Water Pollution Studies

Federal Aid Project F-243-R14

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Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Division of Wildlife

Fish Research Section

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The results of the research investigations contained in this report represent work of the authors and may or may not have been implemented as Division of Wildlife policy by the Director or the Wildlife Commission.

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State: Colorado

Study No. F243R-11

Title: Water Pollution Studies

Period Covered: July 1, 2006 to June 30, 2007

Project Objective: To develop quantitative chemical and toxicological data on the toxicity of pollutants to aquatic life, investigate water pollution problems in the field, and provide expertise in aquatic chemistry and aquatic toxicology.

STUDY PLAN A: TOXICITY STUDIES

Job A.1. Feminization of Fish by Wastewater Treatment Plant Effluents

Job Objective:

Determine whether feminization of rainbow trout and/or fathead minnows occurs following exposure to wastewater treatment plant effluents and/or receiving waters. If effects are found, tests will be conducted to measure the relative magnitude of feminization. Attempts will be made to identify possible compounds contributing to estrogenic activity and estimates made on the contribution of each compound. Feminized fathead minnows will be raised to sexual maturity and spawned to determine reproductive effects of exposure to estrogenic compounds.

Job A.2. Toxicity of Metals to Fish

Job Objective:

Measure the acute (96 hour) and chronic (60 day) effects of zinc, copper and/or cadmium exposure on hatching, survival and growth of different life stages of mottled sculpin, longnose dace and/or other sensitive species. Results from these experiments will compare toxicity thresholds to USEPA metal criteria to ensure that these species are protected.

Job A.3. Effects of Dietary Exposure of Metals to Fish

Job Objective:

Measure the effect of zinc, copper, cadmium and/or selenium from dietary sources

on the survival and growth of fish in the laboratory. Evaluate the sensitivity of dietary-exposed organisms to waterborne exposure. Relate dietary levels that cause diminished performance in the laboratory with levels found in dietary sources in metal impacted areas such as the upper Arkansas River, Clear Creek and the Eagle River.

Job A.4. Toxicity of Unionized Ammonia to Fish at Cold Water Temperatures

Job Objective:

Determine effects of temperature on toxicity of unionized ammonia to rainbow trout and fathead minnows, or to other warm-water species at optimal and very cold (less than 5ºC) water temperatures.

STUDY PLAN B: TECHNICAL ASSISTANCE

Job B.1. Development of a Field Test for Rotenone

Job Objective:

To develop a test for rotenone that can measure sub-piscidal concentrations in water, can be completed in an hour, and can be used in the field.

Job B.2. Water Quality Assistance to Division of Wildlife Personnel and Other State and Federal Agencies

Job Objective:

To provide expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Division of Wildlife and other state and federal personnel as requested. Conduct short or long term experiments to produce toxicity data when such data in the literature are lacking or inadequate.

Job B.3. Regulatory and Legal Assistance

Job Objective:

To provide technical assistance to legal and regulatory agencies toward the development, implementation, and enforcement of water quality standards needed to protect or enhance the aquatic resources of Colorado.

ACCOMPLISHMENTS

Job A.1.

The project continues to provide equipment and support for an onsite bioassays conducted by the University of Colorado. The objective of the study is to detect and quantify estrogenic activity in wastewater treatment plant effluents from the cities of Boulder and Vail. Exposures are finished and fish are currently being processed for changes in biochemistry and histopathology as a result of exposures.

A graduate student at Colorado State University, funded by DOW Aquatic Research, continues to study the effects of 17-β estradiol (E_2) on the reproduction of the red shiner (*Cyprinella lutrensis*). Experiments have been conducted to quantify changes in histopathology of male gonads, male mating behavior, and male secondary sexual characteristics (body color and tubercle number and size). The preliminary results of these experiments have been presented at the Colorado/Wyoming Chapter Meeting of the American Fishery Society and at the Chapter meeting for the Society for Environmental Toxicology and Chemistry (see abstract below). Currently, experiments are being conducted to determine the effects of estrogen exposure on male and female mating behavior during spawning, and the resulting egg quality and quantity, and hatching success after spawning.

Title: The effects of an estrogenic endocrine disruptor on male red shiner (*Cyprinella lutrensis*) reproduction: behavior, secondary sexual traits, development, and productivity

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Abstract: Endocrine disrupting compounds (EDC's) are found worldwide in both aquatic and terrestrial ecosystems and can lead to developmental and reproductive disruption in fishes. The estrogenic EDC 17β-estradiol (E2) is a natural hormone found in most wastewater effluent-treated waters. We exposed adult male red shiners *Cyprinella lutrensis* to E2 at concentrations of 2.4 ng/L and 120 ng/L. After 4 weeks of exposure, mate choice tests were conducted, male mating behavior was assessed, and morphological and histopathological characteristics of males were measured. Plasma concentrations of vitellogenin and hepatosomatic index values were higher in exposed males, indicating EDC exposure. We observed reduced male mating behaviors, fewer and less developed nuptial tubercles, and reduced spawning coloration at the highest concentration of E2. Spermatogenesis was reduced and gonadosomatic index was lower in exposed males. Changes in behavior, secondary sexual traits, and male reproductive development may influence mating opportunities and success. Subsequently, we studied the reproductive productivity of males exposed to E2 at a concentration of 120 ng/L by measuring the number of fertilized eggs and hatching larvae. Our studies suggest that exposure to E2

influences reproductive behavior, secondary sexual traits, developmental processes, and productivity, potentially having consequences for population growth.

Job A.2.

Efforts to develop a reproducing laboratory culture of mottled sculpin have been unsuccessful. Manipulation of temperature and photoperiod resulted in formation of spawning colors in the males and eggs in females. However, aggressive and territorial behavior of males guarding artificial nests resulted in the deaths of several females. Remaining individuals were anesthetized with MS222 and injected with chorionic gonadotropin. Eggs were removed from females seven days later and mixed with macerated testis in milt extender. The eggs were treated with 2000 ppm formalin for 15 minutes and incubated in a McDonald jar. Despite the formalin treatment, all eggs became infected with fungus.

Field-collected young-of-year mottled sculpin from the Dolores River and White River were used to investigate the acute toxicity of zinc, cadmium and copper. Toxicity of zinc to field-collected young-of-year longnose dace was also determined. ***Results of toxicity tests are presented below.***

Job A.3.

Two experiments were initiated during this segment to investigate the toxicity of dietary selenium to fathead minnows and Arkansas darters. Results from the tests, which are ongoing, will be presented next segment.

Job A.4.

No activities during this segment.

Job B.1.

No activities during this segment

Job B.2.

Toxicity tests were conducted to measure the toxicity of zinc, copper, and cadmium to the mayfly *Rhithrogena hageni*. Toxicity of zinc to several other mayfly, stonefly and caddisfly taxa was also determined. The objectives of the tests were to evaluate the protectiveness of USEPA criteria and to create data for use in developing site-specific water quality standards for protection of aquatic organisms. Results are reported below. CDOW also conducted a study to evaluate the use of brown trout metrics as biocriteria for development of metals standards in the Eagle River and upper Arkansas River (***see report to EPA presented below***).

Job B.3.

DOW participated as Party Status in several Water Quality Control Commission Rulemaking and Administrative Action Hearings, including the Rulemaking Hearing for Temperature Standards (January 8, 2007) and the Rulemaking Hearing for the Arkansas and Rio Grande River basins (June 12, 2007). We have also participated in workgroups associated with these and other water quality issues, including the Water Quality Forum, the Temperature Group, the Aquatic Life Workgroup, and the Consortium for Research and Education on Emerging Contaminants (CREEC). We continue to serve on BTAG (Biological Technical Assistance Group) committees for the Arkansas River mine site and for the Standard Mine on Coal Creek near Crested Butte, where we provide expertise and data. We represent DOW on CDPHE's Technical Advisory Committee for mercury contamination in fish tissues. Mercury action limits are being set and protocols for notifying the public of potential health hazards are being developed. We assisted DOW biologists in coordinating their fish collection with CDPHE chemical analysts to assess risks to anglers at numerous reservoirs around the State. DOW also presented our role in the mercury issues to the South Platte Watershed Forum in October 2006.

DOW worked with the CDPHE, EPA and the Attorney General's Office other water quality issues, including Natural Resource Damage Claims for the upper Arkansas River and the Rocky Mountain Arsenal superfund sites, and development of an updated "Fish Kill and Pollution Spills" Directive for our agency. DOW wrote several letters of support for academic researchers and agencies who are seeking nationally-sponsored funding to conduct experiments with heavy metals and endocrine disruptors.

Toxicity of Copper, Cadmium and Zinc to Benthic Invertebrates

ABSTRACT

Case studies of rivers in the Rocky Mountain region have found reduced mayfly abundances immediately downstream of point-source inputs of metals. However, little laboratory data exist that could be used in the development of metal criteria and standards to protect these organisms. Heptageniid mayfly nymphs have been suggested as sensitive indicators of metal contamination in streams based on biomonitoring studies, experimentation *in situ* and experimentation in microcosms. This report describes laboratory tests conducted to evaluate the sensitivity of *Rhithrogena hageni*, a heptageniid mayfly, to waterborne copper, cadmium, and zinc. Zinc toxicity tests were also conducted with nymphs of Ephemeroptera (*Baetis tricaudatus*, *Drunella doddsi*, *Drunella grandis*, *Cinygmula* sp., *Epeorus* sp.,and *Ephemerella* sp.), Plecoptera (Chloroperlidae and *Capnia* sp.) and Trichoptera (*Lepidostoma* sp.). Tests were conducted in soft water (hardness = 40-50 mg/L) at about 12°C. Toxicity endpoints were survival and frequency of moulting.

Three zinc toxicity tests were conducted with *R. hageni*. Median 96 hr lethal concentrations (LC_{50}) for the zinc toxicity tests were >16.4, 39.2, and 50.5 mg/L. Copper and cadmium LC_{50}s were 0.137 and 10.5 mg/L, respectively. Mortality continued after the initial 96 hours of exposure. The number of moults per survivor per day was significantly decreased by exposure to these metals in solution. In general, concentrations that decreased moulting were similar to concentrations that decreased *R. hageni* nymph survival.

