured lability, via the DGT technique, was used to understand the proportion of DOC-bound zinc and its relationship to toxicity to *Chlorella* sp. The work presented in this chapter has been published in the below cited publication.

Highlights

- The influence of DOC on zinc toxicity was dependent on concentration and source.
- DOC high in aromatic humic-like components increased zinc toxicity at the EC50 level.
- The influence of pH on zinc toxicity was DOC source dependent, with pH not influencing toxicity when DOC high in humic-like components were present.
- Zinc lability could not explain the observed increases in toxicity in the presence of DOC.

Price, G. A. V., Stauber, J. L., Jolley, D. F., Koppel, D. J., Van Genderen, E. J., Ryan, A. C., & Holland, A. (2023). Natural organic matter source, concentration, and pH influences the toxicity of zinc to a freshwater microalga. *Environmental Pollution*, *318*, 120797. https://doi.org/10.1016/J.ENVPOL.2022.120797

I developed the experimental design and conducted all bioassays. I completed all chemical and statistical analyses, data visualisation and interpretation. Aleicia Holland completed the DOC analysis. I prepared the manuscript for publication. All authors contributed to study conceptualisation and editing of the manuscript before submission.

4.1 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 carbon (DOC), 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). DOC 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 DOC are location-specific and unique to different aquatic systems, meaning that the ameliorative capacity of DOC can vary for different DOC sources and seasons and different metals (Macoustra et al., 2019, 2020; 2021a; 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, 2022a).

There is a general acceptance that DOC influences zinc toxicity; however, this is based on relatively few studies, mostly focused on freshwater invertebrates. Heijerick et al. (2003) investigated the influence of DOC on the chronic toxicity of zinc to *Daphnia magna* and found a negative linear relationship between DOC concentration and chronic (reproduction) zinc toxicity. Hyne et al. (2005) found that increasing DOC resulted in a small decrease in the acute toxicity of zinc to C*eriodaphnia dubia*, with results only becoming significant in the presence of 10 mg $C.L^{-1}$.

There are currently **no** studies that have investigated the **independent** influence of natural DOC 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 DOC parameter for microalgae (CCME, 2018). There are several studies that have validated bioavailability models that include a DOC component via speciation modelling tools, but it is difficult to elucidate the specific influence of DOC in these studies as other water chemistry parameters (e.g., pH and major ions) covaried with DOC concentration (De Schamphelaere et al., 2005).

Metal uptake in aquatic organisms may be dependent on the concentration of the free metal ion or, in some cases, the concentrations of labile inorganic and organic 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 (WHAM, version 7) can be used to estimate metal speciation at equilibrium under a set of water chemistry parameters. WHAM also considers the binding of metals to fulvic and humic acids, fractions of DOC, in waters (Tipping et al., 2011). However, limited studies have assessed the use of WHAM in the presence of natural Australian DOC (Macoustra et al. 2021a, 2019), with only one study having assessed the use of WHAM for zinc speciation in the presence of Australian DOC (Trenfield et al., 2021).

Under kinetic conditions, metal assimilation into the organism is much faster than metal diffusion to the cell surface (Sunda & 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 & 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 & Zhang, 1994; Zhang & 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 DOC characteristics from Australian sources and DOC 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 DOC 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 DOC on zinc toxicity to a freshwater microalga, which is needed to develop bioavailability-based guidelines for zinc in freshwaters.

4.2 METHODS

4.2.1 Natural DOC collection

Natural DOC 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). DOC 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).

Manton Dam and Appletree Creek DOC concentrates have been optically characterised and were described in detail by Holland et al. (2018). Manton Dam DOC is a circumneutral DOC composed of mainly humic-like (35%) and fulvic-like (46%) substances, with a minor component of protein-like (19%) substances. In contrast, Appletree Creek DOC 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 DOC composition showed minor differences in composition between the time of collection and the time of toxicity testing, and these are shown in Appendix C. Detailed DOC characterisations are reported in Table C-1 – C-3 and fluorescence excitation emission scans are provided in Figure C-1.

4.2.2 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% (v/v) HNO_3, 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_3 (Merck) and thoroughly rinsed with UPW before use.

4.2.3 Organism culturing and toxicity testing

Growth inhibition toxicity tests were conducted using a tropical freshwater green microalga *Chlorella* sp. (Stauber & Apte, 1996). Algae were cultured in Jaworski's medium at 2/5 strength (Thompson et al., 1988) at 27 ± 1 °C on a 12: 12 light/dark cycle (75 µmol photons.$m^{-2}.s^{-1}$). 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 $CaCO_3.L^{-1}$. The test protocol followed the Organisation for Economic Co-operation and Development test guideline 201 (2011a) with modifications as described by Franklin et al. (2005). Both natural DOC concentrates (Manton Dam stock: 138 mg $C.L^{-1}$; Appletree Creek stock: 148 mg $C.L^{-1}$) were diluted with the synthetic test waters to produce a series of DOC concentrations (nominally 0, 2, 5, 10 and 15 mg $C.L^{-1}$, Table 4.1). 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 $NO_3^-.L^{-1}$ ($NaNO_3$) and 0.15 g $PO_4^{3-}.L^{-1}$ (KH_2PO_4) stocks giving final concentrations of 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. Each DOC treatment consisted of a zinc concentration series from 0 (Control) to 5,000 µg $Zn.L^{-1}$ using a stock solution of $ZnCl_2$ (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 $g.L^{-1}$ (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 culture medium. The algal concentrate was spiked into each test flask to give a final cell density of 2 – 4 x 10^3 $cells.mL^{-1}$ (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 $photons.m^{-2}.s^{-1}$ 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 ± SD, n = 20) and the median effect concentration (EC50) was within internal database limits of 2.6 ± 1.1µg $Cu.L^{-1}$ (mean ± SD, n = 20). Controls in DOC tests needed to have a growth rate of >1.2 doublings per day within the 72-h test period and a coefficient of variation <20%. 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 DOC (IHSS) to provide a reference with a standard DOC source (Mager et al., 2011; Trenfield, et al., 2011). A 100 $mg.L^{-1}$ stock of Suwannee River DOC was diluted with

synthetic test waters to produce a series of DOC concentrations (2.5, 4.4 and 8.7 mg C.L^{-1}), as described previously for the Appletree Creek and Manton Dam DOC 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.

4.2.4 Measured zinc lability and ultrafiltration

Zinc lability was measured using the diffusive gradients in thin-films (DGT) technique (Davison & 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^{2+}), simple inorganic metal complexes and any readily labile organic metal complexes (Koppel et al., 2021; Van Leeuwen et al., 2005). Chelex-100-based binding resins (Na form, 200 – 400 wet mesh) and polyacrylamide diffusive gel were synthesised 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), as described in Section 3.2.5 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 DOC 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^{-1}. DGT pistons were deployed for 72 h. Once retrieved, the Chelex binding gel layer was placed in 1M HNO_3 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 Zhang and Davison (1995). Throughout the present study, DGT measured zinc is referred to as DGT-labile zinc. DGT-labile zinc measurements are provided in Table C-8.

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 minutes, and filtrate was subsampled. Ultrafiltration samples were taken at the start and end of each test from 4 to 8 zinc concentrations in each toxicity 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_3 (Tracepur, Merck) and stored below 4 °C until analysis.

4.2.5 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_3 and ultrapure water) 0.45 µm syringe filters (polyethersulfone membrane, Sartorius). All metal samples were acidified to 0.2% (v/v) HNO_3 (Tracepur, Merck) and stored below 4 °C until analysis. Throughout the present study, dissolved zinc is defined as measured and filtered (<0.45µm) zinc unless otherwise stated. All ultrafiltered, dissolved, and total metal samples were analysed by inductively coupled plasma-atomic emission spectroscopy (ICP-AES, Agilent 730ES) that had a minimum instrument detection limit of 0.16 µg $Zn.L^{-1}$ calculated as three times the standard deviations of the acidified blank solutions. Matrix-matched multi-element 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, as MOPS present in the test solution would contribute to a measured DOC concentration. Samples were filtered through acid-rinsed 0.45 µm filters (polyethersulfone, Sartorius), and acidified with concentrated sulfuric acid (H_2SO_4) in glass amber vials. Samples were stored below 4 °C until analysis by the non-purgeable organic carbon method (TOC-L series, Shimadzu).

Additional subsamples were collected from each test treatment at the start and end of each test for physicochemical measurements. Measurements included conductivity (model 30/10 FT, YSI) and dissolved oxygen (Oximeter 330, WTW) and pH (probe ROSS 815600, Thermo Fischer). Two additional subsamples were collected at 24 and 48 h from each test treatment for pH measurement. All instruments were calibrated before use in accordance with manufacturer instructions.

4.2.6 WHAM estimated metal speciation

WHAM (version 7) was used to estimate zinc speciation for all zinc exposures under all test conditions. Input parameters included dissolved zinc, pH, temperature, major ions (Mg^{2+}, Ca^{2+}, K^+, Na^+, Cl^-, SO_4^{2-}, CO_3^{2-}, NO_3^- and PO_4^{3-}), and concentrations of colloidal fulvic and humic acid (mg $C.L^{-1}$) based on measured proportions of fulvic and humic acid in each DOC. Humic- and fulvic-like proportions in both DOC (Table C-1) were determined using fluorescence excitation emission scans followed by parallel factor (PARAFAC) analysis (Holland et al., 2018). An open atmosphere assumption (pCO_2 = 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 (Table C-5 and C-6, discussed section 4.3.2), specifically at the EC10 and EC50 concentrations (Table C-9 and C-10, discussed section 4.3.4) and for all experimental replicates (Table C-11 and C-12). DOC-bound zinc is discussed in section 4.3.4. DOC-bound zinc is defined as the concentration of zinc bound to fulvic and humic acids as calculated in the speciation modelling (Table C-10).

4.2.7 Statistical analysis and modelling

Statistical analyses were performed using the R statistical software (v4.3.0; R Core Team, 2023) 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 C-4. 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 inflection 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 present 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 mid-point 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 DOC 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-Wilk 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 $\alpha = 0.05$.

4.3 RESULTS AND DISCUSSION

4.3.1 Quality assurance and control

All tests met the acceptability criteria. Control growth rates in DOC tests were acceptable in all tests (>1.2 doublings per day) (Table 4.1). It was noted that all tests with added DOC had increased control growth rates relative to the no added DOC 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 DOC 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^{-1}, indicating that the microalgal cultures had repeatable and comparable sensitivity across tests.

4.3.2 Influence of DOC concentration and source on zinc toxicity

In the absence of DOC, *Chlorella* sp. was sensitive to zinc exposure, with an EC10 and EC50 of 1.3 ± 0.3 and 48 ± 4 µg.L^{-1}, respectively. In the presence of Suwannee River DOC, EC10 and EC50 values were significantly (EC10: $p < 0.0001$, EC50: $p < 0.0001$) increased relative to tests with no added DOC, with EC10 and EC50 values of 6.8 ± 0.9 and 72 ± 5 µg.L^{-1}, respectively (Table 4.1). Increasing Suwannee River DOC concentration led to further significant (EC10: $p < 0.0001$, EC50: $p < 0.0001$) increases in both the EC10 and EC50 values, with an approximate 15- and 2.6-fold change across the <1 to 8.7 mg C.L^{-1} range, respectively (Figure C-2 and C-3). These results are as expected, as DOC presence is typically associated with a decrease in bioavailable metal concentrations through complexation (Wood et al., 2011). Suwannee River DOC has also been shown to have similar ameliorative effects on other metals and organisms, with Mager et al. (2011b) reporting reduced lead toxicity to *Ceriodaphnia dubia* in the presence of 4 mg C.L^{-1} Suwannee River DOC 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 DOC from 0.5 to 41 mg C.L^{-1}.

The influence of natural Australian DOC on the toxicity of zinc to *Chlorella* sp. differed from the results of the Suwannee River DOC reference tests and were dependent on the source of the DOC (Figure 4.1, Table 4.1). However, it is important to note that the Suwannee River reference tests were not chemically buffered by MOPS.

In the presence of Manton Dam DOC, DOC concentration had a small yet significant ($p<0.05$) influence on zinc toxicity relative to results in the absence of DOC, with the trend of this influence differing between EC10 and EC50 values. The EC10 results showed a significant increase ($p = <0.0001$) from 1.6 ± 0.3 to 6 ± 1 µg.L^{-1} as DOC concentration increased from no added DOC (<1 mg C.L^{-1}) to 15.1 mg C.L^{-1}, respectively (Figure 4.2, Table 4.1). The EC50 results showed a small ameliorative capacity of the Manton Dam DOC across the tested range, with no significant difference ($p = 0.312$) between EC50 values in the no added DOC test (EC50 = 112 ± 8 µg.L^{-1}) and the highest DOC concentration test at 15.1 mg C.L^{-1} (EC50 = 126 ± 11 µg.L^{-1}). Interestingly, there were small but significant decreases in EC50 values between the no added DOC treatment and the low concentration Manton Dam DOC treatments, with EC50 values of 71 ± 6 ($p = 0.012$) and 86 ± 5 ($p = 0.033$) µg.L^{-1} in the 2.5 and 5.4 mg C.L^{-1} treatments, respectively. There were also significant increases ($p <0.0001$) in EC50 values between low (2.5 mg C.L^{-1}) and high (15.1 mg C.L^{-1}) concentration Manton Dam DOC treatments suggesting that there may be some trend of protective effects with increasing DOC concentration.

Despite these small observed increases in toxicity in low Manton Dam DOC treatments, overall, there appears to be limited influence of Manton Dam DOC 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^{-1} range. This Manton Dam DOC 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 DOC concentration range of <1 to 8.4 mg C.L^{-1}. This greater ameliorative capacity is consistent with copper having a larger binding affinity to DOC compared to zinc (Tipping et al., 2011).

The EC10 results in the presence of Appletree Creek DOC followed a similar trend to the ones observed for Suwannee River and Manton Dam DOC. The EC10 values showed a small but significant increase ($p <0.0001$) from 1.6 ± 0.3 to 3 ± 1 µg.L^{-1} as DOC concentrations increased from <1 (no added DOC) to 13.0 mg C.L^{-1}, respectively (Figure 4.2, Table 4.1). Unexpectedly, the EC50 results in the presence of Appletree Creek DOC contrasted with the overall trends observed for the Manton Dam and Suwannee River DOC (Figure 4.1 and 4.2). The EC50 values in the presence of all Appletree Creek DOC treatments were significantly ($p <0.05$) lower, relative to the no added DOC results, suggesting that the presence of Appletree Creek DOC increased zinc toxicity. The concentration of Appletree Creek DOC did not influence the magnitude of the increase in toxicity, with EC50 values ranging from 17 ± 3 to 25 ± 6 µg.L^{-1} (which were not significant $p = 0.070$), across a DOC concentration range of 2.0 to 13.0 mg C.L^{-1}.

In the presence of Appletree Creek DOC, concentration-response model slope parameters were significantly influenced relative to the slope parameter of the no added DOC concentration-

response models (Figure 4.1). Significant steepening ($p < 0.0001$) of the slope parameter (Table C-4) 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 DOC (Figure 4.1) 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 DOC 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 4.1).

Our results suggest that the increased toxicity of zinc in the presence of Appletree Creek DOC may be due to direct uptake of the Zn-DOC complexes, particularly any lipid-soluble complexes, or due to the formation of a ternary (DOC-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 (Aristilde et al. 2012; Errecalde et al. 1998; Lamelas et al. 2005). Errecalde et al. (1998) investigated the influence of citrate on the toxicity and uptake of zinc and cadmium in *R. subcapitata*. The presence of citrate (100 $\mu mol.L^{-1}$) caused an increase in toxicity (compared to no added citrate) for both metals, with EC50 values for the metal ions Cd^{2+} and Zn^{2+} decreasing by 7- and 6-fold, respectively. The uptake of Cd^{2+} was also increased in the presence of citrate (uptake experiments were not conducted for Zn^{2+}). Aristile 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 µM 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 DOC may be causing effects similar to those described by Errecalde et al. (1998) and Aristile 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 DOC.

Experiments to measure zinc uptake, extracellular or intracellular zinc in the presence of DOC would help provide more evidence as to the cause of the enhanced toxicity in the presence of Appletree Creek DOC. 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 DOC 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 DOC has also been shown for nickel. Holland et al. (2017) found that in the presence of natural DOC from the Amazon Basin in Brazil, nickel toxicity to Cardinal tetras (*Paracheirodon axelrodi*) was increased, relative to tests without DOC and this was dependent on pH. Of the natural DOCs 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 DOC characteristics of the Brazilian black water are very similar to that of Appletree Creek DOC 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-DOC 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 DOC. This can be tested with WHAM speciation modelling by comparing the percentage of DOC-bound copper at low concentrations (1 µg $Cu.L^{-1}$, representing the maximum measured concentration of copper in Appletree Creek DOC) in the presence of low and high zinc concentrations (10 and 5,000 µg $Zn.L^{-1}$). Speciation modelling shows that only at low DOC concentrations (2 mg $C.L^{-1}$) does the percentage of DOC-bound copper change greatly, decreasing from 99% to 42% DOC-bound in the presence of 10 and 5,000 µg $Zn.L^{-1}$, respectively. Modelled percentages of DOC-bound copper at higher DOC concentrations varied between 71 and 100% (Table C-5 and C-6). The decrease to 42% DOC-bound copper in the low DOC concentration cannot explain how increased toxicity was seen uniformly across all Appletree Creek DOC concentrations (Table 4.1) 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 µg $Cu.L^{-1}$ in the presence of 2.1 mg $C.L^{-1}$ of Appletree Creek DOC for the same algae species, suggesting the displacement of low concentrations of copper from Appletree Creek DOC 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 & De Schamphelaere, 2018). For a metal mixture to be causing the increased toxicity in the presence of Appletree Creek DOC, large synergistic interactions would be needed.

No matter the cause, the increased toxicity of zinc in the presence of DOC is contrary to expectations that DOC will ameliorate toxicity to algae and highlights the importance of assessing

the influence of different natural DOC sources, especially with the renewed focus on developing empirically derived bioavailability-based water quality guidelines (Mebane et al., 2020).

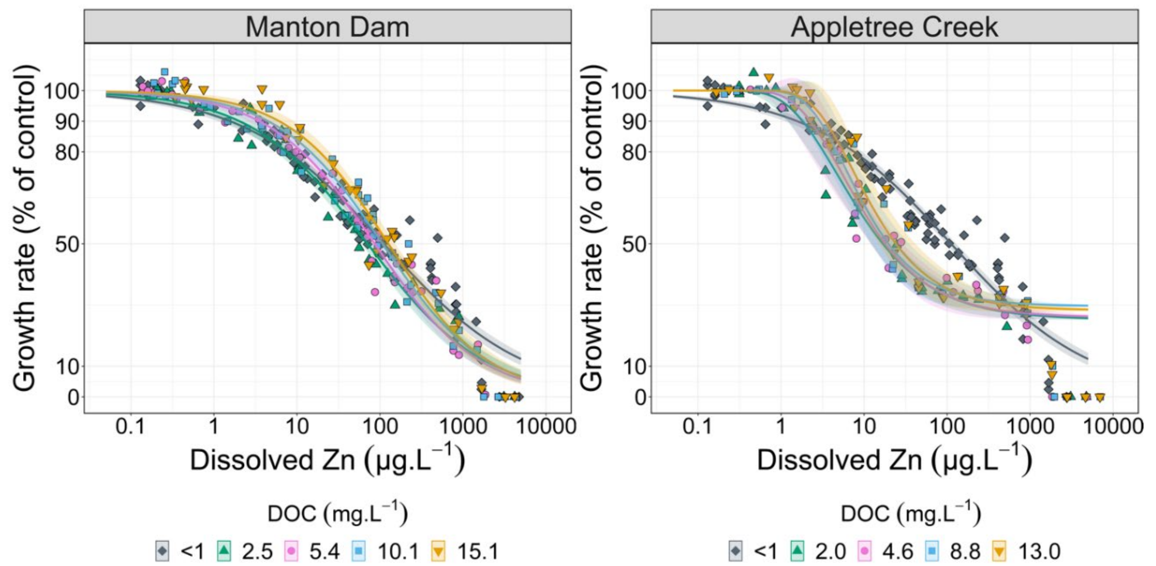

Figure 4.1: Concentration-response curves indicating the effect of Manton Dam DOC (LHS panel) and Appletree Creek DOC (RHS panel) at increasing concentrations of DOC (<1–15 mg.L^{-1}) on the growth rate of *Chlorella* sp. when exposed to dissolved zinc. All tests were conducted at a constant pH of 7.6. 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 DOC have biphasic response curves and the models are fitted to the first lower asymptote.

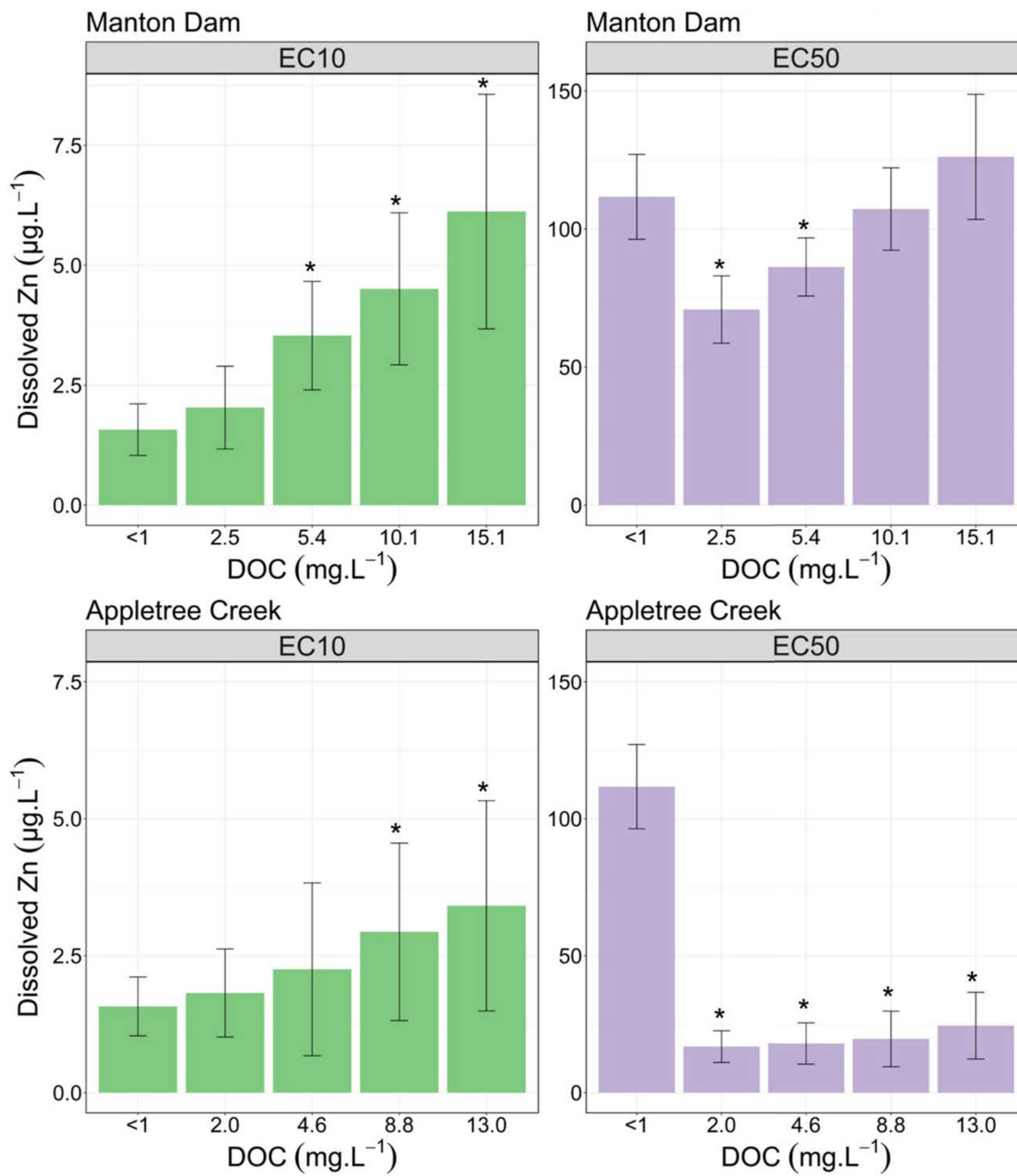

Figure 4.2: Comparison of EC10 and EC50 values for zinc toxicity on the growth rate of *Chlorella* sp. when exposed to dissolved zinc as a function of dissolved organic carbon (DOC) concentration for Manton Dam DOC (upper panel) and Appletree Creek DOC (lower panel) at a fixed pH of 7.6. Error bars indicate the calculated lower and upper 95% confidence intervals. Note variable y-axis scales. * Indicates significant difference ($p < 0.05$) from the no added DOC treatment (<1 mg.L^{-1}).

Table 4.1: The 72-h effect concentrations (EC10/EC50) for growth rate inhibition of *Chlorella* sp. exposed to zinc under different DOC 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 Table C-7.

DOC Source	**DOC (mg C.L^{-1})**	**pH**	**Mean control growth rate (doublings/day)**	**Dissolved (µg.L^{-1})**	
				EC10	**EC50**
Control (no added DOC, 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 DOC, unbuffered)	<1	7.5–8.2[a]	1.88	1.3 (0.61–2.1)	48 (39–57)
Suwannee River Reference DOC	2.5	7.5–8.2[a]	2.10	6.8 (5.3–8.2)	72 (64–81)
	4.4	7.5–8.2[a]	2.10	15 (12–19)	80 (72–89)
	8.7	7.5–8.2[a]	2.14	20 (14–26)	127 (107–146)

[a] pH range of unbuffered tests represents the start and end pH values.

4.3.3 Influence of pH on zinc toxicity in the presence of DOC

The influence of pH on zinc toxicity in the presence of DOC was dependent on the DOC source. In the presence of Appletree Creek DOC, changes in pH did not influence either the EC10 or EC50 values (Table 4.1, Figure 4.3). Toxicity increased at the EC50 level relative to no added DOC 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 (Figures 4.1 and 4.4) from 100 to 1,000 µg $Zn.L^{-1}$; 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 DOC (as hypothesised in section 4.3.2) is unaffected by proton competition at the algal binding site. This is in contrast to results previously discussed in Chapter 2 (Price et al., 2021).

In the presence of Manton Dam DOC, 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 (Figure 4.3). 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 DOC (Price et al., 2021). Given, the general minimal influence of Manton Dam DOC concentration on zinc toxicity (Figure 4.2), the decreased EC50 value may be solely a product of changed pH with little interaction from the added DOC. 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 DOC, proton competition is likely to occur at both algal cell binding sites and at the DOC binding ligand functional groups. As such, pH may not influence zinc toxicity in the presence of DOC in the same manner as it would in the absence of DOC. 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 (Figure 4.4).

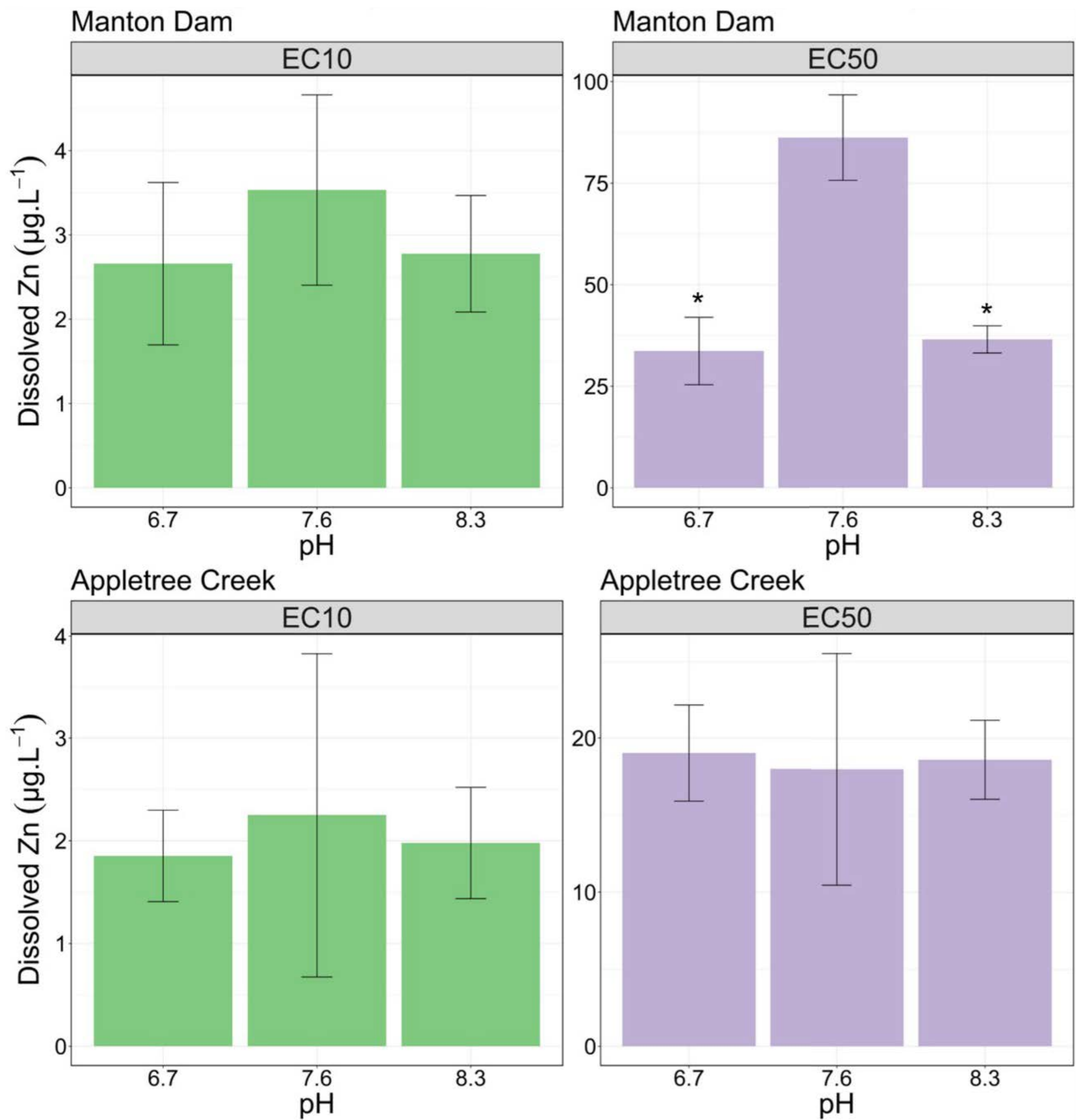

Figure 4.3: Comparison of EC10 and EC50 values for zinc toxicity on the growth rate of *Chlorella* sp. when exposed to dissolved zinc as a function of pH in the presence of 5.5 mg $C.L^{-1}$ Manton Dam DOC (upper panel) and 4.9 mg $C.L^{-1}$ Appletree Creek DOC (lower panel). Error bars indicate the calculated lower and upper 95% confidence intervals. Note variable y-axis scales. * Indicates significant difference ($p < 0.05$) from the pH 7.5 treatment.

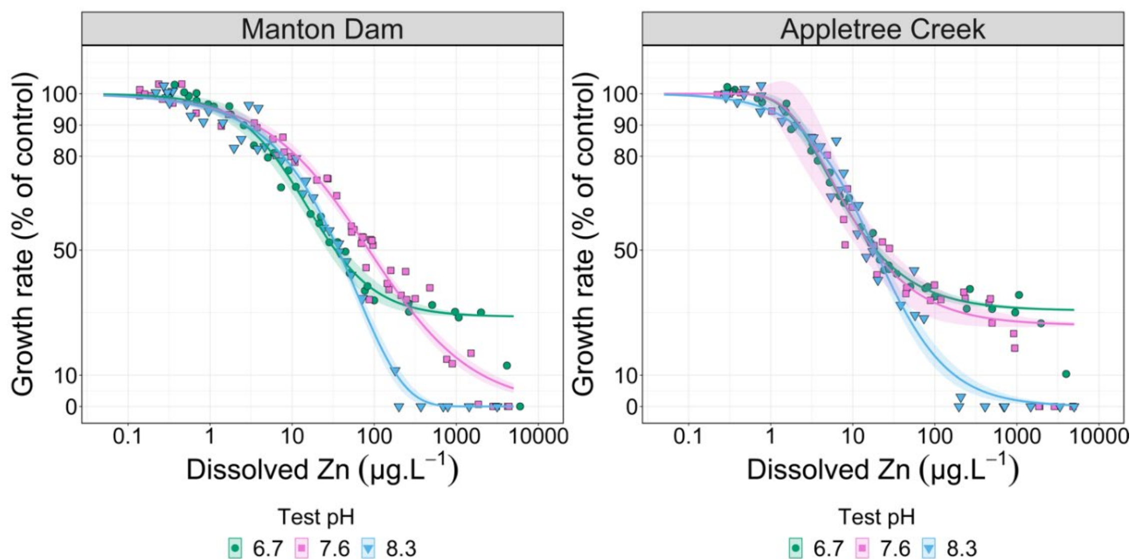

Figure 4.4: Concentration-response curves indicating the effect of three pH values (6.7, 7.6 and 8.3) on the growth rate of *Chlorella* sp. when exposed to dissolved zinc in the presence of 5.5 mg.L^{-1} of Manton Dam DOC (LHS panel) and 4.9 mg.L^{-1} Appletree Creek DOC (RHS panel). Shaded ribbons represent 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.

4.3.4 Zinc lability in freshwaters in the presence of organic carbon

DGT-labile zinc concentrations were measured in four to five zinc treatments (0 – 400 µg.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 DOC sources and at each DOC concentration (Figure 4.5). A summary of all DGT-labile and ultrafiltered zinc concentrations is provided in Table C-8. Concentrations provided in Table C-8 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 DOC-bound zinc and free ion zinc (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 Zn^{2+} and an increase in DOC-bound zinc with increasing concentrations of DOC for both Manton Dam and Appletree Creek. WHAM input and output data is provided in Table C-9 – C-12.

Previous work with Manton Dam DOC and copper (Macoustra et al., 2019) found that DOC 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 DOC concentration increased from an average of 78% of measured dissolved zinc being DGT-labile at

2.5 mg $C.L^{-1}$ down to 67% at 15.1 mg $C.L^{-1}$; however, this difference was not significant ($p = 0.195$) (Table C-8). Similarly, small decreases in ultrafiltered zinc as a proportion of measured dissolved zinc occurred as Manton Dam DOC concentration increased. Percentages of ultrafiltered zinc as a proportion of measured dissolved zinc decreased from 101% to 92% across the DOC concentration range of 2.5 to 15.1 mg $C.L^{-1}$ but again, this difference was not significant ($p = 0.304$) (Table C-8). The limited influence of Manton Dam DOC on the labile and ultrafiltered zinc proportions agrees with the general limited effect on EC50 values across the tested DOC 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 DOC concentration was increased from 2.5 to 15.1 mg $C.L^{-1}$. At the EC10 concentration a decrease in Zn^{2+} from 0.4 to 0.2 µg $Zn^{2+}.L^{-1}$ (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 µg $Zn^{2+}.L^{-1}$ (a change from 35 to 9% of dissolved zinc) was found. Conversely, DOC-bound zinc (as calculated by WHAM, Table C-10) at the EC10 and EC50 concentrations increased as DOC concentration was increased from 2.5 to 15.1 mg $C.L^{-1}$. At the EC10 concentration an increase in DOC-bound zinc from 1.4 to 5.7 $µg.L^{-1}$ (a change from 69 to 94% of dissolved zinc) was found, whereas at the EC50 concentration DOC-bound zinc increased from 27.4 to 105 $µg.L^{-1}$ (39 to 83% of dissolved zinc).

These decreases in Zn^{2+} concentrations and increases in DOC-bound zinc are consistent with the slight decreases in DGT-labile zinc concentrations. This is consistent with the premise that the presence of DOC-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 DOC-bound zinc to DGT-labile zinc suggests that at least a portion of DOC-bound zinc is DGT-labile. This is particularly evident at higher DOC concentrations. For example, at 15.1 mg $C.L^{-1}$ of Manton Dam DOC, 67% of dissolved zinc was DGT-labile, while speciation modelling suggests that 83 to 94% of zinc should be DOC-bound across the EC10 to EC50 range.

In the presence of Appletree Creek DOC, DGT-labile zinc concentrations decreased with increasing DOC concentration (Figure 4.5 and Table C-8). These results agree with those of other studies using Appletree Creek DOC with copper and nickel, where the DGT-lability of both metals decreased in the presence of increasing DOC concentration (Macoustra et al., 2019; 2021a). 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 DOC (Figure 4.2), with DGT-labile zinc as a proportion of measured dissolved zinc decreasing with increasing Appletree Creek DOC (87% DGT-labile zinc at 2.0 mg $C.L^{-1}$ to 58% DGT-labile zinc at 13.0 mg $C.L^{-1}$).

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 µg $Zn^{2+}.L^{-1}$ (a change from 20 to 3% of dissolved zinc) was found with increasing DOC. At the EC50 concentration a decrease in Zn^{2+} from 5.2 to 1.5 µg $Zn^{2+}.L^{-1}$ (a change from 31 to 5% of dissolved zinc) was found with increasing DOC. DOC-bound zinc at the EC10 concentration increased from 1.2 to 3.2 µg.L^{-1} (a change from 64 to 94% of dissolved zinc) and increased at the EC50 concentration from 7.8 to 22.3 µg.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 DOC-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 DOC may be occurring via a mechanism (such as increased uptake/ bioavailability of Zn-DOC complexes) that is not directly related to changes in speciation in solution as measured by DGT or speciation modelling, as discussed previously in section 4.3.2.

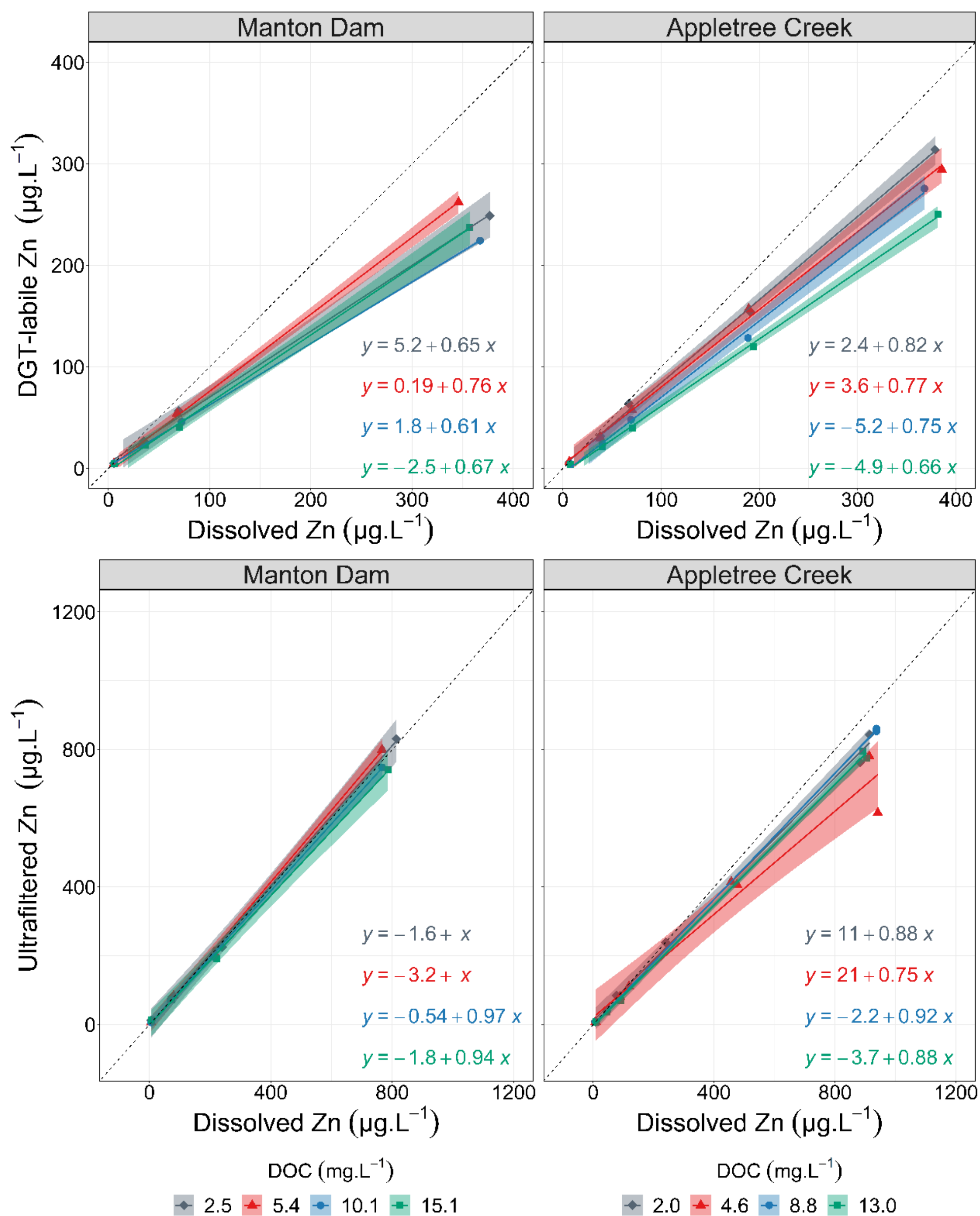

Figure 4.5: Upper panels show the comparison of DGT-labile zinc to dissolved zinc concentrations in the presence of increasing concentrations of Manton Dam DOC (LHS panel) and Appletree Creek DOC (RHS panel). Lower panels show the comparison of ultrafiltered (<3kDa) zinc concentrations to dissolved zinc concentrations in the presence of increasing concentrations of Manton Dam DOC (LHS panel) and Appletree Creek DOC (RHS panel). Shaded ribbons represent the 95% confidence intervals. Dashed line represents the 1:1 relationship.

4.4 CONCLUSIONS

DOC source was important in determining the influence of DOC concentration and pH on zinc toxicity to *Chlorella* sp. In the presence of DOC 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 DOC could not be explained by changes in zinc speciation as determined by WHAM, DGT-labile zinc or ultrafiltration measurements across the DOC concentration range. Increased toxicity may be due to the formation of Zn-DOC 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 DOC on chronic zinc toxicity to a freshwater microalga and provides high quality data useful for incorporating DOC source and concentration into bioavailability-based water quality guidelines.

Chapter 5: Assessing the relevance of the factor-of-2 validation method

Bioavailability-based toxicity models for metals often have their performance assessed by whether they can predict toxicity data within a factor of 2 of their paired observed toxicity data. This method was developed and verified using only median effect values (EC50) for acute fish and daphnia data, however, toxicity models have been developed for a much broader range of effect levels and species. This chapter collated and analysed a large toxicity dataset from repeated tests under the same laboratory conditions to assess the relevance of the factor-of-2 validation method across a range of species, contaminants, and endpoints. The work presented in this chapter has been published in the below cited publication.

Highlights

- The factor-of-2 method was broadly applicable for metal toxicity to a range of species for EC50 data.
- EC10 datasets highlighted larger variability at low effect levels, suggesting the need for a factor-of-3 rule.
- Methods of model validation will depend on the application of the toxicity model.

Price, G. A. V., Stauber, J. L., Stone, S., Koppel, D. J., Holland, A., & Jolley, D. (2022). Does toxicity test variability support bioavailability model predictions being within a factor of 2? Environmental Chemistry, 19(4), 177–182. https://doi.org/10.1071/EN22050

I conceptualised and designed the study and completed all data collation, statistical analyses, data visualisation and interpretation. I prepared the manuscript for publication. All authors contributed to editing of the manuscript before submission.

5.1 INTRODUCTION

In the last several decades there has been increased development, use, and interest in incorporating metal bioavailability models into regulatory water quality guidelines/criteria (Brix et al., 2020). Simple univariate regression models, such as the hardness-adjustment algorithm (USEPA, 1985) have been used in water quality guidelines since the late 1980s. However, over the following decades more complex models, such as the biotic ligand model (BLM) (Di Toro et al., 2001) and multiple linear regression (MLR) models (Brix et al., 2017), which incorporate multiple water chemistry parameters, have been developed to better predict metal toxicity to aquatic organisms.

Recently, bioavailability models have been developed using low effect levels (e.g., data based on effect concentrations that cause a 10% (EC10) and/or 20% (EC20) effect) based on chronic toxicity data. These inherently have higher uncertainty than models based on higher effect levels, such as EC50 values, as there is typically greater uncertainty at the EC10 and EC20 values in a concentration-response model.

The increase in complexity, type and use of these models has resulted in a need for validation methods to test a model's predictive capacity. Garman et al. (2020) outlined several methods of model performance evaluation, including regression slope bias analysis and whether model predictions are within a factor of 2 (i.e., range of 4) of the observed toxicity estimate (e.g., EC50). Use of the factor-of-2 rule was first proposed by Di Toro et al. (2001) and Santore et al. (2001) and was based on a single dataset using a 96-h acute lethality test on larval fathead minnows (*Pimephales promelas*) exposed to copper (Erickson et al., 1996).

Several recent papers have called for the need for further assessment of the among-test variability for tests conducted under the same conditions in order to determine if the factor-of-2 rule is widely applicable (Garman et al., 2020; Peters et al., 2021). This rule has been examined by two other studies. Santore and Ryan (2015) assessed variation in *Daphnia magna* zinc acute lethality tests, while Meyer et al. (2018) examined a larger toxicity dataset where *D. magna* neonates were exposed separately to cadmium, copper, nickel or zinc. Additionally, Meyer et al. (2018) reanalysed *P. promelas* data from Erickson et al. (1996). Both studies found that the factor-of-2 rule was generally applicable across the two species.

No study has yet investigated the suitability of the factor-of-2 rule for microalga, despite several bioavailability models being recently developed (Croteau et al., 2021; DeForest et al., 2018; Peters et al., 2021) nor for low effect levels or chronic toxicity data for any organism. Peters et al. (2018) suggested that a factor-of-3 rule (i.e., a range of 9) may be more appropriate for low effect level and chronic data given the inherent increased uncertainty associated with these. Assessing the suitability of validation techniques like the factor-of-2 rule for these types of

toxicity data is important as they are often preferred over acute EC50 data for water quality guideline development (Batley et al., 2018).

In this study, we report an analysis of the appropriateness of the factor-of-2 rule and the proposed factor-of-3 rule using an extensive collection of repeated toxicity datasets including freshwater and marine invertebrates and microalgae. Acute and chronic data across a range of endpoints at low and high effect levels were assessed. The results of these analyses serve as an important reference point for developing and evaluating bioavailability model performance.

5.2 METHODS

5.2.1 Data sources

Toxicity estimate data were taken from a previously unpublished internal quality control reference toxicant database comprised of standardised tests used to assess test repeatability and organism culture performance over time (Price et al., 2022b). Test species consist of both freshwater and marine organisms. Additional data was sourced from published reference toxicant data in Stone et al. (2022) and Meyer et al. (2018).

Metal toxicity datasets were defined as having the same endpoint, test duration, test vessel, initial organism density (e.g., cell density for microalgae) and test water (laboratory prepared waters with the same chemical characteristics). A minimum of 5 datapoints (i.e., ECx values) were needed per dataset and only tests with measured concentrations were included. In total 29 datasets representing 12 species (including microalgae, invertebrates, and fish), 547 toxicity tests, 3 contaminants (copper ($n = 21$), nickel ($n = 6$) and zinc ($n = 2$)), acute ($n = 11$ datasets) and chronic ($n = 18$) endpoints, and EC10 ($n = 7$ datasets) and EC50 ($n = 22$ datasets) data were collated.

5.2.2 Calculations and statistics

All statistical analyses were performed using the R statistical software (v4.3.0; R Core Team, 2023) with figures produced using the extension packages *ggplot2* (Wickham, 2016) and *ggpubr* (Kassambara, 2020).

All data was tabulated into datasets to calculate means, standard deviations, and percentiles. Upper-lower prediction ratios (ULPRs) were used to assess toxicity dataset variability and were calculated as per Meyer et al. (2018) (Equation 5.1 and 5.2). Both 90%- and 95%-ULPRs were calculated for each dataset using untransformed and $\log_{10}$-transformed data. For the log-transformations all toxicity estimates within a dataset were transformed prior to calculations as per Meyer et al. (2018). Percentiles were calculated as shown in Equation 5.3, using the mean

toxicity estimate within a dataset, the z-score of normal distribution (Z) and the standard deviation (σ) of the dataset.

$$95\% - ULPR = \frac{97.5^{th}\ percentile}{2.5^{th}\ percentile} \quad (5.1)$$

$$90\% - ULPR = \frac{95^{th}\ percentile}{5^{th}\ percentile} \quad (5.2)$$

$$Percentile = mean\ \pm (Z \times \sigma) \quad (5.3)$$

Untransformed and transformed data were tested for normality using the Shapiro-Wilk test. ULPRs were compared to a range of 4 and 9, which are the ranges of deviation from observed toxicity values that the factor-of-2 and factor-of-3 rules suggest is a satisfactory fit for bioavailability-based toxicity models.

Several of the lower percentile calculations (i.e., 2.5^{th} and 5th) for the untransformed data were less than 0 and therefore ULPRs could not be calculated. Additionally, several untransformed datasets were not normally distributed ($p<0.05$). Therefore, for consistency log-transformed results were discussed in the present study as all log-transformed results were normally distributed (except *Ceriodaphnia dubia* exposed to copper) and the transformation negates the issues of negative values at lower percentile calculations.

Applying a 95% prediction limit to assess toxicity variability may be unrealistic when considering bioavailability-based toxicity models developed with data pooled from numerous studies, laboratories and timepoints. This is often the case with most models in the literature predicting far less than 95% of data within a factor of 2 (Besser et al., 2021; Brix et al., 2021; Santore et al., 2021), with Peters et al. (2021) suggesting a model be deemed acceptable if 50% of data lies within a factor of 2 and 90% within a factor of 3 for lower effect levels. Based on this, the present study will discuss the results in terms of a 90%-ULPR and results for 95%-ULPRs are provided in Table D-1 to serve as a direct comparison to other studies.

Additionally, median, geomeans, coefficient of variation (CV) and maximum/minimum ratios (MMR) were calculated with all results provide in Table D-1. Significant differences between ULPRs were tested using the non-parametric unpaired Wilcoxon test following normality testing with the Shapiro-Wilk test. Standard deviation (SD) was used to specify variability (i.e., ± 1SD) and ULPRs are expressed as median (interquartile range) throughout this chapter.

5.3 RESULTS AND DISCUSSION

Across all datasets the range of 90%- and 95%-ULPRs were 1.6 – 12.7 (median: 3.2 (2.8 – 4.4)) and 1.7 – 20.6 (median: 3.9 (3.1 – 5.8)), respectively. All calculations and results for both untransformed and log-transformed data are provided in Table D-1.

5.3.1 EC10 versus EC50

In general, the ULPRs for the EC10 data in the present study do not support the factor-of-2 rule but do support a factor-of-3 rule. The ULPRs for the EC50 data support the factor-of-2 and agree with the findings of Meyer et al. (2018).

