BEFORE THE ILLINOIS POLLUTION CONTROL BOARD

IN THE MATTER OF:)
)
REVISIONS TO WATER QUALITY)
STANDARDS FOR TOTAL DISSOLVED)
SOLIDS IN THE LOWER DES PLAINES RIVER FOR)   R06 - 24
EXXONMOBIL OIL CORPORATION:)   (Site Specific Rule - Water)
PROPOSED 35 Ill. Adm. Code 303.445)

NOTICE OF FILING

Dorothy M. Gunn, Clerk
Anand Rao
Illinois Pollution Control Board
James R. Thompson Center
100 West Randolph Street, Suite. 11-500
Chicago, Illinois 60601

William Richardson, Chief Legal Counsel
Illinois Department of Natural Resources
One Natural Resource Way
Springfield, IL 62702

Matthew J. Dunn
Division Chief, Environmental Enforcement
Illinois Attorney General
100 W. Randolph Street, 12th Floor
Chicago, IL 60601

Jeffrey C. Fort
Letissa Carver Reid
Elizabeth A. Leifel
Sonnenschein, Nath and Rosenthal LLP
7800 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6404

Susan M. Franzetti
Franzetti Law Firm, P.C.
10 S. LaSalle Street, Suite 3600
Chicago, IL 60603

Dennis L. Duffield
Director of Public Works and Utilities
City of Joliet
Department of Public Works and Utilities
921 E. Washington Street
Joliet, Illinois 60603

PLEASE TAKE NOTICE that I have today filed with the Office of the Clerk of the

Pollution Control Board the attached **PRE-FILED TESTIMONY AND RESPONSE TO**

BOARD REQUEST on behalf of the Illinois Environmental Protection Agency, a copy of which is

herewith served upon you.

ENVIRONMENTAL PROTECTION AGENCY
OF THE STATE OF ILLINOIS

By: _Thomas M Gr_

Thomas Andryk
Assistant Counsel
Division of Legal Counsel

DATED: May 31, 2006
Illinois Environmental Protection Agency
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276
(217) 782-5544

<center>THIS FILING IS SUBMITTED ON RECYCLED PAPER</center>

IN THE MATTER OF:)
)
REVISIONS TO WATER QUALITY   )
STANDARDS FOR TOTAL DISSOLVED   )   R06 - 24
SOLIDS IN THE LOWER DES PLAINES RIVER)   (Site Specific Rule - Water)
FOR EXXONMOBIL OIL CORPORATION:)
PROPOSED 35 Ill. Adm. Code 303.445   )

Pre-Filed Testimony and Response to Board Request

The undersigned, as one of its attorneys, hereby provides the following information as to Illinois
EPA's Pre-Filed Testimony and its Response to the Board's Request that it address the
applicability or inapplicability of 35 Ill. Adm. Code Section 102.210(c) prior to hearing, stating
on behalf of Respondent, Illinois Environmental Protection Agency, as follows:

I.   TESTIMONY OF SCOTT TWAIT

My name is Scott Twait and I have been employed by Illinois EPA for over 9 years. I have been
assigned to the Water Quality Standards Unit for all of those years and have participated in
adjusted standards, site-specific water quality standards rulemakings, and variances. I hold a B.S
degree in Civil Engineering from the University of Illinois where I specialized in Environmental
Engineering.

My testimony today will be in support of the ExxonMobil Oil Corporation site-specific relief
from the total dissolved solids (TDS) Secondary Contact and Indigenous Aquatic Life standard
(35 IAC 302.407) and the TDS General Use standard (35 IAC 302.208(g)) in the Des Plaines
River.

The petitioner is adding a Catalytic SO_2 Additive Technology (DESOX) system followed by a
wet gas scrubber (WGS) and a Selective Catalytic Reduction (SCR) system to remove SO_2 and
NO_x from air emissions as part of a consent decree with USEPA and Illinois EPA. The addition
of the DESOX will allow the removal of SO_2 from the emissions by transferring sulfur, in stable
form, from the regenerator to the reactor, where it is released as hydrogen sulfide for
downstream recovery as elemental sulfur, thereby reducing sulfate in the plant wastewater and
minimizing dissolved solids discharged to the Des Plaines River. The DESOX, WGS, and SCR
will remove 95% of SO_2 and 50% NO_x at 130,000 and 9,800 pounds per day respectively. As
indicated in our November 15, 2005 meeting, ExxonMobil is adding a third tank onto the
activated sludge WWTP and will configure the process to provide an anoxic zone to denitrify,

THIS FILING IS SUBMITTED ON RECYCLED PAPER

therefore, total nitrogen loading to the stream will be reduced rather than increased as a result of the air scrubbing. Loading of sulfates and TDS will be increased to the receiving stream; however, sulfates will meet water quality standards after mixing. TDS will not always meet the water quality standards, due to seasonal loading of chlorides found in road salt from the Chicago metropolitan area that has affected concentrations upstream of ExxonMobil.

The subject facility discharges to the Des Plaines River at a point where 1503.0 cfs of flow exists upstream of the outfall during critical 7Q10 low-flow conditions. The Des Plaines River is classified as a Secondary Contact and Indigenous Aquatic Life Use Water at the point of discharge and is a General Use Water downstream of the I-55 bridge. The Des Plaines River is rated a "C" stream under the Agency's Biological Stream Characterization (BSC) program. The Des Plaines River, Waterbody Segment, G-24, is found on the 2004 Illinois 303(d) List. The uses impaired for this segment was aquatic life and fish consumption. The potential causes of impairment given for the segment at that time were copper, sedimentation/siltation, other flow regime alterations, total suspended solids (TSS), DDT (statistical guideline), PCBs, (statistical guideline), mercury (statistical guideline), and total phosphorus (statistical guideline). The potential sources associated with the impairment are industrial point sources, municipal point sources, urban runoff/storm sewers, hydrologic/habitat modification, flow regulation/modification, contaminated sediments, and source unknown. The additional constituents to be discharged by ExxonModbil, sulfate and TDS, therefore have no bearing on the 303(d) status of the waterbody.

The Illinois Department of Natural Resources was contacted on November 17, 2005 with regard to the presence of any threatened or endangered species that may be impacted by this standard change. IDNR terminated the consultation process on December 19, 2005 with a finding of no threatened and endangered species or natural areas affected.

The Agency cannot grant mixing for a discharge if the receiving stream is not meeting the water quality standard. Since the necessary NPDES permit would require the recognition of mixing in the Des Plaines River and the Des Plaines River has occasionally violated water quality standards for TDS, the Agency cannot issue an NPDES permit that will accommodate this new ExxonMobil discharge. Mixing for sulfate is allowable, however, and will extend into the General Use portion of the river.

The petitioners have demonstrated that TDS is not toxic to aquatic life at the concentrations that will be found in the river provided that sulfate is the predominant anion. Toxicity test results on TDS with the chloride to sulfate ratio that will result from the proposed discharge indicate that even the most sensitive species tested can easily tolerate the levels likely to be found in the receiving waters.

In the petition for the site-specific rulemaking, the petitioners discussed compliance alternatives that were all rejected due to cost and/or technical infeasibility. We believe that the petitioners have shown that there are no cost-effective compliance alternatives.

The Agency is in the process of proposing to change the General Use water quality standard for sulfates and eliminate the General Use standard for TDS but has not yet filed its petition before the Board. New aquatic life toxicity data indicates the level of sulfate that sensitive species tolerate. This information was not available when the original water quality standards were adopted for sulfate and TDS. Our new understanding of sulfate toxicity can be coupled with the existing chloride standard to predict a protective level of TDS. Given the hardness of 205 mg/L as $CaCO_3$ and the maximum chloride concentration of 450 mg/L known for the Des Plaines River, the proposed water quality standard based on the aquatic life toxicity of sulfate is 1,138 mg/L. If we add up the major anions, we get 450 + 1,138 = 1,588 mg/L TDS. Adding in the major cations, a TDS concentration of about 3,000 mg/L is protective. Therefore, it has been demonstrated that the 1,686 mg/L TDS requested as relief by ExxonMobil is well within the TDS toxicity threshold. The 1,686 mg/L TDS in the stream in this case consists of chloride and sulfate, plus adding in the sodium, magnesium, calcium, and all the minor ions. This site-specific rulemaking will not result in aquatic life toxicity. For the above conclusions we relied on the attached studies.

This site-specific rulemaking, consisting of a new calculation of the protective level of TDS, is consistent with 40 CFR 131.11(b)(1)(ii). Specifically, a federal site-specific water quality criterion would be allowed in this case because sensitive species of aquatic life have been demonstrated to be protected by the new standard through laboratory toxicity tests. USEPA Region 5 has given preliminary approval of the ExxonMobil site-specific standard under its obligation to review state water quality standards under the Clean **Water Act.**

The Agency is currently reviewing the Secondary Contact and Indigenous Aquatic Life water quality standards for the Lower Des Plaines River through the Use Attainability Analysis (UAA) process. This site-specific rulemaking should remain in effect if the water quality standard for TDS is not revised to at least 1,686 mg/L for the Lower Des Plaines River under the UAA.

There are no other existing dischargers, in this stretch of the river, which have an elevated discharge of TDS. The Channahon WWTF, BASF, ExxonMobil tank farm, Loder Cronklaan, and Dow Chemical polystyrene plant are the Des Plaines River dischargers downstream of the subject facility. Channahon is the only municipal discharge and the TDS expected in an STP discharge would be expected to be 500 - 600 mg/L, so in effect, they are a diluter. The BASF plant, visible from the I-55 bridge, discharges process water and storm water, which are not expected to have elevated TDS. There is also another ExxonMobil facility that is a tank farm and/or a pipeline terminus. They have a boiler blowdown, but this would be minor in size and not likely to have extremely high TDS wastewater. Loder Cronklaan makes vegetable oil products and has no likelihood for high TDS wastewater. Finally, a Dow Chemical polystyrene plant, which has cooling and sanitary wastewater has no potential for high TDS wastewaters of significant size. None of these industries is categorized by the IEPA as a major discharger. None of these dischargers exhibited a need for water quality based effluent limits, past or present. Regardless of the dischargers to this section of the Des Plaines River, the water quality standard that is proposed is more stringent than what the Agency believes is protective of aquatic life.

This site-specific rulemaking will not result in aquatic toxicity, there are no economically or technically feasible alternatives, and is approvable by USEPA. I recommend that the IPCB support the petitioners request for site-specific rulemaking for relief from the water quality standards for TDS at 35 IAC 302.208(g) and 302.407 as written in the petition.

II.   ILLINOIS EPA RESPONSE TO BOARD REQUEST

The Agency respectfully submits the following as its response to the Board's inquiry regarding the applicability of 35 Ill. Adm. Code 102.210. Section 102.210 (c) of the Board's regulations requires the proponent of the rulemaking to provide a descriptive title or other description of any published study or research report used in developing the rule, the identity of the person who preformed the study and a description of where the public may obtain a copy of any such study or research report. If the relied upon report or study was conducted by the agency, the agency also has an obligation to make the underlying data available upon request.

In the instant case, the Proponent of the rulemaking had the obligation to identify studies and reports that it relied upon. The Agency notes that the proponent referenced on-going investigation by the Agency and conclusions reached by the Agency from that investigation. To its Petition for a Site-Specific Rule, Exxon Mobil attached a Jan. 9, 2004 report by Dr. Soucek and citations to other reported toxicity research but alluded to Agency investigations. The Agency is not the proponent of the rulemaking but in this instance, given the long history of Agency efforts to investigate the TDS water quality standard and the need to expedite this rulemaking, the Agency offers the following information to the Board.

Pursuant to Section 303 (c), of the Clean Water Act, 33 U.S.C. § 1313 (c), the Agency has a continuing duty to investigate and propose updates to its Water Quality Standards as science becomes more refined or conditions change. The Agency has been investigating the impact of TDS on aquatic life for several years. It convened a work group, and submitted a preliminary draft justification documents for regulatory changes and proposed language for comment to the work group. Although the Agency is not submitting the draft proposal for comment and justification for regulatory change, entitled *Draft Justification for Changing Water Quality Standards for Sulfate, Total Dissolved Solids and Mixing Zones (January 21, 2004)* as presented to the work group, with this proposal for a site-specific rule, the Agency has made the draft available to the public and believes that the proponent may have relied on the conclusions in the justification document. This regulatory proposal is still in development; the Agency notes that the preliminary draft justification is out-dated in some respects but the conclusions are valid. The Agency plans to submit a rulemaking proposal to the Board at some time in the future to the Board after the conclusion of the regulatory development process.
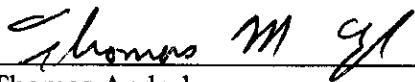
A further study by Dr. Soucek, finalized after the instant proposal, supported the Jan. 9, 2004 Soucek report. Entitled *Effects of Water Quality on Acute and Chronic Toxicity of Sulfate to Freshwater Bivalves,* ***Ceriodaphnia dubia, and Hyalella*** azteca, it is available to the public at the Agency's offices and is being submitted, with several quarterly reports, as an attachment to Scott Twait's prefiled testimony. The Agency notes that this study was not relied upon by the

proponent as it was not completed at the time of the proposal. It, however, was relied upon and considered by the Agency in reaching its conclusion to support the proposal.

The Agency supports the request for relief requested by Exxon-Mobil in its Petition for a Site-Specific water quality standard and respectfully submits this response and additional information in an effort to expedite this time sensitive rulemaking.

ILLINOIS ENVIRONMENTAL PROTECTION AGENCY

By:_____

Thomas Andryk
Assistant Counsel
Division of Legal Counsel

DATED: May 31, 2006

Illinois Environmental Protection Agency
1021 North Grand Avenue East
Post Office Box 19276
Springfield, Illinois 62794-9276
(217)782-5544

STATE OF ILLINOIS	)	
	)	
	)	SS
COUNTY OF SANGAMON	)	
	)	

PROOF OF SERVICE

I, the undersigned, on oath state that I have served the attached **PRE-FILED TESTIMONY AND RESPONSE TO BOARD REQUEST** and **NOTICE OF FILING** upon the person to whom it is directed, by placing a copy in an envelope, with proper first class postage pre-paid, addressed to:

Dorothy M. Gunn, Clerk
Anand Rao
Illinois Pollution Control Board
James R. Thompson Center
100 West Randolph Street, Suite 11-500
Chicago, Illinois 60601

William Richardson, Chief Legal Counsel
Illinois Department of Natural Resources
One Natural Resource Way
Springfield, IL 62702

Matthew J. Dunn
Division Chief, Environmental Enforcement
Illinois Attorney General
100 W. Randolph Street, 12th Floor
Chicago, IL 60601

Jeffrey C. Fort
Letissa Carver Reid
Elizabeth A. Leifel
Sonnenschein, Nath and Rosenthal LLP
7800 Sears Tower
233 South Wacker Drive
Chicago, Illinois 60606-6404

Dennis L. Duffield
Director of Public Works and Utilities
City of Joliet
Department of Public Works and Utilities
921 E. Washington Street
Joliet, Illinois 60603

Susan M. Franzetti
Franzetti Law Firm, P.C.
10 S. LaSalle Street, Suite 3600
Chicago, IL 60603

and mailing it from Springfield, Illinois on May 31, 2006 with sufficient postage affixed as indicated above.

Nancy JD Sampson

SUBSCRIBED AND SWORN TO BEFORE ME

this 31 day of May , 2006

Cynthia L. Wolfe
Notary Public

THIS FILING IS SUBMITTED ON RECYCLED PAPER

Effects of Water Quality on Acute and Chronic Toxicity of Sulfate
to Freshwater Bivalves, *Ceriodaphnia dubia,* and *Hyalella azteca.*

First Quarterly Report

Submitted to:
Edward Hammer and Dertera Collins
United States Environmental Protection Agency
Region 5, Water Division, 77 West Jackson Boulevard
Chicago, Illinois 60604

December 21, 2004
Illinois Natural History Survey, Champaign, IL
U.S. EPA Region 5

Background

While there are no Federal water quality criteria (WQC) for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium (U.S. EPA 1999), several states, including Minnesota, Indiana, and Illinois, are at various stages in the process of developing standards for sulfate. Water quality standards are developed to protect designated uses, aquatic life uses in this case, but the economic impacts of these standards are important considerations as well. For example, after existing sulfate standards were enacted in Illinois, the Illinois Pollution Control Board adopted exceptions to the standards to provide relief for a number of coalmines that were enduring severe economic hardship. In fact, ~60% of coalmines have expired permits in Illinois because of violations of the sulfate standard, and ~50% of those have been expired for more than three years (T. Frevert, pers. comm.). The current "General Use" standard of 500 mg/L in Illinois is based on the value thought to be protective of livestock. Consultation with appropriate authorities revealed that livestock were capable of tolerating much higher levels of sulfate. In light of these factors, the Illinois Environmental Protection Agency (IL EPA) is actively pursuing an update of the sulfate standards based on scientific research, and is close to proposing an updated standard (R. Mosher, IL EPA, pers. comm.).

Sodium is one of the most common major cations in high TDS effluents, but calcium and chloride are usually present in mine-impacted waters as well. While major ion or 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, studies (funded by IL EPA, and IL Coal Association) were conducted by Soucek (2004; In Press Environmental Toxicology and Chemistry) to (1) generate LC50s (lethal concentration to 50% of a sample population) and LC10s (lethal concentration to 10% of a sample population) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia, Chironomus tentans, Hyalella azteca,* and *Sphaerium simile*) in U.S. Environmental Protection Agency's (US EPA, 1993) moderately hard reconstituted water (MHRW) and (2) determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate to *Ceriodaphnia dubia* and *Hyalella azteca* (Soucek, 2004). In these previous studies (Soucek, 2004), the mean LC50s, expressed as mg SO_4^{2-}/L, in moderately hard, reconstituted water (MHRW; U.S. EPA, 1993) ranged from: 512 to 1,4134 mg/L. The LC50 generated for the amphipod, *Hyalella* (512 mg/L) was surprisingly low, given that it is known as a euryhaline organism (Ingersoll et al., 1992), but as will be discussed below, water quality data, including other cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

The composition of the dilution water used during testing in the Soucek (2004) study had a dramatic effect on the toxicity of sulfate to *Hyalella*. Whereas the 96-hour LC50 in MHRW was 512 mg/L, the LC50 increased to 2,855 mg/L when using a "Reformulated Moderately Hard Reconstituted Water" (RMHRW, Smith et al., 1997). The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. Both dilution waters were similar in terms of hardness (~90-106 mg/L as $CaCO_3$), alkalinity, and pH, but RMHRW had a higher chloride concentration and different calcium to magnesium ratio than that in MHRW. An additional experiment conducted at the Illinois Natural History Survey (INHS) laboratory, but not included in the Soucek (2004) report, indicated that when sulfate (~2,800 mg/L) and hardness (106 mg/L) were held constant, percent survival of *H. azteca* was positively correlated with chloride concentration (up to 67 mg Cl⁻/L). These experiments illustrate the need to further characterize the interacting effects of chloride and sulfate on aquatic organisms.

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 the previous study (Soucek 2004), increased hardness reduced the toxicity of sulfate to *Hyalella* and had a dramatic effect on the 48-hour LC50 for *C. dubia*, increasing from 2,050 at a hardness of 90 to > 2,900 mg/L at hardness values higher than 194 mg/L as $CaCO_3$. Others have observed reduced toxicity of saline solutions due to increased hardness as well (e.g., Dwyer et al. 1992; Mount et al. 1997).

While a great deal of progress was made in the understanding of sulfate toxicity under varying water quality conditions, several important data gaps remain. In the previous studies (Soucek 2004), the fingernail clam, *Sphaerium simile*, had a lower LC10 than that of *C. dubia*, but because of the temporal nature of its availability, this bivalve was only tested in MHRW. It remains unclear whether or not a mollusk will have the same physiological response as two crustaceans to increased chloride or hardness in these experiments with sulfate. The principal inorganic anion of crustacean blood, or 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 (Bayliss and Harris, 1988). However, in the unionid mussel, *Toxolasma texasiensis*, chloride and bicarbonate are equally important anions in the hemolymph (see McMahon and Bogan 2001). Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations to the extent that some crustaceans do. If this is the case, the protective effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifest in some unionoidean bivalves. The hardness effect observed in the Soucek (2004) study may be more widespread among aquatic phyla, because calcium simply reduces gill permeability (Lucu and Flik, 1999; Pic and Maetz, 1981). However, McMahon and Bogan (2001) state that unionoideans "generally lose capacity for osmotic and volume regulation above 3-4 ppt" (salinity). TDS is a rough measure of salinity, and the TDS of a sample of RMHRW with 2000

mg/L sulfate is 2.9 g/L or ppt (Soucek unpublished data). Further experiments with freshwater bivalves are required to determine if there is an absolute TDS level that is tolerable, or if the limit depends upon water quality characteristics such as chloride concentration and hardness.

An additional data gap is the fact that all of the tests conducted in the Soucek (2004) study were acute exposures of 48 to 96 hours. Sublethal effects of sulfate in longer-term exposures are unknown to this date. Given that, under the new limit proposed by IL EPA, a continuous, long-term release at a given concentration will be allowed chronic testing should be conducted to determine potential sublethal impacts.

The purpose of the present study is to further provide data to support an appropriate sulfate criterion for the protection of aquatic life in Illinois. Therefore, the objectives of the current study are to build on previous studies conducted to support development of a sulfate criterion for protection of aquatic life by (1) determining the effects of hardness on toxicity of sulfate to bivalves, (2) determining the toxicity of sulfate to juvenile unionid mussels, (3) determining the short-term (7 days) chronic toxicity of sulfate to *Ceriodaphnia dubia*, (4) determining the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*, and (5) determining the effects of hardness on toxicity of sulfate to *Hyalella* at a critical chloride level, i.e., the chloride concentration at which sulfate is significantly less toxic to *Hyalella* as determined in #4 above.

Project Objectives

Based on existing data gaps described above, and because of the desire to expedite the process of updating sulfate standards in several states with limits based on scientific research, the following tasks will be conducted:

Examine hardness and chloride effects on acute sulfate toxicity to bivalves.
The first experiments conducted in this task will include testing the acute toxicity of sulfate to *Sphaerium simile* in RMHRW (Smith et al., 1997). The other two organisms tested in this water had markedly different responses compared to those observed in MHRW; whereas the 96-hour LC50 in MHRW for *Hyalella* was 512 mg/L, the LC50 increased to 2,855 mg/L in RMHRW. The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. However, both of these organisms are crustaceans. Sulfate LC50s will be generated for *S. simile* at two chloride concentrations (5 and 30 mg/L) and three hardness levels (100, 200, 300 mg/L). Because they will be conducted with organisms collected from the field, some of the tests may be eliminated depending on availability of test organisms at the collection site. In addition, we will generate sulfate LC50s using freshwater unionid mussel juveniles as test organisms, to

determine if this family is more sensitive to sulfate than the family Sphaeriidae (fingernail clams).

Conduct 7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia.*
To test the hypothesis that the acute safe level is similar to the chronic safe level, we will conduct 7-day chronic, survival/reproduction tests with *Ceriodaphnia dubia* in both MHRW and RMHRW. Tests will be conducted according to ASTM methods (2002b), and endpoints generated will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*. EC20s and EC50s for survival and reproduction will be generated as will No Observable Adverse Effects Concentrations (NOAEC) and Least Observable Adverse Effects Concentrations (LOAEC) for both endpoints.

Determine the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia,*
Chloride standards currently exist in Illinois (500 mg/L, R. Mosher, pers. comm.), and the State plans to additionally regulate for sulfate in order to address the major non-chloride component of TDS in these waters. Because IL EPA proposes to eliminate the TDS standard, opting instead to regulate sulfate and chloride, the interacting toxic effects of these two anions must be characterized. Soucek (manuscript accepted with minor revisions) has already shown that incrementally increasing chloride from 5 to 67 mg/L reduces sulfate toxicity to *Hyalella*. We will further characterize this interaction by determining sulfate LC50s over a wide range of chloride concentrations (10, 15, 20, 25, 100, 300, 500 mg/L) with hardness held constant. These tests will be conducted with both *H. azteca* and *C. dubia.*

Test effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level.
Having determined the critical chloride concentration required for *Hyalella* to show some resistance to sulfate (task 3), we will determine how hardness affects the toxicity of sulfate to *Hyalella* at this chloride concentration by generating LC50s at hardness values of 100, 200, 300, 400, and 500 mg/L (as $CaCO_3$) and a constant chloride concentration.

Methods

Invertebrates selected for testing include *Ceriodaphnia dubia, Hyalella azteca, Sphaerium simile* (Pelecypoda, Sphaeriidae), and a juvenile freshwater unionid mussel (species to be determined). The cladoceran, *Ceriodaphnia dubia,* was cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1993) methods. Prior to testing, *C. dubia* were fed a diet of *Pseudokirchneriella subcapitata* (also known as *Raphidocelis subcapitata* or *Selenastrum capricornutum*) and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture at a rate of 0.18 ml each per 30-ml water, daily. Cultures were maintained at 25 °C, and a 16:8 (L:D) photoperiod in Moderately Hard Reconstituted Water (MHRW; U.S. EPA, 1993). Amphipods, *Hyalella azteca,* were cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1994a) methods in RMHRW at 22 °C and a 16:8 (L:D) photoperiod for at least 7 d prior to testing. *Hyalella* were fed a diet of *Pseudokirchneriella subcapitata* and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture as well. Sphaeriid clams were and will be collected from Spring Creek, near Loda, Illinois, (Iroquois County) and acclimated to MHRW at 22 °C and a 16:8 (L:D) photoperiod for 5-7 d prior to testing. Clams collected from this site were previously identified to species by Dr. Gerald Mackie, of the University of Guelph, Department of Zoology, Guelph, Ontario, Canada.

For toxicity testing, a pure (99%) grade of anhydrous Na_2SO_4 served as the source of sulfate. A concentrated solution of this salt as well as a sample of laboratory deionized water, will be acidified to pH <2.0 and analyzed for priority metal concentrations at the Illinois State Water Survey (Champaign, IL) using inductively coupled plasma-atomic emission spectrometry according to U.S. EPA (1994b) methods to determine if samples are contaminated with trace metals.

For static, non-renewal acute toxicity tests, conducted according to ASTM E729-96 methods (2002a), treatments are comprised of a 75% dilution series (i.e., the 100% concentration is 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. For the *C. dubia* and *H. azteca* tests, five to six concentrations were tested, with four replicates tested per concentration, five organisms per replicate. Tests with *C. dubia* were conducted for 48 h with a 16:8 (L:D) photoperiod at 25 °C. *H. azteca* and *S. simile* were exposed for 96 h at 22 °C and a 16:8 (L:D) photoperiod. *C. dubia* and *H. azteca* were exposed in 50-ml glass beakers with 5 organisms per beaker, and for *H. azteca*, 1 g of quartz sand was added to each beaker to serve as substrate. Clam tests were and will be conducted in 150-ml glass beakers (no substrate). All clams used are juveniles. *C. dubia* used in tests were less than 24-h old, and *H. azteca* were ~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 *Simile*. For results to be acceptable, controls must have had at least a 90% survival rate.

Chronic testing will be conducted according to guidelines described in ASTM E 1295-01 (2002). Ten replicates will be used per sulfate concentration with one organism per replicate. Endpoints will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*.

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, dissolved oxygen, alkalinity and hardness. 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 (RDP, Dayton, OH, USA) model 58 meter with a self-stirring BOD probe. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA, USA) model MC226 conductivity/TDS meter. Alkalinity, and hardness were measured (beginning of tests only) by titration as described in APHA et al. (1998). Samples from each treatment will be analyzed to confirm sulfate concentrations by ion chromatography at the INHS Aquatic Chemistry Laboratory, Champaign, IL.

LC50 values were calculated using the Spearman-Karber method. To increase confidence in LC50 values, three assays were/will be conducted for each objective (i.e., either organism or chloride X hardness combination). This will provide a stronger estimate of the mean LC50 value for each species.

In experiments testing effects of hardness of sulfate toxicity, hardness will be increased by adding enough $CaSO_4$ and $MgSO_4$ to achieve the nominal hardness values. Then Na_2SO_4 will be added as was done with the standard MHRW. Whole carboys will be made at each elevated hardness level and this water will used as both diluent and control; therefore, each concentration within a given test will have the same hardness (i.e., $[Ca^{2+}]$ and $[Mg^{2+}]$ will not change with dilution). In experiments testing the influence of chloride on the toxicity of sulfate to *H. azteca*, chloride, as NaCl, was added at appropriate concentrations to solutions with a hardness of ~100 mg/L.

Progress to date:

<u>Hardness and chloride effects on acute sulfate toxicity to bivalves.</u>

In September, enough organisms were collected to conduct only two tests. One was conducted in USEPA MHRW and the other in RMHRW. LC50s based on nominal concentrations were 1643 mg SO_4/L for MHRW and 1864 mg SO_4/L. The LC50 for MHRW is on the low end of the range previously generated by Soucek (2004), but these values are not yet based on measured concentrations.

Because of rainfall and excessively high river levels in October and November, further sphaeriid collections were impossible and remaining tests will be conducted in the Spring as soon as clams are again accessible. In addition, tests with unionid glochidia and juveniles will be conducted by USGS in the Spring of 2005 when test organisms are available from gravid females.

<u>Effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*.</u>

Nearly all of the tests with *Hyalella* for this objective have been completed, and a trend in the results has developed based on LC50s calculated from nominal sulfate concentrations (Table 1 and Fig. 1). It appears that increasing chloride concentration from 10 to 33 mg/L results in a sharp decrease in sulfate toxicity. In the Soucek (2004) study, RMHRW had a chloride concentration of 33, and this water resulted in a mean LC50 of 2,855 mg SO_4/L. The 33 mg Cl/L treatment in this study resulted in a mean LC50 of 1,825 mg SO_4/L, but it is important to remember that the solutions in these tests have a lower calcium concentration than in RMHRW (23 mg/L compared to 32 mg/L in RMHRW) despite having the same hardness. In the current study, a Ca:Mg ratio of 2.33:1 (compared to 5.40:1 in RMHRW) was selected to reflect the median ratio for streams in Illinois. Calcium is probably more important in mediating sulfate toxicity than magnesium, thus accounting for the relatively large difference in LC50 values despite nearly identical hardness values. It is my hope that further experiments will support this contention.

Above 33 mg Cl/L, LC50 values rose again slightly in the 100 mg Cl/L treatment but then dropped in the 300 and 500 mg Cl/L tests (Table 1 and Fig. 1). If these data are viewed in a slightly different way, another interesting trend appears (Figs. 2 and 3). I compared conductivities and total dissolved solids (TDS, calculated) at LC50 concentrations for the different chloride treatments, and found that for the lower concentrations, both conductivity and TDS of the LC50 concentration increased with increasing chloride, as was the case for sulfate concentration, until a threshold was reached. Then, at the higher chloride concentrations (100 to 500 mg/L), no further benefit was provided by chloride above 100 mg/L. In fact, it appears that at 100 mg Cl/L, a threshold of 4,300 µS/cm or 3.1 g/L (conductivity and TDS, respectively) is reached. At higher chloride concentrations, less sulfate is required to reach the critical conductivity and TDS values, so LC50s in terms of sulfate decrease at 300 and 500 mg Cl/L. In other words, it appears that for *Hyalella*, in the range of 100 to 500 mg Cl/L, toxicity is reached at a fixed conductivity or TDS and if there is more chloride, less sulfate is required to reach this threshold and vice versa.

Table 1. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. All tests were conducted at 22 °C for 96 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L CaCO₃, and the Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.) LC50s were generated using the Spearman-Karber method.

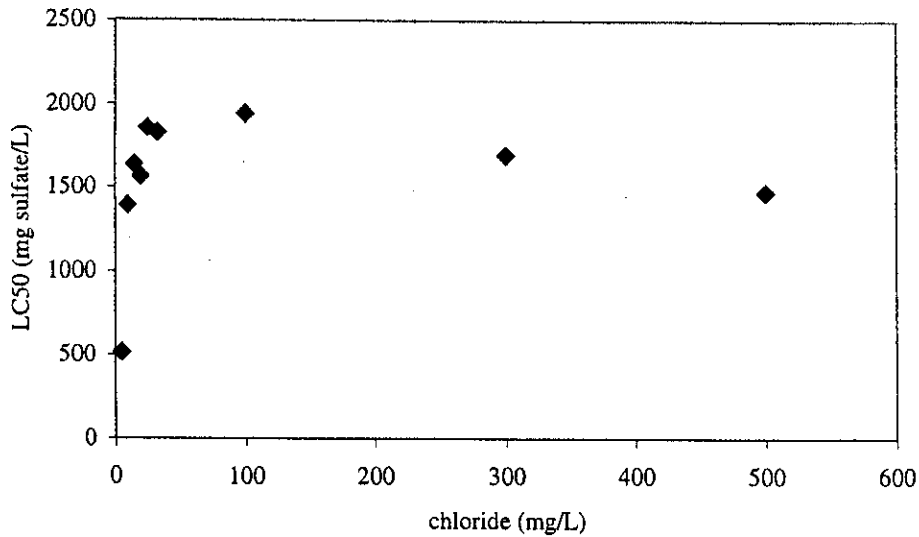| Chloride (mg/L) mg/L	number of tests conducted	Mean LC50 mg sulfate/L
10	3	1,387
15	2	1,632
20	3	1,562
25	3	1,854
33	2	1,825
100	3	1,938
300	3	1,691
500	2	1,469

Figure 1. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. See table one for details on test conditions.
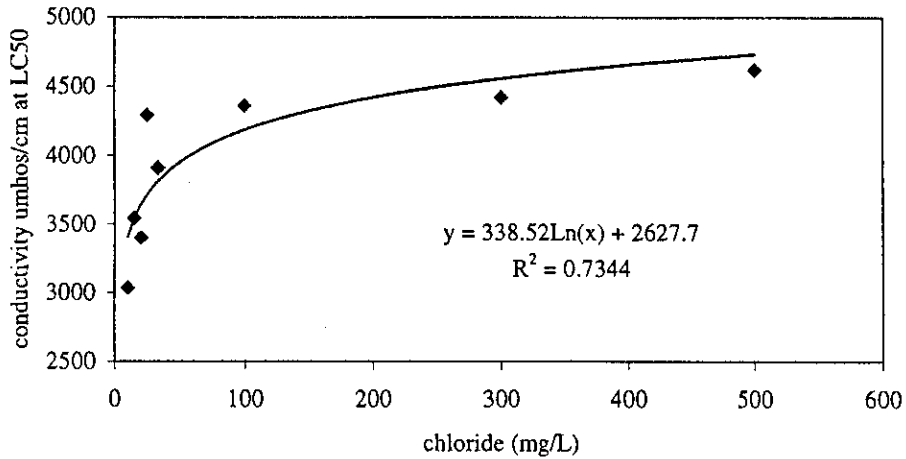
Figure 2. Relationship between chloride concentration and solution conductivity at the LC50 concentration in toxicity tests sulfate with *Hyalella azteca*. Conductivity values were calculated from equations generated with linear regression of measured conductivities at test sulfate concentrations.
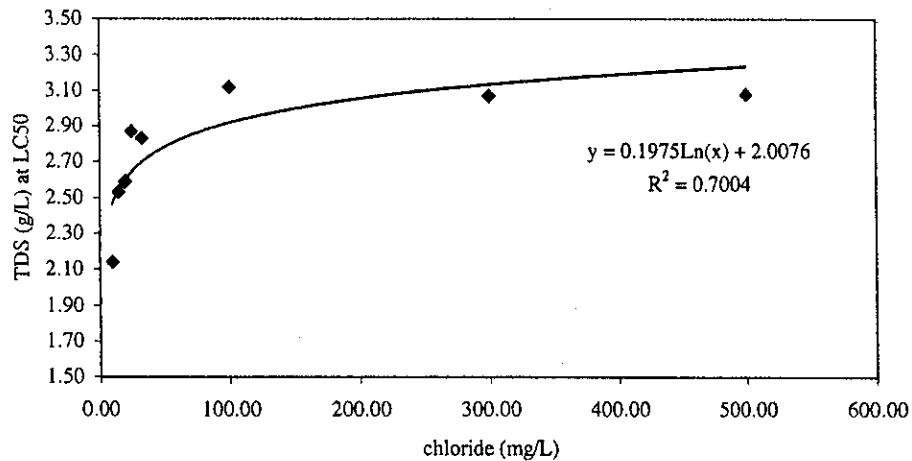

Figure 3. Relationship between chloride concentration and total dissolved solids at the LC50 concentration in toxicity tests sulfate to *Hyalella azteca*. Total dissolved solids values were calculated using nominal concentrations of all ions present in solution (excluding H+ and OH-) at LC50 concentrations.

