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Applicability of Chronic Multiple Linear Regression Models for Predicting Zinc Toxicity in Australian and New Zealand Freshwaters

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Abstract: Bioavailability models, for example, multiple linear regressions (MLRs) of water quality parameters, are increasingly being used to develop bioavailability-based water quality criteria for metals. However, models developed for the Northern Hemisphere cannot be adopted for Australia and New Zealand without first validating them against local species and local water chemistry characteristics. We investigated the applicability of zinc chronic bioavailability models to predict toxicity in a range of uncontaminated natural waters in Australia and New Zealand. Water chemistry data were compiled to guide a selection of waters with different zinc toxicity-modifying factors. Predicted toxicities using several bioavailability models were compared with observed chronic toxicities for the green alga *Raphidocelis subcapitata* and the native cladocerans *Ceriodaphnia* cf. *dubia* and *Daphnia thomsoni*. The most sensitive species to zinc in five New Zealand freshwaters was *R. subcapitata* (72-h growth rate), with toxicity ameliorated by high dissolved organic carbon (DOC) or low pH, and hardness having a minimal influence. Zinc toxicity to *D. thomsoni* (reproduction) was ameliorated by both high DOC and hardness in these same waters. No single trophic level–specific effect concentration, 10% (EC10) MLR was the best predictor of chronic toxicity to the cladocerans, and MLRs based on EC10 values both over- and under-predicted zinc toxicity. The EC50 MLRs better predicted toxicities to both the Australian and New Zealand cladocerans to within a factor of 2 of the observed toxicities in most waters. These findings suggest that existing MLRs may be useful for normalizing local ecotoxicity data to derive water quality criteria for Australia and New Zealand. The final choice of models will depend on their predictive ability, level of protection, and ease of use. *Environ Toxicol Chem* 2023;42:2614–2629. © 2023 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

Keywords: Metal; Bioavailability model; Water quality guidelines; Zinc; Models

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INTRODUCTION

Although it is well known that water quality parameters such as hardness, pH, and dissolved organic carbon (DOC) influence metal bioavailability and consequently toxicity to freshwater biota, only recently have multiple toxicity-modifying factors

(TMFs; not just hardness) been proposed for incorporation into freshwater criteria for metals, for example, nickel (Stauber et al., 2021) in Australia and New Zealand. Note that water quality criteria (also called benchmarks, thresholds, and predicted no-effect concentrations in other jurisdictions) are referred to as default guideline values (DGVs) in Australia and New Zealand, and this term shall be used throughout. The Australia and New Zealand Governments (ANZG, 2018) toxicant DGV derivation methodology (Warne et al., 2018) allows for the use of bioavailability models such as multiple linear regression models (MLRs) or biotic ligand models (BLMs) to normalize the ecotoxicity data for use in species sensitivity distributions (SSDs) for the derivation of guideline values for metals. Although such methods can be used, it is recommended that they be validated using water quality conditions and species relevant for Australia and New Zealand (Warne et al., 2018). Bioavailability models developed for the Northern Hemisphere cannot be adopted for Australia and New Zealand without validation due to 1) the high level of endemism found in local freshwaters, and 2) distinct water chemistry characteristics, such as higher magnesium-to-calcium ratios and different DOC sources and quality (Peters et al., 2018; Holland, Stauber et al., 2018), which can influence metal bioavailability and toxicity.

The DOC–metal relationships used in the bioavailability models developed for Northern Hemisphere waters may or may not be applicable to surface freshwaters in Australia and New Zealand, due to climatic and vegetation differences, the presence of temporary waters resulting from droughts and seasonal inundation, different DOC sources, and anthropogenic and agricultural activities that introduce salinity and acid into arid environments. Recent research has shown that Australian dissolved organic matter (DOM) composition varies between different water types (Holland, Stauber et al., 2018). Naturally acidic waters are dominated by humic-like DOM, which is highly aromatic and of greater molecular weight compared with DOM from circumneutral waters, which is more fulvic-like. The DOM from Australian naturally acidic waters also appears to be more aromatic and of a greater molecular weight than is commonly found in European and North American systems (Holland, Stauber et al., 2018). Studies of the effects of these different DOM compositions on the toxicity of copper and nickel to freshwater algae have found that some DOM, particularly that associated with terrestrial inputs into rainforest streams high in humic-like DOM, were more protective at the same DOC concentrations than other DOM (Macoustra et al., 2019, 2020, 2021). Zinc speciation is less likely to be affected by DOM concentration and quality than copper speciation (Tipping et al., 2011), but the influence of DOM in local waters of Australia and New Zealand on zinc bioavailability has not been previously investigated.

A revised set of freshwater DGVs for zinc for Australia and New Zealand is currently being developed, with the aim of incorporating bioavailability corrections. Canada has recently implemented a zinc guideline with an MLR incorporating pH, hardness, and DOC; however, a complete dataset was only available for the effects of pH, hardness, and DOC on toxicity to rainbow trout. The rainbow trout MLR was applied to all trophic levels, including algae and invertebrates, because it was sufficiently conservative to be protective of most species (Canadian Council of Ministers of the Environment [CCME], 2018). A closer examination of the effect of TMFs on four species (*Raphidocelis subcapitata* [formerly known as *Pseudokirchneriella subcapitata* and *Selenastrum capricornutum*], *Ceriodaphnia dubia*, *Daphnia magna*, and *Oncorhynchus mykiss*) in natural waters showed that each species responded differently (De Schamphelaere et al., 2005). For example, *R. subcapitata* was most sensitive to zinc in high-pH, high-hardness waters, whereas *O. mykiss* was most sensitive in low-pH, low-hardness waters. Consequently, trophic level–specific MLRs are preferred over a single (unified) MLR for all species, to provide more accurate predictions of zinc toxicity.

The BLMs are an alternative to trophic level–specific MLRs (incorporating pH, hardness, and DOC) to develop bioavailability-based guidelines for zinc in Australia/New Zealand. DeForest and Van Genderen (2012) developed a unified zinc BLM that could predict both acute and chronic toxicity over a wide range of zinc bioavailabilities for Northern Hemisphere species. However, in keeping with the approach of the US Environmental Protection Agency (USEPA), they only included invertebrates and fish, not plants or algae, which have been shown to be among the most sensitive taxa to zinc. For example, *Chlorella* sp. was the most sensitive species to zinc at the index condition (pH 7.5, hardness 30 mg $CaCO_3$/L, and DOC 0.5 mg/L) in the SSD being used to derive a revised DGV for zinc in Australia and New Zealand.

DeForest et al. (2023) developed both species-specific and pooled MLRs, together with BLMs, to predict the acute and chronic toxicity of zinc to a range of freshwater biota. They found that hardness had the most consistent influence on zinc toxicity among species, whereas DOC and pH had a variable influence. Models based on pH, hardness, and DOC were sufficiently predictive of observed toxicity, without the need for inclusion of additional parameters (e.g., alkalinity). All the models they developed (DeForest et al., 2023) were a significant improvement over the use of the existing hardness algorithm (USEPA, 1987) currently used to adjust zinc freshwater DGVs in Australia and New Zealand (Australia and New Zealand Environment and Conservation Council/Agriculture and Research Management Council of Australia and New Zealand, 2000).

Our study formed part of a larger study investigating the applicability of zinc chronic bioavailability models for predicting zinc toxicity in a range of uncontaminated natural waters (spiked with zinc) with varying pH, hardness, and natural DOC. Recent research in a companion study by Price et al. (2023) reported the development of a new chronic zinc MLR for a local algal species (*Chlorella* sp.) and tested its applicability to predict zinc toxicity in seven of the same Australian waters used in the present study. We extended this research to compare predicted toxicity with observed tocity in chronic tests with the green alga *R. subcapitata* and the native cladocerans *Daphnia thomsoni* and *Ceriodaphnia* cf. *dubia*. The two cladoceran species were chosen because they were local isolates from New Zealand and Australia-Pacific, respectively, and the

Northern Hemisphere *C. dubia* species has been shown to be one of the most sensitive species to zinc. The *R. subcapitata* was chosen to represent a ubiquitous species and to enable the application of an existing algal MLR to local waters.

METHODS

Compilation of Australian and New Zealand water chemistry data

To characterize water chemistry conditions in Australia and New Zealand and to help identify natural waters for testing, data for three TMFs (pH, DOC [mg/L], and water hardness [$CaCO_3$ mg/L]) were collated from state and local government organizations throughout Australia and New Zealand (Supporting Information, Table S1).

Data for hardness and pH were collated from 6652 and 18 694 Australian freshwater sites, respectively from 1940 to 2020 (Supporting Information, Table S2). There were fewer data for DOC, with available data from 1925 sites from 1991 to 2020. There were no records of DOC from Queensland and very limited DOC data for South Australia and Tasmania. The total numbers of records collated for each TMF are given in the Supporting Information, Table S2. Locations of sites that provided the TMF data around Australia are shown in the Supporting Information, Figure S1.

Similar to the Australian water chemistry data, there was no single database with compiled data for pH, hardness, and DOC in New Zealand freshwaters. Water quality data were compiled from the National River Water Quality Network (NRWQN) of the National Institute of Water and Atmospheric Research (NIWA) supplemented by related studies on the same rivers (Close & Davies-Colley, 1990; Scott et al., 2006), together with data from regional and local council monitoring programs in Auckland, Greater Wellington, Christchurch, and Southland (all in New Zealand; Holland, Kleinmans et al., 2018; Margetts & Marshall, 2018; Perrie et al., 2012) and other studies (Collier, 1987; Moore & Clarkson, 2007).

