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Development of a multiple linear regression model for chronic nickel toxicity to *Ceriodaphnia dubia*: performance of bicarbonate vs. pH as toxicity modifying factors

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Abstract

Multiple linear regression models were developed to predict chronic nickel (Ni) toxicity to *Ceriodaphnia dubia* under an expanded range of conditions relative to published datasets. Test conditions were expanded to study Ni toxicity under very high hardness (up to 1,020 mg/L as $CaCO_3$) and bicarbonate (up to 366 mg/L HCO_3) by conducting tests in synthetic waters and mine-influenced site waters. Toxicity modifying factors (TMFs) identified by the models were hardness, dissolved organic carbon, and either pH or bicarbonate. Because high pH and high bicarbonate co-occur in some mine-influenced waters and their relative importance as TMFs for Ni is unclear, we compared the performance of models with each candidate TMF. The model with bicarbonate performed better than the model with pH and showed closer alignment between site-specific and published datasets, supporting bicarbonate as a TMF in both datasets. The model with bicarbonate performed well across the expanded range of conditions and is expected to be more robust than previous Ni models under the high hardness and bicarbonate conditions studied. Comparison of TMF effects on Ni toxicity to other invertebrates indicated stronger support for a bicarbonate TMF effect than pH for some species, including *C. dubia*. Further support came from site-specific testing that indicated bicarbonate is not toxic to *C. dubia* at the concentrations in our dataset. These findings suggest bicarbonate may play an important role in modifying chronic Ni toxicity to *C. dubia* in alkaline waters. More work is needed to understand the mechanism for bicarbonate and pH TMF effects and why TMF effects are species-specific to reduce uncertainties associated with collinearity in model datasets and in applying models across species.

Keywords: nickel, bicarbonate, bioavailability, metal toxicity

Introduction

Laboratory studies of chronic nickel (Ni) toxicity show a wide range of sensitivity among aquatic species, with effects concentrations spanning more than two orders of magnitude (European Chemicals Agency [ECHA], 2008; Mebane et al., 2020; Santore et al., 2021). Invertebrates are consistently reported to be the most sensitive taxon to Ni, similar to other divalent metals (Mebane et al., 2020). Invertebrates exhibit a wide range of sensitivities to Ni, with species such as *Chironomus riparius* among the least sensitive (10% effect concentration [EC10] >700 µg/L) and others such as *Ceriodaphnia dubia* among the most sensitive (EC10 < 5 µg/L) and best studied test species (Croteau et al., 2021; ECHA, 2008; Mebane et al., 2020; Peters et al., 2021).

Mechanisms underlying the chronic toxicity of Ni are not well understood but seem to relate primarily to the dissolved Ni^{2+} ion. Brix et al. (2017) identified five possible mechanisms with varying levels of empirical support. Three of these pathways involve disruption of major ion and essential metal homeostasis via Ni^{2+} interfering with transport proteins for calcium, magnesium, or iron. The fourth, which hypothesizes an allergic-type response to Ni^{2+} at the gill surface, was considered unlikely. The fifth involves generation of reactive oxygen species, either directly or by interfering with iron homeostasis. The physiological effects of these mechanisms could include reduced calcium/magnesium availability, impaired respiration, or cytotoxicity and tumor formation. Ultimately, these effects would all result in impaired growth and reproduction and/or alterations in energy metabolism.

Consistent with the hypothesis that toxicity is caused by the free Ni^{2+} ion, Ni toxicity is ameliorated by hardness (Chapman et al., 1980; Keithly et al., 2004; Meyer et al., 1999) and this effect has been attributed to competition for ion transport sites

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between Ni^{2+} and the much more abundant calcium and magnesium ions that comprise hardness (Deleebeeck et al., 2008a; Meyer et al., 1999; Playle, 1998). Ni toxicity is also ameliorated by dissolved organic carbon (DOC; Doig and Liber, 2006; Hoang et al., 2004), which has been attributed to complexation of free Ni^{2+} into a less bioavailable form (Richards et al., 1997). Effects of pH on chronic Ni toxicity are less consistent among species and less well understood (ECHA, 2008; Santore et al., 2021), but when present, generally involve a nonlinear increase in Ni toxicity with increasing pH (Croteau et al., 2021; Nys et al., 2016a; Peters et al., 2018). Although less well studied, increasing bicarbonate concentration (HCO_3^-; hereafter referred to as HCO_3) has also been associated with increased Ni toxicity (Deleebeeck et al., 2008a; Nys et al., 2016a; Puttaswamy and Liber, 2012; Santore et al., 2021), which has been hypothesized to relate to bioavailable inorganic Ni complexes (Deleebeeck et al., 2008a; Hoang et al., 2004; Nys et al., 2016a) or toxicity of bicarbonate itself (Santore et al., 2021).

Several predictive toxicity models have been developed to account for effects of various toxicity modifying factors (TMFs) for Ni, which can be broadly categorized into two model types: semi-mechanistic biotic ligand models (BLMs) and empirical multiple linear regression (MLR) models (Croteau et al., 2021; Deleebeeck et al., 2008a,b; Nys et al., 2016a; Peters et al., 2021; Santore et al., 2021; Stauber et al., 2021). Although these two types of models differ in many ways, they both attempt to combine information about chemical speciation (either explicitly in the case of BLMs or implicitly in the case of MLRs) and pathways for Ni uptake into organisms (via calibration of either model type to toxicity data) to explain and predict the effect of TMFs on toxicity. Croteau et al. (2021) recently compared the performance of MLR models and BLMs for Ni following methods presented in Garman et al. (2020) and Brix et al. (2020) and found the two model types to be comparable.

The background for the present study was a need to predict Ni toxicity to sensitive invertebrates in mine-influenced receiving waters in our study area in the Elk Valley, southeast British Columbia, Canada. We selected *C. dubia* as a model organism because of its sensitivity and extensive toxicity dataset. Existing Ni MLR models for *C. dubia* or other invertebrates (Croteau et al., 2021; Peters et al., 2021) did not adequately cover the range of site TMF conditions in our study area, particularly the very high hardness and bicarbonate that can occur in areas near mining. The objective of the present study was to develop a new chronic Ni MLR model from a pooled dataset of published data and new site-specific data developed in this study, to expand the domain of TMFs. As discussed in other studies, the relative contribution of pH and/or bicarbonate as a TMF for Ni is unclear; therefore, given the relatively high pH and bicarbonate conditions of some site waters, we explicitly considered and compared these candidate TMFs in our modeling. As part of evaluating bicarbonate as a TMF, we compiled published toxicity data and conducted chronic toxicity testing with *C. dubia* to evaluate the potential for bicarbonate to be a toxicant under site conditions. In a companion paper (de Bruyn et al., 2025), we evaluate the field application of the laboratory-based Ni MLR model for *C. dubia* for predicting the sensitivity of stream benthic invertebrate communities in our study area.

Materials and methods

Site-specific toxicity testing

Toxicity tests with *C. dubia* were conducted using site waters collected from mine-influenced areas in the Elk Valley, southeast British Columbia, Canada, and in synthetic waters formulated to increase the ranges and combinations of TMFs that could be included in model development. Six rounds of toxicity testing were conducted between 2018 and 2020 to expand the testing domain to higher hardness and bicarbonate than had previously been studied (Table 1; Supplementary material 2). In brief, testing followed standard protocols for the *C. dubia* 3-brood test (Environment Canada, 2007; U. S. Environmental Protection Agency [USEPA], 2002). Site test waters were chosen to reflect a range of TMF conditions while maintaining sufficiently low concentrations of other mine-related constituents (e.g., sulfate, nitrate) that could potentially confound the ability to attribute toxicity to Ni (Supplementary material 2). Synthetic test waters were formulated with a calcium: magnesium ratio similar to that of site water (Supplementary material 2). Total ion concentrations were constrained to be less than estimated thresholds for direct effects to *C. dubia* from high hardness (≤1,000 mg/L as $CaCO_3$) and alkalinity (≤300 mg/L as $CaCO_3$) calculated from multi-ion toxicity models (Mount et al., 2019). Nickel was added to base waters as $NiCl_2$ to provide concentration gradients (including a base water control with no Ni added) bracketing estimated 50% effect concentrations (EC50) for survival and reproduction (Supplementary material 2). All chemicals used for water formulation and Ni spiking were reagent grade. Laboratory culture water controls were included to assess test validity against protocol acceptability criteria. All test concentrations were measured for total and dissolved Ni at the beginning and end of the test (with two exceptions discussed in Supplementary material 2). Major ion concentrations (calcium, magnesium, sodium, potassium, sulfate, chloride, hardness, total alkalinity, and bicarbonate) and DOC were measured at least once for each test water, and pH was measured daily in the tests. When multiple measurements were taken, average concentrations were used for concentration–response analysis and modeling (see MLR analysis). Most tests were conducted by Nautilus Environmental (Calgary, Alberta, Canada). A subset of synthetic waters was tested in multiple laboratories. Laboratories reported Ni effects concentrations based on measured dissolved Ni concentrations and calculated relative to the test water controls using linear and nonlinear regression models chosen based on best fit of the model using Comprehensive Environmental Toxicity Information System (CETIS; Tidepool Scientific Software, 2014, 2018; see Supplementary material 2 for details).