Tests have been conducted with *Ceriodaphnia* at three chloride concentrations, and thus far, it appears the sulfate toxicity increases when chloride increases from 100 to 300 to 500 mg/L (Table 2). This objective has not progressed as far as would have been expected because a problem occurred in that the *C. dubia* cultures were either not producing healthy neonates or were not reproducing at all. A number of weeks were spent isolating the problem and it was discovered through the process of elimination that the *Pseudokirchneriella* culture being used as food for the organisms was causing toxicity. This problem has been rectified and the contaminated food has been replaced with *Pseudokirchneriella* from Aquatic Research Organisms (Hampton, New Hampshire). As a result, *C. dubia* cultures are again robust and producing ample healthy neonates for testing. The tests shown in table 2 were recently conducted using these organisms.

Table 2. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. All tests were conducted at 22 °C for 96 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L $CaCO_3$, and the Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.) LC50s were generated using the Spearman-Karber method.

Chloride (mg/L) mg/L	Mean LC50 mg sulfate/L
100	2,357
300	1,895
500	1,400

Effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level
and
7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia,*
These objectives will be completed during the first quarter of 2005.

Projected accomplishments for first quarter, 2005
After finishing the remaining tests with *C. dubia* and *H. azteca* examining effects of chloride on sulfate toxicity, the remaining two objectives: examining the effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level, and 7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia,* will commence. It is my expectation that the chronic testing and most, if not all of the tests examining hardness effects will be completed in the first quarter of 2005. Then, in the Spring, when bivalves are available, the last remaining objective will be completed. The remaining time in the project period will then be used for data analysis and report preparation.

Literature cited

American Public Health Association (APHA), American Water Works Association, Water Environment Federation. 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.

ASTM (American Society for Testing and Materials). 2002a. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E729-96. American Society for Testing and Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials). 2002b. Standard guide for conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*. E1295-01. American Society for Testing and Materials, Philadelphia, PA, USA.

Bayliss D, Harris RR. 1988. J Comp Physiol 158:81-90

Dwyer FJ, Burch SA, Ingersoll CG, and Hunn JB. 1992. Environ Toxicol Chem 11:513-520.

Ingersoll CG, Dwyer FJ, Burch SA, Nelson MK, Buckler DR, and Hunn JB. 1992. Environ Toxicol Chem 11:503-511.

Lucu C, and Flik G. 1999. Am J Physiol 276:R490-R499.

McMahon, RF, and Bogan, AE. 2001. Mollusca: Bivalvia, in JH Thorp and AP Covich (eds.) Ecology and Classification of North American Freshwater Invertebrates, 2nd Edition. Academic Press, San Diego, CA. pp 352-353.

Mount DR, Gulley DD, Hockett JR, Garrison TD, and Evans JM. 1997. Environ Toxicol Chem 16:2009-2019.

Pic P, and Maetz J. 1981. J Comp Physiol B 141:511-521.

Soucek, DJ. 2004. Effects of hardness, chloride, and acclimation on the acute toxicity of sulfate to freshwater invertebrates. Final Report to: Illinois Environmental Protection Agency and Illinois Coal Association.

Smith ME, Lazorchak JM, Herrin LE, Brewer-Swartz S, and Thoeny WT. 1997. Environ Toxicol Chem 16:1229-1233.

U.S. EPA. 1985. Guidelines for deriving numerical water quality criteria for the protection of aquatic organisms and their uses. PB85-227049. Washington, DC.

U.S. EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 4th ed. EPA/600/4-90/027F, Cincinnati, OH.

U.S. EPA. 1994a. Methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates. EPA/600/R-94/024. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA. 1994b. Methods for the determination of metals in environmental samples. EPA/600/R-4/111. U.S. Environmental Protection Agency, Cincinnati, OH.

U.S. EPA. 1999. National Recommended Water Quality Criteria – Correction. EPA 822-Z-99-001, Washington, DC.

Zipper CE. 2000. Coal mine reclamation, acid mine drainage and the Clean Water Act, in R. Barnhisel, W. Daniels, and R. Darmody (eds) Reclamation of Drastically Disturbed Lands. American Society of Agronomy, Madison, WI.

Effects of Water Quality on Acute and Chronic Toxicity of Sulfate
to Freshwater Bivalves, *Ceriodaphnia dubia,* and *Hyalella azteca.*

Second Quarterly Report

Submitted to:
Edward Hammer and Dertera Collins
United States Environmental Protection Agency
Region 5, Water Division, 77 West Jackson Boulevard
Chicago, Illinois 60604

April 10, 2005
Illinois Natural History Survey, Champaign, IL
U.S. EPA Region 5

Background

While there are no Federal water quality criteria (WQC) for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium (U.S. EPA 1999), several states, including Minnesota, Indiana, and Illinois, are at various stages in the process of developing standards for sulfate. Water quality standards are developed to protect designated uses, aquatic life uses in this case, but the economic impacts of these standards are important considerations as well. For example, after existing sulfate standards were enacted in Illinois, the Illinois Pollution Control Board adopted exceptions to the standards to provide relief for a number of coalmines that were enduring severe economic hardship. In fact, ~60% of coalmines have expired permits in Illinois because of violations of the sulfate standard, and ~50% of those have been expired for more than three years (T. Frevert, pers. comm.). The current "General Use" standard of 500 mg/L in Illinois is based on the value thought to be protective of livestock. Consultation with appropriate authorities revealed that livestock were capable of tolerating much higher levels of sulfate. In light of these factors, the Illinois Environmental Protection Agency (IL EPA) is actively pursuing an update of the sulfate standards based on scientific research, and is close to proposing an updated standard (R. Mosher, IL EPA, pers. comm.).

Sodium is one of the most common major cations in high TDS effluents, but calcium and chloride are usually present in mine-impacted waters as well. While major ion or 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, studies (funded by IL EPA, and IL Coal Association) were conducted by Soucek (2004; In Press Environmental Toxicology and Chemistry) to (1) generate LC50s (lethal concentration to 50% of a sample population) and LC10s (lethal concentration to 10% of a sample population) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia, Chironomus tentans, Hyalella azteca,* and *Sphaerium simile*) in U.S. Environmental Protection Agency's (US EPA, 1993) moderately hard reconstituted water (MHRW) and (2) determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate to *Ceriodaphnia dubia* and *Hyalella azteca* (Soucek, 2004). In these previous studies (Soucek, 2004), the mean LC50s, expressed as mg SO_4^{2-}/L, in moderately hard, reconstituted water (MHRW; U.S. EPA, 1993) ranged from: 512 to 1,4134 mg/L. The LC50 generated for the amphipod, *Hyalella* (512 mg/L) was surprisingly low, given that it is known as a euryhaline organism (Ingersoll et al., 1992), but as will be discussed below, water quality data, including other cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

The composition of the dilution water used during testing in the Soucek (2004) study had a dramatic effect on the toxicity of sulfate to *Hyalella*. Whereas the 96-hour LC50 in MHRW was 512 mg/L, the LC50 increased to 2,855 mg/L when using a "Reformulated Moderately Hard Reconstituted Water" (RMHRW, Smith et al., 1997). The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. Both dilution waters were similar in terms of hardness (~90-106 mg/L as $CaCO_3$), alkalinity, and pH, but RMHRW had a higher chloride concentration and different calcium to magnesium ratio than that in MHRW. An additional experiment conducted at the Illinois Natural History Survey (INHS) laboratory, but not included in the Soucek (2004) report, indicated that when sulfate (~2,800 mg/L) and hardness (106 mg/L) were held constant, percent survival of *H. azteca* was positively correlated with chloride concentration (up to 67 mg Cl⁻/L). These experiments illustrate the need to further characterize the interacting effects of chloride and sulfate on aquatic organisms.

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 the previous study (Soucek 2004), increased hardness reduced the toxicity of sulfate to *Hyalella* and had a dramatic effect on the 48-hour LC50 for *C. dubia*, increasing from 2,050 at a hardness of 90 to > 2,900 mg/L at hardness values higher than 194 mg/L as $CaCO_3$. Others have observed reduced toxicity of saline solutions due to increased hardness as well (e.g., Dwyer et al. 1992; Mount et al. 1997).

While a great deal of progress was made in the understanding of sulfate toxicity under varying water quality conditions, several important data gaps remain. In the previous studies (Soucek 2004), the fingernail clam, *Sphaerium simile*, had a lower LC10 than that of *C. dubia*, but because of the temporal nature of its availability, this bivalve was only tested in MHRW. It remains unclear whether or not a mollusk will have the same physiological response as two crustaceans to increased chloride or hardness in these experiments with sulfate. The principal inorganic anion of crustacean blood, or 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 (Bayliss and Harris, 1988). However, in the unionid mussel, *Toxolasma texasiensis*, chloride and bicarbonate are equally important anions in the hemolymph (see McMahon and Bogan 2001). Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations to the extent that some crustaceans do. If this is the case, the protective effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifest in some unionoidean bivalves. The hardness effect observed in the Soucek (2004) study may be more widespread among aquatic phyla, because calcium simply reduces gill permeability (Lucu and Flik, 1999; Pic and Maetz, 1981). However, McMahon and Bogan (2001) state that unionoideans "generally lose capacity for osmotic and volume regulation above 3-4 ppt" (salinity). TDS is a rough measure of salinity, and the TDS of a sample of RMHRW with 2000

mg/L sulfate is 2.9 g/L or ppt (Soucek unpublished data). Further experiments with freshwater bivalves are required to determine if there is an absolute TDS level that is tolerable, or if the limit depends upon water quality characteristics such as chloride concentration and hardness.

An additional data gap is the fact that all of the tests conducted in the Soucek (2004) study were acute exposures of 48 to 96 hours. Sublethal effects of sulfate in longer-term exposures are unknown to this date. Given that, under the new limit proposed by IL EPA, a continuous, long-term release at a given concentration will be allowed chronic testing should be conducted to determine potential sublethal impacts.

The purpose of the present study is to further provide data to support an appropriate sulfate criterion for the protection of aquatic life in Illinois. Therefore, the objectives of the current study are to build on previous studies conducted to support development of a sulfate criterion for protection of aquatic life by (1) determining the effects of hardness on toxicity of sulfate to bivalves, (2) determining the toxicity of sulfate to juvenile unionid mussels, (3) determining the short-term (7 days) chronic toxicity of sulfate to *Ceriodaphnia dubia*, (4) determining the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*, and (5) determining the effects of hardness on toxicity of sulfate to *Hyalella* at a critical chloride level, i.e., the chloride concentration at which sulfate is significantly less toxic to *Hyalella* as determined in #4 above.

Project Objectives

Based on existing data gaps described above, and because of the desire to expedite the process of updating sulfate standards in several states with limits based on scientific research, the following tasks will be conducted:

<u>Examine hardness and chloride effects on acute sulfate toxicity to bivalves.</u>
The first experiments conducted in this task will include testing the acute toxicity of sulfate to *Sphaerium simile* in RMHRW (Smith et al., 1997). The other two organisms tested in this water had markedly different responses compared to those observed in MHRW; whereas the 96-hour LC50 in MHRW for *Hyalella* was 512 mg/L, the LC50 increased to 2,855 mg/L in RMHRW. The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. However, both of these organisms are crustaceans. Sulfate LC50s will be generated for *S. simile* at two chloride concentrations (5 and 30 mg/L) and three hardness levels (100, 200, 300 mg/L). Because they will be conducted with organisms collected from the field, some of the tests may be eliminated depending on availability of test organisms at the collection site. In addition, we will generate sulfate LC50s using freshwater unionid mussel juveniles as test organisms, to

determine if this family is more sensitive to sulfate than the family Sphaeriidae (fingernail clams).

Conduct 7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia.*
To test the hypothesis that the acute safe level is similar to the chronic safe level, we will conduct 7-day chronic, survival/reproduction tests with *Ceriodaphnia dubia* in both MHRW and RMHRW. Tests will be conducted according to ASTM methods (2002b), and endpoints generated will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*. EC20s and EC50s for survival and reproduction will be generated as will No Observable Adverse Effects Concentrations (NOAEC) and Least Observable Adverse Effects Concentrations (LOAEC) for both endpoints.

Determine the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia.*
Chloride standards currently exist in Illinois (500 mg/L, R. Mosher, pers. comm.), and the State plans to additionally regulate for sulfate in order to address the major non-chloride component of TDS in these waters. Because IL EPA proposes to eliminate the TDS standard, opting instead to regulate sulfate and chloride, the interacting toxic effects of these two anions must be characterized. Soucek (manuscript accepted with minor revisions) has already shown that incrementally increasing chloride from 5 to 67 mg/L reduces sulfate toxicity to *Hyalella*. We will further characterize this interaction by determining sulfate LC50s over a wide range of chloride concentrations (10, 15, 20, 25, 100, 300, 500 mg/L) with hardness held constant. These tests will be conducted with both *H. azteca* and *C. dubia*.

Test effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level.
Having determined the critical chloride concentration required for *Hyalella* to show some resistance to sulfate (task 3), we will determine how hardness affects the toxicity of sulfate to *Hyalella* at this chloride concentration by generating LC50s at hardness values of 100, 200, 300, 400, and 500 mg/L (as $CaCO_3$) and a constant chloride concentration.

Methods
Invertebrates selected for testing include *Ceriodaphnia dubia, Hyalella azteca, Sphaerium simile* (Pelecypoda, Sphaeriidae), and a juvenile freshwater unionid mussel (species to be determined). The cladoceran, *Ceriodaphnia dubia,* was cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1993) methods. Prior to testing, *C. dubia* were fed a diet of *Pseudokirchneriella subcapitata* (also known as *Raphidocelis subcapitata* or *Selenastrum capricornutum*) and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture at a rate of 0.18 ml each per 30-ml water, daily. Cultures were maintained at 25 °C, and a 16:8

(L:D) photoperiod in Moderately Hard Reconstituted Water (MHRW; U.S. EPA, 1993). Amphipods, *Hyalella azteca*, were cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1994a) methods in RMHRW at 22 °C and a 16:8 (L:D) photoperiod for at least 7 d prior to testing. *Hyalella* were fed a diet of *Pseudokirchneriella subcapitata* and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture as well. Sphaeriid clams were and will be collected from Spring Creek, near Loda, Illinois, (Iroquois County) and acclimated to MHRW at 22 °C and a 16:8 (L:D) photoperiod for 5-7 d prior to testing. Clams collected from this site were previously identified to species by Dr. Gerald Mackie, of the University of Guelph, Department of Zoology, Guelph, Ontario, Canada.

For toxicity testing, a pure (99%) grade of anhydrous Na_2SO_4 served as the source of sulfate. A concentrated solution of this salt as well as a sample of laboratory deionized water, will be acidified to pH <2.0 and analyzed for priority metal concentrations at the Illinois State Water Survey (Champaign, IL) using inductively coupled plasma-atomic emission spectrometry according to U.S. EPA (1994b) methods to determine if samples are contaminated with trace metals.

For static, non-renewal acute toxicity tests, conducted according to ASTM E729-96 methods (2002a), treatments are comprised of a 75% dilution series (i.e., the 100% concentration is 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. For the *C. dubia* and *H. azteca* tests, five to six concentrations were tested, with four replicates tested per concentration, five organisms per replicate. Tests with *C. dubia* were conducted for 48 h with a 16:8 (L:D) photoperiod at 25 °C. *H. azteca* and *S. simile* were exposed for 96 h at 22 °C and a 16:8 (L:D) photoperiod. *C. dubia* and *H. azteca* were exposed in 50-ml glass beakers with 5 organisms per beaker, and for *H. azteca*, 1 g of quartz sand was added to each beaker to serve as substrate. Clam tests were and will be conducted in 150-ml glass beakers (no substrate). All clams used are juveniles. *C. dubia* used in tests were less than 24-h old, and *H. azteca* were ~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 *Simile*. For results to be acceptable, controls must have had at least a 90% survival rate.

Chronic testing will be conducted according to guidelines described in ASTM E 1295-01 (2002). Ten replicates will be used per sulfate concentration with one organism per replicate. Endpoints will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*.

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, dissolved oxygen,

alkalinity and hardness. 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 (RDP, Dayton, OH, USA) model 58 meter with a self-stirring BOD probe. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA, USA) model MC226 conductivity/TDS meter. Alkalinity, and hardness were measured (beginning of tests only) by titration as described in APHA et al. (1998). Samples from each treatment will be analyzed to confirm sulfate concentrations by ion chromatography at the INHS Aquatic Chemistry Laboratory, Champaign, IL.

LC50 values were calculated using the Spearman-Karber method. To increase confidence in LC50 values, three assays were/will be conducted for each objective (i.e., either organism or chloride X hardness combination). This will provide a stronger estimate of the mean LC50 value for each species.

In experiments testing effects of hardness of sulfate toxicity, hardness will be increased by adding enough $CaSO_4$ and $MgSO_4$ to achieve the nominal hardness values. Then Na_2SO_4 will be added as was done with the standard MHRW. Whole carboys will be made at each elevated hardness level and this water will used as both diluent and control; therefore, each concentration within a given test will have the same hardness (i.e., $[Ca^{2+}]$ and $[Mg^{2+}]$ will not change with dilution). In experiments testing the influence of chloride on the toxicity of sulfate to *H. azteca*, chloride, as NaCl, was added at appropriate concentrations to solutions with a hardness of ~100 mg/L.

Progress to date:
Hardness and chloride effects on acute sulfate toxicity to bivalves.
In September, enough organisms were collected to conduct only two tests. One was conducted in USEPA MHRW and the other in RMHRW. LC50s based on nominal concentrations were 1643 mg SO_4/L for MHRW and 1864 mg SO_4/L. The LC50 for MHRW is on the low end of the range previously generated by Soucek (2004), but these values are not yet based on measured concentrations.

Currently, water levels have dropped sufficiently for collection of sphaeriids, and enough individuals for two tests were collected on April 9, 2005. Tests will be initiated with these organisms on April 11, 2005, and more clams will be collected in subsequent weeks for further tests.

Effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia.*
All of the tests with *Hyalella* for this objective have been completed, and a trend in the results
has developed based on LC50s calculated from nominal sulfate concentrations (Table 1 and Fig.
1). It appears that increasing chloride concentration from 10 to 33 mg/L results in a sharp
decrease in sulfate toxicity. In the Soucek (2004) study, RMHRW had a chloride concentration
of 33, and this water resulted in a mean LC50 of 2,855 mg SO_4/L. The 33 mg Cl/L treatment in
this study resulted in a mean LC50 of 1,825 mg SO_4/L, but it is important to remember that the
solutions in these tests have a lower calcium concentration than in RMHRW (23 mg/L compared
to 32 mg/L in RMHRW) despite having the same hardness. In the current study, a Ca:Mg ratio
of 2.33:1 (compared to 5.40:1 in RMHRW) was selected to reflect the median ratio for streams in
Illinois. Calcium is probably more important in mediating sulfate toxicity than magnesium, thus
accounting for the relatively large difference in LC50 values despite nearly identical hardness
values.

Above 33 mg Cl/L, LC50 values rose again slightly in the 100 mg Cl/L treatment but then
dropped in the 300 and 500 mg Cl/L tests (Table 1 and Fig. 1). If these data are viewed in a
slightly different way, another interesting trend appears (Fig. 2). I compared total dissolved
solids (TDS, calculated) at LC50 concentrations for the different chloride treatments, and found
that for the lower concentrations, TDS of the LC50 concentration increased with increasing
chloride, as was the case for sulfate concentration, until a threshold was reached. Then, at the
higher chloride concentrations (100 to 500 mg/L), no further benefit was provided by chloride
above 100 mg/L. In fact, it appears that at 100 mg Cl/L, a threshold of ~3.1 g/L TDS is reached.
At higher chloride concentrations, less sulfate is required to reach the critical TDS value, so
LC50s in terms of sulfate decrease at 300 and 500 mg Cl/L. In other words, it appears that for
Hyalella, in the range of 100 to 500 mg Cl/L, toxicity is reached at a fixed TDS and if there is
more chloride, less sulfate is required to reach this threshold and vice versa.

Table 1. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca.* All tests were conducted at 22
°C for 96 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test
had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L $CaCO_3$, and the
Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.)
LC50s were generated using the Spearman-Karber method.

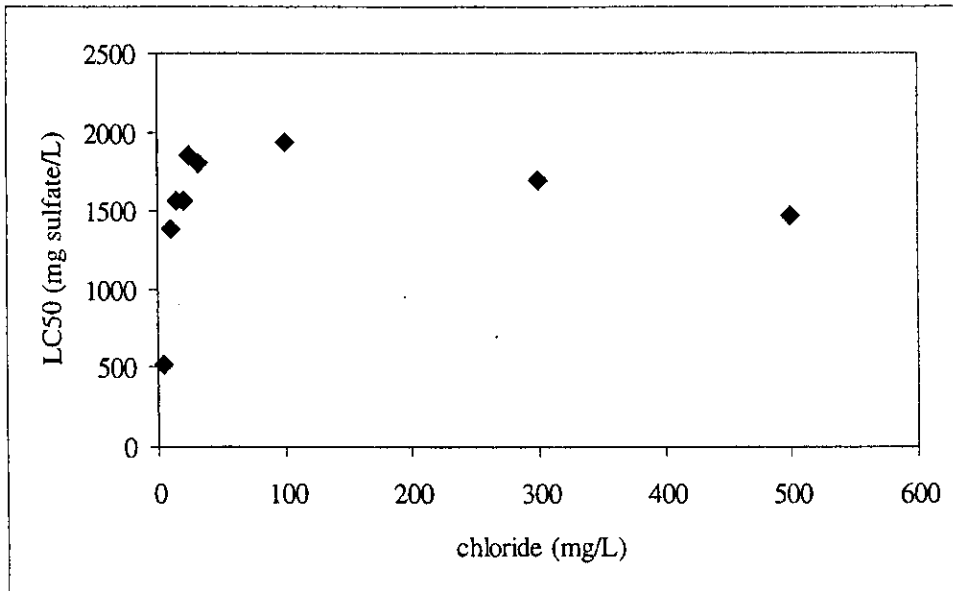| Chloride (mg/L) mg/L	number of tests conducted	Mean LC50 mg sulfate/L
10	3	1,387
15	3	1,563
20	3	1,562
25	3	1,854
33	3	1,799
100	3	1,938
300	3	1,691
500	3	1,470

Figure 1. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. See table one for details on test conditions.
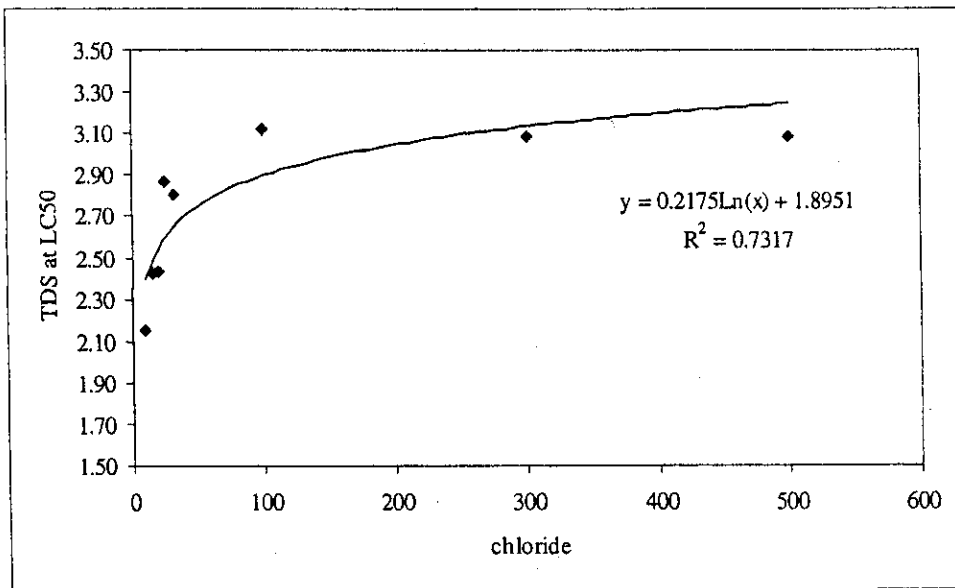

Figure 2. Relationship between chloride concentration and total dissolved solids at the LC50 concentration in toxicity tests sulfate to *Hyalella azteca*. Total dissolved solids values were calculated using nominal concentrations of all ions present in solution (excluding H+ and OH-) at LC50 concentrations.

All of the tests with *Ceriodaphnia* for this objective also have been completed. The trend of decreased toxicity with increasing chloride at the lower end of the range as seen for *Hyalella* is not as clear with *Ceriodaphnia* (Table 2 and Fig. 3). It appears that at chloride concentrations between 10 and 100 mg/L sulfate toxicity is fairly constant (with some noise in the data), with LC50 values between 2,200 and 2,500 mg SO_4/L (nominal concentrations). In the Soucek (2004) study, *Ceriodaphnia* did not respond as strikingly as *Hyalella* when tested in RMHRW compared to MHRW so these results may not be entirely surprising.

As was the case with *Hyalella*, LC50 values in terms of sulfate were lower at the 300 and 500 mg Cl/L concentrations compared to the lower chloride concentrations (Table 2 and Fig. 3). Comparing TDS (calculated) at LC50 concentrations for the different chloride treatments at the lower range of chloride concentrations, TDS values at LC50 concentrations were higher for *Ceriodaphnia* (3.5 – 3.8 g/L) than they were for *Hyalella* (2.4 – 3.1 g/L). The overall trend was different for *Ceriodaphnia* as well with an overall linear trend of decreasing TDS at LC50 with increasing chloride concentration (Fig. 4).

Table 2. Influence of chloride concentration on toxicity of sulfate to *Ceriodaphnia dubia*. All tests were conducted at 25 °C for 48 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L $CaCO_3$, and the Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.) LC50s were generated using the Spearman-Karber method.

Chloride (mg/L) mg/L	number of tests conducted	Mean LC50 mg sulfate/L
10	3	2,469
15	3	2,289
20	3	2,419
25	3	2,272
100	3	2,417
300	3	1,914
500	3	1,496

Figure 3. Influence of chloride concentration on toxicity of sulfate to *Ceriodaphnia dubia*.
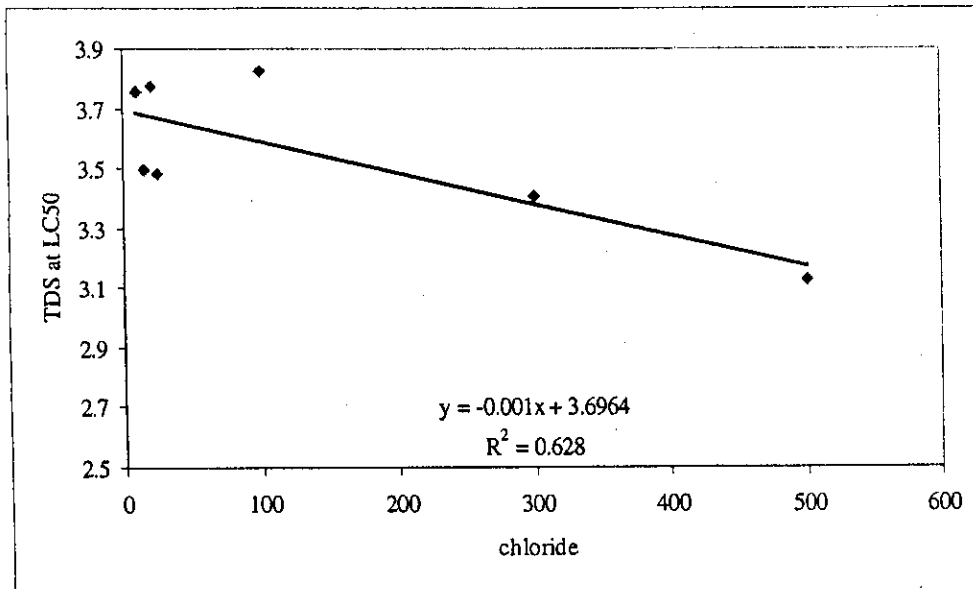See table 2 for details on test conditions.

Figure 4. Relationship between chloride concentration and total dissolved solids at the LC50 concentration in toxicity tests sulfate to *Ceriodaphnia dubia*. Total dissolved solids values were calculated using nominal concentrations of all ions present in solution (excluding H+ and OH-) at LC50 concentrations.

Effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level

A number of tests have been completed for this objective, but not all of them. Thus far, as was seen with *Ceriodaphnia dubia* in the Soucek (2004) study, a general trend of decreased toxicity with increased hardness is developing with the data (Fig. 5). Only one test has been conducted at hardness = 500 mg/L, and that value is rather low, but it is expected that the overall linear trend will smooth out once all of the tests are completed

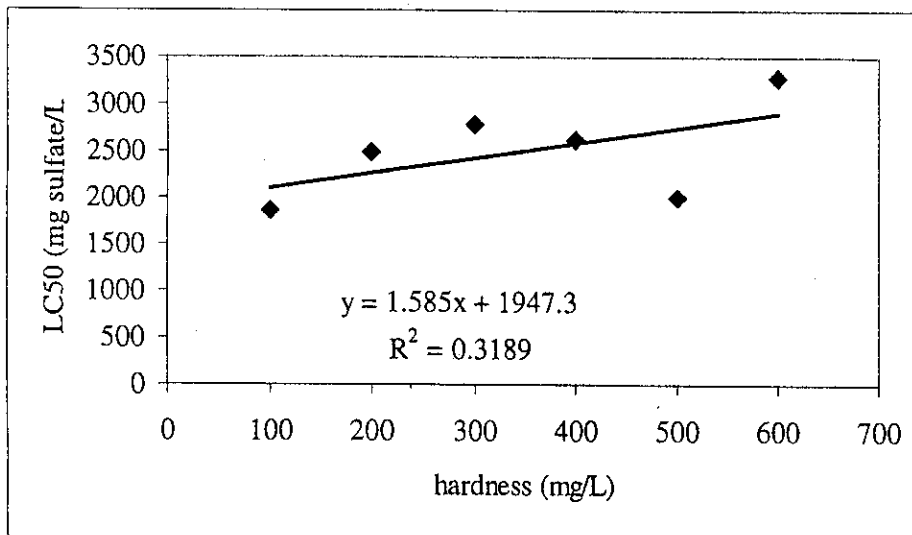$$y = 1.585x + 1947.3$$
$$R^2 = 0.3189$$

Figure 5. Influence of hardness on toxicity of sulfate to *Hyalella azteca* at chloride = 25 mg/L. See table one for details on test conditions.

7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia.*

All of the tests for this objective have been completed (three chronic tests in each dilution water), and results were quite consistent from test to test. As depicted in figures 6-9, chronic toxicity in terms of survival and reproduction was less in RMHRW compared to MHRW. Table 3 summarizes the Least Observable Adverse Effects Concentrations (LOAEC) and No Observable Adverse Effects Concentrations (NOAEC) for each dilution water. While LOAECs for reproduction are quite low, it should be noted that I have had a continuous, self sustaining, reserve culture of *Ceriodaphnia dubia* in MHRW spiked with 1,000 mg SO_4/L since at least August of 2004. Organisms used in chronic testing for this study were cultured in MHRW or RMHRW as appropriate.

Table 3. Summary of mean NOAEC and LOAEC concentrations for survival and reproduction of *Ceriodaphnia dubia* in 7-day static-renewal three brood toxicity tests with sulfate. Values are in terms of mg sulfate/L. All tests were conducted at 25 °C. Chloride and sulfate values shown are nominal concentrations. MHRW = Moderately Hard Reconstituted Water (USEPA 1993). RMHRW = Reformulated Moderately Hard Reconstituted Water (Smith et al., 1997)

Dilution water	number of tests conducted	Survival		Reproduction	
		NOAEC	LOAEC	NOAEC	LOAEC
MHRW	3	1,727	2,273	780	934
RMHRW	3	2,264	3,000	906	1,195

Projected accomplishments for second quarter, 2005

After finishing the remaining tests with *H. azteca* examining effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level, one objective remains: Hardness and chloride effects on acute sulfate toxicity to bivalves. As stated above, a first batch of fingernail clams has been collected and testing will begin immediately, to be finished as soon as possible given water levels remain appropriate for further collections. Juvenile unionid mussels will be available approximately May 1, 2005 and testing at the USGS, Columbia Environmental Research Center, Columbia, MO, will take approximately one week.

In addition, all values reported herein are based on nominal sulfate and chloride concentrations. Sample analysis is on-going and thus far, all measured values have been within approximately 5% of nominal values, so results reported here are not expected to change substantially when chemical analysis is completed. The remaining time in the project period will then be used for data analysis and report preparation.

Figure 6. Mean percent survival of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.
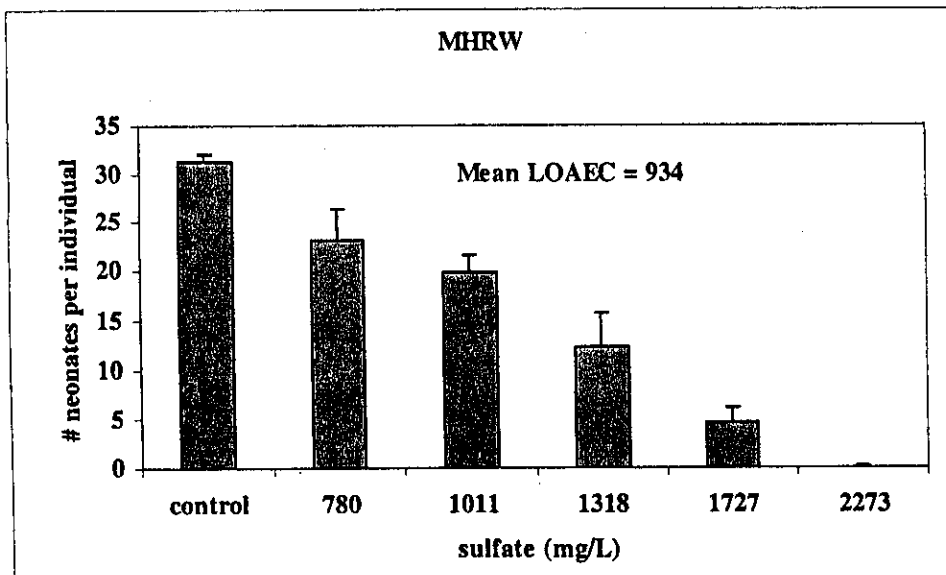
Figure 7. Mean reproduction of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.
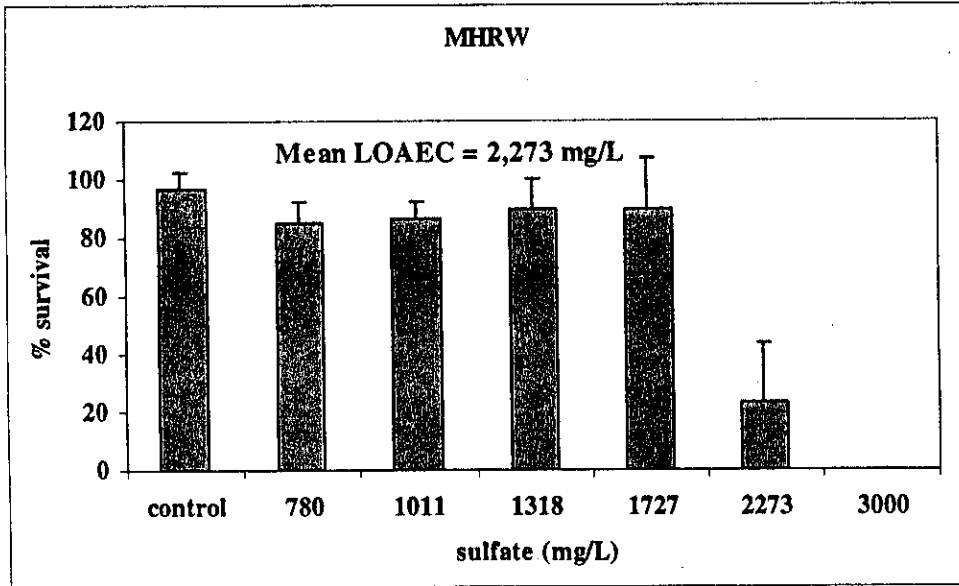
Figure 8. Mean percent survival of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Reformulated Moderately Hard Reconstituted Water (RMHRW). Bars and error bars indicate means and standard deviations for three separate tests.

