BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

IN THE MATTER OF:	)	
	)	
PROPOSED AMENDMENTS TO:	)	
35 Ill. Adm. Code 302.102(b)(6), 302.102(b)(8)	)	R07-09
302.102(b)(10), 302.208(g), 309.103(c)(3),	)	(Rulemaking - Water)
405.109(b)(2)(A), 405.109(b)(2)(B), 406.100(d);	)	
REPEALED 35 Ill. Adm. Code 406.203, PART 407; and	)	
PROPOSED NEW 35 Ill. Adm. Code 302.208(h)	)	

NOTICE OF FILING

Dorothy Gunn, Clerk
Illinois Pollution Control Board
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Springfield, Illinois 62702-1271

ALSO SEE ATTACHED SERVICE LIST

PLEASE TAKE NOTICE that I have today filed with the Office of the Clerk of the Pollution Control Board the **Illinois Environmental Protection Agency's Additional Information and Documents**, a copy of which is herewith served upon you.

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY

By:_____
Sanjay K. Sofat, Assistant Counsel
Division of Legal Counsel

Dated: April 6, 2007
Illinois Environmental Protection Agency
1021 North Grand Avenue East
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REPEALED 35 Ill. Adm. Code 406.203, PART 407; and	)	
PROPOSED NEW 35 Ill. Adm. Code 302.208(h)	)	

ADDITIONAL INFORMATION AND DOCUMENTS

THE ILLINOIS ENVIRONMENTAL PROTECTION AGENCY (the "Agency" or "Illinois EPA") respectfully submits this additional information and documents for the Illinois Pollution Control Board's (the "Board") R07-9 rulemaking proceeding. The Agency files this additional information and documents in support of the Agency's proposal and to address the questions raised by Dr. Anand Rao, a Senior Environmental Scientist for the Board, at the March 7, 2007 hearing. The Agency commends the efforts of the Environmental Law & Policy Center, the Sierra Club, and Prairie Rivers Network in providing helpful questions at this hearing. The Agency also thanks the Board for holding this hearing on this important rulemaking proposal. In response to Dr. Rao's questions, the Agency provides the following responses:

Dr. Rao: "IEPA has granted wet weather dischargers allowed mixing zones for sulfate and sometimes chloride with consideration to upstream flows in the past few years. Can you be a little more specific and tell us what was the receiving stream and what particular source received this permit?" Transcript ("hereinafter Tr."), p.55.

An example of a facility receiving this type of mixing consideration is Mine X. The receiving stream for the mine effluent is a small channelized ditch. The hardness value measured in the

mine effluent was lower than the values measured in the ditch upstream of the mine discharge. The Agency used average effluent hardness of 373 mg/L to calculate the sulfate standard. To calculate the chloride concentration downstream of the mine effluent, the Agency used upstream and effluent chloride values and average effluent and upstream flows. The calculated chloride concentration downstream of the mine effluent was 277 mg/L. The proposed sulfate standard equation from Section 302.208(h)(2)(A) was then used to calculate the allowable sulfate concentration in the ditch, 1903 mg/L. This concentration is much higher than the maximum effluent concentration from the mine; therefore, no mixing is needed to meet the standard.

The average chloride concentration, however, was 567 mg/L in the effluent. In order to determine the allowed mixing during wet weather to assess attainment of the water quality standard of 500 mg/L, actual flow values from the effluent and the ditch were compared. The lowest dilution ratio during discharge was 1.29 to 1 with much higher ratios at other times. Given the upstream average chloride of 29.2 mg/L, the allowable effluent chloride concentration is 1107 mg/L using 100% of the stream flow at this conservative wet weather discharge condition. The permit contains a special condition that prohibits the discharge during dry weather if the effluent has potential to violate any applicable water quality standards.

Dr. Rao: "Can you tell me how many mine discharge permits currently exist in the State that are affected by these rules?" Tr., p.73.

There are 19 active coal mines in Illinois at the present time. The Agency believes that all of these mines have discharges that have the potential to exceed either the Board's existing sulfate or the chloride water quality standards in their final effluent. Other mine related discharges exist at mine reclamation sites, coal ash disposal sites, and related facilities not associated with one of the active mines. These sources total approximately 90 NPDES permits, and most of these

discharges would also not meet one or both of these standards in the final effluent.

Dr. Rao: "Has the Illinois State Water Survey identified a map of these 7Q1.1 streams; how many 7Q1.1 streams are there?" Tr., p.80.

No, the Illinois State Water Survey has not developed a map depicting the 7Q1.1 hydrologic statistics. The Agency, however, does believe that sufficient data and information exist to identify these 7Q1.1 zero flow streams. As part of the permit application, the applicant will provide the basic information necessary for identifying these small streams. Where necessary, the Agency would rely on the expertise of a hydrologist at the Illinois State Water Survey ("ISWS") in identifying the 7Q1.1 zero flow streams. The Agency believes that many thousands of small headwater streams throughout the State would be classified as 7Q1.1 zero flow streams. The exact boundaries of these streams would be determined based on watershed area, rainfall patterns, and soil type.

Dr. Rao: "Has Dr. Soucek's report undergone peer review?" Tr., p.83.

Dr. Soucek has recently published three peer reviewed papers concerning the research he conducted under contract from the Illinois EPA, the Illinois Coal Association, and USEPA. The first paper, *Effects of Hardness, Chloride, and Acclimation on the Acute Toxicity of Sulfate to Freshwater Invertebrates*, presents research that was funded by the Illinois EPA and the Illinois Coal Association. This paper published in *Environmental Toxicology and Chemistry* in 2005 and is now included as Exhibit 1. This paper is a summary of Dr. Soucek's final report to the Illinois EPA and the Illinois Coal Association which was added as Exhibit Q in the "Facts in Support" document (See Attachment I of the Agency's proposal).

Dr. Soucek has also recently published two papers that the Illinois EPA was previously unaware of, as both papers were published following the first hearing. The paper entitled,

Comparison of Hardness- and Chloride-Regulated Acute Effects of Sodium Sulfate on Two Freshwater Crustaceans, presents research funded by USEPA and is a summary of Dr. Soucek's final report submitted to USEPA, which was included as Exhibit U of the Agency's proposal. This publication has now been included as Exhibit 2. The second publication entitled, *Bioenergetic Effects of Sodium Sulfate on the Freshwater Crustacean, Ceriodaphnia dubia*, was published in *Ecotoxicology*, and is now included as Exhibit 3. This paper summarizes research funded by USEPA and mostly includes data that was reported in the final report to USEPA (See Exhibit U of the Agency's proposal).

Respectfully Submitted

ILLINOIS ENVIRONMENTALPROTECTION AGENCY

By: _____

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EXHIBIT

1

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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^{2-}/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^{2-}/L at hardness = 90 mg/L to 3,516 mg SO_4^{2-}/L at hardness = 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^{2-}/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 Min® (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 22°C 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 22°C 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^{2-}/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 µg/L) and zinc (9 µg/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 25°C and the *C. tentans* tests at 22 °C. *H. azteca* and *S. simile* were exposed for 96 h at 22°C 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 (7–14 d 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 Accumet® (Fisher Scientific, Pittsburgh, PA, USA) model AB15 pH meter equipped with an Accumet gel-filled combination electrode (accuracy $<\pm 0.05$ pH at 25°C). 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 Toledo® (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 22°C. Mean percent survivorship values for each treatment were compared using analysis of variance with JMP-IN® 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 = lethal concentration to 50% of a sample population.
[c] LC10 = lethal concentration to 10% of a sample population.
[d] MHRW = moderately hard reconstituted water [20].
[e] RMHRW = 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 ($p = 0.0205$), 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 = 100, SO_4^{2-} = 1,460 mg/L treatment. However, in the hardness = 200 and hardness = 300 mg/L treatments, 15% and 60% of test organisms survived, respectively.

Whereas the mean LC50 for *C. dubia* in standard MHRW (hardness = 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) = 2.685(hardness) + 1595.5, $r^2 = 0.959$. 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) = 526.24(ln[hardness])−574.81 (r^2= 0.8713).

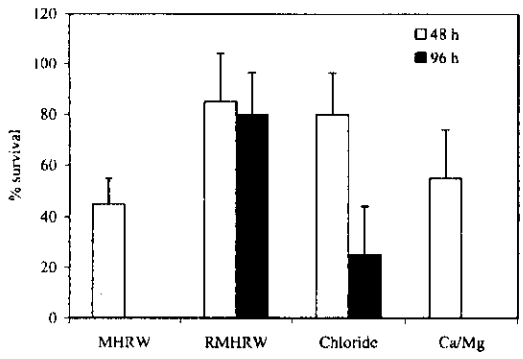

Fig. 1. Effect of various components of reformulated, moderately hard, reconstituted water (RMHRW) on percent survival of *Hyalella azteca* in elevated (2,500 mg SO_4^{2-}/L) sulfate solutions. The chloride and Ca/Mg treatments consisted of standard moderately hard reconstituted water (MHRW) with chloride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

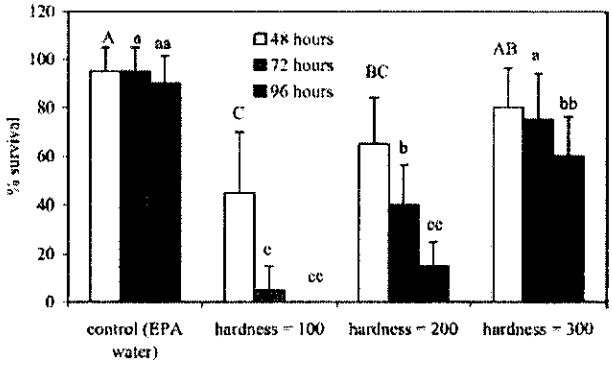

Fig. 2. Effect of hardness on toxicity of elevated sulfate to *Hyalella* in moderately hard reconstituted water. Average measured sulfate concentration was 1,460 mg/L (standard deviation = 25) for the three treatments excluding the control (106 mg/L sulfate). EPA = Environmental Protection Agency. Different 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 = 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).