Selection, collection, and analyses of natural waters

Australian waters. Natural surface freshwaters were collected from eight unimpacted waterways (i.e., not affected by point sources or diffuse pollution from intensive agriculture or urban land use) across Australia, selected to cover a range of climatic, geographical, and state jurisdictions, as well as a range of TMFs. These waters included Woronora River and Jingellic Creek from New South Wales; Ovens River from Victoria; Teatree Creek, Waterpark Creek, and Limestone Creek from Queensland; Magela Creek from Northern Territory; and Blackwood River from Western Australia (Supporting Information, Figure S2). Co-ordinates of the sample locations are given in the Supporting Information, Table S3.

Approximately 25 L of water were collected from each site in 5-L high-density polyethylene containers that had been previously acid washed and rinsed in ultrapure water (18 MΩ.cm, Milli-Q®; Millipore) and site water. The samples were kept on ice until arrival at the laboratory, where samples were filtered through a pre-rinsed 0.45-µm in-line polyethersulfone filter (Waterra). The filtered water samples were stored at 4 °C in the dark until they were used in the bioassays, within 1 month of collection.

Samples were analyzed for a range of parameters at an external commercial accredited laboratory (ALS Environmental, Smithfield, NSW, Australia). Total hardness (as $CaCO_3$) and alkalinity (including hydroxide, carbonate, bicarbonate, and total, measured as $CaCO_3$) were measured using titration. All anions (sulfate and chloride) and nutrients (ammonia, NOx, total Kjeldahl nitrogen, total phosphorus, and reactive phosphorus) were analyzed using a discrete analyser. The DOC was analyzed using an automated Total Organic Carbon analyzer, and inorganic carbon was analyzed using an automated carbon analyzer with infrared detector for CO_2 measurement, according to ALS in-house protocols. All metals and other cations were analyzed using either inductively coupled plasma–atomic emission spectrometry (ICP-AES; Agilent 730 ES) or ICP–mass spectrometry (ICP–MS; Agilent 8800) at Commonwealth Scientific and Industrial Research Organisation (CSIRO), Lucas Heights (NSW, Australia). All sample batches analyzed had the following quality control measurements: laboratory duplicates, method blanks, laboratory control spikes and recoveries, and matrix spike recoveries.

Subsamples of the stored freshwaters were collected and analyzed for total hardness, dissolved metals (0.45 µm filtered), other cations, and DOC using the methods just described immediately prior to and during toxicity testing.

New Zealand waters. Five natural waters were collected for toxicity testing to cover a range of chemistries broadly representative of most rivers and streams in New Zealand (Supporting Information, Table S4). As with the Australian waters, the natural waters were from unimpacted sites.

Water samples from the five selected rivers were collected in several 10-L low-density polyethylene containers (precleaned with 10% nitric acid, Milli-Q water and rinsed with site river water) to obtain 30–40 L of water. Additional samples were collected in laboratory-supplied bottles for chemical analyses. All samples were shipped on ice overnight to the NIWA Hamilton Laboratory, New Zealand, where they were refrigerated (<4 °C in the dark) for up to 10 days before filtration through a pre-rinsed glass fiber filter in preparation for acclimation of test organisms and ecotoxicity testing (bulk samples), or transferred to the chemical analysis laboratory (Hill Laboratories, Hamilton, New Zealand) within 24 h. All natural water samples were analyzed by Hill Laboratories (which is accredited by International Accreditation New Zealand) for pH, anions and cations, dissolved (0.45-µm filtered) metals (arsenic, cadmium, chromium, copper, lead, nickel, zinc, aluminium, iron), dissolved and total nutrients, and DOC (as dissolved nonpurgeable organic carbon). Methods of analysis are given in the Supporting Information, Table S5, and are in most cases consistent with those used for Australian waters.

Chronic toxicity tests with Australian and New Zealand natural waters

Of the eight Australian natural waters collected, six were tested in a companion study (Price et al., 2023) for chronic toxicity to the tropical alga *Chlorella* sp. after spiking with a range of zinc concentrations. In the present study, four of the zinc-spiked waters were tested without acclimation of test organisms in 7-day chronic reproduction tests with *C.* cf. *dubia*. The five New Zealand freshwaters, each spiked with zinc, were tested using *R. subcapitata* (72-h growth rate inhibition) and *D. thomsoni* (21-day reproduction).

Raphidocelis subcapitata toxicity tests. The *R. subcapitata* are unicellular, crescent-shaped (40–60 m^3) green algae of the class Chlorophyceae. A long-term culture is held at the NIWA Hamilton Ecotoxicology Laboratory, originally sourced from the Culture Collection of Algae University of Texas at Austin (UTEX 1648 strain). The algal culture was maintained in 250-mL glass flasks in filter-sterilized (<0.2 µm) aged UV-treated Milli-Q water with added nutrients as described by Environment Canada (2007). Culture conditions were a temperature of 25 °C with 100 rpm rotation under continuous "cool-white" fluorescent lighting 4000 ± 400 lux.

Growth rates of *R. subcapitata* over 72 h were determined in the five zinc-spiked natural waters according to Organisation for Economic Co-operation and Development (OECD) Test No. 201 (2011) and Environment Canada (2007) protocols. The *R. subcapitata* cells were harvested during exponential growth, washed three times with 15 mg/L $NaHCO_3$, and added to a hypernutrient solution in a 1:2 ratio (Supporting Information, Table S6) before aliquoting 30 µL to the microplate wells for a final density of 10 000 cells/mL (with test solution added). Test solutions were made by adding zinc sulfate (100 mg/L stock solution prepared from $ZnSO_4{\cdot}7H_2O$, either Analar [BDH] or ACS grade [Sigma]) to 0.2 µm of filtered natural waters. Test solutions were prepared with 10 nominal concentrations that ranged from 0.002 to 1 mg/L or 0.004 to 2 mg/L depending on the natural water tested. Eight replicate plates were made for each natural water, three for analyzing cell density at 24, 48, and 72 h (one plate/timestep, each with five replicates/plate) and five plates to provide sufficient volume for chemical analyses. At test initiation and after 72 h, subsamples of zinc-spiked natural waters were filtered (<0.45-µm capsule filters; Microscience) and then preserved with 1% nitric acid for analysis of dissolved zinc, calcium, and magnesium by ICP–MS by Hill Laboratories.

The tests were carried out at a temperature of 25 ± 1 °C under continuous "cool-white" fluorescent lighting 4000 ± 400 lux. All microplates (with lids) were sealed in polyethylene plastic bags for the 72-h test duration to reduce cross-contamination. The pH was measured at 0 and 72 h using a Horiba Scientific B-71X pH meter. Algal cell densities were determined at 24, 48, and 72 h by flow cytometry (FACSCalibur or Accuri; BD). The specific growth rate was calculated from the slope (µ) generated by linear regression of the change in cell density (as $\log_{10}$ data) versus time (days). Growth rates as divisions/day were calculated by dividing the specific growth rate by ln(2). Growth rates (as divisions/day) were expressed as a percentage of the control growth rate. Test acceptability criteria included <20% coefficient of variation in control growth rates and >2 doublings/day in controls. A summary of the toxicity test method is given in the Supporting Information, Table S7.

A 72-h cell yield reference toxicant test with zinc sulfate diluted in aged UV-treated Milli-Q water was run in parallel with the natural waters. The 72-h median effect concentration (EC50) values based on cell yield were compared with the long-term database to measure the sensitivity and condition of the current test organisms.

Daphnia thomsoni toxicity tests. *Daphnia thomsoni* is a freshwater microcrustacean belonging to the order Cladocera (water fleas) and is native to New Zealand. The *D. thomsoni* were collected from a known population in Fernhollow Pond, Waikato, New Zealand and have been maintained in the NIWA Hamilton Ecotoxicology Laboratory since 2019. Culture conditions and acclimation procedures prior to testing are described in the Supporting Information.

Chronic toxicity was tested with *D. thomsoni* using a 21-day survival and reproduction endpoint. Standard toxicity testing protocols developed for *D. magna* (NIWA, 2004) based on OECD Test No. 211 (2012) were used and are summarized in the Supporting Information Table S8. Tests were performed under static-renewal conditions in 60-mL polystyrene containers. On test initiation, one <24-h-old neonate from the individual culture setup (i.e., before the test) was added to each test container, with 10 replicates for each zinc treatment concentration and 20 control replicates/test.

At the end of the test, the total number of live offspring produced/adult (live or dead) and the total number/surviving adult were calculated. Any adults that appeared to be male (based on their small size and lack of offspring) were excluded from the analysis. The test was deemed acceptable if control organisms had ≥80% survival (i.e., <20% immobile) and the mean number of control female offspring/surviving adult was >60.

The endpoints (OECD, 2012) included: 1) the total number of living female offspring produced/adult animal (excluding any that died accidentally or inadvertently or were male), and 2) the number of living female offspring produced/surviving adult animal (also excluding any that died accidentally or inadvertently or were male). In addition, the survival of adults was also calculated.

Subsamples of each test concentration were collected and filtered (0.45 µm) for dissolved metals analyses at the start (day 1) and end (day 21) of each test. Subsamples of both old and fresh media were collected during each of the eight water renewals and composited separately. The zinc concentrations used in the statistical analyses were a time-weighted mean (OECD, 2019) of the concentrations measured at the test start, end, and in the two composites.