Two zinc toxicity tests were conducted with *Baetis tricaudatus*. The median lethal concentrations after 96 hours were 10.0 mg/L and 13.2 mg/L. Two tests with *Capnia* sp. had 96 hr LC_{50} values of 5.37 and 6.04 mg/L. *Cinygmula* sp. had a 96 hr LC_{50} of 68.8 mg/L. In several tests, 96 hr LC_{50} values for zinc could not be calculated due to low mortality. As a result, LC_{50}s are expressed as greater than values. These tests included *Ephemerella* sp. (>68.8 mg/L), *Drunella doddsi* (>64.0 mg/L), Chloroperlidae (>68.8 mg/L), and *Lepidostoma* sp. (>48.5 mg/L). Tests conducted with *Drunella grandis* and *Epeorus sp.* were unsuccessful due to excessive mortality in the control group.

Toxicity thresholds were much greater than expected based on invertebrate population responses to metals in biomonitoring and field studies. Possible explanations of the unexpected observations are discussed.

INTRODUCTION

Aquatic insects are often used to assess biological impacts of trace metal pollution. Case studies of contaminated rivers in the Rocky Mountain region have found reduced mayfly abundances immediately downstream of point-source inputs of metals (Winner et al. 1980, Cain et al. 1992, Clements et al. 2000). Heptageniid mayflies have been recognized as especially sensitive to metals in streams (Leland et al. 1989, Peckarsky and Cook 1981, Clements 1994, Clements and Kiffney 1995, Clements et al. 2002) and in microcosm experiments (Clements 2004). *Rhithrogena hageni* has been identified as a strong indicator of metal contamination (Nelson and Roline 1993, Kiffney and Clements 1994; Clements et al. 2000). Kiffney and Clements (1994) found that the concentration of zinc required to reduce heptageniid abundances in stream microcosms was well below the hardness-based ambient aquatic life criterion for zinc (USEPA 1996).

Ambient aquatic life water quality criteria and standards are generally derived from results of laboratory toxicity tests conducted with only one species and one toxicant (USEPA 1985). However, acceptable data from such tests are lacking for mayflies. Results of a single study were used for the development of U.S. aquatic life water quality criteria for cadmium (USEPA 2001). No mayfly toxicity data were used for the development of water quality criteria for copper or zinc (USEPA 1985, USEPA 1996). The objective of this study was to provide toxicity data for use in developing metals criteria and state standards. We conducted a series of experiments to determine the lethality of copper, cadmium and zinc to *R. hageni.* Zinc toxicity tests were also conducted with other common Rocky Mountain aquatic insect fauna, including nymphs of Ephemeroptera (*Baetis tricaudatus*, *Drunella doddsi*, *Drunella grandis*, *Cinygmula* sp., *Epeorus* sp.,and *Ephemerella* sp.), Plecoptera (Chloroperlidae, and *Capnia* sp.) and Trichoptera (*Lepidostoma* sp.). Experiments I-III are described in more detail below.

CULTURE

Initial efforts of this project focused on establishment of a laboratory culture of aquatic nymphs for use in toxicity testing. However, numerous attempts to establish a laboratory culture were unsuccessful. These efforts included attempted collection of fertilized adult females during mayfly hatches and trying to get adult females to oviposit in basins of water near light traps during mayfly hatches. Field-collected nymphs of three species of mayflies (*Baetis tricaudatus, Epeorus longimanus, and Rhithrogena hageni*) were raised in the laboratory to post-emergent adults but attempts to artificially inseminate adults produced eggs that were nonviable. Eggs of *Baetis* sp and Trichoptera sp were scraped from cobble substrate and successfully hatched in large numbers in the laboratory.

Fungal infection was a problem which was solved by treatment of eggs with 2 ppm neutral buffered formalin for 15 minutes. Newly hatched nymphs were fed several types of food including finely ground fish food of several types (including trout chow, algae pellets, flake fish food of several types), spirulina, finely homogenized alfalfa, YTC (yeast-trout chow-cerophil), cultures of the algae chlorella, cultures of the diatom navicula, as well as several mixtures of the above foods. However, in all cases, long-term survival was generally too poor for use in

experiments. The cause of the poor survival is unknown, whether due to a nutritional deficiency, infection, or an abiotic component of culture conditions. As a result of the failure to establish nymph cultures, toxicity tests were conducted using field-collected organisms. Organisms were collected from streams with low or undetectable levels of metals.

I. Acute toxicity of aqueous copper, cadmium, and zinc to the mayfly *Rhithrogena hageni*

Stephen F. Brinkman and Walter D. Johnston

MATERIALS and METHODS

Collection and Handling

Rhithrogena hageni nymphs for the zinc static-renewal test were collected from shallow riffles in the East Fork of the Arkansas River (Lake County CO, USA) in March, 2005. Nymphs for the flow-through tests were collected from the Cache la Poudre River (Larimer County, CO, USA) in October through December of 2005. Analyses of water samples collected from the East Fork of the Arkansas and Cache la Poudre Rivers were below detection limits (<0.2 µg/L Cd, <2.0 µg/L Cu, and <10 µg/L Zn). Cobble substrate was gently removed from the stream and nymphs were collected using small, fine paint brushes. Nymphs were gathered at least 7 days prior to each test. Individuals were identified to genus in the field using a taxonomic key from Ward and Kondratieff (1992). Species identity was verified by two taxonomic experts (Robert Zuellig and Boris Kondratieff, Colorado State University).

Nymphs were transported in a 30-liter cooler along with cobble substrate from the collection site. The transport unit was aerated with airstones connected by nylon tubing to a battery-operated pump. Holding temperature was maintained near ambient conditions (4-6 ºC) during transport to the Colorado Division of Wildlife aquatic toxicology laboratory in Fort Collins, Colorado, USA. Nymphs were carefully transferred with a paint brush to glass holding tanks containing site water. Holding tanks were placed in a temperature-controlled incubator and aerated. Water was gradually replaced (50% per day) with test dilution water and the incubator temperature was gradually increased (2ºC per day) to test temperature (10-12ºC).

Static-Renewal Zinc Test Methods

Dilution water consisted of on-site well water and reverse osmosis water mixed to a hardness of approximately 45 ppm. Exposure water was prepared from an appropriate dilution of stock solution prepared using zinc sulfate heptahydrate. Exposure water was replaced (90%) every 48 hours. Target zinc concentrations were 16.0, 8.0, 4.0, 2.0, 1.0 and 0 mg/L. Each exposure level was replicated twice. Ten mayfly nymphs were randomly assigned to each experimental unit. Nymphs were not fed during the experiments. Mortality, defined as failure to respond to repeated prodding, was observed and recorded every 24 hours. Dead nymphs and moulted exoskeletons (exuvia) were preserved in 70% isopropyl alcohol for later identification.

Exposure chambers consisted of 1.25 L, cylindrical, polypropylene containers equipped with an air-lift system constructed from half-inch polyvinyl chloride (PVC) pipe. Water collected from the center of the container flowed down through the PVC pipe immersed in a

temperature-controlled water bath, then up to the top of the container where an elbow diverted the flow in a circular pattern (Figure I.1). The air-lift maintained dissolved oxygen levels at saturation levels and provided continuous, circular flow in the exposure chamber. Two 5 x 5 cm, unglazed, ceramic tiles were placed in each unit to serve as substrate.

Physical and chemical characteristics of exposure water were measured daily for the first 96 hours of the test. Hardness and alkalinity were determined titrimetrically according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH and temperature. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. Electronic meters were calibrated prior to each use.

Water samples were collected every 48 hours for dissolved metal analysis. Samples were collected from the freshly prepared solutions and from the exposure chambers just prior to renewal. Water was passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH < 2. Metal concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source. Sample splits and spikes were collected and prepared during each sampling event to verify reproducibility and analytical recovery.

Flow-through Test Methods

Two flow-through zinc toxicity tests were conducted. Source water for the zinc toxicity tests consisted of a mixture of onsite well water and reverse osmosis water. A conductivity controller maintained a constant mixture with water hardness near 45 mg/L. Dechlorinated municipal tap water (Fort Collins, CO, USA) was used for the cadmium and copper tests, due to logistical constraints. Both diluent sources had similar water quality characteristics (Table I.1). Source water supplied a continuous-flow serial diluter (Benoit et al. 1982) constructed of teflon, polyethylene, and polypropylene components. The diluter delivered five concentrations of metal toxicant with a 50% dilution ratio and a control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mL/min. Food-grade vinyl tubing delivered test solutions to exposure chambers. Metal stock solutions were prepared by dissolving a calculated amount of metal sulfate salts in deionized water. A concentrated stock solution was delivered to the diluter by peristaltic pump at a rate of 2.0 mL/min. Exposure chambers were the same as used in the static-renewal tests but were modified to allow exposure solution to overflow through a fine nylon screen into a temperature controlled water bath. Two 5 x 5 cm, unglazed, ceramic tiles were placed in each unit to serve as substrate.

The first zinc flow-through zinc test was not replicated and used twelve randomly assigned nymphs per exposure level. The other flow-through tests for zinc, cadmium and copper used four replicates with ten nymphs randomly assigned to each of the 24 exposure chambers. Each chamber was assigned a treatment level using a randomized complete block design, with chamber placement within the water bath as the blocking factor. Mortality, defined as failure to

respond to repeated prodding, and occurrence of exuvia were recorded daily. Nymphs were not fed during the experiments.

Physical and chemical characteristics of exposure water were measured daily for the first 96 hours of the test. Hardness and alkalinity were determined titrimetrically according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH and temperature. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. Electronic meters were calibrated prior to each use.

Water samples were collected daily for dissolved metal analysis during the first 96 hours of the test. Exposure water was passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH < 2. Chambers with no remaining survivors were not sampled. Metal concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source. Sample splits and spikes were collected and prepared during each sampling event to verify reproducibility and analytical recovery.

Median lethal concentrations (LC_{50}) of cadmium and zinc were estimated using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978). Spearman-Karber could not be used to estimate copper LC_{50} due to the nature of the observed concentration- response relationship; therefore probit analysis was used. Both survival at termination and moult frequency (moults per survivor per day) were analyzed with one-way ANOVA. Survival data were arcsine-transformed prior to ANOVA. The assumptions of normal error distribution and homogeneous group variances were tested using Shipiro-Wilk's test and Levene's test, respectively.

Treatment means were compared to the control using Williams' one-tailed test (Williams 1971, Williams 1972) with type-I error rate fixed at 0.05 to determine no-observed-effect concentrations (NOEC) and lowest-observed-effect concentrations (LOEC). When possible, we calculated the geometric mean of the NOEC and LOEC as a coarse estimate of chronic toxicity. Survival data of the cadmium and copper tests did not meet assumptions of normality or homogeneity of variance and were analyzed using Steel's nonparametric Many-One Rank Test.