There were seven datasets based on EC10 values, which is much less than the 22 datasets based on EC50 values reflecting that EC50 values are more common acceptability criteria in reference toxicant testing (see examples in Chapter 3 (Price et al., 2022a) and Stone et al. (2022)). The seven available EC10 datasets were comprised of 92 toxicity tests across four species (two invertebrates and two microalgae) and two contaminants (copper and nickel) with both acute and chronic data. The median 90%-ULPR for all EC10 datasets was 5.6 (3.5 – 7.1) (Figure 5.1). Of the seven datasets, only two had 90%-ULPRs <4 (complying with the factor-of-2 rule); however, six of the seven datasets had 90%-ULPRs <9 complying with the factor-of-3 rule as suggested by Peters et al. (2018). The one dataset that fell outside the factor-of-3 rule had a 90%-ULPR of 12.7 for *Chlorella* sp. copper EC10 data. Meyer et al. (2018) reported similar increases in variability for *Daphnia magna* cadmium EC50 data, which was explained by age-related differences in cadmium sensitivity to *D. magna* neonates. However, this is unlikely the case for *Chlorella* sp. with this greater variability likely to reflect *Chlorella* sp. being highly sensitive to copper with a median EC10 value of 0.5 μg $Cu.L^{-1}$, which is close to the instrument detection limits (inductively coupled plasma – atomic emission spectroscopy (ICP-AES)).

For the EC50 data, 22 datasets comprising 455 toxicity tests across 12 species (invertebrates, microalgae and fish) and three contaminants (copper, nickel and zinc) were available. In comparison to the EC10 ULPRs, the EC50 ULPRs were lower and less variable (Figure 5.1), with the median 90%-ULPR for all EC50 datasets being 3.2 (2.8 – 4.1) (Table 5.1). Of the 22 datasets, 18 datasets had 90%-ULPRs <4 and all 90%-ULPRs were <9. The ULPRs for the EC50 data in this study support the factor-of-2 rule and agree with the findings of Meyer et al. (2018).

Several contaminant- and species-matched datasets were available, which allowed for the comparison of EC10 and EC50 variability from the same tests (i.e., EC10 and EC50 values derived from the same concentration-response curve), rather than across all datasets. One matched dataset was available for a freshwater microalga, *Chlorella* sp., exposed to copper and three

matched datasets were available for the marine copepod *Acartia sinjiensis*, exposed to copper (both acute and chronic) and nickel.

When comparing the *Chlorella* sp. copper matched EC10 and EC50 ULPRs, the EC10 90%-ULPR was much larger, at 12.7, while the EC50 90%-ULPR was 4.9. Larger EC10 ULPRs compared to the matched EC50 ULPRs were also found in the *A. sinjiensis* data (Table 5.1). These matched comparisons provide further evidence to support comments of Peters et al. (2018) about larger EC10 variability, especially given these matched EC10 and EC50 values are estimates from the same concentration-response models.

Table 5.1: The 90% upper-lower prediction ratios for all EC10 and EC50 datasets and for the contaminant- and species-matched datasets. n = total datapoints in dataset.

Dataset	**n**	**90%-ULPR**	
		Based on EC10	**Based on EC50**
All data	93/455[a]	5.6 (3.5 – 7.1)[b]	3.2 (2.8 – 4.1)[b]
Chlorella sp. (copper)	11	13	4.9
Acartia sinjiensis (copper – acute)	37	5.7	2.7
Acartia sinjiensis (copper – chronic)	5	8.4	3.4
Acartia sinjiensis (nickel – chronic)	9	2.8	1.4

[a] EC10 datapoints/EC50 datapoints
[b] median (IQR)

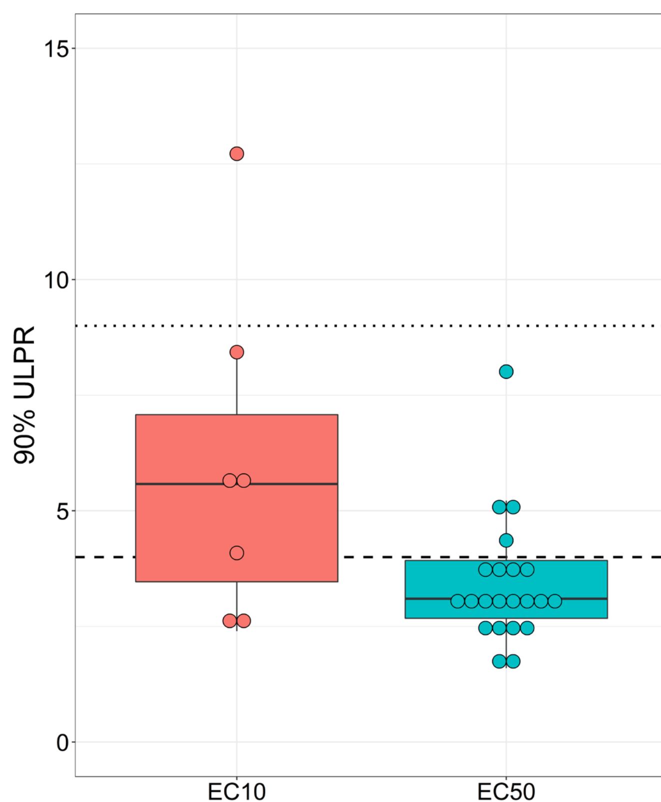

Figure 5.1: Boxplots showing the 90% upper-lower prediction ratios (ULPR) for all EC10 and EC50 datasets. Boxplots span the interquartile range, with median shown, whiskers are 1.5*IQR. Dots show ULPRs for individual datasets. Dashed line indicates the factor-of-2 rule threshold of 4 and the dotted line indicates the factor-of-3 threshold of 9.

5.3.2 Chronic versus acute

Comparing chronic datasets to acute datasets at both the EC10 and EC50 level did not result in any differences between ULPRs (Figure 5.2). When considering all datasets, the 90%-ULPR for EC50 values was not significantly different between chronic and acute datasets ($p = 0.08$). The overall median 90%-ULPR for the acute and chronic EC50 values was 2.8 (2.4 – 3.1), and 3.3 (3.0 – 4.0), respectively. For the EC10 values, there were limited datasets available for acute toxicity ($n = 2$); however, ULPRs were similar for both the acute and chronic datasets, as shown in Figure 5.2.

Only several datasets ($n = 3$) were available to compare contaminant- and species-matched acute and chronic ULPRs, with data available for the marine copepod *A. sinjiensis* and the marine urchin *Heliocidaris tuberculata*. *A. sinjiensis* acute and chronic copper EC50 ULPRs were similar, with acute and chronic EC50 90%-ULPRs of 2.9 and 3.3, respectively. Matched acute and chronic EC50 ULPRs for *H. tuberculata* had similarly small differences between the two datasets, with acute and chronic EC50 90%-ULPRs of 2.8 and 4.0, respectively. The acute and chronic copper EC10 ULPRs for *A. sinjiensis* had a larger difference compared to the EC50 ranges above, with acute and chronic EC10 90%-ULPRs of 5.7 and 8.4, respectively.

The comparisons with all datasets and the EC50 contaminant- and species-matched datasets suggest that acute and chronic test variability are similar. In addition, the median values for the acute and chronic 90%-ULPRs were both <4, broadly suggesting that the data, regardless of whether it is chronic or acute, supports the factor-of-2 rule. The EC10 contaminant- and species-matched datasets suggest differences may be present between acute and chronic EC10 data, as the chronic ULPR range is much larger than the acute range. However, this is likely related to the greater variability in EC10 data (discussed earlier), rather than specific differences between acute and chronic data. Furthermore, this variability is based on a single species and contaminant. More data would be useful to assess the differences in variability at the EC10 level for matched acute and chronic data.

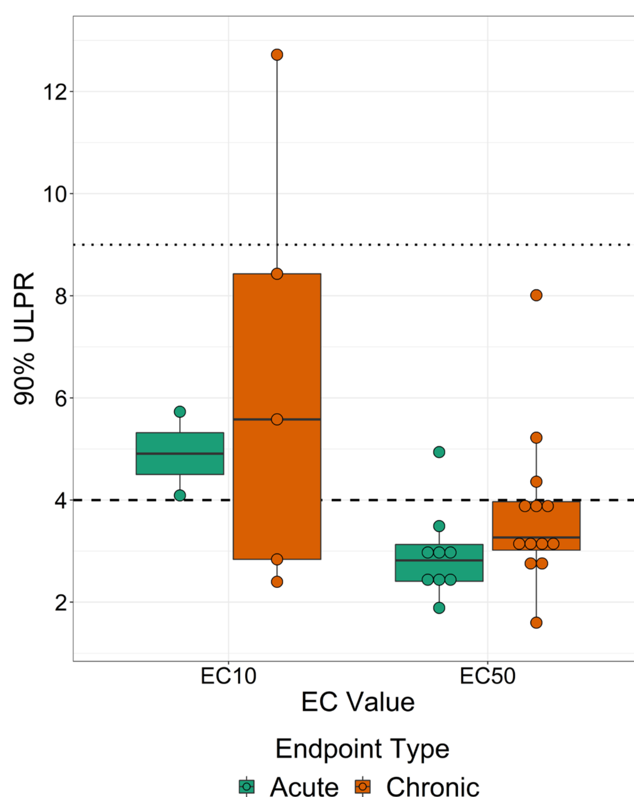

Figure 5.2: Boxplots comparing the acute and chronic 90% upper-lower prediction ratios (ULPR) for all EC10 and EC50 datasets. Boxplots span the interquartile range, with median shown, whiskers indicate the 1.5*IQR. Dots show ULPRs for individual datasets. Dashed line indicates the factor-of-2 rule threshold of 4 and the dotted line indicates the factor-of-3 threshold of 9.

5.3.3 Microalgae

Of the 10 EC50 microalgal datasets, 5 were freshwater species and 5 were marine species. The median EC50 90%-ULPRs for the freshwater and marine species were 4.0 (3.8 – 4.4) and 2.9 (2.7 – 3.1), respectively. This was not significantly different ($p = 0.095$), but the marine species did generally appear to have lower ULPRs (Figure D-1). As freshwater microalgal species are the current focus of algae bioavailability modelling the discussion will focus on these results (Croteau

et al., 2021; DeForest et al., 2020). All data analysis and results for the marine species are provided in Table D-1, and the results were similar to the freshwater species.

The 90%-ULPR for freshwater microalgal EC50 data ranged from 3.2 to 8.0, with a median value of 4.0 (3.8 – 4.4). Based on the median, the factor-of-2 rule may be suitable, with 3 of the 5 datasets with ULPRs <4; however, a factor-of-3 rule appears more applicable with all ULPRs <9.

Comparing the freshwater microalgae and non-algal species ULPRs shows a small, yet significant difference (p = 0.027), with non-algal species having a slightly lower median 90%-ULPR of 2.9 (2.4 – 3.3) compared to 4.0 (3.8 – 4.4) for microalgae (Figure 5.3). When comparing the microalgae ULPRs to other commonly used taxa for bioavailability modelling, such as daphnids and fish, the ULPRs for microalgae were similar. The only daphnid dataset in the present study was for *C. dubia* exposed to copper, which had a 90%-ULPR of 3.1. The *D. magna* data used in Meyer et al. (2018) had a median 90%-ULPR of 2.4 and *P. promelas* had a median 90%-ULPR of 3.5. In general, the microalgal test variability does not appear to be much larger than non-algal species.

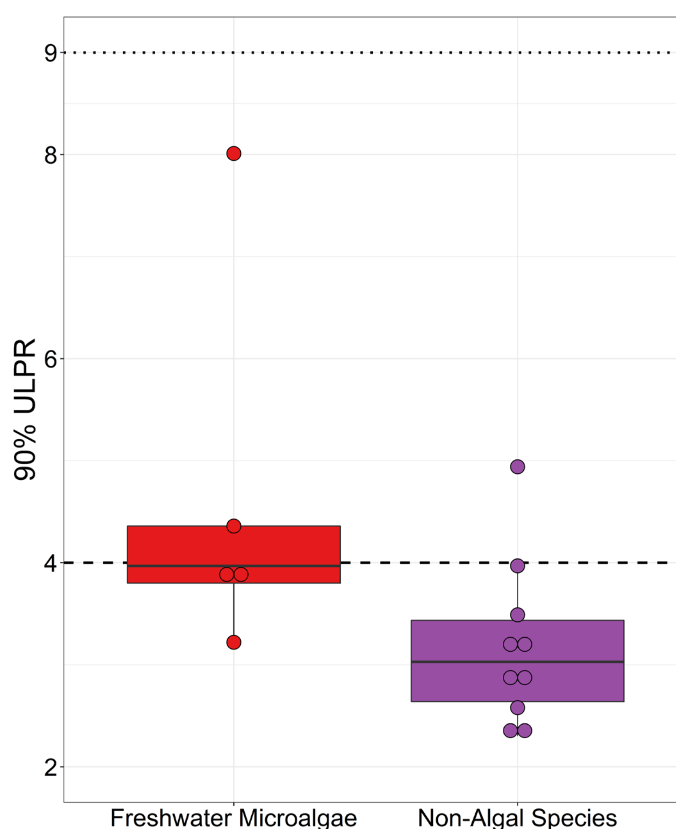

Figure 5.3: Boxplots comparing the 90% upper-lower prediction ratios (ULPR) for all freshwater microalgae and non-algae EC50 datasets. Boxplots span the interquartile range, with median shown, whiskers indicate the 1.5*IQR. Dots show ULPRs for individual datasets. Dashed line indicates the factor-of-2 rule threshold of 4 and the dotted line indicates the factor-of-3 threshold of 9.

5.4 RECOMMENDATIONS

Reference toxicant data is routinely generated during contaminant toxicity studies; however, the data are rarely published. Publication of such data would enable expansion of the present study to other organisms, contaminants and effect levels allowing for further assessment of validation techniques for bioavailability modelling. This would also allow for inter-laboratory comparisons which is important given most bioavailability models are developed using data from numerous sources. However, care is needed when making such comparisons as different laboratories do not necessarily use the same culture and/or testing media.

5.5 CONCLUSIONS

The data in the present study indicated that the factor-of-2 rule is broadly applicable for metal toxicity to a range of species for EC50 data, generally agreeing with the previous analysis by Meyer et al. (2018). The EC10 data highlighted that larger variability exists in low effect levels and supported the use of the factor-of-3 rule as recommended by Peters et al. (2018). Overall, either the factor-of-2 or factor-of-3 rule could be applied to microalgal data and the rule chosen for model performance evaluation may depend on the application of the bioavailability model. Given that most bioavailability models are developed using data from numerous sources, future assessments of inter-laboratory variability for matched tests (i.e., the same species and conditions) would be valuable. However, this may be difficult as differences in sensitivities can arise from small changes in water chemistry between laboratories and strains of the same species.

Chapter 6: Development and validation of zinc toxicity prediction models

This chapter developed and validated multiple linear regression models for predicting chronic zinc toxicity to *Chlorella* sp. using three toxicity modifying factors: pH, hardness, and dissolved organic carbon concentration. Models were developed at three different effect concentration levels: EC10, EC20, and EC50. Models were independently validated using six different zinc-spiked Australian natural waters with a range of water chemistries. The work presented in this chapter has been published in the below cited publication.

Highlights

- Hardness was an influential toxicity modifying factor in all models developed.
- Autovalidation and residual analysis of all models indicated good predictability with little bias based on individual parameters.
- Models performed poorly when predicting toxicity in natural waters, with models consistently overpredicting toxicity.

Price, G. A. V., Stauber, J. L., Jolley, D. F., Koppel, D. J., Van Genderen, E. J., Ryan, A. C., & Holland, A. (2023). Development and Validation of Multiple Linear Regression Models for Predicting Chronic Zinc Toxicity to Freshwater Microalgae. Environmental Toxicology and Chemistry. https://doi.org/10.1002/etc.5749

I planned and coordinated all field work to collect test samples, with field assistance from Anthony Evans, Jenny Stauber, Darren Koppel, and Aleicia Holland. I conducted all statistical analysis, model development and validation, and conducted all natural water toxicity testing. I completed all chemical analysis, with Aleicia Holland completing the DOC analysis. I prepared the manuscript for publication. All authors contributed to study conceptualisation and editing of the manuscript before submission.

6.1 INTRODUCTION

Zinc toxicity to aquatic organisms is dependent on its bioavailability which is influenced by water chemistry parameters, such as pH, hardness and dissolved organic carbon (DOC). For example, pH determines zinc speciation, and protons (H^+) and hardness ions (Ca^{2+} and Mg^{2+}) compete with zinc for biological uptake at the biotic ligand, while DOC complexes zinc thereby altering its bioavailability (Adams et al., 2020).

Currently the Australian and New Zealand water quality guidelines for zinc only account for the influence of hardness on bioavailability via a hardness algorithm (Australian and New Zealand Governments, 2018b). This algorithm is largely based on acute toxicity data derived from North American fish species and has recently been shown to not be appropriate for freshwater microalgae (Price et al. 2022a). Increased attention towards bioavailability-based water quality guidelines has occurred with the development of the biotic ligand model (BLM), which predicts the toxicity of a metal to a species based on water chemistry parameters. The model predicts the amount of accumulation that occurs at the biotic ligand by accounting for changes in metal speciation and the presence of competitive effects from other ions in solution (Di Toro et al., 2001). More recently, there has been interest in the development of simpler empirical bioavailability models, such as multiple linear regression (MLR) models as they can be easier to use. Brix et al. (2017) suggested that there may be perceptions amongst regulators that BLM approaches are too complicated and not sufficiently transparent.

To date, several studies have developed MLR models for predicting bioavailability-based toxicity of metals to freshwater organisms (Brix et al., 2017, 2021, 2023; Croteau et al., 2021; DeForest et al., 2018, 2023; Peters et al., 2021). From the current literature two approaches to data sourcing have been used. Several studies have aggregated large datasets from multiple sources and laboratories (Brix et al. 2017, 2021; Croteau et al. 2021; Peters et al. 2021; DeForest et al. 2023). Others have used data from a single study or laboratory and therefore all toxicity data for a particular species is derived from the same culture and tested under the same conditions using synthetic laboratory test waters (Brix et al., 2023; DeForest et al., 2018). Both approaches have merit. Utilising large, aggregated datasets provides more statistical power in model development and often does not require any additional laboratory work. However, pooling data from different sources also reduces confidence in the comparability of data between studies and introduces issues of co-linearity as studies often varied multiple toxicity modifying factors (TMF) simultaneously. Additionally, pooling studies can also result in an imbalance in the representation of different TMF effects. For example, generally fewer DOC studies are available compared to hardness studies. Relying on data from a single study typically means smaller datasets for model development but often greater confidence in the consistency of data given it was collected under

the same testing conditions and from the one culture. However, of the two MLR models developed using single datasets, neither has been independently validated using natural waters and the MLR models have only previously been developed for one microalga *R. subcapitata* (Brix et al., 2023; DeForest et al., 2018).

This study is the first to develop a zinc bioavailability MLR model for a species other than *R. subcapitata* and the first to be independently validated with natural waters. The objective of this study was to use zinc toxicity data under varying pH, hardness and DOC conditions for the microalga *Chlorella* sp. presented in Chapter 2 – 4 (Price et al. 2021, 2022a, 2023b) to develop empirical models that predict zinc toxicity as a function of these parameters. Models were independently validated using six different zinc-spiked Australian natural waters with a range of water chemistries. Previously developed zinc MLR models for *R. subcapitata* were also validated using the natural waters toxicity data to assess the suitability of these models under Australian water chemistry conditions. These data, together with those presented in the companion paper by Stauber et al. (2023), will be used to develop bioavailability-based zinc water quality guidelines for Australia and New Zealand (ANZG, 2018).

6.2 METHODS

6.2.1 Model development

Data sources

The chronic toxicity of zinc has been tested over a wide range of water chemistries for the freshwater microalga, *Chlorella* sp. These data were sourced from Chapters 2, 3, and 4 (Price et al. 2021, 2022a, 2023b), with each source providing a detailed description of the tests carried out. All tests were conducted using laboratory prepared synthetic freshwaters with pH levels ranging from 6.7 to 8.3, hardness concentrations from 5 to 402 mg $CaCO_3.L^{-1}$ and DOC concentrations of 0 to 15 mg $C.L^{-1}$. Tests were performed at a constant alkalinity (~40 $mg.L^{-1}$) and calcium to magnesium ratio (~0.7) as described in Chapter 3 (Price et al. 2022a). Toxicity data used met the acceptability criteria outlined by Warne et al. (2018b) for use in development of Australian water quality guidelines. All zinc effect concentrations used in this study are expressed as dissolved zinc (measured as <0.45 µm) (Table E-1).

MLR analysis

Multiple linear regression (MLR) analysis was conducted on the chronic toxicity data for *Chlorella* sp. Hardness, pH, DOC, and interactions between these parameters were previously identified as important Toxicity Modifying Factors (TMFs) for zinc and microalgae (CCME, 2018). Multiple linear regression models were developed for the 10, 20 and 50% effect

concentrations (EC10, EC20 and EC50 values) following the methods described by Brix et al. (2017) and DeForest et al. (2018, 2020). All MLR analyses were conducted using the R Statistical Software (v4.3.0; R Core Team 2023). Initial stepwise MLR analyses included independent variables of pH, ln(hardness), and ln(DOC) and ln(EC10) or ln(EC20) or ln(EC50) were the dependent variables. Models with no interaction terms had the generalised form shown in Equation 6.1:

$$\ln(toxicity) = b_0 + [b_1 \times pH] + [b_2 \times \ln(hard)] + [b_3 \times \ln(DOC)] \tag{6.1}$$

where *toxicity* is an effect level (i.e., EC10, EC20 or EC50) in µg $Zn.L^{-1}$; b_0 is the y-intercept; b_1, b_2 and b_3 are the slope parameters for pH, ln(hard) and ln(DOC), respectively, with units of hardness and DOC in $mg.L^{-1}$.

Stepwise MLR analysis was also conducted with interaction terms included as independent variables, in addition to the variables included in Equation 6.1. These interaction models have a generalised form as shown in Equation 6.2. It is noted that only two interaction terms were included (pH x ln(hard) and pH x ln(DOC)), as none of the data sources assessed the influence of DOC on zinc toxicity at varying hardness, so a ln(DOC) x ln(hard) term could not be included.

$$\begin{aligned}\ln(toxicity) = {} & b_0 + [b_1 \times pH] + [b_2 \times \ln(hard)] + [b_3 \times \ln(DOC)] \\ & + [b_4 \times pH \times \ln(hard)] + [b_5 \times pH \times \ln(DOC)]\end{aligned} \tag{6.2}$$

Both the Akaike information criterion (AIC) and Bayesian information criterion (BIC) were used in stepwise MLR analyses to determine which terms to include in the model to create the most parsimonious model at each effect level. Both criteria achieve parsimony and balance specificity and generality through penalising the goodness-of-fit of a model in relation to the number of parameters present in the model. The BIC also accounts for the sample size used in the analysis and is described in further detail by DeForest et al. (2018).

Variance inflation factors (VIF) were calculated for all independent variables to assess collinearity. Variance inflation factors are a quotient that measures the amount of variance in an estimated regression coefficient that is changed by collinearity between parameters used in the model. A low VIF means low collinearity, with VIFs <3 considered acceptable (Zuur et al., 2010). In controlled experiments using laboratory prepared media, each independent variable can be carefully controlled and therefore correlation amongst independent variables is unlikely. In contrast, studies that use field samples may see higher levels of correlation as these independent variables cannot be as easily controlled.

Stepwise regressions were run using the *stepAIC* function from the *MASS* library (Venables & Ripley, 2002) and were assessed both using AIC and BIC. Variance inflation factors were determined using the *vif* function in the *usdm* library (Naimi et al., 2014). Predictive R^2 values

were calculated using leave-one-out cross-validation via the *caret* package (method = "LOOCV") (Kuhn et al., 2021). The predicted R^2 summarises a model's predictive capacity and will always be lower than the corresponding adjusted R^2. A predicted R^2 value that is much smaller than the adjusted R^2 is indicative of model overfitting or a model that is heavily reliant on individual data points.

Four models were developed and considered for each of the EC10, EC20 and EC50 datasets. These four models (at each effect level) were those developed with parameters based on the stepwise regression analysis using either AIC or BIC test statistics with and without interaction terms. For the EC50 dataset an additional four models were developed and considered using a subset of data (n = 18 EC50 values) in which data from Chapter 4 zinc toxicity experiments using DOC sourced from Appletree Creek and Manton Dam (Price et al., 2023b), were excluded. These data were excluded as Chapter 4 (Price et al., 2023b) found that the presence of Appletree Creek DOC increased toxicity at the EC50 level, which is contrary to principles described by the BLM. As such, it was considered that these toxicity enhancing effects are unlikely to be representative of the influence of all Australian DOC, and additional models were developed for comparison. In total, 16 models across the three effect levels were developed and considered in this study. Models developed using all data as described in section 6.2.1 are referred to as 'full data models' and the EC50 models developed excluding the DOC data, as described above, are referred to as 'subset data models' throughout the study.

Validation methods followed procedures described by Garman et al. (2020), Besser et al. (2021) and DeForest et al. (2023). Autovalidation assesses the fit of data used to develop the model. Observed toxicity was plotted versus predicted toxicity data on a 1:1 plot as a visual means to understand how close the model was predicting the observed data. Performance was assessed based on the percentage of observed data that fell within a factor of 2 or factor of 3 of the predicted toxicity values. The factor of 2 is based on inter-test variability on median lethal concentrations (LC50) values for *Pimephales promelas* exposed to copper (Erickson et al., 1996) and *Daphnia magna* exposed to cadmium, copper, nickel or zinc (Santore and Ryan 2015; Meyer et al. 2018). From these studies the factor of 2 has become a standard metric for assessing model predictive capability. Chapter 5 (Price et al. 2022b) found that the factor of 2 metric may not be appropriate for low effect levels (e.g., EC10 and EC20 values), with a factor of 3 metric being more appropriate. This is due to the greater uncertainty at EC10 and EC20 values in a concentration-response model (Peters et al. 2018; Price, et al. 2022b). Chapter 5 (Price et al. 2022b) also found that both the factor of 2 and factor of 3 metric was suitable for microalgae at all effect levels. Based on this, the current study has assessed model performance at all effect levels based on both the factor of 2 and factor of 3 metric.

Model residual analysis, as described by Garman et al. (2020) and implemented by Brix et al. (2021, 2023) and Croteau et al. (2021), was used as an additional metric of performance in model validation. Model residuals were calculated using Equation 6.3. Geometric means and regression slopes of residuals of the observed versus predicted values as a function of either observed and predicted toxicity were assessed to test for consistent over- or under-prediction of the model.

Scoring of model residuals followed methods detailed by Besser et al. (2021). The residual score (RS) was weighted by both the slope (s) parameter of each TMF and the associated p value (p) of the regression of the residual versus each independent variable (i) as shown in Equation 6.4. A total model performance score (MPS) was then used to calculate the overall performance of the model with methods following those described by DeForest et al. (2023). The MPS (Equation 6.5) comprises of six components: (1) the R^2 of the linear regression model of observed versus predicted ECx, (2) the percentage of values predicted within a factor of 2 ($RF_{x,2.0}$) or factor of 3 ($RF_{x,3.0}$) of their paired observed value, and the slope of observed versus predicted ECx residuals versus (3) observed ECx, (4) pH, (5) hardness, and (6) DOC. A MPS was calculated using both $RF_{x,2.0}$ and $RF_{x,3.0}$. The higher the MPS, the greater the model performance (Besser et al., 2021).

$$\ln(\text{residual}) = \ln(ECx_{observed}) - \ln(ECx_{predicted}) \qquad (6.3)$$

$$RS_i = \frac{2}{(1 + 10^{|s_i \times (1-p_i)|})} \qquad (6.4)$$

$$\text{MPS} = \frac{R^2 + RF_x + RS_{obs} + RS_{pH} + RS_{Hardess} + RS_{DOC}}{6} \qquad (6.5)$$

6.2.2 MLR independent validation

Natural water collection and analysis

Natural surface freshwaters were collected from six unimpacted waterways across Australia. These included Woronora River (Tharawal Country) from New South Wales; Ovens River (Waveroo Country) from Victoria; Teatree Creek, Limestone Creek (Darumbal Country) from Queensland; Magela Creek (Mirrar Country) from Northern Territory and Blackwood River (Bibbulman Noongar Country) from Western Australia (Figure E-1). GPS co-ordinates of the sample locations are given in Table E-2. The waterways were selected to cover a range of climatic, geographical and state jurisdictions. Selection was also based on water chemistry previously recorded taking into account TMFs i.e., hardness, pH and DOC concentrations. The hardness, pH, DOC, and conductivity of the natural waters were estimated from online real-time water data sources if available, and from previously published data (Holland et al., 2014; Stauber et al., 2021,

2023). These sites chosen for sampling were targeted for their water chemistries which were generally representative of typical Australian water chemistry ranges.

A YSI multi-probe meter (YSI DSS PRO) was used at each site to confirm that pH and conductivity were within acceptable limits before river water was collected. Water was stored in 5-L high density polyethylene (HDPE) containers which had been previously acid washed and rinsed in ultrapure water (18 MΩ.cm, Milli-Q®; Millipore) and associated river water. Approximately 25 L of water was collected from each site and kept on ice until arrival at the laboratory. The river water was then filtered through a pre-rinsed 0.45 µm in-line flowthrough polyethersulphone filter (Waterra). Filters were pre-rinsed with acid, ultrapure water, and associated river water. The samples were stored at 4°C in the dark until they were used in the toxicity tests. All toxicity tests were conducted within one month of water collection. Additional samples were taken at the time of collection for chemical analysis, which are detailed in Appendix E. Sub-samples of the stored freshwaters were collected and analysed for total hardness, cations, and organic carbon immediately prior to and during toxicity testing (methods provided in Appendix E).

Toxicity testing

Toxicity testing using collected natural waters was conducted to develop an additional dataset for independent validation of MLR models developed in the present study and those available in the literature. The 72-h growth inhibition bioassays were conducted following the same methods used to generate the development dataset (described in detail in Chapter 2 – 4), except natural freshwaters were used instead of a synthetic laboratory water. A buffered (0.5 g MOPS.L^{-1}) and unbuffered test was conducted in each natural water, except Teatree Creek, where only an unbuffered test was conducted as the natural pH of the water was below the buffering range of MOPS. An additional test was conducted on an Ovens River water sample where the pH was increased (named Ovens River – Adjusted) to create potentially high bioavailability conditions. Each test consisted of a zinc concentration series between 0 and 10,000 µg Zn.L^{-1}, with five control replicates and 22 individual zinc treatments. A zinc reference toxicant test in synthetic water was run concurrently with each natural water test. Reference toxicant test water had no additional DOC added. The test was considered acceptable if algal growth rates in reference toxicant tests were 1.7 ± 0.4 doublings per day (mean ± SD, n = 20), control growth rates had a coefficient of variation <20%, and the zinc reference EC50 was within internal database limits of 85 ± 40 µg Zn.L^{-1} (mean ± SD, n = 20).

6.3 RESULTS AND DISCUSSION

6.3.1 MLR models

The VIFs for pH, ln(hardness) and ln(DOC) across all models ranged from 1.0 to 1.1, indicating very low correlation between independent variables. This was expected given all data used came from controlled experiments, where an individual parameter was varied while the others were held constant. This is consistent with models developed from other controlled experiment studies (Brix et al., 2023; DeForest et al., 2018, 2020), where VIFs are generally lower than for models developed from larger databases derived from a range of sources (Brix et al. 2017, 2021).

All models retained hardness as a term, whereas only 7 of the 16 retained DOC and only 4 retained pH. Only two models retained the interactive term of ln(DOC) x pH (Table 6.1). This contrasts with the zinc models of DeForest et al. (2023) developed for *R. subcapitata*, in which only pH and DOC were retained (not hardness) as a significant parameter. On three occasions AIC and BIC methods retained the same model terms and coefficients (shown in Table 6.1 on a single line). On two occasions (EC50 with data subset) there were no differences between models with and without interactive terms, but AIC-selected and BIC-selected models retained different terms and coefficients (Table 6.1).

In general, model adjusted R^2 values were low, ranging from 0.3 to 0.5 for models using the full dataset. Adjusted R^2 values improved in the EC50 models using the subset data, ranging from 0.7 to 0.8. Predicted R^2 values ranged between 0.3 to 0.7, with EC10 models having the lowest (0.3) and EC50 models having the highest (0.7) values. All predicted R^2 values were only slightly lower than their respective adjusted R^2 values, indicating the models were not overfitted. The inclusion of interaction terms either did not improve or only marginally improved model-adjusted R^2 and predicted R^2 values for all EC levels. Therefore, both types of models, with and without interaction terms, were retained for autovalidation.

Table 6.1: Multiple linear regression model statistics for *Chlorella* sp. for 10, 20 and 50% effective concentrations. Hard = hardness, DOC = dissolved organic carbon, Adj. R^2 = adjusted R^2, Pred. R^2 = predicted R^2, AIC = Akaike information criterion, BIC = Bayesian information criterion.

Endpoint	n	Data used	Model	Intercept	Slopes				Adj. R^2	Pred. R^2	AIC	BIC
					pH	ln(Hard)	ln(DOC)	ln(DOC) x pH				
EC10	30	All data	No interaction (AIC)	-0.189	-	0.276 (p=0.006)	0.177 (p=0.06)	-	0.32	0.27	-30.7	-
			No interaction (BIC)	-0.189	-	0.276 (p=0.006)	0.177 (p=0.06)	-	0.32	0.27	-	-26.5
			With interaction (AIC)	0.160	-0.055 (p=0.78)	0.288 (p=0.005)	-2.137 (p=0.15)	0.302 (p=0.12)	0.34	0.28	-29.7	-
			With interaction (BIC)	-0.189	-	0.276 (p=0.006)	0.177 (p=0.06)	-	0.32	0.27	-	26.5
EC20	30	All data	No interaction (AIC and BIC)	0.142	-	0.446 (p<0.001)	-	-	0.41	0.37	-27.7	-24.9
			With interaction (AIC)	0.189	-0.009 (p=0.97)	0.432 (p<0.001)	-2.114 (p=0.18)	0.289 (p=0.16)	0.42	0.34	-25.6	-
			With interaction (BIC)	0.142	-	0.446 (p<0.001)	-	-	0.41	0.37	-	-24.9
EC50	30	All data	No interaction (AIC and BIC)	1.17	-	0.628 (p<0.001)	-	-	0.47	0.43	-14.6	-11.6

		With interaction (AIC and BIC)	1.17	-	0.628 (p<0.001)	-	-	0.47	0.43	-14.6	-11.6
18	Subset data only	No interaction (AIC)	3.97	-0.359 (p=0.17)	0.673 (p<0.001)	0.351 (p=0.080)	-	0.75	0.66	-14.4	-
		No interaction (BIC)	1.15	-	0.684 (p<0.001)	-	-	0.70	0.64	-	-10.4
		With interaction (AIC)	3.97	-0.359 (p=0.17)	0.673 (p<0.001)	0.351 (p=0.080)	-	0.75	0.66	-21.8	-
		With interaction (BIC)	1.15	-	0.684 (p<0.001)	-	-	0.70	0.64	-	-14.8

Autovalidation

Autovalidation was used to validate and assess the fit of data used in the model development dataset. Data were considered acceptable if agreement between observed and predicted EC20 and EC50 values were within a factor of 2. For all values, including EC10 values, agreement within a factor of 3 was also considered (Peters et al. 2018; Price et al. 2022b).

Observed toxicity versus predicted toxicity plots are shown for all models in Figures 6.1 and 6.2. For the EC10 and EC20 models, both models with and without interaction terms generally provided good fit of the data. The inclusion of interaction terms in EC10 models improved the factor of 2 and factor of 3 percentage from 57% and 77% to 80% and 93%, respectively (Figure 6.1, left-hand panel, and Table E-3). Inclusion of interaction terms in EC20 models did not improve the percentage of predictions within a factor of 2, with 77% for both models, but did improve predictions within a factor of 3 marginally from 93% to 97%. EC50 models did not predict data as well as the EC10 and EC20 models. The full dataset (n = 30) model had 53% and 93% of predictions within a factor of 2 and 3, respectively.

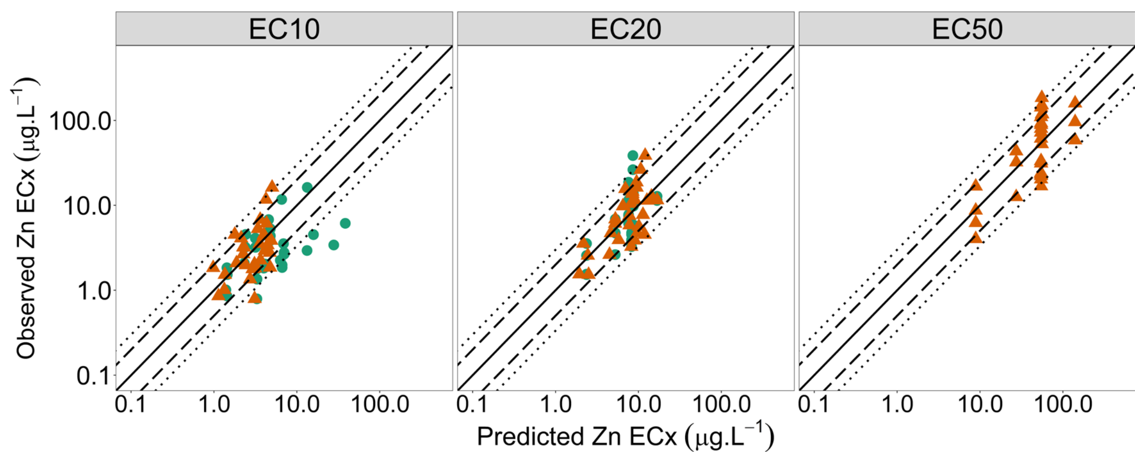

Figure 6.1: Observed versus predicted effect concentration values (ECx) for the multiple linear regression models that were selected in the stepwise regression for the full data models. At the EC10 and EC20 levels both models where interactions terms were included (orange triangles) and excluded (green circles) in stepwise regression are shown. At the EC50 level only the model where interaction terms are shown (orange triangles) as all EC50 models with and without interactive terms were the same. The solid line is the line of perfect agreement between observed and predicted ECx values. Dashed lines indicate a factor of ±2 and dotted lines indicate a factor of ±3.

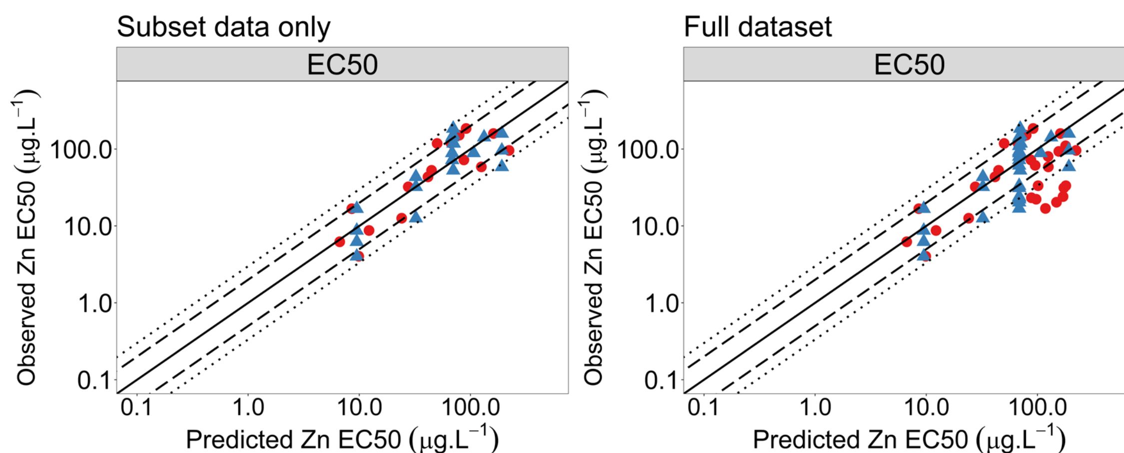

Figure 6.2: Observed versus predicted effect concentration values for the multiple linear regression models that were selected in the stepwise regression for the EC50 subset data models. AIC-selected (red circles) and BIC-selected (blue triangles) models are shown. The left panel shows autovalidation using only the development subset (n = 18) data, the right panel shows autovalidation using the full development dataset (n = 30). The solid line is the line of perfect agreement between observed and predicted EC50 values. Dashed lines indicate a factor of ±2 and dotted lines indicate a factor of ±3.

To assess the EC50 subset data models, factor of 2 and 3 performance were firstly assessed using only the observed data used to develop the model (n = 18). The AIC-selected model had a factor of 2 and factor of 3 percentage of 72% and 100%, respectively, while the BIC-selected model had 61% and 94%. Secondly, the subset data models were assessed using the full development dataset (n = 30). As expected, the performance was reduced, with the AIC-selected model having a factor of 2 and factor of 3 percentage of 57% and 73%, respectively. The BIC-selected model had a slightly lower factor of 2 percentage of 50%, but a slightly higher factor of 3 percentage of 87% relative to the AIC-selected model (Table E-3).

Model residuals (Equation 6.3) were used as an additional metric of performance. Residuals were not homogenous across the entire range of observed and predicted toxicity (Figures E-2 – E-6), with residual slope directionality (i.e., positive, negative) for residuals versus observed ECx tending to be positive at all effect levels. This suggests that there may be a bias in the MLRs leading to underprediction of EC values at higher EC values, which in turn, results in over prediction of toxicity. This trend was not observed for residuals versus TMFs (Figures E-2 – E-6), indicating that the models were accurately capturing the effects of each TMF. Interestingly, the same trend in residuals versus observed ECx was reported by DeForest et al. (2023).

Patterns in model residuals versus TMFs were generally consistent between models with and without interaction terms, except for the EC10 models (Figure E-2). Differences were particularly strong for EC10 DOC residuals, where the model with interaction terms had a slope of 0.0 while the model without interaction terms had a slope of -0.36. This indicates that at increased concentrations of DOC there was a bias to underpredict toxicity, over-attributing the protective effect to the DOC.

Final MLR models

For EC10 and EC20 model comparisons, where models with and without interaction terms were considered, MPS values were consistently higher when interactive terms were included. MPS values for models with interaction terms were higher than models without interactive terms for MPS using a factor of 2 or factor of 3 percentage score (Table E-3). Based on the higher MPS value both the EC10 and EC20 models with interaction terms were selected for further independent validation.

The AIC-selected EC50 model (with or without interaction terms, as they were the same, Table 6.1), using the subset data, had the highest MPS and was selected for further independent validation. All full dataset EC50 models provided the same terms and coefficients and therefore the same MPS value, as such this model was carried through for independent validation.

The models selected are shown below (Equations 6.6 – 6.9). ECx values are expressed in units of $\mu g.L^{-1}$ and DOC and hardness are expressed in units of $mg.L^{-1}$.

EC10:

$$\ln(EC10) = 0.16 + 0.288 \times \ln(hardness) - 2.137 \times \ln(DOC) - 0.055 \times pH + 0.302 \times \ln(DOC) \times pH \quad (6.6)$$

EC20:

$$\ln(EC20) = 0.189 + 0.432 \times \ln(hardness) - 2.114 \times \ln(DOC) - 0.009 \times pH + 0.289 \times \ln(DOC) \times pH \quad (6.7)$$

EC50 (full dataset):

$$\ln(EC50) = 1.173 + 0.628 \times \ln(hardness) \quad (6.8)$$

EC50 (subset data):

$$\ln(EC50) = 3.973 + 0.673 \times \ln(hardness) + 0.351 \times \ln(DOC) - 0.359 \times pH \quad (6.9)$$

6.3.2 Independent Validation using Natural Waters

Test acceptability

Test acceptability criteria for ecotoxicology tests with zinc-spiked natural waters were achieved (data provided in Appendix E). Several unbuffered water tests had slightly higher pH variability

compared to buffered tests and were above the desired ±0.1 unit pH change, with Limestone Creek (unbuffered) and Magela Creek (unbuffered) having a ±0.2 pH variation and Teatree Creek having ±0.3 variation in pH over the test duration. This is still considered very low for chronic algal studies and the data were included in the validation analysis. Further test acceptability data are provided in Appendix E.

Measured toxicity

Zinc was toxic to *Chlorella* sp. in all zinc-spiked natural waters tested (Figure 6.3), with EC10 values ranging from 6.3 to 193 $\mu g.L^{-1}$ and EC50 values ranging from 42 to 603 $\mu g.L^{-1}$ (Table 6.2). Control growth rates were consistent across buffered and unbuffered tests with differences being small between EC10 values and larger for EC50 values. Where larger differences in toxicity between buffered and unbuffered tests were observed (i.e., Blackwood River and Woronora River), the unbuffered tests were consistently more toxic. This was likely due to the increase in pH in the unbuffered tests in the 24-h pre-equilibration period and which is consistent with findings in Chapter 2 (Price et al. 2021) which showed that the toxicity of zinc to this *Chlorella* sp. strain increased with increasing pH. Similar conclusions around the influence of organic buffers on zinc toxicity were found by DeForest et al. (2023) when preparing data sources for MLR development. Based on this, buffered and unbuffered tests were pooled for independent validation.

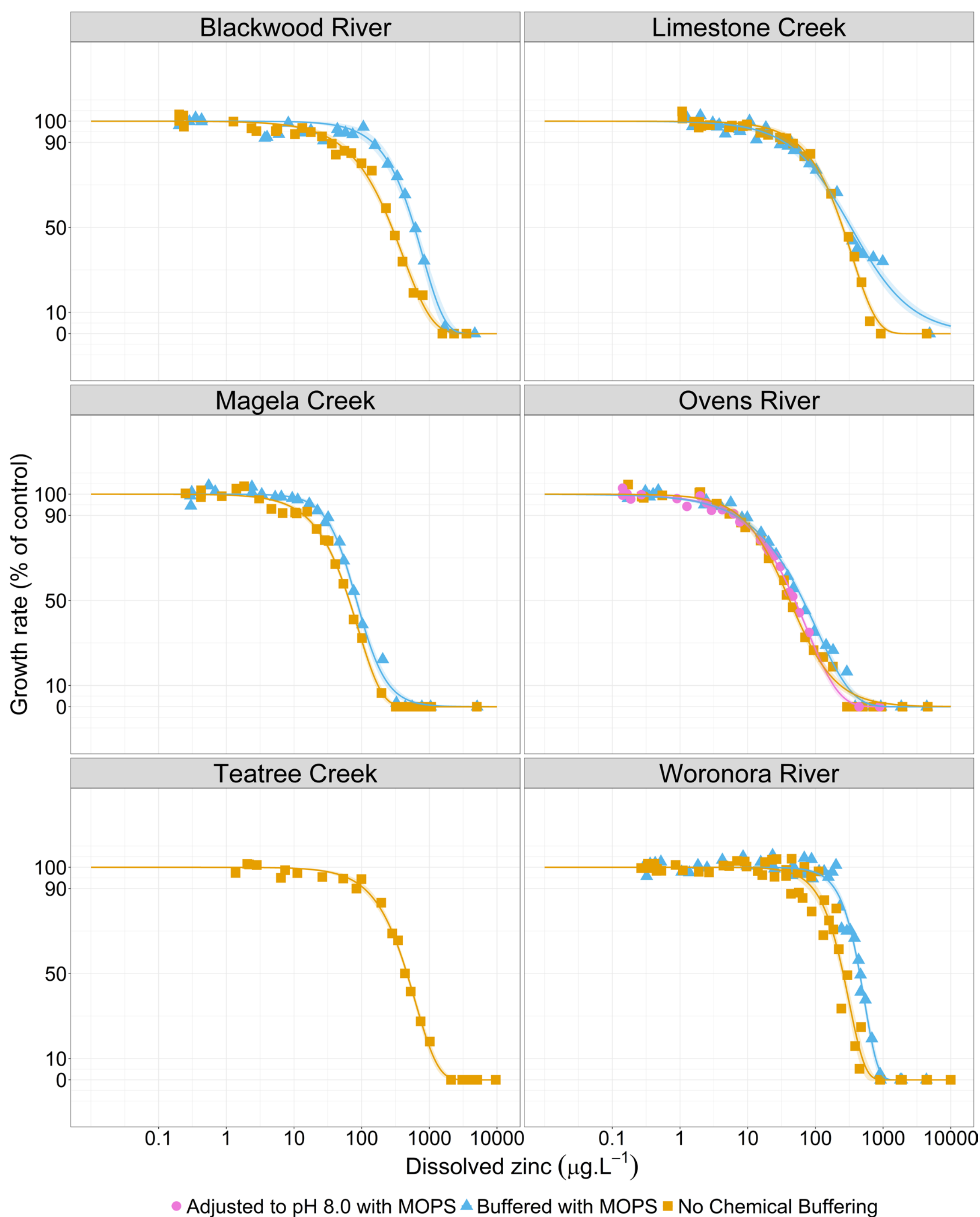

Figure 6.3: The 72-h growth rate inhibition of *Chlorella* sp. (% of control) exposed to zinc concentrations in six different natural freshwaters. Tests were conducted with (blue triangles) and without (yellow squares) chemical buffering (0.5 g MOPS.L^{-1}). An additional pH adjusted test (pink circles) was conducted for the Ovens River sample to theoretically create high zinc bioavailability conditions. Shaded ribbons represent the 95% confidence intervals. Each data point represents one individual replicate response and a corresponding measured zinc concentration. Model parameters are provided in Table E-4. Note that Teatree Creek did not have a buffered test as the natural pH of the water fell outside the buffering capacity of MOPS.

Table 6.2: Zinc toxicity tests with *Chlorella* sp. in Australian natural freshwaters. Summary of water chemistry and toxicity of zinc. pH = test average ± standard deviation. 95% confidence intervals in brackets.

Water	pH	DOC ($mg.L^{-1}$)	Hardness ($mg.L^{-1}$)	EC10 ($\mu g.L^{-1}$)	EC20 ($\mu g.L^{-1}$)	EC50 ($\mu g.L^{-1}$)
Blackwood River Buffered	8.05 (± 0.04)	4.2	355	145 (111–179)	256 (216–295)	603 (553–652)
Blackwood River Unbuffered	8.2 (± 0.1)	4.2	355	42 (34–50)	89 (78–101)	280 (260–299)
Limestone Creek Buffered	7.42 (± 0.02)	20	89	35 (27–43)	81 (68–94)	337 (299–374)
Limestone Creek Unbuffered	8.0 (± 0.2)	20	89	51 (42–60)	96 (85–108)	254(238–269)
Magela Creek Buffered	6.38 (± 0.03)	6.0	3	27 (24–30)	41 (38–44)	84 (79–89)
Magela Creek Unbuffered	6.7 (± 0.2)	6.0	3	14 (12–16)	26 (24–28)	66 (63–69)
Ovens River Buffered	7.47 (± 0.02)	<1	11	6.6 (5.3–7.9)	16 (14–19)	66 (61–71)
Ovens River Unbuffered	7.7 (± 0.1)	<1	11	7.4 (6.1–8.7)	14 (12–16)	42 (39–45)
Ovens River (pH adjusted) Buffered	8.01 (± 0.02)	<1	11	6.3 (5.5–7.0)	14 (13–15)	50 (48–52)
Teatree Creek Unbuffered	6.1 (± 0.3)	25	13	109 (93–124)	194 (176–213)	467 (445–489)
Woronora River Buffered	7.11 (± 0.02)	5.3	18	193 (172–213)	271 (251–290)	452 (433–470)
Woronora River Unbuffered	7.4 (± 0.1)	5.3	18	92 (75–109)	139 (121–156)	257 (238–276)

Ovens River tests showed the lowest ECx values at all effect levels, and this was expected given that there was no measurable DOC, low hardness (11 mg $CaCO_3.L^{-1}$) and high pH (7.47) (relative to other waters tested). However, the increased pH in the adjusted Ovens River test did not increase toxicity at any effect level as expected based on pH terms and coefficient in the MLR models. Magela Creek tests had the second lowest ECx values at all effect levels, despite having a low pH (6.4 – 6.7) and moderate DOC concentration (6.0 $mg.L^{-1}$); however, this water did have very low hardness (3 mg $CaCO_3.L^{-1}$ at the time of testing). These results suggest that low hardness may lead to increased zinc toxicity and this is in general agreement with the inclusion of a hardness term in each MLR model.

