Title:

Acute Toxicity of Major Geochemical Ions to Fathead Minnows (*Pimephales promelas*). Part A: Observed Relationships for Individual Salts and Salt Mixtures

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Data availability:

Data are available through the USEPA Environmental Dataset Gateway (https://edg.epa.gov/metadata/catalog/main/home.page) or by request from the corresponding author (erickson.russell@epa.gov).

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RUNNING HEAD: Observed Major Ion Toxicity to Fathead Minnow

ABSTRACT: This paper reports on the results of a series of experiments on the acute toxicity of major geochemical ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, HCO_3^-/CO_3^{2-}) to fathead minnows (*Pimephales promelas*). Tests of individual major ion salts in various dilution waters demonstrated that the toxicities of Na, Mg, and K salts decrease as the overall ion content of the dilution water increases. For Na and Mg salts, this is attributable to Ca content as previously reported for *Ceriodaphnia dubia*. For K salts, the cause is unclear, but is not due to Na as reported for *C. dubia*. $NaHCO_3$ was also found to be twice as toxic at an unregulated high test pH of 9.3, compared to when the pH was reduced to 8.4. Experiments with binary salt mixtures indicated the existence of multiple independent mechanisms of action. These include K-specific toxicity and Ca/Mg-specific toxicity previously reported for *C. dubia*, but also apparent toxicities related to SO_4 and to high pH/alkalinity in CO_3/HCO_3-dominated exposures.. Previous work with *C. dubia* also suggested a general ion toxicity involving all ions that was correlated with osmolarity. For fathead minnow, similar correlations were observed, but multiple mechanisms were indicated. At higher Ca, this general toxicity could be attributable to osmotic effects, but at lower Ca, osmolarity may be more a covariate than a cause, with this general toxicity being related to a combined effect of ions other than via osmolarity.

KEY WORDS: Aquatic Toxicology, Major Geochemical Ions, *Pimephales promelas*, Fathead Minnow, Toxicity Mechanisms, Mixture Toxicity

INTRODUCTION

Anthropogenic increases of major geochemical ions (Na^+, K^+, Ca^{2+}, Mg^{2+}, Cl^-, SO_4^{2-}, and HCO_3^-/CO_3^{2-}) in freshwater systems are of great concern regarding impacts on aquatic communities (see reviews by Hintz and Relyea, 2019; Cañedo-Argüelles et al., 2019; Berger et al. 2019; Schuler et al., 2019; Cañedo-Argüelles, 2020; Kauschal et al., 2021). Substantial and widespread increases of ions from ambient levels; toxic levels of ions in effluents, runoff, and ambient waters; and impacts on aquatic ecosystems have been reported. Sources of these increases include road salting, irrigation water return, mining leachates and treatment of acid mine drainage, oil and gas production waters, various industrial process effluents, water softening, and saltwater intrusion. This ion enrichment involves complex mixtures that vary widely regarding ion ratios and dominant ions. However, many current environmental standards focus on individual components, often Cl or SO_4, rather than the suite of ions present.

The aquatic toxicity of major ions has been the subject of considerable research, which has established that toxicities of ion mixtures vary widely with their composition and with test water characteristics. For multiple freshwater test species, Mount et al. (1997) established the importance of considering all major ions for assessing toxicities over the broad range of ion mixture compositions that might occur. For perhaps the most studied species, *Ceriodaphnia dubia*, Table 1 lists important toxicological interactions among ions that have been identified (Mount et al., 1997; Soucek and Kennedy, 2005; Soucek, 2007; Soucek et al., 2011; Elphick et al. 2011a,b, Mount et al., 2016; Erickson et al., 2017; Erickson et al., 2018; Mount et al., 2019). Based on the interactions reported in Table 1, Mount et al. (2016) and Erickson et al. (2017) suggested three primary independent mechanisms of action: 1) a K-specific toxicity, 2) a Ca/Mg-

specific toxicity, and 3) a general toxicity related to all ions. Erickson et al. (2018) addressed how these mechanisms, and their dependence on dilution water composition, could be formulated into mathematical models for the acute toxicity of *C. dubia* and Mount et al. (2019) did the same for chronic toxicity. Importantly, fully understanding and describing these relationships requires the use of molar rather than mass concentrations, and is often better based on chemical activities rather than concentrations.

Although there is considerable understanding of major ion toxicity to *C. dubia*, it is less clear whether the same principles govern responses of other freshwater species. Fathead minnows (*Pimephales promelas*) have been used in several studies of major ion toxicity, but the aggregate of these studies does not provide a comparable level of understanding of how this species responds to ion mixtures of widely varying composition. Mount et al. (1997) did extensive testing of the acute toxicity of 10 major ion salts to 2-day-old to 7-day-old fathead minnows, including both individual salt tests and 1:1 (by mass) mixture tests for 20 binary combinations of these salts. Although this study supported some of the mixture interactions in Table 1, it was unclear or incomplete in other regards, including not addressing effects of dilution water chemistry. Other studies on acute toxicity of major ions to fathead minnow (Adelman et al., 1976; Birge et al., 1985; Meyer et al., 1985; Elphick et al., 2011a; Wang et al., 2015) were limited to single-salt testing of NaCl, KCl, Na_2SO_4 and $MgSO_4$, and showed LC50s consistent with Mount et al. (1997), but did not explore the toxic interactions among the major ions or the influence of test water composition. For 7-d fathead minnow embryo-larval tests, Elphick et al. (2011b) did show that the lethality of Na_2SO_4 is dependent on the hardness of the dilution water (although other ions varied in proportion to hardness), and Wang et al. (2015) found that the toxicity of Na_2SO_4 is exacerbated by low K concentrations.

Due to these limitations and uncertainties for existing data, the present study was designed to provide a more extensive and systematic evaluation of the acute toxicity of major ions to juvenile fathead minnow. Tests with single salts determined the effects of dilution water characteristics on toxicity and general potency differences among ions. Binary mixture experiments were used to infer the primary roles of different ions for determining mixture toxicity, in a manner similar to that used previously for *C. dubia* (Erickson et al 2017; Mount et al 2019). The relationship of salt toxicity to osmolarity was also investigated by testing the toxicity of mannitol and the interaction of this toxicity with major ions.

The present paper provides the data from these experiments and the basic evaluations of ion interactions. In a companion paper (Erickson et al. 2022), these findings are used to formulate mathematical models for major ion toxicity to fathead minnows, and we discuss issues regarding their application to risk assessments.

MATERIALS AND METHODS

Test organisms

Fathead minnows were from the in-house culture at the U.S. Environmental Protection Agency (U.S.EPA) Great Lakes Toxicology and Ecology Division (Duluth, MN, USA), which is maintained at 25°C in filtered Lake Superior water (LSW, obtained from an intake located offshore from the laboratory at 46.840° N, 92.004° W). This water typically has a pH of ca. 7.5, a dissolved organic carbon concentration of 1-2 mg C/L, and inorganic constituents as specified in Table 2. For each experiment, a cohort of newly hatched fry (<24 h old) from multiple spawns was isolated, fed brine shrimp nauplii *ad libitum* for 6 days, and used for testing when 7 to 8 days old. Preliminary efforts had evaluated the toxicities of NaCl and $MgSO_4$ to fathead

minnows <1, 3-4, 7-8, and 14-15 days old. The selection of organisms 7-8 days old was based on them not being significantly less sensitive than other ages, being robust compared to newly-hatched fry regarding control survival in the absence of feeding, and being amenable to smaller chambers and less culture effort than older organisms. All procedures involving live fish were conducted according to an institution-approved animal care and use plan.

Test chemicals

Nine major ion salts were used to evaluate the relative toxicities of and the interactions among the major ions, including the chloride salts of all four cations, the sulfate salts for all cations except Ca (due to limited solubility), and the carbonate salts of Na and Mg. Gluconate salts of Na, Mg, and Ca were also tested to provide an anion expected to have limited interaction with the organisms in order to further evaluate correlation of toxicity to the cations. Mannitol was tested to provide a comparison of salt toxicity to that of a compound expected to only affect the external osmotic environment.

All chemicals were obtained from Sigma-Aldrich Chemical Company or Thermo-Fisher Scientific Company. All compounds had a designated hydration and a purity of at least 98%, except for $MgCO_3$ and Mg gluconate, for which the hydration was not specified. The certificates of analysis for these two salts were used to determine the ratio of the anhydrous salt to total salt weight for computing nominal concentrations. The certificate of analysis for $MgCO_3$ also specified the Ca content to be 0.44% of the Mg content (on a molar basis), enough to appreciably affect the background Ca for tests on the toxicity of $MgCO_3$, necessitating additional Ca measurements to document concentrations.

Test waters

149 The dilution water for most tests was amended Lake Superior water (ALSW), which was
150 developed by Mount et al. (2016) to address concerns that ion ratios in some commonly-used
151 dilution waters are atypical of natural waters (e.g., unusually high Mg:Ca ratios in some
152 synthetic waters, low Na in LSW relative to its hardness and alkalinity), which could cause
153 misleading results. ALSW is prepared by amending sand-filtered and UV-treated LSW with
154 major ion salts to provide ion ratios close to median values for U.S. water, while maintaining its
155 alkalinity and other natural constituents. The major ion content of ALSW is provided in Table 2.
156 For some tests, the formula for ALSW was modified to create test waters with different
157 concentrations for some or all ions (Table 2). This included three ways in which Ca was
158 modified. First, Ca was changed in proportion to all other ions, by diluting ALSW with deionized
159 water so that all ion concentrations were reduced to ⅓ of that in ALSW ("⅓X ALSW") and by
160 adding salts so that ion concentrations were increased to 3 times that in ALSW ("3X ALSW").
161 Second, Ca was changed inversely with Mg while keeping water hardness and other ions
162 approximately constant so that the Ca:Mg molar ratio of 2.2 for ALSW was reduced to 0.2
163 ("LoCa:Mg") and increased to 9 ("HiCa:Mg"). Third, several test waters were formulated with
164 altered Ca concentrations ranging from ⅛X to 81X of that in ALSW, reducing Ca by substituting
165 Na for a portion of the Ca in the water formulation and increasing Ca concentrations by adding
166 $CaCl_2$, with other ions approximately constant. To test effects of Na and K concentrations in
167 dilution water, test waters were also formulated with different Na concentrations
168 ("1.6mgNa/L", "10mgNa/L", "30mgNa/L"), with concomitant changes to Cl, and with the K:Na
169 molar ratio altered from 0.14 for ALSW to 0.015 ("LoK:Na") and 3.1 ("HiK:Na"), with negligible
170 changes to other ions.
171 ***Study design***

Seventeen experiments, each comprising 2 to 7 simultaneous toxicity tests (92 total tests), were conducted on the acute (96-h) lethality of single compounds to fathead minnows in various dilution waters. Table 3 specifies the experiments addressing effects of different dilution waters on the lethality of the major ion salts and mannitol. Other experiments compared the toxicities, in ALSW only, of mannitol and of the gluconate salts of Na, Mg, and Ca to the chloride salts of these cations. An additional experiment compared the toxicity of $NaHCO_3$ in ALSW with pH uncontrolled (9.3) to that with pH reduced to 8.4 by elevating CO_2.

Testing the toxicity of mannitol to fathead minnow in dilution waters with different Ca was important to the evaluation of the relationship of salt toxicity to osmolarity and to model development in the companion paper (Erickson et al. 2022). For *C. dubia* the effects of Ca on mannitol were not tested by Mount et al. (2016), so an ancillary experiment was conducted to fill this data gap and is described in the Supporting Information.

Fifteen binary mixture experiments (78 total tests) were conducted in ALSW. Each experiment consisted of multiple toxicity tests, each test having a fixed ratio of the two chemicals. A typical experiment had 5 tests with estimated toxic unit ratios of 1:0, 3:1, 1:1, 1:3, and 0:1, but ratios were sometimes changed or added to better establish chemical interactions. These mixture experiments included all possible combinations involving NaCl, Na_2SO_4, $MgCl_2$, $MgSO_4$, and $CaCl_2$. $NaHCO_3$ and $MgCO_3$ were tested only in mixtures with their respective Cl salts. For K salts, the only mixture tests were of KCl with NaCl and $MgCl_2$, due to the expectation of K-driven toxicity independent of other ions. A final mixture experiment was of NaCl and mannitol to test for concentration additivity on an osmolarity basis.

Toxicity test procedures

194 Static, 96-h, unfed acute toxicity tests were conducted in accordance with ASTM
195 Method E729 (ASTM 2002). Exposures were in 5 oz (ca. 150 ml) polystyrene cups (Fineline
196 Settings, Inc, Middletown, NY) containing 80 ml of test solution and suspended in a
197 temperature-controlled (25 C) water bath using perforated floating foam boards, with glass
198 sheets covering all cups. The test compounds were dissolved in the designated test dilution
199 water (Tables 2 and 3) to produce the highest test concentration, with the dissolution of $MgCO_3$
200 being CO_2-assisted as described in Mount et al. (2016). This high test concentration was serially
201 diluted with the test dilution water to produce a geometric series of 9-10 concentrations with a
202 dilution factor of ca. 0.80X (i.e., 100%, 80%, 64%, 50%...) or 0.707X (i.e., 100%, 70.7%, 50%...).
203 Solutions were prepared immediately prior to test initiation, except for two experiments
204 involving $NaHCO_3$ ($NaCl \times NaHCO_3$ mixture experiment, $NaHCO_3$ toxicity at different pHs), for
205 which solutions were prepared 3 days in advance to allow $CaCO_3$ precipitation to occur and Ca
206 concentrations to stabilize. In other, earlier experiments involving $NaHCO_3$, $CaCO_3$ precipitation
207 caused Ca concentrations to decline by a factor of up to 20 over the course of the 4-day
208 exposures, making it difficult to associate a specific {Ca} with the LC50 for this salt (Note that
209 braces {} are used for chemical activities, versus brackets [] for concentrations).
210 Each concentration was tested in duplicate cups, except quadruplicate cups were used
211 for the control for the last two experiments (on NaCl×mannitol mixture interactions and the
212 effects of Ca on mannitol toxicity) to provide better information on control survival. For the
213 experiment on the effect of added CO_2 on $NaHCO_3$ toxicity, a third cup was added for the
214 control and for three concentrations near the expected LC50 to provide samples for Na and Ca
215 measurements at 48 h, but these destructively sampled cups were not included in effects
216 assessment. Five fathead minnows were added to each cup at test initiation. The number of

surviving organisms in each test chamber was recorded at the end of each 24-h period. Death was determined by the change in appearance from translucent to opaque or no reaction to gentle prodding. At the end of the exposures, surviving fish were euthanized with a buffered solution of non-pharmaceutical grade MS-222 (Finquel).

