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CHRONIC TOXICITY OF MAJOR ION SALTS AND THEIR MIXTURES TO *CERIODAPHNIA DUBIA*

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Abstract

The toxicity of waters with sufficiently elevated concentrations of major geochemical ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , and $\text{HCO}_3^-/\text{CO}_3^{2-}$) to aquatic life is well documented, though the majority of such studies have focused on acute toxicity. In previous work we intensively studied the acute responses of the cladoceran, *Ceriodaphnia dubia*, to major ion salts and their mixtures, culminating in the development of models to predict acute toxicity from ionic composition. To quantitatively evaluate whether the toxicological behavior of major ions observed for *C. dubia* extends to chronic toxicity, we conducted 60 chronic toxicity tests with individual salts and binary mixtures thereof. Chronic responses paralleled those demonstrated previously for acute exposure, specifically: 1) similar relative toxicity of individual salts; 2) different Na salts showing similar potency when exposure is expressed as osmolarity; 3) toxicity of Mg, Ca, and K salts related to cation activity; 4) decreased toxicity of Na and Mg salts when Ca activity is increased at less than toxic concentrations; 5) additive behavior for salt mixtures sharing a common cation; 6) independent behavior for salt mixtures with dissimilar cations, except Mg/Ca mixtures which appeared additive; and 7) expressing exposure on the basis of chemical activity rather than total concentration improved coherence of data across different ion mixtures. Acute-chronic ratios were fairly consistent among salts, with values around 1.8 for acute LC50:chronic EC50, and 2.8 for LC50/EC20 when expressed on an activity basis. Adjusting the previous acute toxicity model for acute-chronic ratios yielded chronic models that predict chronic toxicity within the range of inter-test variability. As these models are informed by a wide range of ion mixtures, they should provide robust assessment tools for waters with a variety of ionic compositions.

Keywords

Aquatic toxicology; Major ions; *Ceriodaphnia dubia*; Toxicity mechanisms; Exposure-response modeling

INTRODUCTION

A variety of anthropogenic activities can increase the concentrations of major geochemical ions (Na^+ , K^+ , Ca^{2+} , Mg^{2+} , Cl^- , SO_4^{2-} , and $\text{HCO}_3^-/\text{CO}_3^{2-}$) in surface waters, and where this enrichment is excessive, adverse biological effects can result [Boelter et al. 1992;

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Dickerson et al. 1996; Kennedy et al. 2003; Pond et al. 2008]. Developing tools to characterize the toxicity of major ions to aquatic organisms can be challenging, because these ions co-occur in a wide range of relative proportions, and the toxicity of these mixtures has been shown to vary as a function of both composition and concentration [Mount et al. 1997, 2016; Erickson et al. 2017].

In previous work, we examined the acute toxicity of major ions to the cladoceran *Ceriodaphnia dubia* with an emphasis on the influence of the dilution water chemistry [Mount et al. 2016] and the interactive toxicity of ion salt mixtures [Erickson et al. 2017]. This work provided several important insights into the toxicology of major ion mixtures to *C. dubia*: 1) toxicity is better expressed based on chemical activities than on concentrations; 2) the toxicities of Mg, Ca, and K salts are primarily attributable to the cation; 3) both the cation and anion contribute to the toxicity of Na salts, which is best expressed based on solution osmolality; 4) Ca is the primary characteristic of the dilution water that influences toxicity of Na and Mg salts; 5) salts sharing a common cation show additive toxicity; 6) toxicities of Mg and Ca are additive, but both are independent of K toxicity; 7) mixtures of Mg or K salts with Na salts show independent action when expressed as cation (Mg, K) activity versus osmolality (to which all the salts contribute), and 8) mixtures of Na salts with Ca salts show toxicity intermediate between additive and independent action. These principles formed the foundation for a mathematical model that was effective in predicting the acute response of *C. dubia* to ion mixtures mimicking ion-enriched field waters reported in the literature [Erickson et al. 2018].

In part because they are comparatively fast and simple to conduct, acute toxicity tests are often used in environmental toxicology as an initial assessment of the potential hazard of aquatic pollutants, and have also been used to explore the interactions of toxicants in mixtures, or in relation to dilution water characteristics that can influence the bioavailability or toxicity of chemicals in water [e.g., Thurston et al. 1981; Santore et al. 2001]. However, most environmental risk assessments are concerned not just with the lethal effects of short-term exposures, but also the lethal or sub-lethal effects under longer term exposure. Because chronic toxicity tests are much more resource intensive, prediction of chronic toxicity often relies on extrapolation from acute effect concentrations. An example is the acute-chronic ratio (ACR; ratio of acute effect concentration to chronic effect concentration [Kenaga 1982]) which is sometimes applied to acute toxicity data to estimate a likely chronic effect concentration for the same or other species, as in the development of U.S. water quality criteria [Stephan et al. 1985]. When applied in the context of a toxicant mixture, the ACR approach carries an implicit assumption that the nature of toxicant interactions, both among mixture constituents and between those constituents and the background water, are the same for acute and chronic toxicity, or at least sufficiently so for the goals of the assessment.

The purpose of the present study was to compare the chronic toxicity of major ions to *C. dubia* with the acute responses assessed previously [Mount et al. 2016; Erickson et al. 2017]. This included not only responses to individual ion salts, but also those to binary mixtures of salts, in combinations designed to evaluate the interaction of different ions within complex mixtures. As a final step, these data were used to re-parameterize the acute toxicity models proposed by [Erickson et al. 2018] to predict chronic response.

METHODS AND MATERIALS

Overview of study design

Thirteen experiments were conducted, each consisting of 2 to 5 chronic toxicity tests structured to evaluate different aspects of major ion toxicity (Table 1) using an experimental approach similar to that in previous acute testing with *C. dubia* [Mount et al. 2016, Erickson et al. 2017]. Individual experiments were structured to evaluate 1) the effect of renewing solutions only on days 3 and 5, rather than daily (Experiment 1); 2) the chronic toxicity of individual major ion salts (Experiments 1–3); 3) how Ca concentration affects the toxicity of NaCl and MgCl₂ (Experiments 4–5); and 4) the interactive chronic toxicity of binary mixtures of major ion salts (Experiments 6–12). A last experiment (13) compared the toxicity of NaCl and MgCl₂ to that of mannitol, Na gluconate, and Mg gluconate.

Preparation of Test Waters

Test waters were prepared from sand filtered and UV-treated Lake Superior water, withdrawn offshore from our laboratory at 46.840° N, 92.004° W. This water was supplemented with Na₂SO₄, NaCl, KCl, CaCl₂·2H₂O and MgCl₂·6H₂O to bring it to a chemistry matching an average North American surface water with hardness of 53 mg CaCO₃/L (see Mount et al. [2016] for more information); this water was the primary control/dilution water used and is called amended Lake Superior water (ALSW; Table 2). In some experiments, variations of ALSW were used that had lower or higher Ca concentrations. The 1/3X Ca water (Table 2) was prepared by diluting Lake Superior water with de-ionized water then adding salts to bring concentrations of other ions back to those in ALSW, with the exception of Na, which was increased to match the decreased equivalents of Ca. Waters with Ca higher than ALSW were prepared by adding appropriate amounts of CaCl₂·2H₂O. All ion salts, gluconate salts, and mannitol were obtained from Sigma-Aldrich Chemical Company or Fisher Scientific, and were ACS reagent grade or better, except for MgCO₃ which was specified as USP quality. The MgCO₃ salt contained some Ca; the certificate of analysis was used to determine the nominal concentration of Mg and the incidental addition of Ca.

