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EFFECTS OF HARDNESS, CHLORIDE, AND ACCLIMATION ON THE ACUTE TOXICITY OF SULFATE TO FRESHWATER INVERTEBRATES

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Abstract-The acute toxicity of sulfate to *Ceriodaphnia dubia*, *Chironomus tentans*, *Hyalella azteca*, and *Sphaerium simile* was assessed to support potential updates of Illinois (USA) sulfate criteria for the protection of aquatic life. The mean lethal concentrations to 50% of a sample population (LC50s), expressed as mg SO_4^{22}/L, in moderately hard reconstituted water (MHRW) were as follows: 512 mg/L for *H. azteca*, 2,050 mg/L for *C. dubia*, 2,078 mg/L for *S. simile*, and 14,134 mg/L for *C. tentans*. At constant sulfate (; 2,800 mg/L) and hardness (106 mg/L), survival of *H. azteca* was positively correlated with chloride concentration. Hardness also was found to ameliorate sodium sulfate toxicity to *C. dubia* and *H. azteca*, with LC50s for *C. dubia* increasing from 2,050 mg SO_4^{22}/L at hardness 5 90 mg/L to 3,516 mg SO_4^{22}/L at hardness 5 484 mg/L. Using a reformulated MHRW with a similar hardness but higher chloride concentration and different calcium to magnesium ratio than that in standard MHRW, the mean LC50 for *H. azteca* increased to 2,855 mg/L, and the LC50 for *C. dubia* increased to 2,526 mg/L. Acclimation of *C. dubia* to 500 and 1,000 mg SO_4^{22}/L for several generations nominally increased mean LC50 values compared with those cultured in standard MHRW.

Keywords-Sulfate Total dissolved solids Osmoregulation *Hyalella* Toxicity

INTRODUCTION

Aquatic ecotoxicological research has primarily focused on the impairment of fauna by contaminants that are toxic at minute concentrations; however, ordinarily benign major ions (e.g., sodium, sulfate) can reach concentrations in wastewater discharges that severely impair sensitive in-stream macroinvertebrates and laboratory test organisms [1—5]. Concentrations of these major ions and therefore, of total dissolved solids (TDS), which is essentially the sum of the concentrations of all common ions (e.g., sodium, potassium, calcium, magnesium, chloride, sulfate, and bicarbonate) in freshwaters, can be elevated by numerous practices, such as reverse osmosis systems, pH modifications, and mining operations [6]; and investigations of major-ion toxicity have involved irrigation drainage water [1,7—9], inundation of freshwater systems by brackish water [3,10], laboratory-formulated salt solutions [11,12], and mining activities [4,5,13].

Coal preparation facilities wash coal to reduce sulfur emissions prior to burning in coal-fired power plants and treat wastewaters for acid-soluble metals. This practice often produces a waste containing sulfuric acid that is usually neutralized by the addition of sodium hydroxide or sometimes quicklime (CaO) prior to release to a receiving system [14]. The result is an effluent containing high concentrations of sulfate, sodium, and/or calcium ions and therefore, TDS. Other ions potentially present at high concentrations because of coal preparation activities include magnesium and chlorides; therefore, the interacting effects of these various ions should be considered. Researchers have found hardness and multiple ''nontoxic'' cations in solution to ameliorate major-ion toxicity ([8,11,15] (http://scholar.lib.vt.edu/theses/available/etd-051499-130633/), and several studies indicate that calcium is more important than magnesium in this regard [16—18].

There are no federal water quality criteria for the protection of freshwater life for TDS, sulfate, or sodium [19], but several states, including Illinois, are developing standards for sulfate to protect aquatic life. Although major-ion (i.e., TDS) toxicity is caused by osmoregulatory stress from the combination of all cations and anions, chloride standards currently exist, and Illinois plans to additionally regulate for sulfate in order to address the major non-chloride component of TDS in these waters. Therefore, the objectives of the current study were to generate lethal concentrations to 50% of a sample population (LC50s) and lethal concentrations to 10% of a sample population (LC10s) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia*, *Chironomus tentans*, *Hyalella azteca*, and *Sphaerium simile*) in the U.S. Environmental Protection Agency (U.S. EPA)'s [20] moderately hard reconstituted water (MHRW) and to determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate. The endpoints generated are described in terms of sulfate concentrations to address regulatory issues; however, it is important to note that in our exposures, sodium was the major cation, and effects observed are probably caused by the combination of all dissolved ions.

MATERIALS AND METHODS

Toxicity of sulfate to freshwater invertebrates in MHRW

Four invertebrates were selected for initial testing. Three of these, *C. dubia*, *H. azteca*, and *C. tentans*, are standard U.S. EPA organisms used to test for either water column or sediment toxicity [20,21]. The fourth, *S. simile*, is a fingernail clam (Bivalvia, Sphaeriidae) that was easily obtained from the field and represented the phylum Mollusca. Reliable toxicity data for sodium sulfate have been generated for *C. dubia* [11], so this organism was used in the present study for comparative

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purposes. Additionally, previous studies have found *C. dubia* to be more sensitive to major-ion or TDS toxicity than other U.S. EPA-recommended test species (e.g., *Daphnia magna*, *Pimephales promelas*) [5,9,11].