In agreement with this were the relatively high ECx values in Blackwood River, which had a high hardness of 355 mg $CaCO_3.L^{-1}$, moderate DOC concentration (4.2 mg $C.L^{-1}$) and high pH (8.0 – 8.2). While Teatree Creek had a relatively low hardness of 13 mg $CaCO_3.L^{-1}$, the relatively high ECx values are likely explained by its high DOC concentration of 25 $mg.L^{-1}$.

Woronora River results were contrary to the expected results based on this hardness dependency. With a low hardness of 18 mg $CaCO_3.L^{-1}$, moderate DOC concentration of 5.3 $mg.L^{-1}$, and a moderate pH of 7.1 to 7.4, toxicity was expected to be relatively high. However, the Woronora River buffered test had the highest EC10 and EC20 values (193 and 271 $\mu g.L^{-1}$, respectively) and the second highest EC50 value (467 $\mu g.L^{-1}$). In addition to these contrasting results based on water chemistry, the relative magnitude of ECx values at all effect levels differed between the natural water results and the synthetic water results from the data sources for MLR development. Zinc EC10 values ranged from 6 to 193 $\mu g.L^{-1}$ in the natural waters, higher than EC10 values of 0.8 to 5 $\mu g.L^{-1}$ in synthetic water. EC20 values ranged from 14 to 271 $\mu g.L^{-1}$ in the natural waters compared to 2 to 19 $\mu g.L^{-1}$ in synthetic water, and EC50 values ranged from 42 to 603 $\mu g.L^{-1}$ in the natural waters compared to 18 to 185 $\mu g.L^{-1}$ in synthetic water.

Predicted toxicity

In addition to the MLR models developed for *Chlorella* sp. in the present study, other models have previously been developed for *R. subcapitata*, a different species of green microalga. These models (Table 6.3) were assessed for their suitability for predicting *Chlorella* sp. toxicity in natural Australian freshwaters and to assess the suitability of cross-species models for microalgae.

Chlorella sp. MLRs

The developed *Chlorella* sp. MLRs (Table 6.3) performed poorly at predicting toxicity in the natural waters. EC10 and EC20 models consistently overpredicted zinc toxicity, predicting 0% of the data within a factor of 2 or 3 (Figure 6.4). Both the EC50 models also predicted natural water toxicity poorly, where the full dataset (n = 30) model predicted 0 and 17% of data within a factor of 2 and 3, respectively. The subset data (n = 18) EC50 model predicted 25 and 33% of data within a factor of 2 and 3, respectively.

Table 6.3: Multiple linear regression model coefficients used in validation analysis. Hard = hardness, DOC = dissolved organic carbon, n/e = not evaluated due to insufficient data, - = term not retained in stepwise regression analysis.

Species	**Model reference**	**Effect level**	**Intercept**	**Slope**			
				pH	**ln(Hard)**	**ln(DOC)**	**ln(DOC) x pH**
Chlorella sp.	Current study	EC10	0.16	-0.055	0.288	-2.137	0.302
		EC20	0.189	-0.009	0.432	-2.114	0.289
		EC50	1.17	-	0.628	-	-
		EC50[a]	3.97	-0.359	0.673	0.351	-
Raphidocelis subcapitata	CCME (2018)	EC50	11.8	-1.122	-	n/e	n/e
	Stauber et al. (2023)	EC50	8.28	-0.75	0.296	0.468	-
	DeForest et al. (2023)	EC10	10.307	-0.992	-	0.378	-
	DeForest et al. (2023)	EC20	11.950	-1.172	-	0.342	-
	DeForest et al. (2023)	EC50	10.925	-0.865	-	0.209	-

[a] subset *Chlorella* sp. model

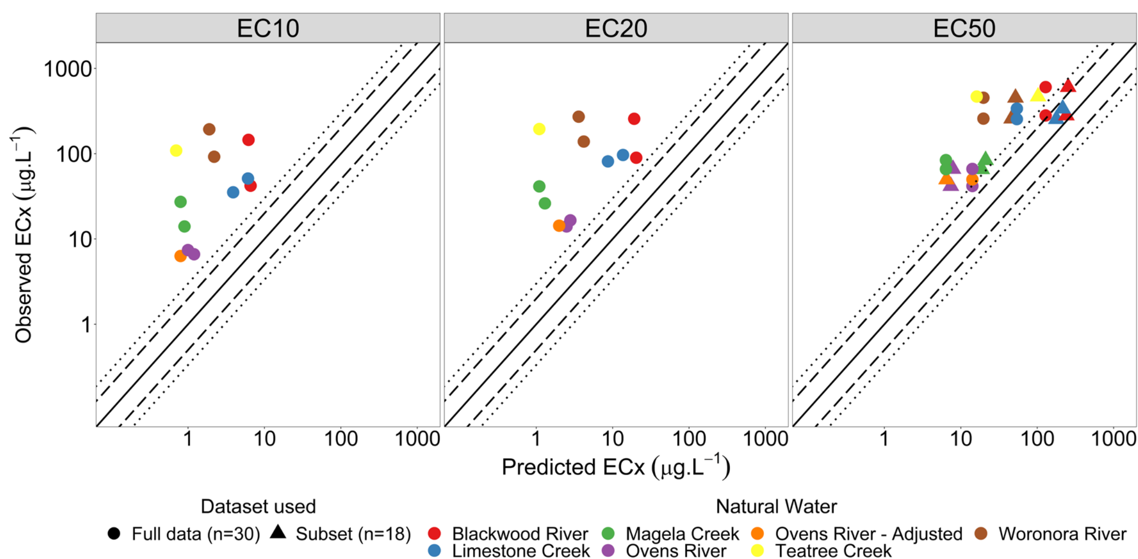

Figure 6.4: Observed toxicity versus predicted toxicity for six Australian natural freshwaters using the *Chlorella* sp. multiple linear regression models with their original sensitivity coefficients. Solid line is the line of perfect agreement (1:1) between observed and predicted ECx values. Dashed lines indicate a factor of ±2 and dotted lines indicate a factor of ±3 deviation from the 1:1 line.

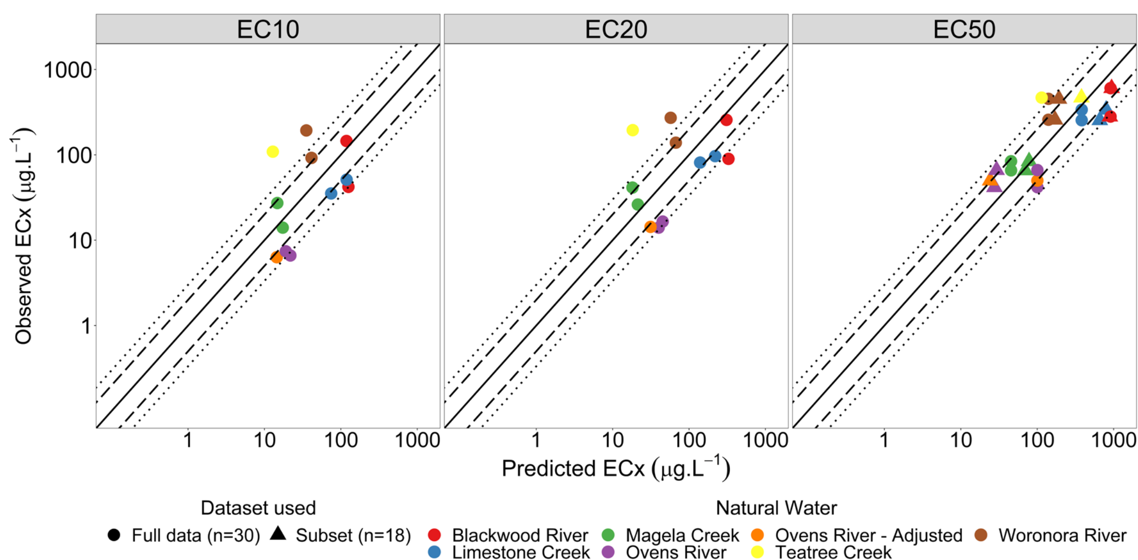

Figure 6.5: Observed toxicity versus predicted toxicity for six Australian natural freshwaters using the *Chlorella* sp. multiple linear regression models with revised sensitivity coefficients. Solid line is the line of perfect agreement (1:1) between observed and predicted ECx values. Dashed lines indicate a factor of ±2 and dotted lines indicate a factor of ±3 deviation from the 1:1 line.

Given all models consistently overpredicted toxicity, sensitivity coefficients (y-intercept) were recalibrated using the natural waters' zinc toxicity data according to methods outlined by (Peters et al. 2021). Observed versus predicted plots with original and updated sensitivity coefficients are shown in Figure 6.4 and 6.5, respectively, and revised coefficient values are provided in Table E-5.

The recalibrated models provided an improved fit to the data, with both the revised EC10 and EC20 models predicting 25 and 75% of data within a factor of 2 and 3, respectively. Both EC50 models were also improved, with the full dataset model predicting 58 and 75% of data within a

factor of 2 and 3, respectively. The subset data model predicted 50 and 92% of data within a factor of 2 and 3, respectively. Model residual plots with original and updated sensitivity coefficients are provided in Figures E-7 – E-14.

While recalibrated sensitivity coefficients did improve the models, it is important to note that this recalibration procedure is typically used for cross-species validation methods (Peters et al., 2021) or when it is believed sensitivity of cultures have significantly shifted with time (Van Regenmortel et al., 2017). Based on the consistency of reference toxicant tests used during the current study, shifts in culture sensitivity are unlikely to explain this consistent overprediction in toxicity to *Chlorella* sp. in the Australian natural waters.

Unaccounted for toxicity modifying factor(s) may be present across all natural waters, causing this consistent overprediction in toxicity. Calcium and magnesium ratios, which are known to be different to those in the Northern Hemisphere (Peters et al. 2021), as a contributor to the overprediction were considered, as were concentrations of sodium, aluminium, iron and manganese, all of which are known to modify metal speciation. Ratios and concentrations are provided in Table E-6. However, there were no consistent trends in the calcium and magnesium ratios among the natural waters, nor were there any consistently elevated concentrations of the four metals listed above.

Elevated control growth rates in natural water testing relative to the concurrently ran reference toxicant tests in synthetic media, suggested that nutrient levels may be influencing toxicity. Control growth rates in natural waters ranged from 2.1 to 2.5 doublings per day compared to 1.7 doublings per day in the reference tests. A broad suite of nutrients (i.e., NH_3, NO_2, NO_3, phosphorous etc.) were analysed for each natural water prior to testing to ensure levels were consistently low. All analytes were consistently below the limit of detection or near limit of detection (Table E-6) except Limestone Creek, which had slightly elevated ammonia (0.16 $mg.L^{-1}$) and Total nitrogen (1 $mg.L^{-1}$). The highest total phosphorus concentration was 0.04 $mg.L^{-1}$ in Teatree Creek. However, it is important to note that all tests including those in natural waters and those used to develop the MLR models were supplemented with nitrogen and phosphorus at the start of each toxicity test, as part of standard toxicity testing protocols (OECD, 2011b). Final concentrations of supplemental NO_3^- and PO_4^{3-} were 1.5 and 0.15 $mg.L^{-1}$, respectively. Therefore, nutrient exposure concentrations for the microalgae were generally consistent across all tests, both natural water and synthetic and does not explain the relative change in toxicity observed, nor does it likely explain the elevated control growth rates. Iron is also a microalgal micronutrient and as mentioned above can modify metal speciation. While dissolved concentrations of iron varied (7 – 500 $\mu g.L^{-1}$, Table E-6) in the natural waters, there were no consistent trends that could explain the change in observed toxicity.

Alternatively, rather than the presence of an unidentified TMF or zinc complexing agent, there may be something present in the natural waters that alters the physiology of the microalgae which indirectly affects zinc toxicity, such as, by a change in mechanism of toxicity. Such physiological changes may be changes in cell membrane permeability (Wood et al., 2011). Further experimental work is required to test this hypothesis.

Another possible explanation for the overprediction is the underlying assumption that models using zinc toxicity modifying relationships derived from synthetic laboratory test waters can be directly applied to natural water samples. Natural waters typically contain a more complex matrix than synthetic laboratory waters and therefore the presence of unknown TMFs may be ameliorating zinc toxicity (as seen by the overprediction of toxicity by the models). The current study is the third study to develop a microalgal MLR solely from synthetic laboratory water toxicity data (Brix et al., 2023; DeForest et al., 2018) and the first study to independently validate the developed MLRs with natural waters. Further research into the suitability of applying synthetic laboratory water-based models to a greater range of natural waters, which potentially include a range of biotic TMFs, is clearly needed.

6.3.3 Comparison to pre-existing MLR models

MLR models developed for *R. subcapitata* were updated to include *Chlorella* sp. specific sensitivity coefficients (Peters et al., 2021). *R. subcapitata* models generally provided a better fit than the *Chlorella* sp. model (with original sensitivity coefficients) for all effect levels and were comparable to the *Chlorella* sp. model with updated sensitivity coefficients (Figure 6.6). The DeForest et al. (2023) EC10 model predicted 42 and 67% of data within a factor of 2 and 3, respectively, and the EC20 model predicted 50% and 75% of data with a factor of 2 and 3, respectively. Of the three EC50 models, the CCME (2018) MLR performed the poorest for both factor of 2 and 3 predictions, with 33% and 58%, respectively. The Stauber et al. (2023) and DeForest et al. (2023) EC50 models performed similarly, with 50 and 83%, and 67 and 75% of data predicted within a factor of 2 and 3, respectively.

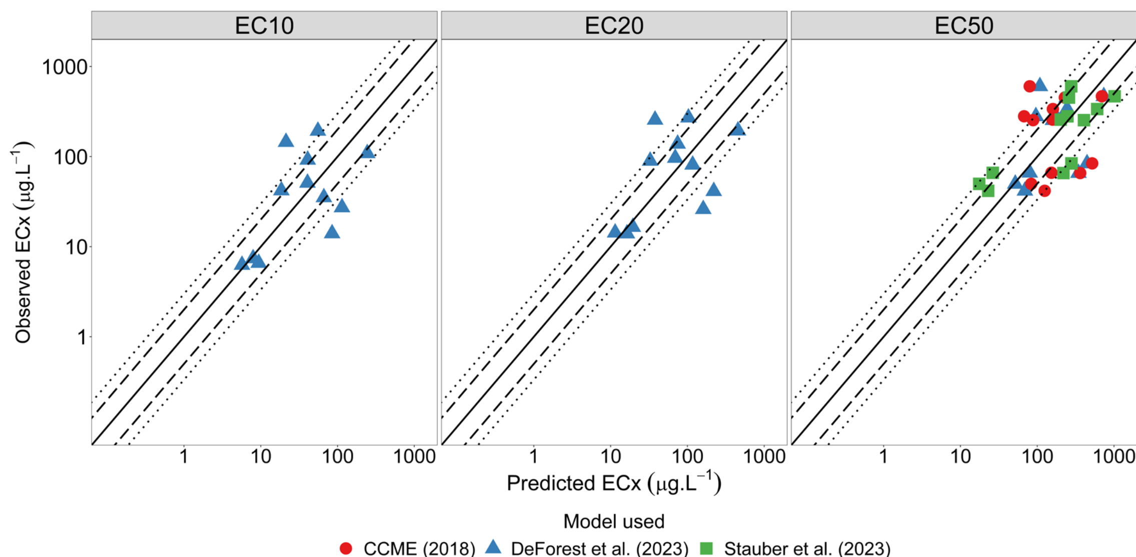

Figure 6.6: Observed toxicity versus predicted toxicity for six Australian natural freshwaters using multiple linear regression models for *R. subcapitata* developed by CCME (2018) (red circles), DeForest et al. (2023) (blue triangles) and Stauber et al. (2023) (green squares). Solid line is the line of perfect agreement (1:1) between observed and predicted ECx values. Dashed lines indicate a factor of ±2 and dotted lines indicate a factor of ±3 deviation from the 1:1 line.

Residual analysis of all *R. subcapitata* models found general biases across all models. The EC10 and EC20 models by DeForest et al. (2023) had low residual bias when plotted against DOC with slopes of -0.031 and -0.043, respectively. The DeForest et al. (2023) EC50 model had a slightly larger model bias when plotted against DOC, with a slope of -0.28. The CCME (2018) EC50 model had biased slopes for all 3 TMFs (DOC: 0.21, hardness: 0.72, pH: 1.1), while the Stauber et al. (2023) EC50 model had low residual bias for DOC, with a slope of 0.045 (Figures E-15–E-17).

Peters et al. (2021) recommended acceptability criteria requiring 50% of data to lie within a factor of 2 and 90% of data within a factor 3. Based on these criteria, none of the tested models would be deemed acceptable; however, both the Stauber et al. (2023) model and the EC20 and EC50 models by DeForest et al. (2023) would pass the 50% within a factor of 2 criterion.

This generally poor performance of the *Chlorella* sp. and *R. subcapitata* models during independent and cross-species validation using natural waters suggests relative changes in zinc toxicity as a function of pH, hardness and DOC may not be consistent across microalgal species nor might these three TMFs be the only significant modifiers of toxicity in Australian natural waters.

Examples of cross-species validation of MLR models for microalgae are limited given the majority of microalgal toxicity data uses a single species (*R. subcapitata*), whereas cross-species comparisons are more common for invertebrates and fish as large toxicity datasets often exist for multiple species (Croteau et al., 2021; Peters et al., 2021). Peters et al. (2021) reported good cross-species validation using *Chlorella* sp. (different strain to present study) for *R. subcapitata* derived

nickel MLRs; however, the validation dataset was small (n = 5) and the range of hardness values used was low.

6.4 CONCLUSIONS

This study presented the first *Chlorella* sp. zinc toxicity MLR models and the first metal toxicity MLR models for microalgae using a development species other than *R. subcapitata*. It was highlighted that while developed models can perform well during autovalidation procedures, assessment of independent datasets using natural waters is critical for assessing predictive power of MLRs.

The findings of the present study showed that zinc toxicity to algae is difficult to predict, even when using species-specific MLRs. Neither the *Chlorella* sp. MLRs nor the existing *R. subcapitata* model accurately predicted zinc toxicity to *Chlorella* sp. in Australian natural waters. Poor independent validation of the *Chlorella* sp. models also suggests that models derived from laboratory waters may not be suitable for predicting toxicity in far more complex matrices like natural waters, and further investigation is needed to elucidate this such as, expanding the toxicity testing dataset for natural waters.

Chapter 7: General discussion and conclusions

The overall aim of this thesis was to improve risk assessment and management of zinc in Australian freshwater ecosystems through the investigation of the influence of water chemistry to the chronic zinc toxicity of the freshwater microalga, *Chlorella* sp.

This thesis provides important insights into the role of water chemistry in modifying zinc toxicity to *Chlorella* sp. and broadens the understanding of zinc toxicity to microalga. This was achieved by assessing the individual and combined influence of key water chemistry parameters, pH hardness and dissolved organic carbon (DOC), on zinc toxicity to *Chlorella* sp. through a series of laboratory toxicity experiments. Changes in chronic zinc toxicity due to varied pH, hardness, and DOC concentration, and source to *Chlorella* sp., were quantified. Relationships between toxicity and water chemistry parameters were used to develop empirical models for the prediction of zinc toxicity under different water chemistries. Derived models were validated using zinc-spiked natural waters with a range of water chemistries.

This chapter summarises the key findings of the research and provides commentary on the implication for and practicalities of regulatory use of bioavailability-based water quality guidelines, in both Australian and international contexts. First, this chapter reviews the research findings presented in Chapters 2 – 6 focusing on toxicity modifying factors, bioavailability measurements and toxicity model development approaches. Secondly, the application and implementation of bioavailability in the Australian and New Zealand guidelines is discussed. Finally, recommendations for future research are presented to address the limitations and gaps identified.

7.1 INFLUENCE OF WATER CHEMISTRY ON ZINC TOXICITY

The research within this thesis has provided high-quality ecotoxicological data quantifying the role of pH (Chapters 2, 3 and 4), hardness (Chapter 3) and DOC (Chapter 4) in modifying chronic zinc toxicity to the freshwater microalga, *Chlorella* sp.

Water pH was tested across a range of 6.7 to 8.3, which is an environmentally relevant range for Australia's freshwater systems (Chapter 2). An increase in pH was generally associated with a linear increase in toxicity (that is, pH had a linear relationship with ECx values), with the greatest toxicity observed at pH 8.3 which is equivalent to the 90th percentile of freshwater pH in Australia (Stauber et al. 2023). This relationship is commonly observed among other algal species and metals; however, the extent of change of toxicity across a given pH range varied significantly between studies (Deleebeeck et al., 2009; Wilde et al., 2006). The relationship between toxicity and pH change was explained by a reduction in competition between hydrogen ions and zinc at the biotic ligand on the algal cell.

The log-linear relationship between hydrogen ion concentration and zinc toxicity was an interesting finding discussed in Chapter 2 (Figure 2.4). This relationship is metal-specific (Brix et al. 2017), highlighting that the influence of pH change on zinc toxicity could not be simply explained as a competitive effect between a hydrogen ion and a zinc ion for a single biological receptor on the algal cell. As previously suggested (De Schamphelaere et al., 2005; Deleebeeck et al., 2009; Heijerick et al., 2002b), competition between the two ions may be occurring at multiple types of binding sites, each with its unique pKa, whereas metals with a linear relationship with hydrogen ion concentrations (such as copper) may be competing for a single binding site.

Cationic competition drives the observed changes in toxicity across the pH range tested in Chapter 2. Had the tested pH range been expanded to values greater than 8.3, however, new relationships may have emerged, as speciation of zinc would likely play a greater role. As pH rises above 8.5, insoluble zinc hydroxide ($Zn(OH)_2$) begins to dominate speciation in the test solutions used in Chapter 2 (Figure 7.1). This would remove zinc from both the dissolved phase and bioavailable fraction. While this is an interesting concept, where perhaps a plateau in increasing toxicity is observed as more zinc enters the particulate phase with increasing pH above 8.5, it likely bears little relevance to toxicity modification in Australian freshwater conditions, given only 0.1% of Australian freshwaters have a pH equal to or greater than 8.5.

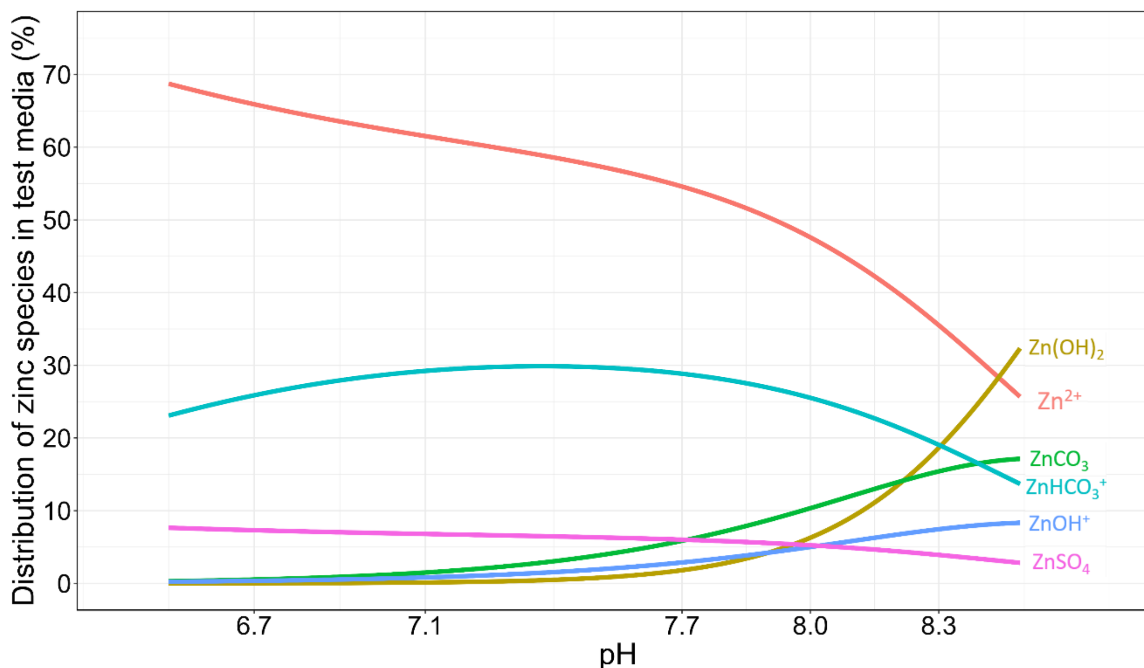

Figure 7.1: Distribution of zinc species in test solution across the tested pH range. Distribution was calculated using WHAM (version 7).

Having a clear understanding of the role of pH on zinc toxicity modification to *Chlorella* sp. was important for exploring the combined influence of hardness and pH on toxicity, the focus of Chapter 3.

The influence of hardness on zinc toxicity at varying pH was investigated because pH and hardness naturally co-vary in freshwaters. A wide range of water hardness is observed in Australian freshwaters, with some regions dominated by very soft (<5 mg $CaCO_3.L^{-1}$) or very hard (>400 mg $CaCO_3.L^{-1}$) waters. Examples of low water hardness regions include the Snowy Mountain alpine region (Ovens River, Chapter 6) and Kakadu National Park (Magela Creek, Chapter 6) while south-west Western Australia exhibited very high water hardness (Blackwood River, Chapter 6).

An increase in hardness generally resulted in a decrease in toxicity across the tested pH range (Chapter 3). The pH did not meaningfully influence the protective nature of increased hardness as there was a consistent effect of hardness-based toxicity modification across the pH range. However, consistent with the findings of Chapter 2, decreased pH was seen to generally ameliorate toxicity across all hardness levels tested.

Chapter 3 showed that the hardness-based algorithms currently used in Australian and New Zealand Freshwater Guidelines (ANZG, 2018) are not protective for *Chlorella* sp. The results indicated that at environmentally relevant high hardness conditions, the hardness-modified zinc guideline was under-protective. This was demonstrated through comparison of the rate of change in toxicity reported in Chapter 3 with the equation used in the hardness-based guideline. Based

on these results, the hardness-based guideline may provide appropriate protection for waters up to a hardness of 93 mg $CaCO_3.L^{-1}$, but at higher water hardness values it begins to overestimate the protective capacity of hardness. This lack of protection had previously been demonstrated for the hardness-based algorithm for Australian water quality guidelines for copper (Markich et al., 2005), resulting in the subsequent removal of the algorithm correction for copper guideline values.

The role of natural Australian DOC concentration and source on zinc toxicity to *Chlorella* sp. at varying pH was investigated in Chapter 4. The two Australian DOC sources selected were distinctly different in chemical composition, with one dominant in humic-like (aromatic and high molecular weight) components and the other dominated by fulvic-like components. This difference in composition appeared to drive differing influences on zinc toxicity to *Chlorella* sp.

Humic-dominated DOC greatly increased zinc toxicity at the EC50 level. While uncommon, DOC-enhanced toxicity has also been observed elsewhere with zinc and microalgae, but also with other metals and test species (Aristilde et al., 2012; Errecalde et al., 1998; Lamelas et al., 2005). A recent study by Hourtane et al. (2022) on the influences of humic acids (HA) on platinum toxicity and bioaccumulation to *Chlorella fusca* and *Chlamydomonas reinhardtii* demonstrated that enhanced toxicity in the presence of HA was correlated with increased bioaccumulation. Subsequent studies, similar to Hourtane et al. (2022), should be conducted to ascertain whether DOC-enhanced toxicity observed in this thesis correlates with increased bioaccumulation. This would aid the hypotheses discussed in Chapter 4 about the role of DOC increasing toxicity.

Interestingly, DOC-enhanced toxicity was not observed at the EC10 level. This appeared to be a consequence of significant change in the slope parameter of the concentration-response model when comparing models with and without DOC addition. A change in slope parameter indicates a rate of response change in the presence of the humic-dominant DOC, thereby indicating the DOC is increasing the rate at which the toxic response is observed as zinc concentration increases. Chemical investigations indicate that this interaction is not a result of zinc speciation changes. There were no clear trends in DGT-labile zinc or ultrafiltered zinc concentrations, or modelled chemical speciation, between treatments that could explain the trends in toxicity (e.g., Zn-DOC complexes, the formation of colloids or precipitates, or shifts in major zinc species).

Hourtane et al. (2022) reported large changes in slope parameters with HA presence and as HA concentration changed. However, trends associated with HA concentration were consistent across effect levels, in contrast to the results of this thesis. The mechanism by which DOC may enhance bioaccumulation and toxicity is still not understood. Several hypotheses were discussed in Chapter 4, including increased bioavailability and uptake through a Zn-DOC complex, or through DOC decreasing the detoxification process of zinc in the microalgae. Unfortunately, further

comparisons to published toxicity studies is challenging because concentration-response curve parameters are generally not published.

The chemical composition of DOC varies around Australia and across seasons, as shown by Holland et al. (2018). Their study examined isolated DOC from nine locations around Australia at different times throughout the year and found that composition varies spatially and seasonally. For example, humic-like components varied from 35 to 60% of the total chemical composition between different waters. The DOC research observed and discussed in this thesis in conjunction with studies such as Holland et al. (2018) highlight the importance of considering DOC source and composition in addition to concentration when assessing risk based on metal bioavailability.

7.2 MEASUREMENTS OF ZINC SPECIATION

This thesis utilised different measurement and modelling techniques of zinc speciation under different water chemistries and examined the efficacy of these techniques in accounting for changes in bioavailability and toxicity, as reflected in changes in test organism response (Chapter 2, 3 and 4). Such methods used included speciation modelling in WHAM, ultrafiltration (<3kDa) and the diffusive gradients in thin-films (DGT) technique. DGT has become increasingly popular since its development in 1994 for measuring metal speciation in a range of environmental compartments (Davison and Zhang 1994) and will be discussed in detail in this section.

The DGT technique was included in this thesis because a body of literature has suggested that the DGT technique samples a more bioavailable fraction of the analyte's species compared to total or dissolved fractions (Gillmore et al. 2021; Koppel et al. 2019; Paller et al. 2019). This feature makes it an attractive surrogate for toxicity testing.

DGT samplers were used concurrently with sampling for standard dissolved (<0.45 μm) metal fractions during toxicity tests (Chapter 2 – 4) to examine whether changes in organism response with changing water chemistry were reflected in corresponding changes in DGT-labile concentrations. Chapters 2 and 3 demonstrated that changes in DGT-labile zinc (operationally defined as zinc accumulated on the Chelex binding resin of the DGT device) had no correlation with changes in observed toxicity as pH and/or hardness varied.

This demonstrates that pH and hardness influences on zinc toxicity are not necessarily related to the lability of zinc species in solution, as determined by DGT measurements. If the modifications to toxicity relate to competition effects associated with hydrogen, calcium, and magnesium ions with zinc ions at biotic ligands, then similar competition is not occurring on DGT binding resins. These data highlight the limitations of using DGT to predict zinc bioavailability to *Chlorella* sp., or to act as a surrogate for toxicity testing for zinc and microalgae especially those waters low in DOC given the large variability in major ion composition in freshwater ecosystems.

The relationship between toxicity and DGT-lability in the presence of different Australian DOC and at varying concentrations of DOC was explored in Chapter 4. The influence of DOC concentration on DGT-lability was dependent on DOC source. The presence and increase in concentration of fulvic-acid dominated DOC (collected from Manton Dam) caused an insignificant reduction in DGT-labile concentrations. This was consistent with the minimal influence of this DOC on toxicity, as measured by EC50 values. Interestingly, in the presence of humic-acid dominated DOC (collected from Appletree Creek), DGT-labile metal concentration decreased as DOC concentration increased, as expected based on findings from the extensive body of literature on DOC-metal complexation. However, this did not explain the changes in toxicity observed, with DOC presence significantly increasing toxicity, contrary to common trends. Given the atypical response of the microalgae to zinc toxicity in the presence of Appletree Creek DOC, it is unsurprising that DGT was not able to accurately predict bioavailability and toxicity. While DGT measurements and toxicity changes were consistent in the presence of Manton Dam DOC, a lack of change in both metrics is not a strong endorsement for DGT accurately predicting the toxicity of zinc.

The DGT technique was not found to be a useful surrogate measure for bioavailability or toxicity in freshwater ecosystems. The Chelex-resin used in this thesis is unlikely to be a true reflection of metal assimilation onto a biotic ligand(s), given its requirement to bind metals as a perfect sink (an assumption of the DGT technique). It is possible to consider other functional groups for a binding resin that are weaker cation exchangers, allowing for a more accurate reflection of ion exchange occurring at the biotic ligand and possibly accounting for competition effects between cations in solution and the target metal. Given the growing body of literature examining the potential use of the DGT technique as a surrogate for bioavailability and toxicity there is clearly interest in surrogate options, and as such, further research exploring the use of different binding resins would be useful. However, it is important to note that while improvements to binding resin functional groups may improve the ability of the DGT technique to reflect binding at a biotic ligand, it is still unlikely to reflect the direct effects of DOC on organisms, which may also influence metal toxicity, as described by Wood et al. (2011).

The use of the DGT technique as a surrogate measurement for toxicity has also been explored for seawater and marine sediments. Based on the limited research available, these environmental matrices appear to act as a better analogue for bioavailability than those found in freshwater studies. Koppel et al. (2019) demonstrated that DGT can predict toxicity to metal mixtures for marine microalgae. The study found that using either DGT-labile or dissolved (<0.45 µm) metal concentrations yielded similar toxicity predictions when using mixture models. Furthermore, both Amato et al. (2014) and Gillmore et al. (2021) investigated the applicability of DGT to predict

metal toxicity to an amphipod in marine sediments. Both studies concluded that DGT-labile metal concentrations were a useful measurement for predicting toxicity.

Better predictability of toxicity in studies using seawater are likely due to the relatively consistent major ion composition present in seawater. Therefore, competition effects from ions such as hydrogen, calcium and magnesium are unlikely to change as significantly as they do in freshwater studies. While DGT may provide adequate prediction of toxicity in seawater, DGT-labile concentrations may not predict toxicity where speciation is not the sole determinant of toxicity, as evidenced by the results presented in this thesis.

It may be more appropriate to consider whether DGT provides an accurate measure of potential metal exposure concentrations in freshwaters. This could act as an additional line of evidence in a detailed risk assessment. There is scope for including such measurements in risk assessment, and these are already outlined in guidance documents for accounting for local water chemistry in the Australian and New Zealand Water Quality Guidelines (ANZG, 2018). Another recent approach using DGT-labile concentrations in water quality frameworks is explored by Amouroux et al. (2023). The study compared a large dataset of dissolved and DGT-labile concentrations for cadmium, nickel, and lead to establish DGT-conversion factors to relate DGT-labile concentrations to the European Water Framework Directive's Annual Average Environmental Quality Standards (AA-EQS). The study found that dissolved concentrations calculated from DGT-labile concentrations via the conversion factor could be appropriate in assigning risk to a marine site. The approach follows similar methods to bioaccumulation factors (BAF) already employed under the framework to compare metal accumulated in biota to the AA-EQS. Therefore, while DGT is not an appropriate tool for predicting bioavailability and zinc toxicity to *Chlorella* sp. in freshwaters, there are many useful potential applications for such measurements in risk assessment and environmental management.

7.3 DEVELOPING BIOAVAILABILITY-BASED MULTIPLE LINEAR REGRESSION MODELS

This thesis has developed and validated multiple linear regression (MLR) models for the prediction of zinc toxicity to *Chlorella* sp. (Chapter 6). The development of these models represents the first developed for any metal using a *Chlorella* sp. and the first models developed for a non-*R. subcapitata* species. A range of model validation techniques were applied to assess model performance. This included the factor-of-3 performance metric proposed and discussed in detail in Chapter 5, which is based on the commonly used factor-of-2 metric. The MLR models developed in this thesis are also the first to be independently assessed using zinc-spiked natural

waters. The natural water toxicity data was also used to assess model performance of other microalgal zinc MLR models in the literature.

Section 7.3 summarises the findings of model development and validation undertaken in this thesis, compares those results to other approaches in the literature and provides commentary on the use of MLR models for metal toxicity prediction compared to other models available.

7.3.1 Model development approaches

While MLR modelling for predicting the toxicity of metals to freshwater organisms is not a new concept, it has been repopularised in recent years. Since the publication of Brix et al. (2017) suggesting that MLR models may be a suitable alternative to the biotic ligand model (BLM), two approaches to data collection for MLR model development have been applied (Table 7.1). First, models can be developed by utilising data from the literature, combining multiple studies from different laboratories, and second, models can be developed from data produced in a single study under identical laboratory conditions.

This thesis aimed to apply the 'single study' approach to develop a set of MLR models from *Chlorella* sp. zinc toxicity data produced as part of the earlier chapters of the thesis (Chapters 2,3 and 4). This provided a relatively large toxicity dataset under consistent laboratory conditions, from the same culture and strain of microalgae, allowing for highly controlled experiments where only one water chemistry parameter (pH, hardness, DOC concentration or DOC source) was varied at a time. In addition to the benefit of these controlled studies, this approach was taken to grow the microalgal zinc toxicity datasets with non-*R.subcapitata* species data. This data can contribute to the development of trophic-level specific MLR models for microalgae, which prior to the completion of the body of work described in this thesis was not possible, as most zinc toxicity data for microalgae was from *R. subcapitata* tests. Trophic-level approaches have been explored for invertebrates (Peters et al., 2021), where multiple invertebrate species were included in a single model.

The main disadvantage of this 'single study' approach is that smaller datasets are typically used in model development. It is often impractical to conduct enough toxicity tests to produce equisized datasets to those produced by combining multiple studies from the literature. Table 7.1 provides a summary of microalgae MLR models developed for metal toxicity prediction and highlights this difference in dataset sizes between the approaches. A clear example of differences in dataset sizes is seen between the study presented in Chapter 6 and a parallel study conducted at the same time by DeForest et al. (2023). In this thesis, MLR models were developed with 30 datapoints (or 18 in the case of the subset models) whereas the DeForest et al. (2023) study, which used the 'combined literature' approach had datasets of 37, 36 and 54 datapoints for the *R. subcapitata*

EC10, EC20 and EC50 models respectively. As discussed in Chapter 6, these larger datasets often provide greater statistical power for model development but can come at the cost of inconsistency across studies and unbalanced datasets, where one parameter may be under- or over-represented. For example, in the DeForest et al. (2023) *R. subcapitata* EC50 dataset, only 17 of the 54 datapoints had reported DOC concentrations, with the 37 datapoints without reported DOC concentrations being estimated. Conversely, in that same EC50 dataset, only one datapoint did not have a reported hardness value. When comparing this to the present study, or the other two 'single study' approach MLR papers (Brix et al., 2023; DeForest et al., 2018, 2020), all datapoints have a reported DOC concentration. All estimated DOC concentrations in DeForest et al. (2023) appear to be at the limit of detection (LOD) and skew the distribution of data, as shown in the DOC panels of Figure 7.2. Based on the measurements of DOC in this thesis, where no DOC sources were added to the test solution, these estimations at the LOD are likely accurate. However, this relies on studies in the literature providing sufficient detail to assume that no DOC sources were added to the test solution. By the experimental design, the DOC concentration distribution in this thesis is also skewed to <1 mg $C.L^{-1}$ (Figure 7.2), as the experiments assessing pH and hardness in Chapters 2 and 3 had no added DOC source.

Interestingly, when comparing the distribution of water chemistry parameters of both approaches to the distribution of parameters of Australian water chemistry data (Stauber et al. 2023), both approaches broadly cover the 10th to 90th percentile range of the three parameters. The few exceptions to this include the Chapter 6 (green in Figure 7.2) DOC distributions, and the hardness distribution in the DeForest et al. (2023) EC10 model.

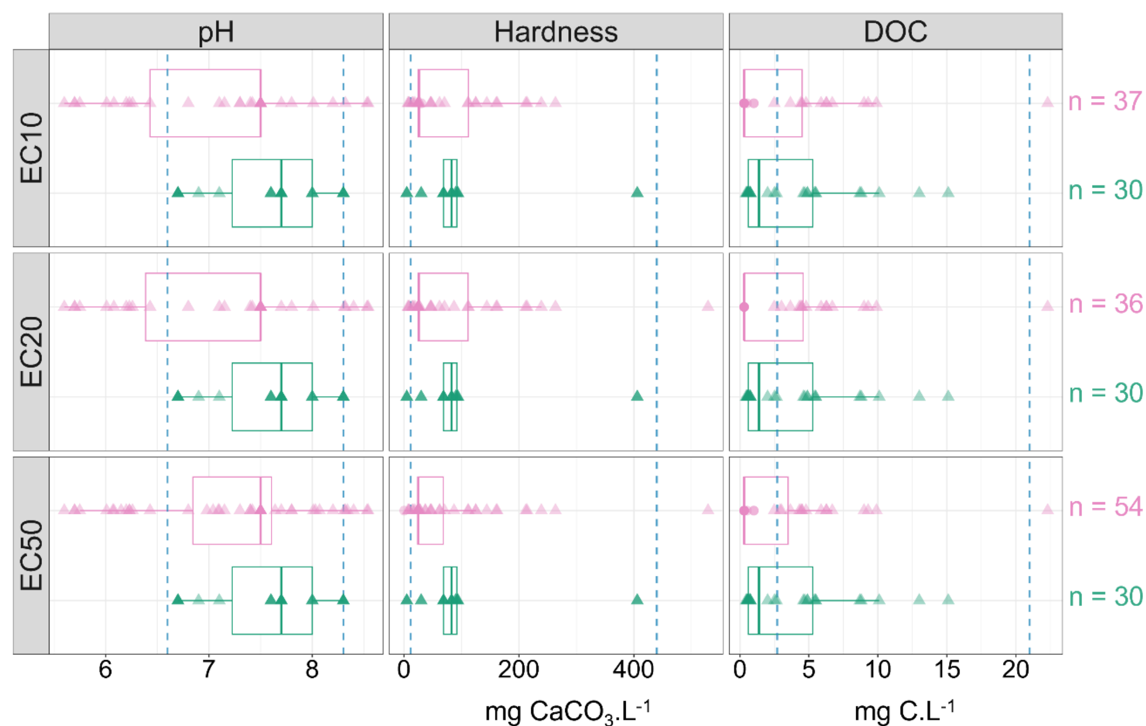

Figure 7.2: Comparison of the distribution of water chemistry parameters used in microalgae multiple linear regression models by DeForest et al. (2023) (pink) and Chapter 6 (green). Triangles represent data that was measured and reported, and circles represent data that was estimated by DeForest et al. (2023). Blue dashed lines represent the 10th and 90th percentile range of parameters within Australian waters based on data reported in Stauber et al. (2023).

As described above and in Chapter 6, both approaches to MLR model development have advantages and disadvantages. When considering which is the most appropriate approach to use, project-specific context is important and should drive decision making. Where regional-specific species data is not of concern, or the region of interest is North America (where most standard test species are found), and large datasets are available in the literature, there is likely reduced benefit to investing resources into producing new toxicity data for model development. However, where regional specificity is important (as is the case in this thesis) or there is limited data available in the literature, as was the case for aluminium (DeForest et al., 2018, 2020) and iron (Brix et al., 2023), resources are likely best placed in producing targeted toxicity data tailored to the needs of model development. Lastly, a third scenario in which a combination of both approaches is used may provide the most robust model outcomes. In this scenario, toxicity data from the literature is used to develop preliminary models that can identify biases or data gaps that can be corrected through small rounds of highly specific toxicity testing. Final models would be developed using both newly generated data and data from previous studies in the literature.

Table 7.1: Compiled data from studies that developed multiple linear regression models for predicting metal toxicity to microalgae.

Reference	**Species**	**Metal**	**Approach**	**Effect level**	**n**	**Intercept**	**pH**	**ln(hard)**	**ln(DOC)**	**ln(DOC) x pH**	**ln(hard) x pH**	**pH x pH**
Chapter 6	*Chlorella* sp.	Zn	Single study	EC10	30	0.16	-0.055	0.288	-2.137	0.302	-	-
Chapter 6	*Chlorella* sp.	Zn	Single study	EC20	30	0.189	-0.009	0.432	-2.114	0.289	-	-
Chapter 6	*Chlorella* sp.	Zn	Single study	EC50	30	1.17	-	0.628	-	-	-	-
Chapter 6	*Chlorella* sp.	Zn	Single study	EC50[a]	18	3.97	-0.359	0.673	0.351	-	-	-
DeForest et al. (2023)	*R. subcapitata*	Zn	Literature	EC10	37	10.31	-0.992	-	0.378	-	-	-
DeForest et al. (2023)	*R. subcapitata*	Zn	Literature	EC20	36	11.95	-1.172	-	0.342	-	-	-
DeForest et al. (2023)	*R. subcapitata*	Zn	Literature	EC50	54	10.93	-0.865	-	0.209	-	-	-
CCME (2018)	*R. subcapitata*	Zn	Literature	EC50	30	11.8	-1.122	-	-	-	-	-
Stauber et al. (2023)	*R. subcapitata*	Zn	Literature	EC50	54	8.28	-0.75	0.296	0.468	-	-	-
Croteau et al. (2021)	*R. subcapitata*	Ni	Literature	EC50	29	3.86	-	0.474	0.221	-	-	-
Peters et al. (2021)	*R. subcapitata*	Ni	Literature	EC10	44	-[b]	-0.20	0.50[c]	0.28	-	-	-
DeForest et al. (2018)	*R. subcapitata*	Al	Single study	EC10	27	-77.28	20.923	4.560	2.342	-0.288	-0.628	-1.27
DeForest et al. (2018)	*R. subcapitata*	Al	Single study	EC20	27	-61.95	17.019	4.007	2.342	-0.204	-0.556	-1.02
Brix et al. (2023)	*R. subcapitata*	Fe	Single study	EC10	25	5.435	0.332	-	0.744	-	-	-
Brix et al. (2023)	*R. subcapitata*	Fe	Single study	EC20[d]	25	6.914	0.173	-	0.541	-	-	-

[a] subset model as described in Chapter 6
[b] Intercept not provided
[c] Peters et al. (2021) separated hardness into magnesium and calcium. This coefficient is based on ln(Mg).
[d] Models were calculated with and without interactions. Only EC20 model without interactions is shown in this table.

7.3.2 Model validation

Validation techniques for metal toxicity prediction models have undergone an iterative process of improvements and change since the original factor-of-2 "rule of thumb" was first proposed by Di Toro et al. (2001) and Santore et al. (2001). Chapter 5 of this thesis explored this factor-of-2 rule and challenged its broad use, given its conceptualisation was based on limited acute EC50 data for *Daphnia magna*. Chapter 5 found that this model validation method may not be applicable across species, such as microalgae, and lower effect levels, such as EC10 and EC20 values. This was a key finding, as several model development studies have applied the factor-of-2 validation method broadly across all species and effect levels and may therefore be penalising models unfairly due to the greater natural variability in certain test species, test endpoints and effect levels. Chapter 6 of this thesis implemented a broad range of validation techniques through autovalidation (validation with development datasets) and independent validation (validation with independent datasets).

There has been substantial evolution in what is considered 'best practice' validation metrics recently. Initial validation steps by Brix et al. (2017) considered the familiar model fit assessment criteria, AIC and BIC, and a visual comparison of predicted vs observed toxicity on a 1:1 line, with factor-of-2 lines also plotted. More comprehensive scoring metrics were discussed and proposed by Garman et al. (2020), where emphasis was placed on both autovalidation and independent validation processes. The study also expanded the validation process beyond a simple comparison of predicted vs observed toxicity to include metrics that considered model bias resulting from individual TMFs. This was achieved by accounting for slope when model residuals were plotted against TMFs. Examples of this are provided in detail in Chapter 6.

Prior to the work of Chapter 5 proper consideration of the broad usefulness of the factor-of-2 rule had only been noted by Peters et al. (2021), where the authors suggested it was unreasonable to expect the same level of certainty in predicting EC10 values from chronic toxicity data compared to EC50 values from acute toxicity data. This speculation was confirmed in Chapter 5 (Price et al. 2022b) which represented one of the few and the largest studies to consider a large dataset of standardised reference toxicant tests for a range of metals, species, and endpoints. This work provided the necessary data to introduce the factor-of-3 rule to the list of validation metrics proposed by Garman et al. (2020). This was particularly important for EC10 datasets and models due to the greater variability associated with lower effect levels, as described in detail in Chapter 5. More recent work by Brix et al. (2021) identified that residual slope scoring processes proposed by Garman et al. (2020) were not particularly sensitive across the range of slopes in their study and subsequently suggested a modified slope scoring tool to reflect a more appropriate sensitivity. The most recent modification to model validation scoring methods was introduced by Besser et

al. (2021) and recently implemented by DeForest et al. (2023) and Chapter 6 of this thesis. This represented a significant improvement in model validation methods, with the inclusion of an uncertainty weighting score by including a residual slope's associated p value. This allowed all TMF residual slopes to be included, but were weighted against their significance in regression analysis. This model performance score (MPS) is discussed and provided in Equation 6.5 of Chapter 6.

A key finding of this thesis was that model autovalidation methods provide little certainty into the ability of the model to predict toxicity to independent data, regardless of the above-mentioned approaches used. This was highlighted in Chapter 6, where autovalidation, plotting of predicted vs observed toxicity data, and the MPS suggested good model performance, yet the models were unable to accurately predict independent toxicity data derived from zinc-spiked natural waters. Possible reasons for these inconsistencies between development and independent natural water toxicity data are discussed at length in Chapter 6. These findings also provide potential concern for other MLR models developed using the 'single study' approach. Chapter 6 (Price et al. 2023a) is the only study currently in the literature with MLR models developed using the 'single study' approach that were independently validated using metal-spiked natural waters. The DeForest et al. (2018, 2020) studies on aluminium MLR development did not include any independent validation. While the more recent iron MLR models development by Brix et al. (2023) did not use natural water validation, the study did use a randomised k-fold cross-validation method. Here, subsets were separated for the development dataset to have all but one subset used as the development data, and the other subset used as the validation dataset. This process is repeated to ensure all subsets are used in validation. While this provides an independent validation method and is better than no independent validation, the method still relies solely on toxicity data derived from synthetic laboratory water. This may not be representative of metal toxicity in a natural water matrix, as discussed in Chapter 6. Further research should investigate how toxicity data derived from synthetic laboratory water may differ from toxicity data derived in natural water. This would ensure that models developed using the 'single study' approach, such as those in this thesis, are representative of toxicity in the natural water bodies for the jurisdictions for which they were developed.