RESULTS

Except for temperature, site water quality parameters were similar to test conditions (**Table I.1**). Water quality parameters were reasonably consistent among the different metal tests. Standard deviations of each parameter indicate constant water quality within each experiment. Dissolved oxygen in the cadmium test was near saturation (Fort Collins elevation 1520 m) but was not measured in the copper or zinc tests. Mean recovery for the QAQC standard was 101%

(range 97-104%) and for spiked samples was 100% (range 97-102%). Mean percent difference between sample splits was <3.3%. Detection limits for the three metals was <0.01 mg/L.

Zinc

No metal-related mortality was observed in the zinc static-renewal test up to the highest concentration tested (16 mg/L) (**Table I.2**). Substantial loss of zinc occurred between freshly prepared solutions and the solutions renewed 48 hours later.

Measured zinc concentrations in the unreplicated Zn flow-through test I were near target levels and consistent over the course of the experiment (**Table I.3**). After 96 hours, exposure to zinc concentrations ≥15.7 mg/L caused reduced survival. The 96 hour median lethal concentration (LC_{50}) was 39.2 mg/L. Continued exposure to ten days reduced survival to 75% and even lower at zinc concentrations ≥4.27 mg/L. Control survival was 100%.

Measured dissolved zinc concentrations in the second zinc flow-through test, Zn flow-through II, were near target levels (**Table I.4**). After 96 hours of exposure, survival was reduced to 25% at 79.4 mg/L, the highest concentration tested. The 96 hour LC_{50} was 50.5 mg/L. After, the initial 96 hours of exposure, nymph survival continued to decline at the higher zinc concentrations (**Figure I.2**). Survival was significantly reduced at zinc concentrations ≥10.8 mg/L at 10 days of exposure when the test was terminated. Survival in the control exposure was 97.5%. Moult frequency was significantly reduced relative to the controls at zinc concentrations ≥10.8 mg/L (**Figure I.3**). The geometric mean of the zinc NOEC and LOEC was 7.59 mg/L.

Cadmium

Measured dissolved cadmium concentrations were consistent over the duration of the test (**Table I.5**). Measured concentrations were near target concentrations at the lower levels but less than target levels at the higher levels, possibly due to sorption or precipitation of cadmium salts. Survival in the control exposure was 100% at test termination after 10 days (**Table I.5**). The 96-hour cadmium LC_{50} was 10.5 mg/L. After 96 hours of exposure, nymph survival remained high at lower cadmium concentrations but continued to decline at concentrations ≥3.52 mg/L (**Figure I.2**). Nymph survival after 7 days was significantly reduced at concentrations ≥3.52 mg/L. Moult frequency/survivor/day decreased as cadmium concentrations increased and was significantly reduced relative to the controls ($p<0.05$) at cadmium concentrations ≥3.52 mg/L (**Figure I.3**). The geometric mean of cadmium NOEC and LOEC was 2.57 mg/L.

Copper

Measured dissolved copper concentrations were consistent over the duration of the test (**Table I.6**). As observed with the cadmium test, measured concentrations were near target concentrations at the lower levels but less than target at the higher levels. After 96 hours of exposure, all test subjects died at 1.57 mg/L and only a few survived at 0.483 and 0.849 mg/L (**Table I.6**). Survival was near 50% at the lowest copper concentration tested, 0.138 mg/L. The 96-hour LC_{50} was 0.137 mg/L. By the seventh day of exposure, all organisms exposed to copper

died (**Figure I.2**). All nymphs in the control exposure survived. Moult frequency was unaffected by copper concentrations ≤0.256 mg/L but was significantly less than controls at concentrations ≥0.483 mg/L.

Table I.1. Mean (S.D.) water quality parameters of exposure water in cadmium, copper, and zinc toxicity tests and collection site water. ND=not determined.

Hardness (mg/L)	Alkalinity (mg/L)	Temperature (°C)	pH (S.U.)	Dissolved Oxygen (mg/L)
East Fork Arkansas River				
84.0	52.4	5.9	7.70	ND
Static-Renewal Zinc Test				
47.7 (0.9)	41.6 (4.5)	10.4 (0.5)	7.67 (0.06)	ND
Cache la Poudre River				
40.2	36.4	5.0	7.95	ND
Flow-Through Copper Test				
44.0 (3.5)	34.5 (1.0)	11.1 (0.4)	7.72 (0.03)	ND
Flow-Through Cadmium Test				
48.0 (2.0)	34.5 (0.6)	12.0 (0.3)	7.66 (0.10)	9.07 (0.15)
Flow-Through Zinc Test I				
49.8	39.7 (1.6)	12.0 (0.1)	7.64 (0.10)	ND
Flow-Through Zinc Test II				
44.4 (1.1)	39.9 (1.3)	11.9 (0.1)	7.77 (0.07)	ND

Table I.2. Mean dissolved zinc concentrations in freshly prepared and renewed solutions and associated mayfly survival (%) at 96 and 7 days. Standard deviations are in parentheses.

Prepared [Zn] (mg/L)	<0.01 (0.001)	1.06 (0.03)	2.10 (0.05)	4.18 (0.09)	8.28 (0.23)	16.4 (0.73)
Renewed [Zn] (mg/L)	0.02 (0.006)	0.87 (0.06)	1.76 (0.12)	3.89 (0.07)	7.43 (0.48)	15.5 (0.16)
96 hr Survival (%)	100 (0)	95 (7)	100 (0)	100 (0)	90 (14)	100 (0)
7 days Survival (%)	90 (14)	95 (7)	100 (0)	100 (0)	85 (21)	90 (0)

Table I.3. Measured dissolved zinc concentrations (mg/L) and associated survival (%) from Zn flow-through test I. Standard deviations in parentheses.

Target Zn Concentration (mg/L)	0	4	8	16	32	64
Measured Zn Concentration (mg/L)	<10 (2)	4.24 (0.33)	8.44 (0.49)	15.7 (0.70)	30.6 (1.22)	64.0 (3.18)
96-h Survival (%)	100	91.7	100	83.4	58.3	33.3
10-d Survival (%)	100	75.0	75.0	25.0	33.3	0

Table I.4. Measured dissolved zinc concentrations (mg/L) and associated survival (%) and moult frequency/survivor/day of *R. hageni* from Zn flow-through test II. Standard deviations in parentheses.

Target Zn Concentration (mg/L)	0	5	10	20	40	80
Measured Zn Concentration (mg/L)	<0.010	5.33	10.8	21.1	39.5	79.4
96-h Survival (%)	97.5 (5.0)	97.5 (5.0)	87.5 (5.0)	85.0 (10.0)	62.5 (12.6)	25.0 (5.9)
10-d Survival (%)	97.5 (5.0)	85.0 (5.8)	45.0* (5.8)	35.0* (12.9)	30.0* (14.1)	5.0* (10.0)
Moult Frequency (%/survivor/day)	9.7 (0.91)	8.3 (0.91)	6.6* (1.8)	6.1* (1.6)	6.5* (2.2)	1.3* (1.3)

*=Significantly less than control ($p<0.05$)

Table I.5. Measured dissolved cadmium concentrations (mg/L) and associated survival (%) and moult frequency/survivor/day of *R. hageni*. Standard deviations in parentheses.

Target Cd Concentration (mg/L)	0	1	2	4	8	16
Measured Cd Concentration (mg/L)	<0.010 (0.005)	0.963 (0.065)	1.88 (0.12)	3.52 (0.21)	7.02 (0.39)	14.3 (0.74)
96-h Survival (%)	100 (0)	100 (0)	95.0 (10.0)	92.5 (9.6)	60.0 (8.2)	42.5 (12.6)
10-d Survival (%)	100 (0)	95.0 (5.8)	92.5 (9.6)	70.0* (14.1)	10.0* (14.1)	7.5* (5.0)
Moult Frequency (%/survivor/day)	9.3 (2.5)	8.3 (1.7)	7.7 (2.3)	6.6* (2.0)	4.9* (0.5)	4.8* (2.2)

*=Significantly less than control ($p<0.05$)

Table I.6. Measured dissolved copper concentrations (mg/L) and associated survival (%) and moult frequency/survivor/day of *R. hageni*. Standard deviations in parentheses.

Target Cu Concentration (mg/L)	0	0.125	0.25	0.5	1	2
Measured Cu Concentration (mg/L)	<0.010 (.003)	0.138 (.010)	0.256 (.018)	0.483 (.023)	0.849 (.047)	1.57 (0.069)
96-h Survival (%)	100 (0)	47.5 (20.6)	27.5 (12.6)	7.5 (5.0)	2.5 (5.0)	0 (0)
8-d Survival (%)	100 (0)	0* (0)	0* (0)	0* (0)	0* (0)	0* (0)
Moult Frequency (%/survivor/day)	11.7 (1.4)	13.1 (1.3)	10.0 (5.2)	4.3* (3.2)	3.6* (1.5)	7.3* (4.7)

*=Significantly less than control ($p<0.05$)