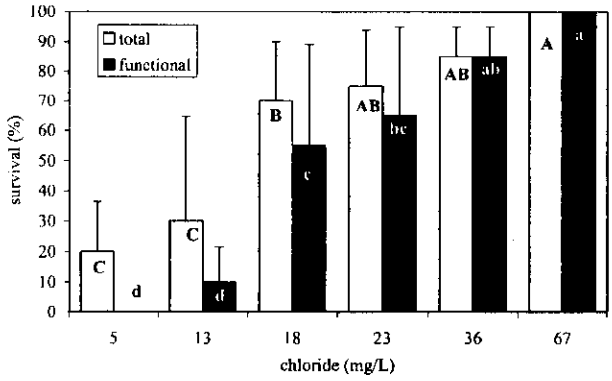

Fig. 3. Effect of increasing chloride concentrations on sulfate toxicity to *Hyalella azteca*. Mean ± standard deviation sulfate concentration for all treatments was 2,846 ± 80 mg SO_4^{2-}/L, mean hardness was 106 ± 2 mg/L as $CaCO_3$, and Ca:Mg was 5.4:1. Different upper- or lower-case letters indicate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

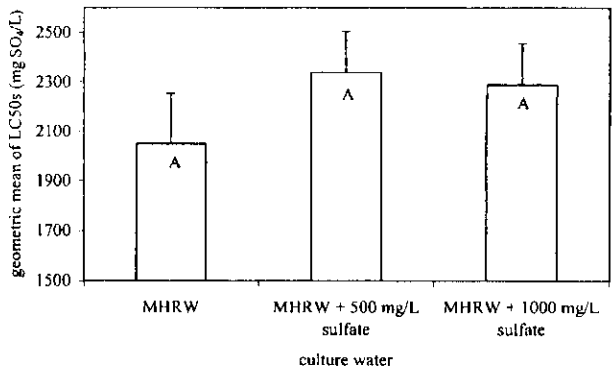

Fig. 4. Effect of acclimation on sulfate toxicity to *Ceriodaphnia dubia*. Organisms were cultured for at least two generations in moderately hard reconstituted water (MHRW). MHRW with 500 mg SO_4^{2-}, or MHRW with 1,000 mg SO_4^{2-}. Three tests were conducted with each population of organisms. Treatments with the same upper-case letters indicate that means are not significantly different ($p < 0.05$). LC50 = 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 (~90–106 mg/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^+ 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^-/L, although, as will be discussed below, hardness (700–800 mg/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^-/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 ± 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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EXHIBIT

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COMPARISON OF HARDNESS- AND CHLORIDE-REGULATED ACUTE EFFECTS OF SODIUM SULFATE ON TWO FRESHWATER CRUSTACEANS

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Abstract—Based on previous observations that hardness (and potentially chloride) influences sodium sulfate toxicity, the objective of the current study was to quantify the influence of both chloride and water hardness on acute toxicity to *Hyalella azteca* and *Ceriodaphnia dubia*. In addition, observed toxicity data from the present study were compared to toxicity predictions by the salinity/toxicity relationship (STR) model. Hardness had a strong influence on sulfate toxicity that was similar for both crustaceans, and nearly identical median lethal concentration (LC50)/hardness slopes were observed for the two species over the tested range. Chloride had a strong but variable influence on sulfate acute toxicity, depending on the species tested and the concentration range. At lower chloride concentrations, LC50s for *H. azteca* strongly were correlated positively with chloride concentration, although chloride did not affect the toxicity of sodium sulfate to *C. dubia*. The opposite trend was observed over the higher range of chloride concentrations where there was a negative correlation between chloride concentration and sulfate LC50 for both species. The widely ranging values for both species and a high correlation between LC50s in terms of sulfate and conductivity suggested that, whether based on sulfate, conductivity, or total dissolved solids (TDS), attempts at water quality standard development should incorporate the fact that water quality parameters such as hardness and chloride strongly influence the toxicity of high TDS solutions. The STR model predicted toxicity to *C. dubia* relatively well when chloride was variable and hardness fixed at approximately 100 mg/L; however, the model did not account for the protective effect of hardness on major ion/TDS toxicity.

Keywords—Sulfate Total dissolved solids *Hyalella* *Ceriodaphnia* Salinity/toxicity relationship model

INTRODUCTION

Currently no federal water quality criteria exist for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium; however, toxicity due to major ions or TDS has received increasing attention in recent years [1,2]. Ordinarily benign major ions (e.g., sodium, sulfate) and, therefore, 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 reach concentrations in wastewater discharges that severely impair sensitive aquatic species [3–7]. Common sources of effluents with elevated TDS include reverse osmosis systems, pH modifications of wastewater, agricultural runoff, gas and oil production, and coal or metal mining operations [1]; investigations of major ion toxicity also have included inundation of freshwater systems by brackish water and laboratory-formulated salt solutions [5–13].

The fact that TDS toxicity is dependent on the ionic composition of a water or effluent has been well established [7,9,12,14,15]. Mount et al. [12] developed statistical models to predict toxicity of high TDS waters to standard test organisms based on ionic composition; seven major ions (Na^+, K^+, Ca^{2+}, Mg^+, Cl^-, HCO_3^-, and SO_4^{2-}) were included in the analysis. In that study, solutions were more toxic when dominated by particular major ions (i.e., K^+, Mg^+, HCO_3^-), and toxicity due to several individual ions, including SO_4^{2-}, to *Ceriodaphnia dubia* and *Daphnia magna* was reduced when solutions contained more than one cation [12]. Several researchers have observed that hardness and/or multiple nontoxic cations in solution ameliorate major ion toxicity [9,12,15], and laboratory experiments with synthesized freshwaters have demonstrated that increasing hardness at a constant calcium-to-magnesium ratio (Ca:Mg) results in decreased sodium sulfate toxicity to *C. dubia* [14].

In experiments with sodium sulfate in laboratory-synthesized freshwaters, Soucek and Kennedy [14] observed that, while composition of dilution water strongly affected sulfate toxicity to *C. dubia*, the magnitude of the effect on *H. azteca* was even greater. Specifically, whereas the median lethal concentrations (LC50s) for *C. dubia* in two diluents with different chloride concentrations and Ca:Mg ratios ranged from 2,050 to 2,526 mg SO_4^{2-}/L, the LC50s for *H. azteca* in the same two diluents were 512 and 2,855 mg SO_4^{2-}/L, respectively [14]. Freshwater organisms use several different osmoregulatory strategies, but most freshwater amphipods and daphnid cladocerans regulate hypertonically with respect to the surrounding medium; this is achieved by active transport of ions, principally chloride, into the hemolymph (see [16–19]). However, even among amphipods, there is a wide range of sodium and chloride influx rates and integument permeabilities, which determine osmoregulatory effectiveness [20,21], so the ionic composition of water may regulate to varying degrees the response of different species to high levels of sodium sulfate.

The observed contrast in responses of *C. dubia* and *H. azteca* to sulfate under different water quality conditions [14] led to the interest in quantifying the relationships between sulfate toxicity and hardness and chloride concentrations for the two distantly related freshwater crustaceans. Therefore, the objectives of the current study first were to determine the influence of hardness on sodium sulfate toxicity to *H. azteca* and to compare its responses to those of *C. dubia* and, second, to quantify the effects of chloride on acute toxicity of sodium

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sulfate to *H. azteca* and *C. dubia* over a wide range of chloride concentrations. The data generated in the first two objectives were useful for investigating the relationship between LC50s calculated in terms of sulfate and those calculated in terms of conductivity to determine the potential utility of a conductivity- or TDS-based water quality standard. The data also presented an opportunity to test the effectiveness of the salinity/toxicity relationship (STR) model developed by Mount and Gulley [22] in predicting acute toxicity of a wide range of sodium, sulfate, chloride, and hardness combinations to *C. dubia*.

MATERIALS AND METHODS

General culturing and testing methods

The cladoceran, *Ceriodaphnia dubia*, was cultured in the laboratory according to U.S. Environmental Protection Agency (U.S. EPA) methods [23]. Amphipods, *Hyalella azteca*, also were cultured in the laboratory according to U.S. EPA methods [24] in a reformulated, moderately hard, reconstituted water described in Smith et al. [25].

For toxicity testing, a pure (99%) grade of anhydrous sodium sulfate (Na_2SO_4) (Chemical Abstract Service [CAS] 7757-82-6) was obtained from Fisher Scientific (Pittsburgh, PA, USA) to serve as the source of sulfate. Previous experiments indicated that the salts and deionized water sources used for our experiments had low to undetectable levels of trace metal contaminants [14,26].

For definitive static, nonrenewal toxicity tests, conducted according to American Society for Testing and Materials E729-96 methods [27], treatments comprised a 75% dilution series (i.e., the 100% concentration was diluted serially 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 in addition to controls with four replicates tested per concentration. Tests with *C. dubia* were conducted for 48 h with a 16:8-h light:dark photoperiod at 25°C, and *H. azteca* were exposed for 96 h at 22°C and a 16:8-h light:dark photoperiod. Both organisms were exposed in 50-ml glass beakers with five organisms per beaker and, for H. *azteca*, 1 g of quartz sand was added to each beaker to serve as substrate. Only one of the 63 tests was fed, and that fed test had an LC50 in the range of two other tests conducted with the same organism in the same water without food. *Ceriodaphnia dubia* used were less than 24-h old, and *H. azteca* were approximately third instar (7–14-d 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 *H. azteca*.

Standard water chemistry characteristics were measured at both the beginning and the end of each exposure period. Temperature, pH, conductivity, and dissolved oxygen were measured using appropriate meters, and alkalinity and hardness were measured (beginning of tests only) by titration as described by American Public Health Association et al. [28]. 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 LC50s were calculated based on both measured sulfate concentrations and measured specific conductivity values for each test concentration using the Spearman-Karber method [29]. To increase confidence in LC50s, three to five assays were conducted with each organism for each water quality combination. This provided a stronger estimate of the mean LC50 for a given set of water quality characteristics for each species. In all, 63 LC50s were generated.