Tests were conducted under ambient laboratory fluorescent lighting with a 16 h light to 8 h dark photoperiod. At the initiation of each test, alkalinity and hardness were measured in an aliquot of the control water, dissolved oxygen (DO) and pH were measured in aliquots of both the control water and the highest test concentration, and conductivity was measured for all test concentrations in one of the replicate test cups. Temperature was measured daily in one chamber of each test. Conductivity, dissolved oxygen, and pH were measured at 24 h, 48 h, and 72 h in one replicate of every test concentration reaching 100% mortality during the preceding time interval, and at 96 h in one replicate of all other concentrations. These measurements were only in test chambers being terminated to avoid the probes perturbing test organisms and/or test water chemistry, while still documenting the chemistry at concentrations causing mortality.

Exposure monitoring

Samples for analysis of the major ions were collected at the start of each experiment from the dilution water(s) and from the highest concentration of each toxicity test. In addition, samples were collected for each test at 24 h from the lowest concentration with 100% mortality, and at 96 h from the concentration nearest the apparent LC50. Additional samples were taken in some experiments to more fully characterize ion concentrations, especially Ca, in treatments near the LC50. The conductivity measurements made on all treatments confirmed

239 that the intended gradient of exposure existed among treatments not sampled for cation
240 analysis.
241 Dilution waters were analyzed for all four cations plus Cl and SO_4. Other samples were
242 analyzed for the test salt cation(s), and also for Ca in tests involving $NaHCO_3$ and $MgCO_3$ (which
243 caused oversaturation of $CaCO_3$), for the Na_2SO_4×$CaCl_2$ and $MgSO_4$×$CaCl_2$ mixture tests (which
244 caused oversaturation of $CaSO_4$), and for the toxicity tests with NaCl, $MgCl_2$, Na_2SO_4, and $MgSO_4$
245 over the series of dilution waters with modified Ca.
246 Samples were filtered through an 0.45 µm nylon syringe filter (Grainger, St. Paul, MN,
247 USA). For cation analyses, samples were acidified to 0.2% (v/v) with concentrated HNO_3 and
248 held at room temperature; for tests with $NaHCO_3$ and $MgCO_3$, this acid addition was increased
249 by an amount calculated to neutralize the extra alkalinity. For Cl and SO_4 analyses, samples
250 were refrigerated. Analyses were by flame atomic absorption spectrophotometry for cations
251 and ion chromatography for anions, using methods and equipment described in Mount et al.
252 (2016). For samples in which SO_4 exceeded 20 mM, there was apparent interference with Ca
253 analysis due to insufficient inhibitor for this interference, resulting in estimated Ca
254 concentrations 10-30% lower than nominal; such measurements were not used.
255 For measurements of ions in dilution waters (ALSW and the modifications thereof in
256 Table 2), the ratios of measured to nominal concentrations had means (% relative standard
257 deviation, sample size) of 1.03 (6%, n=64) for Na, 1.03 (8%, n=63) for K, 1.01 (6%, n=63) for Ca,
258 1.07 (4%, n=63) for Mg, 1.01 (4%, n=57) for Cl, and 1.05 (7%, n=60) for SO_4. For measurements
259 of test salt cations in test solutions near and above the LC50, the ratios of measured to nominal
260 concentrations across all samples were 1.00% (4%, n=402) for Na, 0.98% (3%, n=74) for K,
261 1.02% (5%, n=353) for Mg, and 0.96% (6%, n=41) for Ca, excluding Ca analyses for tests in which

262 $CaCO_3$ or $CaSO_4$ precipitation was a consideration and for tests with $MgCO_3$ due to uncertain
263 nominal Ca. For these test salt cation comparisons (n=870), only 18 measurements (2.1%)
264 deviated by more than 10% from nominal, with a maximum deviation of 19%. Such agreement
265 was considered sufficient to warrant the use of nominal concentrations in the data analyses
266 except as follows.
267 For the $Na_2SO_4 \times CaCl_2$ and $MgSO_4 \times CaCl_2$ mixture experiments, most mixture ratios had
268 visible precipitates and lower-than-nominal measured Ca concentrations at the highest
269 exposure concentrations. At the LC50, precipitation was less evident, but test solutions were
270 calculated to be oversaturated for $CaSO_4$, and there were minor (ca. 10%) reductions in
271 measured Ca from nominal. Therefore, for these experiments, the Ca and SO_4 concentrations
272 to associate with the LC50 were based on interpolation of the average Ca measurements in
273 treatments bracketing the LC50.
274 Measured Ca concentrations were also used in tests with $NaHCO_3$ and $MgCO_3$ due to
275 precipitation of $CaCO_3$ and due to the uncertain Ca content of the $MgCO_3$ salt. At each
276 measurement time, Ca measurements were used to estimate the Ca concentration at the LC50
277 by interpolation. If these estimates declined by ≤25% over time, their average was associated
278 with the LC50. If the temporal variation was greater than this, both the nominal and final Ca
279 concentrations are reported to designate a range for the Ca concentration to associate with the
280 LC50. The same procedure was applied to the Na_2SO_4 toxicity test with the highest Ca elevation
281 (81X), where substantial $CaSO_4$ precipitation at the LC50 was evident.
282 ***Data analysis - LC50 estimation***
283 Median lethal concentrations (LC50s) and their confidence limits were estimated
284 similarly to Mount et al. (2016). For tests with at least 2 partial mortalities, a tolerance

285 distribution analysis was conducted, using a 3-parameter log-logistic model. Parameters were
286 estimated by maximum likelihood analysis using custom software written with Intel Visual
287 Fortran Compiler XE 2015 (Intel Corporation) and Winteracter 13.0 (Interactive Software
288 Services Ltd.). Confidence limits (95%) were calculated using the likelihood ratio method
289 (Williams, 1986). For tests with insufficient partial mortalities for this analysis, the same
290 likelihood ratio method was used to calculate confidence limits for the LC50, which can be
291 assigned in the absence of a unique point estimate for the LC50. For these tests, we assigned
292 the geometric mean of these confidence limits to be the point estimate for the LC50. For cases
293 in which there were no partial mortalities, these confidence limits are >95% and are the
294 bracketing concentrations (i.e., the highest concentration with control survival and the lowest
295 concentration with complete mortality), and the LC50 is equivalent to linear interpolation of
296 survival versus log concentration between these two concentrations.
297 LC50s were initially calculated as a percentage of the highest added concentration in the
298 test, and then converted to molar concentrations of the added salts. These concentrations
299 were then added to the ion concentrations already present in the dilution water and the
300 resultant total ion concentrations were input to the chemical speciation program Visual
301 MINTEQ (version 3.1, https://vminteq.lwr.kth.se/visual-minteq-ver-3-1) to estimate the
302 chemical activity of each chemical species at the LC50. The osmolarity at the LC50 was
303 estimated based on MINTEQ activity estimates using the method described by Robinson and
304 Stokes (1959). The sum of the concentrations of the major inorganic ions, mannitol, and
305 gluconate was also calculated as a potential exposure metric. Confidence limits for LC50s
306 expressed as these various metrics were assigned the same relative values as those for the

307 percent added concentration LC50s, and do not reflect any additional uncertainty associated
308 with the speciation or other calculations.

309 ***Data analysis – Evaluating relationships in binary salt mixture experiments***

310 As discussed in Erickson et al. (2017) and references therein, binary toxicity experiments
311 can be used to infer certain information about toxicity mechanisms, specifically whether the
312 toxicities of the two chemicals show concentration addition (suggesting a shared toxicity
313 mechanism), independent action, synergism, antagonism, or other interactions. Binary mixture
314 toxicity experiments in Erickson et al. (2017) contributed to the inferences about major ion
315 toxicity to *C. dubia* summarized in Table 1, using isobolograms that compared the observed
316 mixture LC50s to isoboles for concentration addition or independent action fitted to the
317 individual salt LC50s for the experiment. In the present study, isoboles are similarly fitted for
318 some data comparisons, but mixture experiment LC50s are also compared to isoboles predicted
319 based on prior results with individual salts. Furthermore, the prior data was just for Cl salts, so
320 that the predictions address consistency of relationships across different anions. Details on the
321 methods for both fitting and predicting isoboles are provided in the Supporting Information.

322 **RESULTS AND DISCUSSION**

323 Tables S1 and S2 in the Supporting Information provide detailed information for single
324 salt and mixture tests, including: control survivals; LC50s (with confidence limits) as total added
325 concentrations of each salt; and estimates at the LC50s for pH, activities for selected ionic
326 species, osmolarity, and total concentrations of solution components (total osmoles). Across all
327 tests, temperature measurements averaged 24.6°C, ranging from 24.1°C to 25.1°C. Dissolved
328 oxygen concentrations were >8 mg/L at test start and were no lower than 7.5 mg/L for
329 treatments with surviving organisms at test end. Additional exposure-effects and water quality

330 data are available at USEPA's Environmental Dataset Gateway
331 (https://edg.epa.gov/metadata/catalog/main/home.page).
332 For four tests, the mortality was <40% at the highest exposure or >50% at the lowest
333 exposure, so the LC50s in Tables S1 and S2 are reported as "greater than" or "less than"
334 concentrations, and the ion concentrations and activities are those for the limiting exposures.
335 For six tests, there was substantial (>25%) loss of Ca from the start to the end of exposure due
336 to precipitation of either $CaCO_3$ or $CaSO_4$, and Table S1 includes chemistries for both the start
337 and end of these tests. The initial test of $MgCO_3$ in 3X ALSW (Experiment 13-21) produced both
338 a "greater than" LC50 and substantial loss of Ca, so this experiment was repeated (Experiment
339 15-32). However, the repeat test for 3X ALSW proved even more problematic and is not
340 reported due to a nonmonotonic and very shallow concentration/exposure relationship
341 apparently caused by irregular trends of $CaCO_3$ precipitation across treatments.
342 ***Effects of dilution water on the toxicity of Mg salts***
343 ***Effects of dilution water Ca.*** For all modifications of Ca in dilution water (Tables 2 and
344 3), Figure 1A provides LC50s for $MgCl_2$, $MgSO_4$, and $MgCO_3$ based on added salt molarity versus
345 Ca concentration. LC50s in Figure 1A increase substantially with Ca concentration for all Mg
346 salts and for all methods for modifying Ca concentration. This adds to a consistent pattern of
347 such effects on major ion toxicity, including Ca effects on major ion toxicity to *C. dubia* (Mount
348 et al., 2016), effects of Ca on Na salt toxicity in the present study, and effects of hardness
349 reported by other authors (e.g., Elphick et al., 2011a,b).
350 The LC50 for the $MgCO_3$ test in 3X ALSW is uncertain because this is a "greater than"
351 value; however, because there was 30% mortality at the highest concentration (Table S1), the
352 LC50 should be no more than 20% higher based on typical slopes of the concentration/effect

353 curves. This LC50 is also uncertain because the Ca changed during the test due to $CaCO_3$
354 precipitation. The Ca concentration to associate with this LC50 is unlikely to be at either
355 extreme of the Ca range, but could be lower than the indicated midpoint of the range to the
356 extent that mortality reflects increased potencies (lower Ca) at the end of the test. Regardless
357 of these uncertainties, this test supports a Ca effect on Mg toxicity.

358 ***Effects of other dilution water characteristics.*** In contrast to the effects of Ca, LC50s for
359 $MgSO_4$ did not change substantially when just Na and Cl were modified in the dilution water
360 (Table S1, Experiment 13-31). Furthermore, if the consistent effects on LC50s of the three
361 methods for changing Ca are due to changes to ions other than Ca, then the LC50 changes
362 should be correlated to these other ions. Instead, increasing LC50s for Mg salts in Figure 1A are
363 associated with increasing Na for the ⅓X/1X/3X ALSW series (see Na concentrations in Table S1
364 for Experiments 13-20, 13-21, 15-32), with decreasing Na for the ⅓X/1X Ca tests (Experiments
365 15-06, 15-18), and with constant Na for the tests with altered Ca:Mg ratio and for Ca elevated
366 above ALSW (Experiments 13-28, 15-06, 15-08). Similar inconsistencies exist for Cl. This further
367 argues against Na and Cl being modifiers for Mg toxicity.

368 However, there are some indications of effects of dilution water on toxicity other than
369 from Ca. At the lowest Ca concentration, the smallest LC50 for $MgSO_4$ in Figure 1A is for a
370 dilution water with a very low ratio of Ca to other ions and is more than 3 times smaller than
371 the LC50 for ⅓X ALSW, for which ion ratios are more typical of natural waters. It is uncertain
372 whether this variation in toxicity is due to the different relative ion concentrations or due to
373 other consequences of testing at very low Ca, where variability among tests was also high for
374 *C. dubia* (Mount et al., 2016; Erickson et al., 2018). However, these variations are of limited

375 practical importance because toxic Mg concentrations are unlikely to co-occur with such low Ca
376 concentrations (Erickson et al. 2018).

377 ***Implications of single salt tests on exposure metrics for Mg salt toxicity***

378 ***Exposure metric for $MgCl_2$ and $MgSO_4$ toxicity.*** In addition to addressing effects of
379 dilution water composition, the data in Figure 1 inform the issue of what exposure metric is
380 best related to Mg toxicity. Based on total added molar concentrations (Figure 1A), $MgSO_4$ is
381 approximately half as toxic as $MgCl_2$ for tests at Ca concentrations from 0.3 to 3 mM. In
382 contrast, based on ion activities (Figure 1B), $MgCl_2$ and $MgSO_4$ have similar toxicities for these
383 same tests, their LC50 confidence limits usually overlapping and differences being less than
384 interexperimental variability. This illustrates how chemical speciation (e.g., complexation of Mg
385 by SO_4) can reduce toxicity on a total chemical basis and how an exposure metric such as {Mg}
386 can better relate to toxicity. At lower Ca concentrations (<0.3 mM), the relative toxicities of
387 $MgSO_4$ and $MgCl_2$ are less clear, due to limited data and to the increasing and variable toxicity
388 as Ca becomes extremely low.

389 ***Exposure metric for $MgCO_3$ toxicity.*** The toxicity of $MgCO_3$ relative to $MgCl_2$ shows a
390 different pattern than $MgSO_4$. Despite Mg complexation by HCO_3/CO_3, $MgCO_3$ is more toxic
391 than $MgCl_2$ on a molar concentration basis (Figure 1A) and becomes even more toxic for the 1X
392 and 3X ALSW dilution waters when expressed as activities (Figure 1B). These LC50s are
393 associated with high pH (9.2) and high alkalinity (≥36 meq/L) (Table S1), which might adversely
394 impact respiratory CO_2 excretion and acid/base balance (Wood, 1992; Truchot and Fogue,
395 1998) in addition to any Mg-related toxicity. *C. dubia* did not exhibit such enhanced toxicity
396 (Mount et al., 2016; Erickson et al., 2017, 2018), which may be due to interspecies differences
397 in respiratory relationships or to alkalinity not reaching these high levels due to the greater

sensitivity of *C. dubia* to Mg. An effect of pH relevant to this was demonstrated for $NaHCO_3$ toxicity, which is discussed in *Effects of dilution water chemistry on toxicity of Na salts*. Due to the limited data and multiple confounded factors (pH, alkalinity, Mg), it is uncertain how best to relate exposures and effects for $MgCO_3$-dominated solutions.