Supplemental bicarbonate toxicity testing (without Ni added) with *C. dubia* was conducted under two hardness conditions (60 and 300 mg/L as $CaCO_3$). Test waters were formulated following similar procedures as Ni tests and with bicarbonate added as $NaHCO_3$ to provide a concentration gradient. Testing and endpoint reporting followed the same standard protocols as Ni testing (see Supplementary material 7 for details).

Published studies

Additional Ni toxicity data for *C. dubia* were compiled from previous studies (Besser et al., 2021; De Schamphelaere et al., 2006; Keithly et al., 2004; Nys et al., 2016b, 2017; Peters et al., 2018; Puttaswamy and Liber, 2012; Wirtz et al., 2004; unpublished Parametrix data reported in De Schamphelaere et al. 2006; Table 1. We relied on the data screening conducted by Croteau et al. (2021) and applied similar screening criteria to more recent data. We compiled 10%, 20%, and 50% effect concentrations reported in these publications or calculated effect concentrations using a logistic model with least-squares nonlinear regression analysis using the USEPA Toxicity Relationship Analysis Program (TRAP; Ver. 1.30; USEPA, 2016; see Supplementary material 2 for

Table 1. Summary of Ni toxicity test data used in multiple linear regression analyses.

Study	*n*	pH	DOC (mg/L)	Hardness (mg/L as $CaCO_3$)	Bicarbonate (mg/L HCO_3)	EC50 (μg/L Ni)
Besser et al. (2021)	6	6.9–8.4	0.4–40	19–356	20–305	13–230
De Schamphelaere et al. (2006)	6	6.6–8.2	3.0–24	15–218	7.9–183	4.9–68
Keithly et al. (2004)	4	7.6–7.8	0.47	50–253	29–30	5.5–12
Nys et al. (2016b, 2017)	4	7.1–7.4	3.5–7.3	84–103	16–28	27–52
Parametrix unpublished in De Schamphelaere et al. 2006	9	8.0–8.7	0.5	42–310	29–234	3.3–17
Peters et al. (2018)	4	7.4–8.0	0.5–10	20–58	32–124	4.4–20
Puttaswamy and Liber (2012)	8	7.9–8.6	0.5	57–88	71–230	2.3–7.0
Wirtz et al. (2004) (in De Schamphelaere et al., 2006)	5	8.3–8.6	0.5–8.4	170–262	97–194	7.0–53
Present study (2018–2020 site waters)	19	7.7–8.2	0.5–4.1	164–793	165–366	4.7–28
Present study (2020 synthetic waters)	36	7.6–8.6	0.4–1.2	81–1,020	63–363	1.9–44

Note. $CaCO_3$ = calcium carbonate; DOC = dissolved organic carbon; EC50 = 50% effect concentration.

details) when raw data could be obtained from the publication or the authors.

Data compilation

Water quality parameters compiled from each published or site-specific Ni study were temperature, pH, DOC, hardness, total alkalinity, major cations (calcium, magnesium, potassium, and sodium), and major anions (bicarbonate, chloride, nitrate, and sulfate), where available. These parameters are common inputs for BLMs and were tested as candidate TMFs for the *C. dubia* Ni MLR model. The average of pH measurements taken during a test (reported for most studies) was used to characterize average exposure conditions. Bicarbonate concentration was calculated from total alkalinity using molar mass conversion. Dissolved organic carbon was the concentration in the test water prior to addition of food in the test and where DOC was not reported a value was assumed (see study summaries in Supplementary material 2).

MLR analysis

Stepwise MLR analysis was conducted in SYSTAT (Ver. 13.2; Inpixon, 2017) to model *C. dubia* reproductive Ni EC50s as a function of candidate TMFs. Methods followed those previously described for other metals (Brix et al., 2020, 2023; Croteau et al., 2021; DeForest et al., 2023). Model derivation focused on the chronic reproductive EC50 because this metric is almost universally reported and represents a more statistically precise and robust metric compared to smaller effect sizes (Croteau et al., 2021). The pooled dataset comprised EC50s from 46 published tests and 55 site-specific tests (Table 1; full dataset in Supplementary material 3). Equations for EC20- and EC10-based models were derived by adjusting the EC50-based MLR model to lower effect sizes using a mean concentration-response slope from site-specific tests (see online supplementary material 4).

In initial MLR analyses, all candidate TMFs were included in backward stepwise regression using the general linear model function. This is an automated function that, starting with all variables, tests the deletion of each variable and then deletes the variable whose loss gives the most statistically insignificant deterioration of model fit. This process is repeated until no further variables can be deleted without a significant loss of model fit. All variables except pH were $\log_{10}$-transformed to stabilize residual variance and linearize slopes.

Backward stepwise regression was initially run without interactions, using the basic model form:

$$y = \beta_1 \times (X_1) + \cdots + \beta_k \times (X_k) + \beta_0 \quad (1)$$

where y is $\log_{10}$ of Ni EC50, X_1 is the first explanatory variable, X_k is *k*th explanatory variable, β_0 is the intercept, β_1 is the slope coefficient for the first explanatory variable, and β_k is the slope coefficient for the *k*th explanatory variable.

An all-subsets regression was also conducted, which provides an analysis of statistical criteria computed for every possible model of every size (Weisberg, 1985). The all-subsets regression method is a more exhaustive search method, which allows flexibility in selecting a model based on a range of statistical criteria (Helsel et al., 2020).

Backward stepwise regression was then conducted using the strongest TMFs (i.e., statistically significant and large standardized coefficients) identified by the initial stepwise and all-subsets analyses, which were bicarbonate, pH, hardness, and DOC. Regression analyses were conducted with either bicarbonate or pH to test which performed better as a TMF because initial MLR analyses selected one or the other parameter but never both. Analyses were conducted with all possible two-way interactions, using the model form:

$$y = \beta_1 \times (X_1) + \beta_2 \times (X_2) + \beta_3 \times (X_3) + \beta_4 \times (X_1 \times X_2) + \beta_5 \times (X_2 \times X_3) + \cdots + \beta_0 \quad (2)$$

where β_4 is the slope coefficient for the interaction between the X_1 and X_2 explanatory variables, β_5 is the slope coefficient for the interaction between the X_2 and X_3 explanatory variables, and so forth. We evaluated consistency in estimated TMF effects by comparing TMF slopes among candidate models and relative to a published Ni MLR model for *C. dubia* that used DOC, hardness, and pH as TMFs (Croteau et al., 2021).

Model evaluation

We evaluated candidate MLR models following guidelines outlined in Garman et al. (2020) and previously applied for Ni and other metals (Brix et al., 2023; Croteau et al., 2021; DeForest et al., 2023). Standard diagnostic tools employed were adjusted R^2, agreement factor 2 (AF2), mean absolute error (MAE), Akaike information criterion (AIC), Schwarz's Bayesian information criterion (BIC), residuals analysis, and variance inflation factors. Further description of metrics is provided in Supplementary material 5 (Table S5-1).