Ceriodaphnia cf. dubia toxicity tests. The test species *C.* cf. *dubia* is a species in the order Cladocera and was isolated from Lake Paramatta, NSW in 1991 by the New South Wales

Environmental Protection Authority's Centre for Ecotoxicology. The *C.* cf. *dubia* were cultured according to the USEPA (2002) protocol with modifications for the Australian isolate, described by Bailey et al. (2000). Details of culture conditions and the static renewal toxicity test protocol are given in the Supporting Information, Table S9.

Two controls (with and without 0.5 g 4-morpholinepropanesulfonic acid [MOPS]/L) and six zinc treatments (with MOPS) were prepared in 300-mL plastic containers and spiked with 30 μL of 100 mg vitamin B_{12}/L and 30 μL of Selenium 0.1 g/L. Zinc (as zinc chloride) was added to each water (10, 25, 50, 100, 250, and 500 μg zinc/L) and pre-equilibrated for 4 h at 25 °C.

To ensure test organism health, a diluted mineral water control was run concurrently with each chronic toxicity test. Tests were deemed acceptable based on 7- or 8-day diluted mineral water control performance if: 1) adult survival was ≥80%; 2) the average number of neonates from surviving adults was ≥15; and 3) ≥60% of surviving adults had three broods of neonates within 7 or 8 days (USEPA, 2002). Note that survival was based on immobilization, after gently prodding immobile cladocerans with a glass pipette. For this reason EC values, rather than lethal concentration (LC) values, are used throughout. In addition to the diluted mineral water control, two natural water controls, with and without MOPS buffer, were included in each test. A 48-h acute copper reference toxicant test in diluted mineral water was conducted concurrently with each chronic *C.* cf. *dubia* test in the natural freshwaters (see the Supporting Information).

Physicochemical parameters including pH, temperature, dissolved oxygen, and electrical conductivity were measured every 24 h throughout each test. On days 0 and 7, and each water renewal day, subsamples were collected from the 10th control and treatment replicate, syringe-filtered through acid-washed 0.45-μm polyethersulfone filters (Sartorius), acidified to 0.2% (vol/vol) HNO_3 (ultrapure HNO_3), and stored at ≤4 °C until analysis for dissolved metals. Matrix-matched calibration standards, blanks, and drift blanks were used for quality assurance.

Statistical analyses

Concentration–response curves were produced using the R Studio environment (Ver. 4.0.2; R Development Core Team, 2016) with the extension package *drc* (Ritz et al., 2015). For the *R. subcapitata* tests, the three-parameter Weibull model was selected, with the upper limit parameter fixed to 100. Concentration–growth rate curves were visualized using the *ggplot2* package (Wickham, 2009). For *D. thomsoni* tests, three-parameter log-logistic models were fitted. Survival data were analyzed using CETIS (Ver. 1.9.7.7). Curves for survival and reproduction were visualized using the *ggplot2* package (Wickham, 2009). For *C.* cf *dubia*, the three-parameter Weibull model was selected based on Akaike Information Criterion and the residual standard error of the model. The endpoint was the number of neonates produced by each live or dead adult *C.* cf. *dubia*. For all tests, concentration–response curves were calculated based on the measured time-averaged zinc concentrations (μg/L). The significance level was set to 0.05 for all statistical tests. The *EDcomp* function within *drc* described in Ritz et al. (2015) was used to test significance ($\alpha = 0.05$) in EC values calculated between each natural water.

Using MLRs to predict toxicity to each test species

To assess the applicability of MLRs for determining the bioavailability of zinc in Australian and NZ freshwaters, a range of chronic zinc MLRs were used to compare the predicted versus observed toxicity of zinc for each test species. Predicted EC values that fell within a factor of 2 of the observed EC values were considered acceptable. However, for EC10 predictions, which have lower confidence, agreement within a factor of 3 was also considered acceptable (Price et al., 2022).

Predicted toxicity to R. subcapitata. Several MLR models have been developed for zinc for *R. subcapitata* (shown in Table 1). The MLR coefficients were used to calculate predicted toxicity (EC) values using the formula in Equation 1, where b_1–b_4 are the coefficients in Table 1.

$$\ln(ECx)_{MLR} = \text{Intercept} + b_1 \times \text{pH} + b_2 \times \ln(\text{Hardness}) + b_3 \times \ln(\text{DOC}) \quad (1)$$

The pH, hardness, and DOC values (Supporting Information, Table S10) were used to calculate the predicted EC values. The pH values used were those measured at the end of the toxicity tests, based on a geometric mean of pH in all test concentrations. Hardness was based on the measured calcium and magnesium concentrations in the control replicate at the end of each test. The DOC was based on that measured in control replicates prior to the test and excludes the addition of algae and nutrient solution. The EC predictions based on these values were compared with the measured (observed) EC values.

Predicted toxicity to D. thomsoni. The chronic zinc MLR models used to predict zinc toxicity to *D. magna* are shown in Table 1. The MLR coefficients were used to calculate predicted toxicity (EC values) using the formula in Equation 2, where b_1–b_4 are the coefficients (slopes) in Table 1.

$$\ln(ECx)_{MLR} = \text{Intercept} + b_1 \times \text{pH} + b_2 \times \ln(\text{Hardness}) + b_3 \times \ln(\text{DOC}) + b_4 \times \text{pH} \times \ln(\text{DOC}) \quad (2)$$

For *D. thomsoni*, the intercept, which is a species-specific sensitivity coefficient, was updated by setting the intercept in Equation 2 to zero, then using Equation 3 to calculate a species-specific coefficient (*Sp*) for each natural water, and then

TABLE 1: Chronic zinc multiple linear regression models used to predict zinc toxicity to *Raphidocelis subcapitata*, *Daphnia thomsoni*, and *Ceriodaphnia* cf *dubia*

					Slope			
Species to which MLR was applied	MLR	MLR reference	ECx	Intercept	pH	Ln (hard)	Ln(DOC)	Ln(DOC) x pH
R. subcapitata	*R. subcapitata*	Canadian Council of Ministers of the Environment (CCME; 2018)	EC50	11.8	−1.12	n/a	n/a	—
		Gadd and Hickey (2023)	EC50	8.28	−0.750	0.296	0.468	—
		DeForest et al. (2023)	EC10	9.82	−0.928	—	0.363	—
		DeForest et al. (2023)	EC20	10.6	−0.983	—	0.331	—
		DeForest et al. (2023)	EC50	10.4	−0.803	—	0.206	—
D. thomsoni	*D. magna*	CCME (2018)	EC10	3.06	n/a	n/a	0.391	—
		Gadd and Hickey (2023)	EC50	6.99	−0.52	0.31	−1.40	0.24
		DeForest et al. (2023)	EC10	4.33	−0.238	0.193	0.373	—
		DeForest et al. (2023)	EC20	4.15	−0.226	0.296	0.422	—
		DeForest et al. (2023)	EC50	4.40	−0.228	0.399	0.430	—
C. cf. *dubia*	*C. dubia*	DeForest et al. (2023)	EC10	1.76	—	0.489	—	—
		DeForest et al. (2023)	EC20	4.19	—	—	—	—
		DeForest et al. (2023)	EC50	3.08	—	0.437	—	—
	D. magna	CCME (2018)	EC10	3.54	—	—	0.391	—
		Gadd and Hickey (2023)	EC50	6.20	−0.52	0.31	−1.4	0.24
		DeForest et al. (2023)	EC10	4.66	−0.238	0.193	0.373	—
		DeForest et al. (2023)	EC20	4.44	−0.226	0.296	0.422	—
		DeForest et al. (2023)	EC50	4.34	−0.228	0.399	0.430	—

For application of the *D. magna* models to *D. thomsoni* and *C.* cf. *dubia*, species-specific intercepts for *D. thomsoni* and *C.* cf. *dubia* are shown.
EC*x* = effect concentration, *x*%; DOC = dissolved organic carbon; MLR = multiple linear regression; n/a = not available.

calculating the geomean of the *Sp* for all natural waters and using that as the intercept (Peters et al., 2021).

$$Sp = \ln(ECx)_{test} - \ln(ECx)_{MLR} \tag{3}$$

The EC concentrations were predicted using these MLR models based on the pH, hardness, and DOC values shown in the Supporting Information, Table S11. Those predictions were compared with the corresponding measured (observed) EC values for *D. thomsoni* for each relevant model and effect level.

Predicted toxicity to C. cf. dubia. Zinc toxicity to *C.* cf. *dubia* was predicted using several pre-existing published and preliminary zinc MLRs for *C.* cf. *dubia* and *D. magna* (outlined in Table 1). The MLRs were developed from EC10, EC20, or EC50 data from tests with Northern Hemisphere cladocerans. The *D. magna* MLRs were adjusted for the *C.* cf. *dubia* species-specific sensitivity coefficient (the y-intercept) using Equation 3.

RESULTS

Range of TMFs in natural waters

Australian waters. More than 2 million water quality data points (pH, hardness, and DOC) were compiled from over 20 000 sites around Australia. The 10th, 50th (median), and 90th percentiles for each of the TMFs in Australian freshwaters are shown in Table 2. The median pH was 7.4, the median hardness was 62 mg $CaCO_3$/L, and the median DOC was 6.7 mg C/L. Pairwise relationships between the TMFs and the corresponding coverage of the 10th–90th percentile ranges are shown in the Supporting Information, Figure S3. Similar to that shown by Brix et al. (2020) for natural waters across the United States, there was a correlation between pH and hardness in Australian natural waters.