Mg toxicity model for isobole predictions. Figure 1B also shows the regression analysis of $MgCl_2$ toxicity data that will be used in isobole predictions for mixture experiments that include Mg salts. This regression analysis used Equation S1 from the Supporting Information, and included data from two $MgCl_2$ toxicity experiments for a range of {Ca} relevant to the mixture experiments ({Ca}<0.4 mM) and from other tests of $MgCl_2$ toxicity in ALSW. The resultant {Mg}-based model for Mg toxicity is:

$$LC50_{\{Mg\}} = 13.2 \times \left(1 - 10^{-\{Ca\}/0.114}\right), \quad R^2 = 0.75 \qquad \text{(Eq. 1)}$$

The pooled relative standard deviation across $MgCl_2$ tests in ALSW (n=9, Tables S1 and S2) for informing the uncertainty of this regression is 8.2%.

Interactions in binary mixtures of Mg salts

$MgCl_2$×$MgSO_4$ binary mixture experiment. Figure 2A presents the isobologram for LC50s based on molarity of added salt. The closeness of the data to a straight line indicates a strong degree of additivity, supportive of Mg-driven toxicity. However, [Mg] at the LC50 steadily increases as the proportion of $MgSO_4$ in the tests increases, reaching nearly 60% greater for the $MgSO_4$-only test than for the $MgCl_2$-only test, indicating total concentration is not an appropriate exposure metric for Mg-based toxicity.

In contrast, LC50s for the $MgCl_2$×$MgSO_4$ mixtures expressed as {Mg} (Figure 2B) are less variable and are consistent with predicted values from the {Mg}-based toxicity model (Equation 1). This prediction includes a small (ca. 10%) reduction of LC50 as the molar fraction of $MgSO_4$

421 increases, due to complexation of Ca by SO_4. Due to the unusually low toxicity for the $MgCl_2$-
422 only exposure in this experiment (which is the lowest black dot in Figure 1B), this Ca effect is
423 not evident in these data. However, a Ca-effect consistent with model predictions is evident in
424 mean LC50s in ALSW across the entire study (Tables S1 and S2), which on the basis of {Mg} drop
425 from 12.1 mM for $MgCl_2$ tests (n=9) to 10.9 mM for $MgSO_4$ tests (n=6).

426 ***$MgCl_2$×$MgCO_3$ binary mixture experiment.*** Figure 2C presents the isobologram for
427 LC50s based on molarity of added salt. Again, the closeness of the data to their linear
428 regression line suggests considerable additivity. However, the Mg concentration at the LC50 for
429 this mixture is 30% lower for $MgCO_3$ than $MgCl_2$, in the opposite direction of that expected
430 from increased complexation and again indicative of total concentration not being a good
431 exposure metric.

432 However, unlike for the $MgCl_2$×$MgSO_4$ mixtures, when LC50s are expressed as {Mg},
433 some $MgCl_2$×$MgCO_3$ mixtures deviate from the {Mg}-based toxicity model predictions (Figure
434 2D). The observed LC50s are reasonably consistent with predicted LC50s up to an equimolar
435 mixture, but show increasingly greater toxicity than expected at higher mole factions of $MgCO_3$.
436 This deviation is associated with pH≥9.1 and {HCO_3} >16 mM, compared to pH≤9.0 and
437 {HCO3}<13 mM for the mixtures with lower mole fractions of $MgCO_3$ (Table S2). This is
438 consistent with the deviations of $MgCl_2$ LC50s from $MgCO_3$ LC50s for 1X ALSW in Figure 1B, for
439 which pH was 9.2 and {HCO_3} was 18 mM (Table S1). This again suggests some effect of the
440 combined high pH and alkalinity in $MgCO_3$-dominated exposures other than that expected just
441 from effects of Ca on Mg-driven toxicity. Equation 1 with {Mg} as the exposure metric is thus
442 useful for mixtures containing up to at least half $MgCO_3$, but the metric for $MgCO_3$-dominated

443 exposures is uncertain. Addressing toxicity in high carbonate exposures is further discussed in
444 the companion modeling paper (Erickson et al. 2022).

445 ***Interactions in binary mixtures of Mg and Ca salts***

446 Figure 3 provides a combined isobologram for the $MgCl_2$×$CaCl_2$ and $MgSO_4$×$CaCl_2$
447 mixture experiments based on Mg and Ca activities, showing good agreement between the Cl-
448 only and combined Cl/SO_4 mixtures, thereby supporting cation-driven toxicity. The linearity of
449 {Mg} versus {Ca} for all but the lowest level of Ca indicates concentration addition, with effects
450 of Ca on Mg toxicity (Figure 1B) causing the LC50 at the lowest Ca to deviate from the line. As
451 for *C. dubia* (Erickson et al., 2017, 2018), this additivity indicates a common mechanism with
452 {Mg} and {Ca} as the exposure metrics. A prediction for this isobole is not provided here
453 because Equation 1 does not address the complete Ca range; rather, this relationship is further
454 addressed in the companion modeling paper (Erickson et al. 2022).

455 ***Effects of dilution water chemistry on toxicity of Na salts and mannitol***

456 ***Effects of dilution water Ca.*** For all modifications of Ca in dilution water (Tables 2 and
457 3), Figure 4A provides LC50s for NaCl, Na_2SO_4, $NaHCO_3$, and mannitol based on added
458 compound concentration versus Ca concentration. Figure 4A also provides the two tests of
459 $NaHCO_3$ toxicity in 1X ALSW for which test solutions were equilibrated prior to exposure with
460 either ambient air or with 1% CO_2.

461 LC50s for NaCl and Na_2SO_4 increase substantially with Ca concentration for all methods
462 for modifying Ca concentration. Relative to the other salts, the data for $NaHCO_3$ are more
463 limited, consisting of three non-equilibrated tests in the ⅓X/1X/3X ALSW series and the pre-
464 equilibrated test at high pH in 1X ALSW, and more uncertain due to the large changes in Ca
465 concentration during the non-equilibrated tests in 1X and 3X ALSW. Nevertheless, based on

466 mid-range Ca concentrations, the Ca-dependence of $NaHCO_3$ is similar to that for NaCl and
467 Na_2SO_4. Only if the LC50s for the non-equilibrated 1X and 3X ALSW tests are related to the
468 lowest Ca concentrations (at the end of the tests) would a Ca dependence not be evident.
469 However, the pre-equilibrated test at high pH in 1X ALSW argues against such an interpretation
470 because it was run at a constant Ca concentration similar to those at the end of the non-
471 equilibrated 1X and 3X ALSW tests, and indicates the LC50 to be considerably lower at this Ca
472 concentration.

473 In contrast to the Ca-dependence of inorganic Na salt toxicity, there is no effect of Ca on
474 mannitol toxicity (Figure 4A). The ancillary experiment for the Ca-dependence of mannitol
475 toxicity *C. dubia* similarly showed no effects (see Supporting Information). If mannitol toxicity is
476 due to osmotic effects on passive water movement across membranes, a lack of an effect of Ca
477 is understandable, as Ca effects should relate more to ion transport (Wood, 1992; Griffith,
478 2017) than to water osmosis.

479 ***Effects of other dilution water constituents.*** In contrast to Ca, Mg has no apparent
480 effect on the toxicity of Na salts. Increasing LC50s for Na salts in Figure 4 are associated with
481 increasing Mg for the variable ALSW tests (see Mg concentrations in Table S1 for Experiments
482 13-19, 13-22, 13-23), with decreasing Mg for variable Mg:Ca ratio tests (Experiment 13-28), and
483 with constant Mg for the variable Ca tests (Experiments 15-02, 15-16, 18-14). These same
484 experiments also showed inconsistent correlations of NaCl salt toxicity to SO_4 in the dilution
485 water and of Na_2SO_4 toxicity to Cl in the dilution water (Table S1), suggesting little or no effect
486 of these anions. Changes in K had no effects on the toxicity of NaCl, but reduced that of Na_2SO_4
487 by 17% and that of $NaHCO_3$ by 30% (Table S1, Experiment 13-24). However, this effect of K on

488 Na_2SO_4 toxicity is of uncertain statistical significance and this effect of K on $NaHCO_3$ toxicity is
489 confounded by uncertainties regarding Ca effects and the impact of high pH/alkalinity.
490 There is an effect of pH on $NaHCO_3$ toxicity, as evidenced by the test with an
491 unregulated pH of 9.3 having an LC50 approximately half of that in the test under an enriched
492 CO_2 atmosphere with pH 8.4 (Figure 4A). This pH effect is also pertinent to the toxicity of
493 $MgCO_3$ being greater than that for $MgCl_2$ and $MgSO_4$ (Figure 1).
494 ***Implications of single salt tests on exposure metrics for Na salt toxicity***
495 ***Added molar salt concentration as exposure metric.*** In addition to addressing effects of
496 dilution water composition, the data in Figure 4 inform the issue of what exposure metric is
497 best related to Na salt toxicity. Using added molar concentration as the exposure metric
498 (Figure 4A) results in considerable differences among the chemicals. Depending on Ca, Na_2SO_4
499 is 2 to 5 times more toxic than NaCl. $NaHCO_3$ is 2 to 3 times more toxic than NaCl when pH is
500 not regulated (9.3-9.5), although similar when pH is reduced. In contrast, mannitol is
501 substantially less toxic than NaCl, by a factor ranging from about 2.5 at higher Ca to 10 at lower
502 Ca.
503 Many of these differences in toxicity based on added molar concentration have
504 plausible explanations. Na_2SO_4 has more Na per mole than NaCl and SO_4 would complex Ca
505 more strongly than Cl, both contributing to greater toxicity than NaCl. For $NaHCO_3$,
506 complexation of Ca would also increase toxicity relative to NaCl. Because $NaHCO_3$ toxicity when
507 pH is reduced corresponds well to that of NaCl and because pH alone up to 9.5 does not cause
508 direct toxicity to fathead minnows (J.R. Hockett, unpublished data), the greater toxicity of
509 $NaHCO_3$ at high pH suggests some joint action of high pH (9.2-9.4) and alkalinity (>25 meq/L),
510 consistent with that for $MgCO_3$ (Table S1). If osmolarity is a factor in Na salt toxicity at higher

511 Ca, mannitol would be less toxic on a molarity basis due to having only one osmole per mole
512 compared to two for NaCl. All these explanations indicate a need for better exposure metrics
513 than total concentration.

514 ***Osmolarity as exposure metric.*** Because prior work with *C. dubia* indicated osmolarity
515 to be a useful exposure metric for Na salt toxicity, Figure 4B plots LC50s based on osmolarity
516 versus {Ca}. Inter-chemical variability is greatly reduced relative to Figure 4A, but does not
517 achieve the same degree of concordance among chemicals as for *C. dubia* (Figure 7C in Mount
518 et al., 2016). At {Ca} > 0.2 mM, LC50s for mannitol, NaCl, and Na_2SO_4 do converge within inter-
519 experimental variability and are similar to internal osmolarities of fish (Hoar and Randall, 1969),
520 so that osmolarity is a plausible exposure metric in this Ca range for Na salt toxicity.

521 However, at lower Ca, osmolarity-based LC50s for NaCl and Na_2SO_4 become increasingly
522 smaller with declining Ca, whereas mannitol LC50s remain constant. This indicates that osmotic
523 effects cannot be the cause of Na salt toxicity at these lower Ca activities, so that osmolarity is
524 not a good mechanistic exposure metric for this range and other exposures metrics must be
525 considered. Furthermore, the osmolarity-based LC50s of NaCl and Na_2SO_4 in Figure 4B also
526 diverge from each other at lower Ca, apparently due to SO_4 toxicity when it is the sole or nearly
527 sole anion (see *Interactions in binary Na salt mixtures*), and differences between $NaHCO_3$ and
528 NaCl toxicities also still exist, further underscoring the need for other exposure metrics.

529 ***Na activity as exposure metric.*** One interpretation for the divergence of Na salt
530 toxicities from mannitol toxicity is that Na salts have specific ion-related toxicities at low Ca that
531 decrease with increasing Ca until the LC50s are high enough to reach an osmolarity-related
532 toxicity and coincide with the LC50 for mannitol. As a first consideration for a different
533 exposure metric at low Ca, Figure 4C replots the data based on {Na}, this being the common ion

for these salts. This further reduces differences in LC50s among the salts at lower Ca compared to Figure 4B, but even on this basis, Na_2SO_4 and $NaHCO_3$ remain more toxic than NaCl at lower Ca, albeit by modest amounts. Although the idea that {Na} is a causal factor independent of anions parallels the inference that the toxicities of Mg, K, and Ca salts are cation-driven, this lack of concordance among the Na salts argues against it. How best to express exposure is further informed by the binary mixture experiments discussed in subsequent sections.

Na toxicity model for isobole predictions. Figure 4C also shows the regression analysis of NaCl toxicity data that will be used in isobole predictions for mixture experiments that include Na salts. The regression analysis used Equation S1 from the Supporting Information, and included data from five NaCl toxicity experiments for a range of {Ca} relevant to the mixture experiments ({Ca}<0.2 mM) and from other tests of NaCl toxicity in ALSW. The resultant {Na}-based model for Na toxicity is

$$LC50_{\{Na\}} = 100 \times \left(1 - 10^{-\{Ca\}/0.126}\right), \quad R^2 = 0.86 \qquad \text{(Eq. 2)}$$

The pooled relative standard deviation across NaCl tests in ALSW (n=13, Tables S1 and S2) for informing the uncertainty of this regression is 7.8%.