Influence of chloride on the toxicity of sodium sulfate

In these experiments, the toxicity of sulfate (with sodium as the major cation) to *H. azteca* and *C. dubia* was measured in freshwater solutions having nominal chloride concentrations of 1.9, 10, 15, 20, 25, 33 (*H. azteca* only), 100, 300, and 500 mg Cl/L. Chloride, as NaCl (CAS 7647-14-5, Fisher Scientific Catalog AC42429-0010), was added at appropriate concentrations to a solution with a hardness of approximately 100 mg/L (molar ratio of Ca:Mg = 1.41; 2.33 in terms of mass). The Ca:Mg ratio was chosen because it is the median value for water bodies sampled in Illinois (R. Mosher, Illinois Environmental Protection Agency, Springfield, IL, USA, personal communication). Whole carboys were made for each elevated chloride level, and this water was used as both diluent and control; therefore, each concentration within a given test had the same chloride concentration (i.e., $[Cl^-]$ 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 to provide a mean LC50 and standard deviation. Exposures were conducted using the same laboratory and calculation methods described above.

After LC50s were calculated as described above, regression analysis was conducted using JMP® software [30] to determine the relationship between chloride concentration and sulfate LC50 for each species; mean LC50s for each chloride concentration were used in these analyses. Because observation of data scatter indicated two different trends were involved depending on the chloride concentration range, two separate analyses were conducted for each species: One for the range of 5 (1.9 mg/L nominal concentration) to 25 mg Cl/L and one for the range of 25 to 500 mg Cl/L. Then, multiple regression analysis with covariance was conducted for the same data ranges using all individual data points to generate an equation for both species, and to determine if the curves were significantly different for the two species.

Influence of hardness on the toxicity of sodium sulfate

In these experiments, the toxicity of sulfate (with sodium as the major cation) to *H. azteca* was tested in six freshwater solutions having nominal hardness values of <100, 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), at a set molar ratio (Ca:Mg = 1.41; 2.33 in terms of mass), to achieve the nominal hardness. Measured hardness values for all tests were similar to target nominal hardness values (±2.2%). A chloride concentration of 25 mg/L was used for all tests investigating the effects of hardness on sodium sulfate toxicity to *H. azteca* based on results from the above-described tests investigating the effects of chloride on sodium sulfate toxicity to *H. azteca* and *C. dubia*. Whole carboys were made for 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 to provide a mean LC50 and standard deviation. Exposures were conducted using the same laboratory and calcu-

lation methods described above. The LC50s for *H. azteca* were compared to previously generated LC50s for *C. dubia* [14], which were conducted in solutions having a Ca:Mg molar ratio of 0.88 and [Cl^-] of 5 mg/L.

After LC50s were calculated as described above, regression analysis was conducted using JMP® software [30] to determine the relationship between hardness and sulfate LC50 for each species. Mean LC50s for each hardness were used in these analyses. Then, multiple regression analysis with covariance was conducted for the same data ranges using all individual data points to generate an equation for both species and to determine if the curves were significantly different for the two species.

Relationship between sulfate LC50s and conductivity LC50s

To investigate variability in conductivity at sulfate LC50 concentrations, linear regression analysis was used, and *C. dubia* data from Soucek and Kennedy [14] were included in the analyses.

Comparison of test results with STR model predictions

To compare results from the present study to toxicity predictions generated by the STR model [22], nominal concentrations of all constituent ions (except H^+ and OH^-, which are not required by the model) were calculated at observed mean sulfate LC50s for *C. dubia* in each test solution type (Cl^- = x, hardness = y, and Ca:Mg = z). The model does not predict toxicity to *H. azteca*. Ions required by the model are Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, HCO_3^-, and SO_4^{2-}. These calculations were possible because adding known salt concentrations to deionized water generated all test solutions. The average of the absolute value of % difference between nominal and measured sulfate concentrations was 2.082%.

The model output includes equivalents of cations and anions and requires that, to have confidence in model output, the difference between the two be less than 15% [22]. The average (±standard deviation) % difference between cations and anions for inputs was 0.09 (±0.01)%, indicating excellent agreement between cation and anion equivalents. Other model outputs included calculated TDS, a NUMCAT value that estimates effective number of cations, LC50 in terms of % of solution, and % survival in 100% of solution. To examine the predictive ability of the STR model over the range of solutions tested, we created scatter plots of predicted % survival versus either chloride concentration or water hardness as appropriate for each species tested. Because all of our input values were ion concentrations calculated at experimentally observed sulfate LC50s, the observed % survival was always 50%. Observed % survival data were plotted as a horizontal line for comparison to STR-predicted data.

RESULTS

Influence of chloride on the toxicity of sodium sulfate

Chloride had variable effects on sodium sulfate toxicity to *C. dubia* and *H. azteca* over the range of 5 to 500 mg Cl^-/L. For *H. azteca*, two different linear trends were observed depending on the chloride range (Fig. 1A and B). Increasing chloride concentration from 5 to 25 mg/L resulted in increasing SO_4^{2-} LC50s (r^2 = 0.8503, p = 0.0258) for *H. azteca* (Fig. 1A), although for *C. dubia*, the slope was not significantly different from zero over this chloride concentration range (r^2 = 0.4906, p = 0.1877). In addition, the LC50s for *C. dubia*

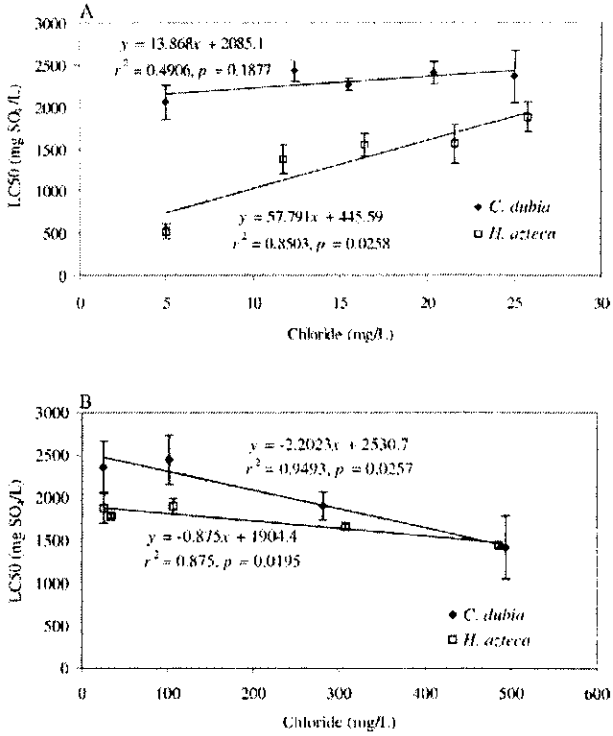

Fig. 1. Influence of chloride concentration over two ranges, (**A**) 5 to 25 mg/L and (**B**) 25 to 500 mg/L, on toxicity of sodium sulfate to *Ceriodaphnia dubia* and *Hyalella azteca*. Hardness was approximately 100 mg/L for all tests and Ca:Mg molar ratios were 1.41 except for the tests at 5 mg Cl^-/L (0.88). LC50 = Median lethal concentration.

were higher than those for *H. azteca* for each chloride concentration over this range. When using a combined data set of individual test LC50s for *C. dubia* and *H. azteca* over this chloride range and at hardness = 100 (n = 33), in a simple linear regression analysis with covariance (with species as a treatment effect and chloride concentration as continuous effect), a strong positive relationship was observed (r^2 = 0.7900, $p < 0.0001$) with both the chloride and treatment (species) effects being significant ($p < 0.0001$; Table 1).

Although a positive relationship between chloride concentration and SO_4^{2-} LC50 was observed for *H. azteca* over the range of 5 to 25 mg Cl^-/L, a significantly negative trend (r^2 = 0.875, p = 0.0195) was observed over the range of 25 to 500 mg Cl^-/L. An even stronger negative relationship (r^2 = 0.9493, p = 0.0257) was observed for *C. dubia* over the same chloride range. When using the combined data set of individual test LC50s for *C. dubia* and *H. azteca* over this chloride range and at hardness = 100 (n = 30), in a multiple linear regression analysis with covariance as described above, a negative relationship was observed (r^2 = 0.6539, $p < 0.0001$), with both the chloride and treatment (species) effects being significant ($p < 0.0001$ and p = 0.0003, respectively; Table 1).

Effects of hardness on toxicity of sulfate to Hyalella *at chloride = 25 mg/L*

A strong linear trend of decreased sulfate toxicity with increased hardness (r^2 = 0.7092, p = 0.0354) was observed for *H. azteca* (Fig. 2). The LC50 values increased from less than 1,900 mg/L at hardness = 100 mg/L, to greater than 4,000

Table 1. Results of multiple regression analysis with covariance for three different subsets of data. Individual median lethal concentrations were used as data points. Data for both species were included in analysis

Term	Estimate	p
[Cl^-] range = 5 to 25 mg/L; hardness approximately 100 mg/L r^2 = 0.7900, n = 33		
Intercept	1,270.23	<0.0001
Chloride	35.14	<0.0001
Species	449.68	<0.0001
Term	Estimate	p
[Cl^-] range = 25 to 500 mg/L; hardness approximately 100 mg/L r^2 = 0.6539, n = 30		
Intercept	2,189.48	<0.0001
Chloride	−1.46	<0.0001
Species	178.92	0.0003
Term	Estimate	p
Hardness range = 100 to 600, Cl = 25 mg/L r^2 = 0.5177, n = 38		
Intercept	1,969.38	<0.0001
Hardness	3.15	<0.0001
Species	−10.38	0.9046

mg/L at a hardness of 500 mg/L. The mean LC50 value at 600-mg/L hardness was lower than that at 500-mg/L hardness. It remains unclear how the trend will continue with increasing hardness above 600 mg/L. When using the combined data set of individual test LC50s for *C. dubia* and *H. azteca* over this hardness range (n = 38) in a multiple linear regression analysis with covariance as described above, a positive relationship was observed (r^2 = 0.5177, $p < 0.0001$; Table 1). The hardness effect was observed to be significant ($p < 0.0001$), but the treatment (species) effect was not (p = 0.9046; Table 1).