Toxicity test procedures

Toxicity test procedures were patterned after the standard chronic method for *C. dubia* described by U.S. EPA [USEPA 2002] with three primary modifications: 1) test solutions were renewed only on days 3 and 5 of the test to reduce the level of effort for conducting multiple simultaneous tests (as justified by Experiment 1 – see Results); 2) the number of replicates per treatment was reduced from 10 to 6; and 3) 9 closely spaced treatment concentrations were used to better support regression analysis of fairly steep response curves (100%, 70%, 50%, 35%, 25%, 17.5%, 12.5%, 8.75%, and 6.25% for all but Experiment 1, which used a 0.8x dilution factor).

Test solutions were prepared from an initial solution consisting of ALSW combined with salt(s) to equal the highest (100%) test concentration; to speed dissolution of MgCO₃ salt, CO₂ gas was bubbled through the solution until the salt dissolved. From the 100% solutions, the remaining concentrations were prepared by diluting proportionally with control water.

Because some solutions enriched with NaHCO_3 or MgCO_3 exceeded theoretical CaCO_3 saturation, test solutions for these salts were prepared 4 days in advance and held under gentle aeration, to permit CaCO_3 precipitation and stabilize pH. Immediately before testing, evaporative losses (from the aeration) were restored by adding DIW to bring each solution back to its original mass.

Test organisms were cultured in-house in ALSW using the individual female method described in [USEPA 2002]. Only broods from females producing at least 10 neonates were used, and these neonates were assigned to treatments using a block method (one brood used to stock the same replicate in every treatment). To minimize variability among experiments, all tests were started using neonates that were less than 8-h-old, which is more restrictive than the <24-h-old (within 8-h range) specification in the standard method.

Toxicity tests were conducted in 30-ml plastic cups (Berry Plastics Corporation) containing 15 ml test solution, held in polystyrene boards, and covered with a sheet of glass. Temperature control came from floating test boards in a temperature-controlled water bath fitted with continuous monitoring and alarms. Mean test temperature (measured daily in test cups) across experiments was 24.6°C with individual test means ranging from 24.3 to 24.8°C ($n=60$), and a spread of no more than 2°C across treatments or days. Food was added to each cup daily in the form of 100 μL each of YCT (a mixture of yeast, cereal leaves, and trout chow) and algae (*Pseudokirchneriella subcapitata* at 3.5×10^7 cells/mL)[USEPA 2002]. Mortality was observed and recorded daily.

Test solutions were renewed only on days 3 and 5 of the test, except for two tests in Experiment 1 that used daily renewal. During solution renewal, adult organisms were transferred by eye dropper to new cups containing fresh test solution, and any neonates produced prior to transfer were counted. On day 7, the experiments were terminated with final recording of survival and neonate production. Technically, the *C. dubia* chronic is a three-brood test rather than a 7-day test (ended when three broods are produced in controls), but by tightly controlling the starting age of organisms, the third brood consistently coincided with the 7th day.

Water chemistry monitoring

Conductivity was measured (Model 2052, Amber Science, Inc.) in every treatment at the start of each test to confirm that the concentration series was prepared properly. In addition, conductivity was measured in new solutions on days 3 and 5 and in old solutions on days 3, 5, and 7 in three treatments representing the lower, middle, and upper thirds of the concentration range. Dissolved oxygen (YSI model #58; Yellow Springs Instruments) and pH (meter model PH150 with A57198 probe; Beckman Coulter, Inc.) were measured in the highest concentration and control at the start of every test, and in old solutions of three treatments on days 3, 5, and 7. Dissolved oxygen averaged 8.3 mg/L with a total range in individual measurements across all tests of 7.5 and 9.3 mg/L. Excluding tests with elevated NaHCO_3 or MgCO_3 , pH averaged 7.8 with a total range of 7.3 to 8.0. For tests with NaHCO_3 or MgCO_3 , additional pH measurements were made in every treatment at the start of the test; in these tests, pH rose with increasing concentration, from 8.4 to 8.7 in the lower concentrations, to around 9 near the EC50, and 9.2–9.4 in the highest concentrations.

Hardness and alkalinity (means in ALSW of 53.9 ± 1.1 and 44.4 ± 1.6 mg/L as CaCO_3 , respectively) were measured by titration [APHA 1998] in an aliquot of dilution water from every test.

Exposure concentration monitoring

In addition to using conductivity to monitor exposure concentrations, concentrations of Na, K, Ca, Mg, Cl, and SO_4 were confirmed analytically in a subset of test solutions. Cations were analyzed using flame atomic absorption spectroscopy (Agilent 240 FS; Agilent Technologies) and anions by ion chromatography (Dionex DX600 or Dionex ICS 5000+DP; Thermo-Fisher Scientific). Each run included a blank, a series of 5–8 calibration standards, a quality control standard, and an independent calibration standard included at both the beginning and end of the run. Replicate analyses were run on 10% of all samples.

At the start of each test, both Cl and SO_4 concentrations were determined in samples from the control and highest test concentration. All four cations were measured in the control sample from every test. In addition, the concentration of the cations from the added salt(s) (e.g., Na and Mg for a mixture test with NaCl and MgCl_2) were measured in the 100% and 35% treatments at the start of the test, and in an intermediate concentration (35% or the treatment judged closest to the apparent EC20) in new solutions on days 3 and 5 and old solutions on days 3, 5, and 7. In tests with elevated NaHCO_3 or MgCO_3 , additional samples were collected to document changes in dissolved Ca concentrations resulting from CaCO_3 precipitation; these included initial samples from the 6 highest treatment concentrations, and new (day 3 and 5) and old (day 3, 5, and 7) old solutions from the three highest concentrations with surviving organisms. All samples for chemical analysis were immediately filtered (0.45 μm nylon syringe filter, Grainger); aliquots for anion analysis were refrigerated, and those for cation analysis were held at room temperature after acidification (0.2% (v/v) concentrated HNO_3 ; more in NaHCO_3 and MgCO_3 experiments as necessary to overcome elevated alkalinity).

Except for Ca concentrations in solutions with elevated NaHCO_3 or MgCO_3 , measured cation concentrations across all test solutions (other than controls) averaged 99.2% of the nominal, with 5th and 95th percentiles of 90.8% and 107.6% ($n=416$). In control solutions, measured ion concentrations (cations and anions combined) also averaged 99.2% of nominal, with 5th and 95th percentiles of 84.8% and 109.7% ($n=94$). In the small number of cases (<1%) where measured concentrations fell outside 80–120% of nominal, measurements of other ions and conductivity measurements were evaluated to determine if there was any further evidence of errors in solution preparation; in no case was such evidence found, indicating that these larger deviations were likely the result of other sources of error. Accordingly, we based all data analysis on the nominal concentrations of the tested solutions. The exception was Ca concentrations in tests with NaHCO_3 or MgCO_3 , in which Ca precipitation was expected, along with minor contamination of Ca in the MgCO_3 salt. Reported Ca concentrations in these tests were based on the more intensive Ca measurements included in those studies.

Exposure-Response analysis

Effects endpoint selection.—In all cases, reproduction per female was a more sensitive endpoint than survival, so all effect endpoints presented are based on reproduction. Further, we decided for several reasons to base our analysis on reproduction of surviving females rather than young per original female. For one, our interest was in determining whether sublethal response to major ion exposure was governed by similar principles as those governing lethality, and were therefore interested in excluding the influence of parental mortality from the chronic effect assessment. In addition, expressing reproduction as young per original female distorts the data distribution in ways that violate statistical assumptions (e.g., zero reproduction for an individual that dies before reproducing, when the death is unrelated to exposure and other replicates have substantial reproduction). Finally, because there were only 6 replicates, a spurious death would exert more influence on the apparent reproductive output and corresponding analysis. However, we did conduct comparative analyses and found that EC20 and EC50 values based on young per original female were very close to those calculated for young per surviving female, with average differences of only +2% and –7% percent for EC20 and EC50 values, respectively.