Figure 9. Mean reproduction of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Reformulated Moderately Hard Reconstituted Water (RMHRW). Bars and error bars indicate means and standard deviations for three separate tests.
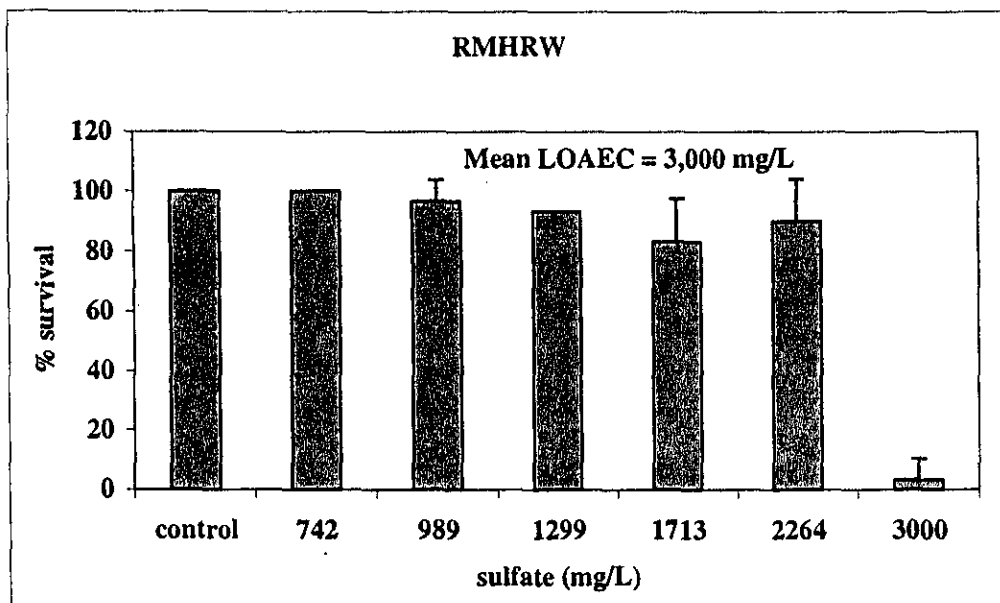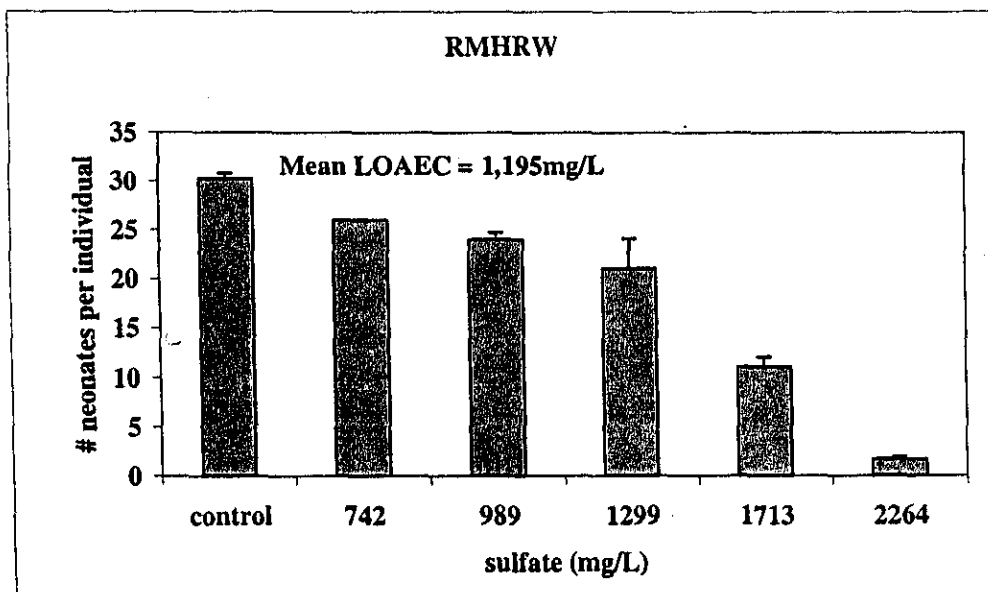
Literature cited

American Public Health Association (APHA), American Water Works Association, Water Environment Federation. 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.

ASTM (American Society for Testing and Materials). 2002a. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E729-96. American Society for Testing and Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials). 2002b. Standard guide for conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*. E1295-01. American Society for Testing and Materials, Philadelphia, PA, USA.

Bayliss D, Harris RR. 1988. J Comp Physiol 158:81-90

Dwyer FJ, Burch SA, Ingersoll CG, and Hunn JB. 1992. Environ Toxicol Chem 11:513-520.

Ingersoll CG, Dwyer FJ, Burch SA, Nelson MK, Buckler DR, and Hunn JB. 1992. Environ Toxicol Chem 11:503-511.

Lucu C, and Flik G. 1999. Am J Physiol 276:R490-R499.

McMahon, RF, and Bogan, AE. 2001. Mollusca: Bivalvia, in JH Thorp and AP Covich (eds.) Ecology and Classification of North American Freshwater Invertebrates, 2nd Edition. Academic Press, San Diego, CA. pp 352-353.

Mount DR, Gulley DD, Hockett JR, Garrison TD, and Evans JM. 1997. Environ Toxicol Chem 16:2009-2019.

Pic P, and Maetz J. 1981. J Comp Physiol B 141:511-521.

Soucek, DJ. 2004. Effects of hardness, chloride, and acclimation on the acute toxicity of sulfate to freshwater invertebrates. Final Report to: Illinois Environmental Protection Agency and Illinois Coal Association.

Smith ME, Lazorchak JM, Herrin LE, Brewer-Swartz S, and Thoeny WT. 1997. Environ Toxicol Chem 16:1229-1233.

U.S. EPA. 1985. Guidelines for deriving numerical water quality criteria for the protection of aquatic organisms and their uses. PB85-227049. Washington, DC.

U.S. EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 4th ed. EPA/600/4-90/027F, Cincinnati, OH.

U.S. EPA. 1994a. Methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates. EPA/600/R-94/024. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA. 1994b. Methods for the determination of metals in environmental samples. EPA/600/R-4/111. U.S. Environmental Protection Agency, Cincinnati, OH.

U.S. EPA. 1999. National Recommended Water Quality Criteria – Correction. EPA 822-Z-99-001, Washington, DC.

Zipper CE. 2000. Coal mine reclamation, acid mine drainage and the Clean Water Act, in R. Barnhisel, W. Daniels, and R. Darmody (eds) Reclamation of Drastically Disturbed Lands. American Society of Agronomy, Madison, WI.

Effects of Water Quality on Acute and Chronic Toxicity of Sulfate to Freshwater Bivalves, *Ceriodaphnia dubia,* and *Hyalella azteca.*

Third Quarterly Report

Submitted to:
Edward Hammer and Dertera Collins
United States Environmental Protection Agency
Region 5, Water Division, 77 West Jackson Boulevard
Chicago, Illinois 60604

October 20, 2005
Illinois Natural History Survey, Champaign, IL
U.S. EPA Region 5

Background

While there are no Federal water quality criteria (WQC) for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium (U.S. EPA 1999), several states, including Minnesota, Indiana, and Illinois, are at various stages in the process of developing standards for sulfate. Water quality standards are developed to protect designated uses, aquatic life uses in this case, but the economic impacts of these standards are important considerations as well. For example, after existing sulfate standards were enacted in Illinois, the Illinois Pollution Control Board adopted exceptions to the standards to provide relief for a number of coalmines that were enduring severe economic hardship. In fact, ~60% of coalmines have expired permits in Illinois because of violations of the sulfate standard, and ~50% of those have been expired for more than three years (T. Frevert, pers. comm.). The current "General Use" standard of 500 mg/L in Illinois is based on the value thought to be protective of livestock. Consultation with appropriate authorities revealed that livestock were capable of tolerating much higher levels of sulfate. In light of these factors, the Illinois Environmental Protection Agency (IL EPA) is actively pursuing an update of the sulfate standards based on scientific research, and is close to proposing an updated standard (R. Mosher, IL EPA, pers. comm.).

Sodium is one of the most common major cations in high TDS effluents, but calcium and chloride are usually present in mine-impacted waters as well. While major ion or 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, studies (funded by IL EPA, and IL Coal Association) were conducted by Soucek (2004; In Press Environmental Toxicology and Chemistry) to (1) generate LC50s (lethal concentration to 50% of a sample population) and LC10s (lethal concentration to 10% of a sample population) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia, Chironomus tentans, Hyalella azteca,* and *Sphaerium simile*) in U.S. Environmental Protection Agency's (US EPA, 1993) moderately hard reconstituted water (MHRW) and (2) determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate to *Ceriodaphnia dubia* and *Hyalella azteca* (Soucek, 2004). In these previous studies (Soucek, 2004), the mean LC50s, expressed as mg SO_4^{2-}/L, in moderately hard, reconstituted water (MHRW; U.S. EPA, 1993) ranged from: 512 to 1,4134 mg/L. The LC50 generated for the amphipod, *Hyalella* (512 mg/L) was surprisingly low, given that it is known as a euryhaline organism (Ingersoll et al., 1992), but as will be discussed below, water quality data, including other cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

The composition of the dilution water used during testing in the Soucek (2004) study had a dramatic effect on the toxicity of sulfate to *Hyalella*. Whereas the 96-hour LC50 in MHRW was 512 mg/L, the LC50 increased to 2,855 mg/L when using a "Reformulated Moderately Hard Reconstituted Water" (RMHRW, Smith et al., 1997). The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. Both dilution waters were similar in terms of hardness (~90-106 mg/L as $CaCO_3$), alkalinity, and pH, but RMHRW had a higher chloride concentration and different calcium to magnesium ratio than that in MHRW. An additional experiment conducted at the Illinois Natural History Survey (INHS) laboratory, but not included in the Soucek (2004) report, indicated that when sulfate (~2,800 mg/L) and hardness (106 mg/L) were held constant, percent survival of *H. azteca* was positively correlated with chloride concentration (up to 67 mg Cl⁻/L). These experiments illustrate the need to further characterize the interacting effects of chloride and sulfate on aquatic organisms.

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 the previous study (Soucek 2004), increased hardness reduced the toxicity of sulfate to *Hyalella* and had a dramatic effect on the 48-hour LC50 for *C. dubia*, increasing from 2,050 at a hardness of 90 to > 2,900 mg/L at hardness values higher than 194 mg/L as $CaCO_3$. Others have observed reduced toxicity of saline solutions due to increased hardness as well (e.g., Dwyer et al. 1992; Mount et al. 1997).

While a great deal of progress was made in the understanding of sulfate toxicity under varying water quality conditions, several important data gaps remain. In the previous studies (Soucek 2004), the fingernail clam, *Sphaerium simile*, had a lower LC10 than that of *C. dubia*, but because of the temporal nature of its availability, this bivalve was only tested in MHRW. It remains unclear whether or not a mollusk will have the same physiological response as two crustaceans to increased chloride or hardness in these experiments with sulfate. The principal inorganic anion of crustacean blood, or 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 (Bayliss and Harris, 1988). However, in the unionid mussel, *Toxolasma texasiensis*, chloride and bicarbonate are equally important anions in the hemolymph (see McMahon and Bogan 2001). Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations to the extent that some crustaceans do. If this is the case, the protective effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifest in some unionoidean bivalves. The hardness effect observed in the Soucek (2004) study may be more widespread among aquatic phyla, because calcium simply reduces gill permeability (Lucu and Flik, 1999; Pic and Maetz, 1981). However, McMahon and Bogan (2001) state that unionoideans "generally lose capacity for osmotic and volume regulation above 3-4 ppt" (salinity). TDS is a rough measure of salinity, and the TDS of a sample of RMHRW with 2000

mg/L sulfate is 2.9 g/L or ppt (Soucek unpublished data). Further experiments with freshwater bivalves are required to determine if there is an absolute TDS level that is tolerable, or if the limit depends upon water quality characteristics such as chloride concentration and hardness.

An additional data gap is the fact that all of the tests conducted in the Soucek (2004) study were acute exposures of 48 to 96 hours. Sublethal effects of sulfate in longer-term exposures are unknown to this date. Given that, under the new limit proposed by IL EPA, a continuous, long-term release at a given concentration will be allowed chronic testing should be conducted to determine potential sublethal impacts.

The purpose of the present study is to further provide data to support an appropriate sulfate criterion for the protection of aquatic life in Illinois. Therefore, the objectives of the current study are to build on previous studies conducted to support development of a sulfate criterion for protection of aquatic life by (1) determining the effects of hardness on toxicity of sulfate to bivalves, (2) determining the toxicity of sulfate to juvenile unionid mussels, (3) determining the short-term (7 days) chronic toxicity of sulfate to *Ceriodaphnia dubia*, (4) determining the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*, and (5) determining the effects of hardness on toxicity of sulfate to *Hyalella* at a critical chloride level, i.e., the chloride concentration at which sulfate is significantly less toxic to *Hyalella* as determined in #4 above.

Project Objectives

Based on existing data gaps described above, and because of the desire to expedite the process of updating sulfate standards in several states with limits based on scientific research, the following tasks will be conducted:

Examine hardness and chloride effects on acute sulfate toxicity to bivalves.
The first experiments conducted in this task will include testing the acute toxicity of sulfate to *Sphaerium simile* in RMHRW (Smith et al., 1997). The other two organisms tested in this water had markedly different responses compared to those observed in MHRW; whereas the 96-hour LC50 in MHRW for *Hyalella* was 512 mg/L, the LC50 increased to 2,855 mg/L in RMHRW. The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. However, both of these organisms are crustaceans. Sulfate LC50s will be generated for *S. simile* at two chloride concentrations (5 and 30 mg/L) and three hardness levels (100, 200, 300 mg/L). Because they will be conducted with organisms collected from the field, some of the tests may be eliminated depending on availability of test organisms at the collection site. In addition, we will generate sulfate LC50s using freshwater unionid mussel juveniles as test organisms, to

determine if this family is more sensitive to sulfate than the family Sphaeriidae (fingernail clams).

Conduct 7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia*.

To test the hypothesis that the acute safe level is similar to the chronic safe level, we will conduct 7-day chronic, survival/reproduction tests with *Ceriodaphnia dubia* in both MHRW and RMHRW. Tests will be conducted according to ASTM methods (2002b), and endpoints generated will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*. EC20s and EC50s for survival and reproduction will be generated as will No Observable Adverse Effects Concentrations (NOAEC) and Least Observable Adverse Effects Concentrations (LOAEC) for both endpoints.

Determine the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*.

Chloride standards currently exist in Illinois (500 mg/L, R. Mosher, pers. comm.), and the State plans to additionally regulate for sulfate in order to address the major non-chloride component of TDS in these waters. Because IL EPA proposes to eliminate the TDS standard, opting instead to regulate sulfate and chloride, the interacting toxic effects of these two anions must be characterized. Soucek (manuscript accepted with minor revisions) has already shown that incrementally increasing chloride from 5 to 67 mg/L reduces sulfate toxicity to *Hyalella*. We will further characterize this interaction by determining sulfate LC50s over a wide range of chloride concentrations (10, 15, 20, 25, 100, 300, 500 mg/L) with hardness held constant. These tests will be conducted with both *H. azteca* and *C. dubia*.

Test effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level.

Having determined the critical chloride concentration required for *Hyalella* to show some resistance to sulfate (task 3), we will determine how hardness affects the toxicity of sulfate to *Hyalella* at this chloride concentration by generating LC50s at hardness values of 100, 200, 300, 400, and 500 mg/L (as $CaCO_3$) and a constant chloride concentration.

Methods

Invertebrates selected for testing include *Ceriodaphnia dubia, Hyalella azteca, Sphaerium simile* (Pelecypoda, Sphaeriidae), and a juvenile freshwater unionid mussel (species to be determined). The cladoceran, *Ceriodaphnia dubia*, was cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1993) methods. Prior to testing, *C. dubia* were fed a diet of *Pseudokirchneriella subcapitata* (also known as *Raphidocelis subcapitata* or *Selenastrum capricornutum*) and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture at a rate of 0.18 ml each per 30-ml water, daily. Cultures were maintained at 25 °C, and a 16:8

(L:D) photoperiod in Moderately Hard Reconstituted Water (MHRW; U.S. EPA, 1993).
Amphipods, *Hyalella azteca*, w

**Effects of Water Quality on Acute and Chronic Toxicity of Sulfate
to Freshwater Bivalves, *Ceriodaphnia dubia,* and *Hyalella azteca.***

Third Quarterly Report

Submitted to:
Edward Hammer and Dertera Collins
United States Environmental Protection Agency
Region 5, Water Division, 77 West Jackson Boulevard
Chicago, Illinois 60604

October 20, 2005
Illinois Natural History Survey, Champaign, IL
U.S. EPA Region 5

Background

While there are no Federal water quality criteria (WQC) for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium (U.S. EPA 1999), several states, including Minnesota, Indiana, and Illinois, are at various stages in the process of developing standards for sulfate. Water quality standards are developed to protect designated uses, aquatic life uses in this case, but the economic impacts of these standards are important considerations as well. For example, after existing sulfate standards were enacted in Illinois, the Illinois Pollution Control Board adopted exceptions to the standards to provide relief for a number of coalmines that were enduring severe economic hardship. In fact, ~60% of coalmines have expired permits in Illinois because of violations of the sulfate standard, and ~50% of those have been expired for more than three years (T. Frevert, pers. comm.). The current "General Use" standard of 500 mg/L in Illinois is based on the value thought to be protective of livestock. Consultation with appropriate authorities revealed that livestock were capable of tolerating much higher levels of sulfate. In light of these factors, the Illinois Environmental Protection Agency (IL EPA) is actively pursuing an update of the sulfate standards based on scientific research, and is close to proposing an updated standard (R. Mosher, IL EPA, pers. comm.).

Sodium is one of the most common major cations in high TDS effluents, but calcium and chloride are usually present in mine-impacted waters as well. While major ion or 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, studies (funded by IL EPA, and IL Coal Association) were conducted by Soucek (2004; In Press Environmental Toxicology and Chemistry) to (1) generate LC50s (lethal concentration to 50% of a sample population) and LC10s (lethal concentration to 10% of a sample population) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia, Chironomus tentans, Hyalella azteca,* and *Sphaerium simile*) in U.S. Environmental Protection Agency's (US EPA, 1993) moderately hard reconstituted water (MHRW) and (2) determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate to *Ceriodaphnia dubia* and *Hyalella azteca* (Soucek, 2004). In these previous studies (Soucek, 2004), the mean LC50s, expressed as mg SO_4^{2-}/L, in moderately hard, reconstituted water (MHRW; U.S. EPA, 1993) ranged from: 512 to 1,4134 mg/L The LC50 generated for the amphipod, *Hyalella* (512 mg/L) was surprisingly low, given that it is known as a euryhaline organism (Ingersoll et al., 1992), but as will be discussed below, water quality data, including other cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

The composition of the dilution water used during testing in the Soucek (2004) study had a dramatic effect on the toxicity of sulfate to *Hyalella*. Whereas the 96-hour LC50 in MHRW was 512 mg/L, the LC50 increased to 2,855 mg/L when using a "Reformulated Moderately Hard Reconstituted Water" (RMHRW, Smith et al., 1997). The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. Both dilution waters were similar in terms of hardness (~90-106 mg/L as $CaCO_3$), alkalinity, and pH, but RMHRW had a higher chloride concentration and different calcium to magnesium ratio than that in MHRW. An additional experiment conducted at the Illinois Natural History Survey (INHS) laboratory, but not included in the Soucek (2004) report, indicated that when sulfate (~2,800 mg/L) and hardness (106 mg/L) were held constant, percent survival of *H. azteca* was positively correlated with chloride concentration (up to 67 mg Cl⁻/L). These experiments illustrate the need to further characterize the interacting effects of chloride and sulfate on aquatic organisms.

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 the previous study (Soucek 2004), increased hardness reduced the toxicity of sulfate to *Hyalella* and had a dramatic effect on the 48-hour LC50 for *C. dubia*, increasing from 2,050 at a hardness of 90 to > 2,900 mg/L at hardness values higher than 194 mg/L as $CaCO_3$. Others have observed reduced toxicity of saline solutions due to increased hardness as well (e.g., Dwyer et al. 1992; Mount et al. 1997).

While a great deal of progress was made in the understanding of sulfate toxicity under varying water quality conditions, several important data gaps remain. In the previous studies (Soucek 2004), the fingernail clam, *Sphaerium simile*, had a lower LC10 than that of *C. dubia*, but because of the temporal nature of its availability, this bivalve was only tested in MHRW. It remains unclear whether or not a mollusk will have the same physiological response as two crustaceans to increased chloride or hardness in these experiments with sulfate. The principal inorganic anion of crustacean blood, or 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 (Bayliss and Harris, 1988). However, in the unionid mussel, *Toxolasma texasiensis*, chloride and bicarbonate are equally important anions in the hemolymph (see McMahon and Bogan 2001). Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations to the extent that some crustaceans do. If this is the case, the protective effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifest in some unionoidean bivalves. The hardness effect observed in the Soucek (2004) study may be more widespread among aquatic phyla, because calcium simply reduces gill permeability (Lucu and Flik, 1999; Pic and Maetz, 1981). However, McMahon and Bogan (2001) state that unionoideans "generally lose capacity for osmotic and volume regulation above 3-4 ppt" (salinity). TDS is a rough measure of salinity, and the TDS of a sample of RMHRW with 2000

mg/L sulfate is 2.9 g/L or ppt (Soucek unpublished data). Further experiments with freshwater bivalves are required to determine if there is an absolute TDS level that is tolerable, or if the limit depends upon water quality characteristics such as chloride concentration and hardness.

An additional data gap is the fact that all of the tests conducted in the Soucek (2004) study were acute exposures of 48 to 96 hours. Sublethal effects of sulfate in longer-term exposures are unknown to this date. Given that, under the new limit proposed by IL EPA, a continuous, long-term release at a given concentration will be allowed chronic testing should be conducted to determine potential sublethal impacts.

The purpose of the present study is to further provide data to support an appropriate sulfate criterion for the protection of aquatic life in Illinois. Therefore, the objectives of the current study are to build on previous studies conducted to support development of a sulfate criterion for protection of aquatic life by (1) determining the effects of hardness on toxicity of sulfate to bivalves, (2) determining the toxicity of sulfate to juvenile unionid mussels, (3) determining the short-term (7 days) chronic toxicity of sulfate to *Ceriodaphnia dubia*, (4) determining the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia*, and (5) determining the effects of hardness on toxicity of sulfate to *Hyalella* at a critical chloride level, i.e., the chloride concentration at which sulfate is significantly less toxic to *Hyalella* as determined in #4 above.

Project Objectives

Based on existing data gaps described above, and because of the desire to expedite the process of updating sulfate standards in several states with limits based on scientific research, the following tasks will be conducted:

Examine hardness and chloride effects on acute sulfate toxicity to bivalves.
The first experiments conducted in this task will include testing the acute toxicity of sulfate to *Sphaerium simile* in RMHRW (Smith et al., 1997). The other two organisms tested in this water had markedly different responses compared to those observed in MHRW; whereas the 96-hour LC50 in MHRW for *Hyalella* was 512 mg/L, the LC50 increased to 2,855 mg/L in RMHRW. The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. However, both of these organisms are crustaceans. Sulfate LC50s will be generated for *S. simile* at two chloride concentrations (5 and 30 mg/L) and three hardness levels (100, 200, 300 mg/L). Because they will be conducted with organisms collected from the field, some of the tests may be eliminated depending on availability of test organisms at the collection site. In addition, we will generate sulfate LC50s using freshwater unionid mussel juveniles as test organisms, to

determine if this family is more sensitive to sulfate than the family Sphaeriidae (fingernail clams).

Conduct 7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia.*
To test the hypothesis that the acute safe level is similar to the chronic safe level, we will conduct 7-day chronic, survival/reproduction tests with *Ceriodaphnia dubia* in both MHRW and RMHRW. Tests will be conducted according to ASTM methods (2002b), and endpoints generated will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*. EC20s and EC50s for survival and reproduction will be generated as will No Observable Adverse Effects Concentrations (NOAEC) and Least Observable Adverse Effects Concentrations (LOAEC) for both endpoints.

Determine the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia.*
Chloride standards currently exist in Illinois (500 mg/L, R. Mosher, pers. comm.), and the State plans to additionally regulate for sulfate in order to address the major non-chloride component of TDS in these waters. Because IL EPA proposes to eliminate the TDS standard, opting instead to regulate sulfate and chloride, the interacting toxic effects of these two anions must be characterized. Soucek (manuscript accepted with minor revisions) has already shown that incrementally increasing chloride from 5 to 67 mg/L reduces sulfate toxicity to *Hyalella*. We will further characterize this interaction by determining sulfate LC50s over a wide range of chloride concentrations (10, 15, 20, 25, 100, 300, 500 mg/L) with hardness held constant. These tests will be conducted with both *H. azteca* and *C. dubia*.

Test effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level.
Having determined the critical chloride concentration required for *Hyalella* to show some resistance to sulfate (task 3), we will determine how hardness affects the toxicity of sulfate to *Hyalella* at this chloride concentration by generating LC50s at hardness values of 100, 200, 300, 400, and 500 mg/L (as $CaCO_3$) and a constant chloride concentration.

Methods
Invertebrates selected for testing include *Ceriodaphnia dubia, Hyalella azteca, Sphaerium simile* (Pelecypoda, Sphaeriidae), and a juvenile freshwater unionid mussel (species to be determined). The cladoceran, *Ceriodaphnia dubia,* was cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1993) methods. Prior to testing, *C. dubia* were fed a diet of *Pseudokirchneriella subcapitata* (also known as *Raphidocelis subcapitata* or *Selenastrum capricornutum*) and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture at a rate of 0.18 ml each per 30-ml water, daily. Cultures were maintained at 25 °C, and a 16:8

(L:D) photoperiod in Moderately Hard Reconstituted Water (MHRW; U.S. EPA, 1993). Amphipods, *Hyalella azteca*, were cultured in-house (Soucek laboratory, INHS) according to U.S. EPA (1994a) methods in RMHRW at 22 °C and a 16:8 (L:D) photoperiod for at least 7 d prior to testing. *Hyalella* were fed a diet of *Pseudokirchneriella subcapitata* and a Yeast-Cereal Leaves-Trout Chow (YCT) mixture as well. Sphaeriid clams were and will be collected from Spring Creek, near Loda, Illinois, (Iroquois County) and acclimated to MHRW at 22 °C and a 16:8 (L:D) photoperiod for 5-7 d prior to testing. Clams collected from this site were previously identified to species by Dr. Gerald Mackie, of the University of Guelph, Department of Zoology, Guelph, Ontario, Canada.

For toxicity testing, a pure (99%) grade of anhydrous Na_2SO_4 served as the source of sulfate. A concentrated solution of this salt as well as a sample of laboratory deionized water, will be acidified to pH <2.0 and analyzed for priority metal concentrations at the Illinois State Water Survey (Champaign, IL) using inductively coupled plasma-atomic emission spectrometry according to U.S. EPA (1994b) methods to determine if samples are contaminated with trace metals.

For static, non-renewal acute toxicity tests, conducted according to ASTM E729-96 methods (2002a), treatments are comprised of a 75% dilution series (i.e., the 100% concentration is 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. For the *C. dubia* and *H. azteca* tests, five to six concentrations were tested, with four replicates tested per concentration, five organisms per replicate. Tests with *C. dubia* were conducted for 48 h with a 16:8 (L:D) photoperiod at 25 °C. *H. azteca* and *S. simile* were exposed for 96 h at 22 °C and a 16:8 (L:D) photoperiod. *C. dubia* and *H. azteca* were exposed in 50-ml glass beakers with 5 organisms per beaker, and for *H. azteca*, 1 g of quartz sand was added to each beaker to serve as substrate. Clam tests were and will be conducted in 150-ml glass beakers (no substrate). All clams used are juveniles. *C. dubia* used in tests were less than 24-h old, and *H. azteca* were ~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 *Simile*. For results to be acceptable, controls must have had at least a 90% survival rate.

Chronic testing will be conducted according to guidelines described in ASTM E 1295-01 (2002). Ten replicates will be used per sulfate concentration with one organism per replicate. Endpoints will include the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*.

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, dissolved oxygen,

alkalinity and hardness. 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 < ± 0.05 pH at 25 °C). Dissolved oxygen was measured using an air-calibrated Yellow Springs Instruments (RDP, Dayton, OH, USA) model 58 meter with a self-stirring BOD probe. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA, USA) model MC226 conductivity/TDS meter. Alkalinity, and hardness were measured (beginning of tests only) by titration as described in APHA et al. (1998). Samples from each treatment will be analyzed to confirm sulfate concentrations by ion chromatography at the INHS Aquatic Chemistry Laboratory, Champaign, IL.

LC50 values were calculated using the Spearman-Karber method. To increase confidence in LC50 values, three assays were/will be conducted for each objective (i.e., either organism or chloride X hardness combination). This will provide a stronger estimate of the mean LC50 value for each species.

In experiments testing effects of hardness of sulfate toxicity, hardness will be increased by adding enough $CaSO_4$ and $MgSO_4$ to achieve the nominal hardness values. Then Na_2SO_4 will be added as was done with the standard MHRW. Whole carboys will be made at each elevated hardness level and this water will used as both diluent and control; therefore, each concentration within a given test will have the same hardness (i.e., $[Ca^{2+}]$ and $[Mg^{2+}]$ will not change with dilution). In experiments testing the influence of chloride on the toxicity of sulfate to *H. azteca,* chloride, as NaCl, was added at appropriate concentrations to solutions with a hardness of ~100 mg/L.

Progress to date:
Hardness and chloride effects on acute sulfate toxicity to bivalves.
Five toxicity tests were conducted with 4-day-old, juvenile unionid mussels (fatmuckets,*Lampsilis siliquoidea*) at the U.S. Geological Survey's Columbia Environmental Research Center. Tests were conducted according to newly developed standard methods (ASTM 2005), and five different diluents were used with varying levels of hardness and chloride. Because of constraints with availability of organisms, one test was conducted for each diluent type. Test water #4 was U.S. EPA's MHRW (USEPA 1993), and the 96-h LC50 in this water (1,727 mg/L) was fairly similar to, but slightly lower than LC50s for *C. dubia* (2,050 mg/L), and the fingernail clam, *S. simile* (2,078 mg/L) in MHRW. When Ca:Mg and chloride concentration were held constant, increased hardness appeared to slightly reduce the toxicity of sulfate to the juvenile mussels (Table 1), but confidence intervals overlapped. Chloride did not appear to have a strong effect either, but increasing chloride concentration from 5 to 33 increased the sulfate LC50 by approximately 100 mg/L (Table 1). Interestingly, while hardness and chloride did not strongly affect sulfate toxicity to freshwater mussels, Ca:Mg ratio appeared to have a substantial

effect. In tests conducted with Ca:Mg = 1.46, 96-h LC50s ranged from 1,727 to 1,822 mg SO_4/L, while at a ratio of 2.33, LC50s ranged from 3,377 to 3,729 mg SO_4/L. This trend suggests that there may be a calcium threshold for unionids, which when reached provides a certain level of protection against sulfate toxicity, but beyond which, additional calcium provides minimal additional protection. Further testing should be conducted to verify these trends.

Table 1. Toxicity of sulfate to juvenile fatmuckets (*Lampsilis siliquoidea*). All tests were conducted at 20 °C for 96 hours with 4-d-old mussels. Mortality was evaluated at 48, and 96 hours. Sulfate LC50 values shown are based on measured concentrations, and all treatments within a given test had the same chloride concentration and hardness. LC50s were generated using the Spearman-Karber method. One test was conducted for each diluent type.

Test	Cl mg/L	Ca:Mg ratio	hardness mg/L ($CaCO_3$)	48 h LC50 (95% C.I.)	96 h LC50 (95% C.I.)
1	25	2.33	100	3183 (2837-3571)	3377 (3205-3558)
2	25	2.33	300	3523 (unrel. var.)	3525 (3330-3731)
3	25	2.33	500	3574 (unrel. var.)	3729 (unrel var.)
4	5	1.46	100	1702 (1598-1812)	1727 (unrel var.)
5	33	1.46	100	2661 (2268-3123)	1822 (unrel var.)

In tests with fingernail clams (*Sphaerium simile*), increasing hardness from ~100 to 200 mg/L did not have a substantial effect on sulfate toxicity, but an additional increase to hardness = 300 mg/L caused a significant increase in the mean 96-h LC50 value (Table 2). When Ca:Mg and hardness were held constant, increased chloride caused a substantial increase in mean LC50 from 2,186 mg/L (Cl = 2 mg/L) to 2,650 (Cl = 33 mg/L) (Table 2).

Table 2. Effects of hardness and chloride on sulfate toxicity to fingernail clams (*Sphaerium simile*). All tests were conducted at 22 °C for 96 hours. Sulfate LC50 values shown are based on nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. LC50s were generated using the Spearman-Karber method. Different capital letters following means indicate means are significantly different ($p < 0.05$).

Diluent type	n	Cl mg/L	Ca:Mg ratio	hardness mg/L ($CaCO_3$)	mean LC50, mg SO_4/L (std. dev)
MHRW	3	2	1.46	84	2186 (388) B
Hard 200 (1.46)	2	2	1.46	193	2190 (151) B
Hard 300 (1.46)	2	2	1.46	269	2926 (340) A
Hard 300 (2.33)	2	2	2.33	280	2590 (76) AB
RMHRW	2	33	5.4	93	2200 (30) B
Cl 33/ hard 100	3	33	1.46	90	2650 (341) AB

Examining sulfate toxicity at different hardness levels reveals that hardness appears to ameliorate sulfate toxicity to a variety of species in at least two different phyla (Arthropoda and Mollusca).

In fact, for three of the species tested in this study, the increase in sulfate LC50 from hardness = 100 mg/L to hardness = 300 mg/L was surprisingly consistent at approximately 900 mg/L (Fig. 1).
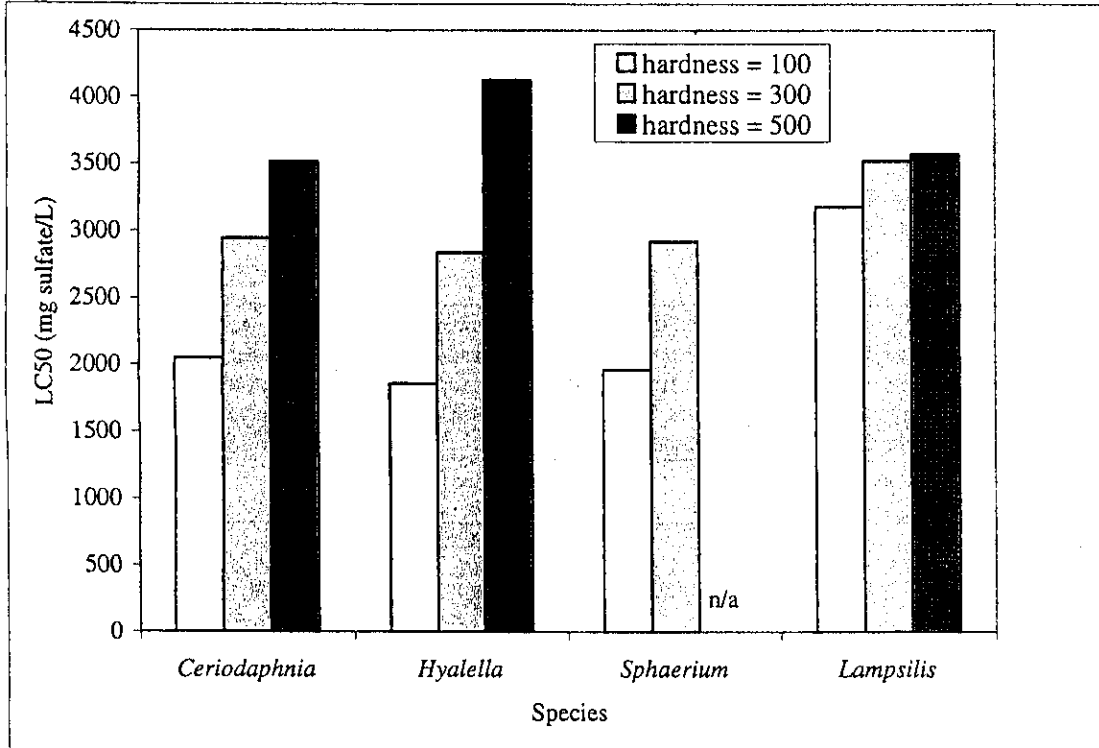

Figure 1. Comparison of LC50s at different water hardness levels for four different species of freshwater invertebrates tested in the laboratory.

Effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia.*
Increasing chloride concentration from 5 to 33 mg/L results in a sharp decrease in sulfate toxicity to *Hyalella* (table 3 and fig. 2). In the Soucek (2004) study, RMHRW had a chloride concentration of 33, and this water resulted in a mean LC50 of 2,855 mg SO_4/L. The 33 mg Cl/L treatment in this study resulted in a mean LC50 of 1,825 mg SO_4/L, but it is important to remember that the solutions in these tests have a lower calcium concentration than in RMHRW (23 mg/L compared to 32 mg/L in RMHRW) despite having the same hardness. In the current study, a Ca:Mg ratio of 2.33:1 (compared to 5.40:1 in RMHRW) was selected to reflect the median ratio for streams in Illinois. Calcium is probably more important in mediating sulfate toxicity than magnesium, thus accounting for the relatively large difference in LC50 values despite nearly identical hardness values.

Above 33 mg Cl/L, LC50 values rose again slightly in the 100 mg Cl/L treatment but then dropped in the 300 and 500 mg Cl/L tests (Table 3 and Fig. 2). If these data are viewed in a

slightly different way, another interesting trend appears (Fig. 3). I compared total dissolved solids (TDS, calculated) at LC50 concentrations for the different chloride treatments, and found that for the lower concentrations, TDS of the LC50 concentration increased with increasing chloride, as was the case for sulfate concentration, until a threshold was reached. Then, at the higher chloride concentrations (100 to 500 mg/L), no further benefit was provided by chloride above 100 mg/L. In fact, it appears that at 100 mg Cl/L, a threshold of ~3.1 g/L TDS is reached. At higher chloride concentrations, less sulfate is required to reach the critical TDS value, so LC50s in terms of sulfate decrease at 300 and 500 mg Cl/L. In other words, it appears that for *Hyalella*, in the range of 100 to 500 mg Cl/L, toxicity is reached at a fixed TDS and if there is more chloride, less sulfate is required to reach this threshold and vice versa.

Table 3. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. All tests were conducted at 22 °C for 96 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L $CaCO_3$, and the Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.) LC50s were generated using the Spearman-Karber method.

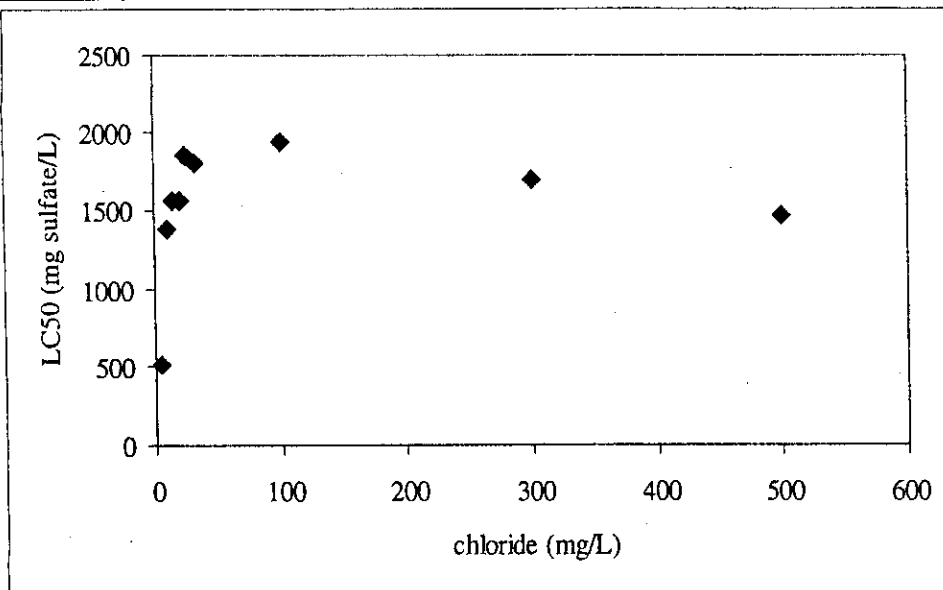| Chloride (mg/L) mg/L	number of tests conducted	Mean LC50 mg sulfate/L
10	3	1,387
15	3	1,563
20	3	1,562
25	3	1,854
33	3	1,799
100	3	1,938
300	3	1,691
500	3	1,470

Figure 2. Influence of chloride concentration on toxicity of sulfate to *Hyalella azteca*. See table 3 for details on test conditions.

Figure 3. Relationship between chloride concentration and total dissolved solids at the LC50 concentration in toxicity tests sulfate to *Hyalella azteca*. Total dissolved solids values were calculated using nominal concentrations of all ions present in solution (excluding H+ and OH-) at LC50 concentrations.

The trend of decreased toxicity with increasing chloride at the lower end of the range as seen for *Hyalella* is not as clear with *Ceriodaphnia* (Table 4 and Fig. 4). It appears that at chloride concentrations between 10 and 100 mg/L, sulfate toxicity is fairly constant (with some noise in the data), with LC50 values between 2,200 and 2,500 mg SO_4/L (nominal concentrations). In the Soucek (2004) study, *Ceriodaphnia* did not respond as strikingly as *Hyalella* when tested in RMHRW compared to MHRW so these results may not be entirely surprising.

As was the case with *Hyalella*, LC50 values in terms of sulfate were lower at the 300 and 500 mg Cl/L concentrations compared to the lower chloride concentrations (Table 4 and Fig. 4). Comparing TDS (calculated) at LC50 concentrations for the different chloride treatments at the lower range of chloride concentrations, TDS values at LC50 concentrations were higher for *Ceriodaphnia* (3.5 – 3.8 g/L) than they were for *Hyalella* (2.4 – 3.1 g/L). The overall trend was different for *Ceriodaphnia* as well with an overall linear trend of decreasing TDS at LC50 with increasing chloride concentration (Fig. 5).

Table 4. Influence of chloride concentration on toxicity of sulfate to *Ceriodaphnia dubia*. All tests were conducted at 25 °C for 48 hours. Chloride and sulfate values shown are nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. Mean hardness for all tests was ~100 mg/L CaCO₃, and the Ca:Mg used was 2.33:1 (mg/L:mg:L) to reflect the median Ca:Mg ratio in Illinois (Clark Olson, IEPA, pers. com.) LC50s were generated using the Spearman-Karber method.

Chloride (mg/L) mg/L	number of tests conducted	Mean LC50 mg sulfate/L
10	3	2,469
15	3	2,289
20	3	2,419
25	3	2,272
100	3	2,417
300	3	1,914
500	3	1,496

Figure 4. Influence of chloride concentration on toxicity of sulfate to *Ceriodaphnia dubia*.
See table 2 for details on test conditions.
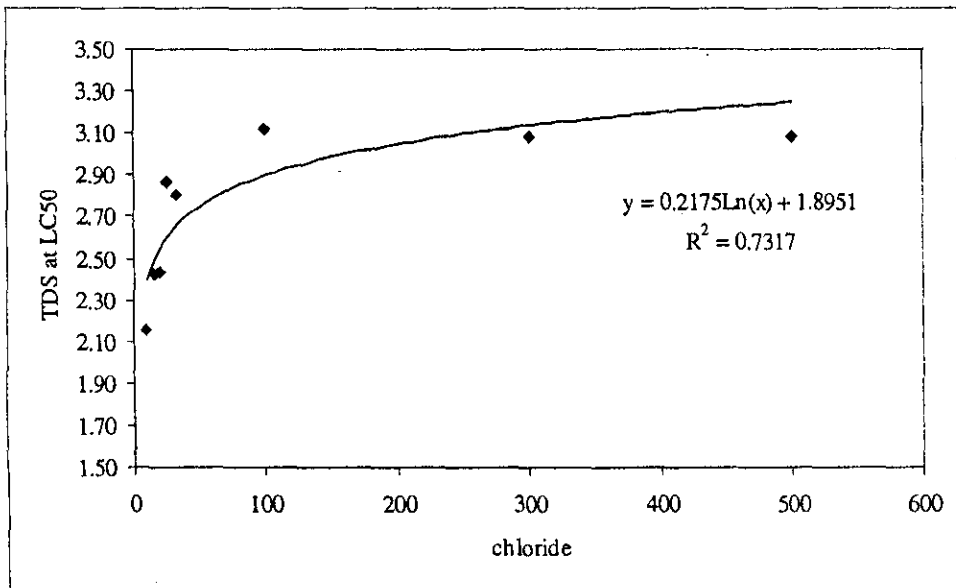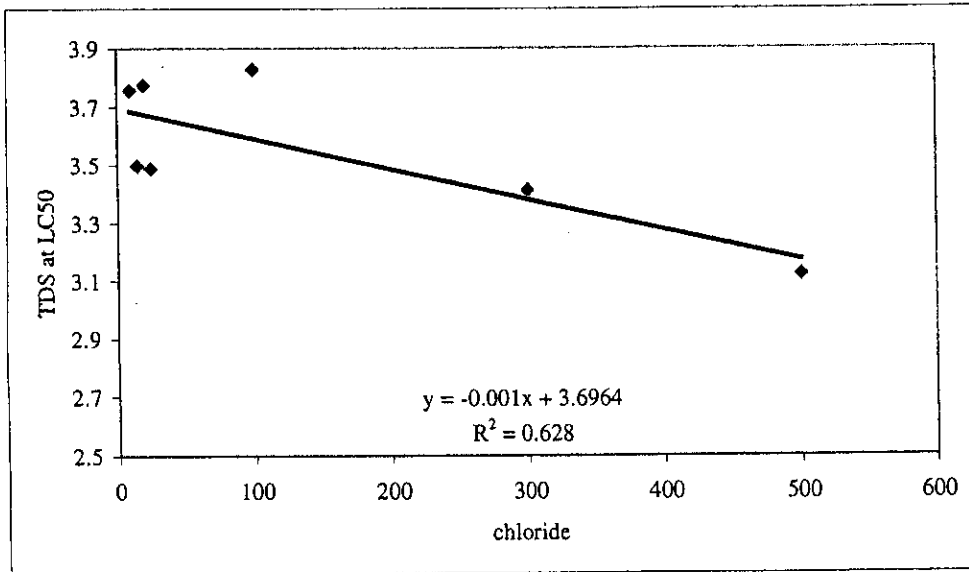
Figure 5. Relationship between chloride concentration and total dissolved solids at the LC50 concentration in toxicity tests sulfate to *Ceriodaphnia dubia*. Total dissolved solids values were calculated using nominal concentrations of all ions present in solution (excluding H+ and OH-) at LC50 concentrations.

Effects of hardness on toxicity of sulfate to *Hyalella* at critical chloride level

As was seen with *Ceriodaphnia dubia* in the Soucek (2004) study, a strong linear trend of decreased sulfate toxicity with increased hardness was observed with *Hyalella* (Fig. 6, 7). LC50 values increased from less than 2,000 mg/L at hardness = 100 mg/L, to greater than 4,000 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, as was the case for *C. dubia*. It remains unclear how the trend will continue with increasing hardness above 600 mg/L and further testing should be conducted with harder waters.
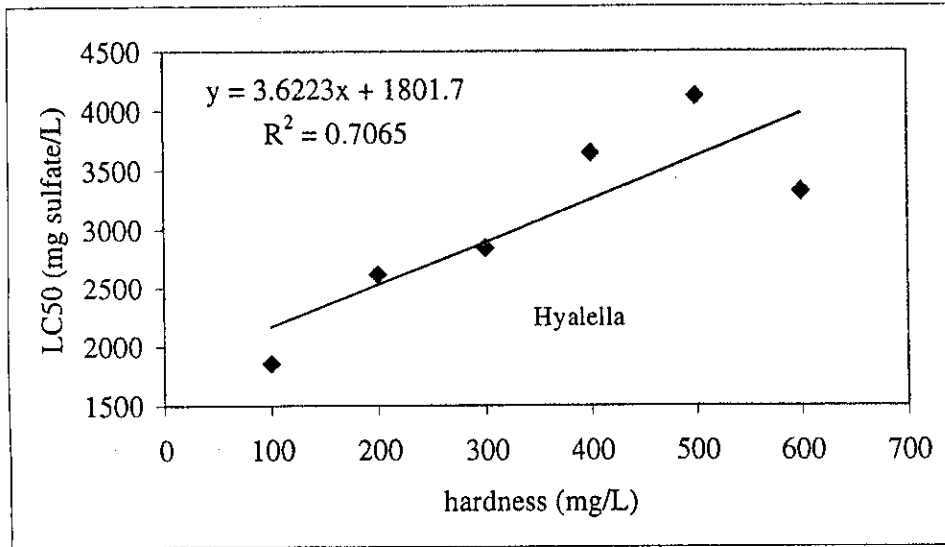
Figure 6. Influence of hardness on toxicity of sulfate to *Hyalella azteca* at chloride = 25 mg/L.
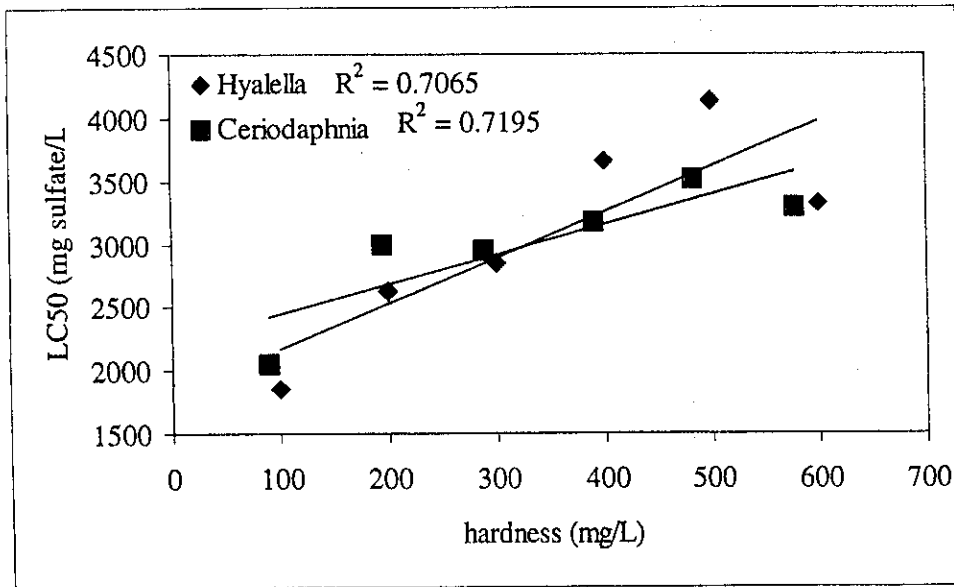
Figure 7. Influence of hardness on toxicity of sulfate to *Hyalella azteca* and *C. dubia*. *C. dubia* data from Soucek 2004).

7-day chronic sulfate toxicity tests with *Ceriodaphnia dubia.*

All of the tests for this objective have been completed (three chronic tests in each dilution water), and results were quite consistent from test to test. As depicted in figures 8-11, chronic toxicity in terms of survival and reproduction was less in RMHRW compared to MHRW. Table 5 summarizes the Least Observable Adverse Effects Concentrations (LOAEC) and No Observable Adverse Effects Concentrations (NOAEC) for each dilution water. While LOAECs for reproduction are quite low, it should be noted that I have had a continuous, self sustaining, reserve culture of *Ceriodaphnia dubia* in MHRW spiked with 1,000 mg SO_4/L since at least August of 2004. Organisms used in chronic testing for this study were cultured in MHRW or RMHRW as appropriate.

Table 5. Summary of mean NOAEC and LOAEC concentrations for survival and reproduction of *Ceriodaphnia dubia* in 7-day static-renewal three brood toxicity tests with sulfate. Values are in terms of mg sulfate/L. All tests were conducted at 25 °C. Chloride and sulfate values shown are nominal concentrations. MHRW = Moderately Hard Reconstituted Water (USEPA 1993). RMHRW = Reformulated Moderately Hard Reconstituted Water (Smith et al., 1997)

Dilution water	number of tests conducted	Survival		Reproduction	
		NOAEC	LOAEC	NOAEC	LOAEC
MHRW	3	1,727	2,273	780	934
RMHRW	3	2,264	3,000	906	1,195

Figure 8. Mean percent survival of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.
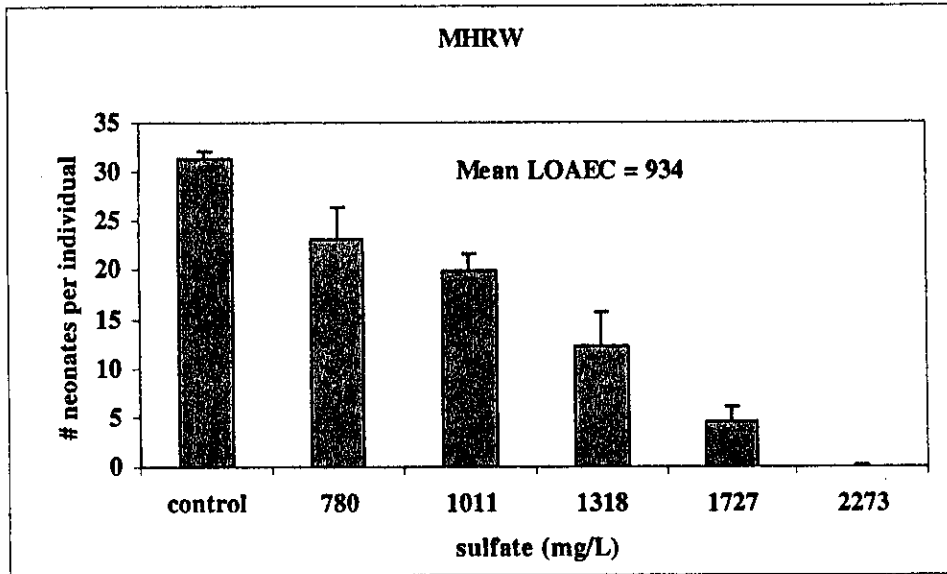
Figure 9. Mean reproduction of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.
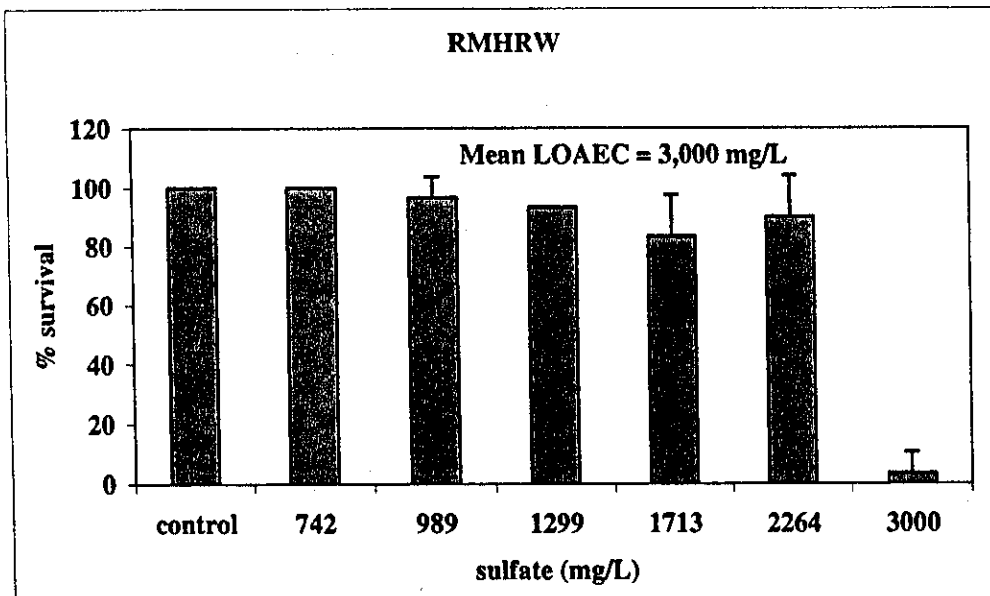
Figure 10. Mean percent survival of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Reformulated Moderately Hard Reconstituted Water (RMHRW). Bars and error bars indicate means and standard deviations for three separate tests.
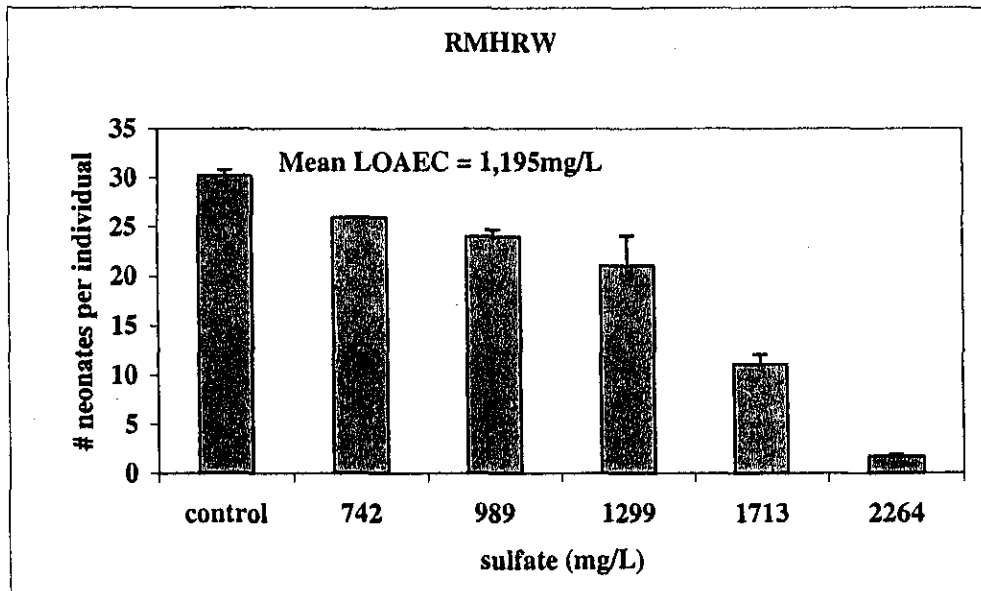
Figure 11. Mean reproduction of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Reformulated Moderately Hard Reconstituted Water (RMHRW). Bars and error bars indicate means and standard deviations for three separate tests.

Comparison of LC50s for fed and unfed organisms
As an additional analysis, I calculated 48-h LC50s using the data generated in the 7-day chronic tests to determine if feeding of test organisms has an effect on sulfate toxicity. Others have shown that feeding algae to cladocerans during toxicity tests may reduce the toxicity of metals because negative charges on algal cells bind and reduce the bioavailability of positively charged metals (e.g., Taylor et al. 1998). In this study, sulfate toxicity to *C. dubia*, was significantly reduced when organisms were fed in both MHRW and RMHRW (Fig. 12). At this point, it is unknown if this observed effect is due to binding of sodium to algal cells, or increased robustness or lowered stress of test organisms that were fed during testing. The most likely explanation is that of increased health of test organisms because sodium and sulfate are highly stable as dissolve ions, but this is conjecture at this point.
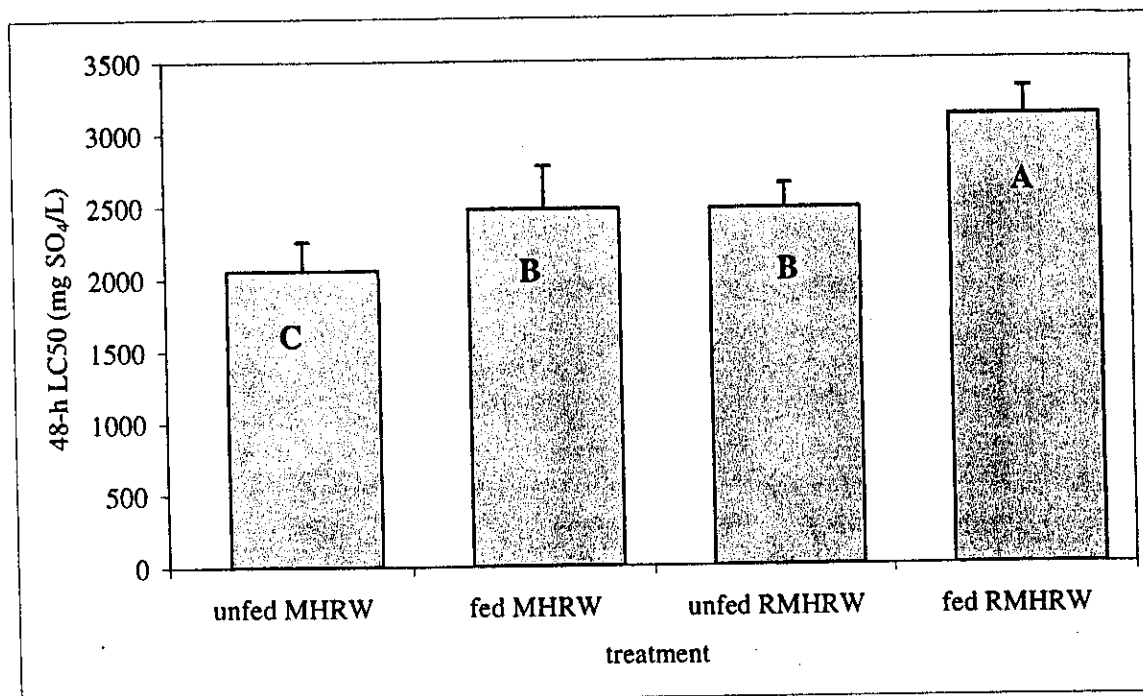
Figure 12. Effects of presence of food on toxicity of sulfate to *Ceriodaphnia dubia* in two types of test waters. Different capital letters indicate that means are significantly different (p<0.05).

LC10/LC50

LC10s were calculated for all the acute tests conducted for this study, and LC10 values were divided by their respective LC50s to determine the steepness of the toxicity curves for sulfate for the organisms tested. The mean quotients varied from species to species, but generally were quite high, ranging from 0.72 o 0.85 (Fig. 13).
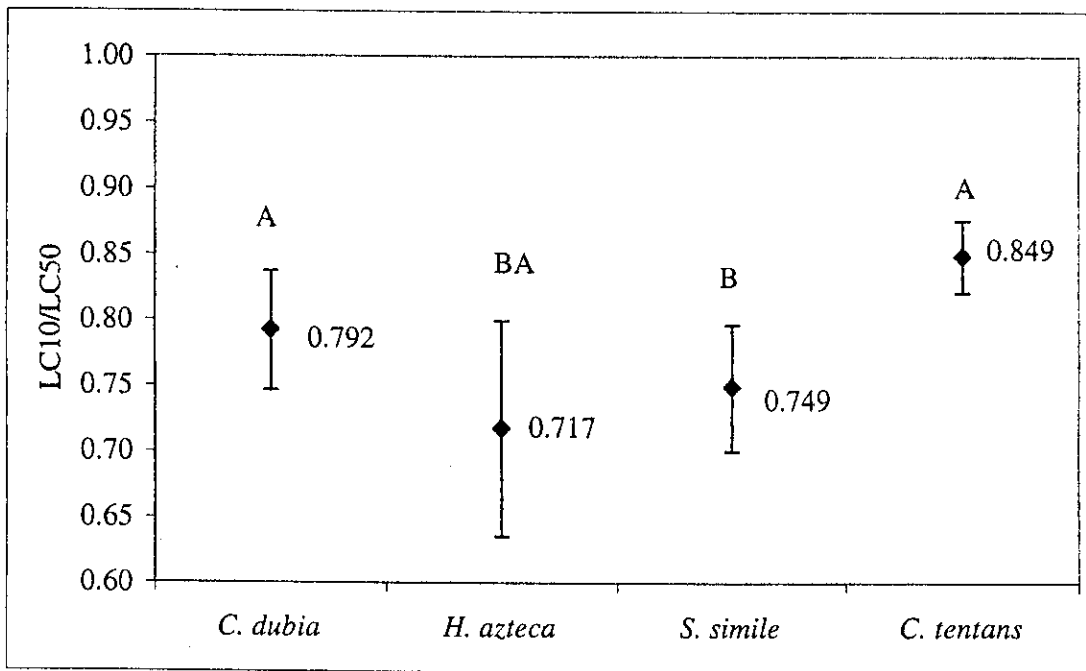
Figure 13. Mean quotients of LC10/LC50 for four species exposed to sulfate in this study. Different capital letters indicated that means are significantly different (p<0.05).

Multiple linear regression analysis

Multiple linear regression analysis was used to generate equations to predict LC50s and LC10s based on the hardness and chloride concentrations of potential receiving waters. Before analyses were conducted, data at hardness greater than 490 and data for *Hyalella* at chloride less than 25 were deleted, because of the uncertainty in trends above hardness =490 and below Cl =25. In order to avoid the assumption that all of the species have the same sensitivities, linear regression with covariance was used (i.e., "species" was introduced as a categorical variable); this model assumes that the species have the same slopes for LC50 vs. hardness and for LC50 vs. chloride, but do not necessarily have the same sensitivities to sulfate. Equations were generated both including and excluding the two data points for *C. tentans*, which had drastically different values compared to the other three species included in the models.

Equations along with R^2 values and intercept and slope estimates are given in appendices 1 and 2. Further analysis and interpretation of these results will be included in the final report for this project.

Remaining tasks- Approximately 50% of the water samples from tests conducted for this study have been analyzed by ion chromatography to verify nominal concentrations. When analyses are completed, all toxicity endpoints will be recalculated using actual concentrations. Samples analyzed to data are generally within 5% of nominal values so toxicity data are not expected to change substantially. In addition, further analysis and interpretation of observed trends will be included in a final report.

Appendix 1. Results of multiple linear regression analysis to generate equations to predict LC50 based on hardness and chloride of a water body. Output were generated using JMP-IN software

LC50 Results without "species" variable with all data points except C. tentans (n = 87)
R-squared = 0.717915   Adjusted R-squared = 0.711198

Parameter	Estimate	Prob. > t
Intercept	1876.7114	<0.0001
Hardness	3.9108	<0.0001
Chloride	-1.5046	<0.0001

LC50 Results with "species" variable with all data points except C. tentans (n = 87)
R-squared = 0.73229   Adjusted R-squared = 0.719231

Parameter	Estimate	Prob. > t
Intercept	1846.096	<0.0001
Species (C. dubia)	91.360858	0.0958
Species (H. azteca)	-91.1015	0.1311
Hardness	3.986	<0.0001
Chloride	-1.4286	<0.0001

LC50 Results without "species" variable with all data points including C. tentans (n = 89)
R-squared = 0.083351   Adjusted R-squared = 0.062034

Parameter	Estimate	Prob. > t
Intercept	2589.4876	<0.0001
Hardness	2.004	0.2214
Chloride	-3.028	0.0425

LC50 Results with "species" variable with all data points including C. tentans (n = 89)
R-squared = 0.964607   Adjusted R-squared = 0.962475

Parameter	Estimate	Prob. > t	
Intercept	4825.2033	<0.0001	
Species (C. dubia)	-2887.746	<0.0001	-2887.746
Species (H. azteca)	-3070.209	<0.0001	-3070.209
Species (S. simile)	-2979.367	<0.0001	-2979.367
Hardness	3.986	<0.0001	
Chloride	-1.4286	<0.0001	

Appendix 2. Results of multiple linear regression analysis to generate equations to predict LC10
based on hardness and chloride of a water body. Output were generated using JMP-IN software

LC10 Results without "species" variable with all data points except C. tentans (n = 87)
R-squared = 0.572252   Adjusted R-squared = 0.562068

Parameter	Estimate	Prob. > t
Intercept	1478	<0.0001
Hardness	2.7286	<0.0001
Chloride	-1.202874	0.0001

LC10 Results with "species" variable with all data points except C. tentans (n = 87)
R-squared = 0.63676   Adjusted R-squared = 0.619041

Parameter	Estimate	Prob. > t		species	estimate
Intercept	1426	<0.0001			
Hardness	2.9485	<0.0001		species	estimate
Chloride	-1.857	0.0003		C. dubia	1587.37
C. dubia	161.37	0.0022		H. azteca	1277.442
H. azteca	-148.558	0.0097		S. simile	??

LC10 Results without "species" variable with all data points including C. tentans (n = 89)
R-squared = 0.069571   Adjusted R-squared = 0.047933

Parameter	Estimate	Prob. > t
Intercept	2056.012	<0.0001
Hardness	1.185	0.3757
Chloride	-2.4361	0.0459

LC10 Results with "species" variable with all data points including C. tentans (n = 89)
R-squared = 0.952131   Adjusted R-squared = 0.949247

Parameter	Estimate	Prob. > t		species	estimate
Intercept	3843.3786	<0.0001			
Hardness	2.848	<0.0001			
Chloride	-1.0857	0.0003		species	estimate
C. dubia	-2255.93	<0.0001		C. dubia	1587.4486
H. azteca	-2565.864	<0.0001		H. azteca	1277.5146
C. tentans	7251.9177	<0.0001		C. tentans	11095.2963
				S. simile	??

Literature cited

American Public Health Association (APHA), American Water Works Association, Water Environment Federation. 1998. Standard methods for the examination of water and wastewater, 20th ed. American Public Health Association, Washington, DC.

ASTM (American Society for Testing and Materials). 2002a. Standard guide for conducting acute toxicity tests on test materials with fishes, macroinvertebrates, and amphibians. E729-96. American Society for Testing and Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials). 2002b. Standard guide for conducting three-brood, renewal toxicity tests with *Ceriodaphnia dubia*. E1295-01. American Society for Testing and Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials). 2005. Standard guide for conducting laboratory toxicity tests with freshwater mussels. E2455-05. American Society for Testing and Materials, Philadelphia, PA, USA

Bayliss D, Harris RR. 1988. J Comp Physiol 158:81-90

Dwyer FJ, Burch SA, Ingersoll CG, and Hunn JB. 1992. Environ Toxicol Chem 11:513-520.

Ingersoll CG, Dwyer FJ, Burch SA, Nelson MK, Buckler DR, and Hunn JB. 1992. Environ Toxicol Chem 11:503-511.

Lucu C, and Flik G. 1999. Am J Physiol 276:R490-R499.

McMahon, RF, and Bogan, AE. 2001. Mollusca: Bivalvia, in JH Thorp and AP Covich (eds.) Ecology and Classification of North American Freshwater Invertebrates, 2nd Edition. Academic Press, San Diego, CA. pp 352-353.

Mount DR, Gulley DD, Hockett JR, Garrison TD, and Evans JM. 1997. Environ Toxicol Chem 16:2009-2019.

Pic P, and Maetz J. 1981. J Comp Physiol B 141:511-521.

Soucek, DJ. 2004. Effects of hardness, chloride, and acclimation on the acute toxicity of sulfate to freshwater invertebrates. Final Report to: Illinois Environmental Protection Agency and Illinois Coal Association.

Smith ME, Lazorchak JM, Herrin LE, Brewer-Swartz S, and Thoeny WT. 1997. Environ Toxicol Chem 16:1229-1233.

Taylor G, Baird DJ, Soares AMVM. 1998. Environ Toxicol Chem 17:412-419.

U.S. EPA. 1985. Guidelines for deriving numerical water quality criteria for the protection of aquatic organisms and their uses. PB85-227049. Washington, DC.

U.S. EPA. 1993. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms, 4th ed. EPA/600/4-90/027F, Cincinnati, OH.

U.S. EPA. 1994a. Methods for measuring the toxicity and bioaccumulation of sediment associated contaminants with freshwater invertebrates. EPA/600/R-94/024. U.S. Environmental Protection Agency, Washington, DC.

U.S. EPA. 1994b. Methods for the determination of metals in environmental samples. EPA/600/R-4/111. U.S. Environmental Protection Agency, Cincinnati, OH.

U.S. EPA. 1999. National Recommended Water Quality Criteria – Correction. EPA 822-Z-99-001, Washington, DC.

Zipper CE. 2000. Coal mine reclamation, acid mine drainage and the Clean Water Act, in R. Barnhisel, W. Daniels, and R. Darmody (eds) Reclamation of Drastically Disturbed Lands. American Society of Agronomy, Madison, WI.

--DRAFT--

**Effects of Water Quality on Acute and Chronic Toxicity of Sulfate
to Freshwater Bivalves, *Ceriodaphnia dubia,* and *Hyalella azteca.***

Final Report

Submitted to:
Edward Hammer and Dertera Collins
United States Environmental Protection Agency
Region 5, Water Division, 77 West Jackson Boulevard
Chicago, Illinois 60604

By
David J. Soucek, Ph.D.
Illinois Natural History Survey
Champaign, Illinois 61820

April 20, 2006

Background

While there are no Federal water quality criteria (WQC) for the protection of freshwater life for total dissolved solids (TDS), sulfate, or sodium (U.S. EPA 1999), several states, including Minnesota, Indiana, and Illinois, are at various stages in the process of developing standards for sulfate. The current "General Use" standard of 500 mg/L in Illinois is based on the value thought to be protective of livestock, but the Illinois Environmental Protection Agency (IL EPA) is actively pursuing an update of the sulfate standards based on scientific research, and is close to proposing an updated standard (R. Mosher, IL EPA, pers. comm.).