Evaluation of the performance of candidate models also considered fit to site-specific versus published data, in addition to general fit to the full dataset. We inspected plots of observed versus predicted EC50s to evaluate model fit for both datasets. We

evaluated residuals of observed versus predicted EC50 as a function of TMFs and observed EC50 to identify whether there were systematic biases in model predictions, and if this differed by dataset.

Model autovalidation

Following Garman et al. (2020), we further evaluated model performance using autovalidation, which characterizes how robustly the model describes the toxicity dataset from which it was parameterized. The autovalidation approach was conducted using a randomization approach called *k*-fold cross-validation to test model stability (Stone, 1974), where k was set to 5, similar to other recent MLR modeling (Brix et al., 2023; DeForest et al., 2023). The complete dataset used for model development was randomized and split into five unique subsets (i.e., autovalidation, not independent validation). The MLR analysis was rerun on 80% of the full dataset (four subsets combined), and the resulting cross-validation model was applied to predict the remaining 20% of the dataset (the excluded subset). Model performance was evaluated using adjusted R^2, AF2, and MAE. Cross-validation was repeated 5 times. Autovalidation was considered successful if the five cross-validation models had similar performance to the original model derived from the full dataset. As described in Brix et al. (2023), the mean, standard deviation, and coefficient of variation (CV) of each TMF coefficient and adjusted R^2 were calculated across the five cross-validation models to provide insight into robustness of model coefficients and fit.

Evidence for bicarbonate and pH as TMFs for Ni effects on invertebrates

A supporting analysis was conducted to evaluate evidence for bicarbonate and pH effects on chronic Ni toxicity to multiple invertebrate test species, including *C. dubia* and *Daphnia magna*. Species were included in this evaluation if the pooled dataset had at least three bounded EC50s from tests spanning at least 1 log unit of bicarbonate and/or 1 pH unit. Reported EC50s were normalized to a common hardness (117 mg/L as $CaCO_3$) and DOC (1.0 mg/L) that reflected the geometric mean of TMF conditions in the combined *C. dubia* dataset, using model coefficients from the *C. dubia* MLR models derived herein (see Supplementary material 6 for details). This "partial normalization" leaves the toxicity data unadjusted for bicarbonate or pH effects. To the extent that TMF effects are present in the data, they should be apparent as a relationship between the partially normalized EC50s and the remaining TMF (bicarbonate or pH) for which the data have not been adjusted. This approach provides a way to test for bicarbonate or pH effects on the toxicity of Ni to these species, and to compare these effects across species, after accounting for differences between studies in hardness and DOC. Partially normalized EC50s were plotted against bicarbonate or pH measured in the individual toxicity tests to visually evaluate slopes and least-squares linear regression (following $\log_{10}$-transformation as described above) was used to test for significant relationships between partially normalized EC50s and bicarbonate or pH using SYSTAT (Ver. 13.2; Inpixon, 2017).

Results and discussion

Domain of TMF conditions

Previous studies of Ni toxicity to *C. dubia* have evaluated a range of TMF conditions (pH 6.6–8.7, DOC 0.4–40 mg/L, hardness 15–356 mg/L as $CaCO_3$, bicarbonate 7.9–305 mg/L HCO_3; Table 1) that did not fully capture the very high hardness and bicarbonate concentrations in our study area (see online supplementary material; Figure S1-1). Site-specific testing expanded the TMF domain to capture more of these high ionic strength conditions (hardness 81–1,020 mg/L as $CaCO_3$, bicarbonate 63–366 mg/L HCO_3; Table 1) but the tested range had to be constrained to avoid multi-ion toxicity effects on *C. dubia* (Mount et al., 2019) and due to solubility limitations of calcium carbonate. Site waters were also distinct in having higher concentrations of bicarbonate per unit pH than have been tested in previous studies (see online supplementary material; Figure S1-1). Published studies showed strong collinearity between pH and bicarbonate (see online supplementary material; Figure S1-1), so if bicarbonate was a Ni TMF, then pH would serve in its place in an empirically based model, whereas site waters did not show the same degree of collinearity.

Ni MLR models

Stepwise and all-subset MLR models always retained DOC as a TMF and also retained hardness or its component ions (calcium and often magnesium; see online supplementary material; Table S5-2), consistent with previous Ni MLR models (Croteau et al., 2021; Peters et al., 2021). However, whereas most previous models have usually identified pH as a TMF, our analysis retained bicarbonate, and only retained pH when bicarbonate was excluded from the analysis (see online supplementary material; Table S5-2). This result suggests that either parameter may describe a TMF effect of increasing Ni toxicity with increasing bicarbonate and/or pH, with the following possible explanations: either bicarbonate is the TMF and pH is collinear enough to serve as a proxy, or vice versa, or both parameters are TMFs but are too collinear to be described separately by the model, or some other parameter is the TMF causing increased Ni toxicity and both bicarbonate and pH are proxies for it. There was higher collinearity between pH and bicarbonate in the published data than our site-specific data (see online supplementary material; Figure S1-1). Identification of bicarbonate as a strong TMF was a novel outcome of our MLR analysis with the expanded dataset, although to our knowledge no previous Ni models have specifically tried to fit bicarbonate as a TMF. Bicarbonate was carried forward in the MLR analyses along with DOC, hardness, and pH. Hardness was chosen over its component ions because it is more commonly and frequently reported. Some models in the initial MLR analysis retained sulfate, possibly due to its correlation with calcium and magnesium in site and synthetic test waters. However, because previous studies have found no effect of sulfate on Ni toxicity (Puttaswamy and Liber, 2012) and there is no mechanistic rationale for sulfate modifying Ni toxicity, it was not carried forward as a TMF.

Additional MLR analyses to directly test the performance of each candidate TMF found that in stepwise regressions without interactions, the significant TMFs were DOC, hardness, and either bicarbonate or pH (Table 2). Whereas when TMF interactions were allowed, only the bicarbonate × hardness or pH × hardness interaction terms were significant, and in both cases the main effect of hardness was not significant. Adjusted R^2 and MAE in models with interactions were the same as models without interactions (see online supplementary material; Table S5-3). As a result, subsequent model development and evaluation focused on the more parsimonious models without interactions. The candidate models retained for further analysis are referred to hereafter as the bicarbonate model (i.e., pH excluded) and the pH model (i.e., HCO_3 excluded).

The DOC slopes of our bicarbonate (0.547) and pH models (0.503) were similar to each other and with the Croteau et al. (2021) model (0.546). However, there was disparity in hardness

Table 2. Summary of *Ceriodaphnia dubia* Ni multiple linear regression models with dissolved organic carbon, hardness, and either bicarbonate or pH.

Model	Intercept	Slopes			
		DOC (mg/L)	Bicarbonate (mg/L HCO_3)	pH	Hardness (mg/L as $CaCO_3$)
Reproduction EC50 bicarbonate model (Present study)	1.124	0.547	−0.520	–	0.411
Reproduction EC50 pH model with fitted $\beta_{Hardness}$ (Present study)	2.974	0.503	–	−0.311	0.217
Reproduction EC50 pH model with fixed $\beta_{Hardness}$ (Present study)	3.435	0.515	–	−0.437	0.465
Survival and reproduction EC25 model (Croteau et al., 2021)	(b)	0.546	–	−0.611[(a)]	0.503

Note. $\beta_{Hardness}$ = hardness slope; $CaCO_3$ = calcium carbonate; DOC = dissolved organic carbon; EC25 = 25% effect concentration; EC50 = 50% effect concentration. Dash (–) indicates parameter was not included in the model run; (a) = transformed from ln space of Croteau et al. (2021) model to $\log_{10}$ space for comparison to parameters derived herein; (b) = intercept not shown because EC50 and EC25 models preclude direct comparison. See also Supplementary Information 4 for additional bicarbonate models and pH models with fixed βHardness to calculate 10% and 20% effect concentrations.