New Zealand waters. Example ranges of the key water quality characteristics of New Zealand freshwaters are shown in Table 3. Most rivers and streams in New Zealand have close to neutral pH values, although there are times when higher pH is measured, particularly during summer months when photosynthesizing periphyton peak in biomass. Most rivers and streams have higher calcium than magnesium concentrations, with ratios of approximately 1:1 to 4:1, with some exceptions, usually related to the geology of the catchment (Close & Davies-Colley, 1990). The DOC is low in most streams although there are sites with median DOC values of 8–33 mg/L, particularly those draining peatlands in Southland and Waikato.

TABLE 2: Median and range (10th−90th percentile; all temporal data from all sites) of toxicity-modifying factors within Australian natural waters

State/territory	pH	Hardness (mg $CaCO_3$/L)	DOC (mg/L)
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	7.4 (6.6–8.3)	62 (12–440)	6.7 (2.7–21)

DOC = dissolved organic carbon; QLD = Queensland; NSW = New South Wales; VIC = Victoria; TAS = Tasmania; SA = South Australia; WA = Western Australia; NT = Northern Terrritory; ACT = Australian Capital Territory.

TABLE 3: Summaries of site medians and 10th–90th percentile values of selected water quality characteristics of New Zealand natural freshwaters

Characteristic	Site medians from NRWQN[a]	Site medians for Auckland streams[b]	Site medians for Southland streams[c]
pH	7.7 (7.2–8.0)	7.3 (7.0–7.7)	7.5 (7.2–7.7)
DOC (mg/L)	1.9 (0.83–3.9)	3.3 (1.7–6.2)	3.3 (1.5–5.9)
Ca (mg/L)	7.8 (4.6–18)	12 (5.5–18)	8.1 (3.8–19)
Mg (mg/L)	1.7 (0.7–3.6)	4.5 (3.4–8.9)	3.3 (1.4–6.4)
Hardness (mg $CaCO_3$/L)	25 (16–56)	50 (28–83)	36 (16–68)
Ca:Mg ratio	4.9 (2.4–12)	2.4 (1.3–3.2)	2.7 (1.9–3.8)

[a]Median values from NRWQN data for 77 sites, pH monitored monthly for 13 years (1989–2012), Ca, Mg, ratio and hardness from monthly measurements in 1989 (Close & Davies-Colley, 1990), DOC data from 6–10 months of monthly monitoring in 2001 and 2002 (Scott et al., 2006).
[b]Based on median values for 12 months (Jan–Dec 2020) of approximately monthly monitoring at 35 sites. Data supplied by Research Investigations Monitoring Unit, Auckland Council.
[c]Based on median values from 5 years (Feb 2015–Feb 2020) of approximately monthly monitoring at 60 sites. Data supplied by Roger Hodson, Environment Southland.
DOC = dissolved organic carbon; NRWQN = National River Water Quality Network.

Physicochemical and chemical parameters in freshwaters collected for ecotoxicity testing

Australian freshwater samples. The database was used to help select eight natural waters from unimpacted sites with a range of TMFs: from pH 5.6 to 7.5, hardness <1 (i.e., less than the limit of detection [LOD]) to 412 mg $CaCO_3$/L, and DOC <0.5 (i.e.,less than the LOD) to 27 mg C/L, with Ca:Mg ratios of 0.23–1.5 (Supporting Information, Table S13). All waters had low concentrations of dissolved metals and nutrients relevant to Australian and New Zealand water quality guidelines (ANZG, 2018; Supporting Information, Table S12–S14). Physicochemical and chemical parameters for each natural water including concentrations of major ions, metals (total and dissolved), and nutrients at the time of collection are given in the Supporting Information, Tables S12–S15.

Major ions and major metals (dissolved aluminum, iron, and manganese) varied substantially across sites. Calcium ranged from 0.2 to 28 mg/L, and magnesium ranged from 0.7 to 83 mg/L. Sodium varied from 1.3 to 329 mg/L. Dissolved aluminum was only detected in Teatree Creek and Waterpark Creek, two waters that had low pH (<6.2). Iron concentration and speciation of total and dissolved fractions varied among sites. Total iron concentrations ranged from 12 (Blackwood River) to 1100 µg/L (Waterpark Creek). Dissolved iron as a fraction of total iron ranged from 6% to 86%. Dissolved metals were generally low across all sites (Supporting Information, Table S14). Exceptions included elevated dissolved nickel (10 µg/L) and zinc (5.0 µg/L) in Limestone Creek, compared with typical background concentrations. Total zinc concentrations were also elevated in Teatree Creek (5.7 µg/L) and Waterpark Creek (4.1 µg/L); however, concentrations in the dissolved phase were low (1.4 and 1.0 µg/L, respectively). The Blackwood River sample had a very high hardness (412 mg $CaCO_3$/L), outside the likely tolerable range of *C.* cf. *dubia* (Lasier et al., 2006) so this sample was not tested.

Nutrients (as nitrogen and phosphorus) were below the LOD (0.01 mg/L) for both nitrite and reactive phosphorus. Ammonia concentrations were low in all samples, ranging from <0.01 to 0.16 mg N/L, nitrate was <0.04 mg/L in all samples except Jingellic Creek (0.47 mg/L), and total nitrogen ranged from <0.01 to 1.0 mg N/L and total phosphorus ranged from <0.01 to 0.04 mg P/L.

New Zealand freshwater samples. Waters were broadly representative of most New Zealand rivers and streams and were selected to have a range of pH from 7 to 8, hardness from 9 to 85 mg $CaCO_3$/L, Ca:Mg ratios of 2–13, and DOC <0.3–7 mg C/L. The characteristics of each natural water at the time of collection are shown in the Supporting Information, Table S16. The pH of Okutua Stream was 4.8, and the water had very low hardness (2.6 mg $CaCO_3$/L, outside the range that would be acceptable for Daphnia survival and reproduction). The water was therefore mixed with the Waihou River water in a 1:1 ratio for ecotoxicity testing.

All waters had relatively low concentrations of nutrients (e.g., nitrate <0.002–0.77 mg/L, dissolved reactive phosphorus less than 0.004–0.064 mg/L) and metals (<1 µg/L), with the exception of the filtered Okutua/Waihou River mixture, which contained 22 µg zinc/L, possibly because of contamination of the water sample during the filtration process due to the high acidity of the Okutua Stream water. The Okutua/Waihou mixture also had a relatively high dissolved aluminum concentration (0.22 mg/L), likely to be in the form of colloidal $Al(OH)_3$.

Observed and predicted toxicity of zinc in Australian freshwaters to *C.* cf. *dubia*

Physicochemical parameters in each Australian natural freshwater over the course of the tests are given in the Supporting Information, Table S17. The use of MOPS buffer ensured reasonable pH control (within ±0.2) in all tests. Acceptability criteria for adult reproduction and survival in all diluted mineral water controls were met (Supporting Information, Table S18), and EC50 values for the copper reference tests agreed with quality assurance acceptability criteria in all tests (Supporting Information, Table S19).

The average number of neonates produced/adult (4–22) in the natural water controls was highly variable compared with the diluted mineral water controls (18–22). Although the acceptability criterion for survival was met in all waters, the reproduction criterion was only met in two natural waters (Woronora River and Jingellic Creek; Supporting Information, Table S18). The reduced reproduction was unlikely to be due to the MOPS buffer, with the exception of Waterpark Creek and Limestone Creek waters, in which reproduction was significantly lower in the presence of the MOPS buffer (Supporting Information, Figure S6). The low pH of the Waterpark Creek sample (pH 6.6) and the high hardness of the Limestone Creek

TABLE 4: Effect concentrations of dissolved (less than 0.45 µm) zinc within each natural freshwater that reduced the percentage of surviving adult *Ceriodaphnia* cf. *dubia* or the number of neonates/adult over a 7-day chronic toxicity test

	Endpoint: Surviving adults			Endpoint: Total neonates/adult		
Natural water	EC10 (µg/L)	EC20 (µg/L)	EC50 (µg/L)	EC10 (µg/L)	EC20 (µg/L)	EC50 (µg/L)
Woronora River	147 (122–172)	149 (124–174)	152 (127–178)	52 (5–99)	73 (27–118)	121 (75–167)
Jingellic Creek	193 (168–219)	194 (169–220)	196 (170–222)	83 (53–113)	101 (74–130)	138 (109–167)
Waterpark Creek	82 (25–138)	99 (76–123)	134 (0–267)	—[a]	—	—
Limestone Creek	239 (192–286)	270 (38–501)	324 (0–1011)	—[a]	—	—

[a]The quality assurance criterion for reproduction was not met in these waters.
The 95% confidence intervals are in parentheses. Note that the Blackwood River sample was not tested because its electrical conductivity exceeded the upper tolerable limit for reproduction in *Ceriodaphnia* cf. *dubia*.
EC*x* = effect concentration, *x*%.

sample (97 mg $CaCO_3$/L) were likely outside the tolerable range for reproduction in this species.

Survival of *C.* cf. *dubia* decreased as the added zinc concentration increased (Supporting Information, Figure S7). The EC values for survival (immobilization) ranged from 82 to 239 µg zinc/L (EC10), 99–270 µg zinc/L (EC20), and 134–324 µg zinc/L (EC50) in the natural waters (Table 4). In most cases, immobilization at high zinc concentrations occurred in the first 48 h. There was no significant difference in all the survival (immobilization) EC10, EC20, and EC50 values between the four natural waters ($p \geq 0.05$).