Interactions in binary mixtures of Na salts

NaCl×Na_2SO_4 binary mixture experiment. Figure 5A presents the isobologram for LC50s based on molarity of added salt. Starting at the NaCl-only LC50 on the abscissa, the data for all the mixtures follow a straight line indicative of additivity, but then abruptly deviate horizontally to the Na_2SO_4-only LC50 on the ordinate. This deviation is consistent with the divergence of NaCl and Na_2SO_4 toxicities at low Ca in Figure 4. Because the only component that increases with this deviation is SO_4, this suggests a SO_4-specific toxicity once SO_4 (or the ratio of SO_4 to

556 another ion) reaches some critical level. Below this critical level, the additive relationship
557 between Na_2SO_4 and NaCl suggests a different mechanism involving only Na or multiple ions.
558 To illustrate this further, Figure 5D presents the data as {Na} versus {SO_4}, with
559 predictions from the {Na}-based toxicity model (Equation 2). The NaCl-only LC50 for this
560 experiment is the lowest in the study (lowest black dot on Figure 4C) and the mixture data also
561 follow the lower limit of the uncertainty band, declining gradually as {Ca} decreases due to
562 complexation by SO_4. However, for the Na_2SO_4-only test, the LC50 abruptly deviates from
563 predictions as SO_4 apparently reaches a toxic level. It is important to note that the adherence
564 of the data to the model line at lower SO_4 does not provide proof that {Na} is the toxicity driver,
565 but only that it is a good correlate; this toxicity could be due to the combined effect of multiple
566 ions as was the apparent case for *C. dubia* (Mount et al., 2016; Erickson et al., 2017). SO_4
567 toxicity is also indicated by study-wide mean LC50s in ALSW from Tables S1 and S2, which on
568 the basis of {Na} drop from 89 mM for NaCl (n=13) to 55 mM for Na_2SO_4 (n=6), twice the drop
569 from 89 mM to 72 mM predicted by the {Na}-based model.
570 ***NaCl×$NaHCO_3$ binary mixture experiment.*** Figure 5B presents the isobologram for
571 LC50s based on molarity of added salt. Although the general linearity of the data suggests a
572 considerable degree of additivity, the low LC50s for $NaHCO_3$-dominated test solutions indicate
573 impacts of other factors. More clarity regarding this is provided by Figure 5E, which plots {Na}-
574 based LC50s versus the mole fraction of $NaHCO_3$ in the mixture, along with predictions from the
575 {Na}-based model of Equation 2. There is considerable $CaCO_3$ precipitation once the mole
576 fraction reaches 20% (Table S2), causing increased toxicity due to low {Ca}. The model does not
577 predict this precipitation, but does predict the general drop of the LC50s when measured Ca
578 concentrations are provided to it. The $NaHCO_3$-only LC50 is not included in the prediction

because the {Ca} for this test is beyond the range for Equation 2 and because of the possible role of elevated pH/alkalinity in such $NaHCO_3$-dominated exposures. Equation 2 with {Na} as the exposure metric is thus appropriate for NaCl×$NaHCO_3$ mixtures containing considerable $NaHCO_3$, but the appropriate metric for $NaHCO_3$-dominated exposures is uncertain.

Interactions in binary mixtures of NaCl and mannitol

A mixture experiment involving NaCl and mannitol (Figure 5C) was conducted to further address whether osmolarity is a useful exposure metric for some aspects of ion toxicity. The dashed line connects the molarity of the mannitol-only LC50 to an estimated molarity of NaCl needed to produce the same level of osmolarity, so represents additive toxicity for an exposure metric of osmolarity. Importantly, mixture toxicities are being elicited at combinations of mannitol and NaCl concentrations which individually would not be toxic, demonstrating considerable additivity; i.e., NaCl contributes to mannitol's mechanism of toxicity. There are upward deviations of mixtures from this line, but these are <20%. At higher proportions of NaCl there is a modest trend toward LC50s with lower osmolarities, suggesting a shift away from osmolarity-driven toxicity.

The relationships in Figure 5C are consistent with the data in Figure 4, which suggested that NaCl has a mechanism that is specifically ion-related and more toxic than mannitol at low Ca, but, as this toxicity decreases with increasing Ca, NaCl LC50s converge with that of mannitol and transition to a mechanism correlated with osmolarity. The mixture test in Figure 5C was conducted at Ca levels near but slightly below this transition, which would explain both the substantial degree of additivity on an osmolarity basis and the deviation to lower osmolarities for the NaCl-dominated tests.

601 Figure 5F plots LC50s on the basis of osmolarity versus {Na} to better illustrate
602 interaction of these two suggested mechanisms, with predictions based on the Ca-dependent
603 {Na}-based model (Equation 2) and an average study-wide, Ca-independent, osmolarity-based
604 LC50 for mannitol-only tests (278 mOsm/L, n=7, Tables S1 and S2). There are undoubtedly
605 complexities to these toxicity relationships that are not fully resolved here and data trends are
606 modest relative to uncertainties, but these predictions are reasonably consistent with the data;
607 namely, osmolarities at the LC50 are roughly constant and similar to mannitol for most
608 mixtures and a transition to a specific ion-related mechanism is indicated at the highest
609 proportions of NaCl. If this mechanistic interpretation is correct, the shape of this isobole
610 would be expected to change at different dilution water Ca concentrations, showing more
611 independence at lower Ca and more complete additivity at higher Ca, which would be a useful
612 element of future study.
613 Given the lack of environmental relevance of exposure to mannitol, the results in
614 Figures 5C and 5F might seem to be of only academic interest. However, there is a need to
615 address interactions of Na salts with salts of other cations, and interpreting such interactions
616 will be informed by these interactions of NaCl and mannitol.
617 ***Interactions in binary mixtures of Na and Ca salts***
618 Figure 6 provides a combined isobologram for LC50s from the $NaCl \times CaCl_2$ and
619 $Na_2SO_4 \times CaCl_2$ mixture experiments, as osmolarity versus {Ca}. It also includes predicted LC50s
620 at {Ca}>3 mM for independent action of osmolarity-based toxicity (the aforementioned study-
621 wide average of 278 mOsm/L for mannitol toxicity) and Ca-specific toxicity (a study-wide
622 average of 15 mM {Ca} for $CaCl_2$ toxicity from Tables S1 and S2). At these higher {Ca}, the
623 observed LC50s are consistent with independent action of these mechanisms; i.e., starting with

624 the $CaCl_2$-only tests at the lower right, Ca activities at the LC50 first remain constant as Na salts
625 are added and then transition to osmolarity-based LC50s similar to those for mannitol. As was
626 the case for *C. dubia* (Erickson et al. 2017), there is an indication of greater-than-expected
627 toxicity when the Ca-specific and osmolarity mechanisms are at roughly equal toxicity (the bend
628 in the prediction line), but the deviation is only for one test and is <20%.

629 The Ca-dependent toxicity at lower Ca is not addressed by the predictions in Figure 6
630 because it involves only two data and these have different mechanisms of toxicity. More
631 complete consideration of this part of the relationship is provided in the companion modeling
632 paper (Erickson et al. 2022).

633 ***Interactions in binary mixtures of Na and Mg salts***

634 ***NaCl×$MgCl_2$ binary mixture experiment***. Figure 7A compares LC50s based on added
635 concentrations of NaCl and $MgCl_2$ to fitted isoboles for concentration addition and independent
636 action. The mixture with the highest NaCl fraction does fall on the independent action line, but
637 the data then veer off to be intermediate to the two isoboles (i.e., "partial addition"). Plotting
638 the data as {Mg} vs {Na} and using the independent action isobole predicted from Equations 1
639 and 2 has no appreciable effect on this pattern (Figure 7B), which was expected due to the
640 strong correlation between added concentrations and cation activities. A primary implication
641 of this is that there is a mechanism of toxicity to which both $MgCl_2$ and NaCl contribute, instead
642 of or in addition to any mechanisms related just to the individual salts.

643 For *C. dubia*, similar plots (Figures 3A-C in Erickson et al., 2017) showed LC50s to also be
644 intermediate between concentration addition and independent action, although closer to the
645 latter. When LC50s were expressed as {Mg} versus osmolarity there was close adherence to
646 independent action (Figures 3D-F in Erickson et al., 2017). This was interpreted as Na salt

toxicity involving a mechanism involving multiple ions, to which the Mg salts also contribute. Osmolarity was selected as the metric for this mechanism because of the close concordance of osmolarity-based salt LC50s with mannitol LC50s, at least at the {Ca} for the mannitol tests; these are also in accord with *D. magna* internal osmolarities indicated in Morris et al. (2021). However, this does not mean that effects are from osmotic stress across all {Ca}.

Because of such relationships for C. dubia, Figure 7C compares LC50s on the basis of {Mg} and osmolarity to a model of independent action for {Mg} (using Equation 1) and osmolarity (278 mOsm/L). The LC50s for the three tests with the highest mole fraction of NaCl have approximately the same osmolarity, consistent with toxicity being related to multiple ions, but are offset from the predictions by 15-25%, indicating osmolarity is not the best metric. This deviation from the predicted osmolarity is consistent with this mixture experiment having {Ca} low enough that NaCl LC50s are reduced compared to higher {Ca} where they closely agree wth mannitol LC50s (Figure 4B).

Because this deviation from mannitol LC50s is not great, osmolarity might still contribute to toxicity, jointly with other mechanisms of toxicity. This possibility is considered in Figure 7D, in which LC50s on the basis of Mg and Na activities are compared to a three-mechanism model that includes not only {Mg}-based and {Na}-based toxicity based on Equations 1 and 2, but also osmolarity-driven toxicity using the study-wide average mannitol LC50 of 278 mOsm/L. This moves the isobole closer to the observed LC50s, but falls short enough of the data that this is not a convincing mechanistic explanation.

$NaCl \times MgSO_4$ and $Na_2SO_4 \times MgCl_2$ binary mixture experiments. These experiments have results similar to that for $NaCl \times MgCl_2$, with LC50s expressed as Mg and Na concentrations or activities showing relationships intermediate to concentration addition and independent

670 action. And because these mixed anion experiments had lower {Ca} due to complexation by
671 SO_4, osmolarity is expected to be even less explanatory of the LC50s. More details on these
672 experiments are provided in the Supporting Information.
673 Overall, the results of the $NaCl \times MgCl_2$, $NaCl \times MgSO_4$ and $Na_2SO_4 \times MgCl_2$ binary mixture
674 experiments demonstrate partial additivity, or some other interaction, between these Na and
675 Mg salts. Therefore, further model development at {Ca} lower than that at which toxicity is
676 credibly related to osmolarity (≈0.2 mM, Figure 4) requires a metric instead of, or in addition to,
677 the {Na} used in Equation 2 which can address interactions of NaCl toxicity with other ions.
678 Based on the information in the present study, a suitable mechanistic metric based on the
679 activities of specific ions is not clear. The implication of this to the development of models for
680 ion toxicity will be further addressed in the companion modeling paper (Erickson et al. 2022).
681 ***$Na_2SO_4 \times MgSO_4$ binary mixture experiment***. In contrast to the three other Na salt × Mg
682 salt mixtures, the isobole for $Na_2SO_4 \times MgSO_4$ on an added salt basis (Figure 7E) is nearly linear,
683 indicative of additive toxicity, although some deviation from the line is suggested at the $MgSO_4$-
684 only test. On a cation activity basis (Figure 7F), the isobole is also largely linear, deviating
685 greatly from the predicted isobole for independent action using Equations 1 and 2, except for
686 the $MgSO_4$-only test. This linear isobole is supportive of the apparent SO_4 toxicity noted
687 regarding Figures 4 and 5. Figure 7G shows how toxicity in this experiment is associated with
688 constant {SO_4}, except at the LC50 in the $MgSO_4$-only test, which suggests a transition to Mg-
689 driven toxicity.
690 ***Interactions of KCl with NaCl and $MgCl_2$***
691 The LC50s for both KCl and K_2SO_4 were similar on the basis of {K} and showed
692 monotonic increases over the ⅓X/1X/3X ALSW dilution water series (Table S1, Experiments 13-

19 and 13-23). For *C. dubia* (Mount et al., 2016), similar increases were shown to be related to the Na concentration in the dilution water, based on tests in waters with variable NaCl concentrations (Figure 8A). However, fathead minnows differ from *C. dubia* in showing no such dependence on Na (Figure 8A). Such interspecies differences might reflect differences in K transporters in fish versus invertebrates (Griffith, 2017). The reason for the effect of different ALSW strengths on K salt toxicity to fathead minnows is unexplained and warrants further testing regarding other attributes of the dilution water, such as Ca concentration.

Figures 8B and 8C show that, like for *C. dubia* (Erickson et al., 2017), K appears to involve a specific mechanism of toxicity that is independent of the toxicity of both NaCl and $MgCl_2$. For *D. magna*, Morris et al. (2021) reported increased K concentrations in the hemolymph as K exposures approached lethality. This would represent disturbance of the normal K gradients between intracellular and extracellular fluid, consistent with a specific action for K that should be independent of other ions. Such independence of K toxicity means that, in the event that K exposure does get to toxic levels, toxicity data for K salts could be used independently of models for the toxicities of Na, Mg, and Ca salts.

SUMMARY

A total of 170 toxicity tests were conducted regarding the acute toxicities of major geochemical salts to fathead minnows, evaluating the impact of dilution water characteristics on these toxicities and the toxicity interactions in binary mixtures of these salts. The relationships among test results supported several inferences regarding mechanisms of toxicity and useful exposure metrics.

Mg and Ca salt toxicities. An important dilution water characteristic affecting the toxicity of Mg salts is Ca content, consistent with previous results for *C. dubia* (Mount et al.,

716 2016; Erickson et al., 2017). Toxicities of Mg and Ca salts are best related to their cation
717 activities and are concentration additive with each other, indicating a shared mechanism, as
718 was also the case for *C. dubia*. However, in contrast to *C. dubia*, the toxicity of $MgCO_3$-
719 dominated exposures is greater than that of $MgCl_2$ and $MgSO_4$ on the basis of {Mg}, this
720 enhanced toxicity possibly reflecting direct effects of combined high pH and alkalinity, requiring
721 separate consideration in risk assessments.

722 ***K salt toxicities.*** In contrast to findings for *C. dubia* (Mount et al., 2016), the toxicity of
723 KCl to fathead minnows is not affected by Na, but is reduced modestly by increasing all ions,
724 with the specific cause of this being uncertain. Toxicities of K salts are also best related to
725 cation activity and are independent of the toxicities of other salts, as was the case for *C. dubia*
726 (Mount et al., 2016; Erickson et al., 2017).

727 ***Na salt toxicities.*** An important dilution water characteristic affecting the toxicity of Na
728 salts is Ca concentration, also consistent with previous results for *C. dubia* (Mount et al., 2016;
729 Erickson et al., 2017). Another notable effect of dilution water is reduction of $NaHCO_3$ toxicity
730 when the high unregulated pH (9.3) in tests with this salt is reduced to 8.4 by increasing CO_2
731 concentration.

732 The toxicities of Na salts and mixtures are not simply related to Na but involve
733 mechanisms related to multiple ions. For *C. dubia*, this was addressed across all Ca
734 concentrations using an exposure metric of osmolarity (Mount et al., 2016; Erickson et al.,
735 2017, 2018), although osmolarity per se was not claimed to be causative at all Ca. The present
736 study indicates the need to consider multiple mechanisms. Osmolarity is possibly causative at
737 Ca concentrations above that of ALSW, where osmolarity-based toxicities of Na salts are similar
738 to that of mannitol. At lower Ca, Ca-dependent Na salt toxicities deviate from Ca-independent

739 mannitol toxicity, indicating different mechanisms. This is also true for further development of
740 the models for *C. dubia* toxicity, given the Ca independence of mannitol toxicity for this species
741 reported in the Supplemental Information.