Relationship between sulfate LC50s and conductivity LC50s

Conductivity LC50s ranged from 1,071 to 8,449 µmhos/cm, and LC50s based on sulfate ranged from 512 to 4,345 mg SO_4^{2-}/L (Fig. 3). When including all data from the present study and *C. dubia* data from Soucek et al. [14], there was a strong positive relationship between sulfate LC50s and conductivity

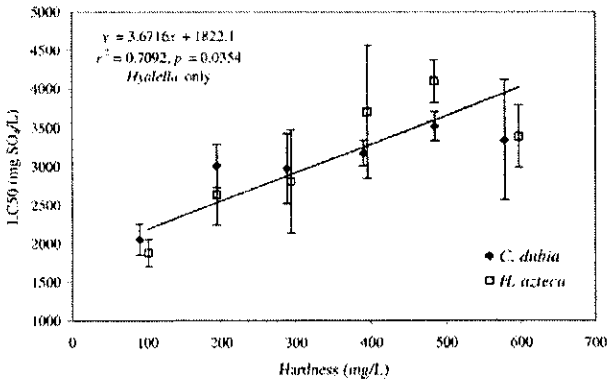

Fig. 2. Influence of hardness on toxicity of sulfate to *Hyalella azteca* and *Ceriodaphnia dubia*. The *C. dubia* data are from Soucek et al. [14]. Concentration for all *H. azteca* tests was approximately 25 mg/L, and Ca:Mg molar ratio was 1.41. LC50 = Median lethal concentration.

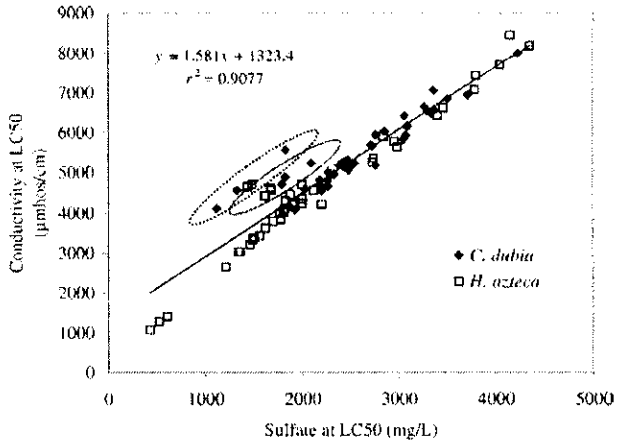

Fig. 3. Relationship between median lethal concentration (LC50) in terms of sulfate (mg/L) and in terms of conductivity (µmhos/cm) for tests with *Hyalella azteca* and *Ceriodaphnia dubia*. In addition to the 63 new tests generated for the present study, 19 tests from Soucek et al. [14] with *C. dubia* were included. Hardness values ranged from 100 to 600 mg/L and chloride ranged from 5 to 500 mg/L. Points enclosed by the solid oval were conducted at 300 mg Cl^-/L and those in the dashed oval at 500 mg Cl^-/L (nominal concentrations).

LC50s (r^2 = 0.9077, $p < 0.0001$; Fig. 3). Twelve data points enclosed in solid and dashed ovals in Figure 3 diverged from the line formed by the remaining points, suggesting sulfate LC50s were lower than would be predicted by conductivity LC50s. These points were for tests with *C. dubia* and *H. azteca* when nominal chloride concentrations were 300 mg/L (solid oval) and 500 mg/L (dashed oval).

Comparison of test results with STR model predictions

All ion concentrations used as input for the STR model were concentrations at the observed mean sulfate LC50 levels for each test solution type (Cl^- = x, hardness = y, and Ca:Mg = z), so observed percent survival in each case was 50%. For tests with *C. dubia* in which hardness was fixed at approximately 100 mg/L and chloride varied from 5 to 500 mg/L, the STR model predicted % survival values ranging from 69.0 to 48.4% (Fig. 4A). Most predictions were greater than 50%, and thus the model slightly underpredicted toxicity in most cases.

For tests with *C. dubia* in which chloride was fixed at approximately 25 mg/L and hardness varied from 100 to 600 mg/L, the STR model predictions were highly variable, ranging from 4.1 to 82.9% survival (Fig. 4B). Only the hardness = 100 mg/L prediction was greater than 50% (82.9%); for hardness values of 200 to 600 mg/L, toxicity was strongly overpredicted, with % survival predictions of 4.1 to 21.8.

DISCUSSION

Chloride had a strong but variable influence on acute sulfate toxicity, depending on the species tested and the concentration range. In multiple linear regression analyses with covariance, including species as a variable, the species term was significant over both the lower (5 to 25 mg Cl^-/L) and the higher (25 to 500 mg Cl^-/L) chloride ranges. The difference between the two species was most striking over the 5- to 25-mg/L chloride range where LC50s for *H. azteca* were strongly positively correlated with chloride concentration and chloride did not affect the response of *C. dubia* (see Fig. 1). *Hyalella* appears

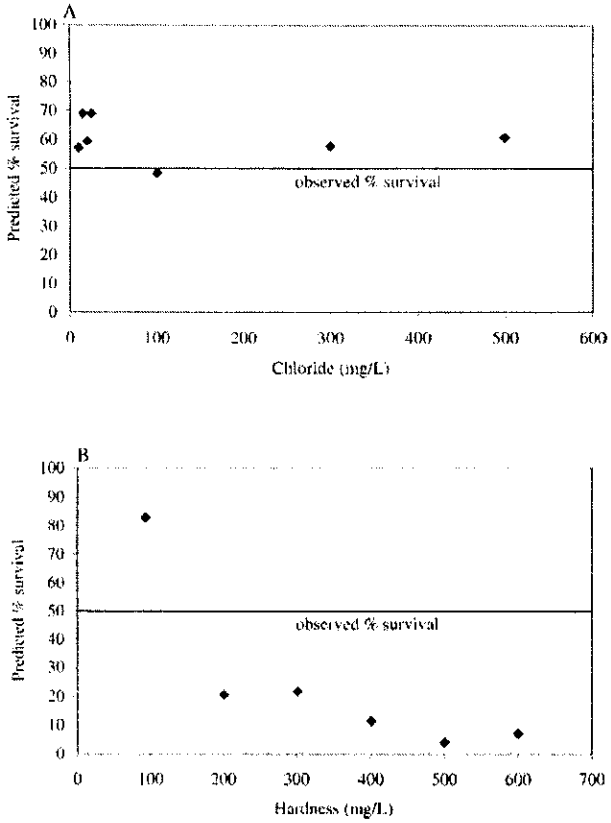

Fig. 4. Percent survival of *Ceriodaphnia dubia* at varying levels of chloride (**A**) and hardness (**B**) as predicted by the salinity/toxicity relationship (STR) model. Points are output predicted by STR and the horizontal line is observed result. Model inputs were ion concentrations at nominal sulfate median lethal concentrations, making observed % survival in each case 50%.

to require a minimal amount of chloride for effective osmoregulation at high sodium sulfate concentrations. Although there are several different osmoregulatory strategies used by freshwater organisms, most freshwater amphipods and daphnid cladocerans regulate hypertonically with respect to the surrounding medium, and this is achieved by active transport of ions into the hemolymph [16–19]. 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 [20]. Even among amphipods, there is a wide range of sodium and chloride influx rates and integument permeabilities that determine osmoregulatory effectiveness [20,21]; therefore, it might not be surprising that the responses of *H. azteca* and *C. dubia* to sodium sulfate were quite different over the lower range of chloride concentrations. Although Borgmann [31] suggested that, under low salinity conditions, bromide was required but chloride was not needed by *H. azteca* for survival, growth, and reproduction, data from the present study suggest that the chloride is quite important in determining the effect of elevated levels of sodium sulfate on that species. Laboratory deionized water and concentrated sodium sulfate solutions were analyzed previously for bromide, and levels were below detection limits [26].

Over the higher range of chloride concentrations (25–500 mg/L), a different trend was observed. Although the slopes of the lines for the two crustacean species were different, there was a negative correlation between chloride concentration and sulfate LC50 for both species. The trend was stronger for *C. dubia*, with a more negative slope (−2.2) compared to *H. azteca* (−0.875), although r^2 values were high and relationships statistically significant for both. These data suggest that, over this range of chloride concentrations, chloride and sodium sulfate toxicity are additive. Chloride LC50s (as NaCl) for *C. dubia* generally range from 900 to 1,200 mg Cl^-/L (e.g., [12]), and so the highest two chloride concentrations in the present study were likely to cause some toxicity without sulfate present.

Hardness had a strong influence on sulfate acute toxicity that was similar for both crustacean species. A number of studies have provided evidence that increasing hardness ameliorates toxicity of waters with high dissolved solids concentrations [7,9,12,15] and Soucek and Kennedy [14] showed quantitatively that, in a sodium-dominated system, sulfate toxicity to *C. dubia* is reduced as hardness progressively increases, albeit with diminishing returns in the hardness = 600 mg/L range. In the present study, the results of multiple linear regression analyses indicated no difference between the sensitivities of the two species over the hardness range of 100 to 600 mg/L as $CaCO_3$. This was in contrast to the results of the tests in which chloride was varied, where the two species had different slopes over both ranges of chloride concentrations examined. In addition, these results are notable because nearly identical slopes were observed for the two species despite the fact that the waters for tests conducted with *C. dubia* had a different chloride concentration (5 mg/L) and Ca:Mg molar ratio (0.88) than those used for tests with *H. azteca* (25 mg Cl^-/L and 1.41 Ca:Mg molar ratio). As an explanation for this phenomenon of hardness ameliorating sulfate toxicity, Soucek and Kennedy [14] proposed that increased calcium concentrations decrease the passive permeability of epithelial cells to water and ions in various aquatic organisms [32,33], reducing passive diffusion and the energy required to osmoregulate and accounting for the decrease in toxicity. Calcium can mitigate hydrogen ion toxicity to aquatic organisms by decreasing membrane permeability to H^+ and stimulating active Na^+ uptake ([see [34]); however, Potts and Fryer [35] found that calcium had little effect on sodium loss in *Daphnia magna*. Although data from the present study support this hypothesis, other explanations are possible and empirical work is needed to determine the mechanism behind the phenomenon.