Sodium is one of the most common major cations in high TDS effluents, but calcium and chloride are usually present in mine-impacted waters as well. While major ion or 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, studies were conducted by Soucek (2004; also published as Soucek and Kennedy 2005) to (1) generate LC50s (lethal concentration to 50% of a sample population) and LC10s (lethal concentration to 10% of a sample population) for sulfate with selected freshwater invertebrates (*Ceriodaphnia dubia, Chironomus tentans, Hyalella azteca,* and *Sphaerium simile*) in U.S. Environmental Protection Agency's (US EPA, 1993) moderately hard reconstituted water (MHRW) and (2) determine the effects of laboratory water composition, water hardness, and test organism acclimation on the acute toxicity of sulfate to *Ceriodaphnia dubia* and *Hyalella azteca* (Soucek, 2004). In these previous studies (Soucek, 2004), the mean LC50s, expressed as mg SO_4^{2-}/L, in moderately hard, reconstituted water (MHRW; U.S. EPA 2002) ranged from: 512 to 14,134 mg/L. The LC50 generated for the amphipod, *Hyalella* (512 mg/L) was surprisingly low, given that it is known as a euryhaline organism (Ingersoll et al., 1992), but as will be discussed below, water quality data, including other cations and anions present, are critical for predicting the responses of freshwater organisms (especially *Hyalella*) to elevated sulfate concentrations.

The composition of the dilution water used during testing in the Soucek (2004) study had a dramatic effect on the toxicity of sulfate to *Hyalella*. Whereas the 96-hour LC50 in MHRW was 512 mg/L, the LC50 increased to 2,855 mg/L when using a "Reformulated Moderately Hard Reconstituted Water" (RMHRW, Smith et al., 1997). The LC50 for *C. dubia* also increased from 2,050 in MHRW to 2,526 mg/L in RMHRW. Both dilution waters were similar in terms of hardness (~90-106 mg/L as $CaCO_3$), alkalinity, and pH, but RMHRW had a higher chloride concentration and different calcium to magnesium ratio than that in MHRW. An additional experiment, not included in the Soucek (2004) report, indicated that when sulfate (~2,800 mg/L) and hardness (106 mg/L) were held constant, percent survival of *H. azteca* was positively correlated with chloride concentration (up to 67 mg Cl/L; Soucek and Kennedy, 2005). These experiments illustrated the need to further characterize the interacting effects of chloride and sulfate on aquatic organisms.

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 the previous study (Soucek 2004), increased hardness reduced the toxicity of sulfate to *Hyalella* and had a dramatic effect on the 48-hour LC50 for *C. dubia,* increasing from 2,050 at a hardness of 90 to > 2,900 mg/L at hardness values higher than 194 mg/L as $CaCO_3$. Others have observed reduced toxicity of saline solutions due to increased hardness as well (e.g., Dwyer et al. 1992; Mount et al. 1997).

While a great deal of progress has been made in the understanding of sulfate toxicity under varying water quality conditions, several important data gaps remained. First, quantification of the effects of hardness on sulfate toxicity to *Hyalella* was needed to determine if the previously observed phenomenon was specific to *Ceriodaphnia*. Another research need was quantification of the effects of a wide range of chloride concentrations on sulfate toxicity to both *Hyalella* and *Ceriodaphnia*. In addition, previous studies (Soucek 2004) indicated that the fingernail clam, *Sphaerium simile,* had a lower LC10 than that of *C. dubia,* but because of the temporal nature of its availability, this bivalve was only tested in MHRW. It remained unclear whether or not a mollusk will have the same physiological response as two crustaceans to increased chloride or hardness in these experiments with sulfate. Another data gap was the fact that all of the tests conducted in the Soucek (2004) study were acute exposures of 48 to 96 hours. Sublethal effects of sulfate in longer-term exposures were unknown. Therefore, the objectives of this study were to build on previous studies conducted to support development of a sulfate criterion for protection of aquatic life by (1) determining the effects of hardness on toxicity of sulfate to bivalves, (2) determining the toxicity of sulfate to juvenile unionid mussels, (3) determining the short-term (7 days) chronic toxicity of sulfate to *Ceriodaphnia dubia,* (4) determining the effects of chloride on acute toxicity of sulfate to *Hyalella* and *Ceriodaphnia,* and (5) determining the effects of hardness on toxicity of sulfate to *Hyalella* at a critical chloride level, i.e., the chloride concentration at which sulfate is significantly less toxic to *Hyalella* as determined in #4 above.

Methods

General culturing and testing methods
Invertebrates selected for testing include *Ceriodaphnia dubia, Hyalella azteca, Sphaerium simile* (Pelecypoda, Sphaeriidae), and a juvenile freshwater unionid mussel (*Lampsilis siliquoidea*). The cladoceran, *Ceriodaphnia dubia,* was cultured in-house (Soucek laboratory, Illinois Natural History Survey) according to U.S. EPA methods (2002). Amphipods, *Hyalella azteca,* also were cultured in house according to U.S. EPA methods (2000) in a "Reformulated Moderately Hard Reconstituted Water" described in Smith et al. (1997). Sphaeriid clams were collected from Spring Creek, near Loda, Illinois, (Iroquois County) and acclimated to MHRW at 22 °C and a 16:8 (L:D) photoperiod for 5-7 d prior to testing. Clams collected from this site were previously identified to species by Dr. Gerald Mackie, of the University of Guelph, Department of Zoology, Guelph, Ontario, Canada.

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) 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 (Soucek 2004).

For definitive static, non-renewal toxicity tests, conducted according to American Society for Testing and Materials (ASTM) E729-96 methods (2002a) treatments were comprised of 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 in addition to controls with four replicates tested per concentration. Tests with *C. dubia* were conducted for 48 h with a 16:8 (L:D) photoperiod at 25 °C, and *H. azteca* and *S. simile* were exposed for 96 h at 22 °C and a 16:8 (L:D) photoperiod. Both crustaceans were exposed in 50-ml glass beakers with 5 organisms per beaker, and for 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). All clams used are juveniles. Only one of the 63 tests was fed, and that fed test had a median LC50 value compared to two other tests conducted with the same organism in the same water type. *Ceriodaphnia dubia* used were less than 24-h old, and *H. azteca* were ~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*.

Chronic testing was conducted according to guidelines described in ASTM E 1295-01 (2002b). Ten replicates were used per sulfate concentration with one organism per replicate. Endpoints included the number of young (both live and dead recorded separately) produced by each first generation *C. dubia*, and survival of first generation *C. dubia*.

Standard water chemistry parameters were measured at both the beginning and the end of each exposure period, including temperature, pH, conductivity, dissolved oxygen, alkalinity and hardness. 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 (RDP, Dayton, OH, USA) model 58 meter with a self-stirring biochemical oxygen demand probe. Conductivity measurements were made using a Mettler Toledo® (Fisher Scientific, Pittsburgh, PA, USA) model MC226 conductivity/TDS meter. Alkalinity, and hardness were measured (beginning of tests only) by titration as described in American Public Health Association (APHA) et al. (1998). 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 based on both measured sulfate concentrations and measured specific conductivity values for each test concentration using the Spearman-Karber method, and sulfate LC10s, LC5s, and LC1s were calculated using probit analysis. To increase confidence in LC50 values, three to five assays were conducted with each organism for each water quality

combination. This provided a stronger estimate of the mean LC50 value for a given set of water quality parameters for each species. In all, 63 new LC50s were generated. After LCXs were calculated using probit analysis, the following quotients were calculated: LC10/LC50, LC5/LC50, and LC1/LC50. Then geometric mean quotients for each species were compared using Student's T-test.

Toxicity testing with freshwater mussels (USGS)

Test conditions and procedures for conducting toxicity tests with newly-transformed juvenile mussels of fatmucket (*Lampsilia siliquoidea*) were in accordance with the recommended test conditions outlined in ASTM E2455-05 (2005, and see Appendix 1). Five 96-h sulfate toxicity tests with juvenile fatmuckets were conducted at three hardness levels (100, 300, and 500 mg/L as $CaCO_3$ with Ca:Mg ratio of 2.33) and two chloride concentrations (5 and 33 mg/L) at one hardness (100 mg/L with Ca:Mg ratio of 1.46) at the U.S. Geological Survey's Columbia Environmental Research Center. A preliminary range-finding test with juvenile fatmuckets indicated that the concentration of 5000 mg sulfate/L resulted in more than 50% mortality and therefore was chosen as the highest concentration for the 96-h toxicity tests. Each of the five tests was conducted with five concentrations of sulfate in a 50% serial dilution and a control with four replicates. Test water and solution were provided by Illinois Natural History Survey, Champaign, IL. 1-d-old juvenile fatmuckets were obtained from laboratory cultures at Southwest Missouri State University, Springfield, MO. When juvenile mussels were received, the water temperature was gradually adjusted to the test temperature (20°C). Shipment water was gradually replaced with test water over a 48-h acclimation period. Five juveniles exhibiting foot movement were impartially transferred into each of 50-ml glass beakers with about 30-ml test solution. Water quality characteristics including pH, conductivity, hardness, and alkalinity in each exposure treatment were determined at the beginning and the end of each test (Table 2) and were close to the nominal. Dissolved oxygen was above 8.2 mg/L during all tests. Sulfate concentrations were measured at INHS as described above. After 48- and 96-h exposure, survival of juvenile mussels was determined. Individuals which exhibited foot movement within a 5-min observation period were classified as alive (ASTM 2005). Median effective concentrations (EC50s) were determined by the Trimmed Spearman-Karber method (TOXSTAT 3.5; WEST 1996). Measured sulfate concentrations were used for EC50 calculation.

Influence of chloride on the toxicity of sodium sulfate

In these experiments, we tested the toxicity of sulfate (with sodium as the major cation) to *H. azteca* and *C. dubia* 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 No.7647-14-5, Fisher Scientific Cat. # AC42429-0010) was added at appropriate concentrations to a solution with a hardness of ~100 mg/L (molar ratio of Ca:Mg = 1.41; 2.33 in terms of mass). 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 level to provide a mean LC50 value 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 (Sall and Lehman, 1996) to determine the relationship between chloride concentration and sulfate LC50 for each species. Mean LC50 values for each chloride concentration were used in these analyses, and two separate analyses were conducted for each species: one for the range or 5 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, we tested the toxicity of sulfate (with sodium as the major cation) to *H. azteca* 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 No. 7778-18-9) and $MgSO_4$ (CAS No. 7487-88-9), at a set molar ratio (Ca:Mg = 1.41; 2.33 in terms of mass) to achieve the nominal hardness values. The Ca:Mg value was chosen because it is the median value for water bodies sampled in Illinois (R. Mosher, IL EPA, pers. com.). 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 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 level to provide a mean LC50 value and standard deviation. Exposures were conducted using the same laboratory and calculation methods described above. LC50 values for *H. azteca* were compared to previously generated LC50s for *C. dubia* (Soucek and Kennedy 2005), which were conducted in solutions having a Ca:Mg molar ratio of 0.88 and [Cl] of 1.9 mg/L.

After LC50s were calculated as described above, regression analysis was conducted using JMP® software (Sall and Lehman, 1996) to determine the relationship between hardness and sulfate LC50 for each species. Mean LC50 values for each hardness level 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 (2005) were included in the analyses. Three data ranges were used to compare sulfate and conductivity LC50 relationships: Cl = 5 to 100 mg/L, Cl = 5 to 300, and Cl = 5 to 500 mg/L.

Comparison of test results with STR model predictions

To compare results from the present study to toxicity predictions generated by the STR model (Mount and Gulley, 1993), we calculated nominal concentrations of all constituent ions (except H^+ and OH^-, which are not required by the model) at observed mean sulfate LC50 levels for each test solution type (Cl = x, hardness = y, and Ca:Mg = z). Ions required by the model include Ca^{2+}, Mg^{2+}, Na^+, K^+, Cl^-, HCO_3^-, and SO_4^{2-}. These calculations were possible because we generated all test solutions using deionized water with known salt concentrations added. In addition, confidence in the use of nominal concentrations for all ions is provided by the fact that 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%. The average (± SD) % difference between cations and anions for out inputs was 0.09 (± 0.01)%, indicating excellent agreement between cation and anion equivalents. Other model outputs included calculated TDS, a "NUMCAT" value, LC50 in terms of % of solution, and % survival in 100% of solution. Because all of our inputs were concentrations at observed LC50, the observed % survival in 100% solution was always 50%. To examine the effectiveness of 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. Model outputs included toxicity predictions for *C. dubia*, *Daphnia magna*, and *Pimephales promelas*. Because *Hyalella* was most similar in sensitivity to *C. dubia*, we compared observed results for *Hyalella* to predicted results for *C. dubia*.

Predictive equations for standard development

A subset of the available data (from this study) was used to calculate slopes describing the effects of hardness and chloride on sulfate toxicity. In consultation with Charles Stephan (USEPA), it was decided that only tests conducted with a Ca:Mg molar ratio of 1.41 (2.33 in terms of mass) would be included for standard development. Further more, because consistent linear relationships between hardness and sulfate were observed for both *C. dubia* and *H. azteca* in the range of ~100 to ~500 mg/L as $CaCO_3$ (see data below), only tests conducted within this range were included for standard development. Finally, because the relationship between chloride concentration and sulfate toxicity was inconsistent below 25 mg/L (see data below), only tests conducted at chloride concentrations of 25 mg/L or higher were included for standard development. This left 42 tests (14 for *C. dubia* and 28 for *H. azteca*) for inclusion in calculation of slopes describing the effects of hardness and chloride on sulfate toxicity. To calculate slopes, multiple linear regression analysis with covariance was conducted with LC50 (based on

measured sulfate concentrations) as the dependent variable and chloride, hardness, and species as independent variables.

RESULTS and DISCUSSION

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 *Hyalella* in particular, two different linear trends were observed depending on the chloride range (Fig. 1a, 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 *Hyalella* (Fig. 1a), while for *C. dubia*, a mildly positive trend was observed, but the relationship was not significant over this chloride concentration range ($R^2 = 0.4906$, p = 0.1877). In addition, the LC50s for *C. dubia* were higher than those for *Hyalella* for each chloride concentration over this range. When using a combined data set of individual test LC50s for *C. dubia* and *Hyalella* 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).

While a positive relationship between chloride concentration and SO_4^{2-} LC50 was observed for *Hyalella* 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 *Hyalella* over this chloride range and at hardness = 100 (n = 30) in a simple 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).

Hyalella appears to require a minimal amount of chloride for effective osmoregulation. While 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 (Dorgelo 1981, Aladin and Potts 1995, Greenaway 1979, Schmidt-Nielsen 1997). 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 (Bayliss and Harris 1986). Even among amphipods, there is a wide range of sodium and chloride influx rates and integument permeabilities which determine osmoregulatory effectiveness (Bayliss and Harris 1986, Taylor and Harris 1986); therefore, it is not surprising that the responses of *H. azteca* and *C. dubia* to sodium sulfate were quite different over the lower range of chloride concentrations. While Borgmann (1996) suggested that under low salinity conditions, bromide was required but chloride was not needed by *H. azteca* for survival, growth and reproduction, data from this study suggest that the chloride is quite

important in determining the response of that organism to elevated levels of sodium sulfate. Laboratory deionized water and concentrated sodium sulfate solutions were analyzed previously for bromide, and levels were below detection limits (Soucek 2004).

Over the higher range of chloride concentrations (25 to 500 mg/L), a different trend was observed than that over the lower concentrations. While 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., Mount et al. 1997), and so the highest two chloride concentrations in this study were likely to cause some toxicity without sulfate present.

Table 1. Results of multiple regression analysis with covariance for three different subsets of data. Individual LC50s were used as data points. Data for both species were included.

[Cl'] range = 5-25 mg/L, hardness ~100 mg/L
R^2 = 0.7900, n =33

Term	Estimate	p
Intercept	1270.23	<0.0001
chloride	35.14	<0.0001
Species	449.68	<0.0001

[Cl'] range = 25-500 mg/L, hardness ~100 mg/L
R^2 = 0.6539, n =30

Term	Estimate	p
Intercept	2189.48	<0.0001
chloride	-1.46	<0.0001
Species	178.92	0.0003

Hardness range = 100-600, Cl = 25 mg/L
R^2 = 0.5177, n = 38

Term	Estimate	p
Intercept	1969.38	<0.0001
Hardness	3.15	<0.0001
Species	-10.38	0.9046

A.

$y = 13.868x + 2085.1$
$R^2 = 0.4906, p = 0.1877$

$y = 57.791x + 445.59$
$R^2 = 0.8503, p = 0.0258$

♦ *C. dubia*
□ *H. azteca*

LC50 (mg SO$_4$/L)

chloride (mg/L)

B.

$y = -2.2023x + 2530.7$
$R^2 = 0.9493, p = 0.0257$

$y = -0.875x + 1904.4$
$R^2 = 0.875, p = 0.0195$

♦ *C. dubia*
□ *H. azteca*
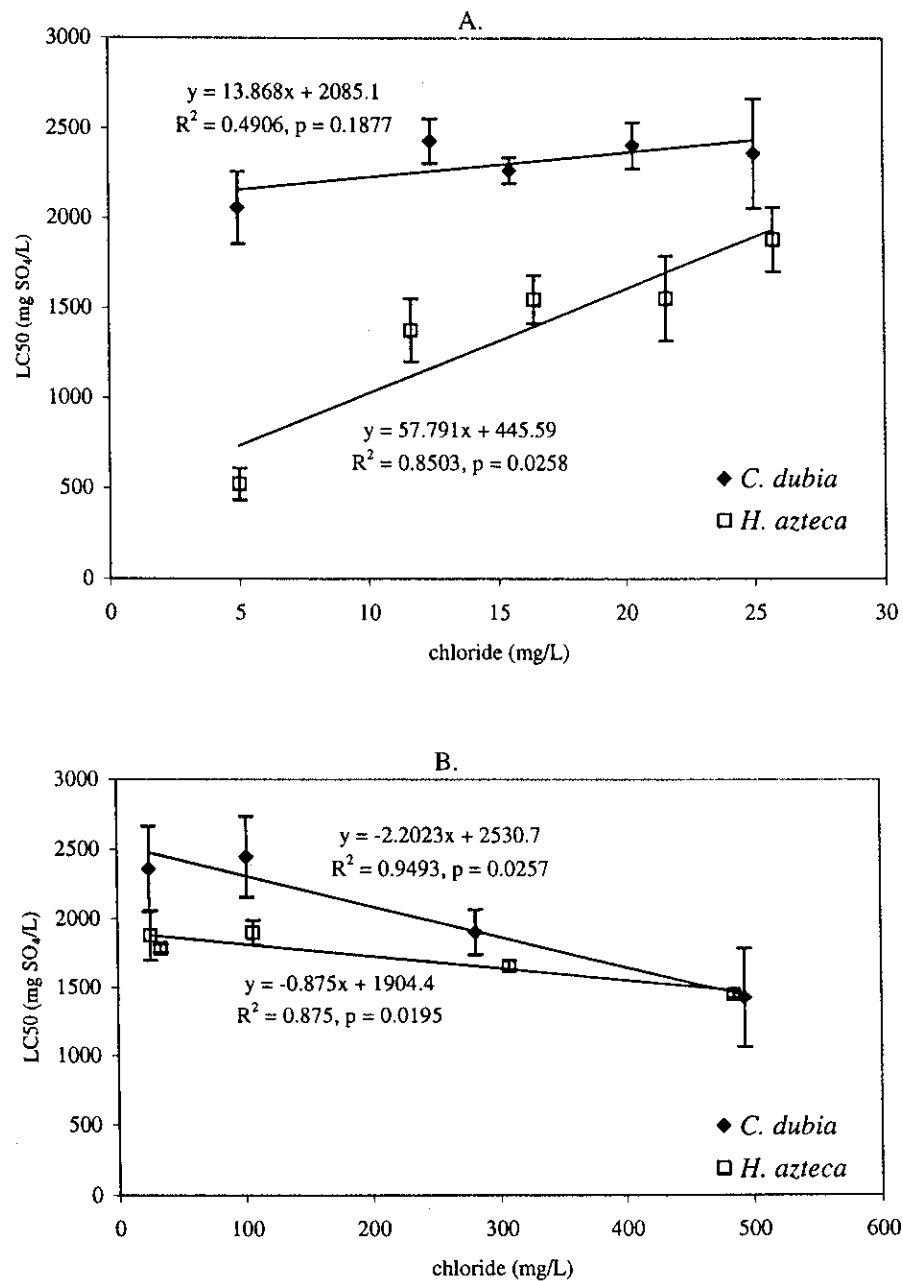
LC50 (mg SO$_4$/L)

chloride (mg/L)

Figure 1. Influence of chloride concentration over two ranges, 5 to 25 mg/L (A) and 25 to 500 mg/L (B), on toxicity of sodium sulfate to *Ceriodaphnia dubia* and *Hyalella azteca*. Hardness was ~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).

Effects of hardness on toxicity of sulfate to Hyalella at chloride = 25 mg/L
When chloride was maintained at 25 mg/L, a strong linear trend of decreased sulfate toxicity with increased hardness (R^2 = 0.7092, p = 0.0354) was observed for *Hyalella* (Fig. 2). LC50 values increased from less than 1,900 mg/L at hardness = 100 mg/L, to greater than 4,000 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 *Hyalella* over this hardness range (n = 38) in a simple 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).

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 (Kennedy et al. 2003, 2005, Dwyer et al. 1992, Mount et al. 1997, Latimer 1999) and Soucek and Kennedy (2005) showed quantitatively that, in a sodium-dominated system, sulfate toxicity to *C. dubia* is reduced as hardness progressively increases. In this study, the results of multiple linear regression analyses indicated no difference between the responses 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 responses (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). Soucek and Kennedy (2005) proposed as an explanation for this phenomenon of hardness ameliorating sulfate toxicity that increased calcium concentrations decrease the passive permeability of epithelial cells to water and ions in various aquatic organisms (Lucu and Flik 1999, Pic and Maetz 1981), 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 Havas and Advokaat 1995); however, Potts and Fryer (1979) found that calcium had little effect on sodium loss in *Daphnia magna*. While data from the present study support this hypothesis, other explanations are possible and empirical work is needed to determine the mechanism behind the phenomenon.
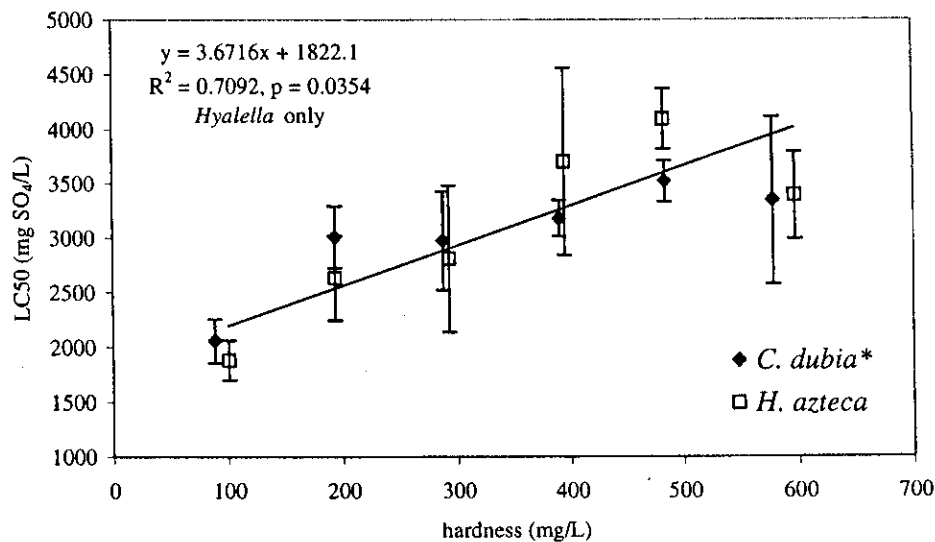
Figure 2. Influence of hardness on toxicity of sulfate to *Hyalella azteca* and *Ceriodaphnia dubia*. *C. dubia* data are from Soucek and Kennedy (2005). Chloride concentration for all H. azteca tests was ~25 mg/L, and Ca:Mg molar ratio was 1.41.

Relationship between sulfate LC50s and conductivity LC50s

Conductivity LC50s ranged from 2,650 to 8,449 µmhos/cm, while LC50s based on sulfate ranged from 1,116 to 4,345 mg SO_4^{2-}/L (Fig. 3). For tests with Cl ≤ 100, sulfate LC50s were strongly correlated with conductivity LC50s (R^2 = 0.9769, p < 0.0001, Fig.3a), while the relationship weakened slightly for Cl ≤ 300 (R^2 = 0.9522, p < 0.0001, Fig.3b), and Cl ≤ 500 (R^2 = 0.8987, p < 0.0001, Fig.3c). Thus, 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 (Ho et al. 1997). Not only did sulfate LC50s range from 1,200 to 4,345 mg/L, but conductivity LC50s ranged from 2,650 to 8,449 µmhos/cm. These wide ranges were observed for just two species with relatively similar sensitivity. 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, but when 300 and 500 mg Cl⁻/L solutions were tested, sulfate LC50s were lower than would be predicted by LC50s based on conductivity.
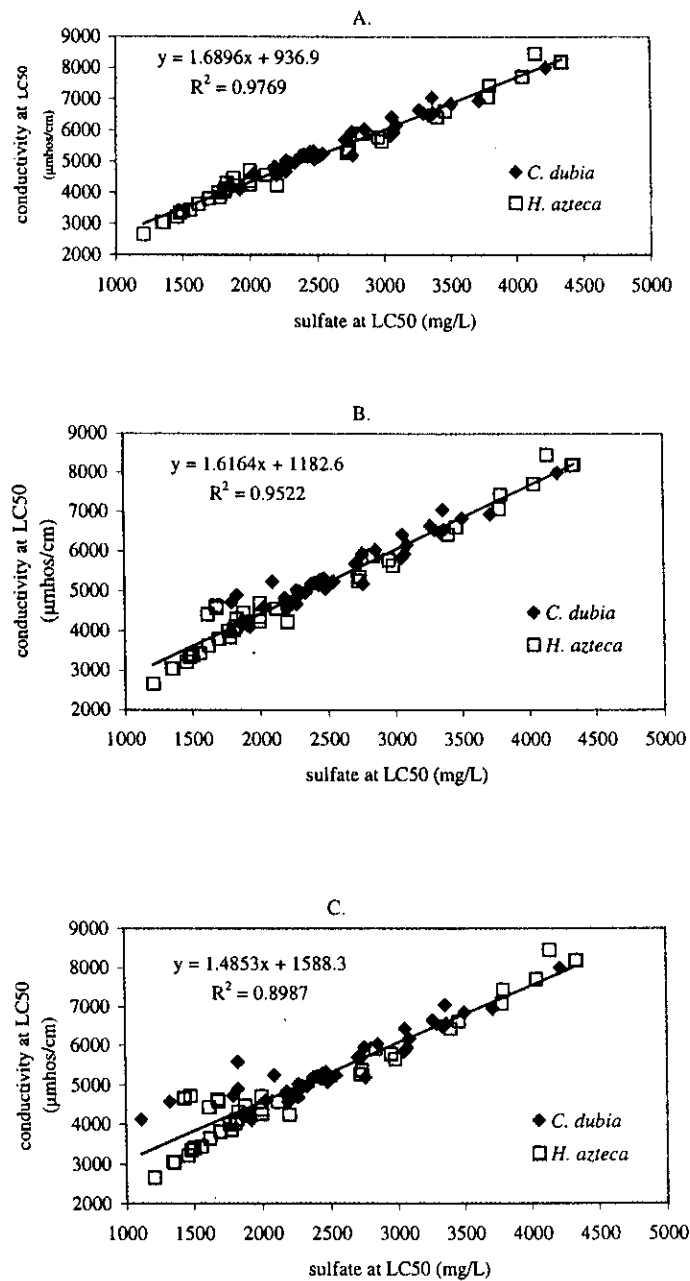
Figure 3. Relationship between LC50s in terms of sulfate (mg/L) and LC50s in terms of conductivity (µmhos/cm) including tests with chloride ranging from 5 to 100 mg/L (A), 5 to 300 mg/L (B), and 5 to 500 mg/L (C). In addition to the 63 new tests generated for this study, 19 tests from Soucek and Kennedy (2005) with *C. dubia* were included. Hardness values ranged from 100 to 600 mg/L and two Ca:Mg molar ratios (0.88 and 0.41) in parts A, B, and C.

Mean LCX quotients for C. dubia and H. azteca

For 12 of the 63 tests conducted, probit analysis would not calculate LCX values because of the distribution of the mortality data. The geometric mean (n = 51) of the LC10/LC50 quotients for both species combined was 0.730 ± 0.092, while the geometric means of LC5/LC50 and LC1/LC50 quotients for both species were 0.668 ± 0.106 and 0.565 ± 0.125, respectively. Using the combined data sets (n = 51) for *C. dubia* and *H. azteca*, each quotient (LC10/LC50, LC5/LC50, LC1/LC50) was used as a dependent variable with hardness, chloride, and species as explanatory variables in a simple linear regression analysis with covariance as described above. In all three cases, only the species effect was found to be significant (p = 0.0187, p = 0.0189, and p = 0.0196, respectively). In pair-wise comparisons, *C. dubia* had significantly higher geometric means than *H.* azteca did for all three quotients (p = 0.0014, p = 0.0014, and p = 0.0015, respectively, Fig. 4), indicating that the dose-response curves for the two crustaceans were significantly different. *Ceriodaphnia dubia* had significantly higher geometric mean LC10/LC50, LC5.LC50, and LC1/LC50 quotients than did *H. azteca*, suggesting that more mortality was seen at sulfate concentrations lower than the LC50 in the *H. azteca* sub-populations than in those of *C. dubia*. It remains unclear whether this observation was due to a difference in the physiological responses or tolerances of the two organisms, or whether the trend was simply a result of the difference in test duration for the two species (48 h for *C. dubia* and 96 h for *H. azteca*). Results of the multiple linear regression analysis indicated that hardness and chloride concentration did not have a significant effect on LCx/LC50 quotients.
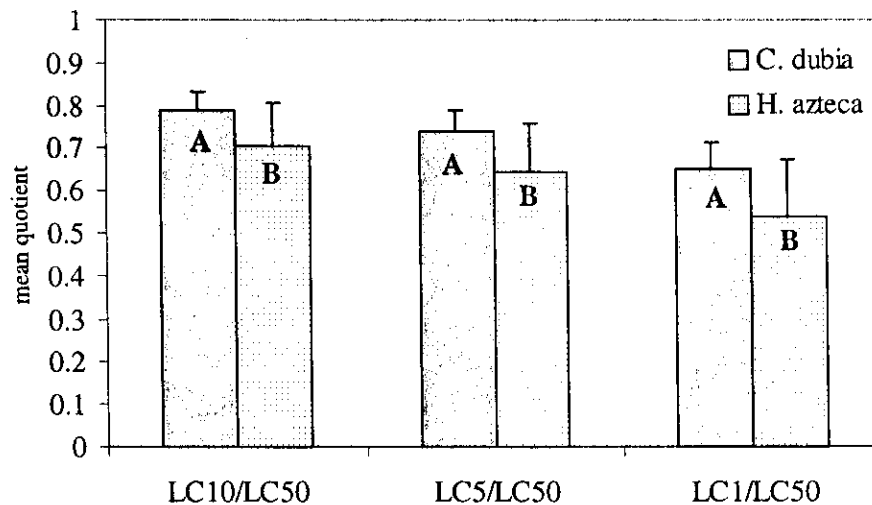
Figure 4. Comparison of geometric means of probit-calculated LC10/LC50, LC5/LC50 and LC1/LC50 quotients for Hyalella azteca (n = 33) and Ceriodaphnia dubia (n = 18). Only new tests generated for this study were included. Measured chloride concentrations ranged from 11 to 525 mg/L, and hardness ranged from 92 to 604 mg/L. Ca:Mg molar ratio for all tests was 1.41. Means with different capital letters are significantly different (p < 0.01).

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 *Ceriodaphnia* in which hardness was fixed at ~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. 5a). Most predictions were greater than 50%, and thus the model slightly under-predicted toxicity in most cases. STR does not predict toxicity for *Hyalella*, so for *Hyalella* test inputs we used the *Ceriodaphnia* predictions from STR. For tests with *Hyalella* in which hardness was fixed at ~100 mg/L and chloride varied from 5 to 500 mg/L, predicted % survival varied more widely than for *Ceriodaphnia* with values ranging from 62.5 to 97.10% (Fig. 5a). However, as chloride concentration increased, the STR model predictions became closer to the observed 50% survival values. In fact, there was a significant negative relationship between chloride concentration and predicted % survival (R^2 = 0.9045, p = 0.0003, n = 8).

For tests with *Ceriodaphnia* in which chloride was fixed at ~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. 5b). Only the hardness = 100 mg/L prediction was greater than 50% (82.9%), while for hardness values of 200 to 600 mg/L, toxicity was strongly over-predicted, with % survival predictions of 4.1 to 21.8. For tests with *Hyalella* in which chloride was fixed at ~25 mg/L and hardness varied from 100 to 600 mg/L, a similar patter was observed with and under-prediction of toxicity at hardness = 100 (88.6% survival) and over-prediction of toxicity at hardness values of 200 to 600 mg/L (42.3 to 0.7% survival, Fig. 5b).

These data indicate that when chloride was variable and hardness was fixed at ~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 ion concentrations at LC50 were used as inputs. With one exception (48%), the model under-predicted toxicity for this data range. This may be because the STR model is largely based on the results of fed tests, which the authors acknowledged had a small influence on test results (Mount et al. 1997). However, I compared 48 h sulfate LC50s in unfed tests using moderately hard reconstituted water (MHRW, USEPA 2002) and Reformulated MHRW (Smith et al. 1997) as diluents with 48 h sulfate LC50s obtained from fed, 7-d chronic tests in the same two diluents (Fig. 6). In both cases, average LC50s for unfed tests were significantly lower than those in fed tests. This factor alone may represent the discrepancy between predicted and observed results for *C. dubia* for these tests. Others have shown that feeding algae to cladocerans during toxicity tests may reduce the toxicity of metals because negative charges on algal cells bind and reduce the bioavailability of positively charged metals (e.g., Taylor et al. 1998). At this point, it is unknown if this observed effect is due to binding of sodium to algal cells, or increased robustness or lowered stress of test organisms that were fed during testing. The most likely explanation is that of increased health of test organisms because sodium and sulfate are highly stable as dissolve ions, but this is conjecture at this point.

For the same range of variables (Cl = 10 to 500 mg/L, hardness ~100 mg/L), the STR model was less accurate at predicting toxicity to *H. azteca*. One reason for this is that the model was not designed using results of tests with *H. azteca* (Mount et al. 1997), and *H. azteca* is generally slightly more sensitive to sodium sulfate than *C. dubia*. When combining all data from this study and those from Soucek and Kennedy (2005), the range of LC50s for the two species are similar (512 to 4,345 mg SO_4^{2-}/L for *H. azteca*, and 1,116 to 4,220 mg SO_4^{2-}/L for *C. dubia*) over the range of hardness and chloride concentrations studied. While the range of LC50s for the two species overlapped, *H. azteca's* average LC50 (2,158 mg SO_4^{2-}/L, n = 43) was significantly lower (p = 0.0316) than *C. dubia's* (2,562 mg SO_4^{2-}/L, n = 42). Thus, as would be expected, the STR model under-predicted toxicity to *H. azteca* when using the *C. dubia* output; however, a difference in sensitivity does not entirely explain the trend shown in figure 5a. Observed survival of *H. azteca* got closer to model predictions as chloride increased from 10 to 500 mg/L, whereas for *C. dubia*, chloride concentration did not appear to influence the accuracy of the model's predictions. This is likely the result of the fact that between 5 and 25 mg Cl/L, *H. azteca's* response to sulfate was strongly influenced by chloride concentration whereas *C. dubia's* was not (Fig 1a). Between 25 and 500 mg Cl/L, the two species have similar responses to sulfate with increasing chloride (Fig. 1b), and the model appears to account for the additive toxicity of the two ions.