The cladoceran, *C. dubia*, was cultured in-house (Soucek Laboratory, Illinois Natural History Survey) according to U.S. EPA methods [20]. The mean LC50 in NaCl reference tests for these *C. dubia* cultures was 2,030 mg NaCl/L, which was comparable to the value of 1,960 mg/L reported in previous studies [11]. The midge, *C. tentans*, also was cultured in-house according to U.S. EPA methods [21]. Prior to testing, larvae were fed a diet of ground Tetra Mint (TetraWerke, Melle, Germany) flake food and rabbit pellets (free of antibiotics). Amphipods, *H. azteca*, were obtained from a commercial source (Aquatic Research Organisms, Hampton, NH, USA) and were acclimated to MHRW at 228C and a 16:8-h (light: dark) photoperiod for at least 7 d prior to testing. Sphaeriid clams were collected from Spring Creek, near Loda, Illinois, USA, and acclimated to MHRW at 228C and a 16:8-h (light: dark) photoperiod for 5 to 7 d prior to testing. Clams were identified to species by Gerald Mackie (University of Guelph, Department of Zoology, Guelph, ON, Canada).

For toxicity testing, a pure (99%) grade of anhydrous sodium sulfate (Na_2SO_4) (CAS 7757-82-6) was obtained from Fisher Scientific (Pittsburgh, PA, USA) to serve as the source of sulfate. A concentrated solution of this salt (19,040 mg SO_4^{22}/L), as well as a sample of laboratory-deionized water, was acidified to pH , 2.0 and analyzed for priority metal concentrations at the Illinois State Water Survey (Champaign, IL, USA) using inductively coupled plasma-atomic emission spectrometry according to U.S. EPA methods [22]. All metals analyzed were below acute standard levels ([19], and R. Mosher, Illinois Environmental Protection Agency, Springfield, IL, USA, personal communication) in the concentrated sulfate sample, and all were below detection limits in the deionized water sample except for iron (37 mg/L) and zinc (9 mg/L). The actual metal concentrations have already been reported [23].

For definitive static, nonrenewal toxicity tests, conducted according to American Society for Testing and Materials E729-96 methods [24], treatments comprised a 75% dilution series (i.e., the 100% concentration was serially diluted by 25%), rather than the standard 50%, because major-ion toxicity tests often cause 100% mortality in one concentration and 0% mortality in the next highest concentration if the spread is too great. Five to six concentrations were tested using MHRW as both the diluent and control, with four replicates tested per concentration. Tests with *C. dubia* and *C. tentans* were conducted for 48 h with a 16:8-h (light:dark) photoperiod, with the *C. dubia* tests being conducted at 258C and the *C. tentans* tests at 22 8C. *H. azteca* and *S. simile* were exposed for 96 h at 228C and a 16:8-h (light:dark) photoperiod. *C. dubia*, *C. tentans*, and *H. azteca* were exposed in 50-ml glass beakers with five organisms per beaker, and for *C. tentans* and *H. azteca*, 1 g of quartz sand was added to each beaker to serve as substrate. Clam tests were conducted in 150-ml glass beakers (no substrate) with three to five organisms per replicate, depending on the animal size. All clams used were juveniles. In the first experiment, clams averaged 4.6 mm in length (anterior to posterior margin), whereas in the second and third tests, they averaged 5.4 and 8.3 mm in length, respectively. This slight difference in size for the last test did not substantially affect toxicity. *C. dubia* used were , 24 h old, *C. tentans* were 10 d old, and *H. azteca* were approximately third instar (7—14d old). Percent survival in each replicate was recorded every 24 h and at the end of the exposure period. A dissecting microscope was used to assess survival of *Hyalella* and *Sphaerium*.

Standard water chemistry parameters, including temperature, pH, conductivity, dissolved oxygen, alkalinity, and hardness, were measured at both the beginning and the end of each exposure period. The pH measurements were made using an Accumett (Fisher Scientific, Pittsburgh, PA, USA) model AB15 pH meter equipped with an Accumet gel-filled combination electrode (accuracy , 60.05 pH at 258C). Dissolved oxygen was measured using an air-calibrated Yellow Springs Instruments (Yellow Springs, OH, USA) model 58 meter with a self-stirring biochemical oxygen-demand probe. Conductivity measurements were made using a Mettler Toledot (Fisher Scientific) model MC226 conductivity/TDS meter. Alkalinity and hardness were measured (beginning of tests only) by titration as described in work by the American Public Health Association [25]. Samples from each treatment were analyzed to confirm sulfate concentrations by ion chromatography at the Illinois Natural History Survey Aquatic Chemistry Laboratory (Champaign, IL, USA).