slopes between our models. The hardness slope of our bicarbonate model (0.411) was close to the Croteau et al. (2021) model (0.503), whereas the fitted hardness slope of our pH model (0.217) was shallower than both these other models and the hardness slopes evident in individual *C. dubia* studies (see online supplementary material; Table S5-4 and Figure S5-1). This suggested that our initial pH model may have misattributed hardness-related variance to other model parameters (i.e., other TMFs or intercept). We therefore refit the pH model using a fixed pooled hardness slope ($\beta_{Hardness}$= 0.465) from available *C. dubia* data (see online supplementary material; Table S5-4), that provided a more comparable hardness slope to the bicarbonate and Croteau et al. (2021) models. The resulting pH model with the fixed $\beta_{Hardness}$ had a DOC slope similar to the other models and a pH slope (−0.437) that was closer to the Croteau et al. (2021) model (−0.611; converted from ln slope of −1.407 to $\log_{10}$ space) than the pH slope of the fitted $\beta_{Hardness}$ model (−0.311). Variability and nonlinearity in pH slopes for Ni have been noted by others (Croteau et al., 2021; Nys et al., 2016a; Peters et al., 2018; Santore et al., 2021) and variability was also evident in the present analysis, suggesting that consideration of alternative modeling methods (e.g., nonlinear) may be warranted to better or more consistently describe the TMF effect of pH. Given the differences in TMF slopes between models, we also assessed the sensitivity of our model predictions to each TMF by plotting how each model responded to individual and co-varying TMFs across three example scenarios of TMFs combinations within the site-specific data range to visually compare how predictions changed between models across the TMF ranges (see online supplementary material; Figure S5-2).

Model evaluation and performance

In general, the bicarbonate model performed similarly to or better than the pH models for almost all performance metrics (Figure 1; Table 3). The most apparent difference in metrics was the lower AIC and BIC for the bicarbonate model, indicating that these criteria selected bicarbonate as the better model over the pH model with fitted $\beta_{Hardness}$. The bicarbonate model showed closer alignment between the site-specific and published datasets (Figures 2 and 3) as the distribution of residuals between datasets was not significantly different for the bicarbonate model, but was for the pH models (two sample *t*-test; SYSTAT Ver. 13.2; Inpixon, 2017; see online supplementary material; Figure S5-3). These results are an indication that bicarbonate is supported as a Ni TMF in both the site-specific and published datasets. The bicarbonate model also had a higher R^2 (0.62) than both pH models (0.51–0.55), indicating that a greater proportion of the variation in EC50s was explained by selecting bicarbonate over pH (Figure 1). The bicarbonate model showed a better fit than the pH models by having similar or higher AF2 and lower MAE for site-specific data and all data combined (Figure 1) and no discernible residual slopes (Figure 2). Variance inflation factors were low for all models (≤1.9; Table 3), although slightly higher for the bicarbonate model than the pH model, indicating that variance of estimated slopes was not unduly inflated by collinearity with the other TMFs in each model. Both iterations of our pH model performed similarly for R^2, MAE, and residuals analysis, but the model with fitted $\beta_{Hardness}$ had higher AF2 than the model with fixed $\beta_{Hardness}$ (Figure 1; Table 3). As discussed above, the pH model with fixed $\beta_{Hardness}$ provided estimates of TMF slopes that were more comparable to the other models.

Residuals tended to become more positive with increasing observed EC50, indicating a systematic bias in all models (Figures 2 and 3; see online supplementary material; Figure S5-6 for pH model with fitted $\beta_{Hardness}$). All models tended to underpredict toxicity for the lowest observed EC50s (i.e., high Ni bioavailability conditions) and overpredict toxicity at the highest EC50s (i.e., low Ni bioavailability conditions), indicating that these models do not explain all of the variance in the datasets and patterns may be influenced by other factors. In a study that reported the same kind of residual plots, a similar pattern has been observed for other MLR models (Peters et al., 2021). We examined the residuals in several ways to try to identify potential reasons for this bias. There were no apparent patterns between residuals and the modeled TMFs, suggesting they are not influencing the residual pattern (Figures 2 and 3), although the pH model sometimes gave slightly different residual slopes for site-specific versus published data. Similarly, there were no patterns for the other candidate TMFs evaluated in the initial MLR analyses or for TMF combinations (e.g., hardness: alkalinity ratio), suggesting that none of these TMFs were missing from the models (see online supplementary material; Figures S5-4 to 5-7). We also evaluated the bicarbonate model with interactions that was not carried forward and observed the same residual pattern for observed EC50, which suggests that an interaction term would not have reduced the unexplained variance (see online supplementary material; Figure S5-5). The residuals for most individual studies in our data compilation showed a similar systematic pattern with observed EC50, suggesting that no particular dataset was driving the overall pattern (see online supplementary material; Figure S5-8). Overall, there was no apparent reason for the systematic bias in our models. Perhaps alternative methods for fitting models (e.g., nonlinear, other TMF transformations) might capture the variance in observed EC50s at either end of the dataset. For the purpose of this study, we retained the current form of the models for simplicity of describing the TMF effects.

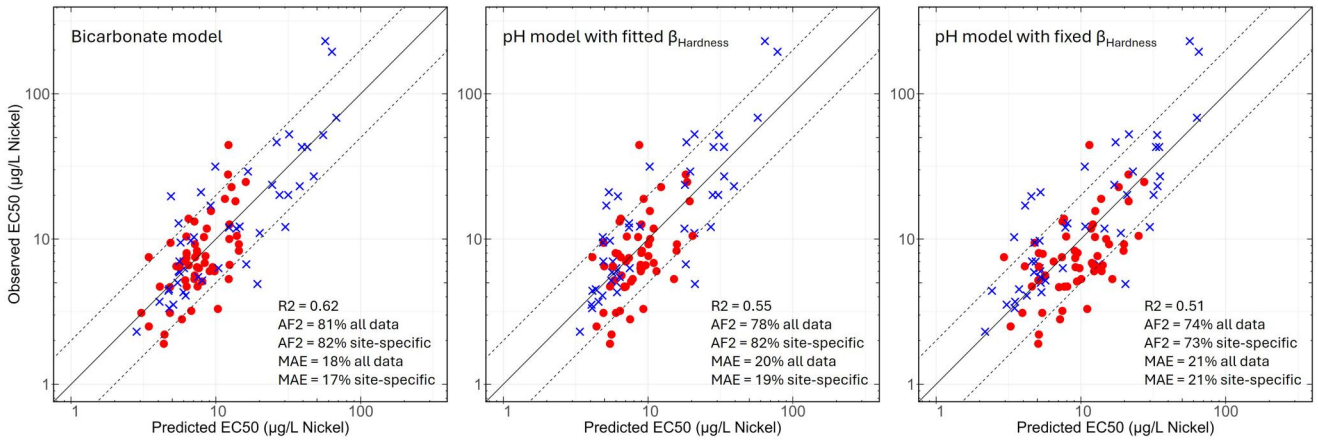

Figure 1. Comparative 1:1 plots of observed and predicted 50% effect concentrations (EC50s) for *Ceriodaphnia dubia* using the bicarbonate model, pH model with fitted hardness slope ($\beta_{Hardness}$), and pH model with fixed $\beta_{Hardness}$, showing the site-specific (red circle) and published (blue x) dataset. Solid line is line of perfect agreement between predicted and observed EC50s. Dashed lines indicate ±a factor of 2. AF2= agreement factor 2; MAE=Mean absolute error.

Table 3. Summary of performance metrics used to evaluate the *Ceriodaphnia dubia* bicarbonate and pH Ni toxicity models.

Metric	Bicarbonate model		pH model with fitted $\beta_{Hardness}$		pH model with fixed $\beta_{Hardness}$	
	Site-specific data	All data	Site-specific data	All data	Site-specific data	All data
Agreement factor 2 (%)	82	81	82	78	73	74
Mean absolute error (%)	17	18	19	20	21	21
AIC	3.3		21		na	
BIC	16		34		na	
Model adjusted R^2	0.62		0.55		na	
Mean corrected R^2	0.64		na		0.51	
Variance inflation factor	1.1 (DOC) 1.9 (Hardness) 1.9 (Bicarbonate)		1.2 (DOC) 1.3 (Hardness) 1.5 (pH)		na	

Note. AIC = Akaike information criteria, BIC = Bayesian information criteria; $\beta_{Hardness}$ = hardness slope; DOC = dissolved organic carbon; na = metric not available based on the SYSTAT module used to fit the model.