The primary data interpretations provided are based on EC50 values, because these values should have less uncertainty than ECs for lower levels of effects, and therefore show more clearly the interactions among ions. However, because lower levels of effect are often of greater interest for risk assessment and regulatory purposes, EC20s were also calculated and are reported in the supplemental material.

Shape of exposure-effects curve.—Most exposure-effects analyses employ a symmetric function (e.g. logistic), with equal rounding at the upper and lower shoulders of the response curve. However, initial inspection of data from the present study suggested that many of the response curves had some asymmetry, showing a broader shoulder at low levels of effect and a sharper transition as they approached zero reproduction. The result of such asymmetry would be to bias EC20 estimates high if data at high effect levels were given equal weight to that at low effect levels in determining the shape of the curve. To more rigorously assess the shape of the response curves, a preliminary meta-analysis was performed across all tests conducted with NaCl (n=7) and MgCl₂ (n=7) in ALSW. For each salt, a logistic equation for young per surviving female was fitted to all treatments and replicates:

$$R = \frac{R_0}{1 + e^{4S(\log C - \log EC50)}}$$

where R is reproduction (number of offspring from a surviving female), C is exposure concentration (mM salt added), and the parameters are the control reproduction R₀, the EC50, and the relative slope S (= d(R/R₀)/dlogC) at the EC50. These analyses were conducted by maximum likelihood analysis using custom software written with Intel Professional Fortran Composer XE 2015 and Winteracter Version 9.2. The complex method of Box [1965] was used for determining the parameter values that maximize the likelihood

for the observed data. Confidence limits for parameter estimates were computed by iterative searches using the likelihood-ratio method [Williams 1986]. For these preliminary analyses, the variability of the offspring count among replicates was assumed to follow a Poisson distribution.

To compare shapes across multiple tests, the data were normalized by dividing the mean R for each treatment by the estimated R_0 for the test and dividing C by the estimated EC_{50} . These normalized data are shown in Figure 1A for NaCl ($n=7$ tests) and in Figure 1B for $MgCl_2$ ($n=7$). For both chemicals, the shape of the relationship is more rounded at low effects than at high effects. This suggested we should either 1) use an asymmetric relationship (adding an additional model parameter); or 2) censor data at high concentrations so that, to the extent possible, the curvature at low concentrations is influenced primarily by data in that part of the curve. Because the asymmetric analysis required more parameters, the latter option (censoring) was chosen (details under *Final analysis method*).

Statistical distribution for replicate variability.—The tests summarized in Figure 1 were also used to address the nature of the variability among replicates, specifically the assumption of a Poisson distribution. For each test and for each treatment, the mean and standard deviation of offspring per surviving female across the replicates were calculated. These individual standard deviations were then pooled for several ranges of reproductive output (mean = 0, >0–5, 5–10, 10–15, 15–20, 20–25, 25–30, and >30). These pooled means and standard deviations are plotted in the inset graphs in Figures 1A and 1B. Also plotted is a line for the expected standard deviation if the assumption of Poisson variability applies. The standard deviations stray from Poisson expectations, being roughly constant except at reproduction below 5 young per female, where they are close to Poisson.

Final analysis method.—The final exposure-effects analyses were as described for the preliminary analysis with two modifications: 1) using a fitted, constant standard deviation when reproduction was >5 per surviving female, with the Poisson assumption still used at lower values, and 2) censoring data at high effects levels. The core censoring rule was to include all treatments up through the first with reproduction less than 50% of control, provided this resulted in at least 3 partial effects defining the shape for the curve near and below the EC_{50} . If this requirement was not met, additional treatments with more than 50% reduction were added to provide more partial effects. In the end, this censoring had little effect on EC_{50} estimation; for 42 tests in which some treatments were censored, the differences between the censored and uncensored EC_{50} estimates averaged <1% and never exceeded 10%. However for EC_{20} s, the expected positive bias of the uncensored estimates was observed; although this only averaged 4%, it was as much as 23% and would be even higher when estimating lower effect concentrations (e.g., EC_{10}).

For 3 of the 60 tests, the highest treatment with surviving females only showed a 20–30% reduction in reproduction, providing insufficient information to fit a curve, and necessitating including some data from non-surviving females at higher concentrations. In these cases, a single replicate from the next higher treatment for the non-surviving female having the highest number of offspring was included in the analysis. This provided an “anchor” at higher effects for the response curve with the minimum possible inclusion of data from non-

surviving females, but entails the assumption that reproduction would have been similar if the female had survived. In 2 other tests, there was only 30% reduction in reproduction at the highest concentration tested, and using these data to extrapolate beyond the highest treatment was similarly inappropriate. In these cases, the analyses used the slope (S) estimated for the same chemical in another test to provide a point estimate for the EC₅₀, but the confidence limits on the EC₅₀ still reflect the full uncertainty of the data without this slope constraint.

Finally, because organisms were assigned to test replicates via “blocking by known parentage”, culture broods that performed anomalously (high mortality rate or consistently low reproduction across treatments) were excluded from data analyses, consistent with standard methodology [U.S. EPA 2002]. Of the 360 broods used in our experiments, 4 (1.1%) were censored for this reason. Replicates were also excluded from data analysis when organisms were identified as male or as being extreme outliers from the other replicates at the $p=0.0001$ significance level ($n=16$, 0.4% of the 3600 total replicates).

Evaluating interactions among ions

For the final exposure effects analyses, exposure was expressed as the percentage of the highest tested concentration, whether single salt or mixture test. EC₅₀s and EC₂₀s based on percentage were converted to concentrations of added salt (“added-salt” EC_p) based on the composition of the 100% test solution (except for Ca in tests of NaHCO₃ or MgCO₃, as noted previously). Total concentrations of individual ions at an EC_p were then computed by adding their concentrations in the dilution water to their concentrations in the added-salt EC_p. These individual ion concentrations and the estimated pHs at the EC_x (by interpolation) were input to Visual MINTEQ (version 3.0, www2.lwr.kth.se/English/Oursoftware/vminteq) to estimate chemical speciation and ion activities. Nominal osmolarities were computed as the sum of all the individual ion concentrations, and actual osmolarities were estimated by the method of Robinson and Stokes [1959] using MINTEQ-calculated ion activities. These various calculated concentrations and activities at the EC_p were used in isobolograms to interpret mixture interactions (e.g., additivity, independence) as discussed in Erickson et al. [2017].

The calculated ion activities and osmolarities at the EC_p were also used in extending the acute ion toxicity model of Erickson et al. [2018] to chronic toxicity. This model consists of two submodels, one representing toxicity based on the joint additive effects of Mg- and Ca-specific toxicity, and the other representing a generalized ion toxicity from the aggregated effects of all ions, using osmolarity as the exposure metric. Because mechanisms represented by these submodels were shown to act independently, the model is applied by making a toxicity prediction with each submodel, with the overall model prediction being the more potent of the two.