742 The toxicities of NaCl, Na_2SO_4, and $NaHCO_3$ at lower Ca also indicate different
743 mechanisms among these salts. For Na_2SO_4-dominated exposures, toxicity is best attributed to
744 {SO_4}, but become additive with NaCl in mixtures that are not SO4-dominated, suggesting
745 Na_2SO_4 also contributes to a mechanism in common with NaCl. Similar to $MgCO_3$, the toxicities
746 of $NaHCO_3$-dominated exposures toxicity are greater than expected based on NaCl toxicity,
747 apparently due to combined effects of high pH and alkalinity as evidenced by the effect of pH
748 on toxicity. $NaHCO_3$ also becomes additive with NaCl in mixtures that are not HCO_3-dominated,
749 suggesting $NaHCO_3$ also contributes to a mechanism in common with NaCl.

750 For Na salt toxicities not attributable to high pH/alkalinity, SO_4, or osmolarity, the
751 appropriate exposure metric is unresolved, despite good correlations with {Na} and osmolarity.
752 Because mixture tests of Na salts with Mg salts indicate some degree of additivity, the exposure
753 metric should not simply be {Na}, but include contributions of other ions. Using osmolarity to
754 address these multi-ion effects would also not be advisable because it suggests that the
755 mechanism involves osmotic effects, which likely is not the case.

756 ***Toxicity modeling implications.*** These results for fathead minnow present a more
757 complicated picture than in Erickson et al. (2018), because *C. dubia* shows neither enhanced
758 toxicity from high pH/alkalinity nor SO_4-related toxicity, and because different mechanisms at
759 different Ca were not then evident for general ion toxicity to *C. dubia*. Application of this
760 information for fathead minnow to toxicity models thus presents more challenges, both in how
761 models should be formulated and what range of exposure conditions should be included.

762 Development of these models and certain implications to risk assessment are addressed in the
763 companion paper (Erickson et al. 2022).

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854 **Table 1.** Interactions for the toxicity of major ions to *C. dubia*

Salts	Exposure Metric(s) Related to Toxicity	Important Interactions
Na Salts	Osmolarity	• Additive toxicity across different Na salts • Toxicity↓ as Ca↑ at nontoxic [Ca]
K Salts	K Activity	• Additive toxicity across different K salts • Toxicity↓ as Na↑ at nontoxic [Na] • Negligible effect of Ca
Mg Salts	Mg Activity	• Additive toxicity across different Mg salts • Toxicity↓ as Ca↑ at nontoxic [Ca]
Ca Salts	Ca Activity	• Additive toxicity across different Ca salts
Na×K Salt Mixtures	Osmolarity K Activity	• Independent toxicity • Both salts contribute to osmolarity • Superimposed on effect of Na on K toxicity
Na×Mg Salt Mixtures	Osmolarity Mg Activity	• Independent toxicity • Both salts contribute to osmolarity • Secondary effects of Ca activity variation
Na×Ca Salt Mixtures	Osmolarity Ca Activity	• Nearly independent toxicity • Both salts contribute to osmolarity • Superimposed on effect of Ca on Na toxicity
K×Mg Salt Mixtures	K Activity Mg Activity	• Independent toxicity • Secondary effects of Ca activity variation
K×Ca Salt Mixtures	K Activity Ca Activity	• Independent toxicity
Mg×Ca Salt Mixtures	Mg Activity Ca Activity	• Additive toxicity • Superimposed on effect of Ca on Mg toxicity

855

856 **Table 2.** Major ion concentrations in various dilution waters used in study.

Dilution Water	Na (mM)	K (mM)	Ca (mM)	Mg (mM)	Cl (mM)	SO_4 (mM)	Alkalinity (meq/L)
LSW	0.070	0.015	0.349	0.120	0.042	0.035	0.859
ALSW	0.282	0.039	0.364	0.168	0.216	0.129	0.859
⅓X ALSW	0.094	0.013	0.121	0.056	0.072	0.043	0.286
3X ALSW[1]	0.845	0.116	1.09	0.488	0.648	0.440	2.54
LoCa:Mg	0.282	0.039	0.075	0.410	0.216	0.150	0.763
HiCa:Mg	0.282	0.039	0.475	0.053	0.216	0.150	0.849
LoK:Na	0.333	0.005	0.364	0.163	0.216	0.155	0.848
HiK:Na	0.082	0.256	0.364	0.163	0.216	0.155	0.848
1.6mgNa/L	0.070	0.039	0.364	0.168	0.042	0.110	0.859
10mgNa/L	0.435	0.039	0.364	0.168	0.407	0.110	0.859
30mgNa/L	1.31	0.039	0.364	0.168	1.278	0.110	0.859
⅓X Ca	0.767	0.039	0.121	0.168	0.216	0.146	0.859
3X Ca	0.282	0.039	1.09	0.168	1.67	0.129	0.859
9X Ca	0.282	0.039	3.28	0.168	6.05	0.129	0.859
27X Ca	0.282	0.039	9.84	0.168	19.2	0.129	0.859
81X Ca	0.282	0.039	29.5	0.168	58.5	0.129	0.859
⅛X Ca	0.919	0.038	0.046	0.168	0.216	0.152	0.858
¼X Ca	0.828	0.038	0.091	0.168	0.216	0.148	0.859
½X Ca	0.645	0.039	0.182	0.168	0.216	0.142	0.859
2X Ca	0.282	0.039	0.729	0.168	0.945	0.129	0.859

857 [1] Major ion concentrations were increased to 3 times that in ALSW, except SO_4 was increased 3.4 times. This was
858 due to concentrations of ions other than these major ions not being altered from that in ALSW. Because these
859 other ions are predominantly anionic, one of the major anions needed to be increased disproportionately.

Table 3. Study design for effects of dilution water compositions on the toxicity of major ion salts and mannitol. × denotes experiment conducted for that combination of chemical and dilution water characteristic; 2× denotes multiple experiments.

Chemical	⅓X,1X,3X ALSW	Varying Ca Conc	Varying Ca:Mg	Varying Na Conc	Varying K:Na
NaCl	2×	2×	×		×
$MgCl_2$	×	×			
Na_2SO_4	×	×	×		×
$MgSO_4$	×	×	×	×	
$NaHCO_3$	×				×
$MgCO_3$	2×				
$CaCl_2$	×				
KCl	×			×	
K_2SO_4	×				
Mannitol		×			

865 **FIGURE CAPTIONS**

866 **Figure 1**. Effect of Ca on the toxicity of Mg salts to *P. promelas* at nontoxic Ca concentrations.
867 Panels A and B provide LC50s for several experiments on the toxicities of $MgCl_2$, $MgSO_4$, and
868 $MgCO_3$ in dilution waters with variable composition, with Panel A expressed as concentrations
869 and Panel B as activities. Error bars are ≥95% confidence limits. Small arrows denote LC50s
870 being slightly less than or greater than the indicated value for tests in which treatments did not
871 bracket the LC50. The dotted blue line indicates the range of Ca concentrations for a test in
872 which significant Ca precipitation occurred. In Panel B, the dashed line is a regression of the
873 $MgCl_2$ data for {Ca}<0.4 mM, including LC50s for other $MgCl_2$ toxicity tests in ALSW (black dots).
874 The gray band provides approximate uncertainty limits for predictions from the regression,
875 based on ±2 standard deviations for the inter-experimental variability of all $MgCl_2$ LC50s in
876 ALSW.

877 **Figure 2.** Effects relationships for binary mixtures of Mg salts. Panel A provides the
878 isobologram for the $MgCl_2$×$MgSO_4$ mixture experiment for LC50s (triangles) based on added salt
879 concentrations. Colors indicate the ratio of salts in the mixture, grading from red for $MgSO_4$ to
880 yellow for $MgCl_2$, and the error bars are ≥95% confidence limits. The dashed line is a linear
881 regression over all data to illustrate concentration additivity. Panel B provides the total Mg
882 activity at the LC50 versus the mole fraction of $MgSO_4$ in the mixture. The solid line denotes
883 LC50 predictions based on the $MgCl_2$ toxicity versus Ca relationship in Figure 1B and the gray
884 band reflects the inter-experimental variability in LC50s. Panels C and D provide comparable
885 information for the $MgCl_2$×$MgCO_3$ mixture experiment.

886 **Figure 3.** Effect relationship for the combined $MgCl_2$×$CaCl_2$ (unfilled triangles) and
887 $MgSO_4$×$CaCl_2$ (dotted triangles) binary mixture experiments. Colors indicate the ratio of salts in

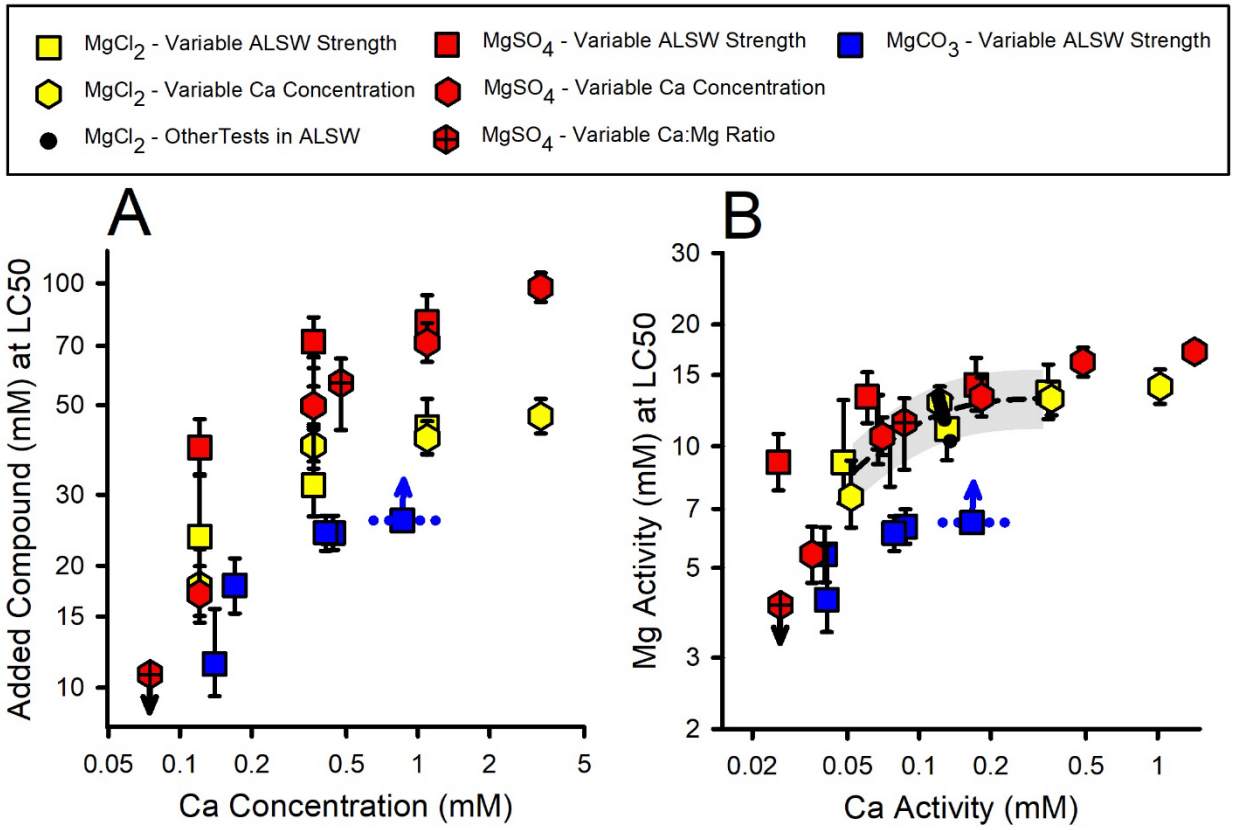
888 the mixtures, grading from red for $MgSO_4$ to yellow for $MgCl_2$, and the error bars are ≥95%
889 confidence limits. Additivity of Mg and Ca toxicity is demonstrated by the linearity of the data
890 except at low Ca, where LC50s are reduced by the dependence of Mg-only toxicity on Ca.
891 **Figure 4.** Effect of Ca and pH on the toxicity of Na salts and mannitol to *P. promelas* at nontoxic
892 Ca concentrations. Panels A-C provide LC50s for several experiments on the toxicities of NaCl,
893 Na_2SO_4, $NaHCO_3$, and mannitol in dilution waters with variable Ca, with Panel A expressed as
894 concentrations of added compound versus Ca concentration, Panel B as osmolarity versus Ca
895 activity, and Panel C as Na activity versus Ca activity. Blue dotted lines indicate the range of Ca
896 concentrations for $NaHCO_3$ tests in which significant Ca precipitation occurred. In Panel B, the
897 black dash-dotted line denotes the average LC50 (no Ca dependence) for mannitol. In Panel C,
898 the dashed line is from a regression analysis of the NaCl data for {Ca}<0.2 mM, including LC50s
899 for other NaCl toxicity tests in ALSW (black dots). The gray band provides approximate
900 uncertainty limits for predictions from the regression, based on ±2 standard deviations for the
901 inter-experimental variability of all NaCl LC50s in ALSW. The light blue circles are for two toxicity
902 tests with $NaHCO_3$ in which test solutions were equilibrated prior to exposure with either
903 ambient air (open circle), for which the pH was 9.3, or with 1% CO_2 (crossed circle), for which
904 the pH was 8.4.
905 **Figure 5.** Effects relationships for binary mixtures of Na salts and mannitol. Panels A-C provide
906 the isobolograms for the NaCl×Na_2SO_4, NaCl×$NaHCO_3$, and NaCl×mannitol experiments, for
907 LC50s based on added compound concentrations. Colors indicate the ratio of compounds in the
908 mixture, based on red for Na_2SO_4, yellow for NaCl, blue for $NaHCO_3$, and black for mannitol.
909 Error bars are ≥95% confidence limits for LC50 estimation. The light blue circles in Panel E are
910 for the separate experiment in which NaHCO3 toxicity is contrasted at the unadjusted high pH

911 (open circle) to a reduced pH (crossed circle). The dashed line in Panel A is a linear regression of
912 data except for the Na_2SO_4-only test. The dashed line in Panel C approximates mixture
913 compositions expected to produce the same level of osmolarity as the mannitol-only test.
914 Panels D-F plot data based on exposure metrics considered relevant to the mixtures. The solid
915 lines denote LC50 predictions based on Ca-dependent NaCl toxicity (regression line in Figure 4C)
916 and Ca-independent mannitol toxicity; for Panel E, the line is limited to not extrapolate beyond
917 the regression limits. The gray band denotes uncertainty based on the inter-experimental
918 variability of LC50s.
919 **Figure 6.** Effect relationship for the combined $NaCl \times CaCl_2$ (unfilled triangles) and $Na_2SO_4 \times CaCl_2$
920 (dotted triangles) binary mixture experiments. Colors indicate the ratio of salts in the mixtures,
921 grading from red for Na_2SO_4 to yellow for NaCl, and the error bars are ≥95% confidence limits.
922 The solid line denotes the predicted relationship at {Ca}>3 mM for independent action of Ca-
923 specific toxicity and osmolarity-driven toxicity, with osmolarity-driven toxicity based on the
924 study-wide average LC50s for all mannitol-only tests and the Ca-specific toxicity being based on
925 the study-wide average LC50s for all $CaCl_2$ tests in ALSW. The gray band denotes uncertainty
926 based on the inter-experimental variability of LC50s. Lower LC50s at the lowest Ca level
927 represent more potent mechanisms than osmolarity-driven that are further addressed in the
928 companion modeling paper.
929 **Figure 7.** Isobolograms for $NaCl \times MgCl_2$ (Panels A-D) and $Na_2SO_4 \times MgSO_4$ (Panels E-G) mixture
930 tests. Panel A compares LC50s for the Cl salt mixtures on the basis of added salt concentration
931 to isoboles (dotted lines) for concentration addition and independent action fit to the single-
932 salt LC50s. Panel B compares Cl salt LC50s on the basis of Na and Mg activities to predictions
933 from a two-mechanism model (solid line) for independent action of these two activities, with

the dashed lines representing the individual mechanisms and the gray band the model uncertainty. Panel C similarly compares Cl salt LC50s on the basis of Mg activity and osmolarity to a model driven by these two metrics. Panel D compares Cl salt LC50s on the basis of Na and Mg activities to a three-mechanism model that also includes osmolarity-driven toxicity (diagonal dashed line). Panel E compares LC50s for the SO_4 salt mixtures on the basis of added salt concentration to a concentration addition isobole (dashed line) fit by linear regression of the data except for the $MgSO_4$-only test. Panel F compares SO_4 salt LC50s on the basis of Na and Mg activities to the two-mechanism model for independent action of these activities. Panel G compares SO_4 salt toxicities on the basis of Mg and SO_4 activity to expected LC50s for Mg-driven toxicity (arrow) and SO_4-driven toxicity (dashed line).