Model autovalidation

We evaluated model stability by characterizing consistency in model parameter selection and variability in parameter coefficients and performance metrics across the five cross-validation models compared with the model based on all data (Table 4 and see online supplementary material; Table S5-5). Parameter selection was consistent, with DOC, hardness, and either bicarbonate or pH selected as TMFs in all cross-validation models. All slope and intercept coefficients for the *k*-fold bicarbonate and pH models were within the model 95% confidence interval for the full dataset, indicating consistency among model runs. Similarly, the average performance metrics across the five cross-validation models were similar to the full dataset, suggesting no discernable difference in model performance depending on the portion of data used (Table 4).

Variability in DOC slopes was low across the five cross-validation models for both the bicarbonate and pH models (CV = 7.2%–8.2%; Table 4). Variability in hardness slopes was higher than for DOC, particularly for the pH model with fitted $\beta_{Hardness}$ (CV = 29%) compared to the bicarbonate model (CV = 17%). This suggests that the pH model had more difficulty correctly attributing TMF-related variance consistent with our earlier suggestion. There was lower variability in the bicarbonate slope (CV = 7.5%) compared to pH slopes (CV = 18%–24%) and using a fixed $\beta_{Hardness}$ resulted in a lower CV (18%) for the pH slope. These differences in CVs highlight some of the increased variability and uncertainty in modeling the pH effect on Ni toxicity compared to other TMFs. Coefficients of variation for model intercepts were similar across models, ranging from 11% for the bicarbonate model to 19% for the pH models. Despite the higher CV for some TMF slopes, performance metrics for the five cross-validation models did not differ by more than 14% from the full dataset for either the bicarbonate or pH models.

Predicted EC50s were relatively consistent across the TMF range between the five cross-validation models and with the model based on all data (see online supplementary material; Figure S5-9). Predicted EC50s for one or two individual k models began to deviate from the other models under TMF conditions that were predicted to decrease Ni bioavailability (higher hardness and DOC, lower bicarbonate and pH).

Evidence for bicarbonate and pH as TMFs for Ni effects in invertebrates

From the review of chronic Ni toxicity data, there were seven invertebrate species with sufficient data to evaluate bicarbonate versus pH effects on Ni toxicity using EC50s partially normalized to hardness and DOC (site-specific *C. dubia* testing plus 21 published studies; see Supplementary material S6). Two of these species, *C. dubia* and *Neocloeon triangulifer*, showed negative slopes with both bicarbonate and pH (Figure 4). For *C. dubia*, this negative relationship is consistent with observations in our MLR models and pH effects to *C. dubia* observed elsewhere (Croteau et al., 2021; ECHA, 2008; Santore et al., 2021). For *N. triangulifer*, this negative relationship is consistent with the analysis by Besser et al. (2021) that showed similar sensitivity to Ni TMF effects as *C.*

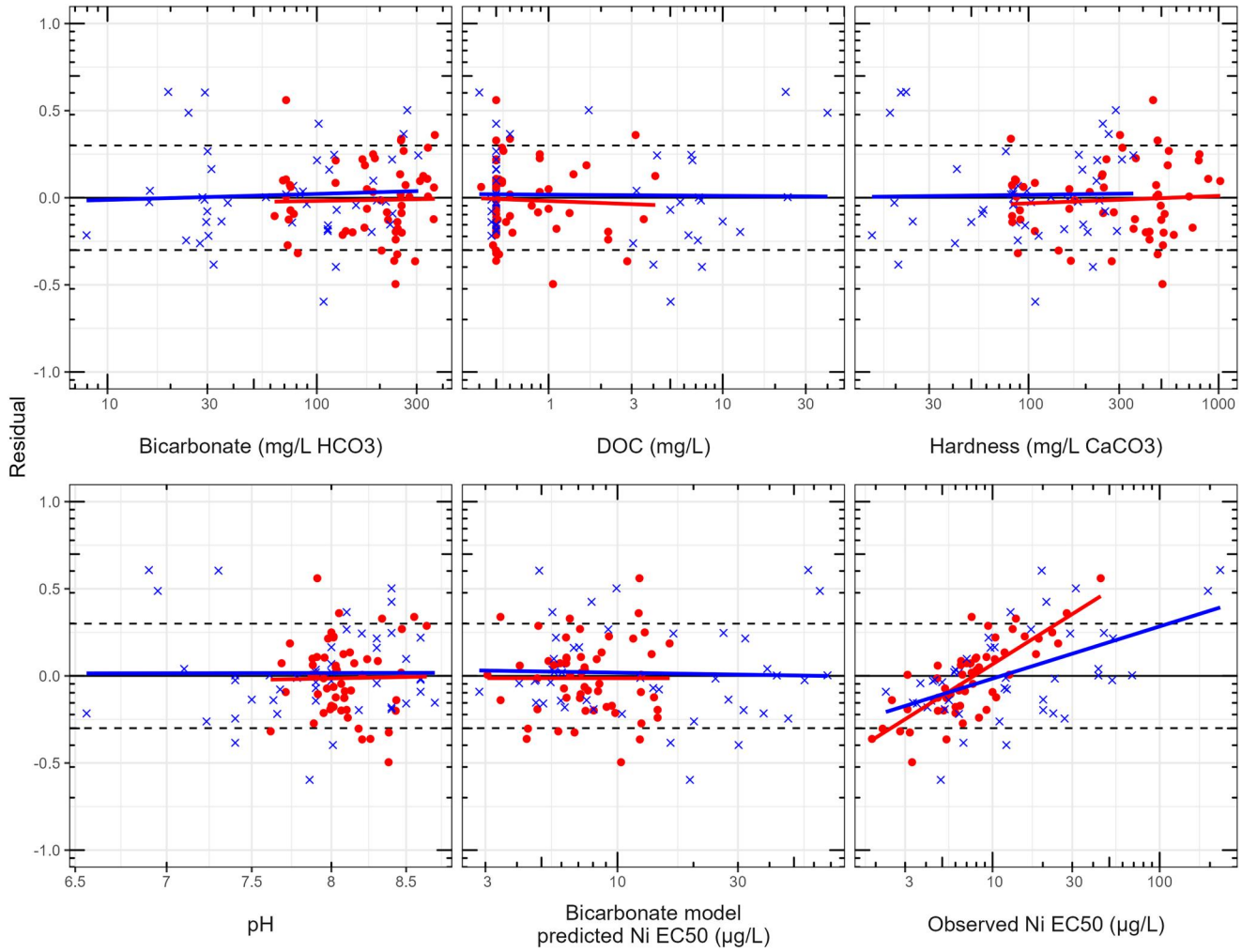

Figure 2. Residuals $\log_{10}$(observed EC50)–$\log_{10}$(predicted 50% effect concentration [EC50]) plotted against toxicity modifying factors and predicted and observed EC50s for the *Ceriodaphnia dubia* bicarbonate model showing the site-specific (red circle) and published (blue x) datasets. Dashed lines indicate residuals within ±a factor of 2. DOC=Dissolved organic carbon.

dubia. The analysis for *N. triangulifer* was not particularly robust given the small dataset ($n=7$) with few bicarbonate and pH conditions tested and apparent sensitivity to the assumptions of this analysis. An analysis of covariance (ANCOVA) of *C. dubia* and *N. triangulifer* data partially normalized with the hardness and DOC coefficients of the pH model found a significant main effect of pH (r^2=0.56; $F_{1,104}$=41; $p<0.001$) but no effect of species ($F_{1,104}$=2.4; $p=0.121$) and no interaction between species and pH ($F_{1,104}$=0.71; $p=0.402$; SYSTAT Ver. 13.2; Inpixon, 2017). The ANCOVA of data partially normalized with the hardness and DOC coefficients of the bicarbonate model found a significant main effect of bicarbonate (r^2=0.63; $F_{1,104}$=43; $p<0.001$) and species ($F_{1,104}$=14; $p<0.001$) but no interaction ($F_{1,104}$=2.7; $p=0.102$). These TMF effects in *C. dubia* and *N. triangulifer* were generally clearer and more consistent for bicarbonate compared to pH in that there were steeper log-scale slopes, more homoscedastic residuals, and higher adjusted R^2 (Table 5).