When chloride was held constant (5 mg/L for *C. dubia* and 25 mg/L for *Hyalella*) and hardness was varied from 100 to 600 mg/L, the STR model was relatively inaccurate in predicting toxicity for both species. This finding is in agreement with Kennedy et al. (2005) who found that the STR model over-predicted toxicity to *C. dubia* in sodium sulfate dominated coal-processing effluents with hardness values in the 700 to 800 mg/L range. Furthermore, the trend of under-prediction at hardness = 100 mg/L followed by increasing degrees of over-prediction at hardness = 200 to 600 mg/L (see Fig 5b) was observed for both species. This is a reflection of similarity with which the two species responded to sulfate with increased hardness levels as depicted in figure 2. These data suggest that the STR model does not account for the protective effect of hardness on major ion/TDS toxicity, but because of the presence of a pattern in the inaccuracy, data from this study may be useful in improving the model.
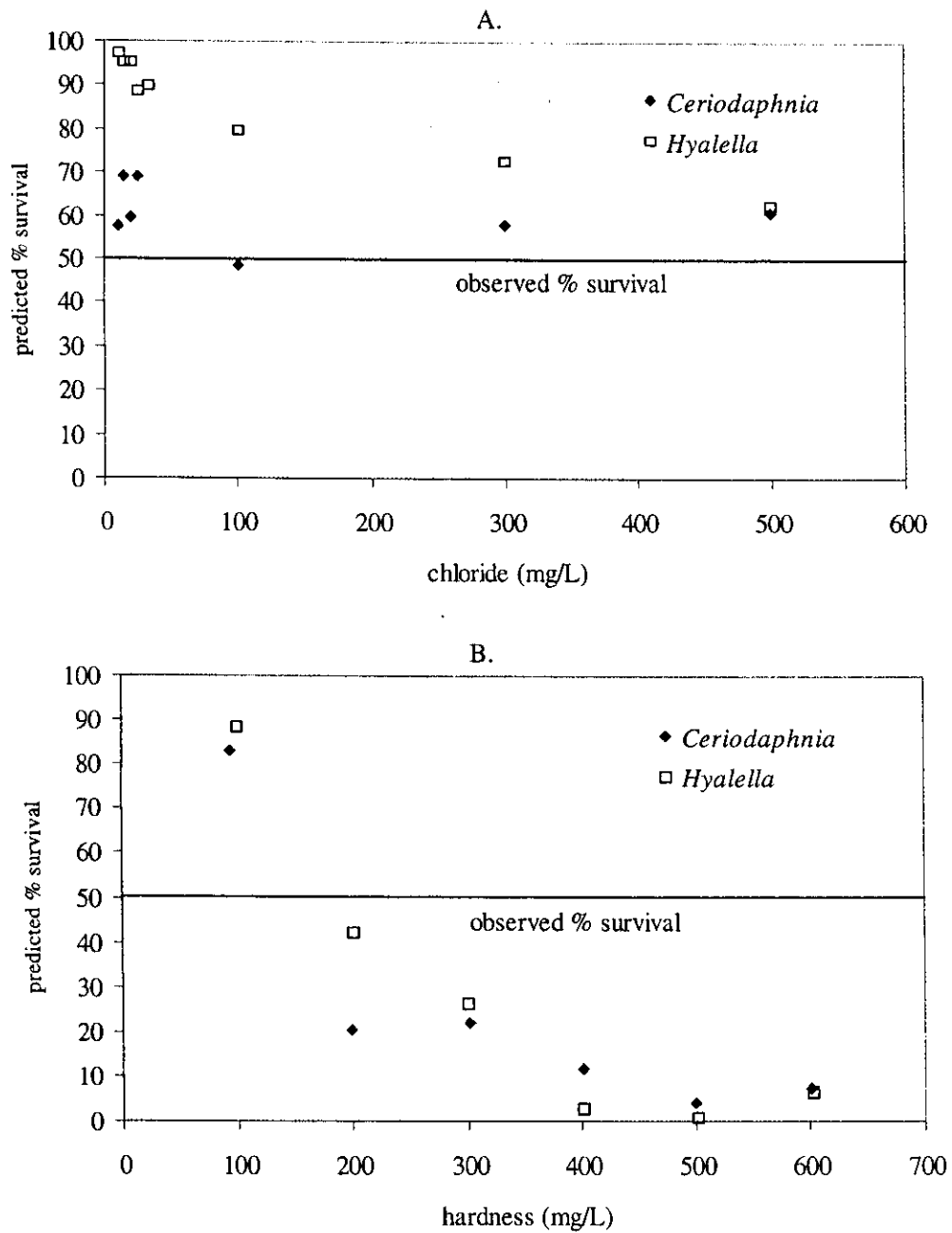
Figure 5. Percent survival of *Ceriodaphnia* and *Hyalella* at varying levels of chloride (A) and hardness (B) as predicted by the STR model. Model inputs were ion concentrations at nominal sulfate LC50s, so observed % survival in each case is 50%. STR does not predict for *Hyalella* so *Ceriodaphnia* predictions were used for *Hyalella* test inputs.
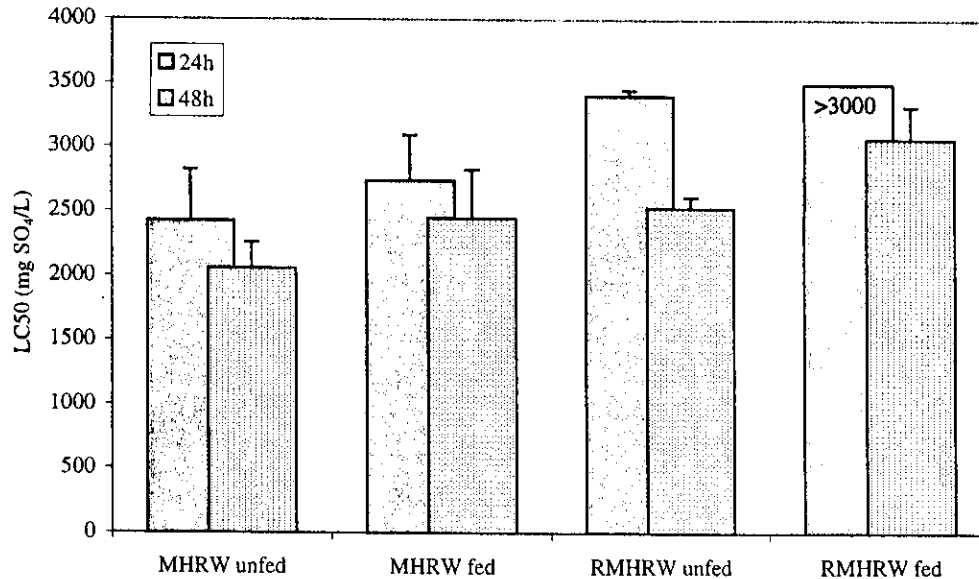
Figure 6. Effects of presence of food on toxicity of sulfate to *Ceriodaphnia dubia* in two types of test waters.

Hardness and chloride effects on acute sulfate toxicity to bivalves.

Five toxicity tests were conducted with 4-day-old, juvenile unionid mussels (fatmuckets, *Lampsilis siliquoidea*) at the U.S. Geological Survey's Columbia Environmental Research Center. When Ca:Mg and chloride concentration were held constant, increased hardness appeared to slightly reduce the toxicity of sulfate to the juvenile mussels (Table 2), but confidence intervals overlapped. Test water #4 was U.S. EPA's MHRW (USEPA 2002), and the 96-h LC50 in this water (1,727 mg/L) was fairly similar to, but slightly lower than LC50s for *C. dubia* (2,050 mg/L), and the fingernail clam, *S. simile* (2,078 mg/L) in MHRW. Chloride did not appear to have a strong effect either, but increasing chloride concentration from 5 to 33 increased the sulfate LC50 by approximately 100 mg/L (Table 2). Interestingly, while hardness and chloride did not strongly affect sulfate toxicity to freshwater mussels, Ca:Mg ratio appeared to have a substantial effect. In tests conducted with Ca:Mg = 1.46, 96-h LC50s ranged from 1,727 to 1,822 mg SO_4/L, while at a ratio of 2.33, LC50s ranged from 3,377 to 3,729 mg SO_4/L. This trend suggests that there may be a calcium threshold for unionids, which when reached provides a certain level of protection against sulfate toxicity, but beyond which, additional calcium provides minimal additional protection. Further testing should be conducted to verify these trends.

Table 2. Toxicity of sulfate to juvenile fatmuckets (*Lampsilis siliquoidea*). All tests were conducted at 20 °C for 96 hours with 4-d-old mussels. Mortality was evaluated at 48, and 96 hours. Sulfate LC50 values shown are based on measured concentrations, and all treatments within a given test had the same chloride concentration and hardness. LC50s were generated using the Spearman-Karber method. One test was conducted for each diluent type.

Test mg/L	Cl ratio	Ca:Mg mg/L (CaCO₃)	hardness (95% C.I.)	48 h LC50 (95% C.I.)	96 h LC50
1	25	2.33	100	3183 (2837-3571)	3377 (3205-3558)
2	25	2.33	300	3523 (unrel. var.)	3525 (3330-3731)
3	25	2.33	500	3574 (unrel. var.)	3729 (unrel var.)
4	5	1.46	100	1702 (1598-1812)	1727 (unrel var.)
5	33	1.46	100	2661 (2268-3123)	1822 (unrel var.)

In tests with fingernail clams (*Sphaerium simile*), increasing hardness from ~100 to 200 mg/L did not have a substantial effect on sulfate toxicity, but an additional increase to hardness = 300 mg/L caused a significant increase in the mean 96-h LC50 value (Table 3). When Ca:Mg and hardness were held constant, increased chloride caused a substantial increase in mean LC50 from 2,184 mg/L (Cl = 2 mg/L) to 2,598 (Cl = 33 mg/L) (Table 3). In the unionid mussel, *Toxolasma texasiensis*, chloride and bicarbonate were found to be equally important anions in the hemolymph (see McMahon and Bogan 2001). Because bicarbonate is readily available via respiration and metabolism, this mussel may not depend on external chloride concentrations to the extent that some crustaceans do. If this is the case, the protective effect of chloride observed for *Hyalella* and *Ceriodaphnia* might not be manifest in some unionoidean bivalves. The hardness effect observed in this study may be more widespread among aquatic phyla, because calcium simply reduces gill permeability (Lucu and Flik, 1999; Pic and Maetz, 1981). However, McMahon and Bogan (2001) state that unionoideans "generally lose capacity for osmotic and volume regulation above 3-4 ppt" (salinity). TDS is a rough measure of salinity, and the TDS of a sample of RMHRW with 2000 mg/L sulfate is 2.9 g/L or ppt (Soucek unpublished data). Further experiments with freshwater bivalves are required to determine if there is an absolute TDS level that is tolerable, or if the limit depends upon water quality characteristics such as chloride concentration and hardness.

Table 3. Effects of hardness and chloride on sulfate toxicity to fingernail clams (*Sphaerium simile*). All tests were conducted at 22 °C for 96 hours. Sulfate LC50 values shown are based on nominal concentrations, and all treatments within a given test had the same chloride concentration and hardness. LC50s were generated using the Spearman-Karber method. Different capital letters following means indicate means are significantly different (p < 0.05).

Diluent type	n	Cl mg/L	Ca:Mg ratio	hardness mg/L (CaCO₃)	mean LC50, mg SO₄/L (std. dev)
MHRW	3	4.5	1.46	99	2184 (419) B
Hard 200 (1.46)	2	6.0	1.46	205	2244 (204) AB
Hard 300 (1.46)	2	4.3	1.46	292	2949 (427) A
Hard 300 (2.33)	2	4.4	2.33	285	2640 (129) AB
RMHRW	2	32.7	5.4	106	2178 (210) AB
Cl 33/ hard 100	3	34.4	1.46	103	2598 (341) AB

Examining sulfate toxicity at different hardness levels reveals that hardness appears to ameliorate sulfate toxicity to a variety of species in at least two different phyla (Arthropoda and Mollusca). In fact, for three of the species tested in this study, the increase in sulfate LC50 from hardness = 100 mg/L to hardness = 300 mg/L was quite consistent at approximately 900 mg/L (Fig. 7).
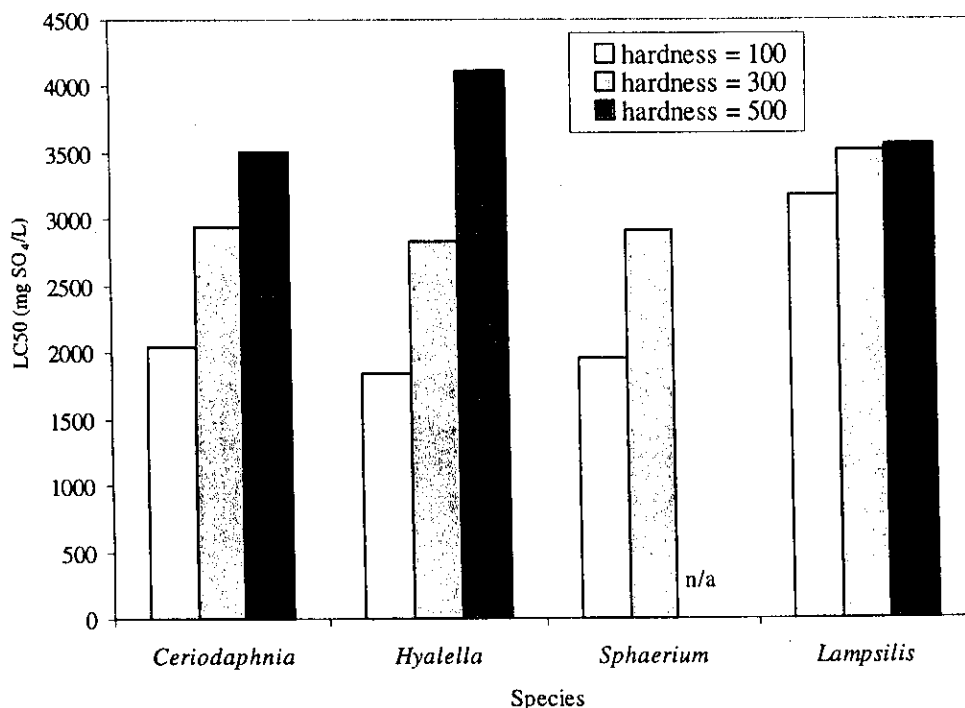
Figure 7. Comparison of LC50s at different water hardness levels for four different species of freshwater invertebrates tested in the laboratory.

7-day chronic sulfate toxicity tests with Ceriodaphnia dubia.

Chronic toxicity in terms of survival and reproduction was less in RMHRW compared to MHRW. The Least Observable Adverse Effects Concentrations (LOAEC) and No Observable Adverse Effects Concentrations (NOAEC) for both survival and reproduction were influenced by dilution water with those for RMHRW being higher than those for MHRW (Fig. 8 and 9). While LOAECs for reproduction are quite low, it should be noted that I have had a continuous, self sustaining, reserve culture of *Ceriodaphnia dubia* in MHRW spiked with 1,000 mg SO_4/L since at least August of 2004. Organisms used in chronic testing for this study were cultured in MHRW or RMHRW as appropriate.

Figure 8. Mean percent survival of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.
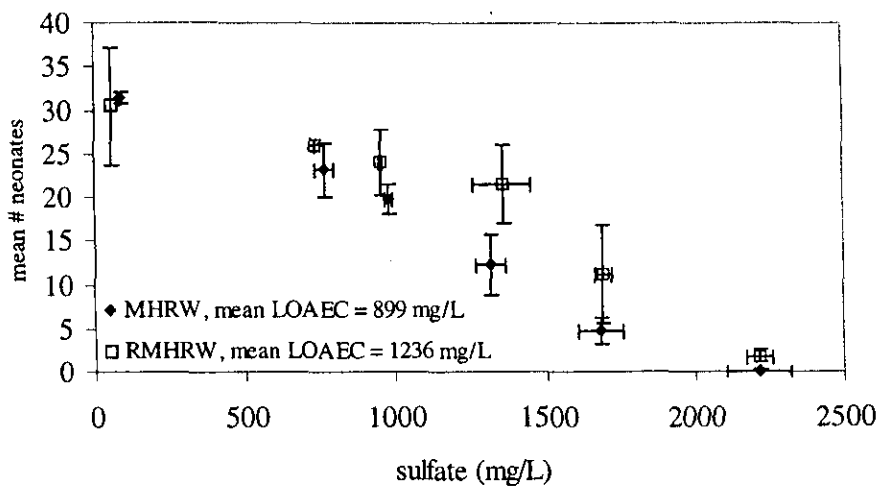
Figure 9. Mean reproduction of *Ceriodaphnia dubia* in 3-brood, static-renewal, chronic toxicity tests with sulfate in Moderately Hard Reconstituted Water (MHRW). Bars and error bars indicate means and standard deviations for three separate tests.

Predictive equations for standard development
Multiple linear regression analysis with covariance with the sub-dataset (n = 42) described
previously using sulfate LC50 as the dependent variable and hardness, chloride and species as
independent variables resulted in the following equation:

$$LC50 = 1646 + 5.508(Hardness) - 1.457(Cl), \quad R^2 = 0.8408, \, p < 0.0001$$

This was the combined model for both species. The intercept for *C. dubia* was 1,828.07 and for
H. azteca it was 1,463.93.

Acknowledgement

Literature cited
Aladin NV, Potts WTW. 1995. Osmoregulatory capacity of the cladocera. *J Comp Physiol B* 164:671-683

ASTM (American Society for Testing and Materials). 2002a. Standard guide for conducting acute toxicity
    tests on test materials with fishes, macroinvertebrates, and amphibians. E729-96. American
    Society for Testing and Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials). 2002b. Standard guide for conducting three-brood,
    renewal toxicity tests with *Ceriodaphnia dubia*. E1295-01. American Society for Testing and
    Materials, Philadelphia, PA, USA.

ASTM (American Society for Testing and Materials) 2005. Standard guide for conducting laboratory
    toxicity tests with freshwater mussels (ASTM E2455-05). ASTM annual book of standards
    volume 11.05, ASTM, West Conshohocken, PA.

American Public Health Association, American Water Works Association, Water Environment Federation.
    1998. Standard methods for the examination of water and wastewater, 20th ed. APHA,
    Washington, DC.

Bayliss D, Harris RR. 1988. Chloride regulation in the freshwater amphipod *Corophium curvispinum* and
    acclamatory effects of external Cl. *J Comp Physiol B* 158:81-90.

Borgmann U. 1996. Systematic analysis of aqueous ion requirements of *Hyalella azteca*: a standard
    artificial medium including the essential bromide ion. *Arch Environ Contam Toxicol* 30:356-363.

Dorgelo J. 1981. Blood osmoregulation and temperature in crustacea. *Hydrobiologia* 81:113-130.

Appendix 1. Summary of test conditions for conducting toxicity tests with juvenile mussels in basic accordance with ASTM (2005a).

Test species:	Fatmucket
Test chemicals:	Sodium sulfate
Test type:	Static
Test Duration:	96 h (also check survival at 48 h)
Temperature:	$20 \pm 1 °C$
Light quality:	Ambient laboratory light
Light intensity:	200 lux
Photoperiod:	16L:8D
Test chamber size:	50 ml
Test solution volume:	30
Renewal of solution:	After 48 h
Age of test organism:	<5 day old
No. organisms per test chamber:	5
No. replicate chambers per concentration:	4
Feeding:	No feeding
Chamber cleaning:	None
Aeration:	None
Dilution water:	Reconstituted water at three hardness levels (100, 300, and 500 mg/L as $CaCO_3$), and at two chloride levels (5 and 33 mg/L) at one hardness level (100 mg/L as $CaCO_3$)
Dilution factor:	0.5
Test concentration:	Five concentrations and a control
Chemical residues:	Sulfate concentrations were determined at the beginning and the end of each test
Water quality:	DO, pH, conductivity, hardness, and alkalinity were determined at the control, medium, and high concentrations of chemicals at the beginning and the end of each test
Endpoint:	Survival (foot movement)
Test acceptability criterion:	>90% control survival

State Water Survey Division

WATER QUALITY SECTION
AT
PEORIA, ILLINOIS

ACUTE TOXICITY OF CHLORIDES, SULFATES, AND TOTAL DISSOLVED SOLIDS TO SOME FISHES IN ILLINOIS

by

Paula Reed and Ralph Evans

September 1981

CONTENTS

ACUTE TOXICITY OF CHLORIDES, SULFATES,
AND TOTAL DISSOLVED SOLIDS
TO SOME FISHES IN ILLINOIS

by Paula Reed and Ralph Evans

INTRODUCTION

This report presents the results of a study undertaken to assess the
acute toxicity to certain fishes of various concentrations of chloride, sul-
fate, and resultant total dissolved solids. A review of the results of the
water quality monitoring program developed by the Illinois State Water Survey
in cooperation with the U.S. Geological Survey during the period 1945-1971
and reported on by Larson and Larson (1957), Harmeson and Larson (1969), and
Harmeson et al. (1973) suggests that chlorides, sulfates, and total dissolved
solids are not significant sources of pollution. After an evaluation of the
Water Survey's water quality data, Nienkerk and Flemal (1976) concluded that
the statewide discharge-weighted mean concentrations for these constituents
are as follows:

<div align="center">

Chloride: 25 mg/l
Sulfate: 70 mg/l
Total dissolved solids: 303 mg/l

</div>

In light of the rules governing maximum permissible concentrations of
these substances in the waters of Illinois these *mean* concentrations are min-
imal. However Nienkerk and Flemal (1976) suggest that sulfate and chloride
are among those mineral constituents most influenced by anthropogenic proces-
ses. Although they speculate that a major source of sulfate in the waters of
northeastern Illinois may be atmospheric fallout and a major source of chloride
in the waters of southeastern Illinois may be the excessive seepage of saline
groundwater, they nevertheless conclude that the principal causes of sulfate
and chloride concentrations exceeding background levels are such activities
as: the use of street de-icing salt, waste disposal, coal mining, and oil
production.

The work of Butts et al. (1976) confirmed that high chloride content in
Illinois streams can be related to oil production and groundwater seepage.
They found for some streams of the Saline River basins that the chloride con-
tent exceeded 500 mg/l about 10 to 45 percent of the time. At the same stream
locations the total dissolved solids exceeded 1000 mg/l about 30 to 60 percent
of the time.

More recently Toler (1980) reported that a reconnaissance of 50 stream
sampling sites on much of the surface-mined area in Illinois revealed sulfate
concentrations ranging from 25 to 4100 mg/l. Indeed, sulfate was the major
mineral constituent in the samples from all sites. On the basis of comparisons
with streams having little or no upstream mining activities he concluded that

1

concentrations of sulfate in excess of 100 mg/l in base stream flow are probably attributable to drainage from mine spoils.

The Illinois Pollution Control Board (1977, with amendments through 1979) recognized the likelihood that excess mineral contributions from human activities are superimposed upon the background concentrations of certain minerals in the state's surface waters. The limitations promulgated by the Board for the three constituents (in milligrams per liter) are:

	Chloride	Sulfate	Total dissolved solids
General stream quality	500	500	1000
Public water supplies	250	250	500

In addition to the general stream standards and the public water supply limitations the Board established the following rule regulating the total dissolved solids concentrations in effluent discharges:

Total dissolved solids shall not be increased more than 750 mg/l above background concentration levels unless caused by recycling or other pollution abatement practices, and in no event shall exceed 3500 mg/l at any time; provided, however, this Rule shall not apply to any effluent discharging to the Mississippi River, which, after mixing as set forth in Rule 201, meets the applicable water quality standard for total dissolved solids.

In this case the background concentration is that of the production water. And although an effluent can contain up to 3500 mg/l of total dissolved solids (more where discharge is to the Mississippi River) the rule does not permit a violation of the general stream quality standard of 1000 mg/l.

The Board's regulations also stipulate, in part:

Any substance toxic to aquatic life shall not exceed 1/10 of the 96-hour median tolerance limit (96-hr.-TL_m) for native fish or essential fish food organisms.

The median tolerance limit (TL_m) is the concentration at which 50 percent of the test specimens survive. It is also referred to as TL50, which is the designation used in this report. A 96-hour bioassay is a desirable minimum length. During this study, an exposure time of 14 days (336 hours) was used.

Of pertinent interest to this study is the validity of the maximum permissible concentrations of chloride (500 mg/l), sulfate (500 mg/l), and total dissolved solids (1000 mg/l) permitted in Illinois water in accordance with the general stream quality rule. The intent of the rule, among others, is to protect the state's waters for aquatic life. This study is also part of a continuing effort to develop information useful to persons and agencies whose activities relate to the enhancement of water quality in the streams and lakes of Illinois.

2

Scope of Study

As part of this investigation certain fishes native to Illinois lakes and streams were exposed to varying concentrations of chloride, sulfate, and resultant total dissolved solids in an effort to ascertain acute toxicity effects. The fishes used as test specimens were largemouth bass fingerlings, bluegill fry, and channel catfish fingerlings. Thirty-three bioassays were performed requiring the use of 3360 test specimens.

The bioassays were of 14-day durations and were performed with various fish sizes and water temperatures. The dilution water was high in the salts of calcium and magnesium with correspondingly high alkalinity.

Plan of Report

The report contains a description of the equipment and methods used for all bioassays; a two-part description of the observed reactions of fishes to chloride and sulfate; and a three-part discussion of the results concerning chlorides, sulfates, and total dissolved solids. All data developed from the bioassays are included in the appendices.

Acknowledgments

This study was conducted under the general supervision of Stanley A. Changnon, Jr., Chief, Illinois State Water Survey, and Dr. William C. Ackermann, Chief Emeritus, Illinois State Water Survey. Many persons of the Water Quality Section assisted in the study. Dave Hullinger and Dana Shackleford provided guidance and assistance in the analysis of chloride, sulfate, and total dissolved solids. Laurie Hebel, Lew Hoffman, and Rick Twait performed analyses, lent direction to the operation of the dilution apparatus, and occasionally maintained continuous 24-hour observations of aquaria. Mr. Maurice Whitacre of the Department of Conservation offered advice on the maintenance of test specimens and supplied many of them. Linda Johnson typed the original manuscript, and Gail Taylor edited it. Illustrations were prepared under the supervision of John W. Brother, Jr.

EQUIPMENT AND METHODS

A modification of a proportional dilutor developed by Mount and Brungs (1967) was used. Water flow was provided through 12 glass test chambers. Each chamber had a volume of 22 liters, and the flow rate, 113 milliliters per minute (ml/min), produced a 95 percent volume displacement every 10 hours. The apparatus permitted the flow of five different concentrations of toxicant into duplicative test chambers, with two chambers available for control purposes. All tests were performed for at least 14 days.

Equipment Modifications and Appurtenances

Previous work by the Water Survey, involving studies of the acute toxicity to fishes of residual chlorine and ammonia (Roseboom and Richey, 1977), copper (Richey and Roseboom, 1978), and zinc (Reed et al., 1980), relied on a syringe style pipettor to inject an exact amount of toxicant from the container of a stock solution to the mixing bowl of the dilutor apparatus. This toxicant feed system is satisfactory when dealing with toxicant concentrations of small magnitude. Since this study involved the use of toxicants generally exceeding 10,000 mg/l in test tanks, another method of delivery had to be devised. The dilution apparatus used consisted of a chemical metering pump supplied by Fluid Metering, Inc., which derives its feed of stock solution from a 200-liter container. The system operates in the following manner.

During the cycling of the dilutor, the timer activates the water solenoid valve to open and begin filling the dilution water chambers as it simultaneously engages the chemical metering pump to start pumping toxicant from the stock solution container into the toxicant bowl. As water from the dilution water chambers overflows into the water bucket, the bucket fills and descends, thereby engaging the switch and breaking the electrical current. This shuts off the water solenoid valve and the chemical metering pump. As dilution water and toxicant combine in the mixing chambers, the water bucket arm rises to complete the electrical circuit. Then the cycle repeats itself. The advantages of this system are an easily adjustable volume and rate of feed at the pump, a fail-safe design directly timed by dilutor function, an ability to maintain high concentrations of toxicant in a flow-through unit, and a relatively low price for a system comprising a timer, a chemical metering pump, and a water solenoid.

A well on the laboratory site, in the same aquifer as the municipal wells, was the source of water for the dilution apparatus.

Two header boxes were used. The first one is a polyethylene plastic barrel equipped with a thermoregulator which can be set at a desired temperature. Significant cooling from the pre-set water temperature energizes a relay which activates a solenoid-controlled valve on a hot water line. Water flows from the first polyethylene plastic barrel to a second polyethylene plastic header box, where air agitation keeps the contents mixed and provides a sustained dissolved oxygen level.

The following characterize the dilution water used in the bioassays (all values except pH are in milligrams per liter):

Chemical oxygen demand	Not detected	Magnesium	25.3
Ammonia-N	0.09	Iron	0.11
Nitrate-N	3.6	Zinc	.07
Phosphate-P	0.20	pH	8.33
Sulfate	183	Hardness	412
Chloride	87	Alkalinity	291
Copper	.008	Cadmium	.004
Fluoride	0.79	Lead	<.08

4

Stock Solutions and Chemical Analyses

The sodium chloride stock solutions were prepared by dissolving technical grade sodium chloride in dilution water. Due to the rather low toxicity of sodium chloride to fish, large quantities of toxicant were used daily in the dilutor. To accommodate the preparation of the toxicant and to assure its thorough mixing, a circulating pump was used.

At least once during the first 24 hours of each bioassay, and generally daily thereafter, chloride analyses were made by removing a sample from the middle of each test chamber. All chloride determinations were performed in accordance with the argentometric method. Results are expressed in mg/l chloride (Cl^-).

The sodium sulfate stock solutions were prepared by dissolving technical grade sodium sulfate in dilution water. Due to the low solubility of sodium sulfate in $20^\circ C$ dilution water, it became necessary to use dilution water heated to $30-35^\circ C$ to achieve the desired stock concentration. Since sodium sulfate is relatively low in toxicity, large volumes of toxicant were also used daily in the proportional dilutor. A circulating pump was utilized to facilitate the preparation of the toxicant and to assure thorough mixing of the sodium sulfate and dilution water. During the winter months it became necessary to use a submersible thermostat heater and to supply aeration by means of air stones in the stock solution container because the sulfate stock solution had a tendency to stratify.

At least once during the first 24 hours of each bioassay, and generally daily thereafter, sulfate determinations were made by removing a sample from the middle of each test chamber. All sulfate analyses were performed in accordance with the turbidimetric method. A Bausch and Lomb Spectronic 20 was used for all absorbance readings. All results are expressed as mg/l sulfate (SO_4).

All analyses were performed as outlined in *Standard Methods for the Examination of Water and Wastewater* (American Public Health Association, 1975).

Hardness and alkalinity were determined in one control chamber and two other test chambers on three occasions during each bioassay. Analyses for pH were conducted on the same three occasions, but samples were taken from six test chambers rather than three. Dissolved oxygen levels, measured by a Yellow Springs Instrument Model 57 oxygen meter, were recorded daily from all test chambers. Water temperature also was measured daily by a standard graduated centigrade thermometer. Hardness determinations were by the EDTA titrimetric method with Eriochrome Black T as an indicator. Alkalinity and pH were determined by a Metrohm Herisau pH meter, Model 588, with 0.02 N H_2SO_4 as a titrant for alkalinity.

Salinity and conductivity measurements of all test chambers were recorded generally on a daily basis with a Yellow Springs Instrument S-C-T meter, model 33. Analyses for total dissolved solids (TDS) were generally determined daily

Table 1. Test Conditions for Chloride Bioassays

	Average fish weight (grams)	Average fish length (cm)	Range chloride (mg/l)	Range total diss. solids (mg/l)	Range pH (units)	Average alkalinity (mg/l)
Bass						
8-6-79	--	4.1	6460-9718	--	8.40-8.57	184
8-8-79	--	2.8	9665-9713	--	8.45-8.46	184
8-9-79	--	3.1	9587	--	8.43-8.43	--
8-13-79	--	3.1	10199-10947	--	8.52-8.54	201
8-27-79	--	3.9	6119-9493	--	8.45-8.59	210
10-22-79	--	--	10490-14075	18192-21313	--	--
11-5-79	2.11	5.1	5898-15308	9951-24529	8.30-8.48	271
11-12-79	2.01	5.2	9647-9847	16111-16178	8.22-8.38	298
1-21-80	3.75	6.6	5358-11067	9741-19158	8.18-8.40	291
2-4-80	4.38	6.8	6247-11371	10520-18869	8.19-8.39	293
11-10-80	1.92	5.6	5968-14126	10617-23289	8.20-8.70	286
12-2-80	2.26	5.6	6237-14432	10981-23437	8.40-8.61	322
Bluegill						
7-10-79	2.64	5.6	6825-10690	--	8.42-8.70	211
7-16-79	4.51	6.6	6775-10704	--	8.28-8.62	221
7-24-79	7.24	7.3	5971-9161	--	8.08-8.54	186
11-15-79	2.24	5.3	11446-11646	19036-19161	8.21	298
11-26-79	2.31	5.3	5277-11546	9434-18549	8.20-8.40	298
12-10-79	0.33	2.8	5105-11231	9378-19143	8.22-8.41	294
Catfish						
8-18-80	1.54	5.6	5175-13783	8899-21265	7.13-8.42	253
9-2-80	2.37	6.4	5185-13151	8951-20618	8.32-8.48	257
9-9-80	3.51	7.1	13340-13592	21287-21303	8.32-8.33	267

Table 1. Concluded

	Average hardness (mg/l)	Percent dissolved oxygen saturation	Average temperature (°C)	Range salinity	Range species conductivity (micro-MHOS)
Bass					
8-6-79	493	92	21	--	--
8-8-79	480	90	21	--	--
8-9-79	--	92	21.7	--	--
8-13-79	480	97	20.8	--	--
8-27-79	514	93	21.4	--	--
10-22-79	--	--	20.6	--	--
11-5-79	407	92	20.5	--	--
11-12-79	416	93	20.3	--	--
1-21-80	415	85	20.3	9.2-20.8	14000-29800
2-4-80	424	81	20.1	9.2-19.0	13800-27200
11-10-80	515	83	19.4	10.7-23.1	15800-31800
12-2-80	527	81	18.6	10.8-24.3	15500-33200
Bluegill					
7-10-79	365	86	22.4	--	--
7-16-79	393	77	22.7	--	--
7-24-79	429	80	21.4	--	--
11-15-79	416	--	--	--	--
11-26-79	428	94	20.6	8.8-19.5	13200-27800
12-10-79	416	96	20.4	9.0-19.9	13800-28200
Catfish					
8-18-80	383	86	17.8	8.8-21.9	12700-30300
9-2-80	387	81	19.9	8.7-21.6	13000-30500
9-9-80	405	87	20.1	21.0-21.2	30500

from all test aquaria using filtration and residue on evaporation at 103 to 105°C. Some ranges and averages of these analyses along with other pertinent data representing test conditions during each bioassay are included in tables 1 and 2. Illumination for the 16-hour photoperiod was furnished by a combination of Duro-test and Wide Spectrum Gro-lux fluorescent lighting in circuit with a timer.

Test Specimens

Three native Illinois fishes were selected as test specimens for the chloride and sulfate bioassays. They were largemouth bass (*Micropterus salmoides*), bluegill (*Lepomis macrochirus*), and channel catfish (*Ictalurus punctatus*). Table 3 lists the type and number of fishes used, average weight of the fishes, and sources of the fishes for each of the bioassays.

All test specimens were acclimated to the 20°C dilution water for a minimum of 10 days. When necessary, the temperature was increased 1°C per day and maintained at the desired temperature for 10 days. Holding tanks were continually flushed with dilution water to eliminate any metabolic waste.

At the beginning of each bioassay, the temperature, salinity, conductivity, and toxicant concentration for each test chamber were determined. One fish at a time was randomly placed in the different aquaria until each of the 12 chambers held 10 fish. Because of rapid mortality at high concentrations, each test chamber was continuously monitored the first 32 hours, and the exact time of each mortality was recorded. Appendices A, B, C, D, E, and F provide the exact mortality times for largemouth bass, bluegill, and channel catfish. After death, the fish were thoroughly blotted to remove excess moisture, and their lengths and weights were determined.

REACTIONS OF FISHES

It is customary to record the behavior of fishes exposed to toxicants during the performance of bioassay work at the Water Survey. This is done for several reasons. A principal one is the desire to develop information useful to personnel in Illinois who have the responsibility for investigating fish kills and determining the likely causes of fish mortality. Observations under controlled conditions of such factors as behavior during stress, sites of hemorrhaging, changes in pigmentation, and body configuration may make it possible to interpret similar observations under field conditions.

A control group of fish was maintained with each bioassay at the ratio of 20 control fish to 100 test fish. The control fish were kept under exactly the same conditions as the test fish in all respects except for the addition of the toxicant. There was never any occurrence of a mortality in the control tanks at any time during the bioassays. All fish behaved normally and eagerly accepted food.