The acute Mg/Ca toxicity submodel has the mathematical form:

$$\log\{Mg\}_M = \left(\log LC50_{MgOnly@ \{Ca\} = 1} - \frac{S \times \log\{Ca\}_M}{\log\{Ca\}_{Min} \times (\log\{Ca\}_M - \log\{Ca\}_{Min})} \right) + \log \left(1 - \frac{\{Ca\}_M}{LC50_{CaOnly}} \right) \quad (1)$$

where $\{Mg\}_M$ and $\{Ca\}_M$ are the Mg and Ca activities (mM) at the LC50 for any mixture of ions. The first parenthetical expression describes the Ca dependence of the median lethal Mg activity (in the absence of Ca toxicity), which has the parameters $LC50_{MgOnly@ \{Ca\} = 1}$, $\{Ca\}_{Min}$, and S (see Erickson et al. [2018] for details). The second parenthetical expression represents additive toxicity of Mg and Ca, with $LC50_{CaOnly}$ being the median lethal Ca activity in the absence of Mg toxicity.

The acute general ion toxicity submodel has the mathematical form:

$$\log\{Osmo\}_M = \left(\log LC50_{OsmoOnly@ \{Ca\} = 1} - \frac{S \times \log\{Ca\}_M}{\log\{Ca\}_{Min} \times (\log\{Ca\}_M - \log\{Ca\}_{Min})} \right) + \log \left(1 - \left(\frac{\{Ca\}_M}{LC50_{CaOnly}} \right)^p \right)^{1/p} \quad (2)$$

where $\{Osmo\}_M$ and $\{Ca\}_M$ are the osmolarity (mOsm) and Ca activity (mM) at the LC50 for any mixture of ions. The first parenthetical expression describes the Ca dependence of the median lethal osmolarity in the absence of Ca toxicity, with parameters of $LC50_{OsmoOnly@ \{Ca\} = 1}$, $\{Ca\}_{Min}$, and S . The second parenthetical expression describes joint toxicity of osmolarity and Ca that is neither additive nor independent, as regulated by the parameter p (see Erickson et al. [2018] for details).

The limited number and the distribution (across ion mixtures) of the chronic data did not allow completely re-parameterizing these models. Instead, the three parameters related to the shape of curve ($S, \{Ca\}_{Min}, p$) were set to the values for the acute models and the three parameters that directly relate to effect concentrations

$(LC50_{CaOnly}, LC50_{MgOnly@ \{Ca\} = 1}, LC50_{OsmoOnly@ \{Ca\} = 1})$ were modified to represent chronic EC50s. $EC50_{CaOnly}$ was set to the average of the log Ca activity at the EC50 for all tests with Ca-only salts. $LC50_{MgOnly@ \{Ca\} = 1}$ and $LC50_{OsmoOnly@ \{Ca\} = 1}$ were determined based on the average ratio (for all applicable tests with Ca activity = 1 mM) of the Mg activity or osmolarity at the chronic EC50 to the value for the acute model at the same Ca activity. Conceptually, this is calculating ACRs where the acute value is derived from the acute model, rather than from a paired acute test.

RESULTS AND DISCUSSION

For each test ($n=60$), added-salt EC50 and EC20 values with confidence limits are provided in the Supplemental data (Tables S1 and S2, respectively), along with the calculated concentrations of all individual ions at those concentrations, the associated ion activities, the pH estimated from bracketing measurements, and both nominal and estimated osmolarities. Experiment 1 compared results of tests with daily solution renewal with those renewed on days 3 and 5 only, using paired tests with $MgCl_2$ and $CaCl_2$. For $MgCl_2$, EC50 values were almost identical, 4.69 mM (95% CI 4.45 – 4.95) for daily renewal and 4.62 (4.29 – 4.93) for day 3/5 renewal (Table S1; Experiment 1 Tests C-D). For $CaCl_2$, the EC50 for daily renewal (6.99 mM [6.09 – 8.15]) was about 20% lower than the EC50 for the day 3/5 renewal (8.29 mM [7.59 – 9.11]), but with overlapping confidence limits (Table S1; Experiment 1, Tests A-B). EC20 values for these tests showed the same patterns (Table S2). Control reproduction was similar under both renewal protocols, averaging 33.3 ± 1.86 (mean $\pm$ 1 SD) and 31.2 ± 3.9 in the daily renewal tests and 34.3 ± 9.5 and 35.2 ± 5.1 when renewed on day 3/5 only. Combined with the demonstrated stability of measured exposure concentrations (see *Exposure Concentration Monitoring* in Methods), and the consistency in control reproduction, we felt these data support the day 3/5 renewal method as being adequate for our purposes and used it for the remainder of the testing reported here.

Relative acute and chronic toxicity of single salts

Chronic toxicity (as the EC50 for reproduction per surviving female) of 9 individual major ion salts is shown in Figure 2. For this analysis, data for all single salt tests in ALSW were collated across all experiments, with Figure 2 showing the mean of those values and the error bars representing the total range of individual values, along with the number of tests conducted on that salt (immediately above each symbol). Shown for reference are similarly aggregated *C. dubia* 48-h LC50 values from the combined data sets of Mount et al. [2016] and Erickson et al. [2017]. These tests were conducted in the same laboratory, using the same dilution water and organism source, but were independent experiments separated in time. Figure 2A compares these values expressed as the molar concentration of salt added. Across salts, chronic potency follows the same general pattern found for acute toxicity, with Na salts showing the highest effect concentrations, KCl the lowest, and Mg and Ca salts intermediate in toxicity. Numbers across the top of the panel are the calculated ACR_{EC50} (average LC50/average EC50) values, which fall within a fairly narrow range (1.5 to 2.8) across the eight salts with defined LC50s.

In our previous work on acute toxicity [Mount et al. 2016; Erickson et al. 2017, 2018], we showed that toxicities of major ions are better expressed on the basis of chemical activity than concentration. Figure 2B shows the same data as 2A, but values are expressed using the exposure metrics proposed by Mount et al. [2016] and Erickson et al. [2017]: osmolarity for Na salts, and individual cation activity for salts of K, Mg, and Ca. Expressing exposure in this way affects ACR_{EC50} values, in part because the effects of chemical complexation and activity are not linear; i.e., if total salt concentration doubles, ion activity goes up by less than 2x. Further, complexation and activity correction differentially affect ions; for example, Mg is more sensitive to complexation by SO_4 than is Na, which is in part responsible for the

ACR_{EC50} for MgSO₄ being reduced by a much greater degree than for Na₂SO₄ when expressed on an activity basis, rather than a concentration basis. Effects of other ions (especially HCO₃⁻ and CO₃²⁻, but also SO₄²⁻) on Ca activity also influence ACR_{EC50} values, because Ca affects the toxicities of Na and Mg salts [Mount et al. 2016; Erickson et al. 2017].

The overall range in activity-based ACR_{EC50} values in Figure 2B is relatively small, from 1.4 to 2.3. Whether the differences within that range are reflective of true differences is not simple to discern, because the acute and chronic data are not paired so as to produce multiple independent estimates of the ACR; they are instead single ratios of average acute and chronic values. The largest of the ACR_{EC50} values in Figure 2B is for NaHCO₃ (2.3), and this value may be inflated due to differences in pH and CaCO₃ precipitation between the acute and chronic tests. Solutions for acute tests were not aged before testing, so CaCO₃ precipitation caused notable decreases in Ca activity during testing (see Mount et al. [2016] for a detailed discussion). In contrast, solutions for chronic testing were aged before the test, yielding lower initial Ca activity at the same HCO₃⁻ concentrations, and smaller changes in Ca activity during the test. The ACR_{EC50} value for CaSO₄ is indefinite because of insufficient acute toxicity, and the ACR_{EC50} values for KCl (1.4) and MgCO₃ (2.0) have added uncertainty because they are calculated from only single chronic EC50 values (differences in pH and Ca speciation may also be a factor for MgCO₃). There is no suggestion of a difference in activity-based ACR_{EC50} between salts acting through a general ion (osmolarity) mechanism (NaCl = 1.9; Na₂SO₄ = 1.8), and those involving the Mg-Ca mechanism (MgCl₂ = 1.7; MgSO₄ = 2.0; CaCl₂ = 1.8). The geometric mean of these five values is 1.8.