All LC50 values were calculated using either the Spearman-Karber method or probit analysis. To increase confidence in LC50 values, three assays were conducted with each organism, except that only two were conducted for *C. tentans* because of their relative tolerance and low variation in LC50s for the first two tests. This provided a stronger estimate of the mean LC50 value for each species. Geometric means are reported because they are less affected by extreme values. In addition, LC10 values were calculated for all species. With the exception of those for *H. azteca*, all LC50 values presented are geometric means of the Spearman-Karber LC50s for a given species, generated from measured sulfate concentrations. The *H. azteca* data did not permit use of the Spearman-Karber method, so probit analysis was used. The LC10 values presented were generated using probit analysis (the Spearman-Karber program does not calculate LC10s) with the combined data from all tests for a given species.

Influence of dilution water composition on sulfate toxicity

Based on observations of others that *H. azteca* had much better control survival in water-only whole-effluent toxicity tests using modified laboratory water [26], experiments were conducted to determine sulfate LC50 values for *C. dubia* and *H. azteca* using the alternate water type referred to as reformulated moderately hard reconstituted water (RMHRW). Reformulated moderately hard reconstituted water is similar to MHRW with two basic differences: The nominal chloride concentration in RMHRW is nearly 18-fold higher than that in MHRW, and the calcium and magnesium salt concentrations are adjusted so that RMHRW has a Ca:Mg molar ratio of 3.25:1, whereas MHRW has a Ca:Mg molar ratio of 0.88:1 (Table 1). A minor modification in the present study was that anhydrous $CaSO_4$ (CAS 7778-18-9) was used for both RMHRW and MHRW. The nominal concentrations shown in Table 1 take this modification into account. Mean LC50s and LC10s were generated for both species in this water using the same laboratory and calculation methods as described above, with the only exception being the changed diluent/control water.

An additional experiment was conducted with *H. azteca* to attempt to isolate the two basic differences between MHRW and RMHRW. In this experiment, only one nominal sulfate

Table 1. Nominal chemical composition of two laboratory waters used in testing with *Hyalella azteca* and *Ceriodaphnia dubia*

Component (units)	MHRW[a]	RMHRW[b]
K^+ (mg/L)	2.1	2.1
Na^+ (mg/L)	26.3	26.3
Ca^{2+} (mg/L)	17.6	32.7
Mg^{2+} (mg/L)	12.1	6.1
SO_4^{2-} (mg/L)	90.2	59.2
Cl^- (mg/L)	1.9	33.9
HCO_3^- (mg/L)	69.7	69.7
Hardness (mg/L as $CaCO_3$)	94	107
Ca/Mg (molar ratio)	0.88	3.25
pH[c]	7.9	7.9
Conductivity (S/cm)[d]	295	341

[a] MHRW = moderately hard reconstituted water [20].
[b] RMHRW = reformulated moderately hard reconstituted water [26].
[c] The average pH for all treatments during all tests was 8.0 ± 0.2 (standard deviation), and dissolved oxygen never dropped below 6.5 mg/L.
[d] Conductivity of samples in MHRW varied depending upon SO_4^{2-} concentration and followed a linear trend described by the formula: Conductivity (S/cm) = 1.7111[SO_4^{2-} (mg/L)] + 717.15, r^2 = 0.9963.

concentration (2,500 mg/L) was tested with various base waters. The first of these was MHRW; the second was RMHRW; the third, called chloride, had the same chloride concentration (33.9 mg/L) as RMHRW (Table 1) but the same Ca:Mg molar ratio (0.88:1) as MHRW; and the final medium, called Ca/Mg, had the same Ca:Mg molar ratio (3.25:1) as RMHRW, but the same chloride concentration (1.9 mg/L) as MHRW. *Hyalella* was exposed to these four treatments for 96 h at 228C. Mean percent survivorship values for each treatment were compared using analysis of variance with JMP-IN t software [27].

Influence of hardness on the toxicity of sodium sulfate

In these experiments, we tested the toxicity of sulfate (with sodium as the major cation) to *C. dubia* in six freshwater solutions having nominal hardness values of , 100 (standard U.S. EPA MHRW), 200, 300, 400, 500, and 600 mg/L (as $CaCO_3$). Hardness was increased by adding enough $CaSO_4$ (CAS 7778-18-9) and $MgSO_4$ (CAS 7487-88-9) in the same molar ratio as that in U.S. EPA MHRW (Ca/Mg = 0.88) to achieve the nominal hardness values. Then Na_2SO_4 was added, as was done with the standard MHRW. Whole carboys were made at each elevated hardness level, and this water was used as both diluent and control; therefore, each concentration within a given test had the same hardness (i.e., [Ca^{2+}] and [Mg^{2+}] did not change with dilution). The only parameters that varied within a particular test were sodium, sulfate, and conductivity. At least three tests were conducted for each hardness level to provide a mean LC50 value and standard deviation. Exposures were conducted using the same laboratory and calculation methods described above, with the only exception being the hardness of the diluent. An additional assay was conducted with *H. azteca* at only one sulfate concentration (1,460 mg/L) and three different hardness levels (90, 200, and 300 mg/L as $CaCO_3$). *Hyalella* was exposed to sulfate at each of these hardness levels for 96 h, and mean percent survival was compared between treatments using analysis of variance with JMP-IN [27].