In the present study, LC50s in terms of conductivity were highly correlated with LC50s in terms of sulfate for both species, except when extremely high chloride concentrations were used (300 to 500 mg/L). The plots of conductivity LC50s and sulfate LC50s clearly illustrate the contention that knowledge of the contribution of various major ions is critical to effectively managing produced waters or effluents with high concentrations of dissolved solids [2]. Not only did sulfate LC50s range from 512 to 4,345 mg/L, but conductivity LC50s ranged from 1,071 to 8,449 μmhos/cm. These wide ranges were observed for just two species with relatively similar sensitivities. Clearly, any attempt at water quality standard development, whether based on TDS, conductivity, sodium, or sulfate, should incorporate the fact that the water quality parameters like hardness and chloride strongly regulate the toxicity of high TDS solutions. Finally, the conductivity/sulfate plots provide further evidence that chloride and sulfate toxicity are additive. When chloride was less than or equal to 100 mg/L,

sulfate toxicity was strictly related to conductivity; however, when 300 and 500 mg Cl^-/L solutions were tested, sulfate LC50s were lower than predicted by LC50s based on conductivity.

When chloride was variable and hardness fixed at approximately 100 mg/L, the STR model was relatively accurate in predicting toxicity to *C. dubia*; predicted survival ranged from 48 to 69%, and observed survival was 50% in each case because calculated ion concentrations at observed sulfate LC50 were used as inputs. With one exception (48%), the model underpredicted toxicity for this data range. This might be because the STR model largely is based on the results of fed tests, which the authors acknowledged had a small influence on test results [12]. However, Soucek ([36]; http://www.pca.state.mn.us/news/eaw/buffalolake-item23.pdf) compared 48-h sulfate LC50s in unfed tests using moderately hard, reconstituted water [23] and reformulated moderately hard reconstituted water [25] as diluents with 48-h sulfate LC50s obtained from fed, 7-d chronic tests in the same two diluents. In both cases, average LC50s for unfed tests were significantly lower than those in fed tests. This factor alone might explain the discrepancy between predicted and observed results for *C. dubia* for these tests.

When chloride was held constant (5 mg/L) and hardness was varied from 100 to 600 mg/L, the STR model was relatively inaccurate in predicting toxicity to *C. dubia*, with a trend of underprediction at hardness = 100 mg/L, followed by increasing degrees of overprediction at hardness = 200 to 600 mg/L. This finding is in agreement with Kennedy et al. [15], who found that the STR model overpredicted toxicity to *C. dubia* in sodium sulfate–dominated coal-processing effluents with hardness values in the 700- to 800-mg/L range. The present study suggests that the STR model does not account for the protective effect of hardness on major ion/TDS toxicity; however, because of the presence of a pattern in the inaccuracy, data from the present study might be useful in improving the model.

CONCLUSION

In conclusion, chloride had a strong but variable influence on the acute toxicity of sulfate, depending on the species tested and the concentration range. Over the 5- to 25-mg/L chloride range, mortality of *H. azteca* decreased with increased chloride concentration and chloride did not affect the response of *C. dubia*. The opposite trend was observed over the higher range of chloride concentrations (25–500 mg/L) where increasing chloride concentrations resulted in increased mortality at given sulfate concentrations for both species. Hardness had a strong influence on sulfate acute toxicity that was similar for both crustacean species, and nearly identical LC50/hardness slopes were observed for the two species despite the fact that test waters for the two species had different chloride concentrations and Ca:Mg ratios. The LC50s in terms of conductivity were highly correlated with LC50s in terms of sulfate for both species. The wide range of values for both conductivity and sulfate LC50s suggests that single-value water quality standards for TDS, conductivity, sodium, or sulfate are not practical, and the fact that water quality parameters like hardness and chloride strongly regulate the toxicity of high TDS solutions should be incorporated into standard development. In addition, both the sulfate LC50/chloride plots and the conductivity/sulfate plots provided evidence that chloride and sulfate toxicity are additive. The STR model predicted toxicity to *C. dubia* relatively accurately when chloride was variable and hardness fixed at approximately 100 mg/L; however, the model does not account for the protective effect of hardness on major ion/TDS toxicity. Data from the present study would be a useful incorporation to the STR model.

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EXHIBIT

3

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Bioenergetic effects of sodium sulfate on the freshwater crustacean, *Ceriodaphnia dubia*

David J. Soucek

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Abstract I tested the hypothesis that if sodium sulfate alters the bioenergetics of *Ceriodaphnia dubia*, concentrations that cause reduced fecundity in the short (7-day) and long (5 generations) term should also cause changes in feeding rate and/or metabolism, measured as oxygen consumption. In addition, to test the hypothesis that an altered bioenergetic level caused by sodium sulfate exposure will affect the response of that organism to another toxicant, I measured the acute toxicity of phenol to *C. dubia* in the presence and absence of both food and sodium sulfate. Sodium sulfate reduced the filter-feeding rate of *C. dubia*, which was associated with significantly reduced oxygen consumption. This decreased energy level appeared to result in a consistent but decreased level of fecundity over a number of generations and the reproductive impairment was dose-dependent. These effects occurred at concentrations much lower than those at which acute (mortality) effects have been observed, a finding that may have regulatory implications. In addition, whereas phenol toxicity to *C. dubia* was exacerbated by the addition of food, increased phenol toxicity, likely induced by an increase in filtering or metabolic rate due to food addition, was negated when sodium sulfate was added to the test medium.

Keywords Sodium sulfate · Fecundity · Respiration · Feeding rate · *Ceriodaphnia dubia*

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Introduction

While a wealth of literature exists on the physiological effects observed in organisms exposed to changes in salinity when sodium and chloride are the dominant ions, i.e., seawater (e.g., Aarset and Aunaas 1990; Arnér and Koivisto 1993; Cowgill and Milazzo 1990; Guerin and Stickle 1992; Richmond and Woodin 1999), only recently has toxicity of effluents dominated by other "major ions" (particularly sodium and sulfate) in freshwater systems received increasing attention (Goodfellow et al. 2000). Common sources of effluents with elevated total dissolved solids (TDS), which in freshwater is essentially the sum of the concentrations of all common ions, include reverse osmosis systems, pH modifications of waste water, agricultural runoff, gas and oil production, and coal or metal mining operations (Goodfellow et al. 2000). Sodium and sulfate are two of the most common dominant ions in effluents associated with the coal industry, and the fact that toxicity of TDS in general and sodium sulfate in particular is dependent on characteristics like hardness, number of major cations, and chloride concentration of a water or effluent has been well established (e.g., Kennedy et al. 2005; Mount et al. 1997; Soucek and Kennedy 2005).

Despite the increasing interest in acute sodium sulfate toxicity, few studies have documented chronic or sub-lethal effects of exposure to sulfate dominated effluents (Chapman et al. 2000; Kennedy et al. 2004, 2005). The purpose of this study was to test the hypothesis that if sodium sulfate alters the bioenergetics of *Ceriodaphnia dubia*, concentrations that cause reduced fecundity should also result in changes in feeding rate and/or metabolism. Bioenergetic models

vary, but most often include a growth or reproduction endpoint (P) that is influenced by energy intake or consumption (C), one or more respiration or metabolic rates (R), and excretion/egestion rates (E) according to the following basic formula: P = C–R–E (e.g., Hayes et al. 2000; McNaught 1989). While metabolic endpoints like oxygen consumption, ammonia excretion, and energy substrate utilization (O:N ratio) have been investigated in numerous studies with invertebrates, particularly with respect to starvation or nutritional status (e.g., Mayzaud and Conover 1988; Pillai and Diwan 2002; Snow and Williams 1971), bioenergetic based studies of cladoceran responses to contaminants are rare (Arnér and Koivisto 1993; Bridgham 1988; McNaught 1989). To test the above stated hypothesis, I determined the short (7-day) and long-term (5 generations) effects of sodium sulfate on *C. dubia* fecundity, and measured the effects of sodium sulfate on filter-feeding and oxygen consumption rates. In addition, the effects of the presence of food during testing on acute toxicity of sodium sulfate were evaluated. Based on results of the first two objectives, I also tested the hypothesis that if sodium sulfate alters the bioenergetic state of *C. dubia*, it will influence the response of that organism to another toxicant with a different mode of action, in this case, phenol. Effects of sodium sulfate on *C. dubia* excretion/egestion rates were not measured in this study.

Materials and methods

General culturing and water chemistry

The cladoceran, *Ceriodaphnia dubia*, was selected as a test organism because of its ubiquitous use in the United States for regulatory permit testing, its greater sensitivity to contaminants compared to other cladocerans, and the large base of data available on its response to other contaminants (see http://cfpub.epa.gov/ecotox/). Previous studies have demonstrated the repeatability of its response to sodium sulfate (Soucek and Kennedy 2005; Kennedy et al. 2005). Organisms were initially obtained from a commercial source (Aquatic Research Organisms, Hampton, NH, www.holidayjunction.com/aro/), and then a continuous culture of the organisms initiated from a single female was maintained in the laboratory for at least 1 year prior to commencement of testing. The organism was cultured in moderately hard, reconstituted water (MHRW) according to U.S. EPA methods (2002). Details on culturing conditions and response to reference toxicants may be found in Soucek and Kennedy (2005). Over the course of the several months during which these experiments were conducted, average pH, conductivity, alkalinity, and hardness for culture water was 8.0 ± 0.1, 300 ± 7 μS/cm, 62 ± 2 mg/l as $CaCO_3$, and 92 ± 2 mg/l as $CaCO_3$, respectively.