The parallel analysis to Figure 2 using EC20 values is provided in Supplemental Information (Figure S1) and shows the same overall patterns. Excluding values for NaHCO₃, KCl, MgCO₃, and CaSO₄ leaves a range in ACR_{EC20} values (average LC50/average EC20) of 2.4 to 3.0 with a geometric mean of 2.8.

Also notable in Figure 2 is that the inter-test variability in the reproduction EC50 was generally larger than was observed previously for the 48-h LC50 (compare sizes of error bars for EC50 and LC50). This difference is even more pronounced for EC20 values (Figure S1). While one might initially presume this stems from variability in reproduction creating more uncertain response curves, it appears more related to inter-test variability in sensitivity, as the confidence intervals for the individual EC50s were small compared to the range in EC50s across tests (Table S1). For MgCl₂, the salt with the most variable EC50s (Figure 2A), the range across EC50 values was a factor of 2.6, but the confidence intervals for individual MgCl₂ EC50s were much narrower, averaging 0.91x to 1.13x of the EC50. Potential reasons for inter-test variability are unclear and did not appear related to variation in fecundity within or between tests, as mean control reproduction was consistently in the 30–35 young per female range, and the highest and lowest EC50s for MgCl₂ had very similar control reproduction of 35.2 ± 5.1 and 33.0 ± 6.8 , respectively.

Interactive toxicity of binary mixtures

A primary goal of the current research was to determine whether the principles governing the acute responses of *C. dubia* to ion mixtures held true for chronic toxicity. In this section we compare results from chronic tests with binary mixtures to those obtained from acute exposures [Erickson et al. 2017]. One of the prominent findings from the acute mixture studies was that salts sharing a common cation show concentration additive behavior. Figure 3 shows three examples for mixtures of NaCl x Na₂SO₄, NaCl x NaHCO₃, and MgCl₂ x MgSO₄. For each mixture, both acute (48-h LC50 from [Erickson et al. 2017]) and chronic (EC50) data are shown, along with lines showing the isoboles for additive toxicity. These isoboles are not statistically fit to all the data, they are simply straight lines connecting the single salt results that lie on each axis. If one defines a toxic unit of a salt to be the concentration at the axis intercept, then all intermediate compositions along those lines sum to one toxic unit (i.e., concentration additivity) and all three mixtures adhere tightly to these isoboles. While additivity is shown whether exposure is based on added salt (panels A-C) or activity (panels D-F), the activity-based isoboles also show similar x and y intercept values, reinforcing the appropriateness of osmolarity (for Na salts) and Mg activity (for Mg salts) as exposure metrics.

Assessing mixtures of CaCl₂ and CaSO₄ is complicated by solubility limitations for CaSO₄, which precluded gathering acute toxicity data for CaSO₄-dominated solutions (Erickson et al. 2017), and because of the strong effects of SO₄ on Ca activity. Rather than evaluating these data as isoboles, they are plotted on the basis of Ca activity at the EC50 over a range of CaSO₄ additions (Figure 4). In addition to data for mixtures of CaCl₂ and CaSO₄, Figure 4 also shows data from other experiments that included tests with either CaCl₂ or CaSO₄ alone. This analysis suggests that toxicity occurs at a similar Ca activity regardless of the relative proportions of CaCl₂ and CaSO₄. This is equivalent to concentration additivity, in that the apparent toxicity of Ca is the same regardless of the salt it originated from.

While mixtures of salts with common cations show additive behavior, mixtures with differing cations show more complex patterns. Figure 5 shows results for mixtures of NaCl and MgCl₂. The isoboles (lines) in these graphs represent the expected behavior of toxicants acting independently; the vertical leg of the isobole indicates that the toxicity expressed as NaCl would be constant when MgCl₂ is below a toxic concentration (y intercept), and the horizontal portion indicates consistent toxicity expressed as MgCl₂ when NaCl concentrations are below its single salt toxicity (x intercept). On an added salt basis (Figure 5A), the acute and chronic response profiles appear similar, but both also show some deviations from the isobole, with a tendency for intermediate mixtures to fall to the left and below the isobole. Adherence to an independence isobole improves when the exposure metrics are changed to osmolarity and Mg activity (Figure 5B). Even though the toxicity of MgCl₂ is attributable to the activity of the cation [Erickson et al. 2017], MgCl₂ also contributes to solution osmolarity. This contribution moves intermediate mixtures of MgCl₂ and NaCl to the right when the x axis is expressed as osmolarity, and provides better alignment with an independent interaction isobole.

Even more complicated behavior results from mixtures of CaCl₂ with salts of other cations (Figure 6). As shown in our previous work [Mount et al. 2016; Erickson et al. 2017], at

relatively low concentrations of Ca, increasing Ca decreases the toxicity of both Na and Mg salts, but as Ca concentrations rise further, they also contribute to toxicity, causing Na and Mg salt concentrations at the EC50 to decline. Figure 6A combines data from two experiments with mixtures of NaCl and CaCl₂, one in which the two were tested over a wide range of proportions (circles; Experiment 8) and one that focused on the ameliorative effect of Ca at lower concentrations (diamonds; Experiment 4). These two chronic experiments combine to yield a relationship paralleling the acute studies, and this consistency between acute and chronic is maintained when exposure is expressed as Ca activity and osmolarity (Figure 6C). For mixtures of MgCl₂ and CaCl₂, the same two types of experiments were performed, and the overall result was similar whether the plots are based on concentration (Figure 6B) or cation activity (Figure 6D).

Chronic toxicity of mannitol, Na gluconate, and Mg gluconate

In our previous acute studies, d-mannitol was used to raise the osmolarity of exposure without introducing ions that would cross the respiratory membrane, as a means to evaluate whether external osmolarity itself was a plausible cause of toxicity. Similarly, Na gluconate and Mg gluconate were used to introduce cations without an accompanying anion capable of crossing the respiratory membrane, which helped evaluate the relative roles of cation and anion in producing toxicity. In acute testing, both mannitol and Na gluconate showed toxicity similar to NaCl (when expressed as osmolarity) and Mg gluconate had toxicity similar to MgCl₂ (when expressed as Mg activity). In chronic tests, all three of these compounds exerted greater toxicity than expected. The EC50 for mannitol in ALSW was 17.1 mOsM (Table S1, Test 6C), while the EC50 value for NaCl in the same experiment was 24.5 mOsM; the exposure-effects relationship for mannitol also had a substantially lower slope than for NaCl, suggesting a different mechanism. For Na and Mg gluconates, reliable EC50s could not be calculated due to irregular and/or incomplete response curves, but were apparently well below those for NaCl and MgCl₂; in particular, Mg gluconate was at least 5-fold more chronically toxic than MgCl₂. We concluded that these organic compounds might cause sublethal chronic effects by mechanisms beyond those of the major ions, and neither support nor contradict the utility of osmolarity and Mg activity for describing the chronic toxicity of major ions to *C. dubia*.