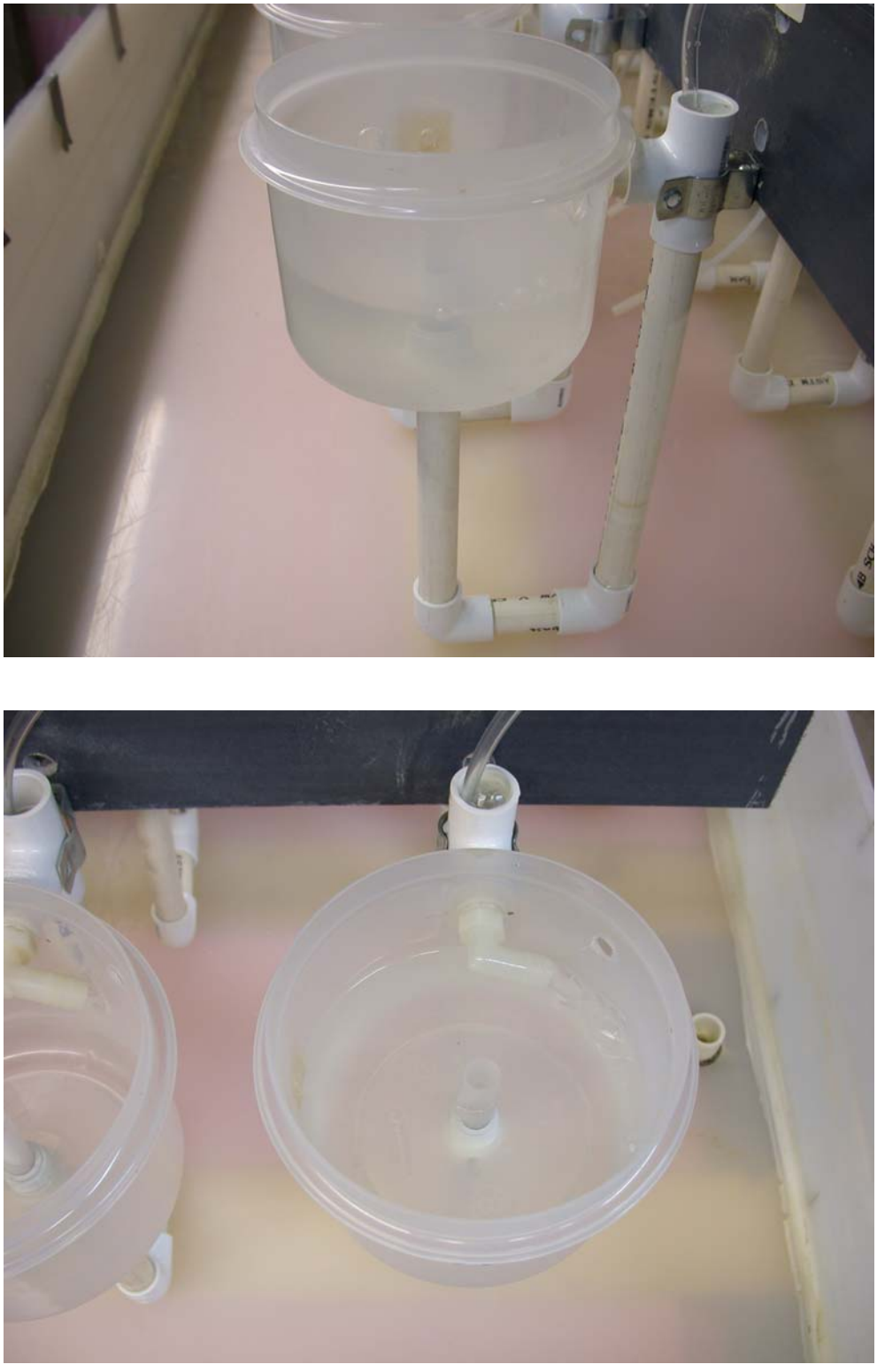


Figure I.1. Flow-through exposure chamber. Aeration in pipe creates an air lift that pumps water to the tee fitting. Exposure solution from air lift creates clockwise circular flow. Screen in center prevents test organisms from getting entrained in air lift.

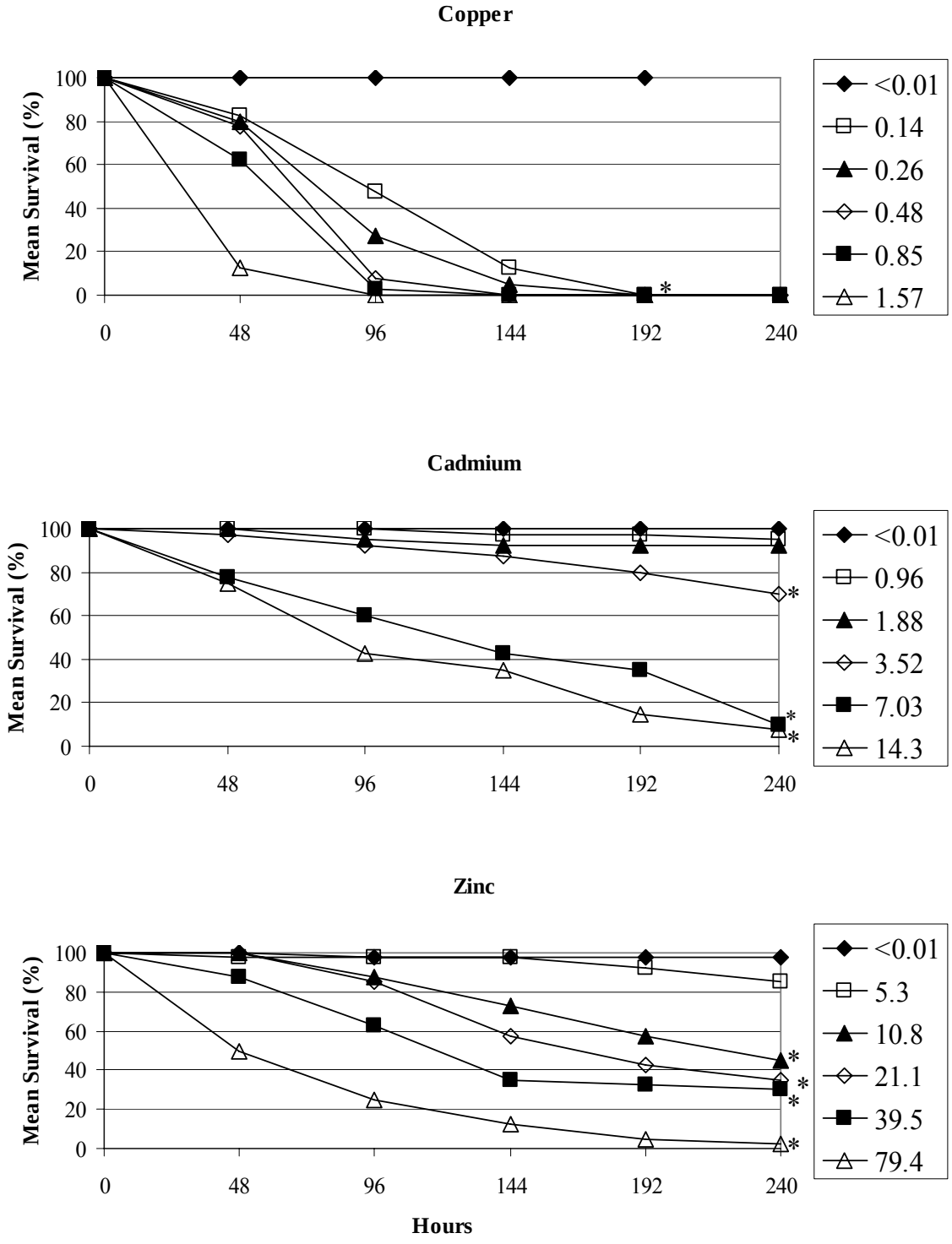


Figure I.2. Mean survival (%) of *R. hageni* versus duration of exposure to copper, cadmium, and zinc (mg/L). Asterisks indicate significantly less than control ($p<0.05$).

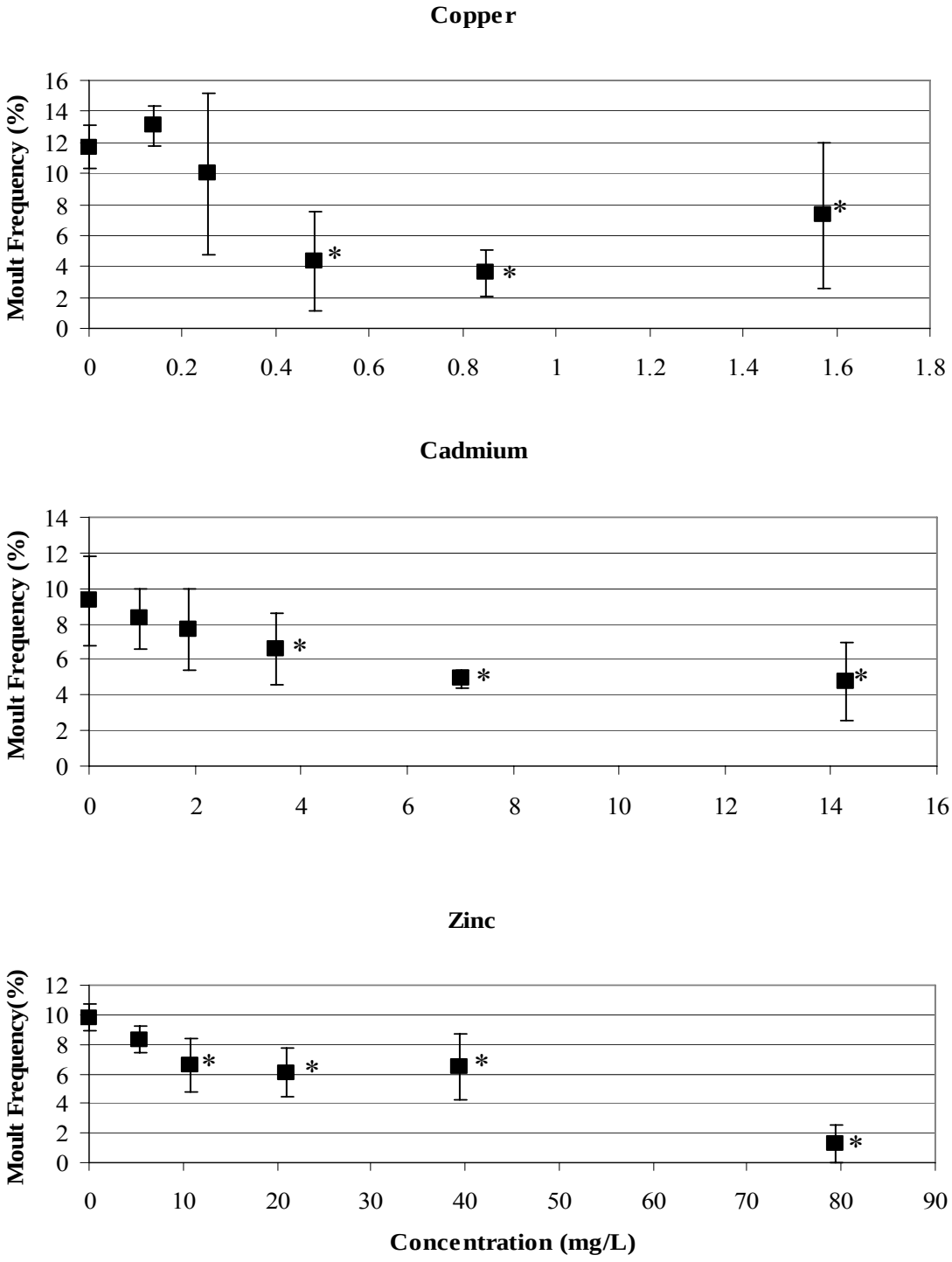


Figure I.3. Moult frequency per day versus concentrations of copper, cadmium, and zinc. Asterisks indicate significantly less than control ($p<0.05$).