Reproduction in *C.* cf. *dubia* decreased as the added zinc concentration increased (Supporting Information, Figure S8). The EC10 values ranged from 52 to 83 µg zinc/L, EC20 values from 73 to 101 µg zinc/L, and EC50 values from 121 to 138 µg zinc/L (Table 4). The EC values for Waterpark and Limestone Creeks, which did not meet the QA criterion for reproduction, could not be calculated. Although the natural waters differed somewhat in their water chemistries (Supporting Information, Table S15), there were no significant differences between the natural waters for the zinc EC10 or EC50 values for *C.* cf. *dubia* reproduction.

Observed EC values for two waters only were compared with those predicted using several MLR models (reproduction) for *C.* cf. *dubia* and *D. magna* (Table 1), with correction for *C.* cf. *dubia* species-specific sensitivity (*y*-intercept). The *C. dubia* and *D. magna* EC10 MLRs (DeForest et al., 2023), as well as the Gadd and Hickey (2023) *D. magna* MLR, only predicted the toxicity of zinc (EC10) in the Woronora River sample within a factor of 2, but within a factor of 3 for both waters (Table 5). Only the CCME EC10 *D. magna* MLR predicted the toxicity of zinc within a factor of 2 of observed EC10 values for both waters. The DeForest et al. (2023) EC20 MLRs also predicted zinc toxicity within a factor of 2 for both waters. Predicted zinc EC50 values were also within a factor of 2 for both waters using the *C. dubia* EC50 MLR (DeForest et al., 2023).

Observed and predicted toxicity of zinc in New Zealand freshwaters to R. subcapitata

All tests met the acceptability criteria with a coefficient of variation of <20% for control cell density, an increase in control cell density of more than 16-fold, and a growth rate of >2 doublings/day (Supporting Information, Table S20). Growth rates were higher in all natural waters than in the control water and were highest in the Hoteo River and Okutua/Waihou Rivers mixture. The zinc EC50 values for inhibition of 72-h cell yield in the reference toxicant tests were all within the expected range of 14 ± 12 µg/L (mean ± 2 SD of the 10-year long-term dataset; NIWA, unpublished data).

The pH values in the natural waters increased over the 72-h test duration by 0.1 – 0.5 units, but were still within the acceptable range for the algal growth rate test (OECD, 2011). Measured dissolved zinc concentrations in the waters were within 20% of nominal concentrations in the reference, Waihou, and Clutha River samples. However, zinc concentrations in the Okutua/Waihou water samples, and at the lowest zinc concentrations of the Hoteo River and Mahurangi River tests, were >20% higher than the nominal zinc concentrations (Supporting Information, Table S21). Therefoe, measured dissolved zinc concentrations were used in all data analyses.

Zinc was toxic to *R. subcapitata* in all five zinc-spiked natural waters tested, with EC10 and EC50 values ranging from 6.3 and 67 µg/L in the Waihou River water to 43 and 133 µg/L in the Okutua/Waihou Rivers water mix (Table 6). The EC50

TABLE 5: Observed versus multiple linear regression-predicted chronic zinc EC10, EC20, and EC50 values (µg zinc/L) for *Ceriodaphnia* cf. *dubia* in natural freshwaters

		Predicted EC*x* based on different MLRs			
Natural water	Observed EC*x*	DeForest et al. (2023) *C. dubia*	DeForest et al. (2023) *D. magna*	CCME *D. magna*	Gadd and Hickey (2023) *D. magna*
EC10					
Woronora	52	24	61	65	57
Jingellic	83	27	40	45	38
EC20					
Woronora	73	66	77	—[a]	—[a]
Jingellic	101	66	51	—[a]	—[a]
EC50					
Woronora	121	77	94	—[a]	—[a]
Jingellic	138	86	63	—[a]	—[a]

[a]No model available.
CCME = Canadian Council of Ministers of the Environment; EC*x* = effect concentration, *x*%; MLR = multiple linear regression.

TABLE 6: Effect concentration (EC) values for *Raphidocelis subcapitata* growth rate in the reference test and in each natural New Zealand freshwater tested (95% confidence intervals in parentheses)

River	EC10 (µg Zn/L)	EC20 (µg Zn/L)	EC50 (µg Zn/L)
Reference[a]	21 (19–23)[b]	39 (36–42)[b]	99 (96–103)[b]
Waihou	6.3 (4.7–7.8)	16 (13–19)	67 (61–73)
Clutha	7.7 (6.5–8.9)	18 (16–20)	68 (64–72)
Mahurangi	20 (19–21)	41 (39–43)	122 (119–126)
Okutua/ Waihou mix	43 (41–46)	68 (65–71)	133 (128–137)
Hoteo	6.9 (5.7–8.2)	16 (14–19)	61 (56–65)

[a]Reference water is UV nanopure water with nutrient solution.
[b]The EC values reported in reference water are for cell yield inhibition.

value was highest in the Okutua/Waihou River water mix (and significantly different from that from all other rivers), which, compared with the other waters, had low hardness but the highest DOC (7.3 mg C/L) of all waters tested and the lowest pH, suggesting that zinc toxicity was ameliorated by moderate DOC or low pH. The Waihou and Clutha Rivers had similar EC50 (67 and 68 µg zinc/L, respectively, with no significant difference) and DOC values (0.9 and 0.7 mg/L, respectively; Supporting Information, Table S10), but the Clutha water had higher hardness and double the calcium concentration, suggesting that hardness had little effect on this species. The toxicity of zinc to the alga in the Hoteo River water was similar to that in the Waihou and Clutha Rivers (no significant difference in the EC10 values between these rivers), and lower than expected based on the higher DOC and hardness of the Hoteo River water compared with the Waihou and Clutha River waters. This may be due to the lower pH of this water (7.6 compared with 7.9–8.0 for the Waihou and Clutha Rivers, respectively).

Using the DeForest et al. (2023) MLR for *R. subcapitata*, four of five predicted EC10 and three of five predicted EC20 values were within a factor of 2 of the observed EC values (Figure 1). Predictions for the Hoteo River were more than a factor of 2 higher than the measured EC10 and EC20 values, and predictions for the Mahurangi EC20 were more than a factor of 2 lower than the measured EC20. The predicted EC50 values based on the EC50 model of DeForest et al. (2023) were within a factor of 2 of measured values for four of five natural waters, except the Mahurangi sample, in which predicted zinc toxicity was >2 times lower than the measured value. Using the CCME (2018) model for EC50 values of *R. subcapitata*, the predicted values for all five natural waters were more than a factor of 2 lower than the measured EC50 values. This model is based on pH only and had relatively low predictive power (adjusted R^2 of 0.488; CCME, 2018). The Gadd and Hickey (2023) EC50 model predicted EC50 values within a factor of 2 of the measured values for only two of the five waters, with predictions for the Waihou, Clutha, and Mahurangi Rivers more than a factor of 3 lower than the measured values (Figure 1).

Plots of the residuals (observed:predicted EC values) versus each TMF for each model used are shown in the Supporting Information, Figure S11). Residuals for each model, except the CCME model, were reasonably similar for all EC values over the range of TMFs used, suggesting low model bias. However, it should be noted that data were limited (five natural waters), so this should be interpreted with caution.

Observed and predicted toxicity of zinc in New Zealand freshwaters to D. thomsoni

All tests were considered acceptable according to the requirements of 80% survival of adults and an average of at least

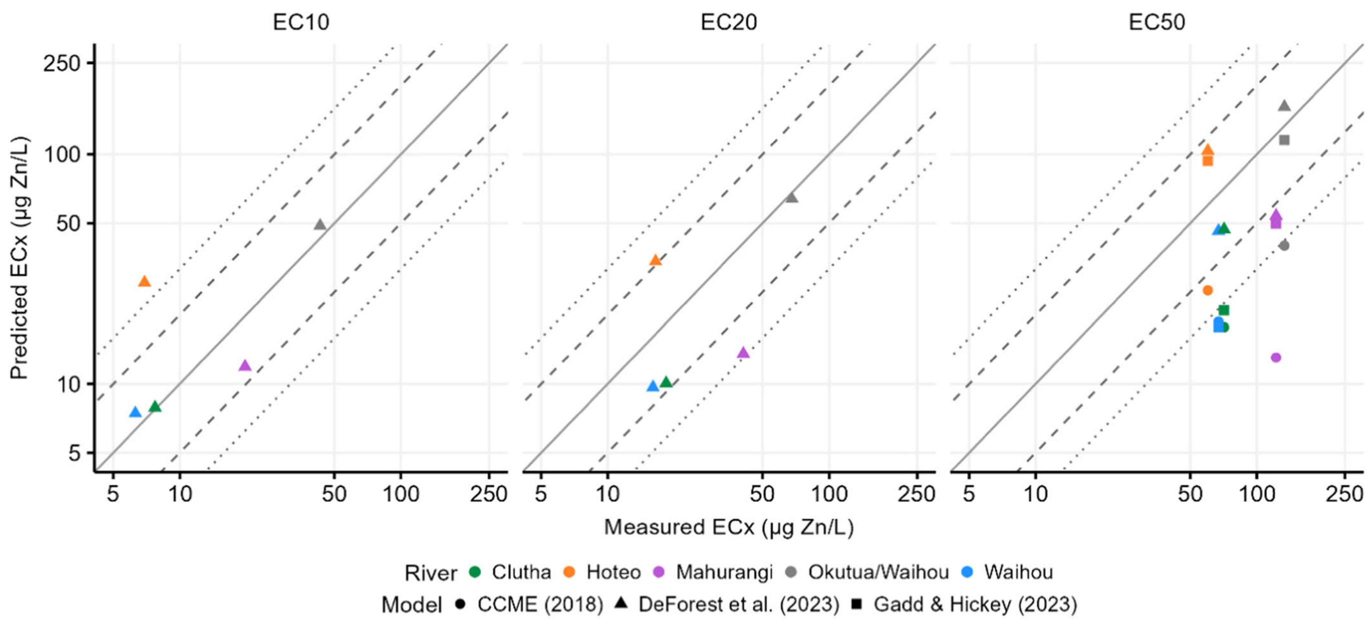

FIGURE 1: Observed effect concentration, *x*% (EC*x*) values for *Raphidocelis subcapitata* versus EC*x* values predicted based on multiple linear regression models. Solid gray line indicates 1:1 line where measured is equal to predicted; dashed lines are at a factor of 2 and dotted lines at a factor of 3 above or below the 1:1 line. CCME = Canadian Council of Ministers of the Environment.