The remaining species did not show the same pattern of TMF effects evident for *C. dubia* and *N. triangulifer*. *Lymnaea stagnalis* showed a negative but nonsignificant slope with bicarbonate, but did not show a negative slope with pH (Figure 4). The other four species, *Brachionus calyciflorus*, *Chironomus dilutus*, *D. magna*, and *Hydra viridissima*, did not show a negative slope with either bicarbonate or pH (Figure 4). For *D. magna*, the lack of relationship is consistent with observations that this species is less sensitive to TMF effects of pH or bicarbonate than *C. dubia* (ECHA, 2008; Nys et al., 2016a) and/or that pH is not a significant TMF for this species (Croteau et al., 2021; Santore et al., 2021). The slope patterns for *L. stagnalis* and *B. calyciflorus* are inconsistent with the conclusion of Nys et al. (2016a) that Ni toxicity to these species was higher (i.e., lower EC50) at higher pH (i.e., would show a negative slope). For *H. viridissima* and *C. dilutus*, we are not aware of previous analyses of TMF effects of bicarbonate or pH specific to these species. This analysis for multiple invertebrate species showed that when there is not an apparent bicarbonate TMF effect, there also is not an apparent pH TMF effect.

Previous studies have focused on pH as a Ni TMF, but pH and bicarbonate were correlated in many of those studies, which makes it difficult to determine which is the TMF. Observations of a nonlinear relationship with pH suggest an additional factor beyond the effect of pH on Ni speciation (i.e., via [Ni^{2+}]) and proton (H^+) competition (which is vanishingly small at pH >8) is influencing Ni toxicity (Deleebeeck et al., 2008a,b; Nys et al., 2016a). Nys et al. (2016a) tested the effect of pH 8.2 versus 8.7 on chronic Ni toxicity to multiple invertebrate, plant, and algae species and reported that Ni toxicity was almost always significantly higher at pH 8.7. In that study, pH was adjusted using $NaHCO_3$ with equivalent measured bicarbonate concentrations of

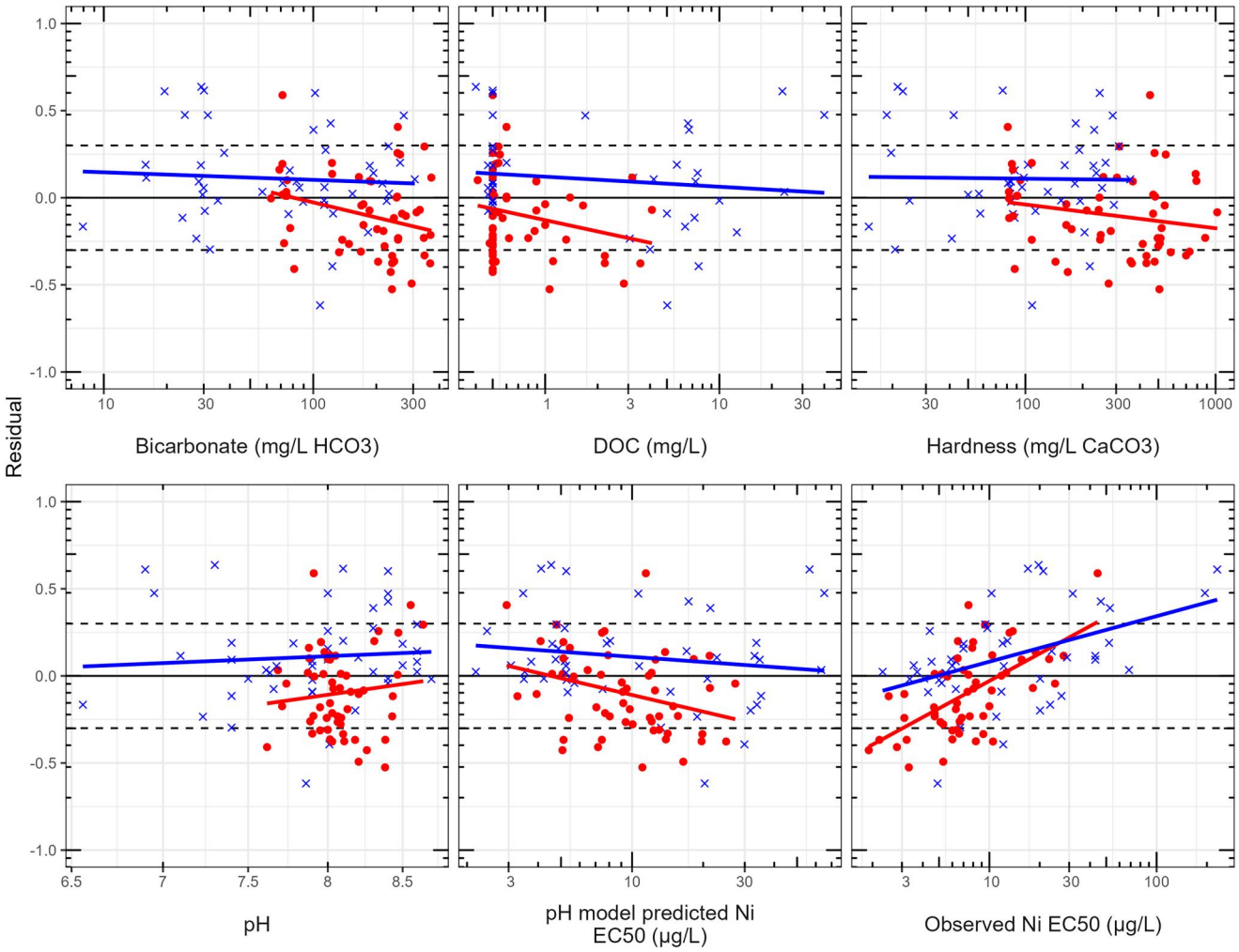

Figure 3. Residuals $\log_{10}$(observed 50% effect concentration [EC50])–$\log_{10}$(predicted EC50) plotted against toxicity modifying factors and predicted and observed EC50s for the *Ceriodaphnia dubia* pH model with fixed $\beta_{Hardness}$ showing the site-specific (red circle) and published (blue x) datasets. Dashed lines indicate residuals within ±a factor of 2. DOC=Dissolved organic carbon.

Table 4. Summary of *k*-fold validation results for *Ceriodaphnia dubia* Ni multiple linear regression with hardness, dissolved organic carbon, and bicarbonate or pH.

Metric	Bicarbonate model				pH model with fitted $\beta_{Hardness}$				pH model with fixed $\beta_{Hardness}$			
	All data	mean	SD	CV (%)	All data	mean	SD	CV (%)	All data	mean	SD	CV (%)
Intercept	1.12	1.10	0.12	11	2.97	2.86	0.55	19	3.44	3.32	0.64	19
β_{DOC}	0.55	0.54	0.04	8.2	0.50	0.50	0.04	7.2	0.52	0.51	0.04	7.7
$\beta_{Hardness}$	0.41	0.41	0.07	17	0.22	0.22	0.06	29	0.47	–		
$\beta_{Bicarbonate}$ or β_{pH}	−0.52	−0.51	0.04	7.5	−0.31	−0.30	0.07	24	−0.44	−0.42	0.08	18
Model adjusted R^2	0.62	0.62	0.03	4.8	0.55	0.55	0.06	12	na			
Mean corrected R^2	0.64	0.65	0.02	3.4	na				0.51	0.51	0.07	14
AF2 (%)	81	81	7.6	9.4	78	74	6.5	8.8	74	73	8.8	12
MAE (%)	18	19	2.3	12	20	22	1.4	6.6	21	23	2.6	11

Note. AF2 = agreement factor; $\beta = \log_{10}$ slope; CV = coefficient of variation; DOC = dissolved organic carbon; MAE = mean absolute error; na = metric not available based on the SYSTAT module used to fit the model; SD = standard deviation. The output of all parameters from the five cross-validation models is provided in Table S5-5 (see online supplementary material).

approximately 110 and 200 mg/L. Their results suggested that the effect of pH (or bicarbonate) had a steeper slope above pH 8.2 and that this effect was consistent across several test species. Nys et al. (2016a) hypothesized that the increased toxicity was related to speciation and inorganic Ni complexes (e.g., $NiHCO_3$ and $NiCO_3$) which become more prevalent at higher pH and could be bioavailable and contribute to chronic Ni toxicity. Testing and speciation modeling by Puttaswamy and Liber (2012) also suggested a potential role of bicarbonate on increasing Ni toxicity to daphnids. Both studies acknowledged that the mechanism of how pH and/or bicarbonate influences Ni toxicity is not understood. While the Nys et al. (2016a) study showing a steeper slope at pH >8.2 was based on testing at two pH levels spanning 0.5 pH units, their observation that this effect was consistent across a range of test species from different taxa suggested a bioavailability effect on Ni toxicity consistent with bicarbonate as a TMF.