II. Acute toxicity of zinc to the nymphs of several mayflies and a stonefly

Stephen F. Brinkman and Walter D. Johnston

MATERIALS and METHODS

Collection and Handling

Nymphs were collected from the Cache la Poudre River, Larimer County, CO which has no history of metal contamination. *Baetis* nymphs were collected in August 2005 and again in September 2005. *Drunella doddsi* and *Drunella grandis* were collected in October 2005. *Ephemerella sp.*, *Cinygmula* sp. and Chloroperlidae nymphs were collected in May 2006.

Cobble substrate was gently removed from the stream and adhered nymphs were collected using small, fine paint brushes. *Baetis* nymphs were sampled using a D-frame net and removed from the benthos with a turkey baster. Nymphs were transported in a modified Coleman cooler with cobble substrate-filled plastic containers. Oxygen and water flow were supplied by a battery-operated pump, flexible tubing and air-stones. Holding temperature was kept near native stream conditions with ice or a 12 V Chiller (Coolworks Inc, San Rafael, CA). At the laboratory, nymphs were carefully transferred with paint brushes to aerated, glass holding tanks and held at 12 °C for a 48-hour acclimatization period prior to test initiation. Individuals were identified to genus in the field using a taxonomic key from Ward and Kondratieff (1992).

Test Methodology

The zinc toxicity tests used a mixture of onsite well water and reverse osmosis water. A conductivity controller maintained a constant mixture with water hardness near 45 mg/L (Table II.1). The dilution water supplied a continuous-flow serial diluter (Benoit et al. 1982) constructed of teflon, polyethylene, and polypropylene components. The diluter delivered five concentrations of metal toxicant with a 50% dilution ratio and a control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mL/min. Food-grade vinyl tubing delivered test solutions to exposure chambers. Zinc stock solutions were prepared by dissolving a calculated amount of $ZnSO_4 \bullet 7H_2O$ in deionized water and delivered to the diluter by peristaltic pump at a rate of 2.0 mL/min.

Exposure chambers consisted of 1.25 L, cylindrical, polypropylene containers equipped with an air-lift system constructed from half-inch polyvinyl chloride (PVC) pipe. Water collected from the center of the container flowed down through the PVC pipe immersed in a temperature-controlled water bath, then up to the top of the container where an elbow diverted the flow in a circular pattern (Figure I.1). The air-lift maintained dissolved oxygen levels at saturation levels and provided continuous, circular flow in the exposure chamber. Two 5 x 5 cm,

unglazed, ceramic tiles were placed in each unit to serve as substrate. In the *Baetis* tests, an 8cm x 8cm square of nylon screen (18mesh) was also added.

The number of organisms and replicates varied depending on abundance of nymphs and ability to collect individuals in the field. The two *Baetis* tests used ten organisms per exposure chamber and were replicated 4 times each (total of 40 organisms per exposure level). *D. doddsi*, *D. grandis*, and Chloroperlidae tests used ten organisms per exposure level with no replication of exposures. The *Ephemerella* sp. and *Cinygmula* sp. tests used eight and six nymphs per exposure level, respectively with no replication of exposures. The tests with *D. doddsi* and *D. grandis* were run concurrently. *Ephemerella* sp., *Cinygmula* sp. and Chloroperlidae tests were also run concurrently. Mortality, defined as failure to respond to repeated prodding were recorded daily. Nymphs were not fed during the experiments.

Hardness and alkalinity were determined titrimetrically according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was calibrated prior to each use and used to measure pH and temperature. Water samples for zinc analysis were passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH < 2. Chambers with no remaining survivors were not sampled. Metal concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST-traceable QAQC standard from an outside source. Sample splits and spikes were collected and prepared during each sampling event to verify reproducibility and analytical recovery.

Median lethal concentrations (LC_{50}) were estimated using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978).

RESULTS

Water quality characteristics were relatively similar among tests (**Table II.1**). High levels of zinc in the exposures interfered with the titrimetric hardness values. Consequently, only results from the control exposure are shown. Although dissolved oxygen was not measured during the tests, previous experience has demonstrated that the air lift maintains dissolved oxygen at saturation levels.

Baetis nymphs would occasionally emerge as adults during the toxicity tests. Lids on the exposure chambers prevented the adults from escaping and the bodies of the adults could be found floating on the surface of the exposure solution. As a result, the survival rates were adjusted by excluding emerged individuals from the number of exposed organisms. In general, survival of *Baetis* nymphs decreased as zinc concentration increased (**Tables II.2 and II.3**). In the first test, 100% mortality did not occur in the highest concentration tested. Exposure concentrations in the second *Baetis* test were increased and complete mortality was observed. In general, survival rates were similar between the two *Baetis* tests. The median lethal

concentrations after 96 hours were 10.0 mg/L and 13.2 mg/L for the first and second test, respectively.

Survival of *Drunella doddsi* continued to be high after 96 hrs at all concentrations of zinc tested (**Table II.4 and Figure II.1**). A 96 hr LC_{50} for this species could not be calculated but must exceed 64 mg/L, the highest concentration tested. As duration of exposure increased past the initial 96 hr exposure, survival of *D. doddsi* nymphs decreased to 60% at 64 mg Zn/L.

Survival of *Drunella grandis* was poor at all exposure levels including the control (**Table II.4**). Intact bodies of mortalities were usually not observed though parts of legs and heads could be seen. This species is partially predaceous (Ward and Kondratieff 1992) and antagonistic (Walter Johnston, personal observation) which was probably the cause of poor survival rather than zinc exposure or unsuitable testing conditions. An LC_{50} could not be calculated for this species.

Insufficient mortality of *Ephemerella* sp. nymphs occurred and we were unable to calculate a 96 hr LC_{50} for *Ephemerella* sp. nymphs (**Table II.5**). However, after 168 hr of exposure, 87.5% of nymphs exposed to 68.6 mg Zn/L died. The 168 hr LC_{50} was 45.9 mg/L.

At 96 hours of exposure, 50% of *Cinygmula* sp. nymphs died at 68.6 mg/L (**Table II.5**). After 168 hours, the LC_{50} decreased to 53.2 mg/L.

All Chloroperlidae stonefly nymphs survived for 168 hours at all zinc concentrations tested up to 68.6 mg/L (**Table II.5**).

Endpoints for each of the tests are summarized in **Appendix Table A.1.**

Table II.1. Mean (s.d.) water quality characteristics for toxicity tests conducted with invertebrate nymphs. Due to interference from zinc, mean hardness and conductivity values are reported for control exposures only. ND=Not determined.

Hardness (mg/L)	Alkalinity (mg/L)	pH (S.U.)	Temperature (°C)	DO (mg/L)	Conductivity (µS/cm)
Baetis tricaudatus Flow-through August 2005					
42.3 (1.0)	29.4 (1.6)	7.60 (0.09)	11.3 (0.4)	ND	78 (2)
Baetis tricaudatus Flow-through September 2005					
40.4 (1.6)	27.4 (0.7)	7.52 (0.06)	11.3 (0.3)	ND	ND
D. doddsi, D. grandis, Flow-through October 2005					
49.8	39.7 (1.6)	7.64 (0.10)	12.0 (0.1)	ND	105
Cinygmula sp., *Ephemerella* sp., and Chloroperlidae, Flow-through April 2006					
51.1 (1.1)	39.4 (2.1)	7.63 (0.04)	12.6 (0.4)	ND	ND

Table II.2. Mean Dissolved zinc exposure concentrations and associated *Baetis tricaudatus* 96 h survival for test conducted in August 2005.

Mean Dissolved Zn (mg/L)	<0.01 (0.001)	2.19 (0.21)	4.60 (0.97)	8.74 (0.18)	14.7 (0.33)	30.0 (0.40)
Mean Survival (%)	92.9 (14.3)	85.0 (17.3)	83.5 (11.1)	31.2 (36.2)	50.5 (24.8)	31.2 (17.6)

Table II.3. Mean Dissolved zinc exposure concentrations and associated *Baetis tricaudatus* 96 h survival for test conducted in September 2005.

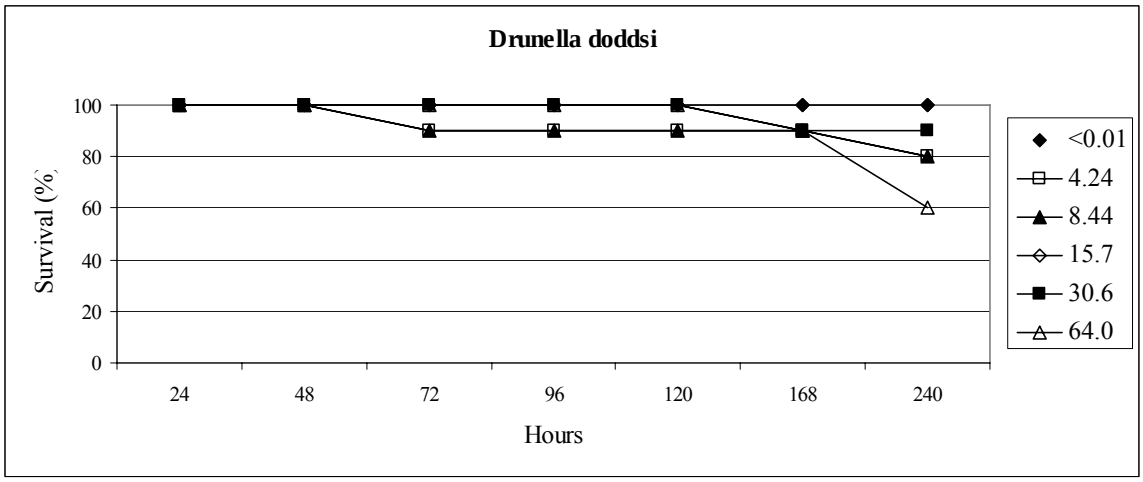
Mean Dissolved Zn (mg/L)	<0.01 (0.004)	3.76 (0.30)	7.62 (0.63)	15.5 (1.1)	28.8 (2.3)	62.0 (6.1)
Mean Survival (%)	80.6 (13.6)	67.9 (9.3)	46.7 (19.4)	45.0 (23.8)	22.1 (8.6)	0 (0)

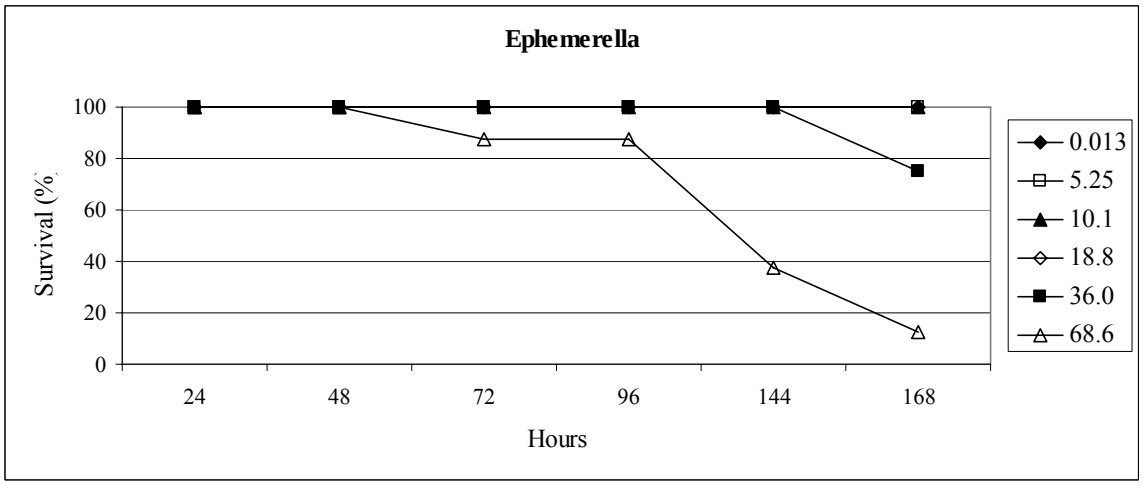
Table II.4. Mean dissolved zinc exposure concentrations and associated survival for *Drunella doddsi* and *Drunella grandis* nymphs after 96 and 240 hours.