For toxicity testing, a pure (99%) grade of anhydrous sodium sulfate (Na_2SO_4, CAS No. 7757-82-6) was obtained from Fisher Scientific (Pittsburgh, PA, USA). Previous experiments indicated that the salts and deionized water sources used for experiments had low to undetectable levels of trace metal contaminants (see Soucek and Kennedy 2005). Standard water chemistry characteristics, including temperature, pH, conductivity, dissolved oxygen, alkalinity and hardness, were measured at both the beginning and the end of each exposure period according to standard methods (American Public Health Association et al. 1998). With the exception of conductivity, the addition of sodium sulfate to test solutions had a negligible effect on the above listed water quality parameters. Samples from each experimental treatment were analyzed to confirm sulfate concentrations by ion chromatography at the Illinois Natural History Survey Aquatic Chemistry Laboratory, Champaign, IL, USA. For longer-term experiments like culturing of *C. dubia* in water with 1,000 mg SO_4^{2-}/l for five generations, conductivity was measured daily in culture water and sulfate concentration was calculated based on the following formula for sulfate in MHRW obtained in previous studies: $[SO_4^{2-}] = (0.503 \times \text{conductivity}(\mu S/cm)) - 135.04$, $R^2 = 0.9949$, $n = 59$ (Soucek unpublished data).

Three-brood survival/reproduction bioassays

To generate dose-response curves illustrating the effects of sodium sulfate on *C. dubia* fecundity or reproduction, 7-day, three-brood chronic bioassays were conducted according to guidelines described in American Society for Testing and Materials (ASTM) E 1295-01 (2002a). Briefly, one <24-h-old neonate was placed in each of ten replicate 50-ml beakers for each of six sulfate concentrations, including a control (no additional sulfate added). Treatments were comprised of a 75% dilution series (i.e., the highest concentration was serially diluted by 25%). Each test organism was fed at a rate identical to that used for culturing (U.S. EPA 2002), and test solutions were renewed daily. Neonates produced by the first generation test organisms (first brood usually occurred on the third or fourth day of the test) were counted daily and the tests lasted until at least 60% of test organisms had produced three broods (7 days in each case). Endpoints included the

number of young produced by each first generation *C. dubia*, and survival of first generation *C. dubia*. Tests were conducted in two different dilution waters: MHRW and a "reformulated MHRW" or RMHRW (Smith et al. 1997) which had a similar hardness and pH but different Ca:Mg ratio and chloride concentration. RMHRW was tested in addition to MHRW because of previous studies indicating that sulfate toxicity was reduced in this water (Soucek and Kennedy 2005). Three tests were conducted in each water type to obtain average values for percent survival and reproduction. For survival, Fisher's Exact test was used to compare treatments to controls and for reproduction Dunnet's test was used (WEST, Inc. and Gulley 1996).

An additional benefit of conducting 7-day chronic bioassays is that calculation of 48-h LC50s (lethal concentration to 50% of a sample population) from these data allowed a comparison of acute toxicity when tests are fed to previously generated data for the same two diluents in unfed tests (Soucek and Kennedy 2005). Survival was evaluated for each chronic test at 48 h and LC50s were calculated using the Spearman-Karber method (Hamilton et al. 1977). Mean 48-h LC50s for fed tests in MHRW and RMHRW were compared to mean LC50s for unfed tests in the same waters with ANOVA and Student's *T*-test for post-hoc comparisons using JMP® software (Sall and Lehman 1996).

Long-term measurement of reproductive rate

To investigate the long-term, generational effects of exposure to sodium sulfate on *C. dubia* fecundity, organisms were cultured according to U. S. EPA (2002) methods with the only modification being that the culture water contained elevated sodium sulfate. The concentration of 1,000 mg SO_4^{2-}/l was selected because of its proximity to reproductive impairment thresholds generated in the three-brood chronic bioassays in MHRW. Control (MHRW) and treatment (MHRW + 1,000 mg SO_4^{2-}/l) cultures were initiated simultaneously using the same cohort of neonates produced by females cultured in MHRW. One adult organism was held in each of twenty 50-ml culture beakers, and neonates were counted and removed daily. Neonates to start a new generation were selected from a female that had produced at least three broods and had not reproduced on the previous day. After the first generation, only neonates produced in MHRW were used to start a new MHRW culture and likewise for the MHRW + 1,000 mg SO_4^{2-}/l treatment. Five generations were cultured in each treatment (control, elevated sulfate). The endpoint evaluated was the total number of neonates produced per female after 8 days (usually three or four broods per female). Because *C. dubia* neonates are clones, there is dependence between generations, thus the unit of measure was a clonal line. Therefore, a repeated-measures ANOVA was used to test whether there was a difference between the treatment and control, and if that difference varied from generation to generation (the time component).

Filter-feeding experiment

To determine the effects of sodium sulfate on *C. dubia* filter-feeding rates, a simple clearing experiment was conducted. MHRW was used as a control and MHRW + 1,000 mg SO_4^{2-}/l was the exposure treatment. 50-ml beakers were filled with 30 ml of each test solution; four replicates were used for each treatment. To each beaker, 1.0 ml of the *Pseudokirchneriella subcapitata* solution used for daily feeding was added, and the solution was thoroughly mixed by stirring with a disposable transfer pipet. Next, eight 48-h-old *C. dubia* were added to each beaker. Two additional treatments in the experiment consisted of the same two solutions prepared as described above (four replicates each) with no organisms added. After 24-h, all solutions were again thoroughly mixed and 3-ml aliquots were removed from the center of each beaker and placed in cuvettes for measurement of light absorbance by chlorophyll (665 nm wavelength) using a Uvikon XL dual-beam spectrophotometer (BioTek Instruments, Wonooksi, VT). Absorbance readings were fit into a previously calculated regression equation to determine concentration (dry mass per unit volume) of algae present. The difference in algal concentration between the treatments with and without organisms for a given test solution was used as the amount of algae consumed by *C. dubia*. Consumption data were expressed on a μg algae h^{-1} $individual^{-1}$ basis. Treatment means were compared with Student's *T*-test using JMP® software (Sall and Lehman 1996).

Oxygen consumption experiment

Oxygen consumption was measured according to previously published methods (e.g., Correa et al. 1985; Rockwood et al. 1990) by placing the test organisms into biochemical oxygen demand (BOD) bottles, and measuring change in dissolved oxygen concentrations after 24 h. MHRW was used as a control and MHRW + 1,000 mg SO_4^{2-}/l was the

exposure treatment. Solutions were poured into eight replicate bottles for each treatment as well as three additional bottles per treatment to which no organisms would be added. Next, 4.5 ml of the *Pseudokirchneriella subcapitata* solution used for daily feeding was added to each bottle, and dissolved oxygen measurements were made using an air-calibrated Yellow Springs Instruments (RDP, Dayton, OH, USA) model 58 meter with a self-stirring BOD probe. Thirty 48-h-old *C. dubia* then were added to each 300 ml BOD bottle. After 24 h, dissolved oxygen was measured again in each replicate BOD bottle and the number of surviving organisms recorded. This exposure period was chosen because it was the minimum period of exposure in some previous studies with invertebrates (Rockwood et al. 1990), although other experimental designs have used shorter exposure periods (e.g., Correa et al. 1985). For each treatment, the average change in oxygen concentration in bottles without organisms was subtracted from the value for each bottle containing organisms to correct for potential differences in oxygen consumption rates not due to test organisms. Oxygen consumption data were expressed on a $\mu g\ O_2\ h^{-1}$ individual^{-1} basis for *C. dubia*. Treatment means were compared with Student's *T*-test using JMP® software (Sall and Lehman 1996).

Multiple chemical bioassay

To test the hypothesis that if sodium sulfate alters the bioenergetic state of *C. dubia*, it will influence the response of that organism to another toxicant with a different mode of action, static, non-renewal acute toxicity tests were conducted according to ASTM E 729-96 methods (2002b) with phenol as the second toxicant. Three test media were used for dilution water and controls: MHRW with no food, MHRW with food (0.45 ml of a *Pseudokirchneriella subcapitata* solution per replicate beaker), and MHRW with food plus 1,000 mg SO_4^{2-}/l. Phenol LC50s were generated for each media type. Treatments were comprised of a 50% dilution series of phenol with the highest concentration being 25 mg/l. Five phenol concentrations were tested in addition to controls with four replicates tested per concentration. Percent survival in each replicate was recorded at 24 h and at the end of the exposure period. Four tests were conducted in each diluent type, and then data for each diluent were combined (n = 80 organisms per test concentration) for LC50 calculation. LC50s with robust 95% confidence intervals were calculated using the Spearman-Karber method (Hamilton et al. 1977).

Results

Three-brood survival/reproduction bioassays

At the end of 7 days, average percent survival of control *C. dubia* (no sodium sulfate added) was 96% in MHRW and 100% in RMHRW, and survival was high (>80%) in all treatments in both diluents up to ~1,700 mg SO_4^{2-}/l (Fig. 1). For RMHRW, high survival (90%) was observed at ~2,200 mg SO_4^{2-}/l as well. Toxicity was greater in MHRW, which had an average Least Observable Adverse Effects Concentrations (LOAEC, defined as the lowest test concentration that produced a statistically significant effect compared to the control) of 2,216 mg SO_4^{2-}/l, whereas RMHRW had a LOAEC of 3,000 mg SO_4^{2-}/l for survival. The mean 7-day LC50 in MHRW was 2,049 mg SO_4^{2-}/l and in RMHRW, it was 2,442 mg SO_4^{2-}/l. In both cases, there appeared to be a threshold response rather than an incremental dose-related response.

Comparison of mean 48-h LC50s generated during 7-day chronic tests (fed tests) to those from previously conducted unfed tests indicated that feeding had a significant effect on acute toxicity of sodium sulfate to *C. dubia* in both diluent types (Fig. 2). The mean "fed" LC50 in MHRW (2,446 mg SO_4^{2-}/l) was significantly higher ($p < 0.05$) than the respective mean "unfed" LC50 (2,056 mg SO_4^{2-}/l), and in the RMHRW diluent the mean "fed" LC50 of 3,065 mg SO_4^{2-}/l was significantly higher than the mean "unfed" LC50 (2,527 mg SO_4^{2-}/l).