Influence of chloride on the toxicity of sulfate

In this experiment, we tested the toxicity of sulfate to *H. azteca* in six freshwater solutions having nominal chloride concentrations of 1.9, 10, 15, 20, 32, and 60 mg/L. Chloride, as NaCl (CAS 7647-14-5, Fisher Scientific AC42429-0010), was added at appropriate concentrations to a solution with a hardness of approximately 106 mg/L (Ca/Mg = 3.25, molar ratio) and a nominal sulfate concentration of 2,800 mg/L. The only parameters that varied between treatments were sodium and chloride. In general, tests were conducted using the same laboratory methods as described above for *Hyalella*. Sulfate, chloride, and bromide were measured in test solutions by ion chromatography. *Hyalella* was exposed to sulfate at each of the six chloride levels for 96 h, and mean percent survival was compared between treatments using analysis of variance with JMP-IN[27]. One additional aspect of this experiment that was different from others in this study using *Hyalella* was that the organisms were cultured in RMHRW and not acclimated to MHRW, as in previous experiments, to potentially improve the health of the test organisms [26]. Finally, two test endpoints were recorded. Tests were checked for survival under a dissecting microscope, and total survival included all living individuals, even if they were lying on the bottom and only legs were twitching. Functional survivors included only those individuals that were active and upright or burrowing.

Table 2. Toxicity of sulfate to freshwater organisms in MHRW[a]

Species	*n*	Mean LC50[b] (mg SO_4^{2-}/L)	Range (mg SO_4^{2-}/L)	LC10[c] (mg SO_4^{2-}/L)
Ceriodaphnia dubia	3	2,050	1,869—2,270	1,759
Chironomus tentans	2[d]	14,134	14,123—14,146	11,682
Sphaerium simile	3	2,078	1,901—2,319	1,502
Hyalella azteca	3	512	431—607	262

[a] MHRW = moderately hard reconstituted water [20].
[b] Lethal concentrations to 50% of a sample population (LC50s) are geometric means of all Spearman-Karber values generated for a given organism using measured sulfate concentrations. Control survival was . 90% in all exposures.
[c] Lethal concentration to 10% of a sample population (LC10) values were generated using probit analysis with the combined data from all tests for a given species.
[d] Tests produced similar LC50s and because values were so high, a third test was not conducted.

Influence of acclimation on the toxicity of sulfate to C. dubia

This experiment was designed to determine the effects of acclimation to relatively high sulfate levels on the response of *C. dubia* to sulfate. *C. dubia* were cultured in U.S. EPA MHRW with Na_2SO_4 added to achieve sulfate concentrations of 500 and 1,000 mg/L. After two to three generations had been cultured in these two sulfate concentrations, acclimated organisms were tested in high sulfate solutions using standard MHRW as a diluent and control as described above. Three replicate tests were conducted for each acclimation level to provide a mean LC50 value and standard deviation.

RESULTS

Toxicity of sulfate to freshwater invertebrates in MHRW

Of the four species tested in MHRW, the most sensitive was *H. azteca*, with a mean LC50 of 512 mg SO_4^{2-}/L (Table 2). *C. dubia* and the fingernail clam, *S. simile*, were similar in sensitivity, with mean LC50s of 2,050 and 2,078 mg SO_4^{2-}/L, respectively. *C. tentans* was tolerant to sulfate exposure, with a mean LC50 of 14,134 mg SO_4^{2-}/L. The LC10 values were calculated by analyzing all tests for each species

Table 3. Influence of culture/testing water composition on toxicity of sulfate to *Hyalella azteca* and *Ceriodaphnia dubia*

Species	Water type	Mean[a] LC50[b] (mg SO_4^{2-}/L)	Range	LC10[c] (mg SO_4^{2-}/L)
H. azteca	MHRW[d]	512 B	431—607	262
H. azteca	RMHRW[e]	2,855 A	2,835—2,876	2,185
C. dubia	MHRW	2,050 B	1,869—2,270	1,759
C. dubia	RMHRW	2,526 A	2,436—2,607	2,216

[a] Different capital letters indicate means are significantly different (p , 0.05). Only intraspecific comparisons were tested.
[b] LC50 5 lethal concentration to 50% of a sample population.
[c] LC10 5 lethal concentration to 10% of a sample population.
[d] MHRW 5 moderately hard reconstituted water [20].
[e] RMHRW 5 reformulated moderately hard reconstituted water [26].

simultaneously, and these ranged from 262 mg SO_4^{2-}/L for *Hyalella* to 11,682 mg SO_4^{2-}/L for *C. tentans* (Table 2).