Table 2. Test Conditions for Sulfate Bioassays

	Average fish weight (grams)	Average fish length (cm)	Range sulfate (mg/1)	Range total diss. solids (mg/1)	Range pH (units)	Average alkalinity (mg/1)
Bass						
9-22-80	1.24	4.8	7556-17484	13321-25469	8.39-8.53	267
9-30-80	1.26	4.7	8627-18868	12001-27277	8.39-8.55	265
10-6-80	1.31	4.8	9953-14567	16104-23666	8.44-8.52	291
10-22-80	1.45	5.1	11201-18989	16306-29573	8.60-8.64	305
10-27-80	1.77	5.4	10323-14907	17183-25986	8.41-8.57	316
Bluegill						
5-19-80	0.67	3.5	9801-17483	15400-26024	8.50-8.60	302
6-2-80	0.59	3.5	9418-18009	15460-26611	8.50-8.65	299
6-9-80	1.09	4.1	13483-13844	21467-21456	8.55-8.59	--
Catfish						
6-16-80	1.01	4.7	8845-18205	--	8.48-8.60	--
6-23-80	1.27	4.9	9032-19245	13877-25968	8.49-8.63	296
7-7-80	1.55	5.2	6769-14564	10722-20440	8.41-8.60	262
7-28-80	1.85	5.6	7019-15584	11052-22954	8.46-8.51	257

	Average hardness (mg/1)	Percent dissolved oxygen saturation	Average temperature (°C)	Range salinity	Range species conductivity (micro-MHOS)
Bass					
9-22-80	416	86	20.2	7.0-19.9	10500-28100
9-30-80	420	83	20.3	6.9-16.8	10300-24300
10-6-80	461	84	20.0	9.3-18.3	13700-26500
10-22-80	461	83	20.2	9.0-19.0	13300-26500
10-27-80	493	83	19.9	9.0-19.2	13600-28100
Bluegill					
5-19-80	571	89	21.0	9.5-17.2	15000-26000
6-2-80	.512	84	21.7	8.6-19.1	13500-28100
6-9-80	--	81	20.8	12.8-15.5	19200-23100
Catfish					
6-16-80	507	80	20.9	7.9-17.0	12000-25000
6-23-80	466	84	20.8	8.8-16.2	13500-23700
7-7-80	440	62	21.0	6.3-15.6	9800-23500
7-28-80	397	87	19.4	7.3-14.1	10900-21100

Table 3. Types, Numbers, Weights,
and Sources of Fish Used in Bioassays

Bioassay	Type of fish	No. of fish	Average wt. of fish (grams)*	Sources of fish
Chloride	Bass	1200	2.08 4.08	IDOC, Spring Grove; Opel's Fish Hatchery, Worden, IL
	Bluegill	560	0.33 2.40 5.9	IDOC, Spring Grove; Opel's Fish Hatchery, Worden, IL
	Catfish	260	2.47	Seven Springs Fish Farm, Evansville, IL
Sulfate	Bass	600	1.41	IDOC, Spring Grove; National Fish Hatchery, Hebron, Ohio
	Bluegill	260	0.78	Fender's Fish Hatchery, Baltic, Ohio
	Catfish	480	1.42	Seven Springs Fish Farm, Evansville, IL

* Bass and bluegill used in chloride bioassays fell into several distinct weight groups, as indicated
Note: IDOC = Illinois Department of Conservation

Chloride

At high chloride concentrations, channel catfish exhibited numerous symptoms of stress. At the beginning of each bioassay the fish experienced a definite loss of equilibrium. This was accompanied by respiratory difficulty; opercular movement was rapid and shallow. Many individuals swam frantically at the water surface. As time progressed, the eyes appeared glazed and respiration became increasingly labored. In addition, the catfish assumed a variety of positions in the water column. Some performed short bursts of swimming in a zigzag fashion at the surface of the water. Others lay on their sides on the bottom of the tank. Some of the fishes underwent a stiffening of their bodies and maintained a position perpendicular to the bottom of the tank. Certain individuals hung at the surface in this rigid position while others stood on their tails.

A few channel catfish experienced muscle spasms and twitching along with tail chasing. Afterwards their bodies became rigid, and death soon followed. Certain physical characteristics that accompanied the catfish mortalities were produced by chloride. They included hemorrhaging in the gills, in the brain, and at the base of the pectoral fins. Curvature of the body was a common reaction to the toxicant. Death was determined by lack of reaction to prodding and the cessation of gill movement.

The appetite of the channel catfish during the bioassay was a function of the concentration of the chloride. In concentrations above 10,000 mg/l chloride, the fishes completely ignored food. In the moderate range of approximately 7500-9000 mg/l chloride, their appetites fluctuated. Initially, the chloride produced a suppression of the appetite. Later, after perhaps some acclimation to the chloride, there was a slight improvement in appetite. Concentrations at or below 5000 mg/l chloride slightly decreased the appetite of the catfish initially, but after awhile all fish eagerly accepted food.

10

The stress patterns of the bluegill exposed to chloride concentrations in excess of 10,000 mg/l were similar to those of the channel catfish. Initially respiration was sluggish and there was a general darkening of body color. The fish experienced a loss of equilibrium, lying on their sides at the surface and floating sideways. Others attempted short dives downward in the water column and later floated back to the top. Coughing and regurgitation were experienced by some bluegill in distress.

As time progressed, some of the fishes underwent a frenzied, convulsive type of activity. Other bluegill became rigid and maintained a vertical position in the water. The eyes appeared glazed. Death usually occurred within nine hours and produced certain distinctive features, including flared gills, severe curvature of the spine, and hemorrhaging in the gills and at the pectoral fins.

At concentrations less than 10,000 mg/l chloride, the same stress patterns occurred as noted before, but with less severity. Deaths seemed to occur more quietly. There was apparent hemorrhaging at the gills as well as the tail, at the base of the dorsal fin, and in the head.

The appetites of the bluegill exposed to chloride varied inversely with the concentrations. In the higher concentrations, the fishes ignored food completely. In lesser chloride concentrations, the bluegill initially would refuse to eat, but as time continued there was a gradual improvement in appetite from a poor to fair status. At concentrations of less than 5000 mg/l chloride, all bluegill ate normally.

The largemouth bass exposed to chloride concentrations in excess of 9000 mg/l revealed stress behavior patterns similar to those of the bluegill and channel catfish. At the beginning of each bioassay, the fish would hover at the water surface with respiratory problems. They exhibited a loss of equilibrium by lying on their sides in the water column. Some bass attempted to right themselves by diving down towards the bottom of the aquarium, but they nearly always rose back to the surface. Certain individuals reacted to the chloride through spinal curvature; in a couple of severe cases, the body was almost L-shaped and there was evidence of internal hemorrhaging.

Other signs of distress included coughing, regurgitation, and gulping of water. As the fish neared death, respiration became more labored. Some experienced tremors or muscle spasms resulting in rapid bends or flips.

Upon expiration the largemouth bass exhibited certain distinctive characteristics as a result of their exposure to chloride. These included flared gills, gaping mouth, loss of pigmentation, and hemorrhaging in the gills, mouth, head, and at the base of the pectoral and caudal fins.

At chloride concentrations less than 9000 mg/l the stress symptoms were the same as those at the higher concentrations, but they generally took longer to occur and were less severe. The appetites of the largemouth bass also were inversely correlated to the concentration of chloride. At the high concentra-

tions, the chloride suppressed all appetites, but as the percent of toxicant present decreased, there was an initial absence of eating and then a gradual improvement in their eating habits. Lower concentrations of chloride did not adversely affect the appetites of the largemouth bass. Most ate well from the beginning to the end of the bioassay. In fact some were eating as well as the controls. This might indicate an acclimation to chloride.

Sulfate

Bluegill exhibited numerous symptoms of stress when exposed to sulfate concentrations in excess of 15,000 mg/l. Typically there was an immediate loss of equilibrium and general body control. Some fishes were observed lying on their sides at the surface, others were doing "barrel rolls," and still others were seen diving to the bottom of the tank and floating back to the top. All were experiencing respiratory difficulty as they rapidly beat their pectoral fins. As the bioassay continued, many bluegill preferred to stay near the bottom of the aquarium and exhibited very little movement. Breathing became more labored and sluggish.

Some noticeable symptoms of distress from the sulfate toxicant included spinal curvature, tremors, flared gills, gaping mouth, and hemorrhaging in the gills and head. Most bluegill underwent a change in pigmentation. Some experienced a darkening of body color, while others were pale in color upon death. In one instance, a fish displayed dark vertical bands above the lateral line and light ones below. Spiny rayed fins were erect. In sulfate concentrations greater than 10,000 mg/l but less than 15,000 mg/l, the stress behavior was similar to that in the higher sulfate concentrations. Upon introduction to the toxicant, many exhibited disorientation and visited the surface briefly. Some were seen swimming sideways. Respiration was sluggish and was accompanied by a rapid beating of the pectoral fins. There was a change in pigmentation, with some becoming darker and others becoming lighter in color. Apparently the sulfate solution irritated the muscle and nerve tissues of certain bluegill to such an extent that they reacted by twitching and trembling. As they neared death and were severely distressed, the fishes stayed on the bottom of the tank.

At concentrations less than 10,000 mg/l sulfate there was a drastic decrease in mortalities. Apparently after the initial shock was over, the bluegill gradually acclimated to the toxicant. All mortalities involved distress characteristics exactly like those which occurred at the higher concentrations.

Channel catfish appeared to react to the sulfate toxicant in a manner similar to the bluegill. At the onset of each bioassay, there was a loss of equilibrium. Some were seen stiffening their bodies and hanging vertically in the water column at the surface. Opercular movement was rapid and shallow as the catfish tried to compensate for the shock and introduction into a different fluid medium. Some fishes were so distressed by the sulfate toxicant that they vomited. As time progressed respiration became increasingly difficult and many rested on the bottom of the tank. Schooling behavior was somewhat erratic at this point.

12

Certain distressed individuals underwent a tail chasing phenomenon and death tremors. Upon their expiration, many catfish displayed an open or gaping mouth, flared gills, erect spiny-rayed fins, curvature of the body, and hemorrhaging at the base of the pectoral, dorsal, and caudal fins and in the head.

In sulfate concentrations in excess of 15,000 mg/l, the largemouth bass exhibited stress symptoms similar to those of the bluegill and channel catfish. Initially they hovered at the water surface with breathing difficulties. There was a rapid fluttering of the pectoral fins as they tried to adjust to the toxicant. All experienced a loss of balance as they entered the sulfate solution. Many rolled back and forth in a barrel roll fashion or simply lay on their sides at the surface. Later it was noted that some fish had spinal curvature. Muscle twitching was also displayed by a few individuals. Generally, most mortalities occurred within 12 hours at the higher concentrations. Many bass revealed gaping mouths, flared gills, and hemorrhaging at the head and operculum.

At sulfate concentrations in the moderate range, between 10,000 mg/l and 15,000 mg/l, the same stress symptoms were observed but appeared to be less severe. As usual, the fishes experienced breathing difficulty at the beginning of each bioassay. A loss of equilibrium followed, with some individuals lying on their sides. Several were observed swimming upside down. Many bass appeared darker in color as the bioassay continued. Death in the moderate sulfate range was accompanied by distress characteristics similar to those in the higher concentrations. These included curvature of the body, an erect dorsal fin, open mouth, flared gills, and hemorrhaging at the operculum and in the gills. Most mortalities occurred within 24-48 hours. The appetites of the largemouth bass exposed to these sulfate concentrations were non-existent or very poor. Many completely ignored food or consumed a little food now and then.

At less than 10,000 mg/l sulfate, the bass appeared to be okay and acted normally after an initial adjustment period. Appetites were usually good, and in fact many were eating as well as the controls. This might indicate an acclimation to the sulfate toxicant at this level.

RESULTS AND DISCUSSION

To estimate the median lethal time -- the time at which 50 percent mortality will occur in a particular test chamber -- the percent mortality for that chamber and its duplicate is plotted against the observed time of mortality. Figure 1 illustrates the procedure, showing that 50 percent mortality occurred in duplicate chambers in 329 minutes (the median lethal time) at the chloride concentration of about 10,900 mg/l. In this manner median lethal times and corresponding chloride concentrations have been determined for each bioassay. An acute toxicity curve can then be developed by plotting the median lethal times against the corresponding chloride concentrations, as shown
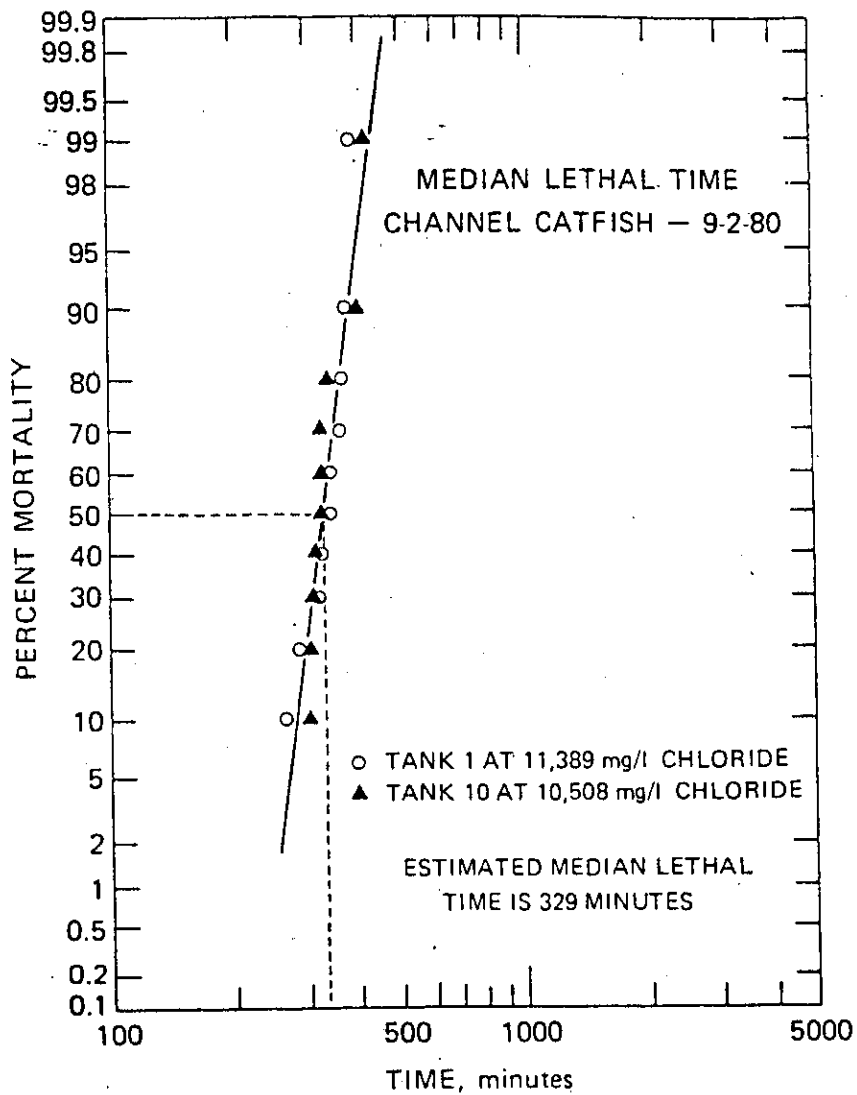
Figure 1. Percent mortality for channel catfish (Cl⁻)

in figure 2. The arrow in figure 2 represents the condition developed from figure 1. If less than 50 percent mortality occurred in a test chamber within 14 days, the time selected for representing the median lethal time is 14 days. For the purposes of this study, 24-hour and 96-hour designations are also included in addition to 14-day times.

From the acute toxicity curves the TL50 value is determined. The TL50 is that concentration at which the curve becomes asymptotic to the time axis.

As mentioned previously, the water pollution regulations in Illinois require an application factor of 1/10 to the TL50 for determining the maximum permissible concentration of any substance toxic to aquatic life. Because the TL50 concentration is derived here from the acute effects of the substance on fishes it is assumed that an allowable concentration of 1/10 the TL50 concentration in Illinois waters will minimize chronic effects related to growth, reproduction, and genetic characteristics of aquatic organisms. Nevertheless the uniform application of the factor (1/10) for *all* toxic substances is a questionable practice without adequate substantiation for Illinois conditions. Under present conditions, however, the 1/10 factor is required and shall remain so until evidence has been developed to justify a reevaluation of its usefulness.

Chloride Bioassays

The reactions of catfish, bass, and bluegill to concentrations of chloride are shown in figures 2, 3, and 4, respectively. It is apparent from these figures that all three species of fish exhibit a similar sensitivity to chloride at water temperatures of about 20°C. The TL50 concentrations range from 8000 to 8500 mg/l chloride with the bass appearing to be slightly more tolerant to chloride than the other two species.

The figures also suggest that there is not a perceptible difference between TL50 concentrations for bioassays with time lengths of 24 hours, 96 hours, or 14 days.

Sulfate Bioassays

The reactions of catfish, bass, and bluegill to concentrations of sulfate are shown in figures 5, 6, and 7, respectively. Here also it is apparent that all three species are similarly sensitive to sulfate at water temperatures of about 20°C. The TL50 concentrations at 14 days range from 10,000 to 11,000 mg/l. Of the three species, bass is the least sensitive to sulfate.

The figures also show that the TL50 concentrations will differ depending on the time length of the bioassay. Generally, the shorter the time length of the bioassay (24 hours versus 96 hours versus 14 days), the higher the resultant TL50, as shown in the figures. A summary of TL50s for figures 5, 6, and 7 appears on page 22.

Figure 5. Acute toxicity curve for channel catfish (Cl⁻)

Figure 3. Acute toxicity curve for largemouth bass (Cl^-)

Figure 4. Acute toxicity curve for bluegill (Cl⁻)

Figure 5. Acute toxicity curve for channel catfish (SO$_4^=$)

Figure 6. *Acute toxicity curve for largemouth bass ($SO_4^=$)*
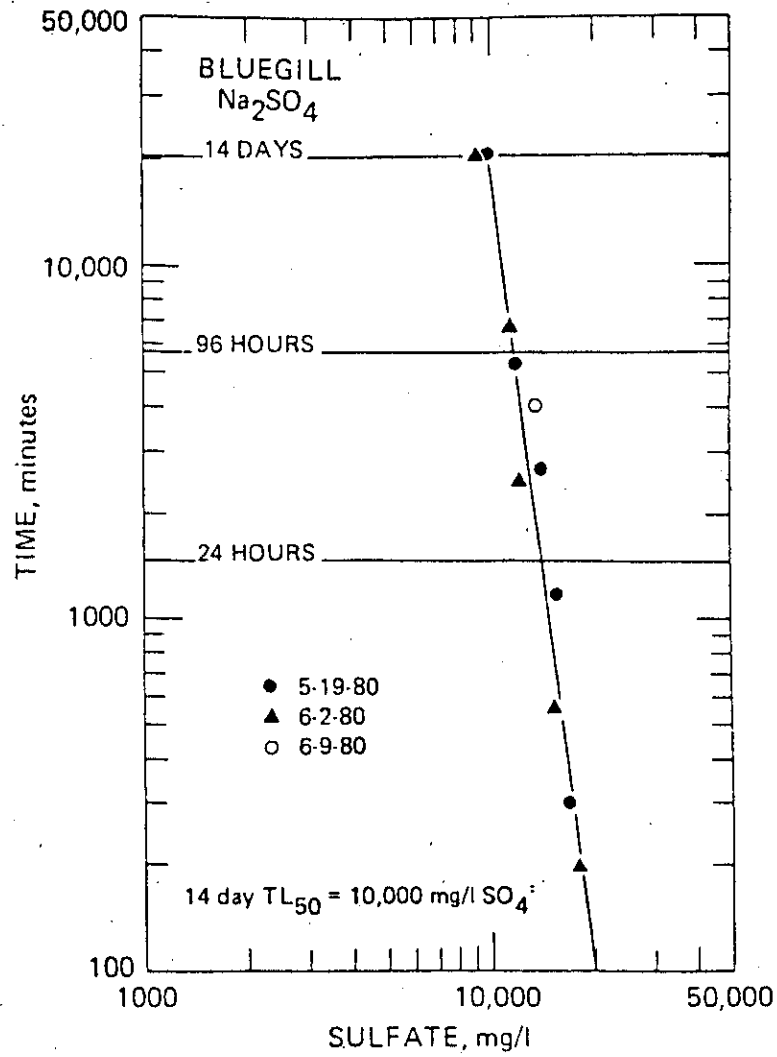
Figure 7. Acute toxicity curve for bluegill ($SO_4^=$)

Summary of TL50s for Figures 5, 6, and 7

(Milligrams per liter)

Time (hours)	Catfish	Bass	Bluegill
24	14,000	15,000	14,000
96	11,000	13,000	12,000
336 (14 days)	10,000	11,000	10,000

Total Dissolved Solids

The assessment of the effects of total dissolved solids on fishes con-
sists basically of considering the chloride and sulfate concentrations in
terms of total dissolved solids for the bioassays performed. Two conditions
are considered. In one case the total dissolved solids are principally made
up of sodium chloride; in the other case they principally consist of sodium
sulfate.

The results for the chloride-oriented total dissolved solids (TDS-Cl^-)
are included in figures 8, 9, and 10 for catfish, bass, and bluegill, respec-
tively. The sulfate-based total dissolved solids (TDS-$SO_4^=$) results are
similarly depicted in figures 11, 12, and 13.

From an examination of figures 8, 9, and 10 it is apparent that all three
species of fish exhibit a similar sensitivity to TDS-Cl^- at water temperatures
of about $20°C$. The TL50 concentrations range from 13,000 to 15,000 mg/l total
dissolved solids. Catfish is the most sensitive; bass is the most tolerant.

An examination of figures 11, 12, and 13 shows that there is more vari-
ability in TL50s among the fishes when exposed to TDS-$SO_4^=$ at about $20°C$.
The TL50 concentrations range from 14,000 to 17,500 mg/l total dissolved sol-
ids. Here again the catfish is more sensitive; the bass and bluegill are about
equally tolerant.