60 juveniles by the end of the test in the controls (Supporting Information, Table S22). However, there was considerable variability in the number of juveniles in control waters for the Clutha and Okutua/Waihou Rivers, with the coefficient of variation greater than 25%. Mean temperature and dissolved oxygen were always within the acceptable range (20 ± 2 °C, dissolved oxygen >3 mg/L or >40% saturation). Although the pH remained within the range that would not cause toxicity to *D. thomsoni* (pH 6–9), it increased by 0.2–0.3 pH units over the 2- to 3-day period between water renewal as the algae in the waters (added for food) photosynthesized. The pH of the Clutha River water varied by up to 1 pH unit over the period before water renewal, likely due to the low buffering capacity of this alpine-sourced water. Measured dissolved zinc concentrations (Supporting Information, Table S23) were used to calculate all EC values.

Survival (immobilization) of *D. thomsoni* was affected by zinc at concentrations of 50–500 μg/L in all natural waters. For most waters, the slopes of the concentration–response curves were steep. Concentration–response models could not be fitted to most waters. No-observed-effect concentrations (NOECs) ranged from 26 to 129 μg/L.

Zinc exposure resulted in reduced reproduction of *D. thomsoni* in all five natural waters tested (Supporting Information, Figure S10). For all natural waters, the number of offspring produced by each adult was highly variable in both the test concentrations and the controls. A three-parameter log-logistic model fitted all datasets based on either total offspring/parent or the offspring from surviving parents only, and EC10, EC20, and EC50 values were calculated for both endpoints for all waters (Table 7), except for the Okutua/Waihou mix. The OECD (2012) protocol recommends using the lowest EC value calculated by these two response variables; however, to compare between waters, the endpoint based on total offspring (regardless of parent survival), was used to compare observed and predicted toxicity.

Based on total offspring produced, the EC10 values ranged from 8 μg/L in the Waihou River water to 72 μg/L in the Okutua/Waihou mix, although there were only significant differences between the Hoteo and Okutua/Waihou EC10 values. Based on EC50 values, toxicity was highest in the Waihou River waters but lowest in the Mahurangi River water (Table 7), with significant differences between EC50 values for most waters. The Waihou River water had the lowest DOC and hardness of the waters tested. The Clutha River water had similar DOC to Waihou River water but higher hardness, with no significant difference between EC values. The higher EC values for the Okutua/Waihou Rivers, with low hardness but highest DOC, suggests that DOC was protective of zinc toxicity.

Observed EC values were compared with predicted EC values using a range of MLRs (Table 1). Without correction for the *D. thomsoni* sensitivity coefficient, the CCME (2018) EC10 model for *D. magna* overpredicted the EC10 values by more than a factor of 2 for four of five natural waters (not shown). The DeForest et al. (2023) *D. magna* model also overpredicted the EC10 values for four of the five natural waters, with two of these being over a factor of 2 larger. Together these results suggest that *D. thomsoni* is more sensitive to zinc than *D. magna*. The two *D. magna* models overpredicted the EC20 values by more than double for the Waihou and Hoteo Rivers, whereas predictions for the other three natural waters were within a factor of 2. In contrast, the EC50 predictions were within, or very close to, being within a factor of 2 for all waters with all models used.

Using the species-specific coefficients for *D. thomsoni*, the predicted EC values were within a factor of 2 of the measured EC values for all EC50 models, with toxicity in some waters slightly overpredicted and some slightly underpredicted with each model (Figure 2). There was very little difference between the EC50 predictions from the Gadd and Hickey (2023) *D. magna* model and the DeForest et al. (2023) *D. magna* model. For the EC20 model (DeForest et al., 2023), four of five waters were predicted within a factor of 2, whereas toxicity in the Clutha River was underpredicted by just over a factor of 2 for the DeForest *D. magna* model. Both EC10 models (CCME, 2018; DeForest et al., 2023) predicted toxicity in three of five waters within a factor of 2—all underpredicted toxicity in the Waihou River water and overpredicted toxicity in the Clutha River water by more than two-fold.

Plots of the residuals (observed:predicted EC values) versus each TMF for each model used are shown in the Supporting Information (Figure S12). Residuals for each model, except the EC10 models, were reasonably similar over the range of TMFs used, suggesting low model bias. However, it should be noted that data were limited (five natural waters) so this should be interpreted with caution. The lower residuals for EC50 models (compared with EC10 models) confirm that these are preferable to use for normalization of ecotoxicity data to water chemistry.

DISCUSSION

The present study builds on our previous attempt (Peters et al., 2018) to compile water chemistry data, specifically pH,

TABLE 7: Effect concentration (EC) values for *Daphnia thomsoni* 21-day reproduction in each natural New Zealand freshwater tested

	Endpoint: Total neonates/adult (μg Zn/L)			Endpoint: Total neonates/surviving adult (μg Zn/L)		
River	EC10	EC20	EC50	EC10	EC20	EC50
Waihou	8 (0–41)	13 (0–52)	34 (10–58)	17 (10–24)	22 (16–28)	33 (26–40)
Clutha	55 (0–113)	58 (7–110)	65 (26–103)	43 (0–90)	46 (0–128)	52 (0–207)
Hoteo	36 (7–64)	55 (23–86)	115 (87–142)	21 (0–59)	45 (0–95)	159 (92–227)
Mahurangi	46 (14–78)	77 (42–113)	188 (130–246)	55 (30–79)	78 (55–101)	145 (110–180)
Okutua/Waihou mix	72 (58–86)	78 (67–90)	91 (83–100)	NC	NC	NC

NC = not calculated because a model could not be fitted.

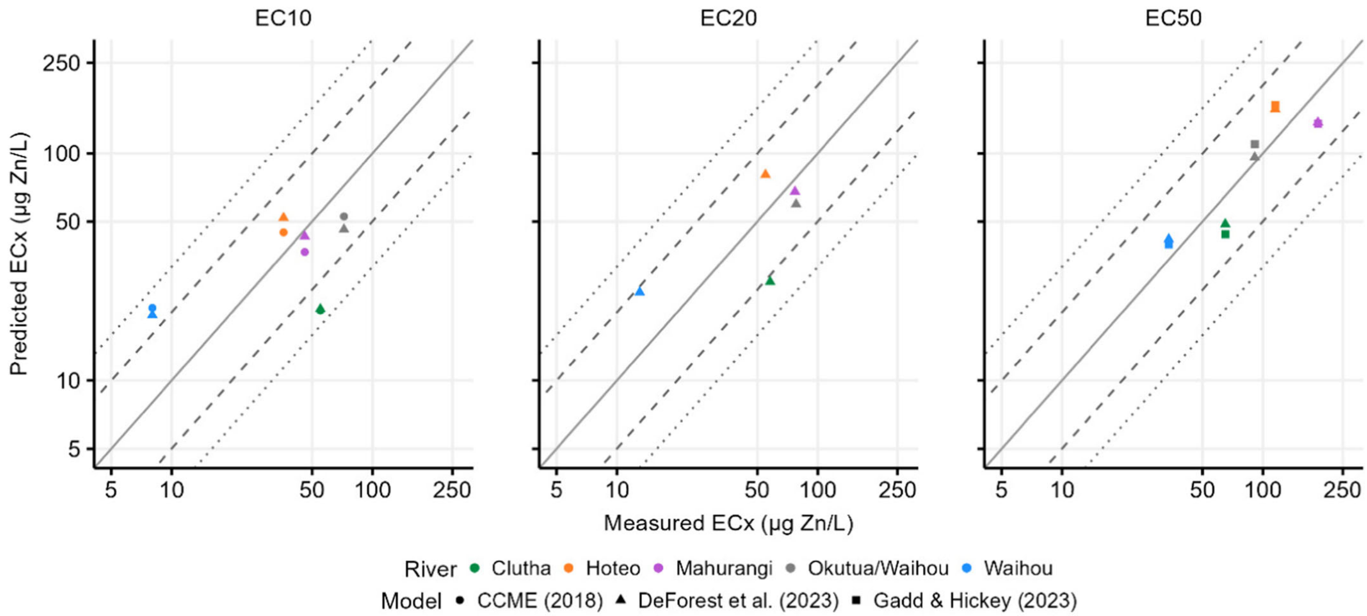

FIGURE 2: Measured (observed) effect concentration, *x*% (EC*x*) values versus predicted values to *Daphnia thomsoni* based on multiple linear regression models. The solid gray line indicates a 1:1 line where measured and predicted concentrations are equal. Dashed lines are at a factor of 2 and dotted lines at a factor of 3 above or below the 1:1 line. CCME = Canadian Council of Ministers of the Environment.

hardness, and DOC, for Australian waters, and for the first time includes a New Zealand freshwater compilation for these TMFs. These data were used to select 13 freshwaters with varying water chemistries across a range of climatic, geographical, and state jurisdictions, to assess the chronic toxicity of zinc to three aquatic species, two of which were local isolates.