Influence of dilution water composition on sulfate toxicity

Testing *H. azteca* in RMHRW produced a strikingly different response compared to results of tests in MHRW (Table 3). The mean LC50 in RMHRW (2,855 mg SO_4^{2-}/L) was more than 5.5-fold higher (p , 0.0001) than that generated using MHRW (512 mg SO_4^{2-}/L), with a . 8-fold increase in the LC10 value. *C. dubia* also had a significantly different (p 5 0.0205), though not as striking, response, with the mean LC50 increasing from 2,050 mg SO_4^{2-}/L in MHRW to 2,526 mg SO_4^{2-}/L in RMHRW. The LC10 for *C. dubia* increased from 1,759 mg / L in MHRW to 2,216 mg SO_4^{2-}/L in RMHRW (Table 3).

In the experiment with *H. azteca* designed to dissect the effects observed in RMHRW, only 45% and 55% of the test organisms exposed to 2,500 mg SO_4^{2-}/L were alive in the MHRW and Ca/Mg treatments, respectively, after 48 h, whereas 85% and 80% survived in the RMHRW and chloride media, respectively (Fig. 1). After 96 h, all of the organisms had died in MHRW and Ca/Mg, whereas 80% still survived in RMHRW and 25% survived in chloride. These data suggest that chloride played the larger role in protecting *H. azteca* against sulfate toxicity and that the different Ca:Mg ratio played a smaller role.

Influence of hardness on the toxicity of sodium sulfate

Increasing water hardness decreased the toxicity of sodium sulfate to *H. azteca* (Fig. 2). In controls, 90% of test organisms survived in MHRW (no sulfate added), whereas after 96 h, all organisms were dead in the hardness 5 100, SO_4^{2-} 5 1,460 mg/L treatment. However, in the hardness 5 200 and hardness 5 300 mg/L treatments, 15% and 60% of test organisms survived, respectively.

Whereas the mean LC50 for *C. dubia* in standard MHRW (hardness 5 90) was 2,050 mg SO_4^{2-}/L, the mean LC50s substantially increased at hardness values of 200 and 300 mg/L (Table 4). Mean LC50s were even higher at the higher hardness values of 390, 484, and 578 mg/L, with a maximum of 3,516 mg SO_4^{2-}/L at a hardness of 484 (Table 4). The LC10s increased as well, from 1,759 mg SO_4^{2-}/L at a hardness of 90 mg/L to 2,173 mg SO_4^{2-}/L, and 2,389 mg SO_4^{2-}/L at hardness values of 200 and 300 mg/L, respectively. Whereas in the 90 through 500 nominal hardness tests, measured sulfate concentrations were very close to nominal sulfate concentrations, measured sulfate in the 600 nominal hardness tests was somewhat lower than nominal sulfate concentrations, suggesting that some precipitation of $CaSO_4$ occurred. Therefore, results may be questionable at this hardness level. If the mean LC10 at that hardness is excluded, a linear relationship exists between water hardness and LC10, described by the formula LC10 (mg SO_4^{2-}/L) 5 2.685(hardness) 1 1595.5, r^2 5 0.959. When the LC10 at a hardness of 578 (nominal hardness of 600) is included, the relationship is better described by a logarithmic function with the formula LC10 (mg SO_4^{2-}/L) 5 526.24(ln[hardness]) 2 574.81 (r^2 5 0.8713).

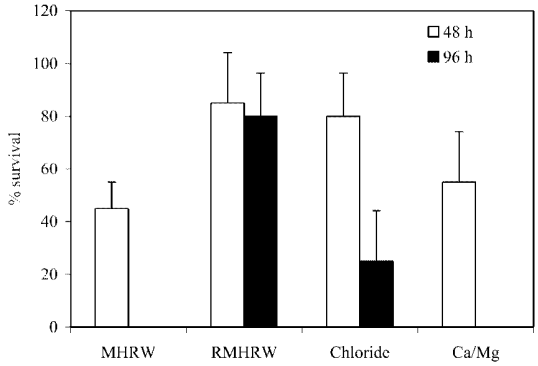

Fig. 1. Effect of various components of reformulated, moderately hard, reconstituted water (RMHRW) on percent survival of *Hyalella azteca* in elevated (2,500 mg SO_4^{2-}/L) sulfate solutions. The chloride and Ca/Mg treatments consisted of standard moderately hard reconstituted water (MHRW) with chloride or Ca:Mg molar ratio adjusted to match RMHRW.