As discussed previously for model development selection, selection of appropriate validation processes is dependent on a project's specific context. While this thesis promotes and encourages using metal-spiked natural waters as an independent validation dataset, this comes at a considerable logistical, time, and financial cost and requires timely transport, filtration, and measurement of physicochemical parameters. When considering samples from hard-to-access remote areas there is also the risk of collecting natural water samples that do not meet specifications for inclusion in an independent validation process, such as those with naturally

elevated nutrients that typically cannot be determined prior to collection. When these barriers are insurmountable, methods such as k-fold cross-validation as utilised by Brix et al. (2023) are a useful alternative.

7.3.3 Considerations of alternate models

While empirical MLR modelling approaches for metal toxicity prediction are not a new concept (Esbaugh et al., 2011, 2012; Welsh et al., 1996), such models have been reproposed on the basis that other model types, namely the biotic ligand model (BLM), are perceived as too complicated for regulators to use (Brix et al., 2017). Furthermore, Brix et al. (2017) suggested that the requirement to measure 10 water chemistry parameters may also act as a barrier to uptake by regulators. The robust science and data needed to underpin water chemistry modified guidelines, a key objective of this thesis, is of little use if end users are unwilling or unable to implement them. The arguments by Brix et al. (2017) for proposed use of empirical-based models (i.e., MLR models) was that they had simpler derivation mechanics, could focus on what was considered key water chemistry parameters (pH, hardness and DOC), and used the same conceptual framework used to derive the already incorporated hardness-based water quality guidelines.

The main difference between MLR models and BLMs is in the methods by which they are developed. MLR models are developed purely by empirical data (often guided by mechanistic information), while BLMs are generally mechanistic or quasi-mechanistic. There have been several studies in recent years comparing MLR models with BLMs for several metals including zinc (DeForest et al., 2023), copper (Brix et al., 2017, 2021), and nickel (Croteau et al., 2021). In general, all studies found that the MLR models performed comparably well, and in some cases better than the BLM. For copper, Brix et al. (2021) found MLR models outperformed BLMs for chronic toxicity data, highlighting that this was mainly due to the copper BLM not being optimised for chronic data, with the BLM in general assuming that TMFs influence acute and chronic toxicity in the same manner. It should be noted that most BLMs are not calibrated using microalgae toxicity data, except for the zinc BLM in DeForest et al. (2023), thereby making microalgae-specific MLR models and BLM comparisons less robust than for invertebrate and fish-specific MLR models. However, as highlighted by Croteau et al. (2021), microalgae data was not available at the time to include in nickel BLM calibrations but was available for inclusion in validation procedures, with validation performance results being acceptable.

In general, there has been less focus on microalgae toxicity data and how it fits into the broader MLR and BLM landscape, as evidenced by its exclusion in the model comparisons of Brix et al. (2017) and (2021). This is largely due to microalgae not being directly incorporated into the calculations of the USEPA Ambient Water Quality Criteria (AWQC). Given most of the research and development in the MLR and BLM space in recent years has been driven by work in the US,

the omission of microalgae is unsurprising. However, it is important to note that while microalgae (and aquatic plants) are not incorporated into those AWQC calculations, assessing the relative sensitivity of organisms used in calculations (i.e., fish, invertebrates, amphibians) to microalgae and aquatic plants is an important step in the AWQC development (USEPA, 1985).

While the benefits of using MLR models, such as their perceived simplicity and relatively comparable performance to the mechanistic BLM, have been highlighted in this chapter and throughout this thesis, there are some potential disadvantages. Given their empirical derivation, the performance of MLR modelling is biased to species with larger datasets. This was evident in the Brix et al. (2017) comparison of copper MLR models and the BLM where the authors found MLR models to perform better than the BLM for species with large datasets (such as the common test species *Daphnia magna* and *Pimephales promelas*) but poorer than the BLM for species with smaller datasets. Furthermore, there have been concerns that MLR modelling may provide a level of reductionism that may put limitations on its predictive capabilities. For example, MLR models describe the influence of each TMF by a linear (or log-linear) trend when these relationships may be non-linear, as demonstrated and discussed by Brix et al. (2020). While non-linearity can be accounted for in MLR modelling through interaction terms, this requires sufficient data to do so and in many cases such data is not available. Importantly, non-linear components in MLR models make extrapolation beyond the range of the development data particularly uncertain.

Another perceived limitation of MLR models (as well as many other aspects of ecotoxicology) is that there is focus only on an effect concentration (ECx), and the rest of the concentration-response curve is often disregarded. This treats all ECx for a given effect level the same and does not consider the role of the slope parameter, which details the rate of toxicity as a function of metal concentration. Examples of concentration-response slope and shape deviating significantly with changing TMFs is shown in Chapter 4, where slope and shape was altered by the presence of DOC, creating a biphasic concentration-response curve rather than the traditional sigmoidal curve. This may be problematic when considering the relationship between a TMF and ECx across different effect levels. However, it is noted that this issue may also arise with BLMs. For example, differences between assumptions in humic-to-fulvic acid ratios in the BLM and in the validation datasets in Brix et al. (2021) was potentially responsible for poor copper BLM performances.

Another potential disadvantage of MLR modelling relative to BLM approaches may be seen when incorporating metal mixtures into toxicity predictions. The BLM is already set up to mechanistically deal with competition effects of other cations, making the possible inclusion of metal mixture effects likely possible without large amounts of data generation. In contrast, to incorporate mixture effects into an MLR model, while relatively straight forward, would likely require a large amount of additional data, and there is currently limited metal mixture toxicity

data under changing water chemistry parameters available. Regardless of the design approach, it would likely require a typically unreasonable amount of new data generation.

Despite the potential and perceived disadvantages or limitations in MLR modelling, the comparisons between MLR models and BLMs to date have shown satisfactory performance and at the very least, improvements on the current hardness-based algorithms in use in several jurisdictions (including the US, Australia and New Zealand). In general, selecting the most appropriate model depends on an array of factors: the application or intended use of the model; the availability of data; the practicality of use; and any policy considerations for a given jurisdiction. Where possible, it would be encouraged to explore both model options and treat each model as complementary of the other.

7.4 IMPLEMENTATION OF BIOAVAILABILITY IN ENVIRONMENTAL MANAGEMENT

The research presented in this thesis adds to the growing body of research around the relationship between water chemistry and zinc toxicity. This data can be used to further incorporate the concepts of bioavailability and TMFs into the Australian and New Zealand water quality guidelines (ANZG, 2018). However, as previously mentioned in this thesis, the development of appropriate data and models to account for bioavailability and toxicity modification in guidelines is only useful if there is uptake by the end users, such as environmental managers and regulators. An important consideration to note is that implementation cannot be legally mandated by the water quality guidelines as they as not legally binding (much like the US and Canada but in contrast to Europe). Rather it is the role of state and/or provincial jurisdictions to adopt and implement such guidelines. This section discusses the implementation of TMF-dependent water quality guidelines in the Australian and New Zealand context, as is the focus of this thesis, and draws on examples from other jurisdictions.

The popularisation of MLR modelling as an alternative to BLM approaches was proposed on the basis of the perceived complexity of the BLM (Brix et al., 2017). While there is truth to this, without appropriate clarity and guidance around the intended use of these models, the choice of modelling approach, be it MLR or BLM, will likely make little difference. A clear example of this is in the lack of implementation guidance with the USEPA release of the copper BLM in the 2007 water quality criteria. Over a decade later, there is still limited uptake and use of the copper BLM. It is unrealistic to assume that without appropriate guidance around the use of bioavailability-based guidelines underpinned by MLR models there will be any greater success in uptake. In Europe, the use of bioavailability in metal water quality assessment has largely been adopted. This success is due to clear guidance documentation, simplified tools (such as bio-met

(Peters et al., 2020)), and a tiered approach that ensures bioavailability assessment is not required at sites with low metal exposures, as determined in early tiers of the approach (Merrington et al., 2023).

Currently, the Australian and New Zealand water quality guidelines are only adjusted for hardness for some metals, such as zinc. However, MLR approach-based guidelines for nickel and zinc have been developed and are in the final stages of the approval process. In preparation for the release of these (and future) guidelines a workshop was held in April 2023 with scientists and regulators from across Australia and New Zealand, and scientists from the UK and US. The objective of the workshop was to discuss the advantages and limitations of bioavailability-based metal guidelines, to better understand the needs and perspectives of regulators regarding implementation, and to identify any further work needed for guideline derivation and implementation for metals. The discussion and outcomes of this workshop are detailed in Stauber and Ryan (2023). Several concerns were raised around the practicalities of measuring the key water chemistry parameters of pH, hardness, and DOC. These included the appropriate use of pH in water systems with strong diurnal pH cycling, how to account for ephemeral systems, and instances in which a particular parameter is lacking. While definitive resolutions to these concerns were not achieved, and perhaps further research is needed to understand the influence of water bodies with varying chemistry with time, there was consensus reached on the best approaches for guideline implementation.

Three approaches were proposed as options for bioavailability-based guideline implementation.

- **Option 1** proposed a Tier 1 assessment where individual samples are compared to a single reference guideline based on the exposure data that has been adjusted to represent a sensitive condition. If Tier 1 fails (i.e., the sample exceeds the reference guideline), the bioavailability of the individual sample should be considered by measuring pH, hardness and DOC.
- **Option 2** proposed adjusting the guideline based on the sample-specific data immediately, without any Tier 1 screening process.

Consensus was that Option 1 was most appropriate as it provided a single number guideline, reduced the total amount of samples requiring water chemistry parameters to be measured, and followed the successful approach currently used in Europe. However, it was acknowledged that further work was needed to develop this single reference guideline, and robust water chemistry data from across Australia and New Zealand was needed for this. Once the water chemistry database is quality checked, an appropriate reference guideline could be established. As there is still work required to develop the reference guidelines, the following third option was agreed upon.

- **Option 3** proposed initial Tier 1 screening against a reference guideline (as per **Option 1**). If the reference guideline was exceeded, a Tier 2 assessment with two options is conducted. Either the metal concentration in the sample can be adjusted based on the sample-specific water chemistry, or the reference guideline can be adjusted for the sample water chemistry (via a simplified tool or look up tables provided in implementation guidance documents). Where the Tier 2 assessment fails, a multiple-lines-of-evidence Tier 3 assessment will be undertaken as outlined in ANZG (2018). A flow diagram illustrating the process is shown in Figure 7.3.

The outcomes of the workshop highlighted the importance and benefit of consultation with end users of bioavailability-based guidelines and will likely lead to improved uptake, implementation, and use of guidelines better able to assess risk of metals in the environment.

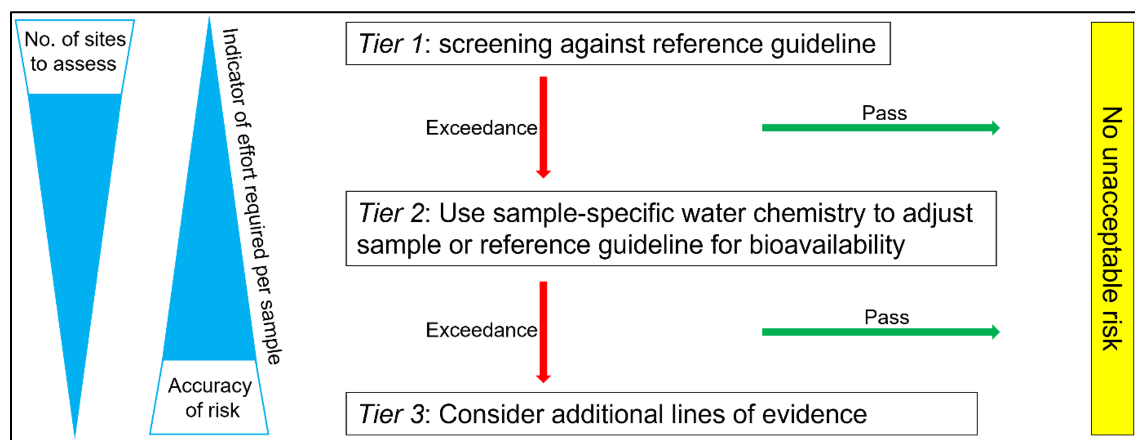

Figure 7.3: Proposed process for the implementation of bioavailability-based guidelines for metals in Australia and New Zealand. Reproduced from Stauber and Ryan et al. (2023).

7.5 FUTURE RESEARCH

This thesis has expanded the knowledge of toxicity of zinc to microalgae and its relationship to pH, hardness, and DOC concentration and source, and has increased microalgae ecotoxicity data for zinc to include more data from an underrepresented species. This thesis has also contributed to a better understanding of and improved validation methods for metal toxicity predictive models by collating, analysing, and publishing large datasets of reference toxicant data to better understand natural variability in ecotoxicological endpoints. This thesis developed the first *Chlorella* sp. MLR for predicting zinc toxicity and expanded the limited number of microalgae models. Based on these outcomes and findings, future work should consider the following:

- Measurements of intra- and extracellular zinc concentrations in *Chlorella* sp. in the presence of humic-dominated DOC to assess whether bioaccumulation explains toxicity interactions. Chapter 4 found the presence of humic-dominated DOC increased zinc toxicity to *Chlorella* sp. As discussed in Chapter 4, other studies with similar findings

found intracellular metal concentrations to correlate with increased toxicity in the presence of DOC.

  - Current studies outside the scope of this thesis are also underway to investigate changes in the proteome and metabolome of *Chlorella* sp. when exposed to zinc in the presence of natural DOC. This may provide insight into the changes in rate of toxicity and concentration-response curve shape in the presence of natural DOC which was observed in Chapter 4.

- Investigate the variability of repeated reference toxicant tests between laboratories, providing better insight into how experimental and biological variability may influence models developed from multiple studies in the literature. Chapter 5 used a large dataset of repeated reference toxicant tests to establish the need to consider a factor-of-3 rule when considering metal prediction model validation and performance. However, this study was conducted using repeated tests from a single laboratory and was unable to examine inter-laboratory variability.
- Assess the comparability of synthetic water toxicity testing and toxicity testing using spiked natural waters. This would provide better insight into the usefulness of a 'single study' approach to MLR development. Chapter 6 developed and validated the first *Chlorella* sp. MLR model for predicting zinc toxicity. The study found that the synthetic laboratory water-derived model could not accurately predict toxicity to zinc-spiked natural waters.

7.6 GENERAL CONCLUSIONS

This thesis was the first to investigate the combined influences of pH and hardness on chronic zinc toxicity to *Chlorella* sp. (Chapter 2 and 3), the first to investigate the independent influence of natural DOC on chronic zinc toxicity to freshwater microalga (Chapter 4); the first to assess the applicability of the factor-of-2 rule for model validation of EC10 and EC20 models; and the first to assess its appropriateness for microalgae toxicity data (Chapter 5). This thesis was also the first to develop a *Chlorella* sp. MLR model for metal toxicity prediction and the first to validate a 'single study'-developed MLR using a zinc-spiked natural water toxicity dataset (Chapter 6).

This thesis improves understanding of zinc toxicity modification by key water chemistry parameters to freshwater microalga and provides high-quality ecotoxicity data and models that underpin new bioavailability-based zinc water quality guidelines for Australia and New Zealand.

This was achieved by successfully meeting the following research objectives:

i. *To assess the influence of key water chemistry parameters on zinc toxicity to a tropical freshwater microalga.*

Varying pH, hardness and DOC concentration and source had substantial influence of chronic zinc toxicity to *Chlorella* sp. as detailed in Chapter 2, 3, and 4. Increasing pH across an environmentally relevant range for Australian freshwaters resulted in an increase in zinc toxicity (EC50). Increases in water hardness had a protective effect on zinc toxicity to *Chlorella* sp. up to 93 mg $CaCO_3.L^{-1}$, with no further increase in protection at higher hardness concentrations. The influence of DOC was dependent on source and composition, with the presence of humic-dominant DOC increasing zinc toxicity to *Chlorella* sp. while fulvic-dominant DOC had little influence on zinc toxicity.

ii. *To determine the relevance of current validation methods for bioavailability modelling.*

The appropriateness of the commonly applied factor-of-2 method for model validation was tested and discussed in this thesis (Chapter 5). This meta-analysis of reference toxicant data for a range of species, metals, effect levels and endpoints represented the largest published assessment of repeated reference toxicant data and provided the necessary information to introduce an alternate validation method, the factor-of-3 method. The study highlighted that different effect levels and species have different inherent validation in toxicity data and that applying certain validation methods may inappropriately penalise models. The findings of Chapter 5 subsequently informed validation methodology for Chapter 6 of this thesis.

iii. *To develop and validate empirical toxicity models to underpin bioavailability-based water quality guidelines for zinc.*

Multiple linear regression models for the prediction of zinc toxicity to *Chlorella* sp. were developed, validated, and discussed in detail in Chapter 6. Models were developed for a range of effect levels and validated against an independent dataset of zinc-spiked natural waters. The developed models were unable to appropriately predict zinc-toxicity to *Chlorella* sp. in zinc-spiked natural waters without a species-sensitivity adjustment. These findings will likely lead to improved understanding in model development using synthetic laboratory waters and how that toxicity data relates to toxicity in spiked natural water.

Environmental risk assessments, through the development and application of water quality guidelines, are continually aiming for improved environmental relevance to ensure robust and defensible management decisions are made. This thesis enhances the understanding of water chemistry's role in zinc toxicity modification to microalgae and provided improved insights into model development and validation that will assist with tailoring freshwater zinc risk assessments that are specific to a given site's water chemistry. This thesis will also contribute to

the development of new freshwater zinc bioavailability-based water quality guidelines for Australia and New Zealand.

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Appendices

Appendix A: The influence of pH on zinc toxicity to *Chlorella* sp.

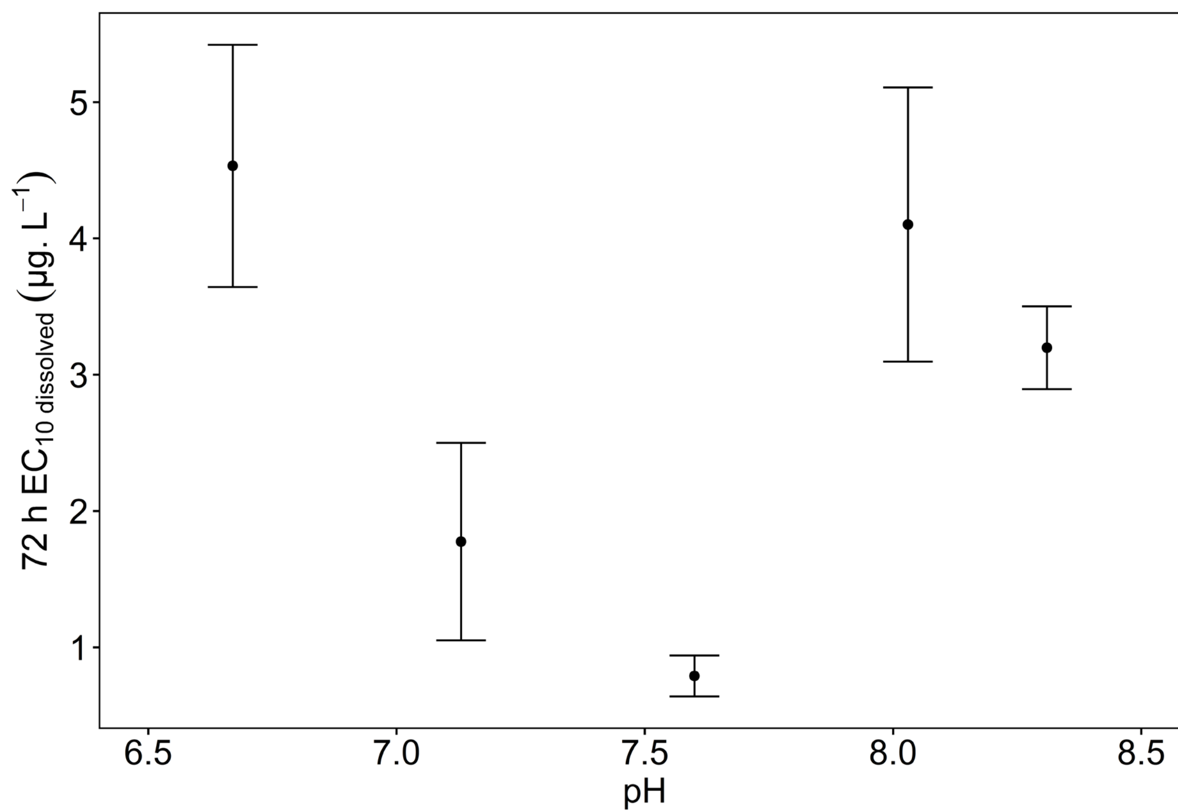

Figure A-1: Relationship between 72 h EC10 concentrations and pH. Error bars indicate +/- standard error of the model estimate.

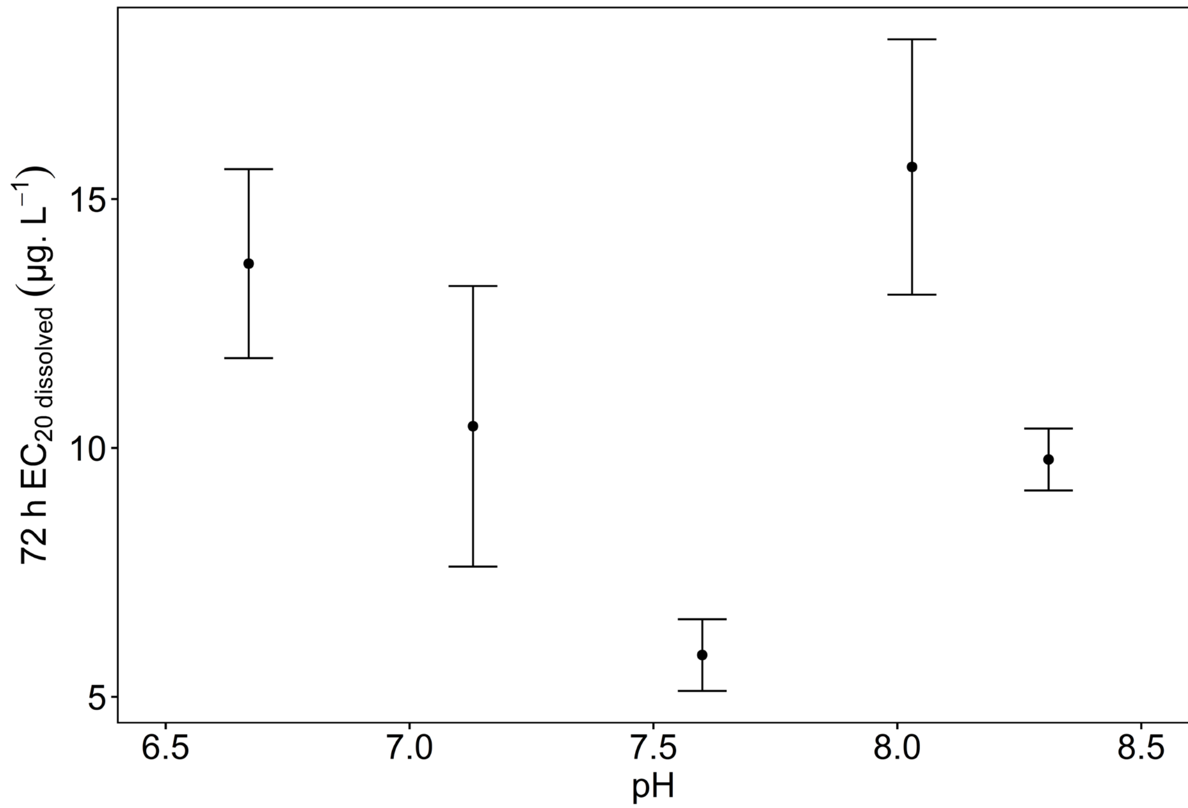

Figure A-2: Relationship between 72 h EC20 concentrations and pH. Error bars indicate +/- standard error of the model estimate.

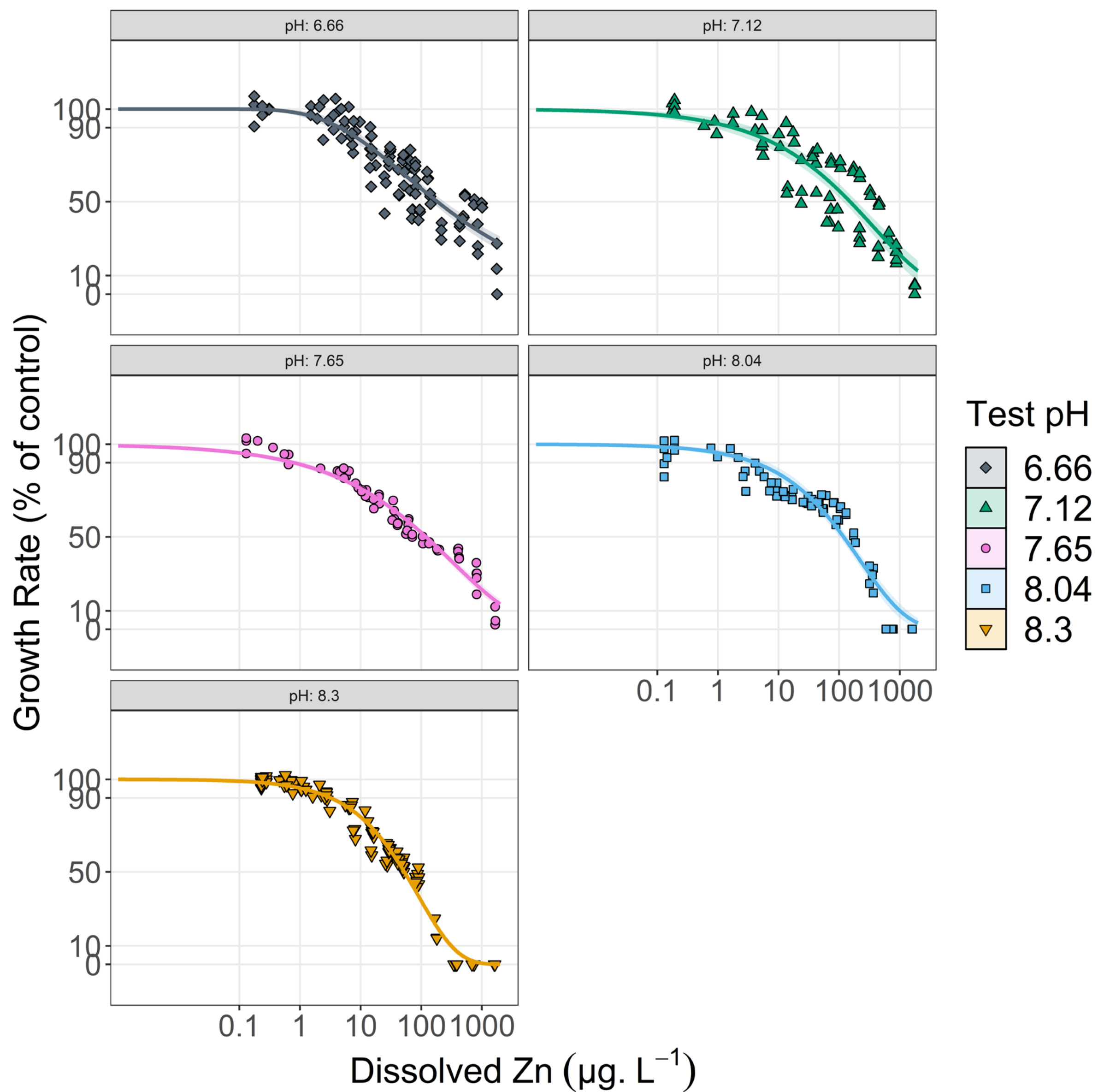

Figure A-3: 72 h growth rate inhibition of *Chlorella* sp. (% of control) exposed to zinc concentrations at five different pH values. Each data point represents one individual replicate response and a corresponding measured zinc concentration. Data is pooled from separate experiments. Replicate responses were normalised to their respective controls for inter-test pooling.

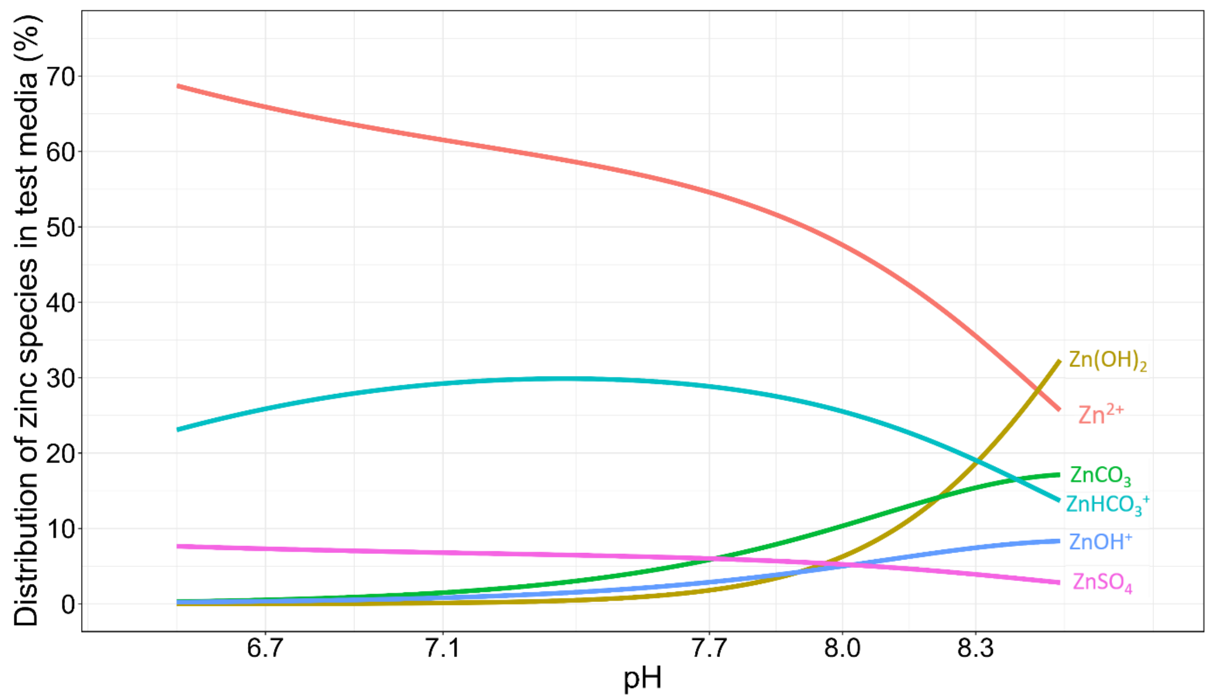

Figure A-4: WHAM7 predicted zinc species distribution across the tested pH range.

Table A- 1: Concentration-response model parameters. n = number of data points in model, b = slope parameter, c = lower limit, d = upper limit, e = inflection parameter, SE = standard error, d.f. = degrees of freedom, E = power of 10

			Parameters											
			b						e					
Test Name	**n**	**Model type**	**Estimate**	**SE**	**t-value**	**p-value**	**c**	**d**	**Estimate**	**SE**	**t-value**	**p-value**	**Residual SE**	**d.f.**
pH 6.5	107	Weibull_2	-0.32	0.02	-15.3	<2.2E-16	0	100	59.5	6.0	9.95	<2.2E-16	10.2	105
pH 7.0	69	Weibull_1	0.42	0.04	11.8	<2.2E-16	0	100	359.5	51.6	6.97	1.8E-09	12.2	66
pH 7.5	69	Weibull_1	0.38	0.01	27.3	<2.2E-16	0	100	317.8	24.0	13.2	<2.2E-16	5.25	64
pH 8.0	69	Weibull_1	0.56	0.04	15.2	<2.2E-16	0	100	227.1	18.7	12.1	<2.2E-16	8.53	65
pH 8.5	111	Weibull_1	0.67	0.02	32.2	<2.2E-16	0	100	91.0	3.3	27.2	<2.2E-16	5.15	108

Table A- 2: Test physicochemical parameters and effect concentrations. n = number of data points in test, DOC = dissolved organic carbon.

Test Name	**No. tests**	**n**	**Hardness ($mg.L^{-1}$)**	**Temp (°C)**	**pH**	**DOC ($mg.L^{-1}$)**	**Ca ($mg.L^{-1}$)**	**Mg ($mg.L^{-1}$)**	**Na ($mg.L^{-1}$)**	**EC10 ($\mu g.L^{-1}$)**	**EC20 ($\mu g.L^{-1}$)**	**EC50 ($\mu g.L^{-1}$)**
pH 6.5	3	107	92.9	27.1	6.7	0.66	15.1	13.8	30	4.5	13.7	184.6
pH 7.0	2	69	92.5	26.2	7.1	0.44	15.2	13.5	30	1.8	10	151.4
pH 7.5	2	69	93.8	26.0	7.6	0.60	15.3	13.5	30	0.79	5.8	119.7
pH 8.0	2	69	93.8	26.0	8.0	0.60	15.3	13.5	30	4.1	15.6	118.1
pH 8.5	3	111	92.9	27.2	8.3	0.69	15.2	13.3	30	3.2	9.8	52.7
Unbuffered	5	82	92.8	26.7	7.5 - 8.3	0.54	15.2	13.3	30	2.7	7.7	45.2

Table A- 3: WHAM7 predicted zinc species distribution across the tested pH range.

Species (%)	**pH 6.7**	**pH 7.1**	**pH 7.7**	**pH 8.0**	**pH 8.3**
Zn^{2+}	60.5	57.5	50.8	42.2	30.1
$ZnOH^{+}$	0.3	0.8	1.9	4.2	6.5
$Zn(OH)_2$	0.02	0.1	0.8	0.05	17.0
$ZnSO_4$	6.7	6.3	5.6	0.05	0.03
$ZnCO_3$	0.4	1.5	4.2	9.5	13.4
$ZnCl^{+}$	<0.01	<0.01	<0.01	<0.01	<0.01
$ZnHCO_3^{+}$	23.3	27.1	25.7	21.9	16.2

Appendix B: The influence of hardness at varying pH on zinc toxicity to *Chlorella* sp..

Table B- 1: Salt masses used for toxicity test media preparation. Salt masses are based on the 'moderately hard' (90 mg.L^{-1}) recipe by USEPA (2002) with modified calcium and magnesium salt masses to alter hardness.

	Salt mass (g) required per 1 L of media prepared			
Test Hardness (nominal) (mg.L^{-1})	**$NaHCO_3$**	**$CaSO_4.2H_2O$**	**$MgSO_4.7H_2O$**	**KCl**
5	0.096	0.0036	0.0072	0.004
30	0.096	0.0212	0.0434	0.004
90	0.096	0.06	0.123	0.004
400	0.096	0.2824	0.5788	0.004

Table B- 2: WHAM7 input values. Temperature and pressure were held constant at 27 °C and 0.00038 atm for all input lines. HA = colloidal humic acid.

Test Name	**Sample ID**	**pH**	**Zn**	**HA**	**Na**	**Mg**	**K**	**Ca**	**Cl**	**NO_3**	**SO_4**	**CO_3**	**PO_4**
			(µg.L^{-1})					**(mg.L^{-1})**					
Hardness 5 - pH 6.5	Control	6.7	0.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Control	6.7	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1	6.7	1.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1	6.7	0.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1	6.7	0.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1	6.7	0.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn3	6.7	2.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn3	6.7	3.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn3	6.7	3.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn3	6.7	3.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn5	6.7	5.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn5	6.7	4.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn5	6.7	5.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 5 - pH 6.5	Zn5	6.7	4.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn7	6.7	6.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn7	6.7	7.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn10	6.7	10.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn10	6.7	9.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn10	6.7	10.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn10	6.7	10.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn15	6.7	15.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn15	6.7	15.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn20	6.7	21.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn20	6.7	19.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn30	6.7	29.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn30	6.7	28.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn30	6.7	28.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn30	6.7	30.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn50	6.7	52.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn50	6.7	53.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn60	6.7	56.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn60	6.7	57.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn100	6.7	59.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn100	6.7	62.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn100	6.7	101.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn100	6.7	101.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn250	6.7	112.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn250	6.7	114.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn500	6.7	223.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn500	6.7	219.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn500	6.7	409.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn500	6.7	406.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1000	6.7	432.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1000	6.7	428.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1000	6.7	828.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn1000	6.7	824.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn5000	6.7	2331.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 6.5	Zn5000	6.7	2293.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)	(mg.L^{-1})									
Hardness 5 - pH 7.5	Control	7.6	0.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Control	7.6	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1	7.6	0.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1	7.6	0.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1	7.6	0.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1	7.6	0.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn3	7.6	2.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn3	7.6	2.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn3	7.6	2.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn3	7.6	3.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5	7.6	4.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5	7.6	4.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5	7.6	4.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5	7.6	4.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn7	7.6	5.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn7	7.6	6.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn10	7.6	8.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn10	7.6	9.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn12	7.6	9.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn12	7.6	11.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn15	7.6	13.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn15	7.6	12.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn20	7.6	18.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn20	7.6	17.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn20	7.6	16.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn20	7.6	16.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn30	7.6	26.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn30	7.6	26.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn40	7.6	32.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn40	7.6	33.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 5 - pH 7.5	Zn60	7.6	52.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn60	7.6	52.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn90	7.6	73.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn90	7.6	69.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn100	7.6	94.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn100	7.6	93.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn150	7.6	110.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn150	7.6	109.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn250	7.6	186.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn250	7.6	181.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn250	7.6	164.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn250	7.6	162.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn500	7.6	375.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn500	7.6	365.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1000	7.6	555.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn1000	7.6	635.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5000	7.6	3058.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 7.5	Zn5000	7.6	3852.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Control	8.3	0.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn1	8.3	0.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn1	8.3	1.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn2	8.3	0.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn2	8.3	0.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn3	8.3	2.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn3	8.3	1.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn3	8.3	1.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn3	8.3	1.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn4	8.3	2.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn4	8.3	1.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 5 - pH 8.5	Zn5	8.3	2.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn5	8.3	1.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn5	8.3	2.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn5	8.3	3.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn8	8.3	4.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn8	8.3	6.0	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn10	8.3	4.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn10	8.3	5.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn10	8.3	6.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn10	8.3	6.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn12	8.3	6.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn12	8.3	6.6	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn15	8.3	8.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn15	8.3	10.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn20	8.3	10.9	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn20	8.3	12.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn30	8.3	16.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn30	8.3	17.8	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn50	8.3	30.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn50	8.3	34.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn60	8.3	38.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn60	8.3	38.2	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn100	8.3	62.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn100	8.3	75.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn100	8.3	62.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn100	8.3	64.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn200	8.3	119.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn200	8.3	120.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn250	8.3	144.1	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn250	8.3	144.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn500	8.3	319.3	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn500	8.3	301.7	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn1000	8.3	557.4	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 5 - pH 8.5	Zn1000	8.3	544.5	0.8	30	0.6	2.1	0.9	1.9	1.5	5	68.6	0.15
Hardness 30 - pH 6.5	Control	6.7	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 30 - pH 6.5	Control	6.7	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Control	6.7	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Control	6.7	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Control	6.7	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Control	6.7	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn1	6.7	1.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn1	6.7	1.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn2	6.7	2.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn2	6.7	1.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn3	6.7	3.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn3	6.7	2.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn3	6.7	2.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn3	6.7	2.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn5	6.7	4.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn5	6.7	4.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn5	6.7	4.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn5	6.7	3.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn7	6.7	6.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn7	6.7	7.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn8	6.7	7.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn8	6.7	7.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn10	6.7	8.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn10	6.7	8.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn15	6.7	16.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn15	6.7	15.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn20	6.7	19.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn20	6.7	19.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn25	6.7	24.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn25	6.7	23.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn50	6.7	44.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn50	6.7	45.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn60	6.7	60.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn60	6.7	60.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn100	6.7	86.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn100	6.7	86.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 30 - pH 6.5	Zn200	6.7	189.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn200	6.7	191.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn250	6.7	280.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn250	6.7	277.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn400	6.7	374.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn400	6.7	376.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn600	6.7	533.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn600	6.7	538.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn750	6.7	816.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn750	6.7	795.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn1000	6.7	999.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn1000	6.7	958.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn2000	6.7	2055.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 6.5	Zn2000	6.7	1948.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Control	7.6	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn1	7.6	0.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn1	7.6	0.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn2	7.6	1.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn2	7.6	1.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn3	7.6	2.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn3	7.6	2.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn3	7.6	2.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn3	7.6	2.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn5	7.6	3.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn5	7.6	4.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn5	7.6	3.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn5	7.6	3.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn7	7.6	5.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn7	7.6	5.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn8	7.6	5.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)	(mg.L^{-1})									
Hardness 30 - pH 7.5	Zn8	7.6	5.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn10	7.6	7.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn10	7.6	7.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn15	7.6	14.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn15	7.6	14.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn20	7.6	16.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn20	7.6	15.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn25	7.6	21.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn25	7.6	21.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn50	7.6	40.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn50	7.6	39.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn60	7.6	54.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn60	7.6	53.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn100	7.6	76.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn100	7.6	76.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn200	7.6	170.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn200	7.6	172.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn250	7.6	250.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn250	7.6	253.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn400	7.6	348.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn400	7.6	331.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn600	7.6	493.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn600	7.6	499.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn750	7.6	715.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn750	7.6	727.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn1000	7.6	914.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn1000	7.6	917.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn2000	7.6	1893.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 7.5	Zn2000	7.6	1905.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Control	8.3	0.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 30 - pH 8.5	Zn1	8.3	0.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn1	8.3	0.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn1	8.3	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn1	8.3	0.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn2	8.3	0.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn2	8.3	0.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn3	8.3	0.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn3	8.3	1.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn3	8.3	1.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn3	8.3	1.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn4	8.3	1.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn4	8.3	1.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn5	8.3	1.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn5	8.3	2.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn6	8.3	3.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn6	8.3	2.9	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn8	8.3	4.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn8	8.3	3.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn10	8.3	5.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn10	8.3	5.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn12	8.3	5.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn12	8.3	5.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn15	8.3	9.6	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn15	8.3	10.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn20	8.3	15.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn20	8.3	13.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn20	8.3	12.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn20	8.3	10.0	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn30	8.3	21.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn30	8.3	20.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn50	8.3	30.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn50	8.3	33.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn75	8.3	52.1	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn75	8.3	48.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn100	8.3	65.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			(µg.L^{-1})	(mg.L^{-1})									
Hardness 30 - pH 8.5	Zn100	8.3	60.7	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn200	8.3	150.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn200	8.3	152.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn250	8.3	202.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn250	8.3	194.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn400	8.3	303.4	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn400	8.3	286.3	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn600	8.3	432.5	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn600	8.3	435.2	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn1000	8.3	715.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 30 - pH 8.5	Zn1000	8.3	685.8	0.5	30	4	2.1	5	1.9	1.5	25	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.4	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Control	6.7	0.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5	6.7	9.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5	6.7	11.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5	6.7	4.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5	6.7	5.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn10	6.7	17.7	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn10	6.7	9.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn10	6.7	10.8	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn10	6.7	9.7	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn20	6.7	19.7	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn20	6.7	18.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn30	6.7	38.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn30	6.7	31.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn40	6.7	36.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn40	6.7	36.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn50	6.7	46.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn50	6.7	49.4	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn60	6.7	60.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn60	6.7	61.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			(µg.L^{-1})					(mg.L^{-1})					
Hardness 400 - pH 6.5	Zn80	6.7	78.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn80	6.7	77.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn100	6.7	105.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn100	6.7	102.7	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn100	6.7	104.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn100	6.7	100.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn250	6.7	216.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn250	6.7	225.5	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn400	6.7	356.8	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn400	6.7	358.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn500	6.7	452.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn500	6.7	442.6	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn750	6.7	672.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn750	6.7	669.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn1000	6.7	910.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn1000	6.7	878.5	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn1000	6.7	880.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn1000	6.7	874.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn2000	6.7	1769.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn2000	6.7	1760.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn2000	6.7	1810.7	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn2000	6.7	1821.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5000	6.7	5029.7	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5000	6.7	4836.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5000	6.7	2270.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 6.5	Zn5000	6.7	2264.8	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Control	7.6	0.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5	7.6	5.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5	7.6	6.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5	7.6	4.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)	(mg.L^{-1})									
Hardness 400 - pH 7.5	Zn5	7.6	4.8	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn10	7.6	8.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn10	7.6	8.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn10	7.6	9.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn10	7.6	9.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn20	7.6	16.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn20	7.6	16.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn30	7.6	26.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn30	7.6	25.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn40	7.6	33.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn40	7.6	32.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn50	7.6	43.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn50	7.6	43.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn60	7.6	57.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn60	7.6	55.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn80	7.6	73.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn80	7.6	69.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn100	7.6	92.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn100	7.6	87.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn100	7.6	98.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn100	7.6	94.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn250	7.6	206.6	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn250	7.6	201.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn400	7.6	345.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn400	7.6	344.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn500	7.6	422.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn500	7.6	416.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn750	7.6	638.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn750	7.6	628.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn1000	7.6	858.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn1000	7.6	810.4	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn1000	7.6	858.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn1000	7.6	865.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn2000	7.6	1696.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn2000	7.6	1641.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)	(mg.L^{-1})									
Hardness 400 - pH 7.5	Zn2000	7.6	1745.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn2000	7.6	1724.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5000	7.6	4592.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5000	7.6	4582.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5000	7.6	2252.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 7.5	Zn5000	7.6	2214.6	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Control	8.3	0.1	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn0	8.3	0.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn0	8.3	0.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn1	8.3	0.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn1	8.3	0.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn3	8.3	1.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn3	8.3	1.3	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5	8.3	3.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5	8.3	2.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5	8.3	2.8	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5	8.3	2.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn10	8.3	6.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn10	8.3	5.5	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn10	8.3	5.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn10	8.3	5.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn20	8.3	11.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn20	8.3	11.9	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn30	8.3	20.6	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn30	8.3	19.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn40	8.3	26.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn40	8.3	26.5	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn50	8.3	32.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn50	8.3	30.6	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn60	8.3	45.7	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15

Test Name	Sample ID	pH	Zn	HA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
			($\mu g.L^{-1}$)					(mg.L^{-1})					
Hardness 400 - pH 8.5	Zn60	8.3	45.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn90	8.3	60.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn90	8.3	60.4	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn100	8.3	71.8	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn100	8.3	66.6	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn150	8.3	112.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn150	8.3	111.2	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn250	8.3	165.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn250	8.3	158.4	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn400	8.3	304.7	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn400	8.3	295.0	0.6	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn500	8.3	343.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn500	8.3	320.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn750	8.3	513.9	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn750	8.3	489.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn1000	8.3	715.3	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn1000	8.3	689.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn2000	8.3	1430.1	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn2000	8.3	1475.2	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5000	8.3	4086.0	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15
Hardness 400 - pH 8.5	Zn5000	8.3	3946.7	0.5	30	55	2.1	71	1.9	1.5	347	68.6	0.15

Table B- 3: Concentration-response model parameters. n = number of data points in model, b = slope parameter, c = lower limit, d = upper limit, e = inflection parameter, SE = standard error, d.f. = degrees of freedom, E = power of 10

			Parameters											
			b						e					
Test Name	n	Model type	Estimate	SE	t-value	p-value	c	d	Estimate	SE	t-value	p-value	Residual SE	d.f.
Hard 5 - pH 6.5	53	Weibull_2	-0.69	0.06	-11.1	3.1E-15	0	100	5.1	0.50	10.1	8.3E-14	9.88	51
Hard 5 - pH 7.5	53	Weibull_2	-0.54	0.05	-10.4	3.2E-14	0	100	8.5	1.1	7.64	5.3E-10	12.3	51
Hard 5 - pH 8.5	51	Weibull_2	-0.61	0.04	-15.3	<2.2E-16	0	100	3.4	0.27	12.7	<2.2E-16	7.86	49
Hard 30 - pH 6.5	50	Weibull_2	-0.57	0.05	-11.4	2.9E-15	6.9	100	13.6	1.6	8.74	1.7E-11	10.1	48
Hard 30 - pH 7.5	50	Weibull_2	-0.44	0.03	-14.6	<2.2E-16	0	100	14.0	1.7	8.35	6.5E-11	9.25	48
Hard 30 - pH 8.5	52	Weibull_2	-0.54	0.03	-17.2	<2.2E-16	0	100	6.4	0.52	12.1	<2.2E-16	7.22	50
Hard 90 - pH 6.5	107	Weibull_2	-0.32	0.02	-15.2	<2.2E-16	0	100	59.5	6.0	9.95	<2.2E-16	10.2	105
Hard 90 - pH 7.5	69	Weibull_1	0.38	0.01	27.3	<2.2E-16	0	100	317.8	24.0	13.2	<2.2E-16	5.25	64
Hard 90 - pH 8.5	111	Weibull_1	0.67	0.02	32.2	<2.2E-16	0	100	91.0	3.3	27.2	<2.2E-16	5.15	108
Hard 400 - pH 6.5	50	Weibull_2	-0.53	0.04	-14.1	<2.2E-16	22	100	20.6	1.5	13.8	<2.2E-16	3.23	47
Hard 400 - pH 7.5	50	Weibull_2	-0.33	0.03	-11.7	1.3E-15	0	100	53.1	8.4	6.29	9.0E-08	10.4	48
Hard 400 - pH 8.5	50	Weibull_1	0.69	0.04	16.5	<2.2E-16	0	100	99.4	6.7	14.9	<2.2E-16	6.72	48

Table B- 4: Physicochemical characteristics of each treatment water. Data were pooled across the number of repeated tests and means of pooled data are shown. Hardness was calculated using measured Ca and Mg concentrations. Alkalinity measured as total alkalinity in $CaCO_3$ measured by titration. Measurements for Ca, Mg, Na, and alkalinity were taken from a bulk test solution sample for each hardness value. pH was measured every 24 h from each test flask, with the mean value reported below.

						Major cations ($mg.L^{-1}$)		
Test Name	No. tests	Hardness ($mg.L^{-1}$)	pH	No. of flasks per test	Alkalinity ($mg.L^{-1}$)	Ca	Mg	Na
Hard 5 - pH 6.5	2	5	6.7	25 – 27	47	0.9	0.6	30
Hard 5 - pH 7.5	2	5	7.6	25 – 27	47	0.9	0.6	30
Hard 5 - pH 8.5	2	5	8.3	25	47	0.9	0.6	30
Hard 30 - pH 6.5	2	31	6.7	25	33	5	4	30
Hard 30 - pH 7.5	2	31	7.6	25	33	5	4	30
Hard 30 - pH 8.5	2	31	8.3	25 – 27	33	5	4	30
Hard 90 - pH 6.5	3	93	6.7	33 – 39	39	15	14	30
Hard 90 - pH 7.5	2	93	7.6	33 – 36	39	15	14	30
Hard 90 - pH 8.5	3	93	8.3	33 – 45	39	15	14	30
Hard 400 - pH 6.5	2	402	6.7	25	36	71	55	30
Hard 400 - pH 7.5	2	402	7.6	25	36	71	55	30

Hard 400 - pH 8.5	2	402	8.3	25	36	71	55	30

Table B- 5: Test results summary. m = number of data points per test.