Ceriodaphnia cf dubia

Other studies with the same local isolate of *C.* cf. *dubia* in diluted mineral water, under the same water chemistry conditions as our laboratory tests, have shown that this species is highly sensitive to zinc (New South Wales Department of Planning and Environment, unpublished data), with an EC10 (and 95% confidence limits) of 44 (0–96) µg zinc/L, an EC20 of 67 (18–120) µg zinc/L, and an EC50 of 134 (103–165) µg zinc/L for chronic reproduction. Cooper et al. (2009) reported a lower EC50 of 22 (12–30) µg zinc/L for the same species in the same diluted mineral water, indicating that this species' sensitivity to zinc is quite variable, even under similar water chemistries.

Previous studies have found that changes in water chemistries can impact the ability of *C. dubia* to reproduce (Armstead et al., 2016; Mebane et al., 2021). For example, Armstead et al. (2016) found that sudden changes in ionic composition due to mining influences inhibited *C. dubia* reproduction. Lasier et al. (2006), who tested four hardness levels ranging from 45 to 100 mg $CaCO_3$/L, found that both a decrease and an increase in water hardness can negatively impact the reproduction and survival of *C. dubia*. Increased DOC has also been observed to have a toxic effect on aquatic species such as *C. dubia* due to a reduction in the bioavailability of calcium ions in the water column (Ivey et al., 2019). Calcium has also been shown to have a protective effect against magnesium toxicity in cladocerans, with a two-fold reduction in magnesium toxicity to *Moinodaphnia macleayi* at magnesium:calcium ratios of <9:1 (van Dam et al., 2010).

Both the Australian waters that tested successfully with *C.* cf. *dubia* (reproduction in Woronora River and Jingellic Creek) had similar water chemistries, so it was not surprising that there was no significant difference between zinc toxicity to *C.* cf. *dubia* reproduction over 7 days. However, reproduction in this species was highly sensitive to changes in water chemistries beyond their tolerable range, and reproduction was highly variable, in agreement with Mebane et al. (2021) and the Southern California Coastal Water Research Project, (2023). These studies suggest that several factors affect the sensitivity and reliability of *C. dubia* tests including pre-exposure conditions (i.e., acclimation, culture conditions, supplementation), general tolerance to changing water chemistry conditions, and sensitivity to specific toxicants such as zinc. In agreement with our study, there seems to be a very low signal-to-noise ratio in the chronic reproduction endpoint in *C. dubia* tests, which currently limits the test's application. Without acclimation, cladoceran reproduction in our study only met quality assurance criteria in two of the Australian waters, so more testing is required, preferably with acclimation, to confirm these results.

Other studies have shown that TMFs such as pH, DOC, and hardness can both increase or decrease or have no effect on the acute and chronic toxicity of zinc to cladocerans (Besser et al., 2021; Nys et al., 2017). Besser et al. (2021) found that pH did not always have a statistically significant effect on chronic zinc toxicity to *C. dubia* and that chronic toxicity endpoints were difficult to calculate for *C. dubia*. In contrast, Nys et al. (2017), who pre-acclimated the test organisms to the different water chemistries, found that as the pH increased over the range 6.4–8.3, the chronic toxicity of zinc to *C. dubia* significantly increased. The same authors also found that in a natural water with 11.4 mg C/L, zinc was less toxic to *C. dubia* reproduction (EC50 of 164 μg/L) compared with a water with only 4.7 mg C/L (EC50 of 43 μg/L). In acute studies, Ivey et al. (2019) found that DOC had no effect on the toxicity of zinc to *C. dubia* survival, and Hyne et al. (2005) found that as water hardness increased from 44 to 374 mg $CaCO_3$/L, zinc EC50 values for survival (immobilization) to *C.* cf. *dubia* increased from 70 to 160 μg/L.

We found that of all the chronic MLRs, the EC10 CCME MLR for *D. magna* gave the best predictions for chronic zinc toxicity to *C.* cf. *dubia* and was slightly better than the *C. dubia* MLRs. None of the models consistently over- or under-predicted zinc toxicity. Similar issues were found for both the chronic *C. dubia* MLR models and the BLM by DeForest et al. (2023), suggesting that other parameters unrelated to bioavailability contribute to variability in chronic zinc toxicity to this species.

However, because we did not acclimate test organisms prior to testing, further testing using acclimated individuals is recommended. Also, given that we tested only a limited number of waters, further testing would be required on a larger number of water samples with diverse chemistries to confirm which model is most suitable for this species.

Daphnia thomsoni

Several chronic zinc MLR models for *D. magna* based on pH, hardness, and DOC were evaluated. Most models could predict toxicity within a factor of 2 of the observed (measured) concentrations when the sensitivity coefficient was updated for *D. thomsoni*, which is more sensitive than the model species *D. magna*.

For the Clutha River water, which had high calcium (11 mg/L) but low hardness (due to a low magnesium concentration of 0.89 mg/L), predicted toxicity to *D. thomsoni* was higher than measured, and outside, or just within, the factor of 2. This calcium:magnesium ratio (13:1) was very different from the ratio of the other natural waters tested (1.3–2.8) and is likely very different from the ratio of waters used in MLR model development. Previous work in BLM development has shown that calcium has a greater effect than magnesium in moderating zinc toxicity to *D. magna* (Heijerick et al., 2002). This may explain why the toxicity was lower in the Clutha River water than predicted and when compared with the Waihou River water, which had a similar pH (geometric mean of 8.1 in test waters) and DOC (<1 mg C/L) and only slight differences in hardness (33 and 21 mg $CaCO_3$/L for the Clutha and Waihou Rivers, respectively). For waters with unusual calcium:magnesium ratios such as this, it is possible that models based on individual calcium and magnesium concentrations may perform better than those based on hardness. Overall, the results suggest that the MLR models are generally applicable to *D. thomsoni*, although their applicability in waters with calcium and magnesium ratios outside of the range used in model development may need to be considered further.

The pH of the Clutha River water varied considerably during the *D. thomsoni* toxicity testing as a result of the low buffering capacity of this unimpacted water. Use of different pH values in the MLR prediction (e.g., from 7.4 to 9.1, based on the total range of pH measurements at any time during the test) resulted in the predicted EC20 being within a factor of 2 of measured, but the predicted EC10 remained lower than measured (although note that the CCME (2018) EC10 model does not include pH as a factor).

Some BLMs have also been developed for zinc for the common toxicity test organism *D. magna* for acute (Heijerick et al., 2002; Santore et al., 2002) and chronic (DeForest et al., 2023; Heijerick et al., 2003) exposures. These BLMs were applied to predict toxicity to *D. thomsoni* in the same five New Zealand waters (Stauber et al., 2022). The BLMs developed for *D. magna* did not predict toxicity within a factor of 2 of the measured concentrations for most New Zealand waters for EC10/NOEC, EC20, or EC50 levels. When the critical accumulation coefficient was updated for the more sensitive *D. thomsoni*, the models predicted EC values within a factor of 2 for three out of the five New Zealand waters. Predictions for the Clutha River were well below the measured values, particularly the EC10 and EC20 values, despite the BLMs using an extended set of water quality data in the model including calcium and magnesium as separate ions.

Rhaphidocelis subcapitata

Zinc was toxic to the standard algal test species *R. subcapitata* in a chronic 72-h cell growth rate test, with EC10 values from 6.3 to 43 μg/L, EC20 values from 16 to 68 μg/L, and EC50 values from 61 to 133 μg/L. These values were at the lower end but within the range of those previously reported for zinc growth rate tests in natural waters with this species (De Schamphelaere et al., 2005; Van Regenmortel et al., 2017), shown in the Supporting Information, Table S24.

The highest toxicities (lowest EC10–EC50 values) were observed in the water samples with low hardness and low DOC and higher pH. However, the water with the highest pH during the test (Mahurangi River) had the second highest EC values (i.e., second lowest toxicity). This water had the highest hardness of the five waters tested. The lowest toxicity was observed in the Okutua/Waihou water with the highest DOC and lowest pH, suggesting that the toxicity is mediated by DOC and/or low pH. The Hoteo River water, with moderate DOC and hardness, showed higher toxicity than expected, possibly due to the lower pH or the poorer fit of the concentration–response curve due to high zinc concentrations in the controls.

The DeForest et al. (2023) model for *R. subcapitata* provided the best predictions for EC50 values. Predicted EC50 values from other models were mostly lower than the measured values by more than a factor of 2. For example, the Gadd and Hickey (2023) EC50 model predicted EC50 values within a factor of 2 of the measured values for only two of the five New Zealand waters, with EC50 predictions for the Waihou, Clutha, and Mahurangi Rivers more than a factor of 3 lower than the measured EC values. The difference (and poorer performance) of this model compared with the DeForest et al. (2023) model is likely due to the different datasets used in the development of each MLR. The Gadd and Hickey (2023) model excluded data with pH <6 and hardness >300 mg $CaCO_3$/L, but included 17 data points from Graff et al. (2003), who tested zinc bioavailability in natural river waters (see Supporting Information II), and these data were not included in development of the DeForest et al. (2023) model. However, because both MLRs underpredicted the EC50 values, that is, overpredicted toxicity to some extent, they could be considered to provide conservative predictions and could still be useful for bioavailability normalization.