Figure 8. Interaction of KCl toxicity with NaCl and $MgCl_2$. Panel A shows different effects of Na in the dilution water on KCl toxicity for C. dubia (+'s) from Mount et al. (2016) and fathead minnow from the present study (×'s). For fathead minnow, KCl toxicity is approximately independent of both NaCl (Panel B, NaCl×KCl experiment) and $MgCl_2$ (Panel C, $MgCl_2$×KCl experiment) toxicities.

950 **FIGURE 1**



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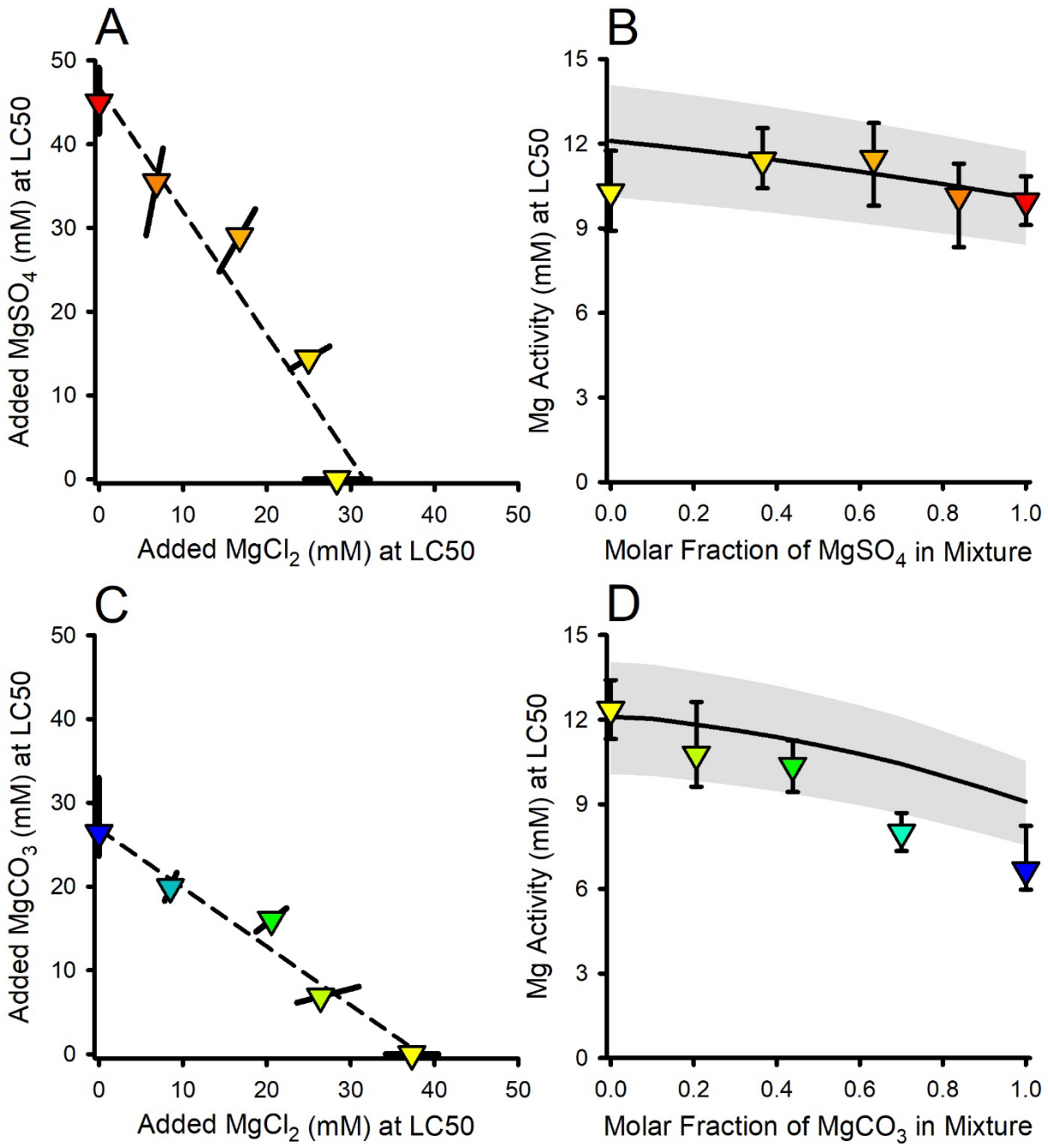
952 **FIGURE 2**



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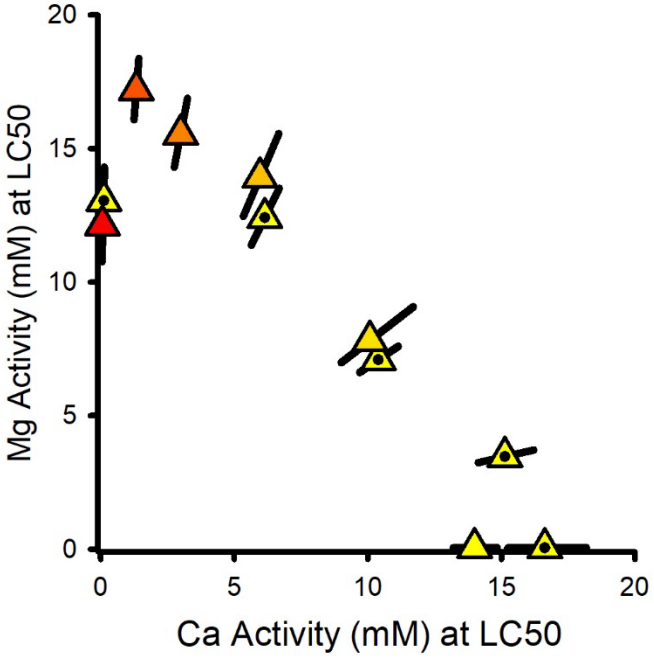
955 **FIGURE 3**



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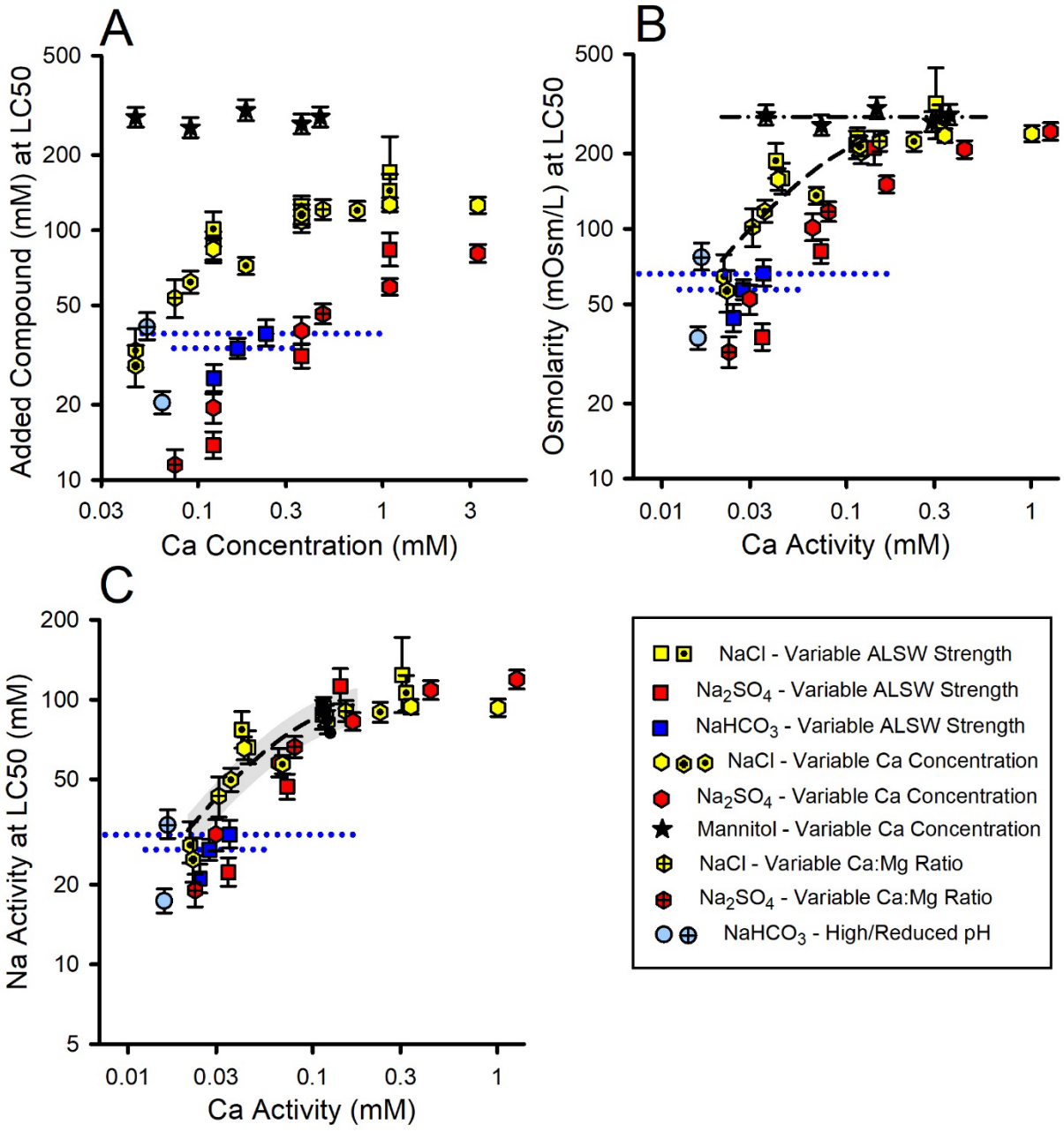
958 **FIGURE 4**



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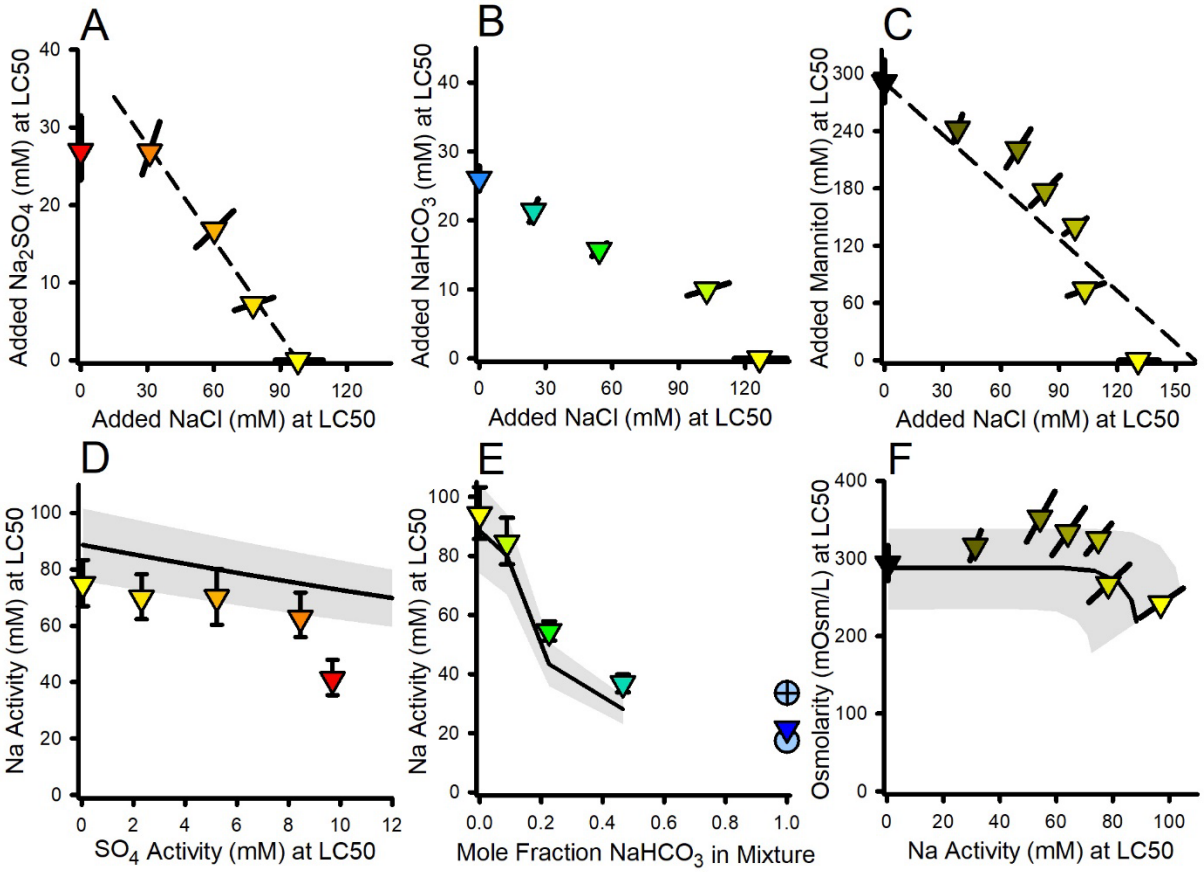
961 **FIGURE 5**