Test Name	No. tests	n	Hardness ($mg.L^{-1}$)	Temp (°C)	pH	DOC ($mg.L^{-1}$)	EC10 ($\mu g.L^{-1}$)	EC20 ($\mu g.L^{-1}$)	EC50 ($\mu g.L^{-1}$)
Hard 5 - pH 6.5	2	53	5	27	6.7	0.75	1.5	2.6	8.7
Hard 5 - pH 7.5	2	53	5	27	7.6	0.75	1.8	3.5	17
Hard 5 - pH 8.5	2	51	5	27	8.3	0.75	0.85	1.5	6.2
Hard 30 - pH 6.5	2	50	31	27	6.7	0.50	3.2	6.9	43
Hard 30 - pH 7.5	2	50	31	27	7.6	0.5	2.1	4.7	32
Hard 30 - pH 8.5	2	52	31	27	8.3	0.5	1.3	2.6	13
Hard 90 - pH 6.5	3	107	93	27	6.7	0.66	4.5	13.7	185
Hard 90 - pH 7.5	2	69	93	27	7.6	0.6	0.8	5.8	120
Hard 90 - pH 8.5	3	111	93	27	8.3	0.69	3.2	9.8	53
Hard 400 - pH 6.5	2	50	402	27	6.7	0.49	5.3	12	96
Hard 400 - pH 7.5	2	50	402	27	7.6	0.49	4.4	13	159
Hard 400 - pH 8.5	2	50	402	27	8.3	0.49	3.9	11	59

Table B- 6: WHAM7 predicted zinc species distribution for each water chemistry conditions tested.

	Species (%)						
Test conditions	Zn^{2+}	$ZnOH^{+}$	$Zn(OH)_2$	$ZnSO_4$	$ZnCO_3$	$ZnCl^{+}$	$ZnHCO_3^{+}$
Hardness 5 - pH 6.5	68.3	0.4	0	0.6	0.6	0	30
Hardness 5 - pH 7.5	56.6	2.6	1.3	0.5	5.6	0	33.4
Hardness 5 - pH 8.5	34	7.7	20	0.3	17.3	0	20.7
Hardness 31 - pH 6.5	67.7	0.4	0	2.8	0.6	0	28.5
Hardness 31 - pH 7.5	56.6	2.5	1.3	2.3	5.3	0	32
Hardness 31 - pH 8.5	34.6	7.6	19.5	1.4	16.6	0	20.2
Hardness 93 - pH 6.5	61.8	0.3	0	6.9	0.5	0	23.8
Hardness 93 - pH 7.5	50.8	1.9	0.8	5.5	4.1	0	25.7
Hardness 93 - pH 8.5	30.8	6.7	17.3	3.4	13.7	0	16.6
Hardness 402 - pH 6.5	61.4	0.3	0	17.4	0.4	0	20.5
Hardness 402 - pH 7.5	54.3	2	1	15.3	3.7	0	23.8
Hardness 402 - pH 8.5	37	6.9	16.6	10.5	12.6	0	16.4

Appendix C: The influence of DOC on zinc toxicity to *Chlorella* sp.

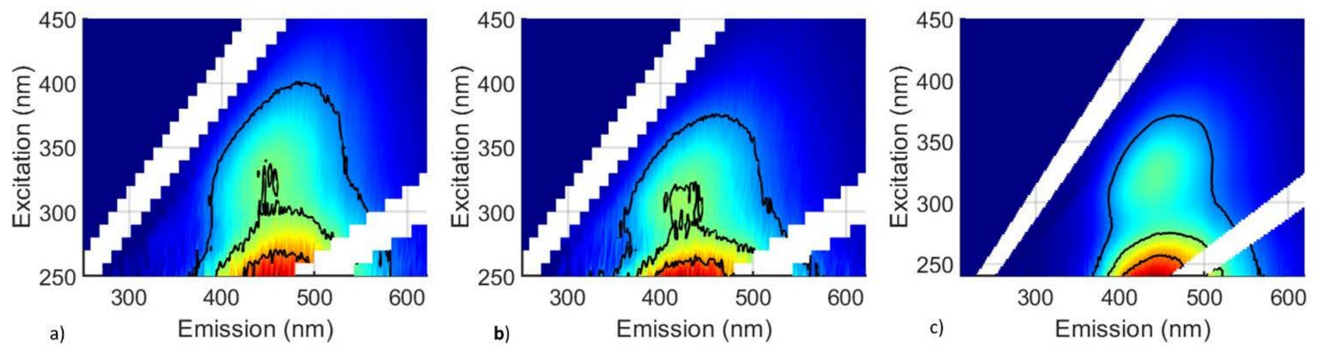

Figure C-1: Fluorescence excitation emission scans of a) Appletree Creek DOC; b) Manton Dam DOC; and c) Suwannee River DOC

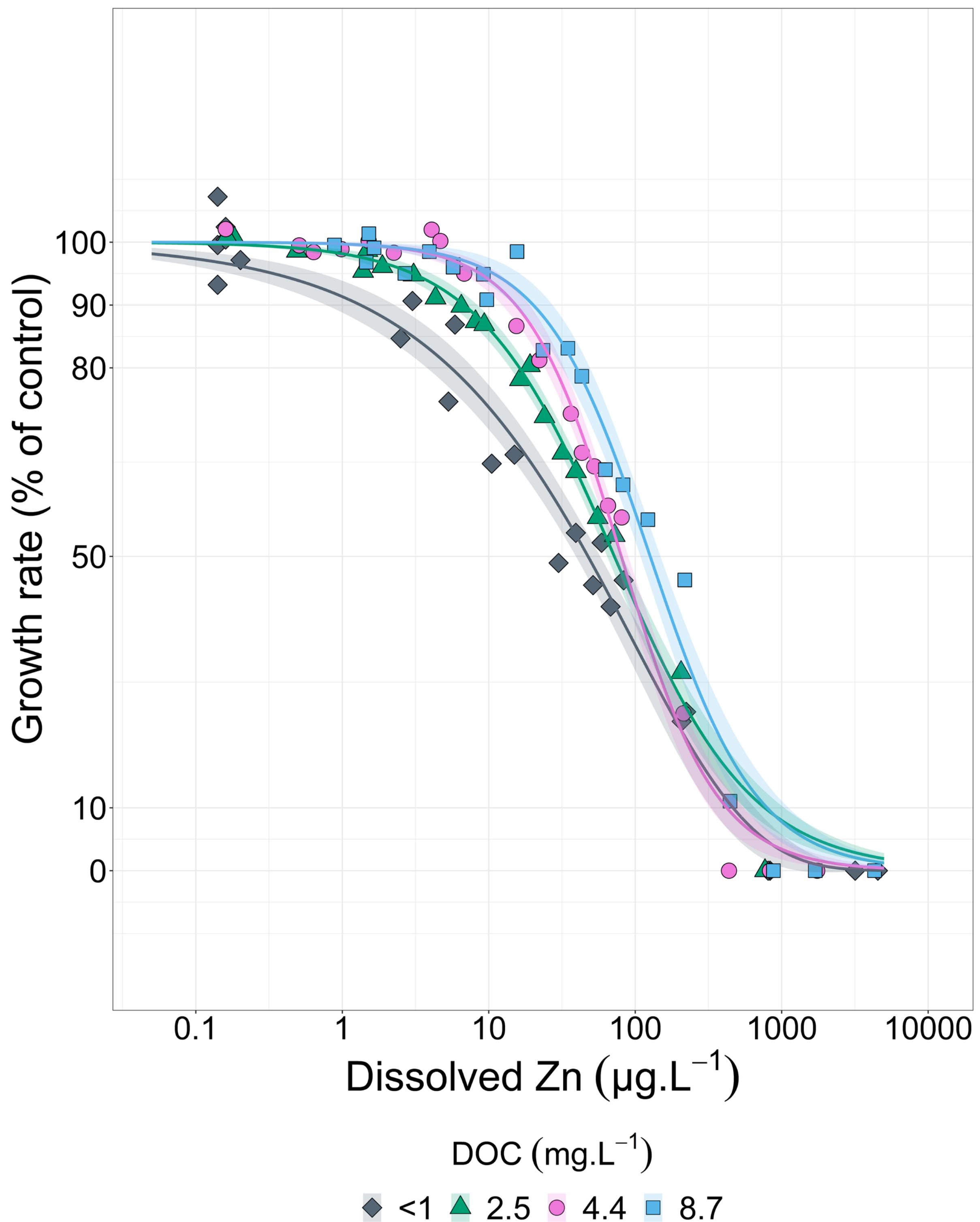

Figure C-2: Concentration-response curves indicating the effect of Suwannee River dissolved organic matter (DOC) at increasing concentrations of DOM (<1–8.7 mg.L^{-1}) on the growth rate of *Chlorella* sp. when exposed to dissolved zinc. Shaded ribbons represent the 95% confidence intervals. Each datapoint 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.

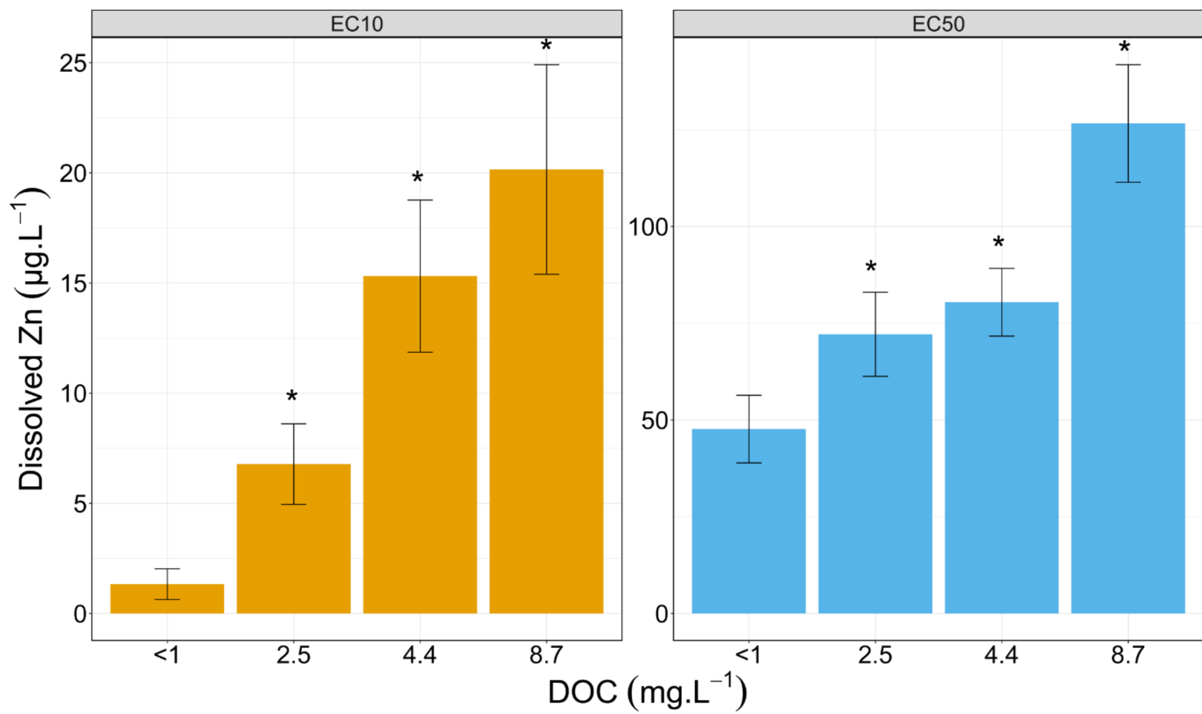

Figure C-3: Comparison of EC10 and EC50 values for zinc as a function of dissolved organic carbon (DOC) concentration Suwannee River DOC. Error bars indicate the calculated lower and upper 95% confidence intervals. Note variable y-axis scales. * Indicates significant difference ($p < 0.05$) from the no added DOC treatment (<1 mg.L^{-1}).

Table C- 1: Dissolved organic carbon characterisation. Data collected using excitation emissions (EEMS). Both naturally collected DOC sources were analysed at time of collection and at the time of toxicity testing. SAC340 = specific absorbance coefficient at 340 nm, SUVA254 = ultra-violet-absorbing molecules, FI = fluorescence index, abs = absorbance.

	Absorbance and/or Fluorescence Indices				**EEMS**		
	SAC340	**SUVA254**	**FI**	**abs**	**% Humic**	**% Fulvic**	**% Protein**
Appletree Creek Stock (2017 Summer/Autumn collection)	44.33	5.38	1.43	4.07	56.03	36.38	7.59
Appletree Creek Stock (2021 toxicity testing)	49.47	5.67	1.32	3.79	58.88	34.67	6.45
Manton Dam Stock (2016 Winter/Spring collection)	11.13	2.29	1.58	7.58	35.40	45.71	18.89
Manton Dam Stock (2021 toxicity testing)	13.59	2.27	1.58	5.96	36.94	47.04	16.02
Suwannee River Dissolved Organic Carbon	22.77	3.05	1.42	4.78	51.47	48.52	0.00

Table C- 2: Dissolved organic carbon concentrate metals analysis. Data collected using undiluted DOC concentrate on the ICP-AES. Two limit of detections (LOD) are listed below as samples were analysed on separate ICP batches, NA = calibration curve was out of range for Al on the Manton Dam batch. Note: both concentrates are diluted between 10 - 100 fold for final toxicity test concentrations, therefore all ion concentrations contributed by DOM addition are below limit of detection of low (i.e. Fe)

	Ag	**Al**	**Cd**	**Cu**	**Fe**	**Mg**	**Ni**	**Pb**	**Zn**
	$\mu g.L^{-1}$								
Limit of Detection	0.13	0.13	0.15	0.74	0.4	1.62	0.39	0.88	0.11
Appletree Creek Concentrate	0.71	31.3	0.18	7.1	550	11	13	<LOD	3.1
Limit of Detection	0.33	NA	0.21	0.99	0.30	2.2	0.63	2.3	0.35
Manton Dam Concentrate	<LOD	NA	<LOD	5.6	155	16	0.91	<LOD	4.1

Table C- 3: Recovery percentages for each DOC at time of collection and concentration.

	Recovery %
Appletree Creek DOC	82
Manton Dam DOC	76

Table C- 4: Concentration-response model parameters. n = number of data points in model, b = slope parameter, c = lower limit, d = upper limit, e = inflection parameter, SE = standard error, d.f. = degrees of freedom, E = power of 10, LL= log-logistic model

Test Name	n	Model type	b Estimate	b SE	b t-value	b p-value	c	d	e Estimate	e SE	e t-value	e p-value	Residual SE	d.f.
Control (No added DOC)	85	LL	0.52	0.02	24.8	<2.2E-16	0	100	111.7	7.7	14.5	<2.2E-16	6.1	83
Manton Dam - 2.5 mg/L	32	LL	0.62	0.04	17.6	<2.2E-16	0	100	70.9	6.1	11.6	0	5.1	30
Manton Dam - 5.4 mg/L	51	LL	0.69	0.03	21.5	<2.2E-16	0	100	86.2	5.3	16.4	<2.2E-16	5.1	49
Manton Dam - 10.1 mg/L	51	LL	0.69	0.04	19.1	<2.2E-16	0	100	107.2	7.5	14.4	<2.2E-16	5.7	49
Manton Dam - 15.1 mg/L	32	LL	0.73	0.05	15.4	8.7E-16	0	100	126.2	11.3	11.1	0	6.0	30
Appletree Creek - 2.0 mg/L	32	Weibull_2	-0.72	0.09	-8.2	4.0E-09	25.2	100	4.8	0.71	6.7	1.9E-07	8.4	30
Appletree Creek - 4.6 mg/L	32	Weibull_2	-0.78	0.15	-5.2	1.5E-05	25.9	100	5.5	1.2	4.5	9.5E-05	10.8	30
Appletree Creek - 8.8 mg/L	36	Weibull_2	-0.92	0.18	-5.1	1.3E-05	29.6	100	6.1	1.2	5.0	1.5E-05	12.8	34
Appletree Creek - 13.0 mg/L	37	Weibull_2	-0.87	0.16	-5.5	3.4E-06	28.3	100	7.5	1.5	4.9	2.2E-05	13.0	35
Manton Dam - 5.5 mg/L pH 6.7	36	LL	1.05	0.10	11.1	7.3E-13	28.7	100	15.0	1.5	10.3	5.7E-12	6.1	34
Manton Dam - 5.5 mg/L pH 8.3	36	Weibull_1	0.73	0.03	21.7	<2.2E-16	0.0	100	60.3	3.2	18.9	<2.2E-16	3.9	34
Appletree Creek - 4.9 mg/L pH 6.7	36	Weibull_2	-0.81	0.05	-15.3	<2.2E-16	30.6	100	4.9	0.32	15.3	<2.2E-16	4.2	34
Appletree Creek - 4.9 mg/L pH 8.3	36	LL	0.98	0.06	16.3	<2.2E-16	0.0	100	18.6	1.3	14.5	<2.2E-16	5.6	34
Control (No added DOC, unbuffered)	24	Weibull_1	0.53	0.04	15.1	4.6E-13	0.0	100	95.6	9.5	10.1	1.0E-09	5.3	22
Suwannee River - 2.5 mg/L	20	LL	0.93	0.05	20.6	6.0E-14	0.0	100	72.1	4.1	17.6	8.9E-13	3.0	18
Suwannee River - 4.4 mg/L	20	LL	1.32	0.08	15.9	4.8E-12	0.0	100	80.4	4.0	20.1	8.9E-14	3.6	18
Suwannee River - 8.7 mg/L	22	LL	1.20	0.09	13.2	2.4E-11	0.0	100	126.6	9.4	13.5	1.8E-11	4.9	20

Table C- 5: WHAM input data for DOC-Cu and DOC-Zn displacement calculations discussed in section 4.3.2. HA = humic acid, FA = fulvic acid.

Test Name	pH	Zn	Cu	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)		(mg.L^{-1})										
Appletree 2mg.L^{-1} Low Zn	7.6	10	1	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2mg.L^{-1} High Zn	7.6	5000	1	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} Low Zn	7.6	10	1	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} High Zn	7.6	5000	1	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10mg.L^{-1} Low Zn	7.6	10	1	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10mg.L^{-1} High Zn	7.6	5000	1	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15mg.L^{-1} Low Zn	7.6	10	1	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 mg.L^{-1} High Zn	7.6	5000	1	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} at pH 6.5 Low Zn	6.7	10	1	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} at pH 6.5 High Zn	6.7	5000	1	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} at pH 8.5 Low Zn	8.3	10	1	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5mg.L^{-1} at pH 8.5 High Zn	8.3	5000	1	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Table C- 6: WHAM output data for DOC-Cu and DOC-Zn displacement calculations discussed in section 4.3.2. Low Zn = 10 $\mu g.L^{-1}$, High Zn = 5000 $\mu g.L^{-1}$, E = power of 10

Species ($\mu g.L^{-1}$)	Water chemistry scenario					
	2 mg C.L^{-1} Low Zn	2 mg C.L^{-1} High Zn	5 mg C.L^{-1} Low Zn	5 mg C.L^{-1} High Zn	10 mg C.L^{-1} Low Zn	10 mg C.L^{-1} High Zn
Total Cu	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00
Total Zn	1.0E+01	5.0E+03	1.0E+01	5.0E+03	1.0E+01	5.0E+03
True solution Cu	7.3E-03	5.4E-01	1.0E-03	2.6E-01	2.4E-04	1.3E-01
True solution Zn	5.9E+00	4.8E+03	2.7E+00	4.4E+03	1.4E+00	3.9E+03
True solution Cu^{2+}	2.7E-04	2.1E-02	3.8E-05	9.8E-03	9.0E-06	5.1E-03
True solution $CuOH^{+}$	4.0E-04	3.0E-02	5.4E-05	1.4E-02	1.3E-05	7.3E-03
True solution $Cu(OH)_2$	4.1E-05	3.1E-03	5.7E-06	1.5E-03	1.4E-06	7.7E-04
True solution Zn^{2+}	3.3E+00	2.7E+03	1.6E+00	2.5E+03	8.1E-01	2.2E+03
True solution $ZnOH^{+}$	1.7E-01	1.4E+02	8.1E-02	1.3E+02	4.2E-02	1.2E+02
True solution $Zn(OH)_2$	1.0E-01	8.5E+01	4.9E-02	7.8E+01	2.5E-02	7.0E+01
DOC-bound Cu Fraction	9.9E-01	4.6E-01	1.0E+00	7.4E-01	1.0E+00	8.7E-01
DOC-bound Zn Fraction	4.1E-01	4.6E-02	7.3E-01	1.2E-01	8.6E-01	2.2E-01

Table C-6 continued.

Species (µg.L^{-1})	Water chemistry scenario					
	15 mg C.L^{-1} Low Zn	15 mg C.L^{-1} High Zn	5 mg C.L^{-1} pH 6.5 Low Zn	5 mg C.L^{-1} pH 6.5 High Zn	5 mg C.L^{-1} pH 8.5 Low Zn	5 mg C.L^{-1} pH 8.5 High Zn
Total Cu	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00	1.0E+00
Total Zn	1.0E+01	5.0E+03	1.0E+01	1.0E+01	5.0E+03	1.0E+01
True solution Cu	8.1E-05	7.8E-02	1.0E-03	7.3E-03	5.4E-01	1.0E-03
True solution Zn	8.7E-01	3.4E+03	3.2E+00	5.9E+00	4.8E+03	2.7E+00
True solution Cu^{2+}	3.0E-06	3.0E-03	7.1E-05	2.7E-04	2.1E-02	3.8E-05
True solution $CuOH^{+}$	4.4E-06	4.3E-03	1.3E-05	4.0E-04	3.0E-02	5.4E-05
True solution $Cu(OH)_2$	4.6E-07	4.5E-04	1.7E-07	4.1E-05	3.1E-03	5.7E-06
True solution Zn^{2+}	4.9E-01	1.9E+03	2.1E+00	3.3E+00	2.7E+03	1.6E+00
True solution $ZnOH^{+}$	2.6E-02	1.0E+02	1.4E-02	1.7E-01	1.4E+02	8.1E-02
True solution $Zn(OH)_2$	1.5E-02	6.1E+01	1.1E-03	1.0E-01	8.5E+01	4.9E-02
DOM-bound Cu Fraction	1.0E+00	9.2E-01	1.0E+00	9.9E-01	4.6E-01	1.0E+00
DOM-bound Zn Fraction	9.1E-01	3.2E-01	6.8E-01	4.1E-01	4.6E-02	7.3E-01

Table C-7: Effect concentration data summaries.

Test Name	EC10 (µg.L^{-1})				EC20 (µg.L^{-1})				EC50 (µg.L^{-1})			
	Estimate	Std. Error	Lower	Upper	Estimate	Std. Error	Lower	Upper	Estimate	Std. Error	Lower	Upper
Control (No added DOC)	1.57	0.27	1.04	2.11	7.59	0.87	5.85	9.32	111.67	7.69	96.38	126.96
Manton Dam - 2.5 mg.L^{-1}	2.03	0.43	1.15	2.91	7.54	1.11	5.28	9.80	70.86	6.10	58.41	83.31
Manton Dam - 5.4 mg.L^{-1}	3.53	0.56	2.40	4.67	11.49	1.28	8.92	14.05	86.21	5.27	75.62	96.79
Manton Dam - 10.1 mg.L^{-1}	4.51	0.79	2.92	6.10	14.52	1.77	10.96	18.08	107.21	7.47	92.19	122.23
Manton Dam - 15.1 mg.L^{-1}	6.12	1.22	3.62	8.62	18.70	2.59	13.41	23.99	126.16	11.34	103.01	149.31
Appletree Creek - 2.0 mg.L^{-1}	1.82	0.40	1.00	2.64	3.26	0.56	2.12	4.40	16.82	2.89	10.93	22.72
Appletree Creek - 4.6 mg.L^{-1}	2.25	0.79	0.64	3.86	3.87	1.03	1.77	5.98	17.99	3.76	10.31	25.68
Appletree Creek - 8.8 mg.L^{-1}	2.93	0.81	1.29	4.58	4.74	1.03	2.64	6.84	19.66	5.08	9.34	29.99
Appletree Creek - 13.0 mg.L^{-1}	3.41	0.96	1.46	5.36	5.62	1.26	3.06	8.19	24.51	6.09	12.14	36.87
Manton Dam - 5.5 mg.L^{-1} pH 6.7	2.66	0.48	1.68	3.64	6.10	0.76	4.55	7.64	33.67	4.15	25.24	42.10

Test Name	EC10 ($\mu g.L^{-1}$)				EC20 ($\mu g.L^{-1}$)				EC50 ($\mu g.L^{-1}$)			
	Estimate	Std. Error	Lower	Upper	Estimate	Std. Error	Lower	Upper	Estimate	Std. Error	Lower	Upper
Manton Dam - 5.5 $mg.L^{-1}$ pH 8.3	2.78	0.35	2.07	3.48	7.75	0.64	6.45	9.04	36.51	1.68	33.09	39.93
Appletree Creek - 4.9 $mg.L^{-1}$ pH 6.7	2.15	0.21	1.72	2.59	3.71	0.27	3.16	4.27	19.17	1.67	15.78	22.56
Appletree Creek - 4.9 $mg.L^{-1}$ pH 8.3	1.98	0.27	1.43	2.53	4.53	0.43	3.65	5.41	18.61	1.28	16.01	21.22
Control (No added DOC, unbuffered)	1.33	0.35	0.61	2.06	5.54	0.98	3.52	7.56	47.65	4.37	38.60	56.71
Suwannee River - 2.5 $mg.L^{-1}$	6.78	0.91	4.95	8.61	16.23	1.47	13.29	19.17	72.13	5.44	61.24	83.02
Suwannee River - 4.4 $mg.L^{-1}$	15.30	1.73	11.85	18.76	28.23	2.20	23.82	32.64	80.41	4.38	71.63	89.19
Suwannee River - 8.7 $mg.L^{-1}$	20.15	2.38	15.39	24.91	39.71	3.36	32.97	46.45	126.60	7.59	111.39	141.81

Table C-8: Metal measurement summaries for diffusive gradient in thin film (DGT) measurements, Ultrafiltration (< 3kDa), dissolved (<0.45 µm) and total zinc.

Test Name	DGT-lability experiments		
	Nominal zinc	Dissolved zinc ($\mu g.L^{-1}$)	DGT-labile zinc
Manton Dam - 2.5 $mg.L^{-1}$	10	5.6	4.9
Manton Dam - 2.5 $mg.L^{-1}$	50	35.0	26.7
Manton Dam - 2.5 $mg.L^{-1}$	100	69.5	56.5
Manton Dam - 2.5 $mg.L^{-1}$	500	376.9	249.0
Manton Dam - 5.4 $mg.L^{-1}$	10	5.5	4.7
Manton Dam - 5.4 $mg.L^{-1}$	50	33.3	22.7
Manton Dam - 5.4 $mg.L^{-1}$	100	67.9	54.2
Manton Dam - 5.4 $mg.L^{-1}$	500	346.0	262.2
Manton Dam - 10.1 $mg.L^{-1}$	10	5.7	4.9
Manton Dam - 10.1 $mg.L^{-1}$	50	34.9	23.2
Manton Dam - 10.1 $mg.L^{-1}$	100	72.4	46.0
Manton Dam - 10.1 $mg.L^{-1}$	500	367.3	224.3
Manton Dam - 15.1 $mg.L^{-1}$	10	5.9	4.5
Manton Dam - 15.1 $mg.L^{-1}$	50	36.5	22.7
Manton Dam - 15.1 $mg.L^{-1}$	100	70.6	40.3
Manton Dam - 15.1 $mg.L^{-1}$	500	357.1	237.4
Appletree Creek - 2.0 $mg.L^{-1}$	10	6.4	6.1
Appletree Creek - 2.0 $mg.L^{-1}$	50	38.2	31.9
Appletree Creek - 2.0 $mg.L^{-1}$	100	66.9	63.8
Appletree Creek - 2.0 $mg.L^{-1}$	250	190.7	154.3

Appletree Creek - 2.0 mg.L^{-1}	500	378.7	314.0
Appletree Creek - 4.6 mg.L^{-1}	10	6.5	6.5
Appletree Creek - 4.6 mg.L^{-1}	50	38.0	29.8
Appletree Creek - 4.6 mg.L^{-1}	100	70.1	57.5
Appletree Creek - 4.6 mg.L^{-1}	250	189.1	157.1
Appletree Creek - 4.6 mg.L^{-1}	500	385.5	294.6
Appletree Creek - 8.8 mg.L^{-1}	10	7.6	4.4
Appletree Creek - 8.8 mg.L^{-1}	50	40.2	24.2
Appletree Creek - 8.8 mg.L^{-1}	100	69.2	48.0
Appletree Creek - 8.8 mg.L^{-1}	250	188.3	128.5
Appletree Creek - 8.8 mg.L^{-1}	500	367.8	275.7
Appletree Creek - 13.0 mg.L^{-1}	10	7.3	4.0
Appletree Creek - 13.0 mg.L^{-1}	50	40.0	21.1
Appletree Creek - 13.0 mg.L^{-1}	100	71.1	39.8
Appletree Creek - 13.0 mg.L^{-1}	250	193.7	120.0
Appletree Creek - 13.0 mg.L^{-1}	500	381.6	250.0

Table C-8: continued.

Test Name	Ultrafiltration experiments			
	Nominal zinc	Dissolved zinc	Ultrafilterable zinc (µg.L^{-1})	Total zinc
Manton Dam - 2.5 mg.L^{-1}	10	6.6	-	7.9
Manton Dam - 2.5 mg.L^{-1}	100	74.0	79.8	83.6
Manton Dam - 2.5 mg.L^{-1}	250	240.8	225.1	239.4
Manton Dam - 2.5 mg.L^{-1}	1000	814.0	830.7	861.0
Manton Dam - 5.4 mg.L^{-1}	10	5.8	6.6	8.3
Manton Dam - 5.4 mg.L^{-1}	100	79.6	83.5	87.9
Manton Dam - 5.4 mg.L^{-1}	250	213.0	209.8	234.0
Manton Dam - 5.4 mg.L^{-1}	1000	766.4	799.6	863.0
Manton Dam - 10.1 mg.L^{-1}	10	6.2	6.6	8.7
Manton Dam - 10.1 mg.L^{-1}	100	68.9	71.5	83.9
Manton Dam - 10.1 mg.L^{-1}	250	211.4	197.8	225.2
Manton Dam - 10.1 mg.L^{-1}	1000	766.4	748.0	862.3
Manton Dam - 15.1 mg.L^{-1}	10	6.2	-	8.6
Manton Dam - 15.1 mg.L^{-1}	100	73.7	71.3	85.7
Manton Dam - 15.1 mg.L^{-1}	250	222.8	191.7	231.4

Test Name	Ultrafiltration experiments			
	Nominal zinc	Dissolved zinc	Ultrafilterable zinc	Total zinc
	($\mu g.L^{-1}$)			
Manton Dam - 15.1 $mg.L^{-1}$	1000	785.5	741.9	860.9
Appletree Creek - 2.0 $mg.L^{-1}$	10	7.3	8.9	9.5
Appletree Creek - 2.0 $mg.L^{-1}$	10	7.7	10.5	9.1
Appletree Creek - 2.0 $mg.L^{-1}$	100	77.8	84.9	84.1
Appletree Creek - 2.0 $mg.L^{-1}$	250	240.4	238.5	219.3
Appletree Creek - 2.0 $mg.L^{-1}$	1000	883.3	763.3	981.5
Appletree Creek - 2.0 $mg.L^{-1}$	1000	914.1	843.2	898.6
Appletree Creek - 4.6 $mg.L^{-1}$	10	7.7	7.0	6.5
Appletree Creek - 4.6 $mg.L^{-1}$	10	8.1	8.1	9.7
Appletree Creek - 4.6 $mg.L^{-1}$	100	118.5	110.8	134.4
Appletree Creek - 4.6 $mg.L^{-1}$	100	88.4	81.0	93.3
Appletree Creek - 4.6 $mg.L^{-1}$	500	456.7	415.1	519.1
Appletree Creek - 4.6 $mg.L^{-1}$	500	475.6	407.4	481.0
Appletree Creek - 4.6 $mg.L^{-1}$	1000	913.1	780.0	1007.6
Appletree Creek - 4.6 $mg.L^{-1}$	1000	941.4	615.8	1052.2
Appletree Creek - 8.8 $mg.L^{-1}$	10	8.9	8.0	9.8
Appletree Creek - 8.8 $mg.L^{-1}$	10	8.3	7.8	9.0
Appletree Creek - 8.8 $mg.L^{-1}$	50	45.0	40.6	44.8
Appletree Creek - 8.8 $mg.L^{-1}$	50	45.8	39.1	44.4
Appletree Creek - 8.8 $mg.L^{-1}$	100	86.9	74.9	90.6
Appletree Creek - 8.8 $mg.L^{-1}$	100	88.3	75.1	87.2
Appletree Creek - 8.8 $mg.L^{-1}$	1000	937.2	860.7	990.1
Appletree Creek - 8.8 $mg.L^{-1}$	1000	936.4	852.7	916.9
Appletree Creek - 13.0 $mg.L^{-1}$	10	8.6	8.1	10.0
Appletree Creek - 13.0 $mg.L^{-1}$	10	9.6	7.5	11.0
Appletree Creek - 13.0 $mg.L^{-1}$	50	45.6	37.0	48.2
Appletree Creek - 13.0 $mg.L^{-1}$	50	45.6	37.5	46.5
Appletree Creek - 13.0 $mg.L^{-1}$	100	88.8	71.6	91.6
Appletree Creek - 13.0 $mg.L^{-1}$	100	91.7	69.4	95.5
Appletree Creek - 13.0 $mg.L^{-1}$	1000	892.0	794.9	937.7
Appletree Creek - 13.0 $mg.L^{-1}$	1000	903.8	774.8	905.5

Table C-9: WHAM input data for EC10 and EC50 comparisons across dissolved organic carbon concentration ranges in Section 4.3.4. Temperature and pressure were held constant at 27 °C and 0.00038 atm for all input lines. HA = colloidal humic acid, FA = colloidal fulvic acid.

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
No DOC Control EC10	7.7	1.6	0	0	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
No DOC Control EC50	7.7	112	0	0	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ EC10	7.6	1.8	2.25	1.45	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ EC50	7.6	17	2.25	1.45	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ EC10	7.6	2.3	5.19	3.34	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ EC50	7.6	18	5.2	3.3	25.9	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 $mg.L^{-1}$ EC10	7.6	2.9	9.89	6.36	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 $mg.L^{-1}$ EC50	7.6	20	9.89	6.36	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ EC10	7.6	3.4	14.6	9.4	25.9	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ EC50	7.6	25	14.58	9.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 EC10	6.7	2.2	5.53	3.56	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 EC50	6.7	19	5.53	3.56	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 EC10	8.3	2	5.53	3.56	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 EC50	8.3	19	5.53	3.56	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Control No DOC EC10	7.6	1.6	0	0	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Control No DOC EC50	7.6	112	0	0	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ EC10	7.6	2	1.76	2.28	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ EC50	7.6	71	1.76	2.28	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ EC10	7.6	3.5	3.8	4.9	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ EC50	7.6	86	3.8	4.9	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ EC10	7.6	4.5	7.16	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ EC50	7.6	107	7.16	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ EC10	7.6	6.1	10.72	13.83	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ EC50	7.6	126	10.72	13.83	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 6.5 EC10	6.7	2.7	3.9	5.04	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 6.5 EC50	6.7	34	3.9	5.04	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 EC10	8.3	2.8	3.91	5.05	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 EC50	8.3	37	3.91	5.05	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Table C-10: WHAM output data for EC10 and EC50 comparisons across dissolved organic carbon concentration ranges in Section 4.3.4, E = power of 10

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Control No DOC EC10	1.6E+00	1.6E+00	8.8E-01	5.8E-02	4.4E-02	0.0E+00
Control No DOC EC50	1.1E+02	1.1E+02	6.2E+01	4.1E+00	3.1E+00	0.0E+00
Appletree 2 $mg.L^{-1}$ EC10	1.8E+00	6.4E-01	3.6E-01	1.9E-02	1.1E-02	6.4E-01
Appletree 2 $mg.L^{-1}$ EC50	1.7E+01	9.2E+00	5.2E+00	2.7E-01	1.6E-01	4.6E-01
Appletree 5 $mg.L^{-1}$ EC10	2.3E+00	4.0E-01	2.3E-01	1.2E-02	7.1E-03	8.3E-01
Appletree 5 $mg.L^{-1}$ EC50	1.8E+01	5.4E+00	3.1E+00	1.6E-01	9.6E-02	7.0E-01
Appletree 10 $mg.L^{-1}$ EC10	2.9E+00	2.6E-01	1.5E-01	7.6E-03	4.6E-03	9.1E-01
Appletree 10 $mg.L^{-1}$ EC50	2.0E+01	3.2E+00	1.8E+00	9.4E-02	5.6E-02	8.4E-01
Appletree 15 $mg.L^{-1}$ EC10	3.4E+00	1.9E-01	1.1E-01	5.7E-03	3.4E-03	9.4E-01
Appletree 15 $mg.L^{-1}$ EC50	2.5E+01	2.7E+00	1.5E+00	7.9E-02	4.8E-02	8.9E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 EC10	2.2E+00	4.3E-01	2.8E-01	1.9E-03	1.4E-04	8.1E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 EC50	1.9E+01	6.5E+00	4.3E+00	2.9E-02	2.2E-03	6.6E-01
Appletree 5 $mg.L^{-1}$ at pH 8.5 EC10	2.0E+00	3.7E-01	1.3E-01	3.5E-02	1.0E-01	8.2E-01
Appletree 5 $mg.L^{-1}$ at pH 8.5 EC50	1.9E+01	6.1E+00	2.2E+00	5.7E-01	1.7E+00	6.8E-01
Manton 2 $mg.L^{-1}$ EC10	2.0E+00	6.3E-01	3.5E-01	1.9E-02	1.1E-02	6.9E-01
Manton 2 $mg.L^{-1}$ EC50	7.1E+01	4.4E+01	2.5E+01	1.3E+00	7.9E-01	3.9E-01
Manton 5 $mg.L^{-1}$ EC10	3.5E+00	6.0E-01	3.4E-01	1.8E-02	1.1E-02	8.3E-01
Manton 5 $mg.L^{-1}$ EC50	8.6E+01	3.5E+01	2.0E+01	1.0E+00	6.3E-01	5.9E-01
Manton 10 $mg.L^{-1}$ EC10	4.5E+00	4.0E-01	2.3E-01	1.2E-02	7.3E-03	9.1E-01
Manton 10 $mg.L^{-1}$ EC50	1.1E+02	2.6E+01	1.5E+01	7.9E-01	4.8E-01	7.5E-01
Manton 15 $mg.L^{-1}$ EC10	6.1E+00	3.6E-01	2.0E-01	1.1E-02	6.5E-03	9.4E-01
Manton 15 $mg.L^{-1}$ EC50	1.3E+02	2.1E+01	1.2E+01	6.3E-01	3.8E-01	8.3E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 EC10	2.7E+00	5.2E-01	3.5E-01	2.3E-03	1.8E-04	8.1E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 EC50	3.4E+01	1.3E+01	8.5E+00	5.7E-02	4.3E-03	6.2E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 EC10	2.8E+00	5.1E-01	1.8E-01	4.9E-02	1.5E-01	8.2E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 EC50	3.7E+01	1.3E+01	4.6E+00	1.2E+00	3.7E+00	6.5E-01

Table C-11: WHAM input data for calculations for all test treatments and replicates. Temperature and pressure were held constant at 27 °C and 0.00038 atm for all input lines. HA = colloidal humic acid, FA = colloidal fulvic acid.

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 2 $mg.L^{-1}$ Test 1	7.6	0.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	0.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	0.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	3.4	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	3.5	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	7.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	7.7	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	28.6	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	28.6	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	49.6	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	49.5	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	80.5	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	77.8	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	156.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	156.1	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	237.7	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	240.4	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	883.3	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	914.1	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 1	7.6	4751.6	1.89	1.21	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	0.5	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	0.7	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	1.1	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	0.9	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	6.5	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	2.4	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	5.4	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	10.4	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	24.7	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	101.2	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	525.1	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 2 $mg.L^{-1}$ Test 2	7.6	3077.3	2.61	1.68	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 5 $mg.L^{-1}$ Test 1	7.6	0.3	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	0.3	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	0.3	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	7.7	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	8.1	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	18.1	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	19.7	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	45.3	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	44.4	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	118.5	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	88.4	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	234.6	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	225.3	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	456.7	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	475.6	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	913.1	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	941.4	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	1904.0	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	1849.2	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 1	7.6	4822.6	5.25	3.38	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	0.4	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	0.2	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	1.0	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	8.6	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	3.5	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	4.9	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	9.8	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	23.0	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	28.0	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	98.9	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	498.3	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ Test 2	7.6	2866.6	5.12	3.29	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 $mg.L^{-1}$ Test 1	7.6	0.2	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 $mg.L^{-1}$ Test 1	7.6	0.2	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 $mg.L^{-1}$ Test 1	7.6	0.3	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		(µg.L^{-1})	(mg.L^{-1})										
Appletree 10 mg.L^{-1} Test 1	7.6	2.8	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	2.4	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	5.1	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	4.4	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	8.9	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	8.3	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	22.1	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	22.5	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	45.0	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	45.8	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	86.9	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	88.3	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	421.1	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	427.4	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	937.2	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	936.4	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	1962.4	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	1863.6	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	2798.6	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	2758.0	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 1	7.6	7013.3	9.73	6.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	0.3	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	0.5	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	0.5	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	1.3	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	1.9	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	3.5	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	7.5	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	17.3	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	34.1	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	140.2	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	472.4	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 10 mg.L^{-1} Test 2	7.6	2774.0	10.05	6.46	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 mg.L^{-1} Test 1	7.6	3.1	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 mg.L^{-1} Test 1	7.6	0.2	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 15 $mg.L^{-1}$ Test 1	7.6	0.2	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	2.2	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	2.3	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	4.0	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	3.9	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	8.6	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	9.6	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	22.5	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	22.6	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	45.6	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	45.6	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	88.8	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	91.7	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	427.7	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	425.2	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	892.0	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	903.8	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	1796.7	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	1842.1	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	2778.3	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	2758.8	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	6937.1	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 1	7.6	4645.9	14.41	9.26	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	0.7	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	1.4	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	1.5	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	1.6	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	2.2	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	7.0	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	8.2	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	18.5	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	34.5	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	134.2	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	478.2	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 15 $mg.L^{-1}$ Test 2	7.6	2799.8	14.76	9.49	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	0.4	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	0.3	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	0.7	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	1.6	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	3.2	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	3.7	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	5.2	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	7.9	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	9.0	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	16.6	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	21.4	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	27.4	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	50.2	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	76.6	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	100.4	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	244.9	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	499.4	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	954.2	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	1969.8	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.7	3982.7	5.41	3.48	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	0.8	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	0.3	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	0.3	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	1.5	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	1.8	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	3.1	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	5.2	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	6.9	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	12.7	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	17.6	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	24.1	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	27.8	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	34.3	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	82.1	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	267.3	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.7	1062.1	5.66	3.64	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	0.4	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	0.3	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	0.8	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	0.8	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	1.4	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	3.1	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	4.0	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	5.4	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	6.9	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	11.4	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	14.6	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	20.2	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	38.4	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	57.3	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	73.7	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	196.7	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	408.7	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	692.6	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	1475.3	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	3338.8	5.51	3.54	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.5	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.5	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.3	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.7	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	1.4	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	2.0	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	3.3	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	6.3	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	7.7	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	11.7	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	15.5	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	17.5	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	55.9	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	207.1	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	706.1	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	4971.8	5.57	3.58	25.89	11.77	41.65	13.77	37	1.5	76.5	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	0.2	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	0.1	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	0.1	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	2.7	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	2.9	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	6.6	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	7.4	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	29.0	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	29.1	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	48.5	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	50.3	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	74.0	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	89.3	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	152.0	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	152.0	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	240.8	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	248.6	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	814.0	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	891.3	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 1	7.6	4189.8	1.65	2.14	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	0.3	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	0.3	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	0.2	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	0.7	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	2.0	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	2.8	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	10.1	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	23.8	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	56.2	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	123.9	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	518.2	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 2 $mg.L^{-1}$ Test 2	7.6	3112.6	1.87	2.42	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ Test 1	7.6	0.2	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		(µg.L^{-1})	(mg.L^{-1})										
Manton 5 mg.L^{-1} Test 1	7.6	0.1	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	0.1	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	3.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	3.5	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	5.8	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	7.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	27.4	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	26.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	56.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	52.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	79.6	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	87.1	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	151.3	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	144.3	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	213.0	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	248.7	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	766.4	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	893.4	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 1	7.6	4220.1	3.66	4.72	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	0.3	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	0.3	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	0.2	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	0.7	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	1.4	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	9.7	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	20.0	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	71.2	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	96.4	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	315.9	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	1513.2	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} Test 2	7.6	3091.4	3.93	5.08	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 mg.L^{-1} Test 1	7.6	0.2	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 mg.L^{-1} Test 1	7.6	0.2	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 mg.L^{-1} Test 1	7.6	0.3	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 mg.L^{-1} Test 1	7.6	3.9	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)	($mg.L^{-1}$)										
Manton 10 $mg.L^{-1}$ Test 1	7.6	3.7	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	6.2	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	7.6	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	26.7	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	28.6	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	55.2	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	53.7	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	68.9	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	83.2	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	141.5	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	140.5	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	211.4	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	236.3	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	766.4	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	900.1	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 1	7.6	4312.3	7.15	9.24	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	0.3	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	0.3	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	0.8	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	2.5	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	1.5	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	6.8	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	11.3	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	40.6	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	93.6	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	506.0	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	1482.8	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 10 $mg.L^{-1}$ Test 2	7.6	3068.9	7.17	9.25	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	0.5	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	0.5	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	0.3	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	3.8	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	3.8	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	6.2	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 $mg.L^{-1}$ Test 1	7.6	10.9	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		(µg.L^{-1})	(mg.L^{-1})										
Manton 15 mg.L^{-1} Test 1	7.6	27.3	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	27.2	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	52.5	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	51.7	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	73.7	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	89.6	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	140.0	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	148.9	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	222.8	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	241.5	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	785.5	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	885.1	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 1	7.6	4190.9	10.52	13.58	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	0.5	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	0.4	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	0.8	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	7.3	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	10.4	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	21.1	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	43.7	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	72.3	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	121.6	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	542.4	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	1687.0	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 15 mg.L^{-1} Test 2	7.6	3248.4	10.91	14.09	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	0.7	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	0.4	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	0.3	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	0.9	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	1.8	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	3.4	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	5.1	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	7.3	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	9.1	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	16.8	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		(μg.L^{-1})					(mg.L^{-1})						
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	21.5	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	28.4	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	50.3	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	76.7	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	100.0	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	264.9	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	508.9	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	968.3	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	2004.3	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.7	4141.8	3.88	5.01	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	0.7	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	0.6	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	0.5	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	1.1	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	1.7	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	2.6	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	6.0	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	6.1	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	11.1	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	22.4	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	35.8	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	44.6	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	82.7	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	262.0	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	1070.0	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 6.5 Test 2	6.7	6000.0	3.93	5.07	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	0.3	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	0.3	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	0.2	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	0.6	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	0.8	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	2.0	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	3.0	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	3.9	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 mg.L^{-1} at pH 8.5 Test 1	8.3	4.6	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Test Name	pH	Zn	HA	FA	Na	Mg	K	Ca	Cl	NO_3	SO_4	CO_3	PO_4
		($\mu g.L^{-1}$)					($mg.L^{-1}$)						
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	11.1	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	14.7	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	18.1	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	37.2	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	52.4	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	72.2	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	200.1	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	370.1	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	692.6	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	1433.4	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3	3149.5	3.82	4.93	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.3	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.3	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.3	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	0.5	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	1.0	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	1.4	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	2.4	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	3.8	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	7.3	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	13.6	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	24.1	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	28.4	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	37.3	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	45.7	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	179.6	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	8.3	788.0	4.00	5.16	23.14	9.78	41.65	11.47	19	1.5	65	68.58	0.15

Table C- 12: WHAM output data for calculations for all test treatments and replicates in Chapter 4, E = power of 10