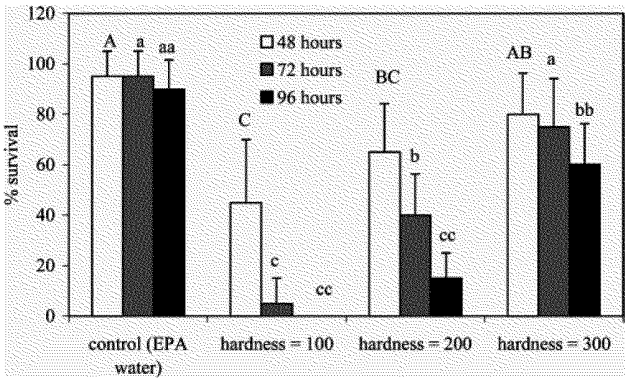

Fig. 2. Effect of hardness on toxicity of elevated sulfate to *Hyalella* in moderately hard reconstituted water. Average measured sulfate concentration was 1,460 mg/L (standard deviation 5 25) for the three treatments excluding the control (106 mg/L sulfate). EPA 5 Environmental Protection Agency. Different upper- or lower-case letters indicate means are significantly different (p , 0.05).

Table 4. Influence of water hardness on toxicity of sulfate to *Ceriodaphnia dubia* in MHRW[a]

Hardness, nominal (actual)	*n*	Mean LC50[b] (mg SO_4^{2-}/L)	Range (mg SO_4^{2-}/L)	LC10[c] (mg SO_4^{2-}/L)
90 (89)	3	2,050	1,869—2,270	1,759
200 (194)	3	3,000	2,706—3,265	2,173
300 (288)	4	2,946	2,383—3,361	2,389
400 (390)	3	3,174	3,073—3,369	2,744
500 (484)	3	3,516	3,338—3,716	2,793
600 (578)	3	3,288	2,761—4,220	2,547

[a] MHRW 5 moderately hard reconstituted water [20].
[b] Lethal concentrations to 50% of a sample population (LC50s) are geometric means of all Spearman-Karber values generated for a given organism using measured sulfate concentrations.
[c] Lethal concentration to 10% of a sample population (LC10) values were generated using probit analysis with the combined data from all tests for a given treatment.

Influence of chloride on the toxicity of sulfate

Sulfate toxicity to *H. azteca* decreased with increased levels of chloride when hardness was held constant (Fig. 3). At the lowest measured chloride concentration tested (5 mg/L), only 20% of the test organisms exposed to 2,846 mg SO_4^{2-}/L were alive after 96 h, and none of these organisms were functionally alive. At 13 mg Cl^-/L, both total and functional survival increased nominally, but not significantly (p . 0.05); however, significant increases in total and functional survival were observed at and above 18 mg Cl^-/L (p , 0.05). Survival was 85% and 100% in the 36 and 67 mg Cl^-/L treatments, respectively. Bromide concentrations in all treatments were below detection limits (, 0.01 mg/L).

Influence of acclimation on the toxicity of sulfate to C. dubia

In this experiment, with *C. dubia* acclimated for several generations to either 500 or 1,000 mg SO_4^{2-}/L, nominal increases in mean LC50 values were observed; however, these means were not significantly greater (p , 0.05) than that for organisms cultured in standard MHRW (Fig. 4).

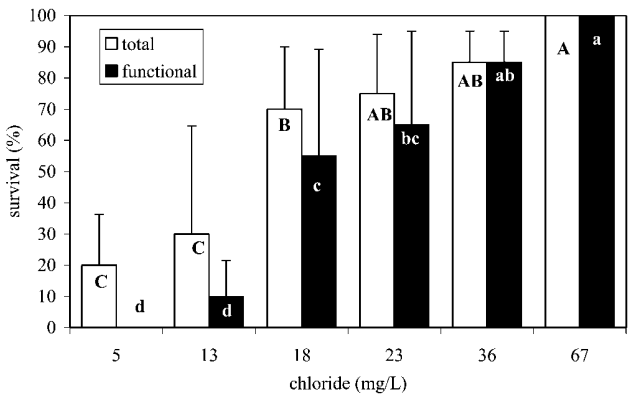

Fig. 3. Effect of increasing chloride concentrations on sulfate toxicity to *Hyalella azteca*. Mean 6 standard deviation sulfate concentration for all treatments was 2,846 6 80 mg SO_4^{2-}/L, mean hardness was 106 6 2 mg/L as $CaCO_3$, and Ca:Mg was 5.4:1. Different upper- or lower-case letters indicate means are significantly different (p , 0.05). Total 5 all survivors including those lying on bottom barely moving; functional 5 survivors that are moving about.