Comparison with other studies

A new chronic zinc MLR for a local species of algae, *Chlorella* sp., has recently been developed (Price et al., 2023) and tested for its applicability to predicting zinc toxicity in seven Australian waters collected in the present study. Observed EC10 and EC50 values in the MOPS-buffered tests ranged from 6.6 to 193 μg/L and 66 to 603 μg zinc/L, respectively, confirming that zinc was less toxic in natural waters compared with laboratory synthetic water. However, there was no clear pattern of zinc toxicity with the physicochemical parameters of the natural waters tested. For example, zinc was most toxic in Magela Creek water, with low pH, low hardness, and low DOC, whereas zinc was least toxic in Blackwood River (high pH, high hardness, moderate DOC, and high conductivity) and Woronora River water (neutral pH, low hardness, and moderate DOC). These newly developed *Chlorella* sp. MLR models based on EC10, EC20, and EC50 values poorly predicted zinc toxicity in our waters, consistently overpredicting zinc toxicity outside the factor of 2 compared with observed toxicity. Only 25%, 25%, and 58% of data were within a factor of 2 for the EC10, EC20, and EC50 models, respectively; however, 75% of the data were within a factor of 3 for all models (Price et al., 2023). Using the *R. subcapitata* MLRs, corrected for the *Chlorella* species-specific sensitivity coefficient, predictions of zinc toxicity were better. Both the Gadd and Hickey (2023) EC50 model and the DeForest et al. (2023) EC20 and EC50 models for *R. subcapitata* best predicted toxicity to *Chlorella* sp., with >50% of predictions within a factor of 2 of observed data. However, no EC10 model met the acceptability criteria, suggesting that further research is required before any algal MLR can confidently be applied to predicting toxicity across local algal species, especially at the EC10 level currently used in SSDs to derive guideline values for zinc.

Heijerick et al. (2003) investigated the effect of changes in pH, hardness, and DOC on the chronic toxicity of zinc to *D. magna*. An increase in pH and/or DOC decreased zinc toxicity, suggesting that there is increased sorption efficiency of zinc to humic substances at higher pH. The DOC value appeared to be the most important TMF, whereas hardness was least important. Lowest zinc toxicity was found in hard water (200–300 mg $CaCO_3$/L), with increased toxicity at lower and higher hardness. In our study, chronic zinc toxicity to the New Zealand cladoceran *D. thomsoni* was also ameliorated at higher hardness and DOC; however, pH influence was less obvious due to the relatively narrow range of pH in the New Zealand waters tested. Reproduction in the Australian isolate of *C. dubia* was not substantially influenced by water chemistry across the range tested, although this was limited by the small number of samples due to the physicochemical tolerance range of this species without pre-acclimation.

Other studies, summarized in the following text, have also found that a range of bioavailability models can predict toxicity (EC10 and EC20 values) to algae and invertebrates within a factor of 2 in most cases, and somewhat better than MLRs used to predict toxicity to our local species. De Schamphelaere et al. (2005) evaluated a range of acute and chronic bioavailability models for predicting zinc toxicity to *R. subcapitata*, *D. magna*, and rainbow trout in eight natural European freshwaters over a range of pH (5.7–8.4), DOC (2.5–23 mg C/L), calcium (1.5–80 mg/L), magnesium (0.70–18 mg/L), and sodium (3.8–120 mg/L) concentrations. Chronic EC10 values varied by >20-fold for the alga (72-h EC10 values of 27–563 μg zinc/L), by 6-fold for the cladoceran (21-day EC10 values from 59 to 387 μg zinc/L), and by 5-fold for the fish (30-day LC10 values from 185 to 902 μg zinc/L). Toxicity was predicted using the chronic zinc BLMs for *D. magna* and rainbow trout, as well as a modified approach combining an empirical regression for zinc toxicity and pH for these waters, together with a Windermere Humic Aqeous speciation model V to predict the algal EC10 values. This was necessary because, for this alga, the effect of pH could not be explained by H^+ competition for a single binding site, rather, multiple binding sites for zinc (and competing ions) were assumed. However, the same waters used for toxicity testing were used for developing the model, that is, autovalidation. The models for each species generally predicted toxicity within a factor of 2 except for two waters with pH values > 8, in which chronic toxicity to *D. magna* was underpredicted by a factor of 3–4.

DeForest and Van Genderen (2012) developed a unified chronic zinc BLM by averaging the biotic ligand binding constants for Zn^{2+} and competing cations (Ca^{2+}, Mg^{2+}, Na^+, and H^+) and optimizing the biotic ligand binding constant for $ZnOH^+$ for a range of invertebrates and one fish (no algae). Using autovalidation, this unified BLM was able to predict EC20 values within a factor of 2 for 45 of 47 tests.

Van Genderen et al. (2020) developed a bioavailability model evaluation and selection procedure, using zinc as a case study. To assess model performance comparisons were undertaken of USEPA hardness-based species equations (USEPA, 1987), CCME zinc-MLR equations for algae, *D. magna*, and rainbow trout (CCME, 2018), and BLMs for algae (Van Regenmortel et al., 2017) and aquatic invertebrates and fish (DeForest & Van Genderen, 2012). Prediction accuracy within a factor of 2 ranged from 19% for hardness only, to 55% for MLRs, and to 87% for the BLMs.

Implications for zinc guidelines in Australia and New Zealand

Current zinc water quality guidelines in Australia and New Zealand only incorporate hardness using the original hardness algorithm (USEPA, 1987), normalized to a hardness of 30 mg

$CaCO_3$/L (ANZG, 2018; Warne et al., 2018). The findings of the present study along with those of others (Heijerick et al., 2002; Rai et al., 1981) indicate that environmentally relevant high-hardness conditions may not continue to provide increased protective effects from zinc toxicity to all freshwater organisms. For example, the hardness algorithm (ANZG, 2018) may provide appropriate increases in guideline values up to 93 mg $CaCO_3$/L, but based on the present results, this may not be appropriate at higher water hardness concentrations. The reason may be the higher slope factor used in the hardness algorithm, at 0.85 (ANZG, 2018), compared with 0.19–0.49 (when hardness was included as a significant factor) in all tested MLR models. Moreover, the use of the algorithm with the ANZG (2018) framework does not allow modification of the guideline value for hardness concentrations below 30 mg $CaCO_3$/L, potentially providing insufficient protection in very soft waters. Australia has both very soft (<10 mg $CaCO_3$/L) and hard waters (>200 mg $CaCO_3$/L) and therefore may be subject to underprotection using the current hardness-correction algorithms. The continued use of the hardness algorithm is therefore not recommended. Inclusion of additional TMFs in MLRs beyond hardness, such as investigated in the present study, to normalize chronic zinc toxicity data, should improve our zinc guidelines, provided they are not used beyond the boundary conditions used in model development. Although the use of BLMs and MLRs in Australia and New Zealand is still in the preliminary stage, there is scope within the framework to use such models provided they are justified and well validated for local conditions and local species.

The zinc freshwater guideline values for Australia and New Zealand are currently being revised, including assessing a range of chronic bioavailability models (MLRs and BLMs) to account for the effect of TMFs on algae, invertebrates, and fish. The final choice of model will depend on their predictive ability, level of protection, and ease of use. No single trophic-level–specific EC10 MLR in our present study was the best predictor of toxicity to algae or invertebrates, and MLRs based on EC10 values both over- and under-predicted zinc toxicity. For the Australian *C.* cf. *dubia*, the *D. magna* MLRs were slightly better for predicting EC10 values than *C. dubia* models. For the New Zealand *D. thomsoni*, only three of the five waters tested were predicted within a factor of 2 using each of the EC10 models, but all predictions were within a factor of 3 (Gadd et al., 2022).

The EC50 MLRs better predicted toxicity to both cladocerans within a factor of 2 for most of the Australian and New Zealand natural waters, but given that EC10 values are used in the SSDs for the derivation of water quality guidelines, decisions would need to be made on whether EC50 models are appropriate for bioavailability corrections for EC10 values. We believe that the use of MLRs based on EC50 values is appropriate for normalizing EC10 values, because there is generally less variability and uncertainty in EC50 values than in EC10 values, and variability in EC50/EC10 ratios is generally low for metals such as zinc.

Our study showed that zinc toxicity to algae is difficult to predict. Neither the *Chlorella* sp. MLRs (Price et al., 2023) nor the existing CCME and Gadd and Hickey MLRs for *R. subcapitata* accurately predicted zinc toxicity to the algae in natural waters. However, the DeForest et al. (2023) model did predict EC10 values for *R. subcapitata* within a factor of 2 for four of the five New Zealand waters tested.

The findings of the present study provide further evidence that the chronic toxicity of zinc to different freshwater organisms varies in different ways for TMFs such as pH, hardness, and DOC. This highlights the importance of considering local taxa with appropriate validation of bioavailability models in natural waters for reliable bioavailability-based guideline value derivation. Our study supports earlier studies with Northern Hemisphere species (DeForest et al., 2023) showing that the TMFs that most influence chronic zinc toxicity to different species are variable. The applicability of MLRs, particularly to algal species, requires further investigation, including additional laboratory studies under controlled test conditions in which water quality parameters are well characterized. This could also include additional natural water testing to push model boundaries or to fill gaps in under-represented water chemistries.

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