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				(µg.L^{-1})		
Appletree 2 mg.L^{-1} Test 1	3.1E-01	8.4E-02	4.8E-02	2.5E-03	1.5E-03	7.3E-01
Appletree 2 mg.L^{-1} Test 1	3.1E-01	8.4E-02	4.8E-02	2.5E-03	1.5E-03	7.3E-01
Appletree 2 mg.L^{-1} Test 1	3.1E-01	8.4E-02	4.8E-02	2.5E-03	1.5E-03	7.3E-01
Appletree 2 mg.L^{-1} Test 1	3.4E+00	1.5E+00	8.8E-01	4.6E-02	2.7E-02	5.5E-01
Appletree 2 mg.L^{-1} Test 1	3.5E+00	1.6E+00	8.9E-01	4.6E-02	2.8E-02	5.5E-01
Appletree 2 mg.L^{-1} Test 1	7.3E+00	3.7E+00	2.1E+00	1.1E-01	6.6E-02	4.9E-01
Appletree 2 mg.L^{-1} Test 1	7.7E+00	4.0E+00	2.3E+00	1.2E-01	7.1E-02	4.8E-01
Appletree 2 mg.L^{-1} Test 1	2.9E+01	1.8E+01	1.0E+01	5.4E-01	3.2E-01	3.6E-01
Appletree 2 mg.L^{-1} Test 1	2.9E+01	1.8E+01	1.0E+01	5.4E-01	3.2E-01	3.6E-01
Appletree 2 mg.L^{-1} Test 1	5.0E+01	3.4E+01	1.9E+01	1.0E+00	6.0E-01	3.2E-01
Appletree 2 mg.L^{-1} Test 1	5.0E+01	3.4E+01	1.9E+01	1.0E+00	6.0E-01	3.2E-01
Appletree 2 mg.L^{-1} Test 1	8.0E+01	5.8E+01	3.3E+01	1.7E+00	1.0E+00	2.8E-01
Appletree 2 mg.L^{-1} Test 1	7.8E+01	5.6E+01	3.2E+01	1.6E+00	9.9E-01	2.8E-01
Appletree 2 mg.L^{-1} Test 1	1.6E+02	1.2E+02	6.8E+01	3.5E+00	2.1E+00	2.3E-01
Appletree 2 mg.L^{-1} Test 1	1.6E+02	1.2E+02	6.8E+01	3.5E+00	2.1E+00	2.3E-01
Appletree 2 mg.L^{-1} Test 1	2.4E+02	1.9E+02	1.1E+02	5.6E+00	3.4E+00	2.1E-01
Appletree 2 mg.L^{-1} Test 1	2.4E+02	1.9E+02	1.1E+02	5.7E+00	3.4E+00	2.0E-01
Appletree 2 mg.L^{-1} Test 1	8.8E+02	7.8E+02	4.4E+02	2.3E+01	1.4E+01	1.2E-01
Appletree 2 mg.L^{-1} Test 1	9.1E+02	8.0E+02	4.6E+02	2.4E+01	1.4E+01	1.2E-01
Appletree 2 mg.L^{-1} Test 1	4.8E+03	4.5E+03	2.6E+03	1.3E+02	8.1E+01	4.8E-02
Appletree 2 mg.L^{-1} Test 2	4.7E-01	1.0E-01	5.9E-02	3.1E-03	1.8E-03	7.8E-01
Appletree 2 mg.L^{-1} Test 2	6.7E-01	1.7E-01	9.4E-02	4.9E-03	2.9E-03	7.5E-01
Appletree 2 mg.L^{-1} Test 2	1.1E+00	3.0E-01	1.7E-01	9.0E-03	5.4E-03	7.2E-01
Appletree 2 mg.L^{-1} Test 2	8.6E-01	2.3E-01	1.3E-01	6.8E-03	4.1E-03	7.3E-01
Appletree 2 mg.L^{-1} Test 2	6.5E+00	2.7E+00	1.5E+00	7.9E-02	4.7E-02	5.9E-01
Appletree 2 mg.L^{-1} Test 2	2.4E+00	8.0E-01	4.5E-01	2.4E-02	1.4E-02	6.7E-01
Appletree 2 mg.L^{-1} Test 2	5.4E+00	2.1E+00	1.2E+00	6.3E-02	3.8E-02	6.1E-01
Appletree 2 mg.L^{-1} Test 2	1.0E+01	4.7E+00	2.7E+00	1.4E-01	8.4E-02	5.5E-01
Appletree 2 mg.L^{-1} Test 2	2.5E+01	1.3E+01	7.5E+00	3.9E-01	2.3E-01	4.7E-01
Appletree 2 mg.L^{-1} Test 2	1.0E+02	6.7E+01	3.8E+01	2.0E+00	1.2E+00	3.4E-01
Appletree 2 mg.L^{-1} Test 2	5.3E+02	4.2E+02	2.4E+02	1.2E+01	7.4E+00	2.1E-01
Appletree 2 mg.L^{-1} Test 2	3.1E+03	2.8E+03	1.6E+03	8.4E+01	5.0E+01	8.6E-02
Appletree 5 mg.L^{-1} Test 1	3.1E-01	2.7E-02	1.5E-02	7.9E-04	4.7E-04	9.2E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^+$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				(µg.L^{-1})		
Appletree 5 mg.L^{-1} Test 1	3.1E-01	2.7E-02	1.5E-02	7.9E-04	4.7E-04	9.2E-01
Appletree 5 mg.L^{-1} Test 1	3.1E-01	2.7E-02	1.5E-02	7.9E-04	4.7E-04	9.2E-01
Appletree 5 mg.L^{-1} Test 1	7.7E+00	1.8E+00	1.0E+00	5.4E-02	3.2E-02	7.7E-01
Appletree 5 mg.L^{-1} Test 1	8.1E+00	1.9E+00	1.1E+00	5.7E-02	3.4E-02	7.6E-01
Appletree 5 mg.L^{-1} Test 1	1.8E+01	5.4E+00	3.1E+00	1.6E-01	9.6E-02	7.0E-01
Appletree 5 mg.L^{-1} Test 1	2.0E+01	6.0E+00	3.4E+00	1.8E-01	1.1E-01	7.0E-01
Appletree 5 mg.L^{-1} Test 1	4.5E+01	1.7E+01	9.8E+00	5.1E-01	3.1E-01	6.2E-01
Appletree 5 mg.L^{-1} Test 1	4.4E+01	1.7E+01	9.5E+00	5.0E-01	3.0E-01	6.2E-01
Appletree 5 mg.L^{-1} Test 1	1.2E+02	5.6E+01	3.2E+01	1.7E+00	1.0E+00	5.3E-01
Appletree 5 mg.L^{-1} Test 1	8.8E+01	3.9E+01	2.2E+01	1.2E+00	7.0E-01	5.6E-01
Appletree 5 mg.L^{-1} Test 1	2.3E+02	1.3E+02	7.2E+01	3.8E+00	2.3E+00	4.6E-01
Appletree 5 mg.L^{-1} Test 1	2.3E+02	1.2E+02	6.9E+01	3.6E+00	2.2E+00	4.6E-01
Appletree 5 mg.L^{-1} Test 1	4.6E+02	2.8E+02	1.6E+02	8.4E+00	5.0E+00	3.8E-01
Appletree 5 mg.L^{-1} Test 1	4.8E+02	3.0E+02	1.7E+02	8.8E+00	5.3E+00	3.8E-01
Appletree 5 mg.L^{-1} Test 1	9.1E+02	6.4E+02	3.6E+02	1.9E+01	1.1E+01	3.0E-01
Appletree 5 mg.L^{-1} Test 1	9.4E+02	6.6E+02	3.8E+02	2.0E+01	1.2E+01	3.0E-01
Appletree 5 mg.L^{-1} Test 1	1.9E+03	1.5E+03	8.5E+02	4.4E+01	2.7E+01	2.2E-01
Appletree 5 mg.L^{-1} Test 1	1.8E+03	1.4E+03	8.2E+02	4.3E+01	2.6E+01	2.2E-01
Appletree 5 mg.L^{-1} Test 1	4.8E+03	4.2E+03	2.4E+03	1.3E+02	7.5E+01	1.3E-01
Appletree 5 mg.L^{-1} Test 2	4.3E-01	4.1E-02	2.3E-02	1.2E-03	7.3E-04	9.0E-01
Appletree 5 mg.L^{-1} Test 2	2.2E-01	1.8E-02	1.0E-02	5.3E-04	3.2E-04	9.2E-01
Appletree 5 mg.L^{-1} Test 2	1.0E+00	1.4E-01	8.0E-02	4.2E-03	2.5E-03	8.6E-01
Appletree 5 mg.L^{-1} Test 2	8.6E+00	2.1E+00	1.2E+00	6.3E-02	3.8E-02	7.5E-01
Appletree 5 mg.L^{-1} Test 2	3.5E+00	6.9E-01	3.9E-01	2.1E-02	1.2E-02	8.0E-01
Appletree 5 mg.L^{-1} Test 2	4.9E+00	1.0E+00	5.9E-01	3.1E-02	1.9E-02	7.9E-01
Appletree 5 mg.L^{-1} Test 2	9.8E+00	2.5E+00	1.4E+00	7.5E-02	4.5E-02	7.4E-01
Appletree 5 mg.L^{-1} Test 2	2.3E+01	7.5E+00	4.2E+00	2.2E-01	1.3E-01	6.7E-01
Appletree 5 mg.L^{-1} Test 2	2.8E+01	9.6E+00	5.5E+00	2.8E-01	1.7E-01	6.6E-01
Appletree 5 mg.L^{-1} Test 2	9.9E+01	4.6E+01	2.6E+01	1.4E+00	8.1E-01	5.4E-01
Appletree 5 mg.L^{-1} Test 2	5.0E+02	3.2E+02	1.8E+02	9.4E+00	5.6E+00	3.6E-01
Appletree 5 mg.L^{-1} Test 2	2.9E+03	2.4E+03	1.4E+03	7.1E+01	4.2E+01	1.7E-01
Appletree 10 mg.L^{-1} Test 1	2.1E-01	7.9E-03	4.5E-03	2.3E-04	1.4E-04	9.6E-01
Appletree 10 mg.L^{-1} Test 1	2.3E-01	8.8E-03	5.0E-03	2.6E-04	1.6E-04	9.6E-01
Appletree 10 mg.L^{-1} Test 1	3.0E-01	1.2E-02	6.7E-03	3.5E-04	2.1E-04	9.6E-01
Appletree 10 mg.L^{-1} Test 1	2.8E+00	2.4E-01	1.4E-01	7.2E-03	4.3E-03	9.1E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Appletree 10 $mg.L^{-1}$ Test 1	2.4E+00	2.1E-01	1.2E-01	6.2E-03	3.7E-03	9.2E-01
Appletree 10 $mg.L^{-1}$ Test 1	5.1E+00	5.4E-01	3.1E-01	1.6E-02	9.6E-03	8.9E-01
Appletree 10 $mg.L^{-1}$ Test 1	4.4E+00	4.5E-01	2.5E-01	1.3E-02	8.0E-03	9.0E-01
Appletree 10 $mg.L^{-1}$ Test 1	8.9E+00	1.1E+00	6.3E-01	3.3E-02	2.0E-02	8.7E-01
Appletree 10 $mg.L^{-1}$ Test 1	8.3E+00	1.0E+00	5.8E-01	3.0E-02	1.8E-02	8.8E-01
Appletree 10 $mg.L^{-1}$ Test 1	2.2E+01	3.7E+00	2.1E+00	1.1E-01	6.6E-02	8.3E-01
Appletree 10 $mg.L^{-1}$ Test 1	2.3E+01	3.8E+00	2.1E+00	1.1E-01	6.7E-02	8.3E-01
Appletree 10 $mg.L^{-1}$ Test 1	4.5E+01	9.6E+00	5.5E+00	2.8E-01	1.7E-01	7.9E-01
Appletree 10 $mg.L^{-1}$ Test 1	4.6E+01	9.8E+00	5.6E+00	2.9E-01	1.7E-01	7.9E-01
Appletree 10 $mg.L^{-1}$ Test 1	8.7E+01	2.3E+01	1.3E+01	6.8E-01	4.1E-01	7.4E-01
Appletree 10 $mg.L^{-1}$ Test 1	8.8E+01	2.3E+01	1.3E+01	6.9E-01	4.2E-01	7.4E-01
Appletree 10 $mg.L^{-1}$ Test 1	4.2E+02	1.8E+02	1.0E+02	5.2E+00	3.1E+00	5.8E-01
Appletree 10 $mg.L^{-1}$ Test 1	4.3E+02	1.8E+02	1.0E+02	5.3E+00	3.2E+00	5.8E-01
Appletree 10 $mg.L^{-1}$ Test 1	9.4E+02	4.9E+02	2.8E+02	1.5E+01	8.8E+00	4.7E-01
Appletree 10 $mg.L^{-1}$ Test 1	9.4E+02	4.9E+02	2.8E+02	1.5E+01	8.8E+00	4.7E-01
Appletree 10 $mg.L^{-1}$ Test 1	2.0E+03	1.3E+03	7.1E+02	3.7E+01	2.2E+01	3.6E-01
Appletree 10 $mg.L^{-1}$ Test 1	1.9E+03	1.2E+03	6.7E+02	3.5E+01	2.1E+01	3.7E-01
Appletree 10 $mg.L^{-1}$ Test 1	2.8E+03	1.9E+03	1.1E+03	5.8E+01	3.5E+01	3.1E-01
Appletree 10 $mg.L^{-1}$ Test 1	2.8E+03	1.9E+03	1.1E+03	5.7E+01	3.4E+01	3.1E-01
Appletree 10 $mg.L^{-1}$ Test 1	7.0E+03	5.8E+03	3.3E+03	1.7E+02	1.0E+02	1.8E-01
Appletree 10 $mg.L^{-1}$ Test 2	2.9E-01	1.1E-02	6.4E-03	3.3E-04	2.0E-04	9.6E-01
Appletree 10 $mg.L^{-1}$ Test 2	5.4E-01	2.4E-02	1.4E-02	7.2E-04	4.3E-04	9.6E-01
Appletree 10 $mg.L^{-1}$ Test 2	5.1E-01	2.2E-02	1.2E-02	6.5E-04	3.9E-04	9.6E-01
Appletree 10 $mg.L^{-1}$ Test 2	1.3E+00	8.5E-02	4.8E-02	2.5E-03	1.5E-03	9.4E-01
Appletree 10 $mg.L^{-1}$ Test 2	1.9E+00	1.4E-01	7.7E-02	4.0E-03	2.4E-03	9.3E-01
Appletree 10 $mg.L^{-1}$ Test 2	3.5E+00	3.2E-01	1.8E-01	9.5E-03	5.7E-03	9.1E-01
Appletree 10 $mg.L^{-1}$ Test 2	7.5E+00	8.7E-01	4.9E-01	2.6E-02	1.5E-02	8.8E-01
Appletree 10 $mg.L^{-1}$ Test 2	1.7E+01	2.6E+00	1.5E+00	7.6E-02	4.6E-02	8.5E-01
Appletree 10 $mg.L^{-1}$ Test 2	3.4E+01	6.4E+00	3.6E+00	1.9E-01	1.1E-01	8.1E-01
Appletree 10 $mg.L^{-1}$ Test 2	1.4E+02	4.1E+01	2.3E+01	1.2E+00	7.4E-01	7.0E-01
Appletree 10 $mg.L^{-1}$ Test 2	4.7E+02	2.0E+02	1.1E+02	5.9E+00	3.5E+00	5.8E-01
Appletree 10 $mg.L^{-1}$ Test 2	2.8E+03	1.9E+03	1.1E+03	5.6E+01	3.4E+01	3.1E-01
Appletree 15 $mg.L^{-1}$ Test 1	3.1E+00	1.7E-01	9.7E-02	5.1E-03	3.1E-03	9.4E-01
Appletree 15 $mg.L^{-1}$ Test 1	2.4E-01	5.8E-03	3.3E-03	1.7E-04	1.0E-04	9.8E-01
Appletree 15 $mg.L^{-1}$ Test 1	1.7E-01	4.0E-03	2.2E-03	1.2E-04	7.1E-05	9.8E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Appletree 15 mg.L^{-1} Test 1	2.2E+00	1.1E-01	6.1E-02	3.2E-03	1.9E-03	9.5E-01
Appletree 15 mg.L^{-1} Test 1	2.3E+00	1.1E-01	6.4E-02	3.4E-03	2.0E-03	9.5E-01
Appletree 15 mg.L^{-1} Test 1	4.0E+00	2.4E-01	1.4E-01	7.2E-03	4.3E-03	9.4E-01
Appletree 15 mg.L^{-1} Test 1	4.0E+00	2.4E-01	1.4E-01	7.1E-03	4.2E-03	9.4E-01
Appletree 15 mg.L^{-1} Test 1	8.6E+00	6.6E-01	3.8E-01	2.0E-02	1.2E-02	9.2E-01
Appletree 15 mg.L^{-1} Test 1	9.6E+00	7.7E-01	4.3E-01	2.3E-02	1.4E-02	9.2E-01
Appletree 15 mg.L^{-1} Test 1	2.3E+01	2.4E+00	1.3E+00	7.0E-02	4.2E-02	9.0E-01
Appletree 15 mg.L^{-1} Test 1	2.3E+01	2.4E+00	1.3E+00	7.0E-02	4.2E-02	9.0E-01
Appletree 15 mg.L^{-1} Test 1	4.6E+01	6.2E+00	3.5E+00	1.8E-01	1.1E-01	8.6E-01
Appletree 15 mg.L^{-1} Test 1	4.6E+01	6.2E+00	3.5E+00	1.8E-01	1.1E-01	8.6E-01
Appletree 15 mg.L^{-1} Test 1	8.9E+01	1.5E+01	8.8E+00	4.6E-01	2.8E-01	8.3E-01
Appletree 15 mg.L^{-1} Test 1	9.2E+01	1.6E+01	9.2E+00	4.8E-01	2.9E-01	8.2E-01
Appletree 15 mg.L^{-1} Test 1	4.3E+02	1.3E+02	7.3E+01	3.8E+00	2.3E+00	7.0E-01
Appletree 15 mg.L^{-1} Test 1	4.3E+02	1.3E+02	7.2E+01	3.8E+00	2.3E+00	7.0E-01
Appletree 15 mg.L^{-1} Test 1	8.9E+02	3.5E+02	2.0E+02	1.0E+01	6.2E+00	6.1E-01
Appletree 15 mg.L^{-1} Test 1	9.0E+02	3.5E+02	2.0E+02	1.0E+01	6.3E+00	6.1E-01
Appletree 15 mg.L^{-1} Test 1	1.8E+03	9.0E+02	5.1E+02	2.7E+01	1.6E+01	5.0E-01
Appletree 15 mg.L^{-1} Test 1	1.8E+03	9.3E+02	5.3E+02	2.8E+01	1.7E+01	5.0E-01
Appletree 15 mg.L^{-1} Test 1	2.8E+03	1.6E+03	9.1E+02	4.8E+01	2.9E+01	4.2E-01
Appletree 15 mg.L^{-1} Test 1	2.8E+03	1.6E+03	9.1E+02	4.7E+01	2.8E+01	4.2E-01
Appletree 15 mg.L^{-1} Test 1	6.9E+03	5.1E+03	2.9E+03	1.5E+02	9.2E+01	2.6E-01
Appletree 15 mg.L^{-1} Test 1	4.6E+03	3.1E+03	1.8E+03	9.3E+01	5.6E+01	3.3E-01
Appletree 15 mg.L^{-1} Test 2	7.1E-01	2.1E-02	1.2E-02	6.2E-04	3.7E-04	9.7E-01
Appletree 15 mg.L^{-1} Test 2	1.4E+00	5.1E-02	2.9E-02	1.5E-03	9.1E-04	9.6E-01
Appletree 15 mg.L^{-1} Test 2	1.5E+00	5.6E-02	3.2E-02	1.6E-03	9.9E-04	9.6E-01
Appletree 15 mg.L^{-1} Test 2	1.6E+00	6.1E-02	3.5E-02	1.8E-03	1.1E-03	9.6E-01
Appletree 15 mg.L^{-1} Test 2	2.2E+00	1.0E-01	5.8E-02	3.1E-03	1.8E-03	9.5E-01
Appletree 15 mg.L^{-1} Test 2	7.0E+00	4.9E-01	2.8E-01	1.5E-02	8.8E-03	9.3E-01
Appletree 15 mg.L^{-1} Test 2	8.2E+00	6.1E-01	3.4E-01	1.8E-02	1.1E-02	9.3E-01
Appletree 15 mg.L^{-1} Test 2	1.9E+01	1.8E+00	1.0E+00	5.2E-02	3.1E-02	9.0E-01
Appletree 15 mg.L^{-1} Test 2	3.5E+01	4.1E+00	2.3E+00	1.2E-01	7.3E-02	8.8E-01
Appletree 15 mg.L^{-1} Test 2	1.3E+02	2.6E+01	1.5E+01	7.8E-01	4.7E-01	8.0E-01
Appletree 15 mg.L^{-1} Test 2	4.8E+02	1.5E+02	8.2E+01	4.3E+00	2.6E+00	7.0E-01
Appletree 15 mg.L^{-1} Test 2	2.8E+03	1.6E+03	9.1E+02	4.8E+01	2.9E+01	4.3E-01
Appletree 5 mg.L^{-1} at pH 6.5 Test 1	3.7E-01	3.8E-02	2.6E-02	1.7E-04	1.3E-05	9.0E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	3.1E-01	3.1E-02	2.1E-02	1.4E-04	1.0E-05	9.0E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	6.9E-01	9.3E-02	6.2E-02	4.1E-04	3.1E-05	8.7E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	1.6E+00	2.8E-01	1.9E-01	1.2E-03	9.2E-05	8.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	3.2E+00	7.0E-01	4.7E-01	3.1E-03	2.3E-04	7.8E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	3.7E+00	8.5E-01	5.6E-01	3.7E-03	2.8E-04	7.7E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	5.2E+00	1.3E+00	8.6E-01	5.7E-03	4.3E-04	7.5E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	7.9E+00	2.2E+00	1.4E+00	9.5E-03	7.2E-04	7.3E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	9.0E+00	2.6E+00	1.7E+00	1.1E-02	8.5E-04	7.1E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	1.7E+01	5.6E+00	3.7E+00	2.5E-02	1.9E-03	6.6E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.1E+01	7.7E+00	5.1E+00	3.4E-02	2.6E-03	6.4E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.7E+01	1.0E+01	7.0E+00	4.6E-02	3.5E-03	6.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	5.0E+01	2.2E+01	1.5E+01	9.7E-02	7.4E-03	5.6E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	7.7E+01	3.7E+01	2.5E+01	1.6E-01	1.2E-02	5.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	1.0E+02	5.1E+01	3.4E+01	2.2E-01	1.7E-02	4.9E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.4E+02	1.5E+02	9.8E+01	6.5E-01	4.9E-02	4.0E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	5.0E+02	3.4E+02	2.3E+02	1.5E+00	1.1E-01	3.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	9.5E+02	7.1E+02	4.8E+02	3.1E+00	2.4E-01	2.5E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.0E+03	1.6E+03	1.1E+03	7.1E+00	5.4E-01	1.8E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 1	4.0E+03	3.5E+03	2.3E+03	1.5E+01	1.2E+00	1.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	7.9E-01	1.1E-01	7.0E-02	4.6E-04	3.5E-05	8.7E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	3.0E-01	2.7E-02	1.8E-02	1.2E-04	9.1E-06	9.1E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	3.4E-01	3.3E-02	2.2E-02	1.4E-04	1.1E-05	9.0E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.5E+00	2.6E-01	1.7E-01	1.1E-03	8.6E-05	8.3E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.8E+00	3.1E-01	2.1E-01	1.4E-03	1.0E-04	8.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	3.1E+00	6.4E-01	4.3E-01	2.8E-03	2.1E-04	7.9E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	5.2E+00	1.2E+00	8.2E-01	5.4E-03	4.1E-04	7.6E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.9E+00	1.8E+00	1.2E+00	7.8E-03	5.9E-04	7.5E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.3E+01	3.8E+00	2.5E+00	1.7E-02	1.3E-03	7.0E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.8E+01	5.8E+00	3.9E+00	2.5E-02	1.9E-03	6.7E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.4E+01	8.6E+00	5.7E+00	3.8E-02	2.9E-03	6.4E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.8E+01	1.0E+01	6.9E+00	4.5E-02	3.4E-03	6.3E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	3.4E+01	1.3E+01	8.9E+00	5.9E-02	4.5E-03	6.1E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	8.2E+01	3.9E+01	2.6E+01	1.7E-01	1.3E-02	5.2E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.7E+02	1.6E+02	1.1E+02	7.0E-01	5.3E-02	4.0E-01
Appletree 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.1E+03	8.0E+02	5.3E+02	3.5E+00	2.6E-01	2.5E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	3.9E-01	4.1E-02	1.5E-02	3.8E-03	1.2E-02	9.0E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	2.8E-01	2.7E-02	9.7E-03	2.5E-03	7.7E-03	9.0E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	7.6E-01	1.0E-01	3.6E-02	9.5E-03	2.9E-02	8.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	7.7E-01	1.0E-01	3.7E-02	9.6E-03	2.9E-02	8.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	1.4E+00	2.4E-01	8.5E-02	2.2E-02	6.7E-02	8.3E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	3.1E+00	6.4E-01	2.3E-01	6.0E-02	1.8E-01	7.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	4.0E+00	8.9E-01	3.2E-01	8.4E-02	2.5E-01	7.8E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	5.4E+00	1.3E+00	4.6E-01	1.2E-01	3.6E-01	7.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	6.9E+00	1.7E+00	6.2E-01	1.6E-01	4.9E-01	7.5E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	1.1E+01	3.2E+00	1.1E+00	3.0E-01	9.0E-01	7.2E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	1.5E+01	4.4E+00	1.6E+00	4.1E-01	1.2E+00	7.0E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	2.0E+01	6.6E+00	2.4E+00	6.2E-01	1.9E+00	6.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	3.8E+01	1.5E+01	5.3E+00	1.4E+00	4.2E+00	6.2E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	5.7E+01	2.4E+01	8.7E+00	2.3E+00	6.8E+00	5.8E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	7.4E+01	3.3E+01	1.2E+01	3.1E+00	9.3E+00	5.6E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	2.0E+02	1.1E+02	3.8E+01	1.0E+01	3.0E+01	4.6E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	4.1E+02	2.5E+02	9.0E+01	2.4E+01	7.1E+01	3.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	6.9E+02	4.6E+02	1.7E+02	4.4E+01	1.3E+02	3.3E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	1.5E+03	1.1E+03	4.0E+02	1.0E+02	3.1E+02	2.5E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 1	3.3E+03	2.8E+03	1.0E+03	2.6E+02	7.9E+02	1.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	4.8E-01	5.3E-02	1.9E-02	5.0E-03	1.5E-02	8.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	4.7E-01	5.2E-02	1.9E-02	4.9E-03	1.5E-02	8.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	2.8E-01	2.7E-02	9.7E-03	2.5E-03	7.6E-03	9.0E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	7.5E-01	9.7E-02	3.5E-02	9.2E-03	2.8E-02	8.7E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	1.4E+00	2.2E-01	8.0E-02	2.1E-02	6.3E-02	8.4E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	2.0E+00	3.6E-01	1.3E-01	3.4E-02	1.0E-01	8.2E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	3.3E+00	6.9E-01	2.5E-01	6.4E-02	1.9E-01	7.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	6.3E+00	1.5E+00	5.4E-01	1.4E-01	4.3E-01	7.6E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	7.7E+00	2.0E+00	7.0E-01	1.8E-01	5.5E-01	7.5E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	1.2E+01	3.3E+00	1.2E+00	3.1E-01	9.3E-01	7.2E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	1.5E+01	4.7E+00	1.7E+00	4.4E-01	1.3E+00	7.0E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	1.7E+01	5.4E+00	2.0E+00	5.1E-01	1.5E+00	6.9E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	5.6E+01	2.3E+01	8.3E+00	2.2E+00	6.6E+00	5.8E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	2.1E+02	1.1E+02	4.0E+01	1.1E+01	3.2E+01	4.6E-01
Appletree 5 mg.L^{-1} at pH 8.5 Test 2	7.1E+02	4.7E+02	1.7E+02	4.5E+01	1.3E+02	3.3E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Appletree 5 $mg.L^{-1}$ at pH 8.5 Test 2	5.0E+03	4.3E+03	1.6E+03	4.1E+02	1.2E+03	1.3E-01
Manton 2 $mg.L^{-1}$ Test 1	2.1E-01	3.8E-02	2.2E-02	1.2E-03	7.0E-04	8.2E-01
Manton 2 $mg.L^{-1}$ Test 1	1.4E-01	2.2E-02	1.3E-02	6.7E-04	4.0E-04	8.4E-01
Manton 2 $mg.L^{-1}$ Test 1	1.4E-01	2.2E-02	1.3E-02	6.7E-04	4.0E-04	8.4E-01
Manton 2 $mg.L^{-1}$ Test 1	2.7E+00	9.5E-01	5.4E-01	2.9E-02	1.7E-02	6.5E-01
Manton 2 $mg.L^{-1}$ Test 1	2.9E+00	1.0E+00	5.7E-01	3.0E-02	1.8E-02	6.5E-01
Manton 2 $mg.L^{-1}$ Test 1	6.6E+00	2.7E+00	1.6E+00	8.2E-02	5.0E-02	5.8E-01
Manton 2 $mg.L^{-1}$ Test 1	7.4E+00	3.2E+00	1.8E+00	9.5E-02	5.8E-02	5.7E-01
Manton 2 $mg.L^{-1}$ Test 1	2.9E+01	1.6E+01	9.1E+00	4.8E-01	2.9E-01	4.5E-01
Manton 2 $mg.L^{-1}$ Test 1	2.9E+01	1.6E+01	9.1E+00	4.8E-01	2.9E-01	4.5E-01
Manton 2 $mg.L^{-1}$ Test 1	4.9E+01	2.9E+01	1.6E+01	8.7E-01	5.3E-01	4.0E-01
Manton 2 $mg.L^{-1}$ Test 1	5.0E+01	3.0E+01	1.7E+01	9.1E-01	5.5E-01	4.0E-01
Manton 2 $mg.L^{-1}$ Test 1	7.4E+01	4.7E+01	2.7E+01	1.4E+00	8.5E-01	3.6E-01
Manton 2 $mg.L^{-1}$ Test 1	8.9E+01	5.8E+01	3.3E+01	1.7E+00	1.1E+00	3.5E-01
Manton 2 $mg.L^{-1}$ Test 1	1.5E+02	1.1E+02	6.0E+01	3.2E+00	1.9E+00	3.0E-01
Manton 2 $mg.L^{-1}$ Test 1	1.5E+02	1.1E+02	6.0E+01	3.2E+00	1.9E+00	3.0E-01
Manton 2 $mg.L^{-1}$ Test 1	2.4E+02	1.8E+02	1.0E+02	5.3E+00	3.2E+00	2.6E-01
Manton 2 $mg.L^{-1}$ Test 1	2.5E+02	1.8E+02	1.0E+02	5.5E+00	3.3E+00	2.6E-01
Manton 2 $mg.L^{-1}$ Test 1	8.1E+02	6.8E+02	3.9E+02	2.0E+01	1.2E+01	1.7E-01
Manton 2 $mg.L^{-1}$ Test 1	8.9E+02	7.5E+02	4.3E+02	2.3E+01	1.4E+01	1.6E-01
Manton 2 $mg.L^{-1}$ Test 1	4.2E+03	3.9E+03	2.2E+03	1.2E+02	7.1E+01	6.7E-02
Manton 2 $mg.L^{-1}$ Test 2	3.0E-01	5.4E-02	3.1E-02	1.6E-03	9.8E-04	8.2E-01
Manton 2 $mg.L^{-1}$ Test 2	2.7E-01	4.7E-02	2.6E-02	1.4E-03	8.5E-04	8.3E-01
Manton 2 $mg.L^{-1}$ Test 2	2.0E-01	3.2E-02	1.8E-02	9.6E-04	5.8E-04	8.4E-01
Manton 2 $mg.L^{-1}$ Test 2	6.6E-01	1.5E-01	8.7E-02	4.6E-03	2.8E-03	7.7E-01
Manton 2 $mg.L^{-1}$ Test 2	2.0E+00	5.8E-01	3.3E-01	1.8E-02	1.1E-02	7.0E-01
Manton 2 $mg.L^{-1}$ Test 2	2.9E+00	9.1E-01	5.2E-01	2.7E-02	1.6E-02	6.8E-01
Manton 2 $mg.L^{-1}$ Test 2	1.0E+01	4.3E+00	2.4E+00	1.3E-01	7.7E-02	5.8E-01
Manton 2 $mg.L^{-1}$ Test 2	2.4E+01	1.2E+01	6.7E+00	3.6E-01	2.1E-01	5.0E-01
Manton 2 $mg.L^{-1}$ Test 2	5.6E+01	3.2E+01	1.8E+01	9.7E-01	5.9E-01	4.2E-01
Manton 2 $mg.L^{-1}$ Test 2	1.2E+02	8.0E+01	4.6E+01	2.4E+00	1.5E+00	3.5E-01
Manton 2 $mg.L^{-1}$ Test 2	5.2E+02	4.0E+02	2.3E+02	1.2E+01	7.3E+00	2.2E-01
Manton 2 $mg.L^{-1}$ Test 2	3.1E+03	2.8E+03	1.6E+03	8.5E+01	5.1E+01	9.1E-02
Manton 5 $mg.L^{-1}$ Test 1	1.9E-01	1.4E-02	7.7E-03	4.1E-04	2.5E-04	9.3E-01
Manton 5 $mg.L^{-1}$ Test 1	1.4E-01	9.3E-03	5.3E-03	2.8E-04	1.7E-04	9.3E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Manton 5 mg.L^{-1} Test 1	1.4E-01	9.3E-03	5.3E-03	2.8E-04	1.7E-04	9.3E-01
Manton 5 mg.L^{-1} Test 1	3.7E+00	6.8E-01	3.8E-01	2.0E-02	1.2E-02	8.2E-01
Manton 5 mg.L^{-1} Test 1	3.5E+00	6.2E-01	3.5E-01	1.9E-02	1.1E-02	8.2E-01
Manton 5 mg.L^{-1} Test 1	5.8E+00	1.2E+00	6.7E-01	3.5E-02	2.1E-02	8.0E-01
Manton 5 mg.L^{-1} Test 1	7.7E+00	1.7E+00	9.6E-01	5.1E-02	3.1E-02	7.8E-01
Manton 5 mg.L^{-1} Test 1	2.7E+01	8.6E+00	4.9E+00	2.6E-01	1.6E-01	6.9E-01
Manton 5 mg.L^{-1} Test 1	2.7E+01	8.3E+00	4.7E+00	2.5E-01	1.5E-01	6.9E-01
Manton 5 mg.L^{-1} Test 1	5.7E+01	2.1E+01	1.2E+01	6.4E-01	3.9E-01	6.2E-01
Manton 5 mg.L^{-1} Test 1	5.3E+01	2.0E+01	1.1E+01	5.9E-01	3.5E-01	6.3E-01
Manton 5 mg.L^{-1} Test 1	8.0E+01	3.3E+01	1.9E+01	9.8E-01	5.9E-01	5.9E-01
Manton 5 mg.L^{-1} Test 1	8.7E+01	3.7E+01	2.1E+01	1.1E+00	6.6E-01	5.8E-01
Manton 5 mg.L^{-1} Test 1	1.5E+02	7.2E+01	4.1E+01	2.2E+00	1.3E+00	5.3E-01
Manton 5 mg.L^{-1} Test 1	1.4E+02	6.8E+01	3.8E+01	2.0E+00	1.2E+00	5.3E-01
Manton 5 mg.L^{-1} Test 1	2.1E+02	1.1E+02	6.2E+01	3.3E+00	2.0E+00	4.9E-01
Manton 5 mg.L^{-1} Test 1	2.5E+02	1.3E+02	7.4E+01	3.9E+00	2.4E+00	4.7E-01
Manton 5 mg.L^{-1} Test 1	7.7E+02	5.1E+02	2.9E+02	1.5E+01	9.2E+00	3.4E-01
Manton 5 mg.L^{-1} Test 1	8.9E+02	6.1E+02	3.5E+02	1.8E+01	1.1E+01	3.2E-01
Manton 5 mg.L^{-1} Test 1	4.2E+03	3.6E+03	2.1E+03	1.1E+02	6.6E+01	1.4E-01
Manton 5 mg.L^{-1} Test 2	2.6E-01	1.9E-02	1.1E-02	5.6E-04	3.4E-04	9.3E-01
Manton 5 mg.L^{-1} Test 2	3.1E-01	2.3E-02	1.3E-02	7.0E-04	4.2E-04	9.3E-01
Manton 5 mg.L^{-1} Test 2	2.4E-01	1.6E-02	9.2E-03	4.9E-04	2.9E-04	9.3E-01
Manton 5 mg.L^{-1} Test 2	6.8E-01	6.7E-02	3.8E-02	2.0E-03	1.2E-03	9.0E-01
Manton 5 mg.L^{-1} Test 2	1.4E+00	1.8E-01	9.9E-02	5.3E-03	3.2E-03	8.7E-01
Manton 5 mg.L^{-1} Test 2	9.7E+00	2.1E+00	1.2E+00	6.3E-02	3.8E-02	7.8E-01
Manton 5 mg.L^{-1} Test 2	2.0E+01	5.4E+00	3.0E+00	1.6E-01	9.7E-02	7.3E-01
Manton 5 mg.L^{-1} Test 2	7.1E+01	2.7E+01	1.5E+01	8.1E-01	4.9E-01	6.2E-01
Manton 5 mg.L^{-1} Test 2	9.6E+01	3.9E+01	2.2E+01	1.2E+00	7.1E-01	5.9E-01
Manton 5 mg.L^{-1} Test 2	3.2E+02	1.7E+02	9.6E+01	5.1E+00	3.1E+00	4.7E-01
Manton 5 mg.L^{-1} Test 2	1.5E+03	1.1E+03	6.3E+02	3.3E+01	2.0E+01	2.7E-01
Manton 5 mg.L^{-1} Test 2	3.1E+03	2.5E+03	1.4E+03	7.6E+01	4.6E+01	1.8E-01
Manton 10 mg.L^{-1} Test 1	1.9E-01	6.2E-03	3.5E-03	1.9E-04	1.1E-04	9.7E-01
Manton 10 mg.L^{-1} Test 1	2.1E-01	7.0E-03	4.0E-03	2.1E-04	1.3E-04	9.7E-01
Manton 10 mg.L^{-1} Test 1	2.6E-01	9.0E-03	5.1E-03	2.7E-04	1.6E-04	9.7E-01
Manton 10 mg.L^{-1} Test 1	3.9E+00	3.3E-01	1.9E-01	9.9E-03	6.0E-03	9.1E-01
Manton 10 mg.L^{-1} Test 1	3.7E+00	3.2E-01	1.8E-01	9.5E-03	5.8E-03	9.2E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				(μg.L^{-1})		
Manton 10 mg.L^{-1} Test 1	6.2E+00	6.0E-01	3.4E-01	1.8E-02	1.1E-02	9.0E-01
Manton 10 mg.L^{-1} Test 1	7.6E+00	7.9E-01	4.5E-01	2.4E-02	1.4E-02	9.0E-01
Manton 10 mg.L^{-1} Test 1	2.7E+01	4.1E+00	2.3E+00	1.2E-01	7.5E-02	8.5E-01
Manton 10 mg.L^{-1} Test 1	2.9E+01	4.5E+00	2.6E+00	1.4E-01	8.2E-02	8.4E-01
Manton 10 mg.L^{-1} Test 1	5.5E+01	1.1E+01	6.2E+00	3.3E-01	2.0E-01	8.0E-01
Manton 10 mg.L^{-1} Test 1	5.4E+01	1.1E+01	6.0E+00	3.2E-01	1.9E-01	8.0E-01
Manton 10 mg.L^{-1} Test 1	6.9E+01	1.5E+01	8.4E+00	4.4E-01	2.7E-01	7.9E-01
Manton 10 mg.L^{-1} Test 1	8.3E+01	1.9E+01	1.1E+01	5.7E-01	3.4E-01	7.7E-01
Manton 10 mg.L^{-1} Test 1	1.4E+02	3.8E+01	2.2E+01	1.1E+00	6.9E-01	7.3E-01
Manton 10 mg.L^{-1} Test 1	1.4E+02	3.8E+01	2.1E+01	1.1E+00	6.8E-01	7.3E-01
Manton 10 mg.L^{-1} Test 1	2.1E+02	6.5E+01	3.7E+01	1.9E+00	1.2E+00	7.0E-01
Manton 10 mg.L^{-1} Test 1	2.4E+02	7.5E+01	4.2E+01	2.2E+00	1.4E+00	6.8E-01
Manton 10 mg.L^{-1} Test 1	7.7E+02	3.5E+02	2.0E+02	1.1E+01	6.4E+00	5.4E-01
Manton 10 mg.L^{-1} Test 1	9.0E+02	4.3E+02	2.5E+02	1.3E+01	7.9E+00	5.2E-01
Manton 10 mg.L^{-1} Test 1	4.3E+03	3.2E+03	1.8E+03	9.6E+01	5.8E+01	2.6E-01
Manton 10 mg.L^{-1} Test 2	3.2E-01	1.1E-02	6.4E-03	3.4E-04	2.0E-04	9.6E-01
Manton 10 mg.L^{-1} Test 2	3.4E-01	1.2E-02	7.0E-03	3.7E-04	2.2E-04	9.6E-01
Manton 10 mg.L^{-1} Test 2	8.2E-01	3.9E-02	2.2E-02	1.2E-03	7.1E-04	9.5E-01
Manton 10 mg.L^{-1} Test 2	2.5E+00	1.9E-01	1.1E-01	5.6E-03	3.4E-03	9.3E-01
Manton 10 mg.L^{-1} Test 2	1.5E+00	8.9E-02	5.0E-02	2.7E-03	1.6E-03	9.4E-01
Manton 10 mg.L^{-1} Test 2	6.8E+00	6.9E-01	3.9E-01	2.1E-02	1.2E-02	9.0E-01
Manton 10 mg.L^{-1} Test 2	1.1E+01	1.3E+00	7.4E-01	3.9E-02	2.4E-02	8.8E-01
Manton 10 mg.L^{-1} Test 2	4.1E+01	7.3E+00	4.1E+00	2.2E-01	1.3E-01	8.2E-01
Manton 10 mg.L^{-1} Test 2	9.4E+01	2.2E+01	1.2E+01	6.6E-01	4.0E-01	7.6E-01
Manton 10 mg.L^{-1} Test 2	5.1E+02	2.0E+02	1.1E+02	6.1E+00	3.7E+00	6.0E-01
Manton 10 mg.L^{-1} Test 2	1.5E+03	8.3E+02	4.7E+02	2.5E+01	1.5E+01	4.4E-01
Manton 10 mg.L^{-1} Test 2	3.1E+03	2.1E+03	1.2E+03	6.3E+01	3.8E+01	3.2E-01
Manton 15 mg.L^{-1} Test 1	4.7E-01	1.1E-02	6.4E-03	3.4E-04	2.1E-04	9.8E-01
Manton 15 mg.L^{-1} Test 1	4.5E-01	1.1E-02	6.1E-03	3.2E-04	2.0E-04	9.8E-01
Manton 15 mg.L^{-1} Test 1	3.4E-01	7.8E-03	4.4E-03	2.3E-04	1.4E-04	9.8E-01
Manton 15 mg.L^{-1} Test 1	3.8E+00	2.0E-01	1.1E-01	5.9E-03	3.6E-03	9.5E-01
Manton 15 mg.L^{-1} Test 1	3.8E+00	2.0E-01	1.1E-01	5.9E-03	3.6E-03	9.5E-01
Manton 15 mg.L^{-1} Test 1	6.2E+00	3.8E-01	2.1E-01	1.1E-02	6.8E-03	9.4E-01
Manton 15 mg.L^{-1} Test 1	1.1E+01	7.8E-01	4.4E-01	2.3E-02	1.4E-02	9.3E-01
Manton 15 mg.L^{-1} Test 1	2.7E+01	2.7E+00	1.5E+00	7.9E-02	4.8E-02	9.0E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Manton 15 mg.L^{-1} Test 1	2.7E+01	2.6E+00	1.5E+00	7.9E-02	4.8E-02	9.0E-01
Manton 15 mg.L^{-1} Test 1	5.2E+01	6.5E+00	3.7E+00	2.0E-01	1.2E-01	8.8E-01
Manton 15 mg.L^{-1} Test 1	5.2E+01	6.4E+00	3.6E+00	1.9E-01	1.2E-01	8.8E-01
Manton 15 mg.L^{-1} Test 1	7.4E+01	1.0E+01	5.9E+00	3.1E-01	1.9E-01	8.6E-01
Manton 15 mg.L^{-1} Test 1	9.0E+01	1.4E+01	7.7E+00	4.1E-01	2.5E-01	8.5E-01
Manton 15 mg.L^{-1} Test 1	1.4E+02	2.5E+01	1.4E+01	7.4E-01	4.5E-01	8.2E-01
Manton 15 mg.L^{-1} Test 1	1.5E+02	2.7E+01	1.5E+01	8.1E-01	4.9E-01	8.2E-01
Manton 15 mg.L^{-1} Test 1	2.2E+02	4.7E+01	2.6E+01	1.4E+00	8.4E-01	7.9E-01
Manton 15 mg.L^{-1} Test 1	2.4E+02	5.2E+01	2.9E+01	1.6E+00	9.4E-01	7.9E-01
Manton 15 mg.L^{-1} Test 1	7.9E+02	2.6E+02	1.5E+02	7.9E+00	4.7E+00	6.7E-01
Manton 15 mg.L^{-1} Test 1	8.9E+02	3.1E+02	1.8E+02	9.3E+00	5.6E+00	6.5E-01
Manton 15 mg.L^{-1} Test 1	4.2E+03	2.6E+03	1.5E+03	7.9E+01	4.8E+01	3.7E-01
Manton 15 mg.L^{-1} Test 2	5.4E-01	1.3E-02	7.3E-03	3.8E-04	2.3E-04	9.8E-01
Manton 15 mg.L^{-1} Test 2	4.4E-01	1.0E-02	5.6E-03	3.0E-04	1.8E-04	9.8E-01
Manton 15 mg.L^{-1} Test 2	7.5E-01	2.0E-02	1.1E-02	5.9E-04	3.5E-04	9.7E-01
Manton 15 mg.L^{-1} Test 2	7.4E+00	4.5E-01	2.5E-01	1.3E-02	8.1E-03	9.4E-01
Manton 15 mg.L^{-1} Test 2	1.0E+01	7.0E-01	3.9E-01	2.1E-02	1.3E-02	9.3E-01
Manton 15 mg.L^{-1} Test 2	2.1E+01	1.8E+00	1.0E+00	5.3E-02	3.2E-02	9.2E-01
Manton 15 mg.L^{-1} Test 2	4.4E+01	4.8E+00	2.7E+00	1.4E-01	8.7E-02	8.9E-01
Manton 15 mg.L^{-1} Test 2	7.2E+01	9.6E+00	5.5E+00	2.9E-01	1.7E-01	8.7E-01
Manton 15 mg.L^{-1} Test 2	1.2E+02	2.0E+01	1.1E+01	5.9E-01	3.6E-01	8.4E-01
Manton 15 mg.L^{-1} Test 2	5.4E+02	1.5E+02	8.5E+01	4.5E+00	2.7E+00	7.2E-01
Manton 15 mg.L^{-1} Test 2	1.7E+03	7.4E+02	4.2E+02	2.2E+01	1.3E+01	5.6E-01
Manton 15 mg.L^{-1} Test 2	3.2E+03	1.8E+03	1.0E+03	5.5E+01	3.3E+01	4.4E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	6.8E-01	8.7E-02	5.8E-02	3.9E-04	2.9E-05	8.7E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	3.7E-01	3.7E-02	2.5E-02	1.7E-04	1.3E-05	9.0E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	3.0E-01	2.8E-02	1.9E-02	1.3E-04	9.6E-06	9.1E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	9.5E-01	1.4E-01	9.0E-02	6.0E-04	4.6E-05	8.6E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	1.8E+00	3.1E-01	2.1E-01	1.4E-03	1.1E-04	8.3E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	3.4E+00	7.0E-01	4.7E-01	3.1E-03	2.4E-04	7.9E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	5.1E+00	1.1E+00	7.7E-01	5.1E-03	3.9E-04	7.7E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	7.3E+00	1.8E+00	1.2E+00	8.2E-03	6.2E-04	7.5E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	9.1E+00	2.4E+00	1.6E+00	1.1E-02	8.2E-04	7.3E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	1.7E+01	5.3E+00	3.5E+00	2.4E-02	1.8E-03	6.8E-01
Manton 5 mg.L^{-1} at pH 6.5 Test 1	2.1E+01	7.2E+00	4.8E+00	3.2E-02	2.5E-03	6.6E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^+$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.8E+01	1.0E+01	6.9E+00	4.6E-02	3.5E-03	6.4E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	5.0E+01	2.1E+01	1.4E+01	9.3E-02	7.1E-03	5.9E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	7.7E+01	3.5E+01	2.3E+01	1.6E-01	1.2E-02	5.4E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	1.0E+02	4.8E+01	3.2E+01	2.2E-01	1.6E-02	5.2E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.6E+02	1.6E+02	1.0E+02	6.9E-01	5.3E-02	4.1E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	5.1E+02	3.4E+02	2.2E+02	1.5E+00	1.1E-01	3.4E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	9.7E+02	7.1E+02	4.8E+02	3.2E+00	2.4E-01	2.7E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	2.0E+03	1.6E+03	1.1E+03	7.3E+00	5.5E-01	1.9E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 1	4.1E+03	3.6E+03	2.4E+03	1.6E+01	1.2E+00	1.3E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.8E-01	8.5E-02	5.7E-02	3.8E-04	2.9E-05	8.8E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	5.5E-01	6.3E-02	4.2E-02	2.8E-04	2.1E-05	8.9E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	4.8E-01	5.3E-02	3.5E-02	2.4E-04	1.8E-05	8.9E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.1E+00	1.6E-01	1.1E-01	7.4E-04	5.6E-05	8.5E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.7E+00	2.9E-01	1.9E-01	1.3E-03	9.9E-05	8.3E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.6E+00	4.9E-01	3.2E-01	2.2E-03	1.6E-04	8.1E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.0E+00	1.4E+00	9.4E-01	6.3E-03	4.8E-04	7.7E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.1E+00	1.4E+00	9.6E-01	6.4E-03	4.9E-04	7.6E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.1E+01	3.1E+00	2.1E+00	1.4E-02	1.0E-03	7.2E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.2E+01	7.5E+00	5.0E+00	3.4E-02	2.6E-03	6.6E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	3.6E+01	1.4E+01	9.0E+00	6.0E-02	4.6E-03	6.2E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	4.5E+01	1.8E+01	1.2E+01	7.9E-02	6.0E-03	6.0E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	8.3E+01	3.8E+01	2.5E+01	1.7E-01	1.3E-02	5.4E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	2.6E+02	1.5E+02	1.0E+02	6.8E-01	5.2E-02	4.2E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	1.1E+03	8.0E+02	5.3E+02	3.5E+00	2.7E-01	2.6E-01
Manton 5 $mg.L^{-1}$ at pH 6.5 Test 2	6.0E+03	5.4E+03	3.6E+03	2.4E+01	1.8E+00	1.0E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.5E-01	3.4E-02	1.2E-02	3.2E-03	9.7E-03	9.0E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.0E-01	2.8E-02	9.8E-03	2.6E-03	7.9E-03	9.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	2.2E-01	1.9E-02	6.7E-03	1.8E-03	5.3E-03	9.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	5.8E-01	6.7E-02	2.4E-02	6.3E-03	1.9E-02	8.9E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	8.3E-01	1.1E-01	3.9E-02	1.0E-02	3.1E-02	8.7E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	2.0E+00	3.4E-01	1.2E-01	3.2E-02	9.6E-02	8.3E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.0E+00	5.7E-01	2.0E-01	5.3E-02	1.6E-01	8.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.9E+00	7.8E-01	2.8E-01	7.4E-02	2.2E-01	8.0E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	4.6E+00	9.8E-01	3.5E-01	9.2E-02	2.8E-01	7.9E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	1.1E+01	2.9E+00	1.0E+00	2.7E-01	8.3E-01	7.4E-01

Test Name	Total Zn	True solution Zn	True solution Zn^{2+}	True solution $ZnOH^{+}$	True solution $Zn(OH)_2$	DOC-bound Zn fraction
				($\mu g.L^{-1}$)		
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	1.5E+01	4.2E+00	1.5E+00	3.9E-01	1.2E+00	7.2E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	1.8E+01	5.4E+00	1.9E+00	5.1E-01	1.5E+00	7.0E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.7E+01	1.3E+01	4.8E+00	1.3E+00	3.8E+00	6.4E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	5.2E+01	2.0E+01	7.3E+00	1.9E+00	5.8E+00	6.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	7.2E+01	3.0E+01	1.1E+01	2.9E+00	8.6E+00	5.8E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	2.0E+02	1.0E+02	3.7E+01	9.8E+00	3.0E+01	4.8E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.7E+02	2.2E+02	7.7E+01	2.0E+01	6.2E+01	4.2E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	6.9E+02	4.5E+02	1.6E+02	4.3E+01	1.3E+02	3.5E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	1.4E+03	1.1E+03	3.8E+02	1.0E+02	3.0E+02	2.6E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 1	3.2E+03	2.6E+03	9.3E+02	2.5E+02	7.4E+02	1.8E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	3.0E-01	2.6E-02	9.4E-03	2.5E-03	7.5E-03	9.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	3.2E-01	2.9E-02	1.0E-02	2.7E-03	8.2E-03	9.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	2.7E-01	2.3E-02	8.3E-03	2.2E-03	6.6E-03	9.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	5.2E-01	5.4E-02	1.9E-02	5.1E-03	1.5E-02	9.0E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	9.6E-01	1.3E-01	4.5E-02	1.2E-02	3.6E-02	8.7E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	1.4E+00	2.1E-01	7.6E-02	2.0E-02	6.1E-02	8.5E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	2.4E+00	4.1E-01	1.5E-01	3.9E-02	1.2E-01	8.3E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	3.8E+00	7.3E-01	2.6E-01	6.9E-02	2.1E-01	8.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	7.3E+00	1.6E+00	5.9E-01	1.6E-01	4.7E-01	7.8E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	1.4E+01	3.6E+00	1.3E+00	3.4E-01	1.0E+00	7.4E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	2.4E+01	7.5E+00	2.7E+00	7.0E-01	2.1E+00	6.9E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	2.8E+01	9.1E+00	3.3E+00	8.6E-01	2.6E+00	6.8E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	3.7E+01	1.3E+01	4.6E+00	1.2E+00	3.7E+00	6.5E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	4.6E+01	1.7E+01	5.9E+00	1.6E+00	4.7E+00	6.4E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	1.8E+02	8.9E+01	3.2E+01	8.4E+00	2.5E+01	5.1E-01
Manton 5 $mg.L^{-1}$ at pH 8.5 Test 2	7.9E+02	5.2E+02	1.8E+02	4.9E+01	1.5E+02	3.4E-01

Appendix D: Assessing the relevance of current toxicity model validation methods

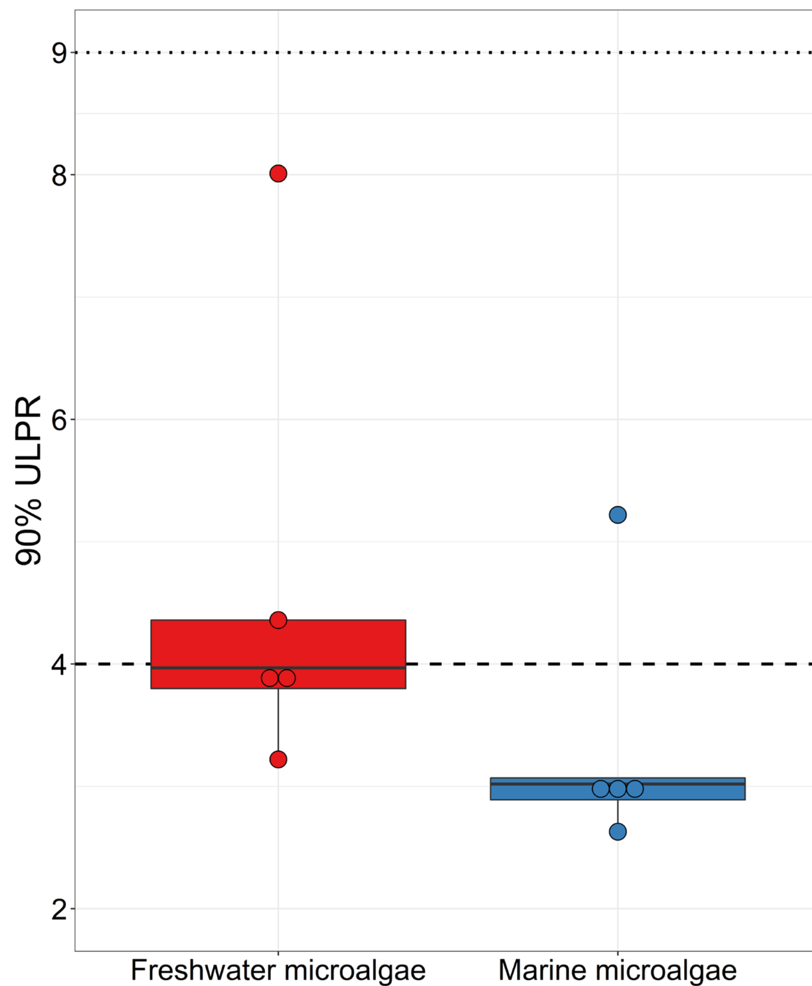

Figure D- 1: Boxplots comparing the 90% upper-lower prediction ratios for all freshwater microalgae and marine microalgae EC50 datasets. Dashed line indicates the factor-of-2 rule threshold of 4 and the dotted line indicates the factor-of-3 threshold of 9.