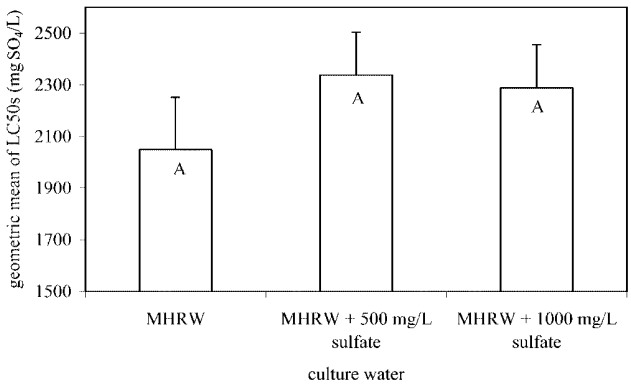

Fig. 4. Effect of acclimation on sulfate toxicity to *Ceriodaphnia dubia*. Organisms were cultured for at least two generations in moderately hard reconstituted water (MHRW), MHRW with 500 mg SO_4^{2-}, or MHRW with 1,000 mg SO_4^{2-}. Three tests were conducted with each population of organisms. Treatments with the same upper-case letters indicate that means are not significantly different (p , 0.05). LC50 5 median lethal concentration.

DISCUSSION

Toxicity of sulfate to freshwater invertebrates in MHRW

The geometric mean for the three tests with *C. dubia* in this study was 2,050 mg SO_4^{2-}/L (Table 2), which compares favorably with the value of 3,080 mg Na_2SO_4/L (equivalent to 2,082 mg SO_4^{2-}/L) generated in previous studies [11]. Values generated in this study for *H. azteca* and *S. simile* were lower than values generated by others for the fathead minnow, *P. promelas* (5,380 mg/L), and *D. magna* (3,096 mg/L) [11]. The midge, *C. tentans*, was relatively insensitive compared with other invertebrates. This finding agrees with the observation of no significant reductions in relative chironomid abundance in waters exceeding 3,000 mg SO_4^{2-}/L below a coal processing discharge facility (A.J. Kennedy, unpublished data). The British Columbia Ministry of Environment, Land and Parks (BCMELP) has an online database (wlapwww.gov.bc.ca/wat/wq/BCguidelines/sulphate/index.html) that includes a variety of sulfate toxicity data for a number species. The values generated by BCMELP for *Hyalella* were quite variable and not similar to that obtained in this study using MHRW; however, with the exception of hardness estimates, water quality data were not presented in the database, so it is difficult to make comparisons with our study. As will be discussed below, water quality data, including cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

Influence of chloride on the toxicity of sulfate

The composition of dilution water used during testing in this study had a dramatic effect on the toxicity of sulfate to *Hyalella*. In fact, the 96-h LC50 in RMHRW was 5.5-fold higher than that generated using MHRW. Both dilution waters were similar in terms of hardness (; 90—106mg/L as $CaCO_3$), alkalinity, and pH, but one potential reason for the difference in response is the difference in chloride concentrations between the two media (see Table 1). Freshwater organisms tend to osmoregulate hypertonically with respect to the surrounding medium, achieved by active transport of ions into the hemolymph [28,29]. The principal inorganic anion of crustacean hemolymph is chloride, and it has been suggested that low chloride concentrations may limit the distribution of at least

one euryhaline amphipod (*Corophium curvispinum*) in freshwaters [30]. *H. azteca* is a euryhaline amphipod [7], and perhaps when encountering high ion (Na^1 and SO_4^{2-}) concentrations in MHRW, it is not able to osmoregulate because of the relatively low concentration of chloride. This same effect was observed, to a lesser extent, with *C. dubia*.

The experiment with *Hyalella*, in which hardness and sulfate were held constant and chloride was variable (see Fig. 3), supports the hypothesis that chloride has a protective effect against sulfate toxicity, because incremental increases in chloride were associated with incremental increases in survival. Borgmann [31] included bromide as one of the ions required by *Hyalella* for long-term survival, stating that chloride is not required; however, chloride is chemically similar to bromide, and results of this study indicate that if chloride is not indeed required, it does appear to at least provide protection from salt toxicity. Bromide was not present at measurable concentrations (, 0.01 mg/L) in our experiments. The results of this study further support the findings of others that MHRW may not be an acceptable medium for water-only testing with *Hyalella* [26].

The fingernail clam, *S. simile*, had a marginally lower LC10 value for sulfate than that of *C. dubia* in MHRW, but the former was not tested in RMHRW because of temporal restrictions in its availability. It remains unclear whether or not mollusks will have the same physiological response as two crustaceans to increased chloride in toxicity experiments with sulfate. In a field study, 76% of transplanted Asian clams (*Corbicula fluminea*) in and below a treated mining discharge survived sulfate levels of approximately 3,600 mg SO_4^{2-}/L with ; 700 mg Cl^{2-}/L, although, as will be discussed below, hardness (700—800mg/L as $CaCO_3$) likely played a role in this system [5]. Chloride is a principal anion in the hemolymph of most bivalves [32], but others have found that in the unionoidean *Toxolasma texasensis*, chloride and bicarbonate are equivalent anionic components [33]. Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations for osmoregulation to the extent that some crustaceans do. If this is the case, the effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifested in some unionoidean bivalves, and further work should be done to clarify this.