There was a dose-related response for reproduction in both diluents, with increasing sodium sulfate causing *C. dubia* to produce fewer neonates (Fig. 3). In fact, simple regression analysis indicated a significant negative linear relationship between sulfate concentration and mean number of neonates per female for both MHRW ($y = -0.0157x + 33.71$, $R^2 = 0.979$) and RMHRW ($y = -0.0134x + 34.76$, $R^2 = 0.906$). The LOAECs for reproduction were substantially lower than those for survival: 899 mg SO_4^{2-}/l for MHRW and 1,236 mg SO_4^{2-}/l for RMHRW. Using these regression equations to calculate EC50s, values of 1,148 mg SO_4^{2-}/l and 1,458 mg SO_4^{2-}/l were obtained for MHRW and RMHRW, respectively.

Long-term measurement of reproductive rate

Organisms that either died due to technician error or did not reproduce at all (two out of 100 individuals in the MHRW and three out of 100 individuals in the MHRW + 1,000 mg SO_4^{2-}/l treatment) were excluded

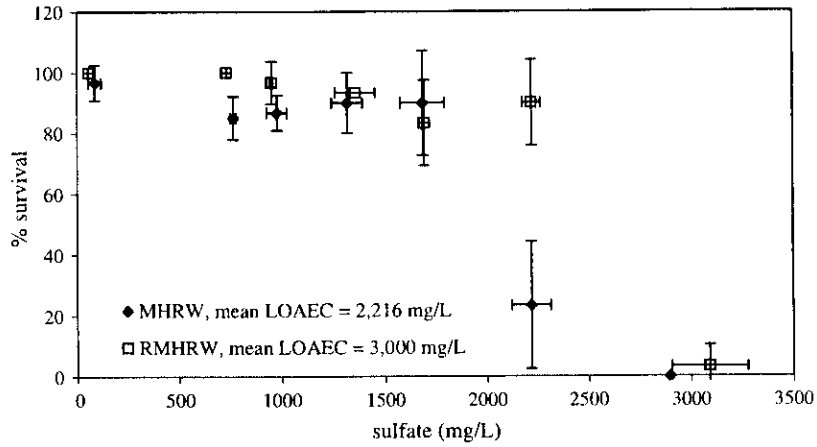


Fig. 1 Mean percent survival of *Ceriodaphnia dubia* after 7 days at various sulfate concentrations in two different diluents. Values shown are means (±standard deviation) for three bioassays. Horizontal error bars are standard deviations for measured sulfate concentrations. MHRW = moderately hard reconstituted water, RMHRW = reformulated moderately hard reconstituted water. LOAEC = least observable adverse effect concentration

Fig. 2 Mean (±standard deviation) 48-h median lethal sulfate concentration (LC50) in fed and unfed tests in two different diluents. Different capital letters indicate means are significantly different ($p < 0.05$). MHRW = moderately hard reconstituted water, RMHRW = reformulated moderately hard reconstituted water

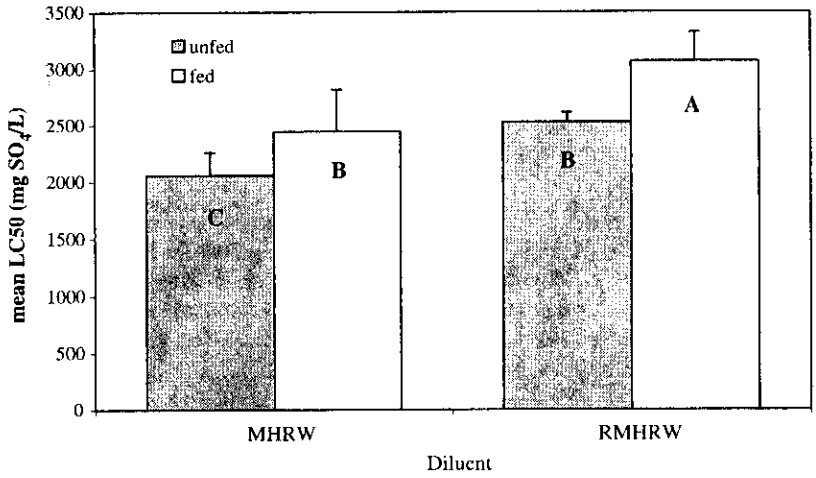
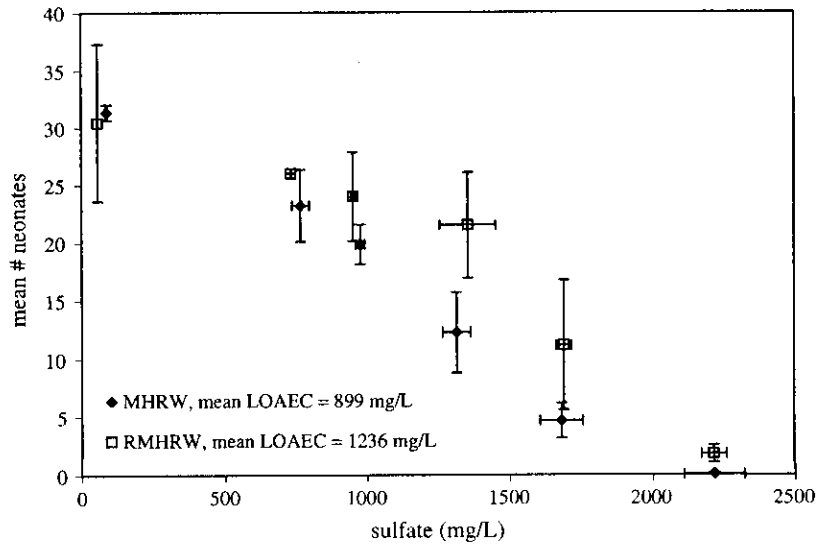


Fig. 3 Mean number of neonates produced per female *Ceriodaphnia dubia* after 7 days at various sulfate concentrations in two different diluents. Values shown are means (±standard deviation) for three bioassays. Horizontal error bars are standard deviations for measured sulfate concentrations. MHRW = moderately hard reconstituted water, RMHRW = reformulated moderately hard reconstituted water. LOAEC = least observable adverse effect concentration



from analyses. *Ceriodaphnia dubia* cultured in 1,000 mg SO_4^{2-}/l produced significantly fewer neonates than those cultured in MHRW according to the repeated measures ANOVA ($F = 359.464$, DF = 36, $p < 0.0001$ for treatment effect, Fig. 4). In addition, sulfate affected *C. dubia* reproduction differently in different generations as both the generation term ($F = 43.760$, DF = 33, $p < 0.0001$) and the generation × treatment interaction term ($F = 4.018$, DF = 33, $p = 0.0092$) were significant. Separate pairwise analysis of treatment effects for each generation also indicated that *C. dubia* cultured in 1,000 mg SO_4^{2-}/l produced significantly fewer neonates after 8 days than controls ($p < 0.0001$ for each generation). Combining the five generations, the overall mean (±standard deviation) number of neonates produced per female after 8 days in MHRW was 62.2 ± 6.6 ($n = 98$), and for the MHRW + 1,000 mg SO_4^{2-}/l it was 50.6 ± 5.3 ($n = 97$).

Filter-feeding and oxygen consumption experiments

Solution type (with or without sodium sulfate) did not have an effect on light absorbance ($p = 0.2892$) or change in oxygen concentration ($p = 0.0739$) when no organisms were added. However, to further control for potential differences in oxygen consumption rates not due to test organisms, the average change in oxygen concentration in bottles without organisms for a given treatment was subtracted from the value for each bottle containing organisms for the same treatment.

Significant differences were observed between treatments for both algal and oxygen consumption (Fig. 5). The ratio of algae to oxygen consumption (in terms of $\mu g/h^{-1}$ individual^{-1}) was 2.10 for the controls, and slightly higher (2.65) for the sulfate exposed organisms. This difference is attributable to the fact that while exposure to 1,000 mg SO_4^{2-}/l reduced feeding rate by 25%, it reduced the respiration rate by 41%.

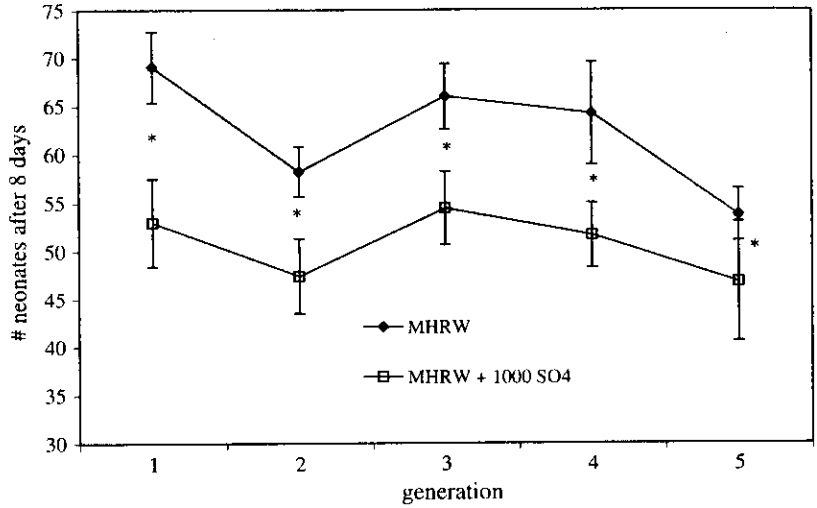


Fig. 4 Mean number (±standard deviation) of neonates produced per female *Ceriodaphnia dubia* (n = 19–20 per treatment per generation) after 8 days over five generations in either controls (MHRW = moderately hard reconstituted water) or treatments exposed at to 1,000 mg SO_4^{2-}/l. Neonates produced by generation one were used for generation two and so on. Asterisks indicate means for a given generation are significantly different ($p < 0.05$, repeated measures ANOVA and post-hoc pairwise tests)

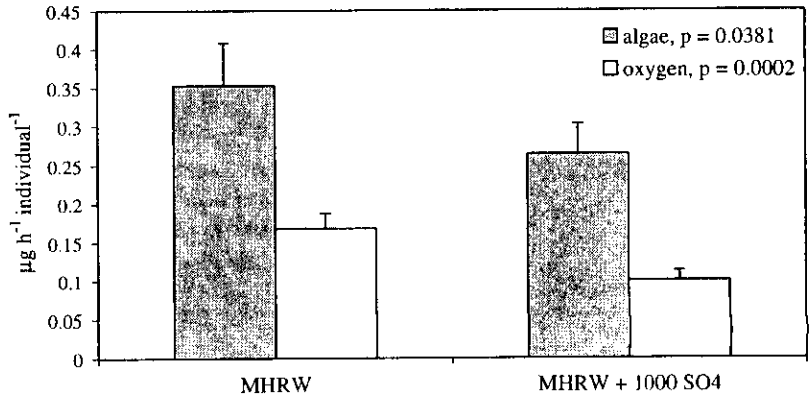


Fig. 5 Mean (±standard deviation) algal and oxygen consumption rates (in µg per hour per individual) for *C. dubia* in controls (MHRW) and exposed to 1,000 mg SO_4^{2-}/l consumption. P-values shown are for Student's t-test comparing control and treatment means for each endpoint separately

Multiple chemical bioassay

The presence or absence of food, and the presence or absence of sulfate had strong effects on phenol toxicity to *C. dubia*. When combining data from four separate tests (n = 80 individuals per test concentration), the LC50 (lower and upper 95% confidence limits) in MHRW with no food added was 4.34 (3.88–4.85) mg phenol/l, whereas when food was added to each beaker, the phenol LC50 was 3.29 mg/l (3.04–3.57). When both food and 1,000 mg SO_4^{2-}/l were added to MHRW, the phenol LC50 was 4.26 mg/l (3.91–4.65).