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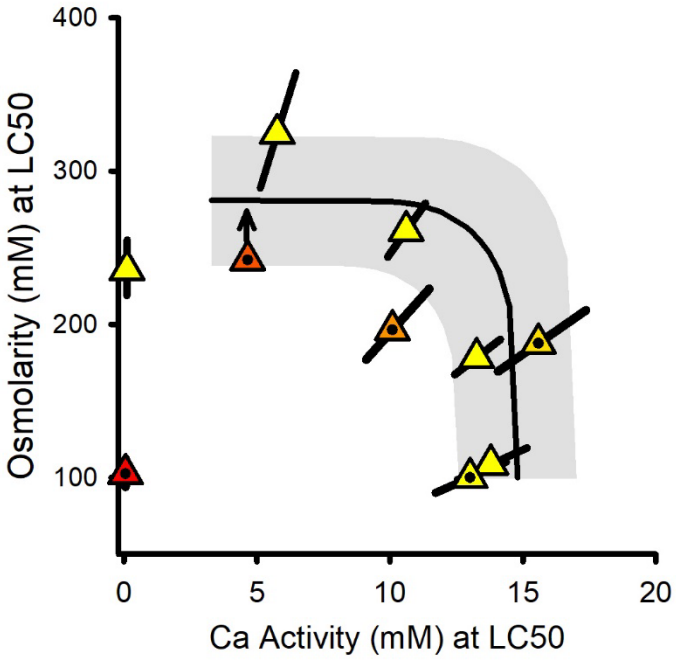
964 **FIGURE 6**



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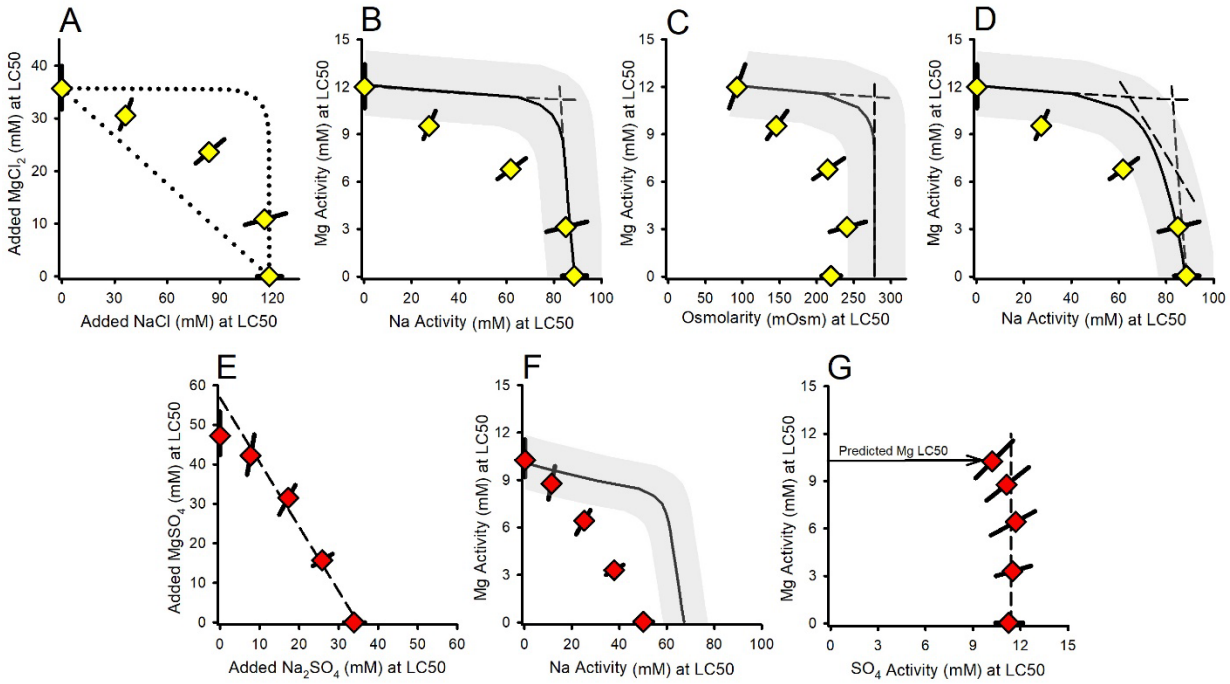
967 **FIGURE 7**



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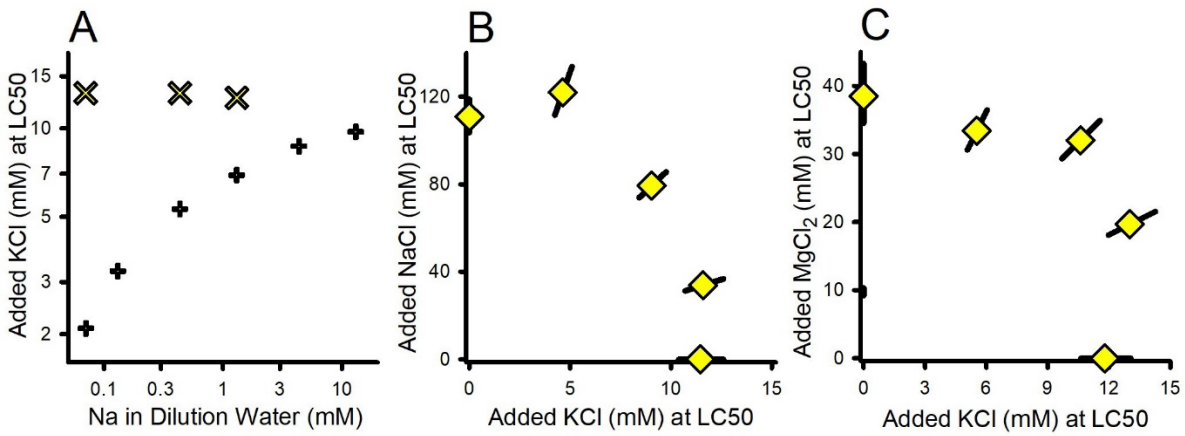
970 **FIGURE 8**



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SUPPORTING INFORMATION FOR:

Acute Toxicity of Major Geochemical Ions to Fathead Minnows (*Pimephales promelas*). Part A: Observed Relationships for Individual Salts and Salt Mixtures.

RJ Erickson, DR Mount, TL Highland, JR Hockett, DJ Hoff, CT Jenson,TJ Norberg-King, BB Forsman

A. Evaluating relationships for binary salt mixture effects concentrations

The evaluation of the toxicity of binary mixtures can be helped by the use of isobolograms – plots of the concentration of one chemical versus that of the other chemical for exposures with a specified level of toxicity. Across a range of concentration ratios for the two chemicals, this defines a relationship (isobole) regarding how toxicity varies with mixture composition and which can inform how the chemicals are interacting. Figure S1 provides a hypothetical isobologram showing two simple isobole possibilities.

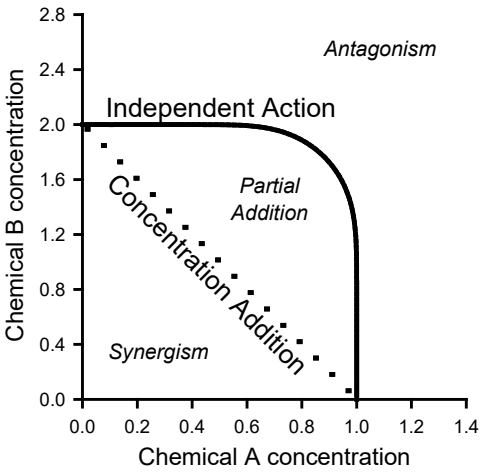


Figure S1. Hypothetical isobologram for toxicity of mixtures of two chemicals, showing different types of interactions.

If the chemicals are concentration additive, the isobole is a straight line connecting the effect concentrations (ECs) of the individual chemicals. For this line, the sum of the toxic units (TUs) equals 1.0 for all mixture ratios ($TU_A+TU_B \equiv [A]/EC_A+[B]/EC_B=1$). Concentration addition can occur if both chemicals affect the same site of toxic action, so that toxicity reflects the

989 combined concentration of chemicals interacting with that site. Such interactions allow for the
990 presence of toxicity when neither chemical alone is high enough to elicit any toxic effects.

991 In contrast, if the chemicals act independently at different sites of action, the isobole
992 will have a vertical component equal to EC_A when chemical B is too low to exert effects and a
993 horizontal component equal to EC_B when chemical A is too low to exert effects (Figure S1).
994 When both chemicals are high enough to exert some effect, the isobole becomes curved due to
995 their combined effects; factors determining this curvature are discussed in Erickson et al. (2017)
996 and references therein. One such factor is the steepness of the exposure-effects curves for the
997 chemicals and the curvature will be broader as the exposure-effects curves becomes more
998 shallow, potentially making independent action difficult to distinguish from concentration-
999 addition.

1000 However, mixture toxicity is often more complex than the simple concentration addition
1001 or independent action in Figure S1. As further described in Erickson et al. (2017) and references
1002 therein, various interactions can make the isoboles more complicated. For example:

1003 (1) If one chemical shares a toxicity mechanism with the other, but also acts by a more
1004 potent mechanism that isn't shared, mixtures can reflect both additive and independent
1005 interactions and lie within the region labeled "partial addition" on Figure S1. An
1006 isobologram based on metrics for the separate mechanisms, rather than the individual
1007 chemical, would then show independent action of the mechanisms (see discussion of
1008 Figure S2 below for an example).

1009 (2) If the presence of one of the chemicals makes the other more toxic, such synergistic
1010 interactions would include any isoboles below the concentration addition line (Figure
1011 S1). If the two chemicals would act independently in the absence of these synergetic
1012 interactions, such isoboles could also be in the "partial addition" region.

1013 (3) If the presence of one of the chemicals makes the other less toxic, such antagonistic
1014 interactions would include any isoboles beyond the independent action line (Figure S1).
1015 If the two chemicals would be concentration additive in the absence of these
1016 antagonistic interactions, such isoboles could also be in the "partial addition" region.

(4) If the toxicities of chemicals A or B depend on some factor in the dilution water (such as the effect of Ca on the toxicity of Mg and Na salts) and this factor is altered by either chemical, the shape and position of the isobole can also be altered.

These more complex interactions are considered in the present study, but depend on first comparing observed mixture toxicities to simple concentration addition and independent action isoboles. The following text describes how those isoboles are calculated.

Fitting isoboles to binary mixture data

Binary toxicity experiments in Erickson et al. (2017) contributed to inferences about major ion toxicity to *C. dubia*, based on simple comparisons of LC50 relationships to the basic isobole shapes expected for concentration addition or independent action. These isoboles were fit to the LC50s in each experiment and Figure S2 provides some examples of this.

Figure S2. Panel A is the isobologram for the acute LC50s of binary mixtures of NaCl and Na_2SO_4; the dashed line represents a concentration addition isobole anchored to the LC50s for the pure salts. Panel B is for binary mixtures of Na_2SO_4 and $MgSO_4$ with the LC50s expressed as molarity of the added salts; the dashed line represents an independent action isobole anchored to the average LC50 of the two tests with the highest proportion of each salt. Panel C revises Panel B using LC50s expressed as Mg activity and osmolarity. Red denotes sulfate salts and yellow denotes chloride salts, with intermediate colors for mixtures. Data are from Erickson et al. (2017).

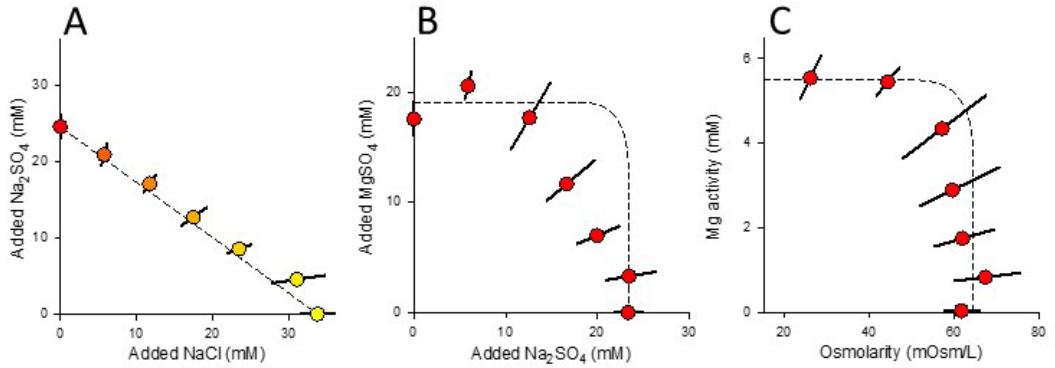


Figure S2A shows a linear relationship for binary mixtures of NaCl and Na_2SO_4, indicative of concentration addition. The dashed line was fit to the data by anchoring it to LC50s for the individual salts, and illustrates that the individual data do not deviate significantly from concentration additivity.

In contrast, Figure S2B for binary mixtures of Na_2SO_4 and $MgSO_4$ clearly deviates from concentration additivity, but also deviates from the fitted independent action isobole. For each salt, the isobole was anchored to the average LC50 for two tests – the pure salt test and the mixture test with the highest fraction of the salt; the curvature of the isobole was calculated as described in Erickson et al. (2017). That the isobole is intermediate between concentration addition and independent action raises the possibility of "partial addition" (Figure S1), which can occur if both salts contribute additively to the same toxicity mechanism, but one salt also acts by a more potent mechanism when its concentration is high enough relative to the other salt. Figure S2C replots the data based on two mechanisms – a Mg-driven toxicity and a general ion toxicity correlated with osmolarity to which both salts contribute (Erickson et al. 2017, 2018). Using these exposure metrics results suggests independent action of the two mechanisms, as opposed to the interaction of the separate chemicals.

Predicting isoboles for binary mixture data

Isobolograms and fitted isoboles such as in Figure S2 helped the evaluation of mixture relationships by Erickson et al. (2017). However, fitting isoboles in this manner does not address some factors that affect isobole shape. In the present study, some isoboles for mixture experiments are fitted to the single-salt toxicities in each experiment as described above, but in other cases isoboles are predicted based on prior information of single salt toxicity, including Ca effects on toxicity. Furthermore, this prior information is just for chloride salt toxicity, so that these predictions would help address whether relationships change across salts with different anions. The following steps describe the procedure for such predictions, using mixtures of $MgCl_2$ and $MgSO_4$ as an example, with Figure S3 conceptually depicting the procedure.

(1) Experiments that addressed the Ca-dependence of $MgCl_2$ toxicity were used to develop a relationship for {Mg}-based LC50s ($LC50_{\{Mg\}}$) to {Ca}. (Braces {} are used here to denote chemical activities, in contrast to brackets [] for concentrations.) This analysis was limited to a {Ca} range relevant to the mixture tests for which toxicity was to be predicted, so did not involve the more extensive modeling described in a companion

paper (Erickson et al. 2022). Rather, the following equation was used to provide an approximate relationship for $LC50_{\{Mg\}}$ over a limited Ca range:

$$LC50_{\{Mg\}} = LC50_{MgMax} \times \left(1 - 10^{-\{Ca\}/\{Ca\}_T}\right) \quad \text{(Eq. S1)}$$

This equation describes an exponential approach of $LC50_{\{Mg\}}$ to a maximum value ($LC50_{MgMax}$) as {Ca} increases. The parameter $\{Ca\}_T$ is the {Ca} at which 90% of $LC50_{MgMax}$ is reached, providing a horizontal reference point for the curve. These two parameters allow both horizontal and vertical adjustments to fit the curvature and position of the data. This analysis was by nonlinear least-squares regression using Sigmaplot 10.0 (Systat Software, Inc.) and is conceptually represented by the solid line on Figure S3A.