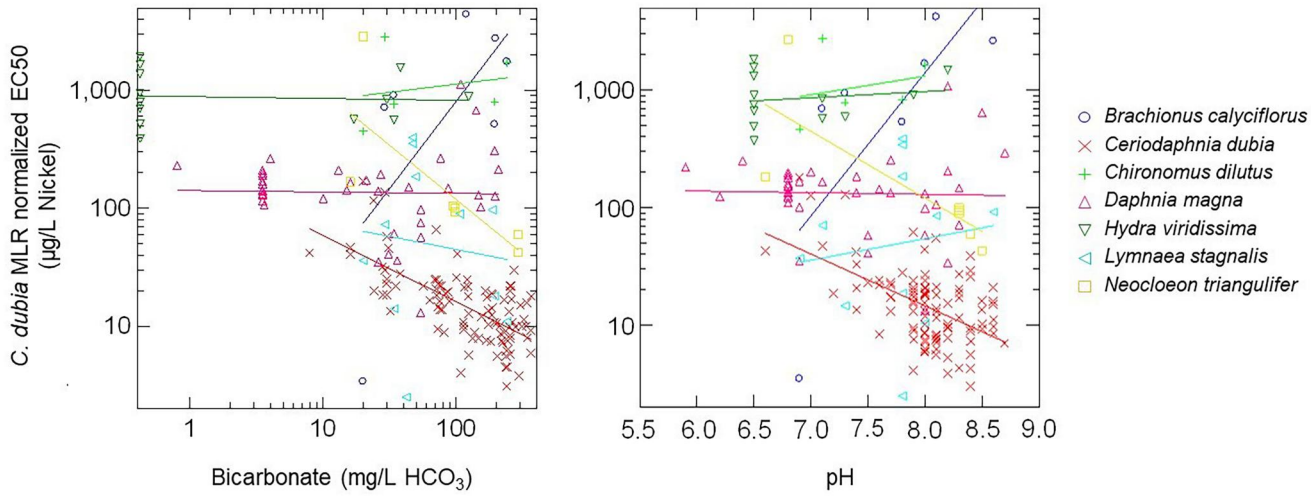

Figure 4. Relationship between 50% effect concentrations (EC50s) and bicarbonate or pH for invertebrates with sufficient data; *Brachionus calyciflorus* (blue circle), *Ceriodaphnia dubia* (red x), *C. dilutus* (light green plus), *Daphnia magna* (magenta triangle), *Hydra viridissima* (dark green triangle), *Lymnaea stagnalis* (cyan triangle), *Neocloeon triangulifer* (yellow square). EC50s were partially normalized to a common hardness (117 mg/L as $CaCO_3$) and dissolved organic carbon (1.0 mg/L) using slopes from our *C. dubia* bicarbonate or pH multiple linear regression models. Data for *B. calyciflorus* are EC20s.

Table 5. Summary of linear regression results for invertebrates with negative slopes between partially normalized effect concentration and both bicarbonate and pH.

Species	EC50 vs. bicarbonate		EC50 vs. pH	
	Slope	Adjusted R^2	Slope	Adjusted R^2
Ceriodaphnia dubia	−0.515	0.399	−0.442	0.274
Neocloeon triangulifer	−0.862	0.442	−0.577	0.515

Note. EC50 = 50% effect concentration.

Nys et al., (2025) also recently incorporated the effect of high pH into an acute Ni toxicity model for invertebrates for pH >8.0, and showed no significant effect up to pH 8.0. They noted that this differed from chronic Ni models for daphnids (Deleebeeck et al., 2008a; De Schamphelaere et al., 2006) where pH was a significant TMF across the entire pH range of the dataset, but had a steeper slope at pH >8.2. Our analysis suggests the TMF effect for *C. dubia* is log-linear over almost two orders of magnitude of bicarbonate concentrations and two pH units. In our *C. dubia* MLR analysis, bicarbonate explained more variance than pH and bicarbonate was always picked over pH in TMF selection and in model performance, supporting its role as a Ni TMF.

Comparison of Ni toxicity using multi-species models is complicated by differences in TMF effects among species. Santore et al. (2021) concluded that the effect of pH on chronic Ni toxicity is inconsistent across test species (fish, amphibians, invertebrates, plants, algae), with most species showing either no effect or increased sensitivity at high pH, and with *C. dubia* appearing to be more sensitive to Ni at high pH than other organisms. Previously, separate BLMs were developed for *C. dubia* and *D. magna* because of the higher intrinsic sensitivity of *C. dubia* and a steeper pH slope for *C. dubia* than *D. magna* (De Schamphelaere et al., 2006; ECHA, 2008; Nys et al., 2016a). Peters et al. (2023) recently showed that a *C. dubia* BLM modified for higher pH per Nys et al. (2016a) was more appropriate for normalizing Ni toxicity data for some invertebrates (*C. dilutus* formerly *C. tentans*, *L. stagnalis*, and *Daphnia lumholtzi*) whereas a *D. magna* BLM with a shallower pH slope was more appropriate for normalizing data for other invertebrates examined in their dataset (*B. calyciflorus*, *H. viridissima*, and other cladocerans). In our evaluation, Ni toxicity to *C. dubia* and *N. triangulifer* showed patterns with both pH and bicarbonate, but these patterns were not apparent for most of the other invertebrate species included in our evaluation. It is unclear what these differences among species indicate about the potential mechanisms for TMF effects of pH and/or bicarbonate, but they may suggest that the TMF effect is not just a pH-driven speciation mechanism (i.e., via $[Ni^{2+}]$) and H+ competition, as is generally thought to be the mechanism for how pH affects the bioavailability of other divalent metals.

Further support for a TMF effect of bicarbonate on Ni toxicity to *C. dubia* comes from results of chronic toxicity testing with bicarbonate (Figure 5) that indicate the bicarbonate ion itself is not toxic to *C. dubia* at the concentrations in our Ni dataset (maximum concentration of 366 mg/L HCO_3). Santore et al. (2021) developed a separate BLM for *C. dubia* that assumed $NiHCO_3$ complexes were bioavailable and found that model performance was significantly improved. However, the authors also suggested the high bicarbonate concentrations that occur at high pH may themselves be toxic and that the increased sensitivity of *C. dubia* at high pH is a potential mixture effect of Ni and bicarbonate. Bicarbonate toxicity can be challenging to measure and is complicated by solubility constraints of laboratory formulated waters and potential for precipitate formation at high concentrations. Our bicarbonate toxicity testing under site water conditions indicated that bicarbonate is not toxic at the concentrations in the *C. dubia* Ni dataset, with an EC10 greater than 500 mg/L (=8.2 mM) HCO_3 (effect concentrations and confidence limits [CL] provided in Table S7-2 [see online supplementary material]). Comparison to published bicarbonate toxicity data showed that tests appeared to separate into two groups (Figure 5; see online supplementary material 7), with tests run by Lasier and Hardin (2010) and Farag and Harper (2014) showing effects at lower concentrations than our site-specific testing and the results of Johnson (2014) and Mount et al., (2019). The reasons for these differences between tests are not known. Analysis of the pooled dataset estimated an EC10 of 471 mg/L (95% CL 427–520) (=7.7 mM [95% CL 7.0–8.5]) HCO_3, EC20 of 544 mg/L (95% CL 507–583; =8.9 mM [95% CL 8.3 = 9.6]) HCO_3, and EC50 of 694 mg/L (95% CL 663–727; =11.4 mM [95% CL 10.9–11.9]) HCO_3, which are higher than