From this assessment it appears that total dissolved solids concentrations
are not a sensitive indicator of acute toxicity for fishes. The tolerance to
total dissolved solids varies with the species of fish and depends upon the
principal anion comprising the dissolved solids.
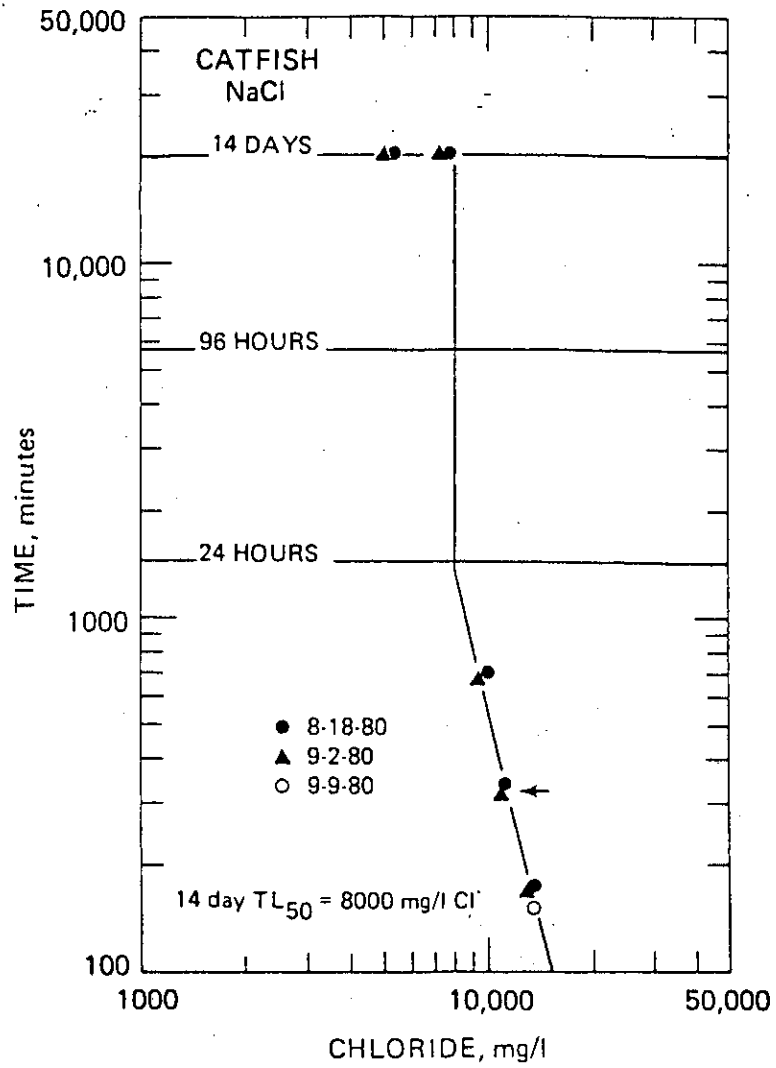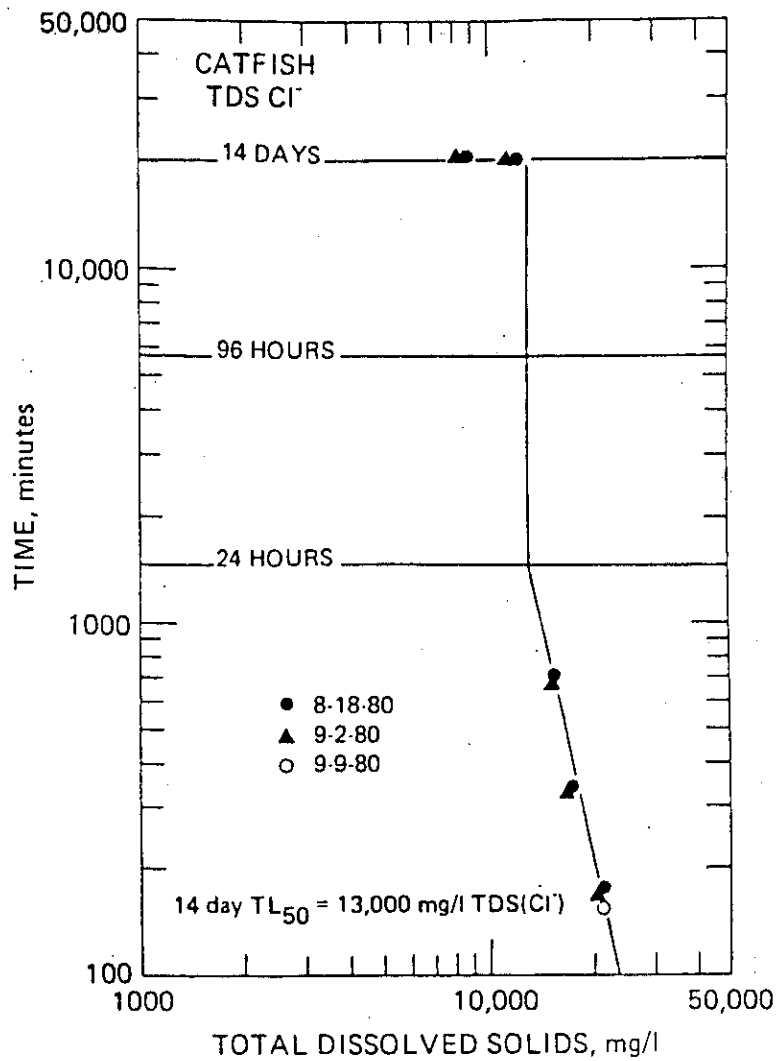
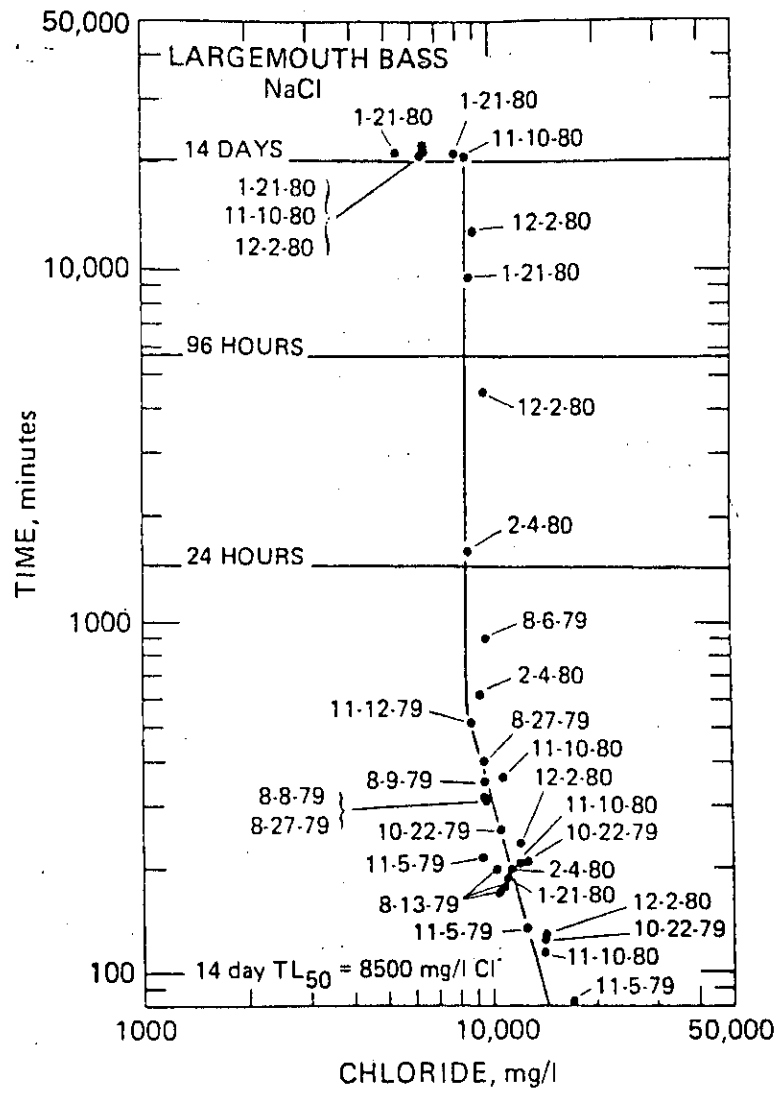*Figure 8.   Acute toxicity curve for channel catfish (TDS-Cl⁻)*
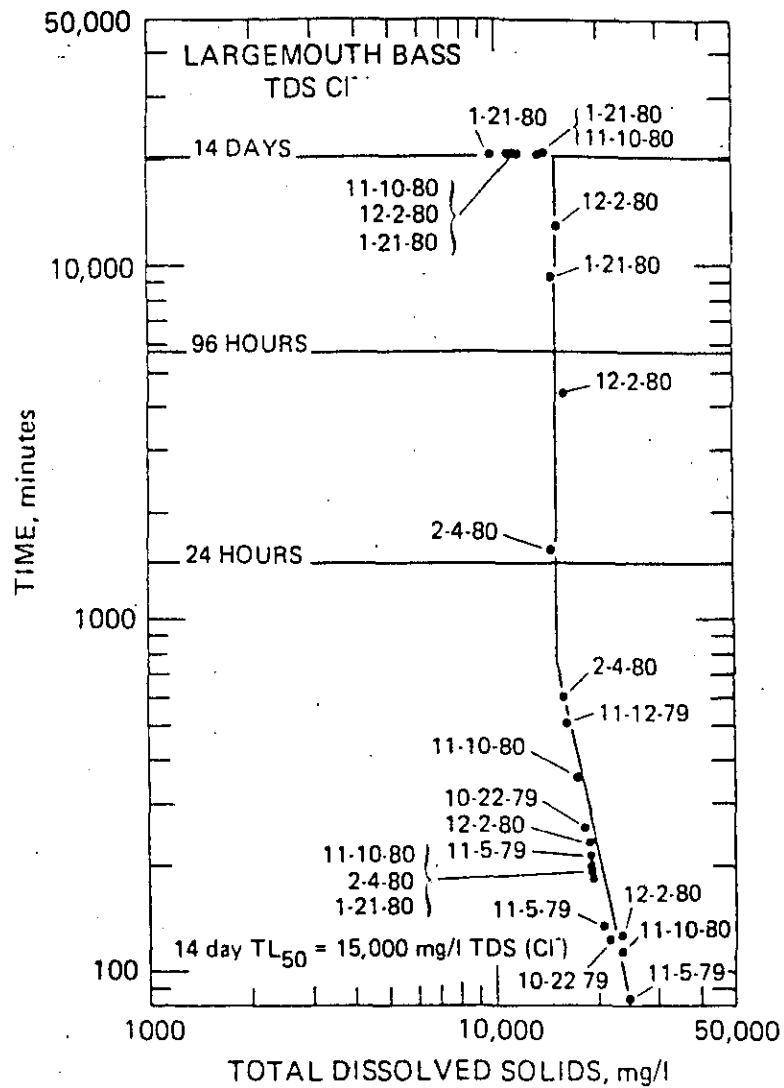
Figure 9. Acute toxicity curve for largemouth bass (TDS-Cl⁻)
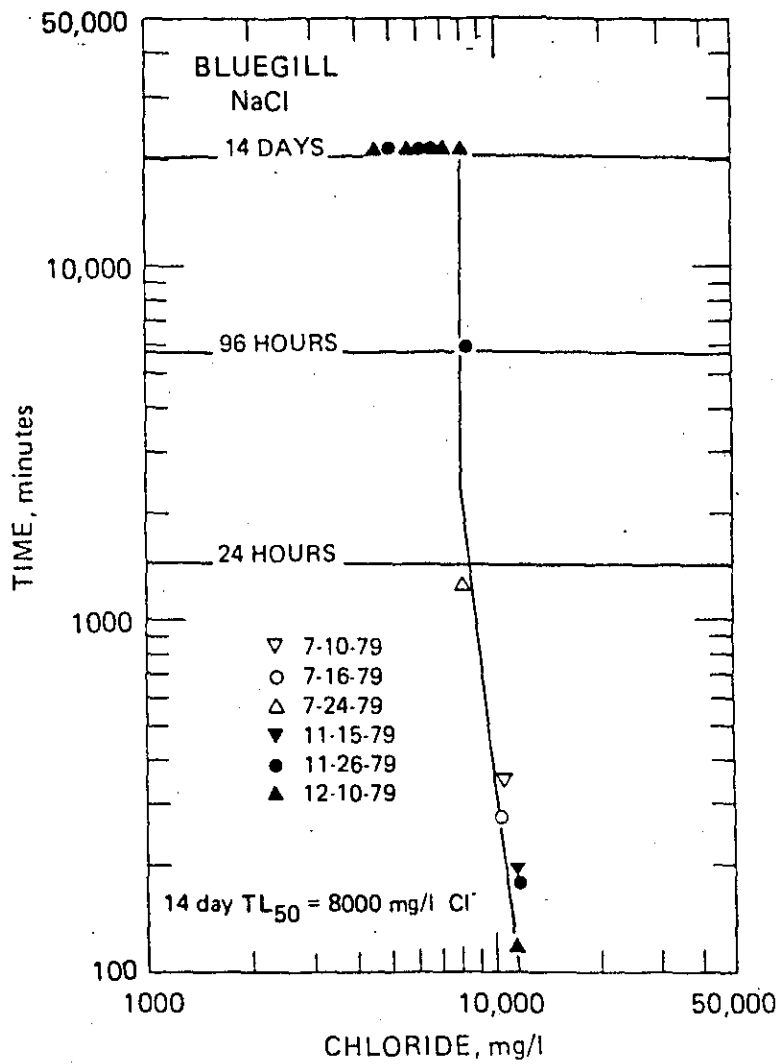
24
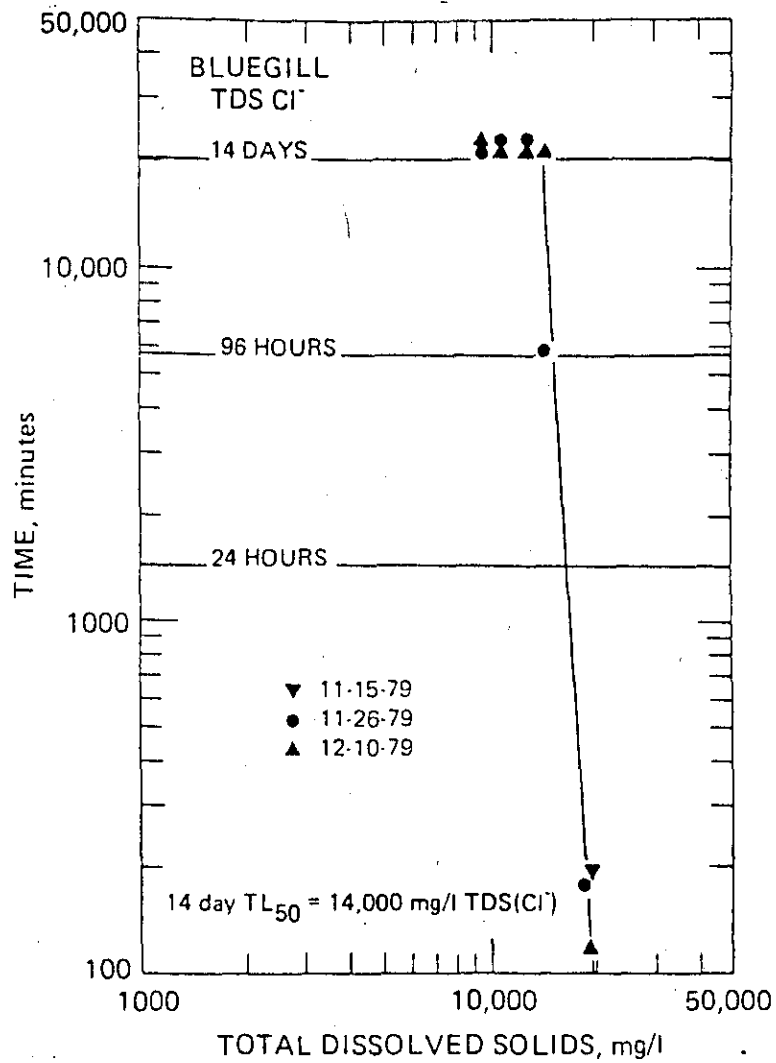
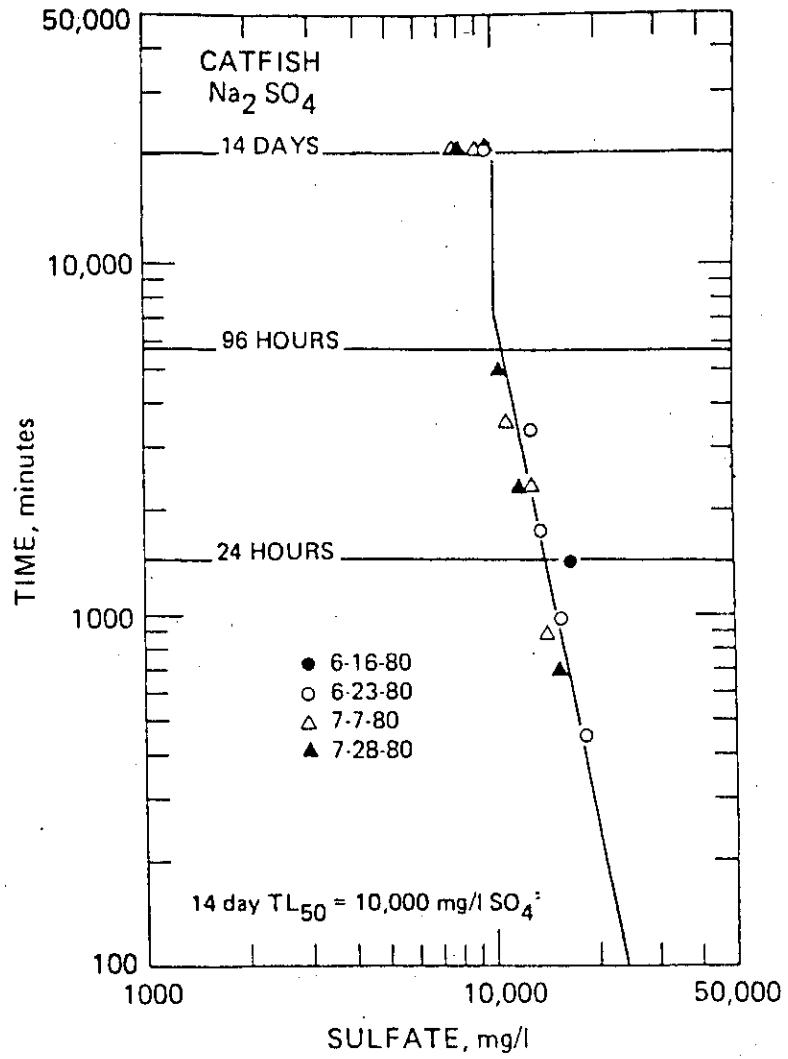*Figure 10. Acute toxicity curve for bluegill (TDS-Cl⁻)*
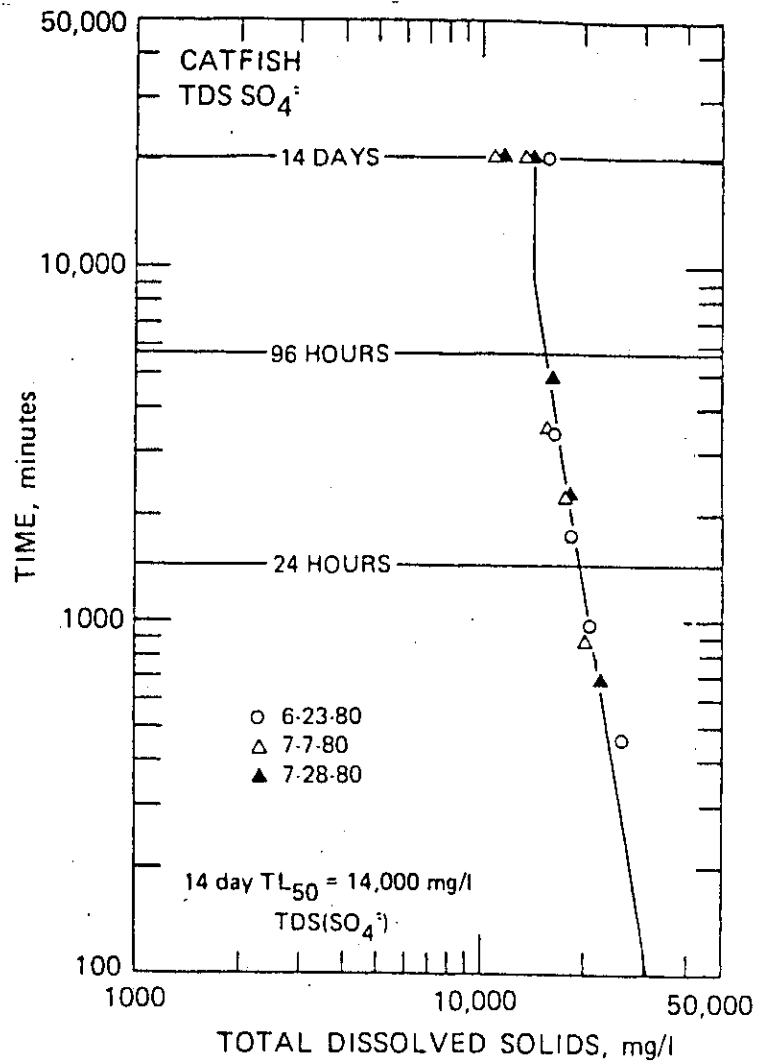
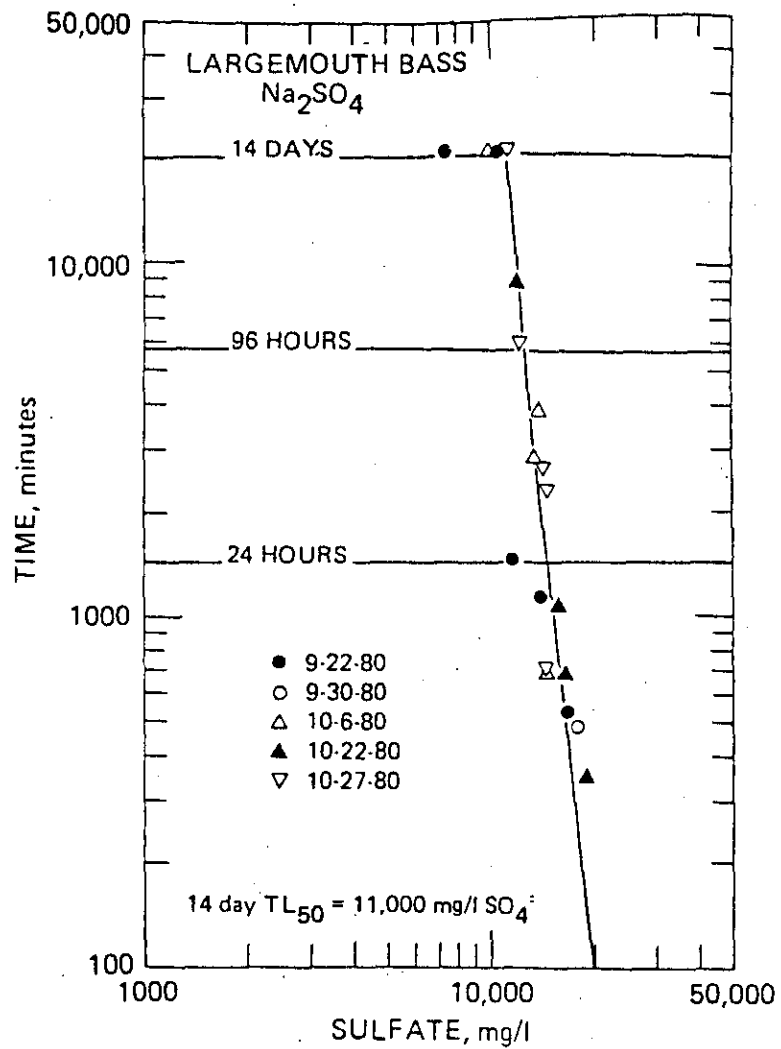*Figure 11. Acute toxicity curve for channel catfish (TDS-$SO_4^=$)*
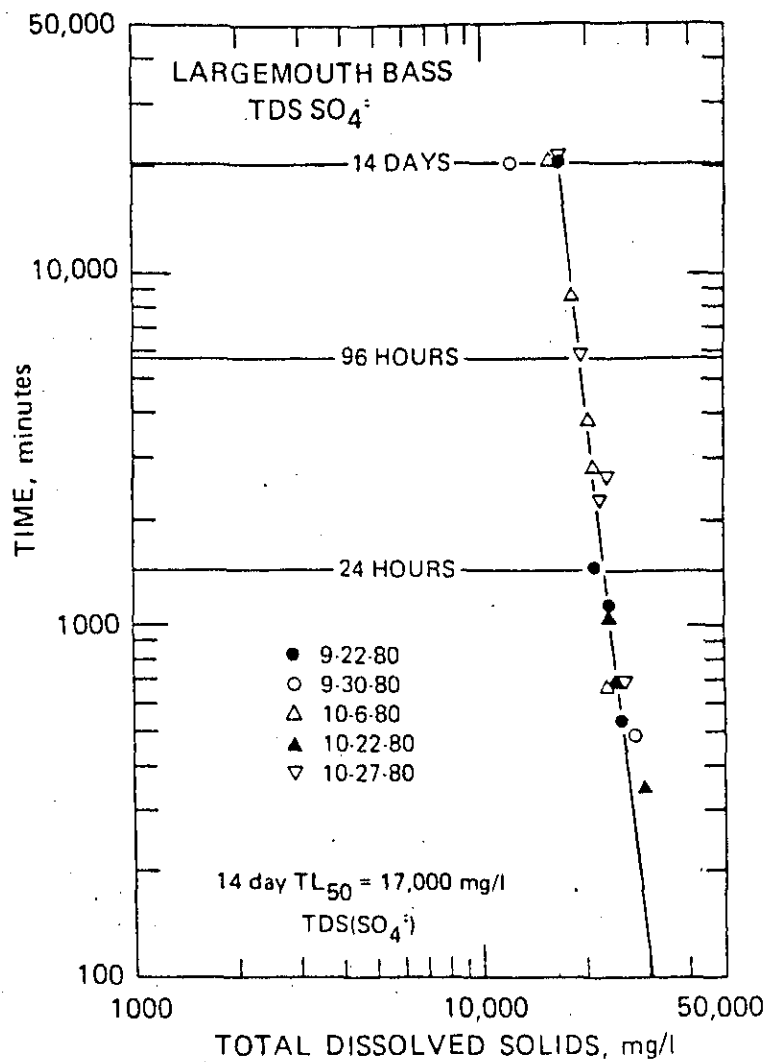
*Figure 12. Acute toxicity curve for largemouth bass (TDS-SO$_4^=$)*
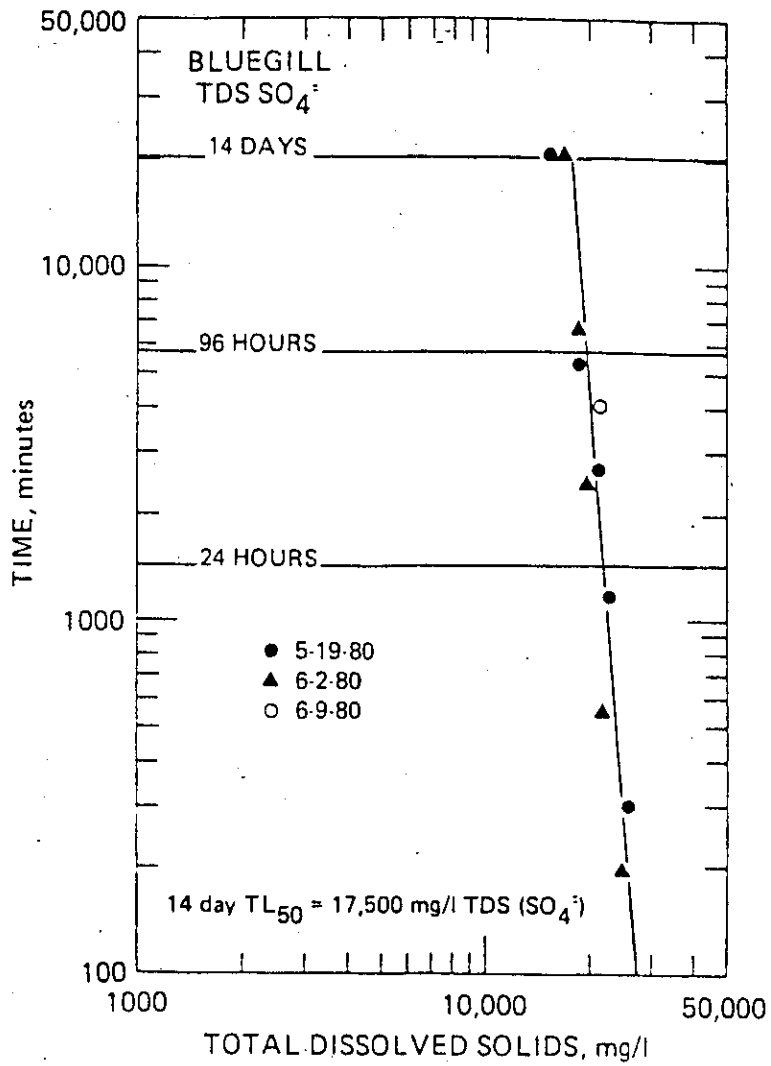
Figure 13. *Acute toxicity curve for bluegill (TDS-SO$_4^=$)*

SUMMARY AND CONCLUSIONS

In developing this summary the factor of 1/10 has been applied to the observed TL50s produced by this study.

- Channel catfish fingerlings, largemouth bass fingerlings, and bluegill fry were subjected to varying concentrations of chlorides and sulfates at water temperatures of about $20°C$ in waters relatively high in alkalinity and the salts of calcium and magnesium.

- Median tolerance limits (TL50) were developed from bioassays performed over a period of 14 days. Resultant toxicity curves permitted the comparison of 24-hr and 96-hr bioassays with the 14-day bioassays.

- The TL50 concentration for chloride ranged from 800 to 850 mg/l. Largemouth bass was the most tolerant of the three species.

- For chloride, there was not a perceptible difference in TL50 concentrations between bioassays with time lengths of 24 hrs, 96 hrs, and 14 days.

- The TL50 concentration for sulfate ranged from 1000 to 1100 mg/l. Largemouth bass was the most tolerant of the three species.

- For sulfate runs there was a difference in TL50 concentrations for time lengths of 24 hrs, 96 hrs, and 14 days. The shorter runs produced more liberal values. For example, the TL50 96-hr concentrations of sulfate ranged from 1100 to 1300 mg/l.

- The TL50 concentration for total dissolved solids where chloride was the principal constituent ranged from 1300 to 1500 mg/l. Channel catfish was the most sensitive of the three fish species.

- The TL50 concentration for total dissolved solids comprised mainly of sulfate ranged from 1400 to 1750 mg/l. Channel catfish was the most sensitive of the three fish species.

The current regulations governing the maximum permissible concentrations of chloride and sulfate in Illinois surface waters (500 mg/l) are more than adequate for the protection of aquatic life. In fact maximum permissible concentrations of 800 mg/l chloride and 1000 mg/l sulfate are more reasonable standards based on the results of this study.

The use of total dissolved solids as an indicator for the protection of aquatic life has little merit without considering the constituent concentrations of the dissolved solids.

29

In terms of relative acute toxicity, fishes are more tolerant to sulfates than chlorides; and generally the channel catfish is more sensitive than largemouth bass or bluegill to total dissolved solids.

The uniform application of the 1/10 factor to all toxic substances is a questionable practice. For some substances it may be too conservative, and for others too liberal. A thorough study of its utility would be worthwhile.

REFERENCES

American Public Health Association, American Water Works Association, and Water Pollution Control Federation. 1975. *Standard methods for the examination of water and wastewater*. American Public Health Association, New York, NY, 1193 p., 14th edition.

Butts, Thomas A., Jack W. Williams, and Ralph L. Evans. 1976. *Water quality evaluation of the Rector Creek -- North Fork Saline River drainage*. Prepared for Soil Conservation Service, U.S. Department of Agriculture, Champaign, IL, by Illinois State Water Survey, 94 p.

Harmeson, Robert H., and T. E. Larson. 1969. *Quality of surface water in Illinois, 1956-1966*. Illinois State Water Survey Bulletin 54, 185 p.

Harmeson, Robert H., T. E. Larson, Laurel M. Henley, R. A. Sinclair, and J. C. Neill. 1973. *Quality of surface water in Illinois, 1966-1971*. Illinois State Water Survey Bulletin 56, 100 p.

Illinois Pollution Control Board rules and regulations. 1977. Chapter 3: Water Pollution (as amended through July 1, 1979). Springfield, IL, 63 p.

Larson, T. E., and B. O. Larson. 1957. *Quality of surface water in Illinois*. Illinois State Water Survey Bulletin 45, 135 p.

Mount, D. I., and W. A. Brungs. 1967. *A simplified dosing apparatus for fish toxicity studies*. Water Research v.1:21-29.

Nienkerk, Monto M., and Ronald C. Flemal. 1976. *Regional distribution of the major dissolved solids in the streams of Illinois*. Water Resources Center, University of Illinois at Urbana-Champaign, Research Report 109, 56 p.

Reed, Paula, Dorothy Richey, and Donald Roseboom. 1980. *Acute toxicity of zinc to some fishes in high alkalinity water*. Illinois State Water Survey Circular 142, 21 p.

Richey, Dorothy, and Donald Roseboom. 1978. *Acute toxicity of copper to some fishes in high alkalinity water*. Illinois State Water Survey Circular 131, 24 p.

Roseboom, Donald P., and Dorothy L. Richey. 1977. *Acute toxicity of residual chlorine and ammonia to some native Illinois fishes.* Illinois State Water Survey Report of Investigation 85, 42 p.

Toler, L. G. 1980. *Some chemical characteristics of mine drainage in Illinois.* U.S. Geological Survey, Open-File Report 80-416, Champaign, IL, 47 p.

Appendix A.   Observations of Percent Bass Mortality,
              Chloride Bioassays*

          Date: 8/6/79
          Average Weight: 0.72 grams
          Water Temperature: 20.8°C

          Chloride (mg/l)   9718   9584
          T.D.S. (mg/l)   DNA   DNA

          % Mortality
              10   434   938
              20   521   1010
              30   576   1067
              40   630   1110
              50   632   1134
              60   675   1164
              70   788   1177
              80   864   1178
              90   866   1570
             100   2131   2131

          Date: 8/8/79
          Average Weight: 0.26 grams
          Water Temperature: 21°C

          Chloride (mg/l)   9665   9713
          T.D.S. (mg/l)   DNA   DNA

          % Mortality
              10   208   295
              20   261   302
              30   294   217
              40   295   218
              50   297   326
              60   302   331
              70   334   361
              80   371   364
              90   382   368
             100   394   382

          * Time of mortality is in minutes
          DNA = data not available

Date: 8/9/79
Average Weight: 0.35 grams
Water Temperature: 21.8°C   -

Chloride (mg/l)	9587	9587
T.D.S (mg/l)	DNA	DNA

% Mortality

10	214	274
20	271	318
30	312	319
40	336	331
50	364	342
60	365	371
70	373	385
80	431	423
90	467	433
100	502	573

Date: 8/13/79
Average Weight: 0.38 grams
Water Temperature: 21.1°C

Chloride (mg/l)	10199	10442	10413	10510	10753	10947	7593	7638
T.D.S. (mg/l)	DNA	DNA	DNA	DNA	DNA	DNA	DNA	DNA

% Mortality

10	153	130	125	133	113	115	--	--
20	154	144	133	136	115	118		
30	160	178	138	162	173	170		
40	177	215	142	171	174	178		
50	184	224	161	172	201	181		
60	205	225	173	177	216	185		
70	226	235	174	187	219	193		
80	227	246	228	243	239	210		
90	280	270	229	254	241	217		
100	312	312	230	260	280	237		

Date: 8/27/79
Average Weight: 0.72 grams
Water Temperature: 21.6°C

Chloride (mg/l)	9262	9493	9480	9393	6119	6127
T.D.S (mg/l)	DNA	DNA	DNA	DNA	DNA	DNA

% Mortality

10	201	255	240	243	--	--
20	310	310	241	244		
30	313	343	248	274		
40	324	355	260	299		
50	362	386	279	311		
60	378	413	315	312		
70	488	454	353	359		
80	494	559	521	370		
90	744	731	529	401		
100	818	1005	530	461		

Date: 10/22/79
Average Weight: DNA
Water Temperature: 20.6°C

Chloride (mg/l)	14075	14075	12647	12375	10549	10490
T.D.S (mg/l)	21313	21481	DNA	DNA	17881	18192

% Mortality

10	95	95	144	185	182	161
20	119	111	161	199	204	162
30	120	125	173	213	237	218
40	125	126	177	219	242	222
50	127	130	197	252	253	267
60	128	131	198	257	267	321
70	129	132	202	261	274	322
80	139	135	204	295	284	337
90	148	136	219	315	385	406
100	149	137	220	331	406	528

Date: 11/5/79
Average Weight: 2.11 grams
Water Temperature 20.5°C

Chloride (mg/l)	15308	14745	12346	12934	11621	11809
T.D.S (mg/l)	24393	24529	20492	20488	18975	18780

% Mortality						
10	69	73	103	88	163	184
20	71	74	107	125	207	186
30	72	82	128	126	209	187
40	73	83	130	138	216	199
50	77	84	131	145	222	204
60	78	85	139	153	226	208
70	80	86	164	154	253	217
80	90	87	165	160	261	223
90	91	88	170	173	271	225
100	101	89	182	182	273	241

Date: 11/12/79
Average Weight: 2.02 grams
Water Temperature: 20.5°C

Chloride (mg/l)	9847	9647
T.D.S (mg/l)	16111	16178

% Mortality		
10	317	345
20	331	358
30	337	405
40	357	425
50	427	564
60	531	571
70	715	674
80	806	701
90	843	739
100	1475	949

. Appendix A.   Continued

Date: 1/21/80
Average Weight: 3.59 grams
Water Temperature: 20.4°C

Chloride (mg/l)	10973	11067	8582	8938	8077	7955	6439	6534	5400	5358
T.D.S (mg/l)	19158	19136	14663	14677	13679	13639	11632	11729	9811	9741

% Mortality										
10	169	152	7230	9028	8305	9030	--	--	--	--
20	171	165	7310	9028						
30	174	175	8304	9028						
40	177	178	8305	9028						
50	181	189	9028	9038						
60	194	191	9788	10016						
70	199	192		10608						
80	206	195		10608						
90	236	196								
100	240	242								

Date: 2/4/80
Average Weight: 4.39 grams
Water Temperature: 20.1°C

Chloride (mg/l)	11371	11184	9078	9499	8709	8725
T.D.S (mg/l)	18869	18814	15858	15858	14662	14624

% Mortality						
10	112	172	300	269	712	466
20	138	183	466	369	976	805
30	170	186	467	466	1148	849
40	186	191	575	488	1379	1014
50	204	203	611	527	1911	1030
60	222	205	651	620	1911	1370
70	223	236	757	635	2890	2517
80	234	237	903	1212	3440	
90	239	251	933	1427	5690	
100	279	252	1200	2755		

Date: 11/10/80
Average Weight: 1.92 grams
Water Temperature: 19.5 C

Chloride (mg/l)	14126	14065	11924	11924	10665	10763	8452	8462	6170	6140
T.D.S (mg/l)	23240	23289	18942	1826	16880	17344	13960	13865	10932	10889

% Mortality

10	94	97	161	175	366	256	--	--	--	--
20	110	101	172	176	367	256				
30	113	106	177	194	368	266				
40	114	108	180	194	372	283				
50	120	110	193	204	394	289				
60	121	116	198	207	400	289				
70	124	117	199	210	415	320				
80	126	123	221	245	415	468				
90	126	128	225	253	421	468				
100	133	133	320	253	531	646				

Date: 12/2/80
Average Weight: 2.26 grams
Water Temperature: 18.5°C

Chloride (mg/l)	14432	14371	12597	11558	9726	9626	9131	9038	6418	6363
T.D.S. (mg/l)	23409	23437	18984	18976	15866	15805	15093	14965	11342	11278

% Mortality

10	89	127	185	228	761	1016	6888	6888	--	--
20	95	133	200	239	784	4702	8938	8084		
30	100	137	205	240	2846	5410		11031		
40	109	139	205	245	4606	5410		11031		
50	109	144	212	246	5410	5410		11031		
60	110	157	215	257	5410	6888		16028		
70	110	159	220	264	5410	6888				
80	133	159	241	267	8084	6888				
90	134	165	248	281						
100	145	187	253	313						

Appendix B. Observations of Percent Bluegill Mortality,
Chloride Bioassays*

Date: 7/10/79
Average Weight: -2.79 grams
Water Temperature: 22.5°C

Chloride (mg/l)	10690	10597
T.D.S (mg/l)	DNA	DNA

% Mortality

10	281	200
20	307	313
30	314	337
40	315	338
50	340	342
60	354	343
70	410	389
80	474	465
90	482	471
100	545	529

Date: 7/16/79
Average Weight: 4.51 grams
Water Temperature: 23.5°C

Chloride (mg/l)	10704	9878
T.D.S. (mg/l)	DNA	DNA

% Mortality

10	167	252
20	179	262
30	185	284
40	194	330
50	199	343
60	240	364
70	248	366
80	254	392
90	266	475
100	276	864

* Time of mortality is in minutes
DNA = data not available

Date: 7/24/79
Average Weight: 7.24 grams
Water Temperature: 21.7°C

Chloride (mg/l)	9103	9161
T.D.S (mg/l)	DNA	DNA

% Mortality

10	680	1017
20	811	1042
30	858	1128
40	894	1187
50	902	1344
60	1133	1516
70	1187	1610
80	1798	1999
90	2536	3100
100	2536	

Date: 11/15/79
Average Weight: 2.13 grams
Water Temperature: 20.0°C

Chloride (mg/l)	11646	11446
T.D.S (mg/l)	19161	19036

% Mortality

10	153	173
20	157	181
30	165	201
40	174	204
50	185	208
60	187	212
70	189	221
80	204	225
90	209	249
100	218	290

Date: 11/26/79
Average Weight: 2.31 grams
Water Temperature: 20.6°C

Chloride (mg/l) 11546 11546 8287 8244 6648 7376 6606 6622 5303 5277
T.D.S (mg/l)   18547 18549 14596 14609 13333 13283 11219 11251 9555 9434

% Mortality
  10   157 152   694 2339
  20   159 153 2596 2340
  30   160 514 2855 4053
  40   177 156 4279 4219
  50   186 167 6657 6657
  60   189 174 6658 9520
  70   197 187 11950 9521
  80   206 196
  90   214 201
 100   232 231

Date: 12/10/79
Average Weight: 0.34 grams
Water Temperature: 20.4°C

Chloride (mg/l) 11184 11231 8212 8166 7425 7518 6150 6173 5151 5105
T.D.S. (mg/l)   19143 19119 14208 14207 13043 12956 11220 11285 9541 9378

% Mortality
  10   94 80 5470   429 17095 4274 12080
  20   96 91 7000 3442 19850 8606
  30   99 109 18430 3512   19850
  40   111 110
  50   128 114
  60   132 126
  70   136 145
  80   139 146
  90   142 149
 100   181 155

Appendix C.   Observations of Percent Catfish Mortality,
Chloride Bioassays*

Date: 8/18/80
Average Weight: 1.54 grams
Water Temperature: 17.8°C

Chloride (mg/l)	13272	13783	11167	11103	9878	9954	7626	7795	5289	5175
T.D.S. (mg/l)	21144	21265	17231	17264	15428	15306	12396	12441	9206	8899

% Mortality

10	150	150	241	303	500	568	7372	15735		
20	163	169	276	303	502	672	10153			
30	165	174	303	316	587	676				
40	172	179	346	323	709	724				
50	172	180	347	323	743	744				
60	177	180	356	356	743	749				
70	182	187	358	363	746	770				
80	184	191	369	425	747	770				
90	198	191	425	438	769	1065				
100	210	194	488	460	975	1180				

Date: 9/2/80
Average Weight: 2.37 grams
Water Temperature: 19.9°C

Chloride (mg/l)	13151	13088	11389	10508	9439	9489	7704	7626	5185	5077
T.D.S. (mg/l)	20566	20618	16671	16718	15124	15086	12253	12108	8951	8721

% Mortality

10	148	153	264	301	467	600				
20	152	153	287	303	490	604				
30	160	153	320	313	508	749				
40	167	163	325	320	549	778				
50	175	165	341	325	600	779				
60	180	167	343	325	631	782				
70	187	174	360	325	705	836				
80	187	174	365	339	793	852				
90	203	174	374	400	820	860				
100	210	190	385	414	890	927				

* Time of mortality is in minutes
DNA = data not available

42

Date: 9/9/80
Average Weight: 3.51 grams
Water Temperature: 20.1°C

| Chloride (mg/l) | 13340 | 13592 |
| T.D.S. (mg/l) | 21287 | 21303 |

% Mortality

10	128	117
20	149	147
30	152	153
40	160	155
50	162	156
60	163	157
70	164	159
80	166	164
90	166	164
100	174	164

Appendix D. Observations of Percent Bass Mortality, Sulfate Bioassays*

Date: 9/22/80
Average Weight: 1.24 grams
Average Temperature: 20.2°C

Sulfate (mg/l)	17484	16468	14031	14132	10984	12406	10210	9907	7870	7556
T.D.S (mg/l)	25351	25469	20668	20635	21820	21837	17073	16992	13499	13321

% Mortality

10	275	402	957	866	1197	1171		2137		
20	416	411	1065	889	1220	1340		3819		
30	466	579	1129	956	1340	1377				
40	467	642	1130	1001	1386	1511				
50	526	661	1197	1171	1400	1607				
60	528	698	1220	1197	1438	1623				
70	588	730	1351	1220	1473	1666				
80	621	743	1367	1243	1717	1723				
90	622	771	1438	1438	1753	1753				
100	640	870	1511	1608	2292	1834				

Date: 9/30/80
Average Weight: 1.30 grams
Average Temperature: 21.6°C

Sulfate (mg/l)	18868	16547
T.D.S (mg/l)	27275	27277

% Mortality

10	171	258
20	303	303
30	343	357
40	431	420
50	621	431
60	701	572
70	832	638
80	876	700
90	941	741
100	1521	950

* Time of mortality
D/A - Data not available

Date: 10/27/80
Average Weight: 1.77 grams
Average Temperature: 19.9°C

Sulfate (mg/l) 14839 14208 14907 13916 14804 14647 12166 11950 10479 10323
T.D.S. (mg/l) 25941 25986 23424 23421 22237 23043 19934 19752 17186 17183

% Mortality

% Mortality										
10	352	325	1541	2297	1571	911	3554	3554	8150	5236
20	528	618	1702	2297	1896	1267	4284	4572		5909
30	541	688	2253	2297	2253	2253	4572	5236		
40	631	722	2253	2297	2253	2253	5236	5874		
50	688	753	2253	2954	2253	2253	6773	6217		
60	893	755	2920	3146	2253	3003	6773	6217		
70	965	817	3141	3165	2857	3285	6773	6773		
80	1019	846	3213	3706	2900	3353		8150		
90	1063	933	3730	3706	2973	3800				
100	1235	1235	4329	4383	3610	4549				

Appendix E. Observations of Percent Bluegill Mortality,
Sulfate Bioassays*

Date: 5/19/80
Average Weight: 0.65 grams
Average Temperature: 21.0°C

Sulfate (mg/l)	15806	17483	14789	16059	13533	14430	11861	11741	9801	9850
T.D.S. (mg/l)	26024	25963	22224	23589	21102	20973	18608	18594	15527	15400

% Mortality

10	106	208	713	138	772	204	1060	715	920	
20	127	225	1060	355	1210	1302	4227	934	9314	
30	158	242	1077	557	1230	1652	4361	5934		
40	216	253	2709	576	1565	2235	5561	6985		
50	228	266	2829	655	1769	4130	6098	7253		
60	333	386	2836	810	5707	6985	8004	9237		
70	652	476	2985	825	6985	8500	8473	9237		
80	675	435	4211	908	7253	9237	9237	10628		
90	854	487	6810	1302	7253	9237	10628	17726		
100	880	743	6810	4130	9237	10628				

Date: 6/2/80
Average Weight: 0.59 grams
Average Temperature: 21.7°C

Sulfate (mg/l)	17296	18009	15565	14546	11956	12058	11131	11477	9730	9418
T.D.S. (mg/l)	23143	26611	21901	22057	19816	19409	18399	18437	15651	15460

% Mortality

10	122	84	424	235	1422	768	712	1945		
20	156	123	500	345	1945	769	1270	1945		
30	201	145	501	346	1945	801	6700	2496		
40	214	167	598	348	2610	1031	8179	9121		
50	218	187	705	495	3387	1409	12483	10060		
60	231	188	712	503	4824	1945	12922	14701		
70	266	238	712	523	8179	1945	15800	15800		
80	268	239	713	801	8180	3387	15800	15800		
90	370	313	767	890	8891	9121	15918	15800		
100	480	399	1407	2402	9800	11213	16618			

* Time of mortality
DNA = data not available

47

Date: 6/9/80
Average Weight: 1.09 grams
Average Temperature: 20.8°C

Sulfate (mg/l)	13483	13844
T.D.S. (mg/l)	21467	21456

% Mortality

10	2359	2770
20	3350	3350
30	3350	3350
40	3925	3350
50	4346	3350
60	5227	4577
70	5227	4577
80	5227	5676
90	5227	5676
100	5804	6500

Appendix F. Observations of Percent Catfish Mortality, Sulfate Bioassays*

Date: 6/16/80
Average Weight: 1.01 grams
Average Temperature: 21.4°C

Sulfate (mg/l)	15426	18205
T.D.S. (mg/l)	DNA	DNA

% Mortality

%		
10	250	310
20	280	378
30	290	434
40	333	464
50	407	497
60	407	517
70	424	517
80	464	549
90	464	549
100	655	826

Date: 6/23/80
Average Weight: 1.28 grams
Average Temperature: 20.8°C

Sulfate (mg/l)	16840	19245	15587	15377	13705	13496	13287	12555	9032	9959
T.D.S. (mg/l)	25968	25947	21105	20585	18625	17656	16329	16406	13877	13966

% Mortality

%										
10	322	328	513	740	619	1102	1333	914	9190	6309
20	339	386	619	889	889	1228	2071	2071	9959	9190
30	386	440	831	889	914	2071	2899	2071		10508
40	409	440	1000	934	1333	2071	3540	2629		14074
50	409	509	1039	946	1333	2071	3540	4970		
60	440	548	1041	1039	2071	2071	4003	4970		
70	440	551	1168	1043	2071	2071	4970	5618		
80	447	621	1401	1212	2071	3540	5822	5724		
90	457	646	1441	1212	2071	3540	5896	6291		
100	718	889	2071	1333	4970	5500	6377	9190		

* Time of mortality
DNA = data not available