Mean Dissolved Zn (mg/L)	<10 (2)	4.24 (0.33)	8.44 (0.49)	15.7 (0.70)	30.6 (1.22)	64.0 (3.18)
Drunella doddsi 96 h Survival (%)	100	90	90	100	100	100
Drunella doddsi 240 h Survival (%)	100	80	80	80	90	60
Drunella grandis 96 h Survival (%)	100	90	100	100	80	40
Drunella grandis 240 h Survival (%)	40	70	80	70	30	10

Table II.5. Mean dissolved zinc exposure concentrations and associated survival for *Ephemerella* sp. *Cinygmula* sp. and Chloroperlidae nymphs after 96 and 168 hours.

Mean Dissolved Zn (mg/L)	0.013 (0.09)	5.25 (0.33)	10.1 (0.34)	18.8 (1.1)	36.0 (2.6)	68.6 (1.1)
Ephemerella sp. 96 h Survival (%)	100	100	100	100	100	87.5
Ephemerella sp. 168 h Survival (%)	100	100	100	100	75	12.5
Cinygmula sp. 96 h Survival (%)	100	100	100	100	100	50
Cinygmula sp. 168 h Survival (%)	83.3	100	100	100	100	16.7
Chloroperlidae 96 h Survival (%)	100	100	100	100	100	100
Chloroperlidae 168 h Survival (%)	100	100	100	100	100	100





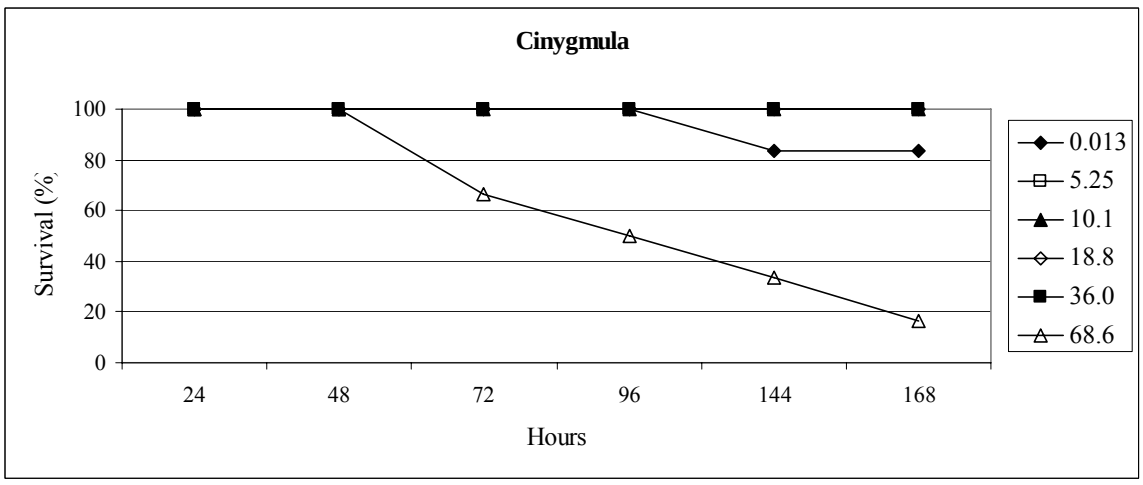


Figure II.1. *Drunella doddsi*, *Ephemerella* sp., and *Cinygmula* sp. nymph survival at different zinc concentrations as a function of duration of exposure.

III. Toxicity of zinc to nymphs of *Capnia* sp., *Lepidostoma* sp. and *Epeorus* sp. nymphs

by Katharine Mitchell, Stephen Brinkman, and Nicole Vieira

MATERIAL and METHODS

Organism Collection and Handling

Capnia sp. (Family: Capniidae) nymphs were collected from natural leaf packs in Buckhorn Creek (Larimer County, CO). The organisms were collected in late October for *Capnia* Test I and again in early December for a second test designated *Capnia* Test II. Collections of organisms were within 2 weeks of the start of the tests. Leaf packs were placed in a pan with site water to loosen invertebrates from the detritus. *Capnia* sp. was identified in the field and gently removed from pan with a turkey baster and placed in a 15-liter cooler with site water, some detritus, and a mesh screen. The cooler was not aerated due to the short transportation time (less than 20 minutes). Individuals were transported to the Colorado Division of Wildlife Aquatic Toxicology Laboratory in Fort Collins, Colorado, USA. The cooler was placed in a temperature-controlled incubator (4^{o}C) with aeration. Site water was gradually replaced (50% per day) with test water (50 ppm hardness dechlorinated municipal tap water) and incubator temperature was increased 2^{o}C per day until test temperature was achieved (12^{o}C).

Lepidostoma sp. (Family: Lepidostomatidae) and *Epeorus* sp. (Family: Heptageniidae) nymphs were collected from Boulder Creek (Boulder County, CO). The organisms were collected in late November, approximately 1 week before exposure. Organisms were gently removed from rocks using a fine paint brush and placed in Styrofoam containers with site water and aeration. Individuals were transported to the Colorado Division of Wildlife Aquatic Toxicology Laboratory in Fort Collins, Colorado, USA. The cooler was placed in a temperature-controlled incubater (4^{o}C) with aeration. Site water was gradually replaced (50% per day) with test water (50 ppm hardness dechlorinated municipal tap water) and incubator temperature was increased 2^{o}C per day until test temperature was achieved (12^{o}C).

Test Methods

All experiments were conducted as static-renewal tests in an incubator with controlled temperature (12^{o}C). Twenty-four 1-Liter polypropylene tri-pour exposure chambers were arranged over 4 shelves, with 6 containers per shelf. Each container contained 500 mL of test water with the appropriate amount of stock solution to obtain the desired concentration. Five zinc concentrations and a control were prepared, with four replicate chambers per concentration. Chambers were arranged such that each concentration appeared on each of the four shelves. The

stock solution was prepared by dissolving 2.98 g of zinc sulfate ($ZnSO_4 \cdot 7H_2O$ Mallincrodt) in 100 mL deionized water to achieve 10,000 mg/L zinc. All test water was cooled to 12°C before being allocated to a container.

Invertebrates were gently transferred from the cooler using a turkey baster or a fine paintbrush and randomly assigned to an exposure chamber. Due to limited number of individuals, the *Epeorus* sp. and *Lepidistoma* sp tests had only 2 replicates each and were conducted concurrently. In *Capnia* Test I, each container received 12 individuals; 10 individuals per container were used in *Capnia* Test II and *Epeorus* sp./*Lepidostoma* sp. Tests. Nymphs were not fed during the experiments. Fifty percent of exposure solution was renewed at 48 hours. The water removed was pooled by treatment and saved for water quality and metals analyses. Mortality, defined as the failure to respond to repeated prodding, and occurrence of exuvia was recorded at 48 and 96 hours. In *Capnia* Test I, the capniids attempted to drift from the onset of the experiment, and were found floating on the surface film of the water. Therefore, plastic mesh was added to the containers in an attempt to minimize drifting. However, the individuals did not cling to the mesh and continued to drift throughout the experiment. No mesh was added for *Capnia* Test II for this reason.

Water quality characteristics of exposure water were measured at 48 and 96 hours from pooled samples. Hardness and alkalinity were determined titrimetrically according to Standard Methods (APHA 1998). A Thermo Orion 635 meter measured pH and temperature. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. Electronic meters were calibrated prior to each use.

Dissolved water samples for metal analysis were collect at 48 and 96 hours from pooled samples with surviving larva. Water samples were also collected from stock exposure water in *Capnia* Test II to ensure there was no sorption or evaporative effects on the metals concentration. Samples were passed through a 0.45 μm filter and immediately preserved with high purity nitric acid to pH <2. Concentrations were measure using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC from an outside source (High Purity Standards, Charleston, SC). Median lethal zinc concentrations (LC_{50}) were estimated using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978).

RESULTS

Measured water quality parameters were similar among tests (**Table III.1**). Water hardness and alkalinity were about 10% than previous tests. Metals analysis on stock and pooled water samples showed no difference in concentrations. No nymphs survived in the three highest zinc concentrations for *Capnia* Test I (**Table III.2**). The calculated LC_{50} value was 5.37 mg/L (95% Confidence Intervals 4.64 – 6.21 mg/L). In *Capnia* Test II, all nymphs died in the highest zinc concentration (**Table III.3**). The 96 h LC_{50} concentration for *Capnia* Test II was 6.04 mg/L

(95% Confidence Interval 4.86 – 7.51). In both *Capnia* tests, there was no discernable difference in exuvia counts among treatments.

Survival of *Lepidostoma* sp. nymphs was high at all zinc exposure levels (**Table III.4**). The 96 hour LC_{50} for *Lepidostoma* sp. was greater that 48.5 mg/L, the highest concentration used.

Survival of *Epeorus* sp. nymphs was very low at all zinc exposure levels including the control (**Table III.4**). An LC_{50} for this species was not calculated.