Influence of hardness on the toxicity of sodium sulfate

Another factor that appears to have a strong effect on the toxicity of sulfate is the presence of other major cations, in this case, calcium and magnesium, measured as hardness. In our sodium-dominated system, increased hardness reduced the toxicity of sulfate to *Hyalella* (see Fig. 2) and had a dramatic effect on the 48-h LC50 for *C. dubia* (see Table 4). Mount et al. [11] obtained a similar result in that when using only Na_2SO_4, the LC50 for *C. dubia* was 2,082 mg SO_4^{2-}/L, but when using a 1:1 ratio of Na_2SO_4 and $MgSO_4$, the LC50 increased to 2,335 mg SO_4^{2-}/L. They were careful to point out that the effect was not necessarily caused by hardness, but rather by multiple major cations, citing that the LC50 (expressed as mg Cl^{2-}/L) for *C. dubia* in NaCl was nearly identical to that in $CaCl_2$, despite the fact that the two solutions had very different hardness values. However, increased calcium is known to decrease the passive permeability of gill epithelia to water and ions in seawater-adapted fish and crabs [34,35]. A similar phenomenon may explain the results of the hardness experiments conducted in this study; i.e., we hypothesize that the increased calcium concentrations at higher hardness levels reduced epithelial permeability, thus reducing passive diffusion and the energy required to osmoregulate and accounting for the decrease in toxicity. In support of this hypothesis, the decreased toxicity of sulfate to *Hyalella* in RMHRW was not entirely explained by the increased chloride concentration (see Fig. 1). The different Ca:Mg ratio also appeared to have an effect, and hardness in RMHRW was similar to that in MHRW (; 106 and ; 90 mg/L as $CaCO_3$, respectively). An alternative hypothesis is that increased calcium is competing for binding sites in a manner similar to that proposed for metals like copper [36]; however, this may be unlikely, because sulfate is an anion and sodium is a monovalent cation. Further experiments are required to test these hypotheses.

Others have observed reduced toxicity of saline solutions because of increased hardness. Dwyer et al. [8] demonstrated that increasing the hardness of an NaCl-dominated irrigation return water reduced its toxicity to striped bass and *D. magna*. A similar phenomenon was observed with a coal processing discharge in Ohio, USA [5,15]. Although this discharge did include elevated sulfate (3,672 mg/L) and chloride (792 mg/L) concentrations, the nature of the toxicity was complicated by other ions in solution. The hardness of the field-collected effluent (; 792 6 43 mg/L as $CaCO_3$) and several synthetic solutions of varying hardness appeared to reduce sodium and sulfate-dominated TDS toxicity in a fashion similar to that observed in the current study, on both an acute and chronic scale [5; Alan J. Kennedy, unpublished data]. In addition, the BCMELP database suggests a hardness effect on sulfate toxicity for both *D. magna* and *Hyalella*. The present study has shown quantitatively that, in a sodium-dominated system, sulfate toxicity is reduced as hardness progressively increases, although results may require further investigation at the highest hardness tested (578 mg/L). Higher hardness levels should be tested to determine whether the relationship remains linear or is logarithmic and reaches an asymptote.

Influence of acclimation on the toxicity of sulfate to C. dubia

We hypothesized that *C. dubia* acclimated to varying levels of sulfate would be less sensitive to sulfate than naive organisms, as implied in other studies addressing TDS acclimation [1,37] and shock [38]. Although the LC50s for the sulfate-acclimated organisms were nominally higher, the means were not significantly different from those of unacclimated organisms. Perhaps more generations of exposure are required before a significant benefit is seen, and further work should be done in this area.

CONCLUSIONS

In conclusion, sulfate toxicity is a complex issue, and a number of factors may interact to determine the responses of various organisms to sodium and sulfate-dominated, saline waters. We have found that in MHRW, *H. azteca* is the most sensitive to sulfate of the four invertebrates tested, followed by *C. dubia* and *S. simile*, then *C. tentans*. Furthermore, we demonstrated that increasing chloride concentration reduces the toxicity of sulfate to *Hyalella*, and increasing water hardness ameliorates sodium sulfate toxicity to *Hyalella* and *C. dubia*. More research is required into the hardness issue to determine whether it was truly calcium that ameliorated sulfate toxicity, because only one Ca:Mg ratio was used in this study when increasing hardness, and other major cations like potas-

sium were not investigated. In addition, the actual mechanism behind the mode of protection from multiple cations should be studied. Finally, the aforementioned issues should be examined at a chronic scale using sublethal and/or multigenerational endpoints as more accurate indicators of population-level effects.

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