Modeling of aggregated data

Figures 3 through 6 suggest that the same principles govern the acute and chronic toxicity of major ion mixtures, and that it is therefore reasonable to predict chronic toxicity to *C. dubia* by modifying the acute toxicity model as described in the Methods. Figure 7A shows all chronic data (large symbols) for Mg salts and their mixtures, Mg/Ca mixtures, Mg/Na mixtures dominated by Mg, and Ca salts and their mixtures. It also shows the acute Mg/Ca submodel (dashed line) and supporting data (small symbols) from Erickson et al. [2018]. The solid line denotes the Mg/Ca submodel for chronic toxicity, adapted from the acute version by: 1) retaining the acute parameter values for $\log\{Ca\}_{Min}(-3.52)$ and $S(2.95)$; 2) modifying $LC50_{CaOnly}$ from 7.74 mM for the acute model to 4.45 mM, the average median lethal Ca activity for tests with CaCl₂ and CaSO₄; and 3) dividing $LC50_{MgOnly@ \{Ca\} = 1}$ for

the acute submodel (8.99) by 1.78, the average ratio of the acute model to the chronic data at {Ca} 1.0 mM. The equation for the chronic EC50 Mg/Ca submodel therefore is:

$$\log\{\text{Mg}\}_{\text{M}} = \left(\log\left(\frac{8.99}{1.78}\right) + \frac{2.95 \times \log\{\text{Ca}\}_{\text{M}}}{3.52 \times (\log\{\text{Ca}\}_{\text{M}} + 3.52)} \right) + \log\left(1 - \frac{\{\text{Ca}\}_{\text{M}}}{4.45}\right) \quad (3)$$

Because of the way the model was adjusted, it is expected that the chronic data should adhere well for the data clustered near {Ca} of 0.2 mM and those at the extreme bottom right. However, the data between these two clusters also track the line closely, well within expected test variability, and consistent with the relationships shown in Figure 6. Also, there are no deviations suggesting systematic biases related to the anions of the test salts. The blue circle for the MgCO_3 test lies close to the line, the diamonds for the $\text{MgSO}_4 \times \text{MgCl}_2$ mixture test do not show a consistent trend related to the anions, and the single salt tests with MgSO_4 and MgCl_2 from different experiments straddle the line with similar variability, as might be expected from Figure 3F.

Figure 7B shows all chronic data (large symbols) for Na salts and their mixtures, Na/Ca mixtures, Mg/Na mixtures dominated by Na, and Ca salts and their mixtures. It also shows the acute general ion toxicity submodel (dashed line) and supporting data (small symbols) from Erickson et al. [2018]. The chronic model (solid line) was adapted from the acute version by: retaining the acute parameter values for $\log\{\text{Ca}\}_{\text{Min}} (-2.43)$, S (0.51), and p (3.6); modifying $\text{LC50}_{\text{CaOnly}}$ from 7.81 mM for the acute model to 4.45 mM as for the Mg/Ca submodel; and dividing $\text{LC50}_{\text{OsmoOnly@}\{\text{Ca}\}=1}$ for the acute submodel (85.4) by 2.21, the average ratio of the acute model to the chronic data at {Ca} 1.0 mM. The equation for the chronic EC50 general ion toxicity submodel is therefore:

$$\log\{\text{Osm}\}_{\text{M}} = \left(\log\left(\frac{85.4}{2.21}\right) + \frac{0.51 \times \log\{\text{Ca}\}_{\text{M}}}{2.43 \times (\log\{\text{Ca}\}_{\text{M}} + 2.43)} \right) + \log\left(\left(1 - \left(\frac{\{\text{Ca}\}_{\text{M}}}{4.45} \right)^{3.6} \right)^{1/3.6} \right) \quad (4)$$

As with the chronic Mg/Ca submodel, alignment of the chronic data with the model is expected near {Ca} of 0.2 mM and for Ca-dominated solutions at the bottom right. But beyond those clusters, the additional data track the model well within expected test variability. The higher SO_4 solutions (red-orange diamonds) of the $\text{NaCl} \times \text{Na}_2\text{SO}_4$ mixture experiment are clustered above the line, but this is true of the higher Cl solutions (yellow and yellow-orange diamonds) for that experiment as well, and two single salt tests with Na_2SO_4 (red circles) are near or slightly below the line. Higher HCO_3/CO_3 solutions (green diamonds) are below the line, but only slightly so for the single salt NaHCO_3 tests (blue symbols); also, these tests involve a degree of uncertainty regarding CaCO_3 precipitation and pH variability. If one uses data for NaCl in ALSW as an index of inter-test variability (yellow symbols at about 0.2 mM {Ca}), we feel that the overall chronic data conform to the model as well as can be expected given the observed variability in repeated tests.

A parallel analysis using EC20s is presented in Supplemental Information (Figure S2), and this figure shows the same general trends and interpretation as the EC50-based plots (Figure 7). Submodel equations for the reproduction EC20s are also provided in the Supplemental Information.

Models based on concentration

While our work has shown that toxicity of major ion solutions to *C. dubia* is better described by chemical activities than by concentrations, the use of chemical activity makes applying the models computationally complex, and requires geochemical software. To simplify application of our toxicity models, Erickson et al [2018] also provided a model based on Mg and Ca concentrations and nominal osmolality (total ion concentration); while these concentration-based models increase uncertainty (especially for the Mg/Ca submodel), they are simpler to apply. For the same reasons, we developed similar concentration-based submodels for chronic toxicity using adjustments of the acute models parallel to those used to adjust the activity-based submodels.

Figure 8 shows the concentration-based data and models in a manner analogous to the activity-based presentation in Figure 7. In panel A, the concentration-based Mg/Ca submodel was derived from the concentration-based submodel from Erickson et al. [2018] by: 1) retaining the acute parameter values for $\log\{Ca\}_{Min}(-2.90)$ and $S(3.37)$; 2) modifying $LC50_{CaOnly}$ from 19.7 mM for the acute model to 9.69 mM, the average median lethal Ca concentration for tests with $CaCl_2$ and $CaSO_4$; and 3) dividing $LC50_{MgOnly@{Ca}=1}$ for the acute submodel (20.1) by 2.22, the average ratio of the acute model to the chronic data at $[Ca] = 2$ mM. The equation for the chronic EC50 Mg/Ca submodel is therefore:

$$\log[Mg]_M = \left(\log\left(\frac{20.1}{2.22}\right) + \frac{3.37 \times \log[Ca]_M}{2.90 \times (\log[Ca]_M + 2.90)} \right) + \log\left(1 - \frac{[Ca]_M}{9.69}\right) \quad (5)$$

Figure 8B presents the data and models for Na salts and their mixtures, Na/Ca mixtures, Mg/Na mixtures dominated by Na, and Ca salts and their mixtures on a concentration basis. Erickson et al. [2018] simplified the concentration-based acute general ion toxicity submodel (dashed line) by assuming complete independence of general ion toxicity from Ca-specific toxicity (Eq. 16 in Erickson et al. [2018]). Cases in which the toxicity might be Ca-dominated are thus covered by the Mg/Ca submodel, and the data points for high Ca solutions (lower right) are shown in Figure 8B only for reference. The chronic submodel (solid line) was adapted from the acute version by retaining the acute parameter values for $\log\{Ca\}_{Min}(-2.39)$ and $S(0.91)$ and dividing $LC50_{OsmoOnly@{Ca}=1}$ for the acute submodel (83.5) by 2.14, the average ratio of the acute model to the chronic data at $[Ca] = 2$ mM. The resulting equation for the chronic EC50 general ion toxicity submodel is:

$$\log\{Osm\}_M = \left(\log\left(\frac{83.5}{2.14}\right) + \frac{0.91 \times \log\{Ca\}_M}{2.39 \times (\log\{Ca\}_M + 2.39)} \right) \quad (6)$$

A parallel analysis using concentration-based EC20s is presented in Supplemental Information (Figure S3), which shows the same general trends and interpretation as the EC50-based plots (Figure 8). Submodel equations for the concentration-based reproduction EC20s are also provided in the Supplemental Information.