Table D- 1: Results summary table for each toxicity dataset. a = database available at (https://doi.org/10.1071/EN22050), b = database available at (https://doi.org/10.1002/etc.5235) c = database available at (https://doi.org/10.1007/s00128-017-2258-4) , PGRT = percent of control growth rate, EC = effect concentration, n = number of replicate tests, MMR = maximum/minimum ratio, sd = standard deviation, cv = coefficient of variation, ULPR = upper-lower prediction ratio.

Source	Species	Organism category	Endpoint	Acute or Chronic	Duration	Contaminant	EC value	n
Internal database [a]	Acartia sinjiensis	Marine copepod	Mobility	Acute	48	copper	EC10	37
Internal database [a]	Chlorella sp.	Freshwater microalgae	PGRT	Chronic	72	nickel	EC10	7
Internal database [a]	Isochrysis sp.	Marine microalgae	PGRT	Chronic	72	copper	EC10	5
Internal database [a]	Heliocidaris tuberculata	Marine urchin	Fertilisation	Acute	1	copper	EC10	18
Internal database [a]	Acartia sinjiensis	Marine copepod	Larval development	Chronic	78	copper	EC10	5
Internal database [a]	Acartia sinjiensis	Marine copepod	Larval development	Chronic	78	nickel	EC10	9
Internal database [a]	Acartia sinjiensis	Marine copepod	Mobility	Acute	48	copper	EC50	56
Internal database [a]	Acartia sinjiensis	Marine copepod	Larval development	Chronic	78	copper	EC50	6
Internal database [a]	Chlorella sp.	Freshwater microalgae	PGRT	Chronic	72	nickel	EC50	7
Internal database [a]	Ceriodaphnia dubia	Cladoceran	Survival	Acute	48	copper	EC50	36
Internal database [a]	Chlorella vulgaris	Freshwater microalgae	PGRT	Chronic	72	zinc	EC50	11
Internal database [a]	Entomoeis punctulata	Freshwater microalgae	PGRT	Chronic	72	copper	EC50	12
Internal database [a]	Raphidocelis subcapitata	Freshwater microalgae	Yield	Chronic	72	copper	EC50	17
Internal database [a]	Isochrysis sp.	Marine microalgae	PGRT	Chronic	72	copper	EC50	7
Internal database [a]	Isochrysis sp.	Marine microalgae	PGRT	Chronic	72	copper	EC50	5
Internal database [a]	Nitzschia closterium (temperate)	Marine microalgae	PGRT	Chronic	72	copper	EC50	29
Internal database [a]	Nitzschia closterium (temperate)	Marine microalgae	PGRT	Chronic	72	copper	EC50	6
Internal database [a]	Nitzschia closterium (tropical)	Marine microalgae	PGRT	Chronic	72	nickel	EC50	8
Internal database [a]	Heliocidaris tuberculata	Marine urchin	Fertilisation	Acute	1	copper	EC50	33
Internal database [a]	Heliocidaris tuberculata	Marine urchin	Larval development	Chronic	72	copper	EC50	8
Internal database [a]	Chlorella sp.	Freshwater microalgae	PGRT	Chronic	72	copper	EC10	11
Stone et al. 2022 [b]	Acartia sinjiensis	Marine Copepod	Larval development	Chronic	78	nickel	EC50	29
Internal database [a]	Chlorella sp.	Freshwater microalgae	PGRT	Chronic	72	copper	EC50	124
Internal database [a]	Chlorella sp.	Freshwater microalgae	PGRT	Chronic	72	copper	EC50	11
Internal database [a]	Acartia sinjiensis	Marine copepod	Mobility	Acute	48	copper	EC50	37
Internal database [a]	Acartia sinjiensis	Marine copepod	Larval development	Chronic	78	copper	EC50	5
Stone et al. 2022 [b]	Acartia sinjiensis	Marine copepod	Larval development	Chronic	78	nickel	EC50	9
Meyer et al. 2018 [c]	Pimephales promelas	Freshwater fish	Survival static synthetic	Acute	96	copper	EC50	7
Meyer et al. 2018 [c]	Pimephales promelas	Freshwater fish	Survival static lake	Acute	96	copper	EC50	8
Meyer et al. 2018 [c]	Pimephales promelas	Freshwater fish	Survival flow lake	Acute	96	copper	EC50	5
Meyer et al. 2018 [c]	Daphnia magna	Cladoceran	Survival	Acute	48	copper	EC50	14
Meyer et al. 2018 [c]	Daphnia magna	Cladoceran	Survival	Acute	48	nickel	EC50	20

Meyer et al. 2018 [c]	Daphnia magna	Cladoceran	Survival	Acute	48	zinc	EC50	7

Table D- 1: continued.

Source	**median**	**min**	**max**	**MMR**	**mean**	**sd**	**cv**
Internal database [a]	17.00	3.80	42.00	11.05	16.47	8.44	0.51
Internal database [a]	33.00	17.00	58.00	3.41	35.57	17.78	0.41
Internal database [a]	1.60	1.10	1.90	1.73	1.50	0.38	0.25
Internal database [a]	26.00	11.00	43.00	3.91	27.00	9.37	0.35
Internal database [a]	1.60	0.74	2.90	3.92	1.77	0.96	0.54
Internal database [a]	6.30	2.80	7.10	2.54	5.78	1.35	0.23
Internal database [a]	39.63	19.00	64.00	3.37	40.46	11.60	0.29
Internal database [a]	2.35	1.60	4.20	2.63	2.50	0.91	0.36
Internal database [a]	194.00	106.00	361.00	3.41	218.22	87.68	0.40
Internal database [a]	6.10	2.70	10.80	4.00	6.28	2.06	0.33
Internal database [a]	558.00	199.80	1267.00	6.34	614.12	367.06	0.60
Internal database [a]	12.65	7.60	23.00	3.03	14.33	5.09	0.36
Internal database [a]	15.00	6.00	25.00	4.17	14.53	5.68	0.39
Internal database [a]	8.50	7.20	17.20	2.39	10.89	3.81	0.35
Internal database [a]	4.81	2.60	5.86	2.25	4.54	1.28	0.28
Internal database [a]	12.20	4.59	31.00	6.75	14.03	6.79	0.48
Internal database [a]	3.10	2.10	5.00	2.38	3.35	1.14	0.34
Internal database [a]	6.35	4.00	9.00	2.25	6.51	1.71	0.26
Internal database [a]	41.00	21.00	49.00	2.33	40.05	10.57	0.26
Internal database [a]	11.75	6.60	20.00	3.03	11.76	4.41	0.38
Internal database [a]	0.47	0.24	2.70	11.25	0.83	0.82	1.00
Stone et al. 2022 [b]	8.50	6.10	10.60	1.74	8.58	1.08	0.13
Internal database [a]	3.10	0.90	8.30	9.22	3.39	1.54	0.45
Internal database [a]	2.50	1.20	5.70	4.75	2.89	1.48	0.51
Internal database [a]	35.00	19.00	64.00	3.37	38.27	11.37	0.30
Internal database [a]	2.30	1.60	4.20	2.63	2.52	1.01	0.40
Stone et al. 2022 [b]	8.20	7.50	10.00	1.33	8.51	0.80	0.09
Meyer et al. 2018 [c]	0.27	0.17	0.32	1.93	0.25	0.06	0.24
Meyer et al. 2018 [c]	0.13	0.06	0.18	2.87	0.12	0.04	0.34
Meyer et al. 2018 [c]	0.08	0.03	0.11	3.34	0.08	0.03	0.40
Meyer et al. 2018 [c]	0.10	0.07	0.15	2.01	0.10	0.02	0.20

Meyer et al. 2018 [c]	1.41	0.93	2.33	2.50	1.60	0.43	0.27
Meyer et al. 2018 [c]	0.97	0.61	1.28	2.12	0.93	0.26	0.28

Table D- 1: continued.

Source	**2.5%ile**	**5%ile**	**10%ile**	**90%ile**	**95%ile**	**97.5%ile**	**80-ULPR**	**90-ULPR**	**95-ULPR**
Internal database [a]	-0.1	2.6	5.7	27.3	30.4	33.0	4.8	11.7	-507.9
Internal database [a]	0.7	6.1	12.8	58.4	64.8	70.4	4.6	10.6	98.6
Internal database [a]	0.8	0.9	1.0	2.0	2.1	2.2	2.0	2.4	3.0
Internal database [a]	8.6	11.6	15.0	39.0	42.4	45.4	2.6	3.7	5.3
Internal database [a]	-0.1	0.2	0.5	3.0	3.3	3.6	5.5	17.0	-35.0
Internal database [a]	3.1	3.6	4.1	7.5	8.0	8.4	1.9	2.2	2.7
Internal database [a]	17.7	21.4	25.6	55.3	59.5	63.2	2.2	2.8	3.6
Internal database [a]	0.7	1.0	1.3	3.7	4.0	4.3	2.7	4.0	5.9
Internal database [a]	46.4	74.0	105.8	330.6	362.4	390.1	3.1	4.9	8.4
Internal database [a]	2.2	2.9	3.6	8.9	9.7	10.3	2.5	3.4	4.6
Internal database [a]	-105.3	10.3	143.6	1084.7	1217.9	1333.5	7.6	118.1	-12.7
Internal database [a]	4.4	6.0	7.8	20.8	22.7	24.3	2.7	3.8	5.6
Internal database [a]	3.4	5.2	7.2	21.8	23.9	25.7	3.0	4.6	7.6
Internal database [a]	3.4	4.6	6.0	15.8	17.2	18.4	2.6	3.7	5.4
Internal database [a]	2.0	2.4	2.9	6.2	6.6	7.1	2.1	2.7	3.5
Internal database [a]	0.7	2.9	5.3	22.7	25.2	27.3	4.3	8.8	37.7
Internal database [a]	1.1	1.5	1.9	4.8	5.2	5.6	2.6	3.6	5.0
Internal database [a]	3.2	3.7	4.3	8.7	9.3	9.9	2.0	2.5	3.1
Internal database [a]	19.3	22.7	26.5	53.6	57.4	60.8	2.0	2.5	3.1
Internal database [a]	3.1	4.5	6.1	17.4	19.0	20.4	2.9	4.2	6.5
Internal database [a]	-0.8	-0.5	-0.2	1.9	2.2	2.4	-8.3	-4.1	-3.1
Stone et al. 2022 [b]	6.5	6.8	7.2	10.0	10.4	10.7	1.4	1.5	1.7
Internal database [a]	0.4	0.9	1.4	5.4	5.9	6.4	3.8	6.9	17.1
Internal database [a]	0.0	0.5	1.0	4.8	5.3	5.8	4.8	11.5	-6126.0
Internal database [a]	16.0	19.6	23.7	52.8	57.0	60.6	2.2	2.9	3.8
Internal database [a]	0.5	0.9	1.2	3.8	4.2	4.5	3.1	4.9	8.4
Stone et al. 2022 [b]	6.9	7.2	7.5	9.5	9.8	10.1	1.3	1.4	1.5
Meyer et al. 2018 [c]	0.1	0.1	0.2	0.3	0.3	0.4	1.9	2.3	2.8
Meyer et al. 2018 [c]	0.0	0.1	0.1	0.2	0.2	0.2	2.6	3.6	5.0
Meyer et al. 2018 [c]	0.0	0.0	0.0	0.1	0.1	0.1	3.1	4.8	8.3

Meyer et al. 2018 [c]	0.1	0.1	0.1	0.1	0.1	0.1	1.7	2.0	2.3
Meyer et al. 2018 [c]	0.8	0.9	1.1	2.1	2.3	2.4	2.0	2.6	3.2
Meyer et al. 2018 [c]	0.4	0.5	0.6	1.3	1.4	1.4	2.1	2.7	3.4

Appendix E: Development and validation of zinc toxicity prediction models

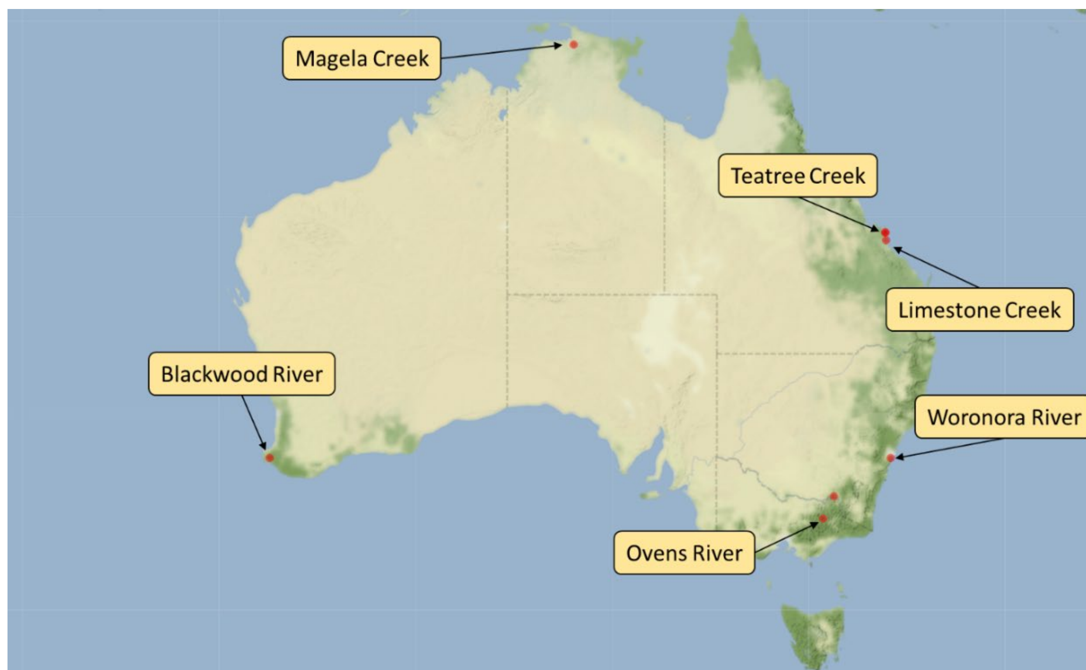

Figure E-1: Sampling locations for independent validation testing with natural waters.

Chemical analysis of natural waters

Additional samples were taken at the time of collection for chemical analyses at an external commercial laboratory (ALS Environmental). Samples were collected in laboratory-supplied and preserved containers and were collected and analysed in accordance with ALS Environmental in-house collection and holding time requirements. Total hardness (as CaCO3) and alkalinity (including hydroxide, carbonate, bicarbonate and total, measured as CaCO3) were measured using PC titration. All anion (sulphate and chloride) and nutrients (ammonia, NOx, total Kjeldahl nitrogen, total phosphorus, and reactive phosphorus) were analysed using a discrete analyser. Dissolved organic carbon was analysed using an automated TOC analyser and inorganic carbon was analysed using an automated carbon analyser with infrared detector for CO2 measurement. All cations were analysed using either ICP-AES or ICP-mass spectrometry (ICP-MS) at CSIRO, Lucas Heights. Additional DOC samples were collected and analysed from test waters at time of testing and determined by the non-purgeable organic carbon method (TOC-L series, Shimadzu) at La Trobe University, Albury-Wodonga Campus. All sample batches analysed had the following quality control (QC) measurements: laboratory duplicates, method blanks, laboratory control spikes and recoveries, and matrix spike spikes and recoveries.

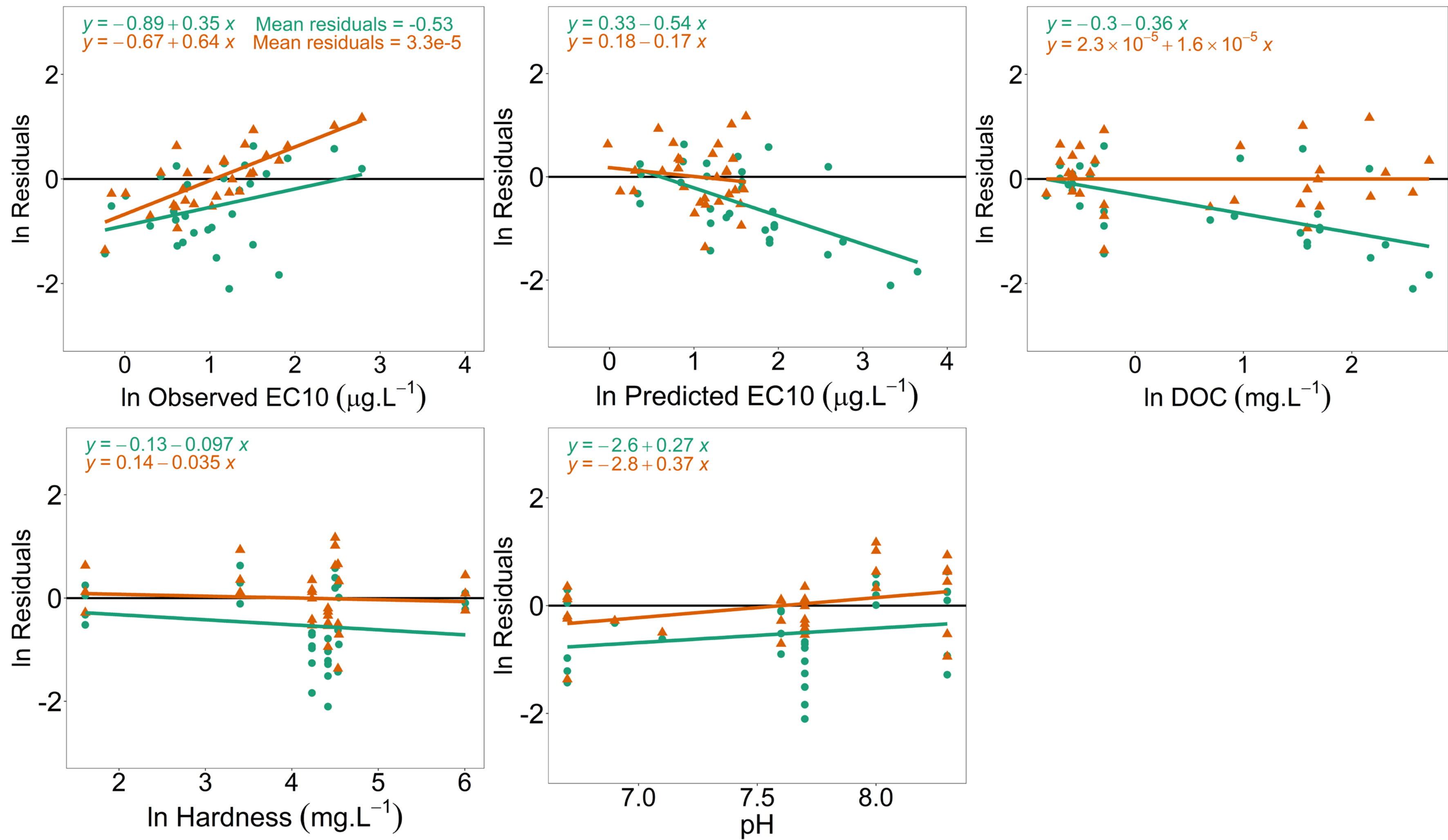

Figure E-2: Model residuals as a function of observed EC10 values, predicted EC10 values, DOC, hardness and pH for EC10 MLR models without (green text, line and circles) and with (orange text, line and circles) interaction terms.

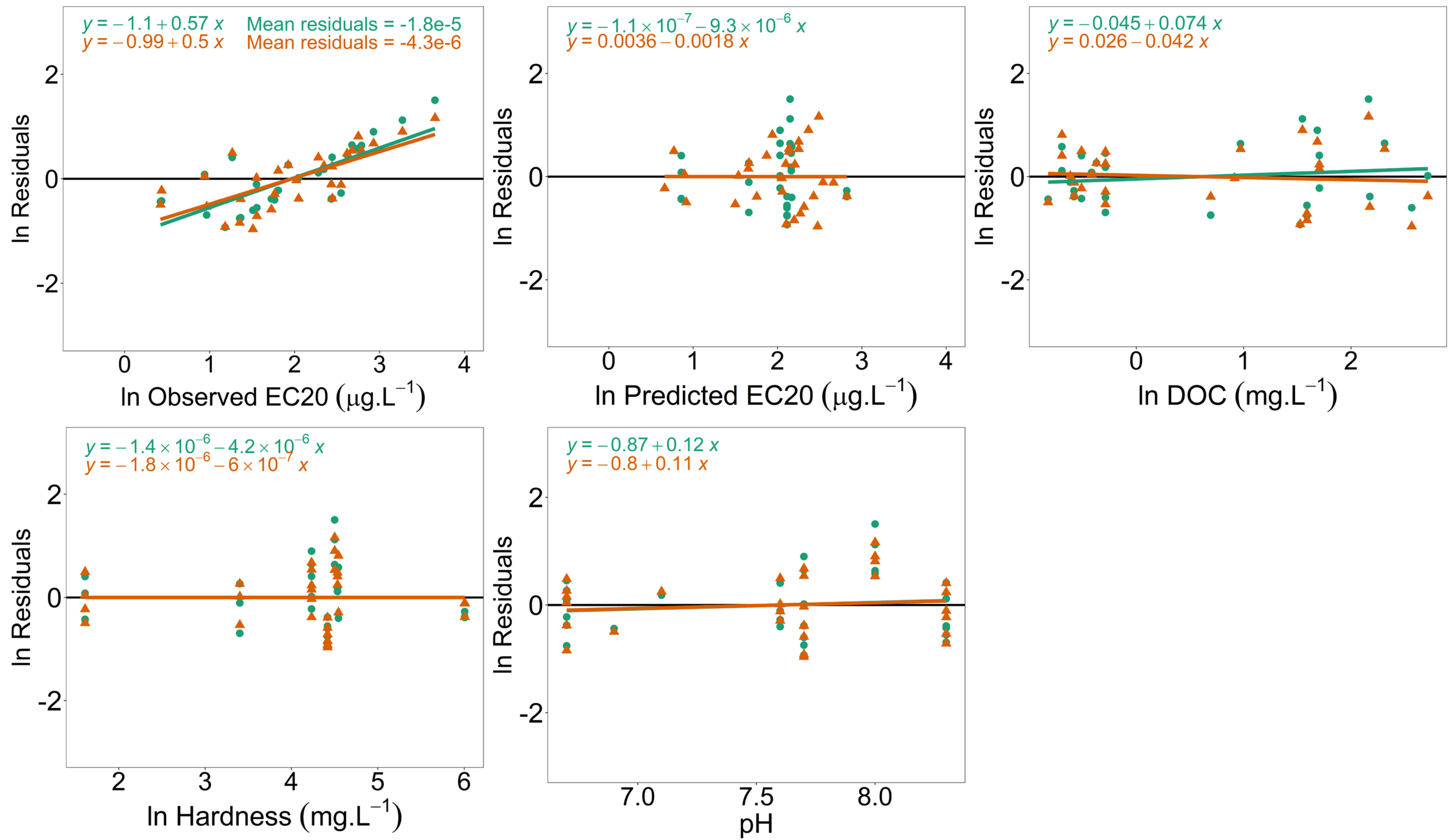

Figure E-3: Model residuals as a function of observed EC20 values, predicted EC20 values, DOC, hardness and pH for EC20 MLR models without (green text, line and circles) and with (orange text, line and circles) interactive terms.

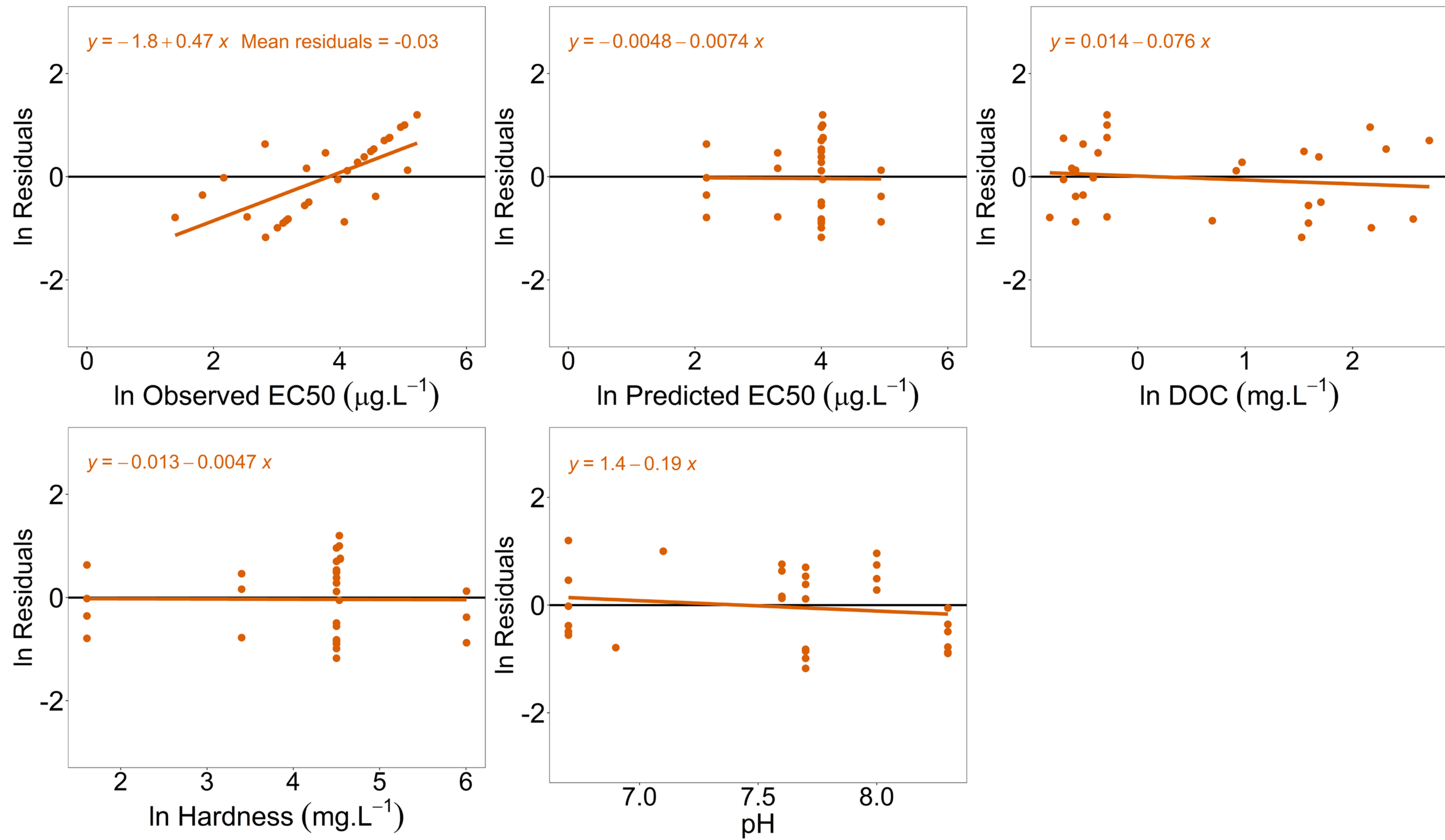

Figure E-4: Model residuals as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH for the EC50 (full dataset) MLR model.

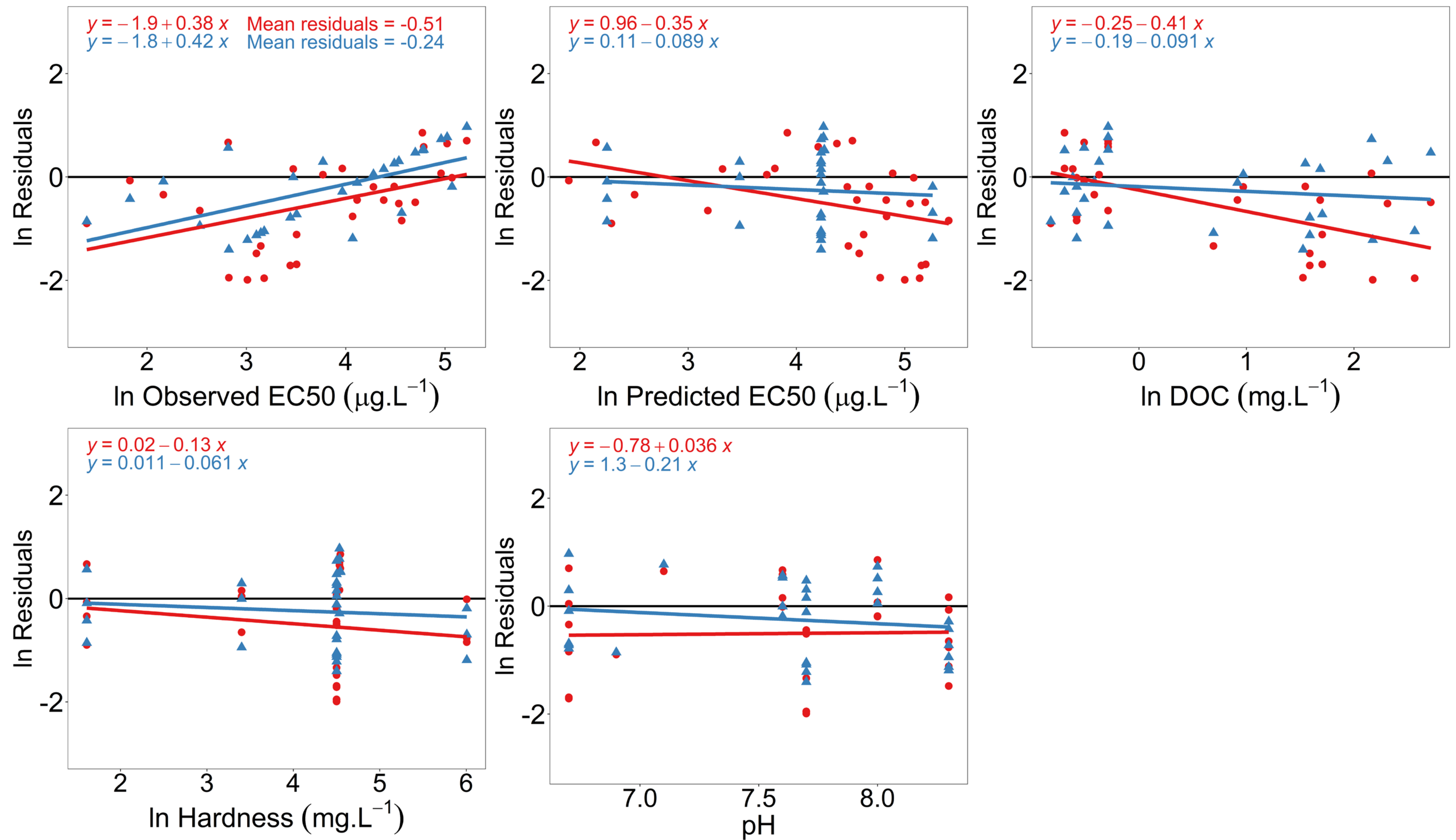

Figure E-5: Model residuals for the EC50 MLR models developed using the data subset (n=18) using the full dataset (n=30) for autovalidation. Model residuals as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH for EC50 MLR AIC-selected (red text, line and circles) and BIC-selected (blue text, line and circles) models.

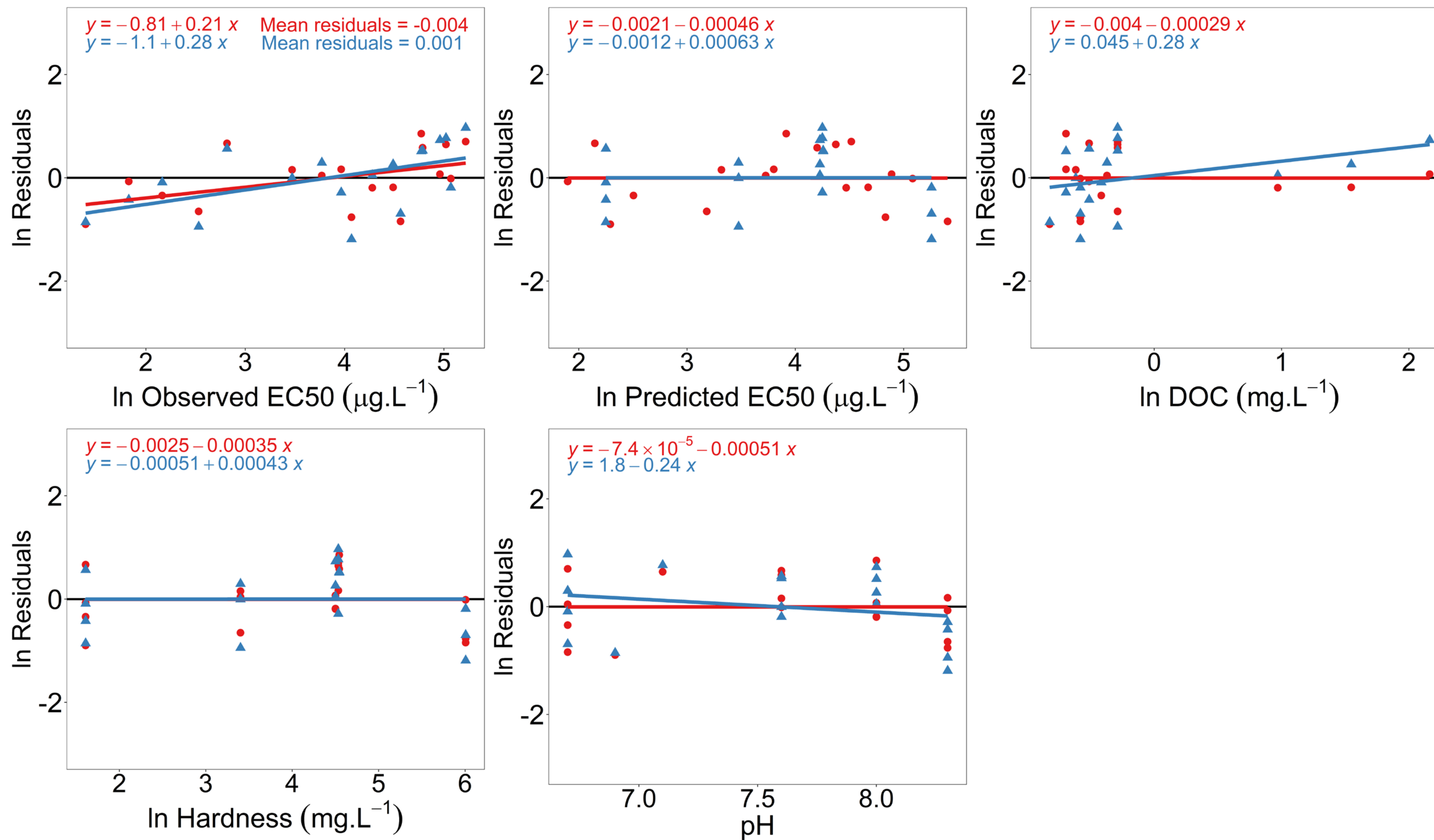

Figure E-6: Model residuals for the EC50 MLR models using the data subset (n=18) for both development and autovalidation. Model residuals as a function of observed EC50 values, predicted EC10 values, DOC, hardness and pH for EC10 MLR AIC-selected (red text, line and circles) and BIC-selected (blue text, line and circles) models.

Independent validation toxicity test acceptability data

All control growth rates were high relative to the synthetic laboratory water controls (Price et al. 2021; 2022; 2023) and had low variation, with all tests having a coefficient of variation below the acceptability criteria of 20%.

Zinc reference toxicant tests had a mean EC50 of 92 (± 35, n =7) $\mu g.L^{-1}$ and mean control growth rates were 1.7 (± 0.1, n =7) doublings/d, indicating that the microalgal cultures had repeatable and comparable sensitivity across tests. Growth rate data in controls presented below is mean ± standard error (n = 5).

Water type	Growth rate in controls (doublings per day)	Control coefficient of variation (%)
Reference toxicant water	1.7 ± 0.1	8
Blackwood River Buffered	2.12 ± 0.03	1
Blackwood River Unbuffered	2.08 ± 0.06	3
Limestone Creek Buffered	2.50 ± 0.05	2
Limestone Creek Unbuffered	2.51 ± 0.08	3
Magela Creek Buffered	2.23 ± 0.08	4
Magela Creek Unbuffered	2.36 ± 0.03	1
Ovens River Buffered	2.34 ± 0.04	2
Ovens River Unbuffered	2.41 ± 0.06	3
Woronora River Buffered	2.16 ± 0.08	4
Woronora Unbuffered	2.20 ± 0.03	1
Ovens River (pH adjusted) Buffered	2.27 ± 0.04	2
Teatree Creek Unbuffered	2.51 ± 0.05	2

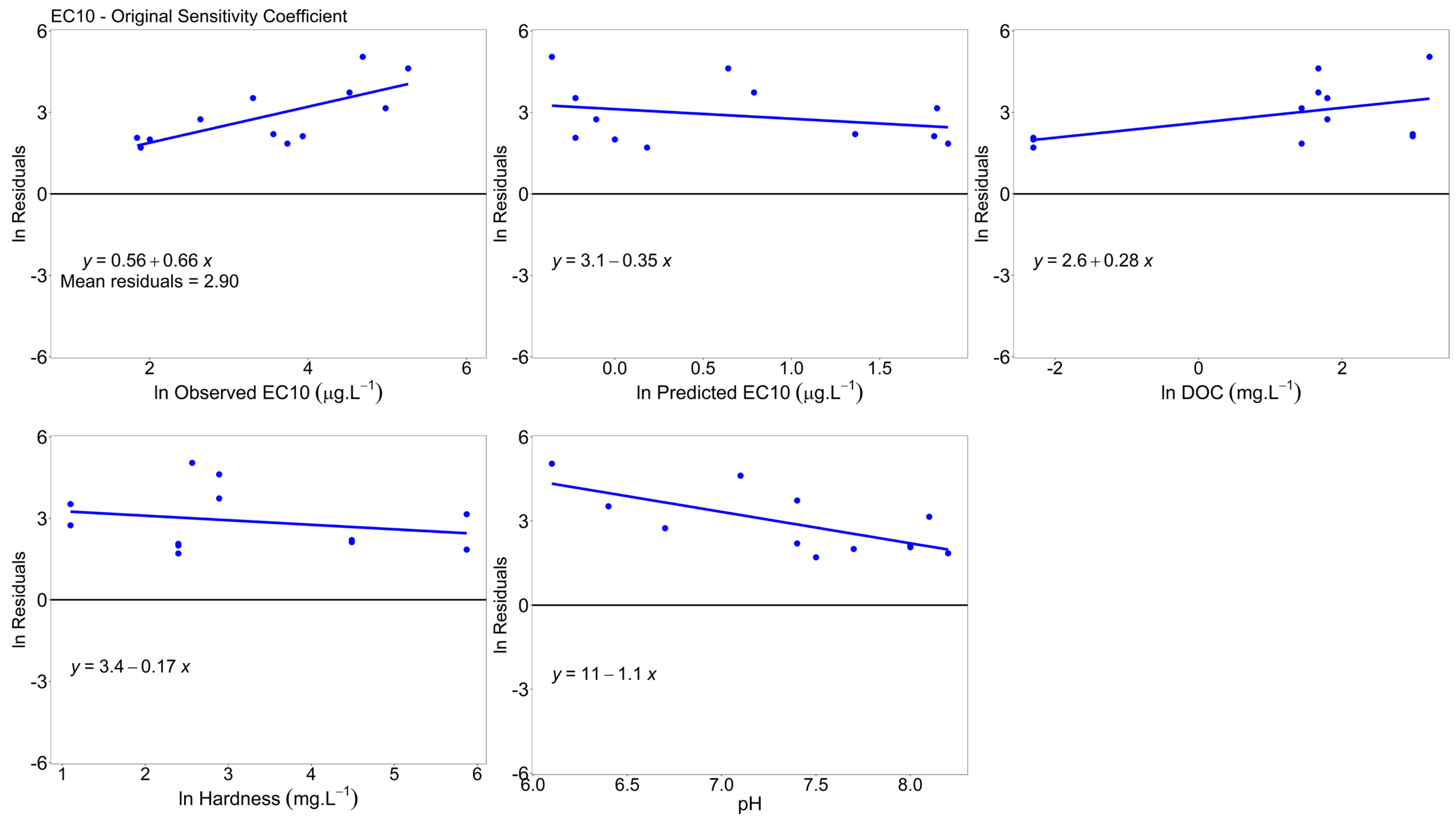

Figure E-7: Model residuals for the EC10 model with original sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC10 values, predicted EC10 values, DOC, hardness and pH.

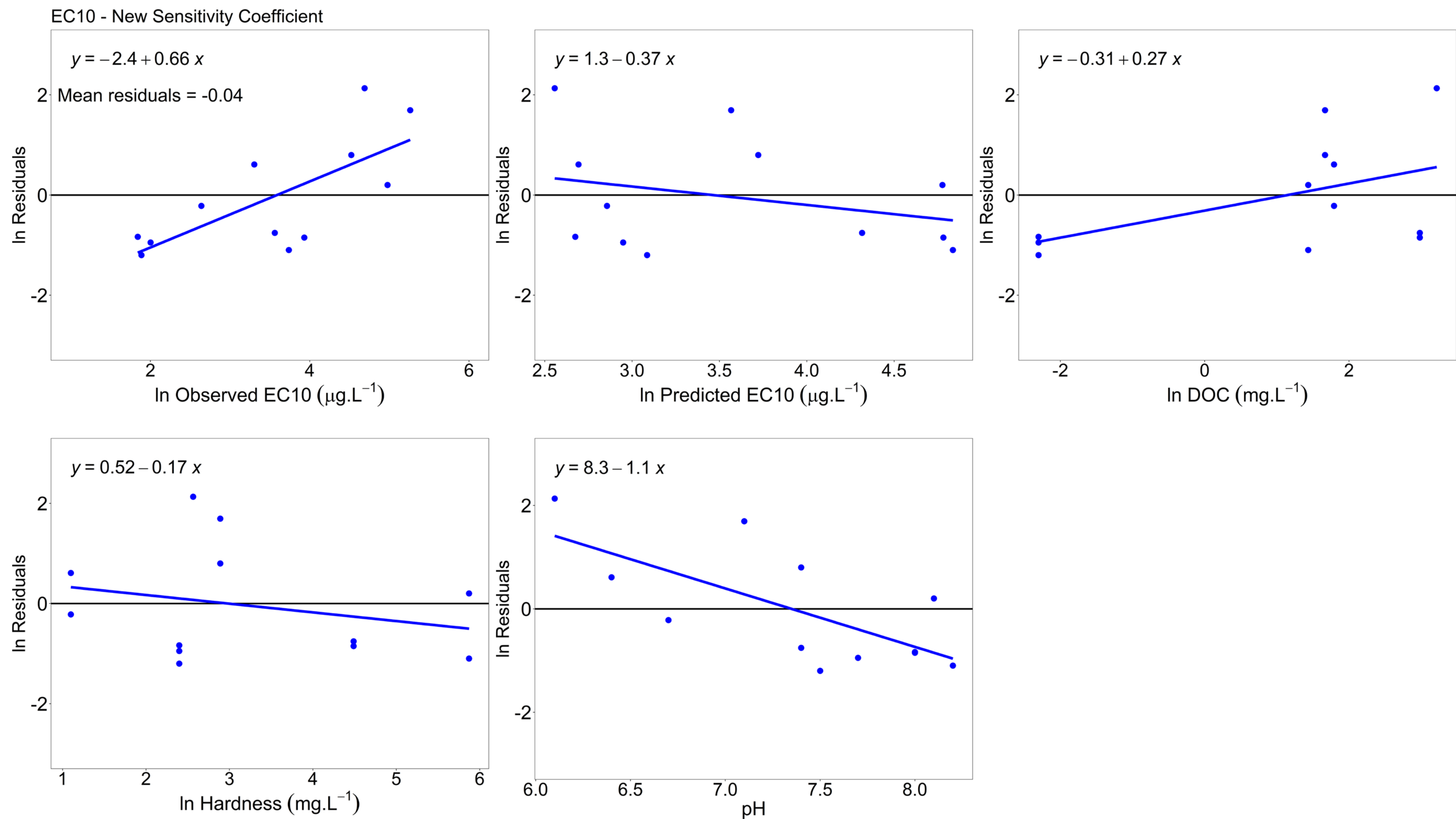

Figure E-8: Model residuals for the EC10 model with updated sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC10 values, predicted EC10 values, DOC, hardness and pH.

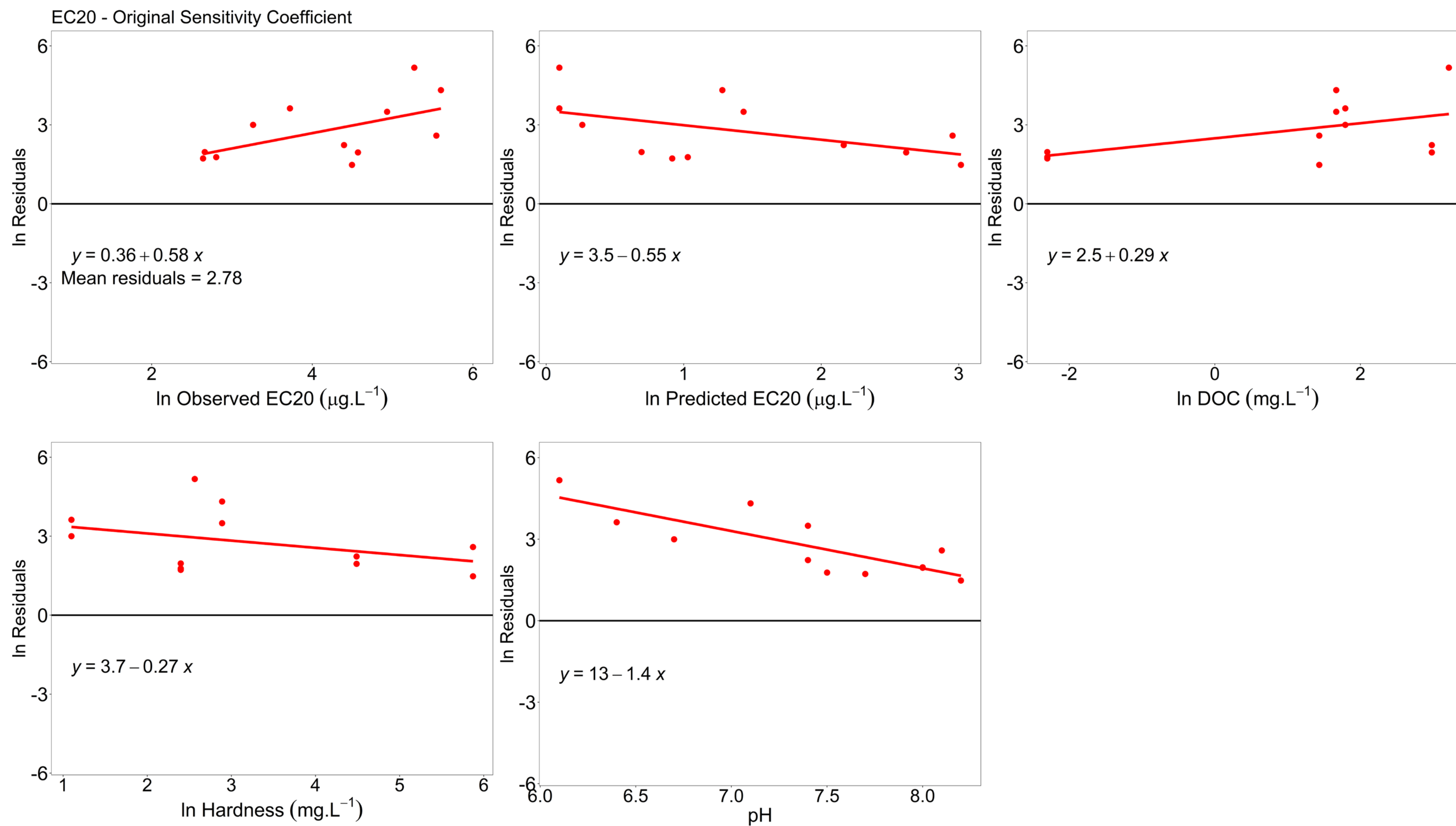

Figure E-9: Model residuals for the EC20 model with original sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC20 values, predicted EC20 values, DOC, hardness and pH.