Discussion

The mean 7-day sulfate LOAECs for *C. dubia* survival in MHRW and RMHRW (2,216 and 3,000 mg SO_4^{2-}/l, respectively), compare well with other published values with sodium sulfate dominated effluents. Kennedy et al. (2005) collected effluent from a coal preparation facility in Ohio, USA, and conducted 7-day chronic tests with *C. dubia* in both the actual effluent and a laboratory prepared effluent meant to simulate the field-collected effluent. Treatments were based on conductivity (µS/cm) and survival LOAECs were in the range of 4,500 to >6,000 µS/cm. These values correspond to sulfate concentrations in the range of 2,100 to >2,800 mg SO_4^{2-}/l according to the regression equation used in this study generated with sodium sulfate in MHRW. However, effluents tested in the Kennedy et al. (2005) study contained low levels of trace metals and aluminum, and had elevated hardness (650–770 mg/l as $CaCO_3$), which is known to ameliorate sulfate toxicity (Soucek and Kennedy 2005) and may change the SO_4^{2-}/conductivity relationship. While the results are not directly comparable to those from the present study because of the differences in ion composition, survival data from the two studies fall within the same approximate range. Additionally, results of previous work indicating that Ca:Mg and chloride concentration influence sulfate toxicity (Soucek and Kennedy 2005) were supported by these results, as toxicity was consistently lower in RMHRW than in MHRW.

Comparison of *C. dubia* survival results in 7-day chronic tests to those in previously conducted unfed 48-h acute tests (Soucek and Kennedy 2005) suggests that feeding organisms during sodium sulfate bioassays improves survivorship. The 48-h LC50s generated in these fed tests were significantly higher than those from unfed tests. In fact, the 7-day LC50 for *C. dubia* in MHRW during the present study was similar to the mean unfed 48-h LC50 from the Soucek and Kennedy (2005) study (2,049 and 2,050 mg SO_4^{2-}/l, respectively). Similar trends were observed in RMHRW. Pieters et al. (2005) documented similar results with *Daphnia magna* exposed to fenvalerate; comparing high and low food treatments, effects of fenvalerate on survival and reproduction were exacerbated by reduced food concentration. It is well known that food, dissolved organic carbon, and other ligands can reduce availability and toxicity of some metals (e.g., Paquin et al. 2003), so feeding is recommended against in acute toxicity bioassays with most freshwater organisms (ASTM 2002b). However, sodium and sulfate are stable as ions and unlikely to bind with the algal food used in these tests. Therefore, a more likely explanation for the increased survival in fed tests in the present study is that fed organisms have more energy to direct toward ionoregulation than starved organisms.

Reproduction of *C. dubia* was strongly influenced by sodium sulfate in three-brood chronic tests. Dose-related, negative relationships between sulfate and reproduction were observed in both diluents. The slope for MHRW was nominally steeper than that for RMHRW, which is consistent with previous findings that increased Ca:Mg ratio and chloride concentration reduces sodium sulfate toxicity to *C. dubia* (Soucek and Kennedy 2005). While LOAECs for reproduction were less than half the values for survival in this study, organisms at the LOAEC concentrations still produced an average of ~20 neonates, or 63–70% of the means for controls.

Kennedy et al. (2004) observed reduced reproduction of *C. dubia* at conductivities ranging from 3,254 to 4,530 µS/cm, while conductivities at LOAECs in the present study ranged from 2,050 to 2,700 µS/cm. The disparity between the two studies may again be a result of the fact that the field collected and mock effluents from the Kennedy et al. (2004) study had much higher hardnesses (650–770 mg/l as $CaCO_3$) compared to those in the present study (90–100 mg/l as $CaCO_3$). In this study, the EC50s in terms of mg Na_2SO_4/l were 1,566 and 2,070 for MHRW and RMHRW, respectively. Cowgill and Milazzo (1990) conducted three-brood chronic tests with *C. dubia* using NaCl as a toxicant and obtained an EC50 of 1,794 mg NaCl/l (hardness of their diluent was 90–110 mg/l). This value falls within the range observed in the present study for Na_2SO_4, suggesting that sulfate and chloride salts of sodium have similar effects on reproduction. This is in contrast to the observation of Mount et al. (1997) that NaCl is more toxic than Na_2SO_4 in terms of 48-h LC50s (1,960 and 3,070 mg/l for the respective salts).

Based on reproductive impairment results of the three-brood chronic bioassays (LOAEC in MHRW = 899 mg SO_4^{2-}/l), the five-generation exposure was conducted at a concentration of 1,000 mg SO_4^{2-}/l, and this concentration of sulfate had a significant and consistent effect on *C. dubia* reproduction relative to controls over the course of the five generations. While the generation and interaction terms indicated that differences in reproduction between controls and sulfate exposed organisms were different from generation to generation, the actual percent differences between controls and sulfate exposed only ranged from 12.9 to 23.3 (mean ± SD = 18.4 ± 3.7). The finding of significant generational differences was likely due to the relatively high statistical power (n = 19–20 per treatment per generation). The fact that sulfate-exposed *C. dubia* reproduced at a relatively constant but significantly lower rate compared to controls suggests a decrease in energy allocated to reproduction due to increased osmoregulation requirements, an explanation also suggested by Arnér and Koivisto (1993) working with *Daphnia magna* in various NaCl exposure levels.

If the observed decrease in reproductive rate in *C. dubia* exposed to sodium sulfate is due to changes in energy allocation, one might expect to observe changes in other bioenergetic endpoints when similarly exposed. That was the case in the present study, as consumption of algae was significantly decreased when exposed to sulfate, as was metabolic rate, measured as oxygen consumption. At the most basic level, following the bioenergetic model of P = C–R–E, and holding excretion constant because it was not measured in this study, a decrease in both algal consumption (C) and respiration (R) would result in reduced growth or reproduction (P). Respiration and reproduction rates in freshwater daphnids have been observed to decrease in response to elevated NaCl (Arnér and Koivisto 1993), and 2,2′-dichlorobiphenyl (Bridgham 1988), although in the latter study, variable results were obtained in two different respiration experiments. Temperature has also been documented to influence respiration rates in *Ceriodaphnia reticulata* (Gophen 1976). Both temperature and food concentration influence daphnid filter-feeding rates as well (Gophen 1976; Mourelatos and Lacroix 1990), and several chemicals, including magnesium, nitrite, lindane, and zinc have been shown to reduce food consumption in daphnids (see McNaught 1989).

The results of this study imply that *C. dubia* populations exposed to sodium sulfate would grow at slower rates than unexposed populations. Because this organism is a low level consumer, this effect may cascade to higher trophic levels. However, the implications of reduced feeding rates observed due to sodium sulfate exposure are not entirely negative. The results of phenol bioassays in the present study indicated that phenol toxicity increased when food was present, perhaps due to a higher metabolic rate and/or a higher filtering rate because filter-feeding increases with increased food concentration (Mourelatos and Lacroix 1990). However, addition of 1,000 mg SO_4^{2-}/l to the test medium, thereby reducing feeding rate, negated the effect of food on phenol toxicity.

Brix et al. (2001) reported similar results when they observed that increased sulfate concentrations caused reduced acute toxicity of selenate to several freshwater species including *C. dubia*, and Hansen et al. (1993) observed reduced bioconcentration of selenate in *Chironomus decorus* and *D. magna* at elevated sulfate concentrations (up to 206 mg SO_4^{2-}/l for *D. magna* and up to 3,235 mg SO_4^{2-}/l for *C. decorus*. The suggested mechanism was related to direct competition at the cell uptake site since the two chemicals were structurally similar, and much is known about the absorption antagonism between selenate and sulfate (reviewed in Hansen et al. 1993), but results from the present study suggest that a sulfate induced lower filtering rate also may be a contributing factor to reduced selenate uptake and toxicity.

In conclusion, this study has shown that sodium sulfate reduces filter-feeding rate in *C. dubia*, which is associated with, but not necessarily the cause of a reduced metabolic rate, measured as oxygen consumption. This decreased energy level appears to result in a consistent but decreased level of reproductive output over a number of generations and the reproductive impairment is dose-dependent. In addition, sodium sulfate toxicity appears to be reduced when test organisms are fed, whereas phenol toxicity to *C. dubia* is exacerbated by the addition of food. Increased phenol toxicity induced by a likely increase in filtering or metabolic rate due to food addition, is negated when sodium sulfate is added to the test medium.

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PROOF OF SERVICE

I, the undersigned, on oath state that I have served the attached **Illinois Environmental Protection Agency's Additional Information and Documents** upon the persons to whom it is directed, by placing a copy in an envelope addressed to:

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Meredith Kelley

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