Relationship to other studies

Erickson et al. [2018] evaluated the predictive ability of their acute models by testing the toxicity of 10 ion mixtures of varying composition, patterned after mixtures observed in studies of surface waters or effluents with elevated major ion concentrations. We did not conduct similar experiments in the present study, but did compare our data and concentration-based models to literature studies of the chronic toxicity of major ions to *C. dubia* (Figure 9), focusing on studies that evaluated toxicity of a single major ion salt over a range of water chemistries [Elphick et al. 2011a,b; Lasier and Hardin 2010] or those that tested waters reconstituted to match a major ion mixture found in a surface water or effluent [Kennedy et al. 2005; Brix et al. 2010; Kunz et al. 2013]. Establishing criteria for assessing concordance of literature data with the present study is challenging, as expected deviations would include both inter-lab and inter-test variability, and some additional sources of potential error. These additional sources of uncertainty include the need to infer ionic composition of test waters where it was not explicitly reported, and the reliance on author-reported effect concentration where treatment level data were not available. It appeared most author reported values were based on reproduction per original female rather than per surviving female, and many used interpolation based on linear exposure concentrations (reported as “IC50” values), rather than log-transformed exposure concentrations. In this light, it seems reasonable to expect that concordance of literature data with model predictions would fall within a range at least some wider than the degree of inter-test variability observed in the present study, which is reflected by the range of values we observed in the multiple tests conducted at 0.36 mM Ca (about 2-fold lowest to highest).

Only three studies involved mixtures where effects of Mg-Ca were predicted to dominate (Figure 9A), but reported toxicity was in general agreement with model predictions. Brix et al. simulated a hardrock mine effluent dominated by Ca and SO₄, whose reported toxicity matched well with the model prediction. Two samples studied by Kunz et al. [2013] were dominated by Ca, Mg, and SO₄; neither of these reached 50% effect at the highest concentration tested, placing both samples above the model prediction. Because high SO₄ reduces the activity of Mg, an effect not accounted for by concentration-based exposure, overestimation of toxicity by the concentration-based model is expected when both Mg and SO₄ are high, as noted for acute toxicity by Erickson et al. [2018].

The remaining literature studies involved Na salts or Na-dominated mixtures, and were thus compared to predictions from the general ion toxicity submodel (Figure 9B). Generally speaking, EC50/IC50 values from these studies were within or only marginally beyond the inter-test variability observed in the present study at 0.36 mM Ca. Elphick et al. [2011a,b] tested both NaCl and Na₂SO₄ in dilution waters with a range of hardness, and the ameliorating effect of Ca was similar in magnitude to that found in the present study. Lasier and Hardin tested the toxicity of NaCl, Na₂SO₄, and NaHCO₃ in each of three dilution

waters with varying Ca and alkalinity. For NaCl and Na₂SO₄, the reported toxicity and Ca dependence was again in general agreement with the present study and model. Larger deviations were observed for tests with NaHCO₃ (blue downward triangles in Figure 9B); we believe these differences are due to the plotted Ca concentrations being based on the initial dilution water (which was all the authors reported), and thus do not reflect reduced Ca resulting from CaCO₃ precipitation during the test (clearly documented in the present study). Three tests with reconstituted waters matched to field waters with high Na were identified: two reported by Kennedy et al. [2005] (Na/SO₄ dominated) and one from Kunz et al. [2013] (Upper Dempsey; primarily Na/SO₄/HCO₃). Deviations from the model for these solutions show a similar degree of scatter as was observed for single salt studies.

Because of the role of Ca in modifying toxicity, methods testing of waters with high carbonate alkalinity should be considered carefully. In our experience, it is quite common for high alkalinity waters from the field to have Ca and alkalinity concentrations above CaCO₃ saturation under atmospheric pCO₂ and, as such, are likely to precipitate CaCO₃ during the course of toxicity testing, as solutions equilibrate with ambient air. The resulting drift in Ca concentration during testing can confound the assessment of major ion toxicity; this drift can be reduced by encouraging precipitation via an aeration period prior to testing if it doing so does not compromise other objectives of the test. In any case, we strongly recommend that toxicity testing with high alkalinity waters include frequent monitoring of Ca concentrations.

Most literature studies of major ion toxicity to *C. dubia* have focused on single salts (almost exclusively Na salts) and expressing toxicity in terms of individual anions, whereas our work suggests that both cations and anions contribute to toxicity, and that their effects on *C. dubia* are aggregated through their contributions to osmolarity (excepting mixtures with sufficient Mg/Ca or K to cause toxicity). However, both Mount et al. [1997] and Lasier and Hardin [2010] used additive models based on anion concentrations (with limited interactions) to describe the toxicity of Na salt mixtures to *C. dubia* with some success. While it lacks the mechanistic underpinning of aggregating mixtures via osmolarity, one can see how such an approach can approximate the behavior observed in our recent ion studies. If the primary cation is Na, then potencies expressed as anion concentrations capture to some degree the contribution of Na to the overall toxicity of the salt, because the added Na is proportional to the anion addition. And while the true osmolarity of ion mixtures does not increase linearly with concentration, an additive model is a loose approximation, absent factors such as effects on Ca activity and its attendant effects on toxicity. However, anion-based approaches can be expected to show greater errors when applied to solutions with more diverse cation composition. For that reason, a Ca-normalized osmolarity approach, combined with a Ca-Mg based model, should provide greater accuracy for more complex mixtures where Na is not the dominant anion, such as in Appalachian waters affected by coal mining, where Ca and Mg dominate over Na [Pond et al. 2008; Cormier and Suter 2013].

While we emphasize the role of osmolarity as an expression of major ion exposure for *C. dubia*, it bears considering that other exposure metrics may prove more appropriate for other species. Work with the mayfly, *Neocloeon triangulifer*, suggests that sensitivity of that species to Na salts is higher than can be explained based on osmolarity alone, and that ion-

specific activities, such as Na, may be better exposure metrics for that species [Soucek et al. 2018]. In our own laboratory, preliminary work with the amphipod, *Hyaletella azteca*, also points to Na rather than osmolality as being a larger determinant of toxicity for Na salts (unpublished data). Such interspecific differences will be important considerations in the development of environmental guidelines that address the sensitivities of full aquatic communities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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SUMMARY AND IMPLICATIONS

This study of chronic responses of *C. dubia* to major ion salts and their mixtures provides strong evidence that the principles governing acute responses of this species to major ion mixtures are applicable to chronic toxicity as well, and given that, predicting chronic toxicity by modifying acute toxicity models seems appropriate. Activity-based ACR_{EC50} and ACR_{EC20} values were fairly consistent across major ion salts, and adjusting the acute models of Erickson et al. [2018] yielded chronic models that represented individual chronic tests with individual salts and binary mixtures, within the limits of inter-test variability. Comparison of chronic concentration-based models to literature data showed generally good agreement, with slightly higher variability as might be expected given the conditions of the comparison. While the activity-based models have the advantage of being structured on demonstrated principles of both toxicological behavior and geochemistry, full application of these models requires a more complete characterization of the ionic composition and geochemical calculations (activity and osmolarity) that are not commonly employed in environmental assessment, and may be less familiar to many practitioners. Concentration-based models may provide a simpler approach without introducing unacceptable uncertainty. We recognize that normalizing toxicity to hardness (rather than Ca) may have history, familiarity and data availability on its side; if needed, it should be possible to adapt our models to be hardness-based, given a set of assumptions regarding how overall water chemistry varies with hardness and the types of ion enrichment of concern.

Figure 1.