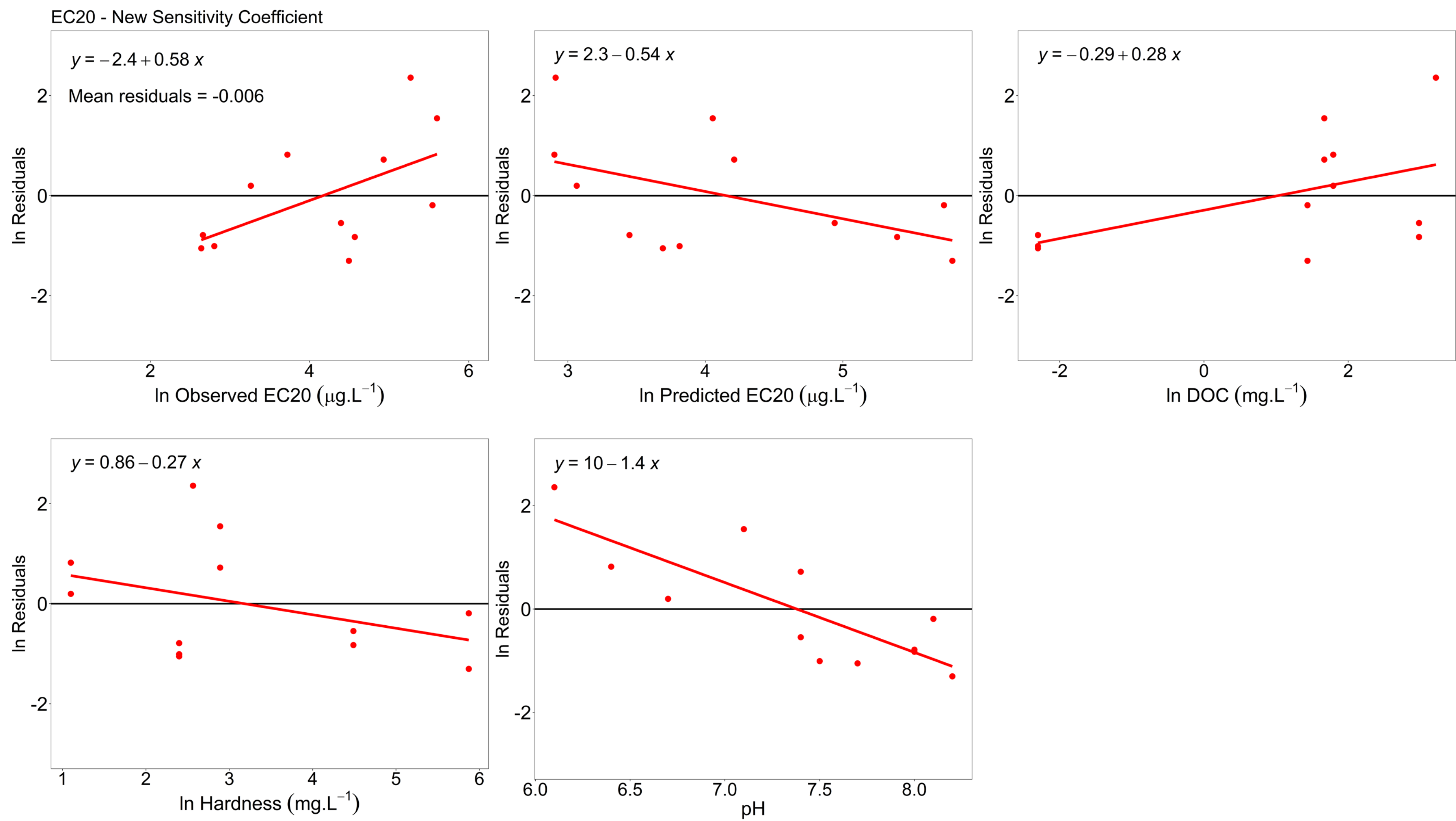

Figure E-10: Model residuals for the EC20 model with updated sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC20 values, predicted EC20 values, DOC, hardness and pH.

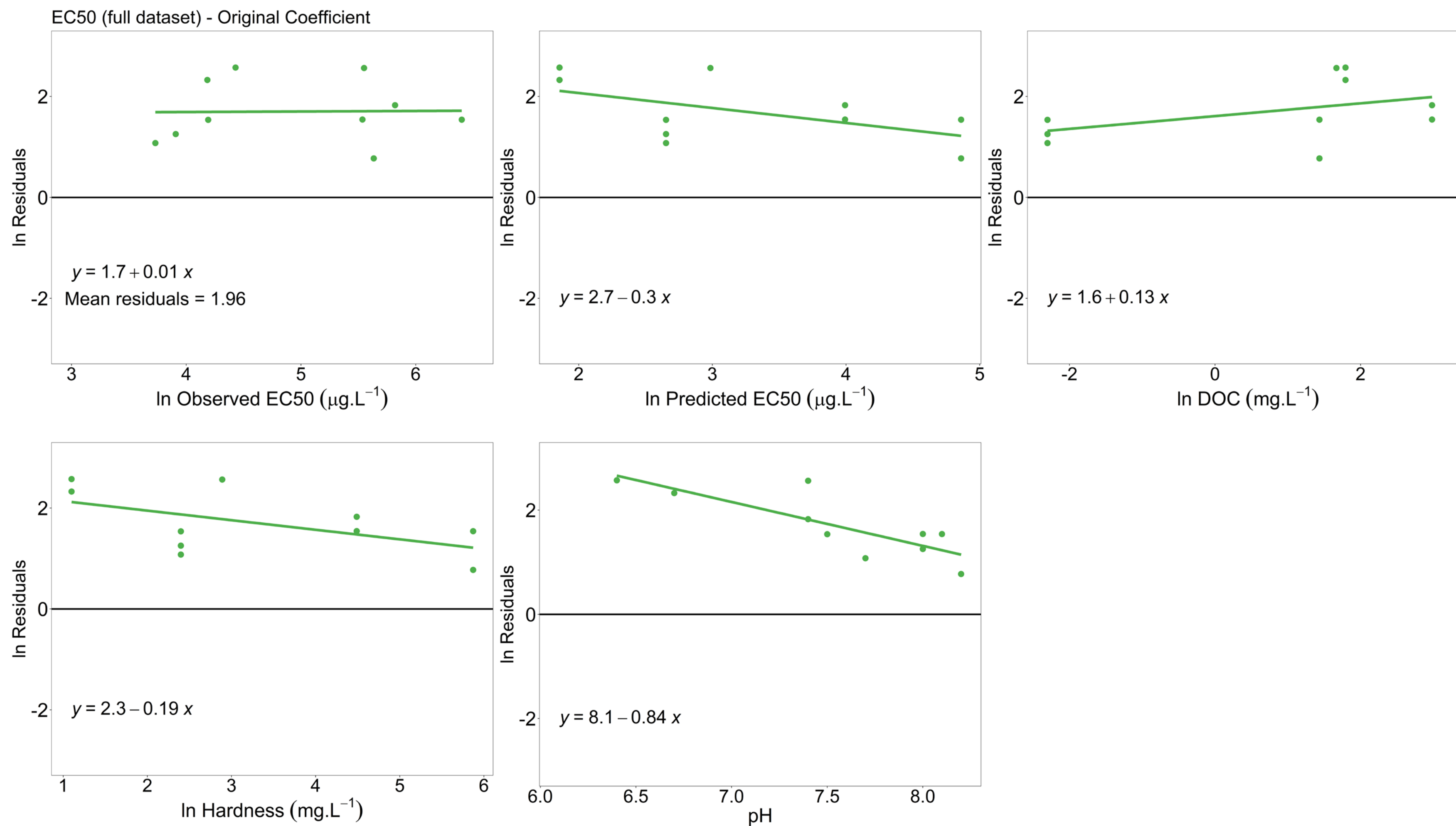

Figure E-11: Model residuals for the EC50 model (full dataset) with original sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH.

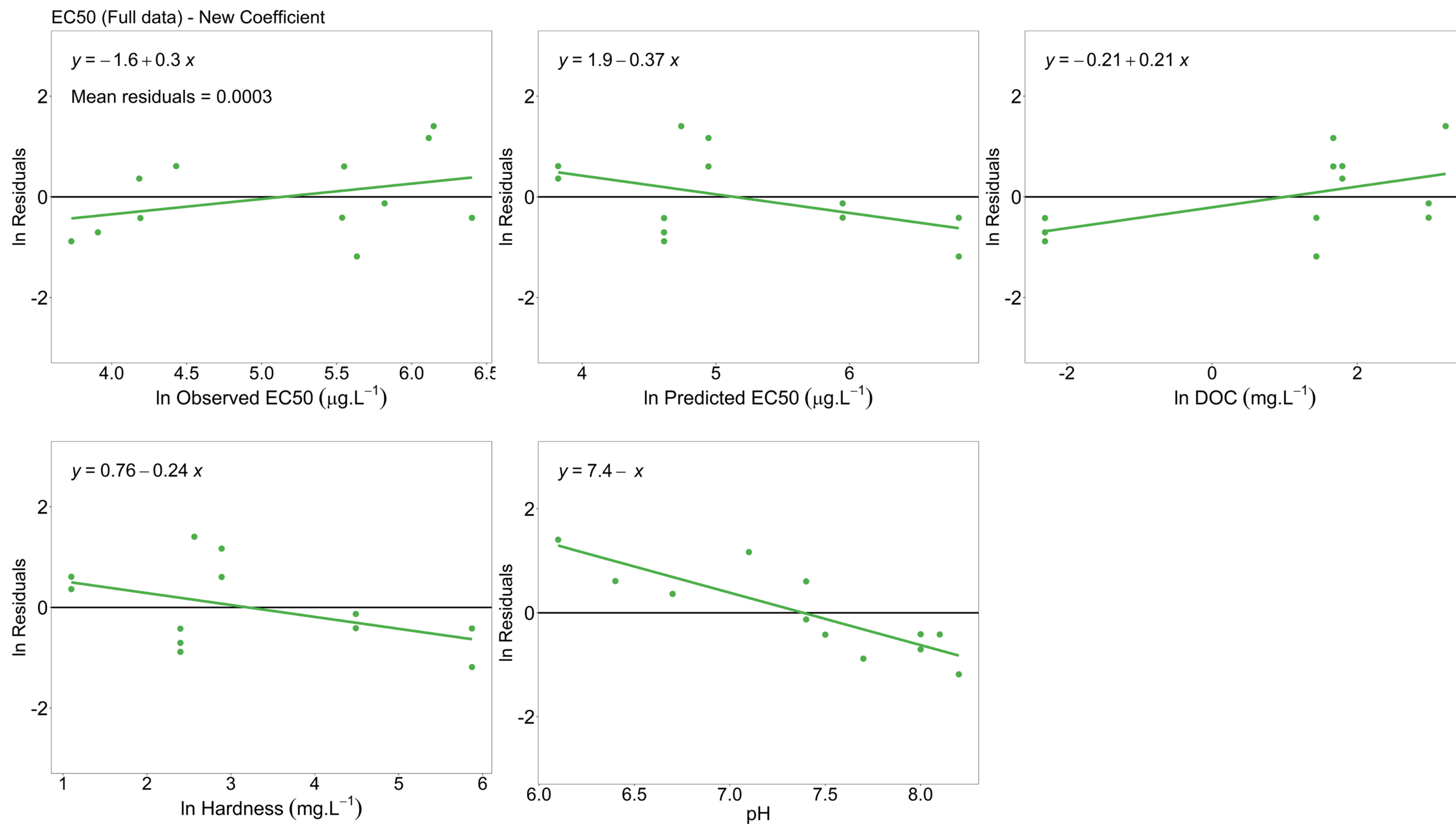

Figure E-12: Model residuals for the EC50 model (full dataset) with updated sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH.

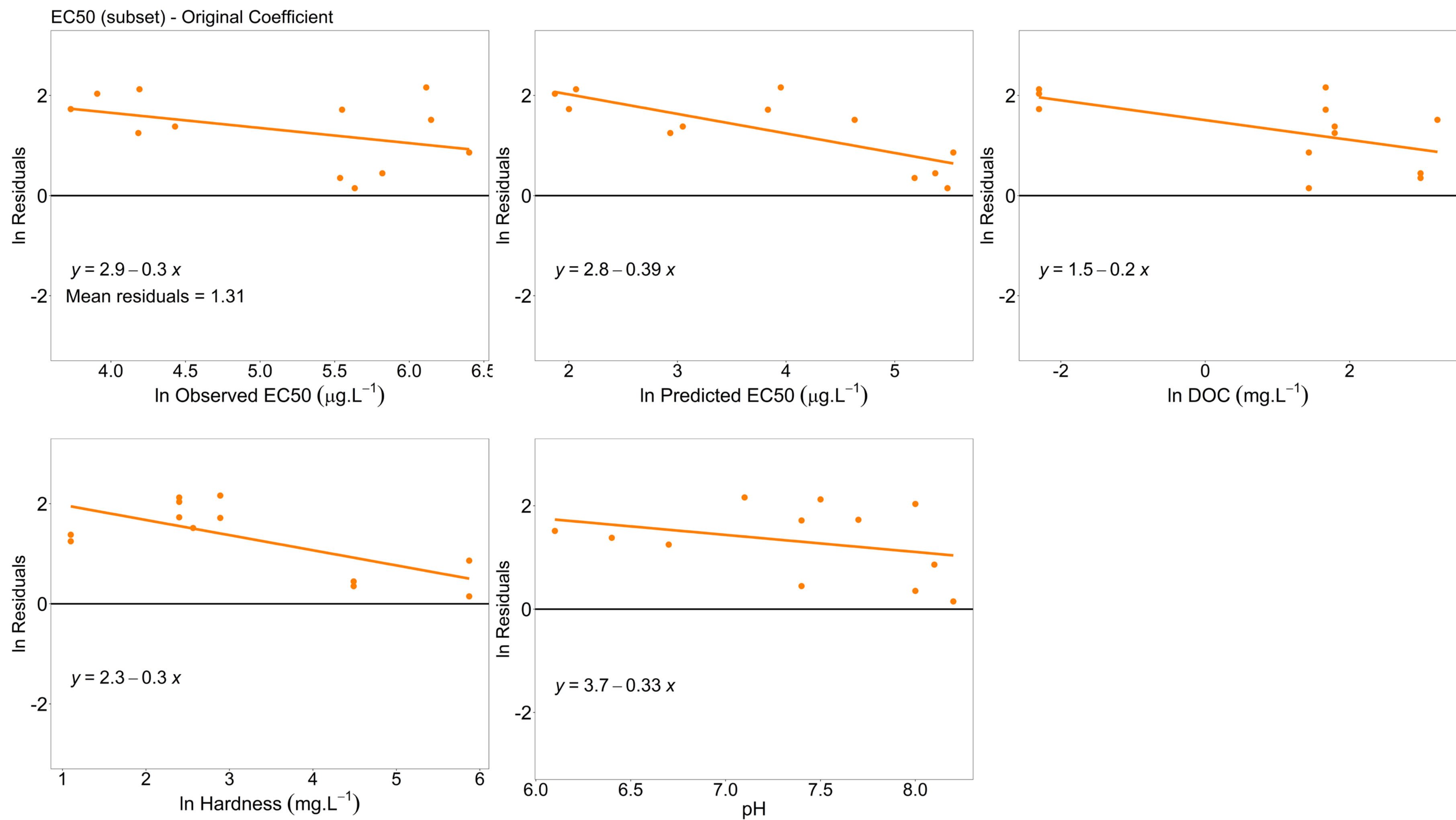

Figure E-13: Model residuals for the EC50 model (subset data) with original sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH.

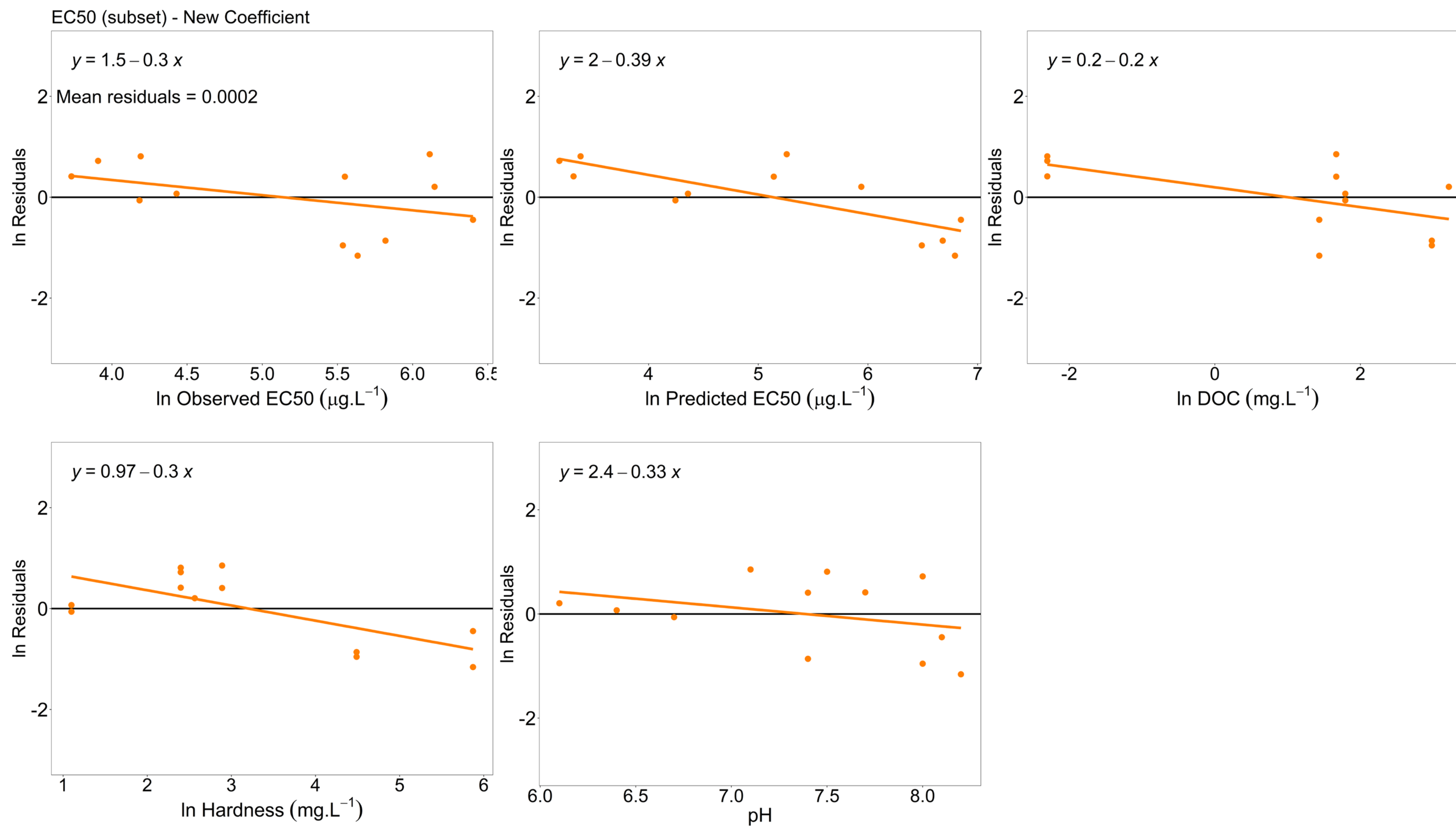

Figure E-14: Model residuals for the EC50 model (subset data) with new sensitivity coefficients using the Australian natural waters for independent validation. Residuals are as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH.

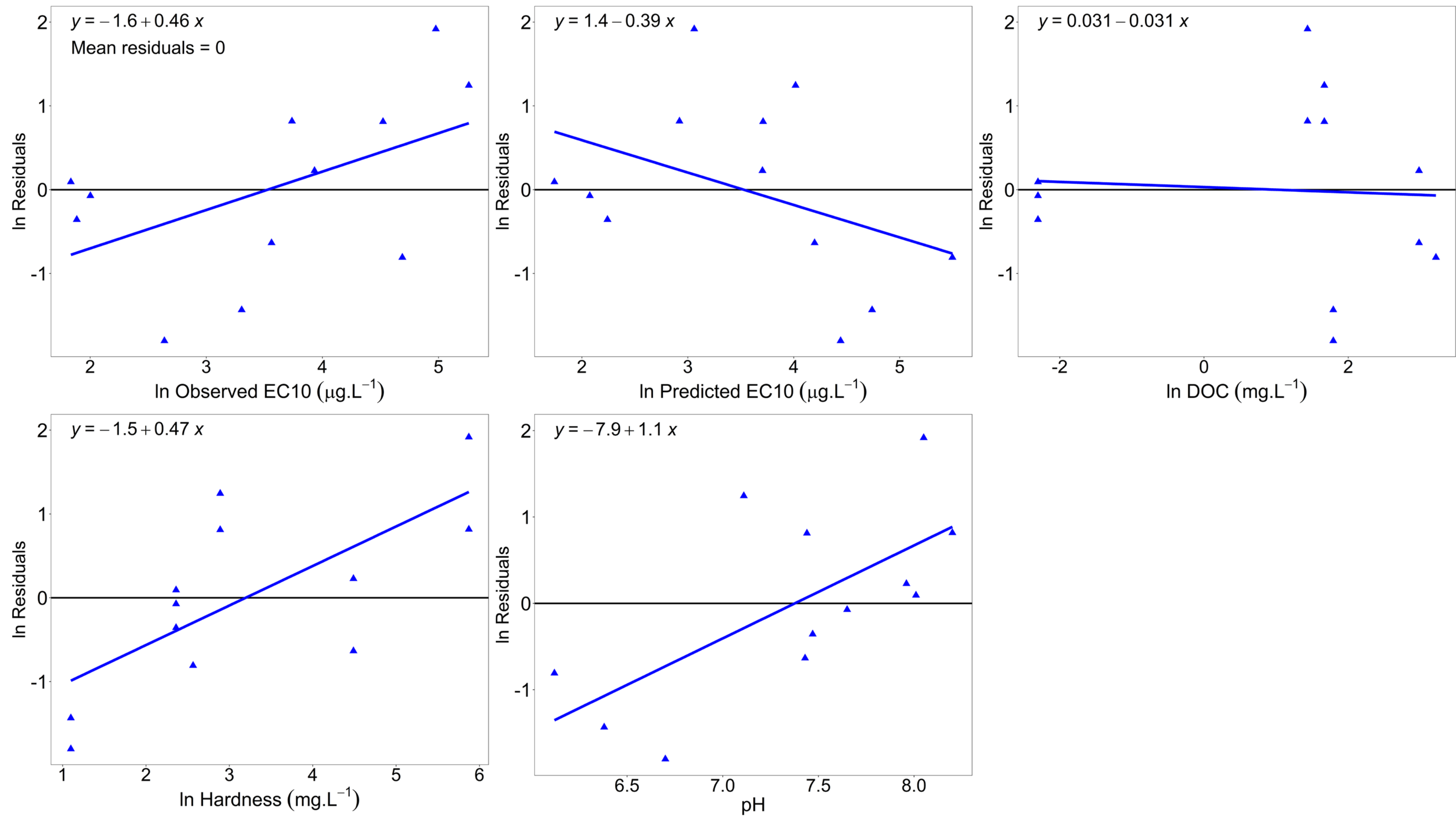

Figure E-15: Model residuals for the DeForest et al. (2023) *R. subcapitata* EC10 model. Residuals are as a function of observed EC10 values, predicted EC10 values, DOC, hardness and pH.

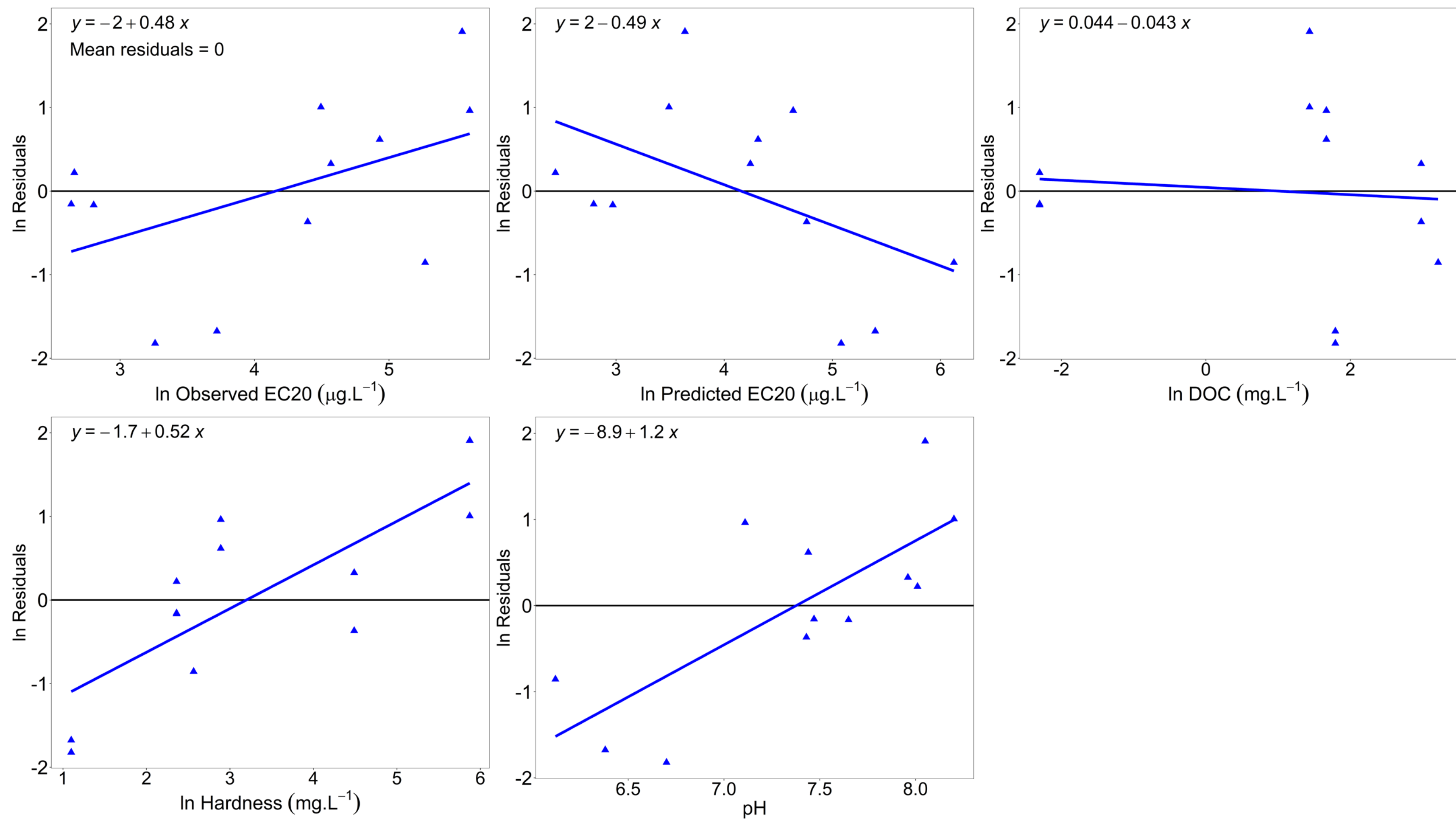

Figure E-16: Model residuals for the DeForest et al. (2023) *R. subcapitata* EC20 model. Residuals are as a function of observed EC20 values, predicted EC20 values, DOC, hardness and pH.

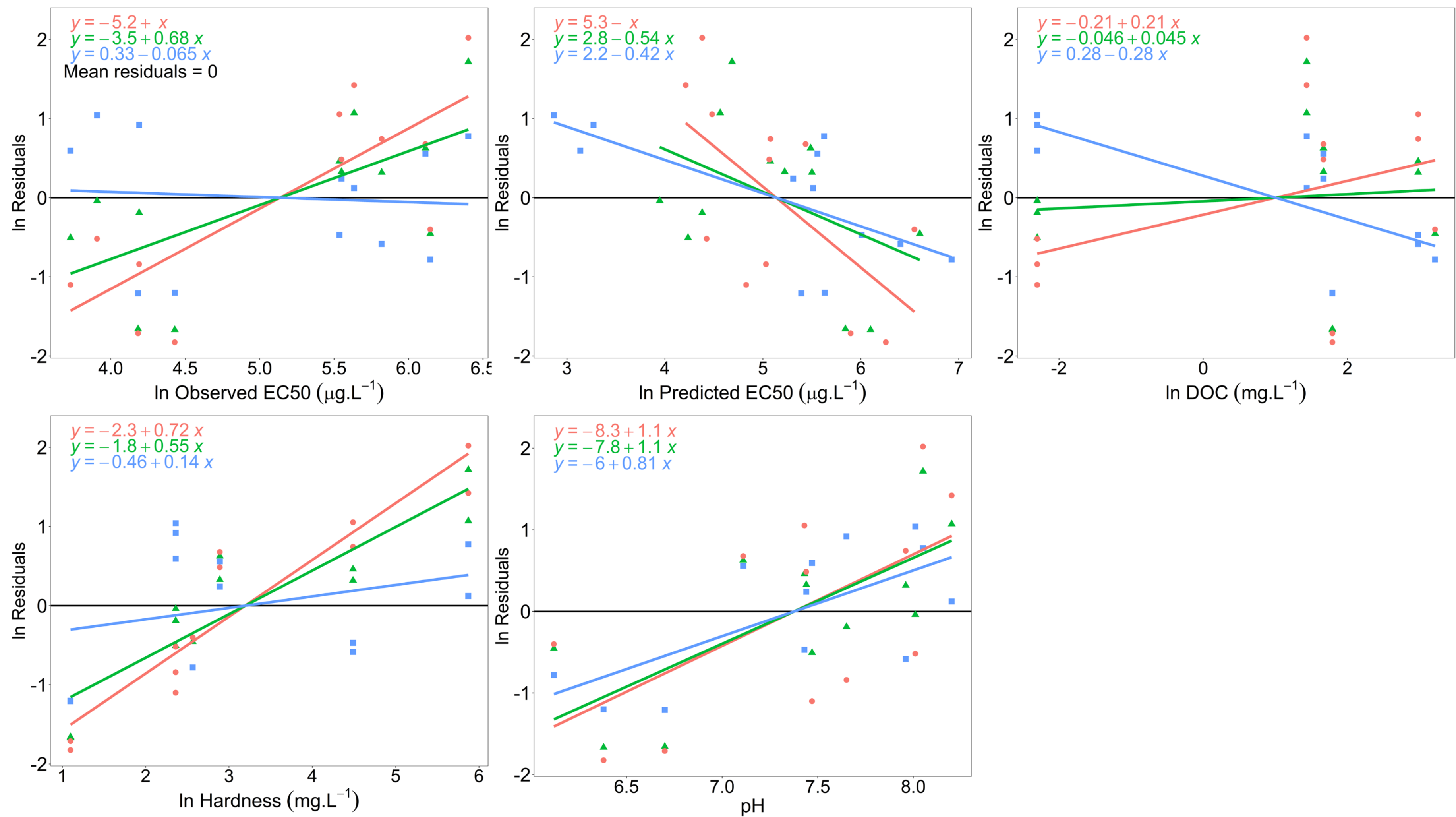

Figure E-17: Model residuals for the CCME (2018) (red circles), DeForest et al. (2023) (blue squares) and Stauber et al. (2023) (green triangles) *R. subcapitata* EC50 models. Residuals are as a function of observed EC50 values, predicted EC50 values, DOC, hardness and pH.

Table E- 1: Summary of all data used for Chlorella sp. MLR model development at EC10, EC20, and EC50 level. EC = effect concentrations, SD = standard deviation, DOC = dissolved organic carbon, MD = Manton Dam, AC = Appletree Creek, SR = Suwannee River

Test ID	n	EC Value	Conc	SD	pH	Ca ($mg.L^{-1}$)	Mg ($mg.L^{-1}$)	Hardness ($mg.L^{-1}$)	DOC Source	DOC ($mg.L^{-1}$)
H5_pH6.5	2	EC10	1.53	0.27	6.7	1.19	0.65	5	No	0.66
H5_pH6.9	1	EC10	1.01	0.28	6.9	1.19	0.65	5	No	0.44
H5_pH7.5	2	EC10	1.84	0.41	7.6	1.19	0.65	5	No	0.6
H5_pH8.5	2	EC10	0.85	0.12	8.3	1.19	0.65	5	No	0.6
H30_pH6.5	2	EC10	3.21	0.57	6.7	4.995	4.27	30	No	0.69
H30_pH7.5	2	EC10	2.09	0.39	7.6	4.995	4.27	30	No	0.54
H30_pH8.5	2	EC10	1.35	0.18	8.3	4.995	4.27	30	No	0.75
H90_pH6.5	3	EC10	4.53	0.89	6.7	15.1	13.8	93	No	0.75
H90_pH7.0	2	EC10	1.78	0.72	7.1	15.2	13.5	93	No	0.75
H90_pH7.5	2	EC10	0.79	0.15	7.6	15.3	13.5	94	No	0.75
H90_pH8.0	2	EC10	4.10	1.01	8	15.3	13.5	94	No	0.5
H90_pH8.5	3	EC10	3.20	0.30	8.3	15.2	13.3	93	No	0.5
H400_pH6.5	2	EC10	5.28	0.49	6.7	70.99	55.48	406	No	0.56
H400_pH7.5	2	EC10	4.37	1.30	7.6	70.99	55.48	406	No	0.56
H400_pH8.5	2	EC10	3.85	0.75	8.3	70.99	55.48	406	No	0.56
M2_pH7.5	2	EC10	2.03	0.43	7.7	11.47	9.78	68.9	MD	2.5
MD5_pH7.5	2	EC10	3.53	0.56	7.7	11.47	9.78	68.9	MD	5.4
MD10_pH7.5	2	EC10	4.51	0.79	7.7	11.47	9.78	68.9	MD	10.1
MD15_pH7.5	2	EC10	6.12	1.22	7.7	11.47	9.78	68.9	MD	15.1
AC2_pH7.5	2	EC10	1.82	0.40	7.7	13.77	11.77	82.9	AC	2
AC5_pH7.5	2	EC10	2.25	0.79	7.7	13.77	11.77	82.9	AC	4.6
AC10_pH7.5	2	EC10	2.93	0.81	7.7	13.77	11.77	82.9	AC	8.8
AC15_pH7.5	2	EC10	3.41	0.96	7.7	13.77	11.77	82.9	AC	13
MD5_pH6.5	2	EC10	2.66	0.48	6.7	11.47	9.78	68.9	MD	5.5
MD5_pH8.5	2	EC10	2.78	0.35	8.3	11.47	9.78	68.9	MD	5.5
AC5_pH6.5	2	EC10	1.85	0.22	6.7	13.77	11.77	82.9	AC	4.9
AC5_pH8.5	2	EC10	1.98	0.27	8.3	13.77	11.77	82.9	AC	4.9
SR5	1	EC10	6.78	0.69	8	-	-	90	SR	2.64
SR10	1	EC10	11.72	1.06	8	-	-	90	SR	4.7
SR15	1	EC10	16.22	2.14	8	-	-	90	SR	8.7
H5_pH6.5	2	EC20	2.56	0.35	6.7	1.19	0.65	5	No	0.66
H5_pH6.9	1	EC20	1.52	0.32	6.9	1.19	0.65	5	No	0.44
H5_pH7.5	2	EC20	3.55	0.62	7.6	1.19	0.65	5	No	0.6
H5_pH8.5	2	EC20	1.54	0.16	8.3	1.19	0.65	5	No	0.6

Test ID	n	EC Value	Conc	SD	pH	Ca (mg.L^{-1})	Mg (mg.L^{-1})	Hardness (mg.L^{-1})	DOC Source	DOC (mg.L^{-1})
H30_pH6.5	2	EC20	6.86	0.95	6.7	4.995	4.27	30	No	0.69
H30_pH7.5	2	EC20	4.72	0.71	7.6	4.995	4.27	30	No	0.54
H30_pH8.5	2	EC20	2.62	0.28	8.3	4.995	4.27	30	No	0.75
H90_pH6.5	3	EC20	13.70	1.90	6.7	15.1	13.8	93	No	0.75
H90_pH7.0	2	EC20	10.44	2.82	7.1	15.2	13.5	93	No	0.75
H90_pH7.5	2	EC20	5.84	0.72	7.6	15.3	13.5	94	No	0.75
H90_pH8.0	2	EC20	15.64	2.56	8	15.3	13.5	94	No	0.5
H90_pH8.5	3	EC20	9.77	0.62	8.3	15.2	13.3	93	No	0.5
H400_pH6.5	2	EC20	11.52	0.76	6.7	70.99	55.48	406	No	0.56
H400_pH7.5	2	EC20	12.77	2.86	7.6	70.99	55.48	406	No	0.56
H400_pH8.5	2	EC20	11.39	1.52	8.3	70.99	55.48	406	No	0.56
M2_pH7.5	2	EC20	7.54	1.11	7.7	11.47	9.78	68.9	MD	2.5
MD5_pH7.5	2	EC20	11.49	1.28	7.7	11.47	9.78	68.9	MD	5.4
MD10_pH7.5	2	EC20	14.52	1.77	7.7	11.47	9.78	68.9	MD	10.1
MD15_pH7.5	2	EC20	18.70	2.59	7.7	11.47	9.78	68.9	MD	15.1
AC2_pH7.5	2	EC20	3.26	0.56	7.7	13.77	11.77	82.9	AC	2
AC5_pH7.5	2	EC20	3.87	1.03	7.7	13.77	11.77	82.9	AC	4.6
AC10_pH7.5	2	EC20	4.74	1.03	7.7	13.77	11.77	82.9	AC	8.8
AC15_pH7.5	2	EC20	5.62	1.26	7.7	13.77	11.77	82.9	AC	13
MD5_pH6.5	2	EC20	6.10	0.76	6.7	11.47	9.78	68.9	MD	5.5
MD5_pH8.5	2	EC20	7.75	0.64	8.3	11.47	9.78	68.9	MD	5.5
AC5_pH6.5	2	EC20	3.92	0.32	6.7	13.77	11.77	82.9	AC	4.9
AC5_pH8.5	2	EC20	4.53	0.43	8.3	13.77	11.77	82.9	AC	4.9
SR5	1	EC20	16.23	1.11	8	-	-	90	SR	2.64
SR10	1	EC20	26.28	1.57	8	-	-	90	SR	4.7
SR15	1	EC20	38.55	3.48	8	-	-	90	SR	8.7
H5_pH6.5	2	EC50	8.70	0.78	6.7	1.19	0.65	5	No	0.66
H5_pH6.9	1	EC50	4.03	0.50	6.9	1.19	0.65	5	No	0.44
H5_pH7.5	2	EC50	16.71	2.13	7.6	1.19	0.65	5	No	0.6
H5_pH8.5	2	EC50	6.21	0.47	8.3	1.19	0.65	5	No	0.6
H30_pH6.5	2	EC50	43.41	6.03	6.7	4.995	4.27	30	No	0.69
H30_pH7.5	2	EC50	32.18	4.04	7.6	4.995	4.27	30	No	0.54
H30_pH8.5	2	EC50	12.57	1.03	8.3	4.995	4.27	30	No	0.75
H90_pH6.5	3	EC50	184.63	23.18	6.7	15.1	13.8	93	No	0.75
H90_pH7.0	2	EC50	151.39	19.65	7.1	15.2	13.5	93	No	0.75
H90_pH7.5	2	EC50	119.70	7.64	7.6	15.3	13.5	94	No	0.75

Test ID	n	EC Value	Conc	SD	pH	Ca (mg.L^{-1})	Mg (mg.L^{-1})	Hardness (mg.L^{-1})	DOC Source	DOC (mg.L^{-1})
H90_pH8.0	2	EC50	118.11	9.15	8	15.3	13.5	94	No	0.5
H90_pH8.5	3	EC50	52.74	1.77	8.3	15.2	13.3	93	No	0.5
H400_pH6.5	2	EC50	95.89	14.85	6.7	70.99	55.48	406	No	0.56
H400_pH7.5	2	EC50	159.07	25.79	7.6	70.99	55.48	406	No	0.56
H400_pH8.5	2	EC50	58.52	3.96	8.3	70.99	55.48	406	No	0.56
M2_pH7.5	2	EC50	70.86	6.10	7.7	11.47	9.78	68.9	MD	2.5
MD5_pH7.5	2	EC50	86.21	5.27	7.7	11.47	9.78	68.9	MD	5.4
MD10_pH7.5	2	EC50	107.21	7.47	7.7	11.47	9.78	68.9	MD	10.1
MD15_pH7.5	2	EC50	126.16	11.34	7.7	11.47	9.78	68.9	MD	15.1
AC2_pH7.5	2	EC50	16.82	2.89	7.7	13.77	11.77	82.9	AC	2
AC5_pH7.5	2	EC50	17.99	3.76	7.7	13.77	11.77	82.9	AC	4.6
AC10_pH7.5	2	EC50	19.66	5.08	7.7	13.77	11.77	82.9	AC	8.8
AC15_pH7.5	2	EC50	24.51	6.09	7.7	13.77	11.77	82.9	AC	13
MD5_pH6.5	2	EC50	33.67	4.15	6.7	11.47	9.78	68.9	MD	5.5
MD5_pH8.5	2	EC50	36.51	1.68	8.3	11.47	9.78	68.9	MD	5.5
AC5_pH6.5	2	EC50	19.02	1.56	6.7	13.77	11.77	82.9	AC	4.9
AC5_pH8.5	2	EC50	18.61	1.28	8.3	13.77	11.77	82.9	AC	4.9
SR5	1	EC50	72.12	4.10	8	-	-	90	SR	2.64
SR10	1	EC50	88.96	3.56	8	-	-	90	SR	4.7
SR15	1	EC50	142.52	8.42	8	-	-	90	SR	8.7

Table E- 2: Natural water sample coordinates.

Sample Site	Latitude	Longitude
Woronora River	-34.1066183	150.94842
Blackwood River	-34.0747222	115.3888056
Ovens River	-36.9001667	147.0706111
Magela Creek	-12.504587	132.818348
Limestone Creek	-23.1732778	150.6858889
Teatree Creek	-22.7461944	150.6425

Table E- 3: Autovalidation and model performance scoring (MPS) results of MLR models. Includes Adjusted R2, Predicted R2, Factor of 2 and 3 percentages and Geometric mean of residuals. Model residual scores for Observed and Predicted toxicity, and DOC, hardness and pH using scoring methods from Garman et al. (2020) and DeForest et al. (2023) and individual parameters and scores are described in the methods section of Chapter 6 in equation 6.4 and 6.5.

Model	Autovalidation parameters			Slopes and p values of Residuals versus							
	R^2	$RF_{x,2.0}$	$RF_{x,3.0}$	Observed ECx	p	pH	p	Hard	p	DOC	p
EC10 no interactions	0.30	0.57	0.77	0.35	0.07	0.27	0.3	-0.1	0.4	-0.36	0.0
EC10 interactions	0.30	0.80	0.93	0.64	0.0	0.37	0.05	-0.03	0.7	0.00	1.0
EC20 no interactions	0.43	0.77	0.93	0.57	0.0	0.12	0.57	0.0	1.0	0.07	0.4
EC20 interactions	0.50	0.77	0.97	0.50	0.0	0.11	0.6	0.0	1.0	-0.04	0.6
EC50[a]	0.53	0.53	0.93	0.47	0.0	-0.19	0.4	0.0	1.0	-0.08	0.5
EC50[a] SR (full dataset) AIC	0.41	0.57	0.73	0.38	0.01	0.04	0.9	-0.1	0.4	-0.41	0.0
EC50[a] SR (full dataset) BIC	0.53	0.50	0.87	0.42	0.0	-0.21	0.4	-0.06	0.6	-0.09	0.4
EC50[a] SR (SR only dataset) AIC	0.79	0.72	1.0	0.21	0.06	0.0	1.0	0.0	1.0	0.00	1.0
EC50[a] SR (SR only dataset) BIC	0.72	0.61	0.94	0.28	0.02	-0.24	0.4	0.0	1.0	0.28	0.1

a – models with or without interactions were the same.

Table E- 3: continued.

Model	Residual scores				Model Performance Scores	
	RS_{obs}	RS_{pH}	RS_{Hard}	RS_{DOC}	MPS - $RF_{x,2.0}$	MPS - $RF_{x,3.0}$
EC10 no interactions	0.64	0.78	0.93	0.60	0.64	0.67
EC10 interactions	0.37	0.62	0.99	1.00	0.68	0.70
EC20 no interactions	0.42	0.94	1.00	0.95	0.75	0.78
EC20 interactions	0.48	0.95	1.00	0.98	0.78	0.81
EC50[a]	0.51	0.87	1.00	0.95	0.73	0.80
EC50[a] SR (full dataset) AIC	0.59	1.00	0.91	0.56	0.67	0.70
EC50[a] SR (full dataset) BIC	0.55	0.85	0.97	0.94	0.72	0.78
EC50[a] SR (SR only dataset) AIC	0.78	1.00	1.00	1.00	0.88	0.93
EC50[a] SR (SR only dataset) BIC	0.70	0.82	1.00	0.73	0.76	0.82

Table E- 4: Concentration-response model parameters. Upper (d) and lower (c) limits fixed to 100 and 0, respectively. n = number of data points in model, b = slope parameter, e = inflection parameter, SE = standard error, d.f. = degrees of freedom, E = power of 10

			Parameters									
			b				e					
Test Name	**n**	**Model type**	**Estimate**	**SE**	**t-value**	**p-value**	**Estimate**	**SE**	**t-value**	**p-value**	**Residual SE**	**d.f.**
Blackwood River Buffered	26	Weibull_2	1.3	0.10	12.8	3.2E-12	795.1	37.6	21.1	<2.2E-16	3.80	24
Blackwood River Unbuffered	27	Weibull_2	1.0	0.05	21.5	<2.2E-16	404.5	15.3	26.5	<2.2E-16	2.97	25
Limestone Creek Buffered	27	Logistic_2	0.97	0.05	19.7	<2.2E-16	336.8	18.7	18.0	8.3E-16	3.52	25
Limestone Creek Unbuffered	27	Weibull_2	1.2	0.05	22.6	<2.E-16	346.7	10.1	34.2	<2.2E-16	2.73	25
Magela Creek Buffered	27	Logistic_2	2.0	0.09	20.9	<2.2E-16	83.9	2.4	34.5	<2.2E-16	2.71	25
Magela Creek Unbuffered	27	Weibull_2	1.2	0.04	27.7	<2.2E-16	88.4	2.4	37.4	<2.2E-16	2.14	25
Ovens River Buffered	27	Weibull_2	0.8	0.03	26.9	<2.2E-16	103.5	4.2	24.5	<2.2E-16	2.99	25
Ovens River Unbuffered	27	Logistic_2	1.3	0.06	22.9	<2.2E-16	41.7	1.6	26.1	<2.2E-16	3.14	25
Ovens River Adjusted	24	Weibull_2	0.91	0.03	33.0	<2.2E-16	74.5	2.1	34.9	<2.2E-16	1.59	22
Teatree Creek	23	Weibull_2	1.3	0.06	22.6	3.9E-16	619.7	15.6	39.7	<2.2E-16	2.34	21
Woronora River Buffered	45	Weibull_2	2.2	0.13	17.3	<2.2E-16	533.3	12.0	44.2	<2.2E-16	4.21	42
Woronora River Unbuffered	54	Weibull_2	1.8	0.16	11.7	4.4E-16	314.0	12.9	24.3	<2.2E-16	6.33	51

Table E- 5: Original and updated sensitivity coefficients for Chlorella MLR models.

Model	**Original Sensitivity Coefficient**	**Updated Sensitivity Coefficient**
EC10	0.16	3.07
EC20	0.19	2.96
EC50 (full dataset)	1.17	3.13
EC50 (subset data)	3.97	2.95

Table E- 6: Major ion, metals, and nutrient data for Australian natural freshwaters. Synthetic water = laboratory waters used in Chlorella sp. reference toxicant tests. N = nitrogen, TKN = Total Kjeldahl nitrogen, P = phosphorus.

Analyte	**Water ID**						
	Synthetic water	**Blackwood River**	**Limestone Creek**	**Ovens River**	**Magela Creek**	**Teatree Creek**	**Woronora River**
Ca ($mg.L^{-1}$)	15	28	11	2.1	0.2	1.3	3.2
Mg ($mg.L^{-1}$)	14	83	17	1.6	0.7	2.4	2.2
K ($mg.L^{-1}$)	2.1	6	6	0.3	0.3	0.9	1.4
Na ($mg.L^{-1}$)	30	329	57	2.8	1.26	22	14
SO_4 ($mg.L^{-1}$)	89	43	1	<1	<1	<1	<10
Cl ($mg.L^{-1}$)	16	881	102	1	2	34	25
Ca:Mg ratio	1.07	0.34	0.65	1.31	0.23	0.54	1.45
Al ($\mu g.L^{-1}$) - Total	<63	70	<63	<63	<63	1480	<63
Al ($\mu g.L^{-1}$) - <0.45 µm	<63	<63	<63	<63	<63	419	<63
Al ($\mu g.L^{-1}$) - <3 kDa	<63	<63	<63	<63	<63	97	<63
Fe ($\mu g.L^{-1}$) - Total	<0.4	12	107	28	44	581	168
Fe ($\mu g.L^{-1}$) - <0.45 µm	<0.4	7	85	22	29	497	114
Fe ($\mu g.L^{-1}$) - <3 kDa	<0.4	3.7	10	1.5	3.4	70	1.6
Mn ($\mu g.L^{-1}$) - Total	<0.2	78	2.1	1.9	1.8	12	35
Mn ($\mu g.L^{-1}$) - <0.45 µm	<0.2	67	1.4	1.6	1.8	11	34
Mn ($\mu g.L^{-1}$) - <3 kDa	<0.2	68	1	1.6	1.5	8.5	31
Ammonia as N ($mg.L^{-1}$)	-	0.03	0.16	<0.01	0.04	<0.01	<0.01
Nitrite as N ($mg.L^{-1}$)	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01
Nitrate as N ($mg.L^{-1}$)	-	<0.01	0.02	0.04	0.04	0.01	<0.01
TKN ($mg.L^{-1}$)	-	0.3	1	<0.1	<0.1	0.6	0.2
Total N ($mg.L^{-1}$)	-	0.3	1	<0.1	<0.01	0.6	0.2
Total P ($mg.L^{-1}$)	-	0.02	<0.02	0.02	<0.01	0.04	<0.01
Reactive P ($mg.L^{-1}$)	-	<0.01	<0.01	<0.01	<0.01	<0.01	<0.01