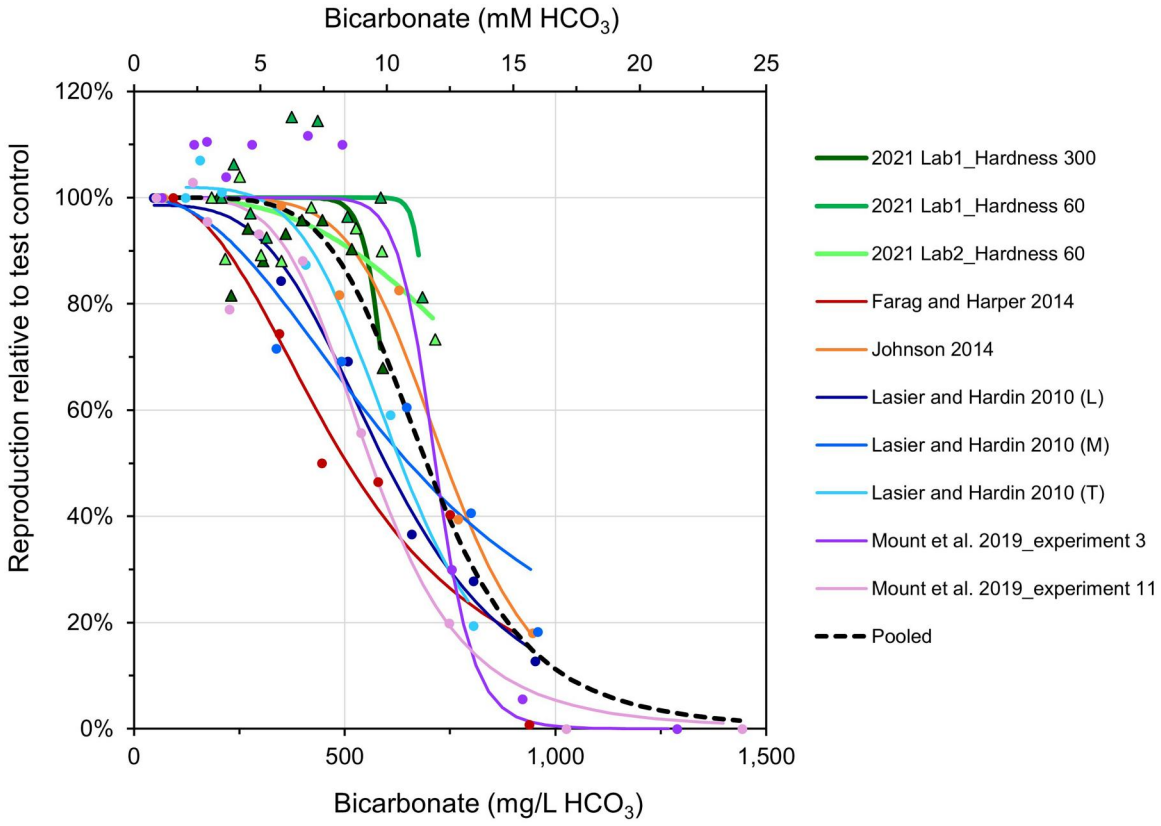

Figure 5. Chronic toxicity of bicarbonate to *Ceriodaphnia dubia* reproduction in site-specific (triangles) and published (circles) studies. Dashed line describes the pooled dataset. Refer to Supplementary material 7 or original studies for study descriptors.

the maximum concentration of 366 mg/L HCO_3 in the Ni dataset. The tests showing toxicity at lower concentrations could support an effect of bicarbonate and Ni in the *C. dubia* Ni dataset (most likely via independent action), but our bicarbonate testing and other published data support the interpretation that bicarbonate in the Ni tests is having a TMF effect, rather than being toxic, even if the mechanism of the TMF effect is not understood.

In addition to our analysis of nickel and bicarbonate interactions for chronic toxicity, the effect of high pH/bicarbonate on acute Ni toxicity to invertebrates discussed in Nys et al. (2025) also indicates that bicarbonate toxicity is not likely to be a driver in our *C. dubia* Ni dataset. Acute toxicity of bicarbonate to *C. dubia* has been reported over a wide range of concentrations (48-hr median lethal concentrations [LC50] of ~700–1,600 mg/L HCO_3; Mount et al., 2016), well above concentrations where bicarbonate is affecting acute Ni toxicity. Given these observations, we suggest the most parsimonious explanation is that bicarbonate is exerting some sort of bioavailability effect on Ni that applies to both acute and chronic Ni toxicity as would be expected.

Our analysis of other invertebrate species suggests that the bicarbonate TMF effect may not be unique to *C. dubia*. Most notably, *N. triangulifer* toxicity data also show changes in Ni sensitivity over a range of pH and bicarbonate conditions. Besser et al. (2021) found there was better fit of their Ni toxicity data for *N. triangulifer* when using the Santore et al. (2021) *C. dubia* BLM compared to a "best overall" BLM calibration (Schlekat et al., 2010) and suggested that *N. triangulifer* may also be more sensitive to bicarbonate and/or pH toxicity than other invertebrates examined in Santore et al. (2021). Our supporting analysis was in agreement with this observation by Besser et al. (2021) of improved fit using a *C. dubia* model, but we interpreted the response to be a TMF effect for reasons discussed above for *C. dubia*. A bicarbonate TMF effect was not apparent for other invertebrate species in the analysis, but given limitations in the size and range of bicarbonate/pH conditions in the datasets for some of those species, it is uncertain whether a bicarbonate TMF effect might be more general across all invertebrates. Taken together, these findings suggest that both BLM and MLR models for *C. dubia*, and possibly other species, could be improved by explicitly considering bicarbonate as a TMF.

Conclusion

Our findings support bicarbonate more strongly than pH as a TMF for Ni toxicity to *C. dubia*, which may explain why previous studies have reported inconsistent results for pH. However, the mechanisms for TMF effects of bicarbonate and/or pH on chronic Ni toxicity to *C. dubia* remain unclear. Similar TMF effects were apparent for some species (e.g., *N. triangulifer*) but not others (e.g., *D. magna*), and the reason for these differences is also unclear. More work is needed to understand both mechanisms for TMF effects and why TMF effects appear to be species-specific to reduce uncertainties associated with the collinearity of bicarbonate and pH in model datasets and in applying bioavailability models more generally across species. Bicarbonate and/or total alkalinity should be measured in future studies along with other TMFs to help characterize the potential influence of bicarbonate on Ni toxicity, as explicitly considering bicarbonate as a TMF may improve both BLM and MLR models for *C. dubia*, and possibly other invertebrate species.

Notwithstanding uncertainty about mechanisms, our MLR model with bicarbonate performed well for *C. dubia* across the expanded range of TMF conditions developed herein, including under conditions tested in previous studies, and is probably more

robust than previous Ni models under very high hardness and bicarbonate conditions.

Supplementary material

Supplementary material is available online at *Environmental Toxicology and Chemistry*.

Data availability

The data underlying this article are available in the article and in its online supplementary material.

Author contributions

Jordana L. Van Geest (Data curation, Investigation, Methodology, Writing—original draft, Writing—review & editing), Jennifer M. Daley (Formal analysis, Investigation, Methodology, Validation, Visualization, Writing—original draft), James Richard Elphick (Investigation, Methodology), Melanie Gallant (Investigation, Methodology), Kevin V. Brix (Investigation, Methodology, Writing—review & editing), Nick Manklow (Project administration), Mariah Arnold (Funding acquisition, Project administration, Writing—review & editing), and Mark Digel (Investigation), and Adrian M.H. de Bruyn (Conceptualization, Formal analysis, Investigation, Methodology, Supervision, Writing—original draft, Writing—review & editing)

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Conflicts of interest

J.L.V.G., J.M.D., J.R.E., M.G., K.V.B., and A.M.H.D.B. have no competing interests beyond receipt of funding from EVR to conduct the studies reported herein. N.A.M., M.C.A., and M.D. are employed by EVR.

Disclaimer

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