Aggregated analysis of exposure response curves for all tests of NaCl (A) and MgCl₂ (B). Different colors identify individual tests conducted over time. Inset graphs show variability of reproduction within ranges of 5 young per female (stars) as compared to a Poisson distribution (dotted line).

Figure 2.

Comparison of acute and chronic toxicity of major ion salts to *Ceriodaphnia dubia*. Red symbols denote 48-h LC50s from Mount et al. (2016) and Erickson et al. (2017) and green symbols denote 7-d EC50s for total reproduction from the present study. Error bars denote minimum and maximum results across replicate tests; the number of tests is indicated by the number above the upper error bar. Arrow for CaSO₄ indicates no acute toxicity at solubility (plotted concentration). The upper panel provides effect concentrations based on the molarity of added salt, while the lower panel uses effect concentrations based on the exposure metrics of osmolarity for Na salts and cation activity for other salts. Acute-chronic ratios (ACR) are provided across the top of each panel.

Figure 3.

Isobolograms for binary mixture experiments with NaCl×Na₂SO₄, NaCl×NaHCO₃, and MgCl₂×MgSO₄, for both acute toxicity (48-h LC50s from Erickson et al. 2017) and chronic toxicity (7-d EC50s for total reproduction from the present study) to *Ceriodaphnia dubia*. Panels A-C show results based on molarity of added salts, while panels D-F use activity-based exposure metrics (osmolarity or cation activity) recommended by Erickson et al. (2017). Colors denote graded mixtures of different anions (yellow=Cl, red=SO₄, blue=HCO₃/CO₃). Lines denote approximate isoboles for simple concentration addition.

Figure 4.

Toxicity of CaCl_2 and CaSO_4 to *Ceriodaphnia dubia*, when tested as mixtures or single salts. Small symbols are 48-h LC50 values from Erickson et al. (2017) and large symbols are 7-d reproduction EC50 values from the present study. The x-axis represents the amount of CaSO_4 present in the mixture, while the y-axis indicates the combined Ca activity from both salts. Colors denote graded mixtures of different anions (yellow= Cl^- , red= SO_4^{2-}).

Figure 5.

Isobolograms for binary mixture experiment with NaCl×MgCl₂, for both acute toxicity (48-h LC50s from Erickson et al. 2017) and chronic toxicity (7-d EC50s for total reproduction from the present study) to *Ceriodaphnia dubia*. Effect concentrations are based on the molarity of added salts in panel A and on osmolarity and Mg activity in panel B. Lines denote approximate isoboles for simple independent action.

Figure 6.

Isobolograms for binary mixture experiments with NaCl×CaCl₂ (panel A/C) and MgCl₂×CaCl₂ (panel B/D), for both acute toxicity (48-h LC50s from Erickson et al. 2017) and chronic toxicity (7-d EC50s for total reproduction from the present study) to *Ceriodaphnia dubia*. Panels A/B are shown on molarity basis while C/D are shown on an cation activity/osmolarity basis.

Figure 7.

Toxicities of Mg salt and Ca salts (and mixtures thereof; panel A), and Na and Ca salts (and mixtures thereof; panel B) to *Ceriodaphnia dubia*, expressed on a chemical activity basis. Small symbols are 48-h LC50s from Mount et al. [2016] and Erickson et al. [2017], while large symbols are reproduction EC50s from the present study. The dashed lines are the acute submodels presented by Erickson et al. [2018]; the solid lines are the acute submodels modified to reflect chronic potency (see text). Exposures with single anions are shown as primary colors, with color gradients reflecting proportional anion mixtures. Single salt exposures are circles while other shapes denote different binary mixtures.

Figure 8.

Toxicities of Mg salt and Ca salts (and mixtures thereof; panel A), and Na and Ca salts (and mixtures thereof; panel B) to *Ceriodaphnia dubia*, expressed on a chemical concentration basis. Small symbols are 48-h LC50s from Mount et al. [2016] and Erickson et al. [2017], while large symbols are reproduction EC50s from the present study. The dashed lines are the acute submodels presented by Erickson et al. [2018]; the solid lines are the acute submodels modified to reflect chronic potency (see text). Exposures with single anions are shown as primary colors, with color gradients reflecting proportional anion mixtures. Single salt exposures are circles while other shapes denote different binary mixtures.

Figure 9.

Concentration-based chronic EC50 values (small symbols) and models (solid line) from the present study compared with literature data (large symbols) for single salts tested over a range of water chemistries [Elphick et al. 2011a,b; Lasier and Hardin 2010] or waters reconstituted to match chemistries of effluents or field samples with elevated major ions [Kennedy et al. 2005; Brix et al. 2010; Kunz et al. 2013]. Legend is for literature data (large symbols); legend for small symbols as in Figure 8. Panel A shows the Mg-Ca submodel and panel B shows the general ion toxicity (osmolarity) submodel; literature data are plotted according to the submodel that predicted greater potency for that chemistry. Symbols with up arrows indicate mixtures that did not reach 50% effect at the highest tested concentration. Symbols with leftward arrows are solutions wherein unmeasured CaCO₃ precipitation likely reduced actual Ca concentrations.

Table 1.

Summary of tests conducted. Dilution water chemistries provided in Table 2.

Experiment #	# of Tests	Description
1	4	MgCl ₂ and CaCl ₂ in ALSW with daily renewal and d 3 and 5 renewal
2	5	NaCl, KCl, Na ₂ SO ₄ , MgSO ₄ and CaSO ₄ in ALSW
3	2	MgCO ₃ and NaHCO ₃ in ALSW
4	4	NaCl in 0.3 Ca, ALSW, 3x Ca, and 9x Ca
5	5	MgCl ₂ in 0.3 Ca, ALSW, 2x Ca, 4x Ca, and 8x Ca
6	5	Mixtures of NaCl and Na ₂ SO ₄ in ALSW
7	5	Mixtures of NaCl and MgCl ₂ in ALSW
8	5	Mixtures of NaCl and CaCl ₂ in ALSW
9	5	Mixtures of MgCl ₂ and MgSO ₄ in ALSW
10	5	Mixtures of MgCl ₂ and CaCl ₂ in ALSW
11	5	Mixtures of NaCl and NaHCO ₃ in ALSW
12	5	Mixtures of CaCl ₂ and CaSO ₄ in ALSW
13	5	NaCl, MgCl ₂ , d-mannitol, sodium gluconate, and Mg gluconate in ALSW

Table 2.

Composition of dilution waters; DIW = deionized water.

Dilution Water Description	Abbreviation	Base Water	Na mg/L	K mg/L	Ca mg/L	Mg mg/L	Cl mg/L	SO ₄ mg/L	HCO ₃ mg/L
Lake Superior Water	LSW	--	1.62	0.60	14.0	2.92	1.50	3.40	52.4
Amended Lake Superior Water	ALSW	LSW	6.48	1.51	14.6	4.09	7.66	14.9	52.4
ALSW with Ca = 1/3x	1/3x Ca	1:2 LSW:DIW	17.6	1.51	4.87	4.09	7.66	14.9	52.4
ALSW with Ca = 2x	2x Ca	LSW	6.48	1.51	29.2	4.09	33.5	14.9	52.4
ALSW with Ca = 3x	3x Ca	LSW	6.48	1.51	43.8	4.09	59.3	14.9	52.4
ALSW with Ca = 4x	4x Ca	LSW	6.48	1.51	58.4	4.09	85.1	14.9	52.4
ALSW with Ca = 8x	8x Ca	LSW	6.48	1.51	117.0	4.09	189.0	14.9	52.4
ALSW with Ca = 9x	9x Ca	LSW	6.48	1.51	131.5	4.09	214.4	14.9	52.4