Median lethal concentrations are summarized in **Appendix Table A.1.**

Table III.1. Mean water quality characteristics for toxicity tests conducted with invertebrate nymphs. Standard deviations are in parentheses. Due to interference from zinc, mean hardness and conductivity values are reported for control exposures only.

Hardness (mg/L)	Alkalinity (mg/L)	pH (S.U.)	Temperature (°C)	DO (mg/L)	Conductivity (µS/cm)
Capnia Test I					
49.0 (5.5)	34.1 (5.2)	7.10 (0.23)	15.4 (2.9)	7.10 (0.22)	101 (15.1)
Capnia Test II					
61.5 (1.7)	46.4 (4.5)	7.44 (0.18)	12.6 (1.1)	7.24 (0.28)	115 (5.1)
Epeorus sp./*Lepidostoma* sp.					
50.0 (5.6)	36.6 (3.3)	7.49 (0.15)	13.1 (0.8)	7.30 (0.35)	108 (23)

Table III.2. Mean Dissolved zinc exposure concentrations and associated survival from *Capnia* Test I.

Mean Dissolved Zn (mg/L)	0.041 (0.015)	3.00 (0.052)	6.71 (1.55)	23.3 (0.32)	42.2 (0.71)	77.7[1]
Mean Survival (%)	85.4 (14.2)	54.2 (4.8)	12.5 (14.4)	0 (0)	0 (0)	0 (0)

[1]Concentration from a single sample. No standard deviation

Table III.3. Mean Dissolved zinc exposure concentrations and associated survival from *Capnia* Test II

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Mean Dissolved Zn (mg/L)	0.05 (0.003)	4.00 (0.042)	7.56 (0.14)	15.88 (0.60)	32.0 (0.78)	65.0 (5.87)
Mean Survival (%)	80.0 (8.2)	57.5 (30.0)	27.5 (9.6)	12.5 (5.0)	5.0 (5.8)	0 (0)

Table III.4. Mean dissolved zinc exposure concentrations and associated survival for *Lepidostoma* sp. and *Epeorus* sp. after 96 hours. Standard deviations are in parentheses.

Mean Dissolved Zn (mg/L)	0.032 (0.005)	3.00 (0.038)	5.52 (0.32)	10.4 (0.55)	22.8 (1.4)	48.5 (5.7)
Lepidostoma sp.	95.0 (7.1)	95.0 (7.1)	100 (0)	100 (0)	95.0 (7.1)	95.0 (7.1)
Epeorus sp.	25.0 (7.1)	15.0 (7.1)	25.0 (7.1)	35.0 (7.1)	10.0 (14.1)	0 (0)

DISCUSSION

The collection, handling, exposure chambers, and techniques generally provided adequate conditions for the short term survival for a variety of mayfly and stonefly nymphs. The exposure setup developed for rheophilic species provided an easy and inexpensive means of creating a constant, circular, and easy to control flow. The airlift system offered an additional benefit of aeration of exposure water. The delivery of toxicant via a continuous flow-through diluter eliminated the problem of loss of test material observed with the static-renewal test. Survival was generally good in the controls and lower exposure levels and often was 100% over the duration of the exposures and test organisms moulted regularly in the control treatments. Exceptions were *Drunella grandis* and the small *Epeorus* sp. nymphs. We believe the poor survival of *Drunella grandis* was related to predation and not poor test conditions or zinc exposure. We base this conclusion on the lack of corpses and because mortality was observed in all exposures including the control. Poor survival of *Epeorus* sp. was probably due to rough handling necessary to dislodge the clinging nymphs from the Styrofoam collection container. Other test organisms were collected in smooth plastic or glass containers and were easy to transfer to exposure chambers.

Test results were generally consistent within species. The two tests conducted with *Baetis tricaudatus* were 10.0 and 13.2 mg/L. For *Capnia* sp. the LC_{50}s were 5.37 and 6.04 mg/L. The 96 hr LC_{50}s for *R. hageni* were >16, 39.2, and 50.5. The latter two values for *R. hageni* were derived from tests using organisms from two different seasons (spring 2005 and fall 2005, respectively), and two different rivers (Arkansas River and Cache la Poudre, respectively). The similarity of results suggests that differences between drainages may be relatively small.

R. hageni nymphs were sensitive to metals in the order: Cu > Cd > Zn. These results are consistent with limited metal toxicity data available for mayflies. *Ephemerella subvaria* was more than six times more sensitive to copper than cadmium (Warnick and Bell 1969). *Epeorus latifolium* was more sensitive to waterborne copper than zinc (Hatakeyama 1989). Clements (2004) found heptageniid abundance in colonized trays was unaffected by Zn up to 30 times the USEPA criterion and only slightly reduced when cadmium was added to zinc. However, addition of copper to the mixture caused sharp decreases of heptageniid abundance.

A sublethal endpoint, moult frequency, was significantly reduced by high concentrations of copper, cadmium, and zinc in *R. hageni*. Moult frequency declined as cadmium and zinc exposure concentrations increased. Moult frequency was reduced at cadmium and zinc concentrations that also reduced survival. In contrast, copper reduced moult frequency at much higher than lethal concentrations.

Reduction of moult frequency, relative to control, varied among the metals. Moult frequency was affected most by zinc and was reduced to about 13% of control at 79 mg/L. Cadmium and copper reduced moult frequency by about one-half to one-third of control. Reduced moult frequency was probably the result of impaired growth and/or development. Increased moult interval (e.g., decreased moult frequency) and reduced growth have been

previously reported for the mayfly, *Epeorus latifolium*, exposed to copper and zinc (Hatakeyama 1989). The long term consequences of increased moult interval are unknown but it might reduce the chance of survival to the adult reproductive stage.

Sensitivity of tested insects to acute waterborne exposure to zinc was in the order: *Capnia sp.>Baetis tricaudatus>Rhithrogena hageni>Drunella doddsi, Ephemerella* sp.*, Cinygmula* sp.*,* Chloroperlidae*, Lepidostoma* sp. (**Table A.1**). In general, the LC_{50}s were relatively high and comparable to some of the most tolerant species used to develop current zinc criteria. Our results indicate that USEPA acute Zn criteria adequately protect nymphs of taxa that we tested. The zinc LC_{50}s of the most sensitive organisms tested, *Capnia* sp. were 5.37 and 6.04 mg/L while the zinc criteria is 0.065 mg/L at a water hardness of 50 mg/L.

Observed Zn tolerance of mayflies tested in this study disagrees with numerous biomonitoring studies that have attributed reduced mayfly nymph abundance to much lower concentrations of zinc. For example, abundance of *R. hageni* and other heptageniid mayflies in the East Fork of the Arkansas River were reduced downstream of the Leadville Mine Drainage Tunnel where zinc concentrations ranged between 0.1-1.0 mg/L Zn (Clements 1994, Nelson and Roline 1996, Clements 2004). Following installation of a treatment plant, zinc concentrations dropped below criteria levels and abundance of *R. hageni* and other heptageniids at downstream sites increased (Nelson and Roline 1996, Clements 2004).

The apparent discrepancy between the laboratory-derived lethal concentrations and concentrations found to decrease abundance in biomonitoring studies could arise from several causes. Synergism may occur in metal mixtures typically found in streams thus increasing toxicity *in situ* (Clements 2004). Also, sensitive taxa may be eliminated or reduced by pulses of metals undetected by grab samples collected during biomonitoring studies. However, it is unlikely that pulses of metals at the concentrations used in our laboratory tests would ever occur in places such as the Arkansas River.

Differences between effect concentrations observed in the laboratory and *in situ* may be due to experimental factors. Insufficient test duration or use of a tolerant life stage could result in laboratory-derived toxicity thresholds that are much greater than those observed in streams. Mortality in our test did not cease at 96 hours but continued to increase until test termination. Longer exposures might result in significantly lower lethal thresholds. In addition, late-instar nymphs may be more tolerant of metals than earlier instars. Use of late-instar nymphs was necessary due to the difficulty of identifying and handling early instars. Smaller individuals were more sensitive to a metal mixture for several mayfly species including *R. hageni* (Kiffney and Clements 1996, Clark and Clements 2006). In metal-impacted streams, reduction or elimination of metal-sensitive early instars may be offset by recolonization of tolerant late instars from clean tributaries or from upstream of the metal source. However, heptageniid mayflies drift only rarely (Rader 1997), therefore recolonization by metal-tolerant instars would be slow.

Indirect effects of metals may also contribute to differences between metal concentrations that cause lethality in the laboratory and decreased abundance in streams. Increased drift of mayflies (Leland et al. 1989, Clements 1999) and increased risk of predation (Clements 1999) in

response to metal exposure could reduce mayfly abundance in metal-impacted streams. Metal-caused shifts in periphyton communities (Hatakeyama 1989, Medley and Clements 1998, Hill et al. 2000), which are a primary food source for scrapers such as heptageniid mayflies (Merritt and Cummins 1996), could influence their abundance.

Perhaps most important is the possible influence of dietary rather than waterborne metal exposure affecting mayfly abundance in metal impacted streams. *Hexagenia rigida* accumulated Cd and Zn primarily from the diet and not from the water (Hare et al. 1991). Copper, cadmium, and zinc concentrations in invertebrates have been found to be more strongly correlated with metal content of *aufwuchs* (periphyton and associated embedded abiotic material that serves as food for grazing benthic invertebrates) than with water or sediment concentrations (Kiffney and Clements 1993, Beltman et al. 1999). Several experiments have documented toxicity of dietary metals to mayflies. Growth and emergence were reduced in *Epeorus* mayflies fed diatoms with elevated levels of copper or zinc (Hatakeyama 1989). *Baetis tricaudatus* mayflies experienced reduced growth when fed metal-contaminated biofilm (Courtney and Clements 2002, Carlisle and Clements 2003) and cadmium-dosed diatom mats (Irving et al. 2003). Reduced growth from dietary exposure to metals may be due to food avoidance (Hatakeyama 1989, Irving et al. 2003, Wilding and Maltby 2006) or reduced food quality (Courtney and Clements 2002, Carlisle and Clements 2003).

Metal toxicity to aquatic species is generally assumed to occur through waterborne exposure. In general, criteria and state standards do no take into account the potential impact of dietary sources of metal. In instances where dietary exposures exert their own toxicity or interact with waterborne exposures, water quality criteria and standards may underprotect organisms in aquatic environments. Future studies on the effects of dietary versus aqueous exposure, for both early and late instars of aquatic invertebrates, may help explain differences between laboratory toxicity tests and field studies.