(2) For a specified mole fraction of $MgCl_2$ and $MgSO_4$, speciation calculations were made using Visual Minteq (version 3.1, https://vminteq.lwr.kth.se/visual-minteq-ver-3-1) for a range of exposure solution strengths to establish a relationship of {Mg} to {Ca} that would straddle the toxicity relationship from step 1 (dashed lines on Figure S3A). In practice this involved more than 10 mole fractions, but is represented by just three mole fractions on Figure S3.

(3) {Mg} and {Ca} at the LC50 for each mole fraction were estimated by the intersection of the solid and dashed lines, designated by the circles on Figure S3A. Determining this intersection required an iterative search using the toxicity and speciation relationships

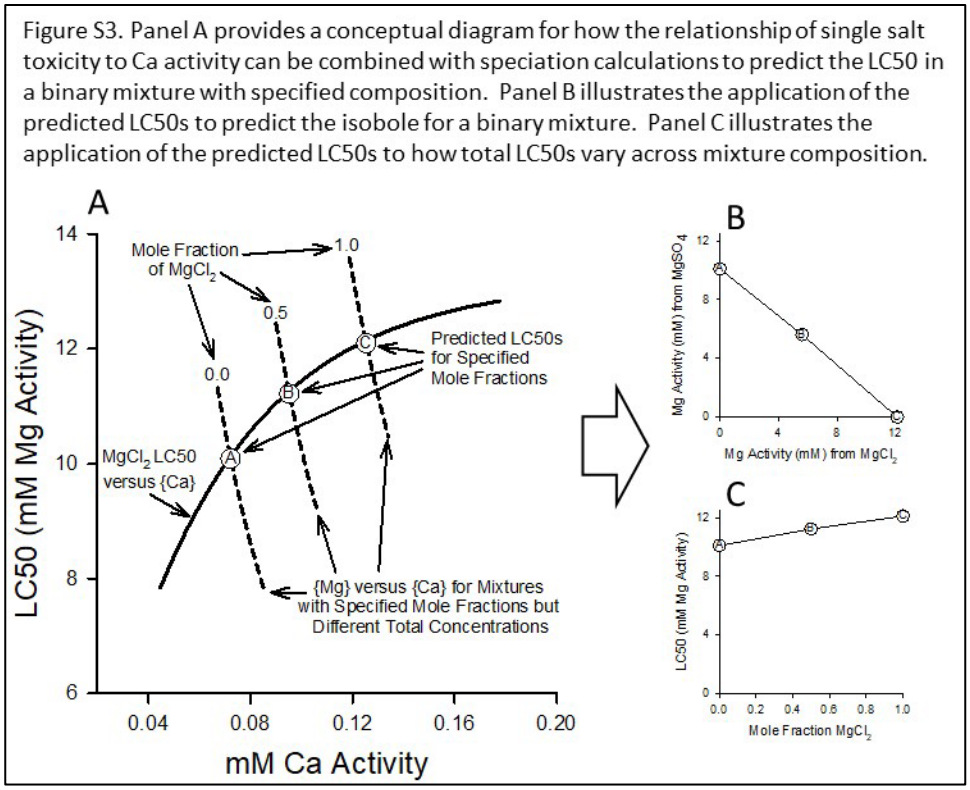


Figure S3. Panel A provides a conceptual diagram for how the relationship of single salt toxicity to Ca activity can be combined with speciation calculations to predict the LC50 in a binary mixture with specified composition. Panel B illustrates the application of the predicted LC50s to predict the isobole for a binary mixture. Panel C illustrates the application of the predicted LC50s to how total LC50s vary across mixture composition.

from steps 1 and 2. These LC50 predictions can be used to formulate predicted isoboles as shown in Figure S3B or predicted toxicity relationships for other types of plots such as Figure S3C.

Comparable relationships were developed for $MgCl_2$×$MgCO_3$ mixtures and for mixtures of Na salts based on NaCl toxicity. To address interactions for mixtures of Na and Mg salts, predictions were made for both the $MgCl_2$-based and NaCl-based toxicity models. These were combined assuming independent action as described in Erickson et al. (2017, 2018). Independent interactions of Mg activity and osmolarity driven toxicities were similarly developed using this MgCl2-based model and osmolarity-based LC50s from mannitol tests.

B. Interactions in NaCl×$MgSO_4$ and Na2SO_4×$MgCl_2$ Mixtures

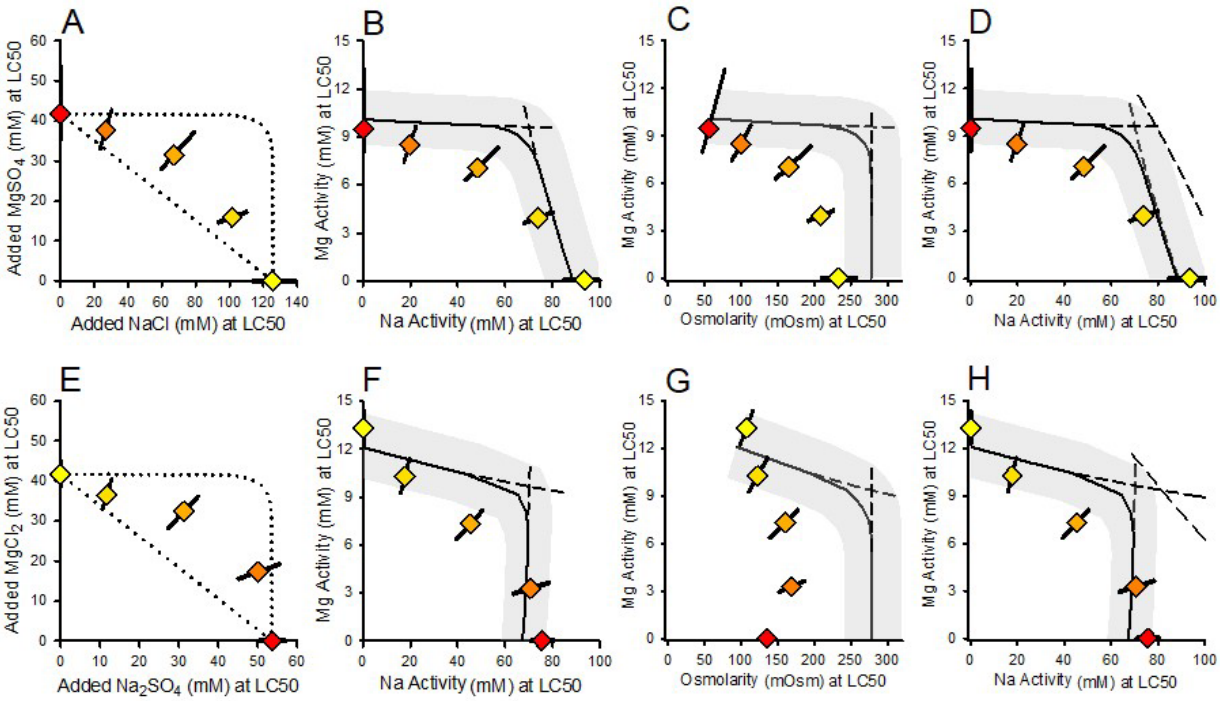
The main text of this paper discusses NaCl×$MgCl_2$ and Na_2SO_4×$MgSO_4$ binary mixture experiments. Interactions similar to that in the NaCl×$MgCl_2$ experiment were observed in the NaCl×$MgSO_4$ and Na_2SO_4×$MgCl_2$ experiments and are provided in Figure S4 using the same series of four plots and predictions described for NaClx$MgCl_2$ in the main text.

(1) Figures S4A and S4E compare LC50s on the basis of added salt concentrations to concentration addition and independent action isoboles fit to the data as described in ***Fitting isoboles to binary mixture data*** above. This shows the data to be intermediate to concentration addition and independent action, suggesting partial addition as discussed for Figure S1 above and as was also the case for NaCl×$MgCl_2$ in the main text.

(2) Figures S4B and S4F compare LC50s on the basis of Na and Mg activities to model predictions based on the Ca-dependent toxicities of Mg and Na salts as described in ***Predicting isoboles for binary mixture data*** above. The slanted legs of the isoboles reflect the binding of Ca by SO4 and the consequent effects on toxicity (the slanted legs of the isobole). This produces slightly better agreement with the data, but partial addition is still evident.

(3) Figures S4C and S4G plot LC50s on the basis of Mg activity and osmolarity to model predictions assuming independent action of the Mg model described in ***Predicting isoboles for binary mixture data*** and an average osmolarity-based LC50s from mannitol toxicity tests. As explained in the main text, osmolarity was considered as a metric due to its efficacy in describing similar partial additivity in NaxMg salt mixture experiments for *Ceriodaphnia dubia* toxicity (Erickson et al.; 2017, 2018). However, as was also the case for NaClx$MgCl_2$ mixtures in the main paper, these predictions are not effective.

Figure S4. Isobolograms for LC50s from NaCl×$MgSO_4$ (Panels A-D) and $Na2SO_4$×$MgCl_2$ (Panels E-H) binary mixture experiments. Panels A and E compare LC50s on the basis of added salt concentration to isoboles (dotted lines) for concentration addition and independent action anchored to the single-salt LC50s. Panels B and F compare LC50s on the basis of Na and Mg activities to predictions from a two-mechanism model (solid line) for independent action of these two metrics, with the dashed lines representing the individual mechanisms. Panels C and G similarly compare LC50s on the basis of Mg activity and osmolarity to a model driven by these two metrics. Panels D and H compare LC50s on the basis of Na and Mg activities to a three-mechanism model that also includes osmolarity-driven toxicity (diagonal dashed line). Red denotes sulfate salt, yellow denotes chloride salt, and intermediate colors denote mixtures. Shaded areas are approximate error limits for predictions based on inter-experimental variability. Confidence limits are ≥95%.



(4) Figures S4D and S4H again plot LC50s on the basis of Na and Mg activities, but compare them to a three-mechanism model that include not only the Mg and Na activity driven models, but osmolarity. For the evaluation of NaCl×$MgCl_2$ mixture toxicity in the main text, osmolarity was found to have some impact on the isobole but to not completely explain the partial addition. For NaCl×$MgCl_2$ and Na_2SO_4×$MgSO_4$ here, there is negligible impact on the isobole. This can be attributed to SO_4 reducing {Ca} and further distancing LC50s from the apparent osmolarity-driven toxicity in Figure 5B in the main text.

C. Effects of Ca on Mannitol Toxicity to Ceriodaphnia dubia

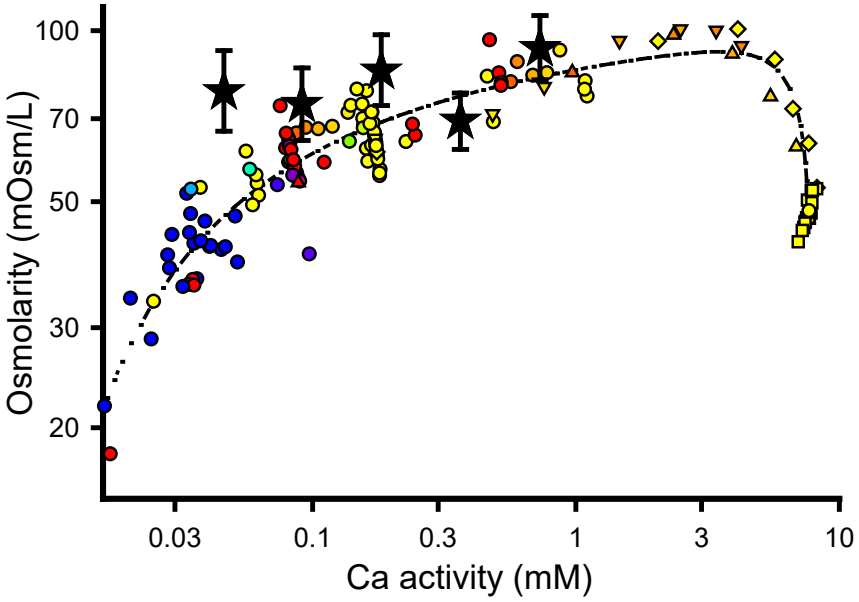
A notable finding regarding major ion toxicity to fathead minnow toxicity in the present study was that, contrary to the effect of Ca on the toxicity of Na salts, there is no effect of Ca on the toxicity of mannitol (Figure 4 in main text). This creates a disparity between the comparative toxicities of Na salts and mannitol at Ca concentrations below and above circa 0.5 mM and was interpreted as meaning there are different mechanisms of toxicity for these Ca ranges (Figures 4B and 4C in main text). This interpretation is supported by the NaCl×mannitol binary mixture experiment (Figure 5C and 5F in main text).

This is contrary to the Ca-dependent, osmolarity-based model developed for *C. dubia* (Figure 3 in Erickson et al., 2018) that applies to all Ca. For this model, there was no claim that osmolarity per se was the cause of toxicity for the entire Ca range, but rather that it was a good correlate for a single relationship. However, the results of the present study with fathead minnows raised the question of whether multiple mechanisms and other metrics should be addressed for this "general ion toxicity" model. In particular, the Ca dependence of mannitol toxicity to *C. dubia* was not evaluated and considered for this model, and would be pertinent to further model development.

Therefore, an experiment was conducted regarding the Ca-dependence of acute mannitol toxicity to *C. dubia*, using dilution waters ranging from 0.125X to 2X of that in Amended Lake Superior Water as described for fathead minnow tests in the main text. 48-h acute toxicity tests with *C. dubia* were conducted and analyzed as described in Mount et al. (2016). Figure S5 provides the results of this experiment, superimposed on the general ion toxicity model and the data used for the development of the model.

As for fathead minnow, this shows the absence of a substantial Ca effect of mannitol toxicity. This absence indicates that, at lower Ca, mannitol and the Na salt cannot share the same mechanism of toxicity. However, the concordance of mannitol and Na salt LC50s at higher Ca and the additivity in NaCl×mannitol mixtures indicates a shared mechanism. These results will be further addressed in the companion modeling paper (Erickson et al. 2022) regarding how the development of models for major ion toxicity to fathead minnow relate to major ion toxicity to *C. dubia*.

Figure S5. Effect of Ca on mannitol toxicity to *Ceriodaphnia dubia*. Stars denote osmolarity-based mannitol LC50s with 95% confidence limits. These are superimposed on the data and model line for the general ion toxicity model developed in Figure 3 of Erickson et al. (2018).



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