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APPENDIX TABLE

Table A.1. Summary of median lethal concentrations (LC_{50}) (mg/L) at 96 hours and at test termination.

	Metal	96 h LC_{50}	LC_{50} at test termination
Rhithrogena hageni	Cd	10.5	4.26 [a]
Rhithrogena hageni	Cu	0.137	<<0.138 [b]
Rhithrogena hageni	Zn static-renewal	>16.4	>16.4[a]
Rhithrogena hageni	Zn	39.2	12.5 [a]
Rhithrogena hageni	Zn	50.5	14.4[a]
Baetis tricaudatus	Zn	10.0	--
Baetis tricaudatus	Zn	13.2	--
Drunella doddsi	Zn	>64.0	>64.0
Ephemerella sp.	Zn	>68.8	45.9 [c]
Cinygmula sp.	Zn	68.8	53.2 [c]
Chloroperlid	Zn	>68.8	>68.8 [c]
Capnia sp.	Zn static-renewal	5.37	--
Capnia sp.	Zn static-renewal	6.04	--
Lepidostoma sp.	Zn static-renewal	>48.5	--

[a] Test terminated at 240 hours.
[b] All organisms died by 192 hours at 0.138 mg/L, the lowest copper concentration tested.
[c] Test terminated at 168 hours.

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Evaluating Brown Trout Metrics for Developing Biocriteria-Based Water Quality Standards in Metal-Impacted Rivers:

Upper Arkansas River and Eagle River

June 2007
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EXECUTIVE SUMMARY

Heavy metals pollution from historic mining operations is a ubiquitous environmental stressor in many Rocky Mountain river basins, impacting approximately 33% of streams and rivers in Colorado (Clements et al. 2000). Nearly half of the Superfund sites in Colorado are associated with mining pollution, including those in the upper Arkansas River and Eagle River drainages. Despite remediation efforts at mine sites, technological limitations combined with uncontrollable non-point sources of metals often result in metals concentrations which exceed Environmental Protection Agency (EPA) water quality criteria (EPA 1999, 2000). For this reason, site-specific water quality standards are developed to protect the existing uses of these rivers, including protection of aquatic life.

The Colorado Department of Public Health and Environment (CDPHE) and EPA recommend a number of options to develop site-specific standards, including technology-based standards, the recalculation procedure which utilizes laboratory toxicity tests for resident aquatic species, "existing quality" standards (e.g. based on economic limitations of remediation or other circumstances), and biocriteria-based standards. The latter tool has been proposed for development of water quality criteria for metals-impacted streams, where biological metrics in contaminated reaches must meet or exceed metric levels at reference sites. Metals concentrations in years where metric attainment is achieved are then used to identify protective "existing quality" metals concentrations. For a biocriteria metric to be useful in this application, it must be sensitive to moderate changes in metals concentrations associated with remediation to demonstrate improvement in water quality, and to ultimately determine whether such improvements provide acceptable protection for aquatic biota. On the other hand, metrics must be fairly insensitive to confounding environmental factors, which might mask relationships with changing metals levels, to be reliable. For instance, changes in stream flow, benthic habitat, stream temperature and prey base may influence biota to an extent where metric responses to metals are not clear, even when attainment is not achieved.

Thus far, application of the biocriteria approach in Colorado's mine-impacted streams has been met with mixed success. Fish metrics, in particular, have proven difficult to compare across years and sites due to inter-annual variability in trout populations. This study explores the utility of several metrics for brown trout, the dominant trout species in many of Colorado's mine-impacted rivers, for use in biocriteria-based metals standards. Our objective was to utilize data in post-remediation years from mine-impacted rivers to determine how brown trout metrics respond to changes in metals concentrations and other environmental conditions. We investigated temporal trends in trout biomass, trout relative weight (condition), and trout densities to evaluate if these three metrics would be reliable metrics for water quality standards development; that is, to determine whether these metrics were responsive to reduced metals loads yet independent of confounding influences of natural fluctuations in environmental conditions. Using *a priori* models and information-theoretic model selection, we compared the relative influences of inter-annual changes of the following variables on trout metrics: zinc, cadmium, and copper toxicity (expressed as a ratio of measured concentrations to hardness-based acute toxicity equations developed for brown trout); peakflows, minimum flows, flows during the metals season and baseflows; and the quality and quantity of the prey base (number of young of year fish, and benthic invertebrate densities and richness).

We compiled existing data from long-term monitoring efforts at Superfund Sites in the Eagle River near Minturn, CO and in the upper Arkansas River near Leadville, CO. The Colorado Division of Wildlife (CDOW), through a partnership with CDPHE and EPA has monitored fish populations, macroinvertebrate communities and water quality in the Eagle River near the Eagle Mine Superfund Site since 1990. Monitoring efforts, which were funded in part by CBS-Viacom International, sought to evaluate the progress of biological recovery in the Eagle River during and after mine remediation efforts. Another objective of monitoring activities was to provide a biological data set that would support the development of biologically-based water quality standards for the Eagle Mine Superfund Site. Positive benefits from major remediation efforts began to take effect in the year 1997. CDOW has also monitored water quality and fish population changes in the upper Arkansas River Superfund Site since 1994, in conjunction with the Bureau of Reclamation (BOR), CDPHE, EPA, and GEI Consultants, Inc, Chadwick Ecological Division (contracted by Resurrection Mine Co.). Chadwick Ecological Division and several academic researchers from Colorado State University monitored changes in benthic invertebrate communities during this time period. A number of remediation activities upstream of the CDOW monitoring sites already took place before 1994. As such, our monitoring was targeted at documenting the long-term effects of those efforts and of ongoing activities in California Gulch, the primary loading source which is still undergoing minor remediation.

Our results suggest that brown trout biomass, relative weight, and densities do not meet our criteria for useful and reliable biocriteria metrics. Previous comparisons between mine-impacted stream reaches and reference sites in post-remediation years in the Eagle River (1997-2005) and the upper Arkansas River (1995 - 2005) have revealed notable improvements in brown trout population measures, especially density and biomass (Vieira et al. 2005; Brinkman et al, 2006; Chadwick Ecological Division 2006). However, in a biocriteria context, these metrics have yielded inconsistent results, where biological conditions at some contaminated sites do not meet reference site conditions in multiple, consecutive years. Our analyses revealed a likely reason for this inconsistency. First, these metrics did not respond well to moderate changes in metals levels, even though toxicity was reduced from levels above brown trout LC50 levels to concentrations well below these values. In addition, temporal patterns in these metrics were confounded by other environmental conditions not related to mining activities, especially peakflow and prey base. Interestingly, these relationships held true in two rivers which differ in hydrological and geomorphological conditions. The Eagle River through the Superfund area is a larger, wider 4th order river on the western slope and the upper Arkansas through the Superfund area is a smaller, colder 3rd order stream which drains to eastern Colorado.

Of the three fish metrics we tested, brown trout density appeared to be the best candidate for use as a biocriteria metric. Density of juvenile and adult fish (excluding young of year) was the most sensitive metric to metals levels in both rivers. For the Eagle River, this pattern is clear using all of the sites combined. For the Arkansas River, this pattern is more evident when the site most affected by metals loading (AR3) is isolated for analysis (Brinkman et al. 2006; Chadwick Ecological Consultants 2006). Even so, alternative approaches to determining protective water quality standards, such as laboratory toxicity tests for brown trout or use of EPA's recalculation procedure will likely result in metals standards which are more protective than those determined from biocriteria. This is certainly true for both the Eagle and Arkansas Rivers, and is a primary

reason why the recalculation procedure is being pursued in favor of using fish biometrics (WQCC 2005, 2007). Since long-term impacts of metals exposure to trout are unknown, it is critical to err on the side of caution and to develop standards which are as protective as possible within the scope of technological and economic feasibility. In general, fish communities in Colorado's mountain streams are not diverse, leaving few options for fish metrics. Benthic invertebrate community metrics were not evaluated in this analysis due to lack of data availability, but may prove to be more reliable tools for setting metals standards in mine-impacted streams, and for ground-truthing standards developed from laboratory-based toxicity tests.

PURPOSE

Heavy metals pollution from historic mining operations is a ubiquitous environmental stressor in many Rocky Mountain river basins, impacting approximately 33% of streams and rivers in Colorado (Clements et al. 2000). Nearly half of the Superfund sites in Colorado are associated with mining pollution, including those in the upper Arkansas River and Eagle River drainages. Despite remediation efforts at mine sites, technological and economic limitations combined with uncontrollable non-point sources of metals often result in metals concentrations which exceed Environmental Protection Agency (EPA) water quality criteria (EPA 1999, 2000). For this reason, site-specific water quality standards are developed to protect the existing uses of these rivers, including aquatic life uses.

The Colorado Department of Public Health and Environment (CDPHE) and EPA recommend a number of tools to develop site-specific standards, including technology-based standards, the recalculation procedure, "existing quality" standards, and biocriteria-based standards. The latter tool has been proposed for development of water quality criteria for metals-impacted streams, where biological metrics in contaminated reaches must meet or exceed metric levels at reference sites. Metals concentrations in years where metric attainment is achieved are then used to identify protective "existing quality" metals concentrations. For a biocriteria metric to be useful in this application, it must be sensitive to moderate changes in metals concentrations associated with remediation to demonstrate improvement in water quality, and to ultimately determine whether such improvements provide acceptable protection for aquatic biota. On the other hand, metrics must be fairly insensitive to confounding environmental factors, which might mask relationships with changing metals levels, to be reliable. For instance, changes in stream flow, benthic habitat, stream temperature and prey base may influence biota to an extent where metric responses to metals are not clear, even when attainment is not achieved.

Thus far, application of the biocriteria approach in Colorado's mine-impacted streams has been met with mixed success. Fish metrics, in particular, have proven difficult to compare across years and sites due to inter-annual variability in trout population metrics. This study explores the utility of several metrics for brown trout, the dominant trout species in many of Colorado's mine-impacted rivers, for use in biocriteria-based metals standards. Our objective was to compile data in post-remediation years from mine-impacted rivers to determine how brown trout metrics respond to changes in metals concentrations and other environmental conditions. We compared trout biomass, trout relative weight (condition), and trout densities to determine if these three metrics would be useful and reliable biocriteria for water quality standards development; that is, whether these metrics were responsive to reduced metals loads while minimally responding to confounding influences of natural fluctuations in environmental conditions. Using *a priori* models and information-theoretic model selection, we compared the relative influences of inter-annual changes in: zinc, cadmium, and copper toxicity (expressed as a ratio of measured concentrations to hardness-based acute toxicity equations developed for brown trout); peakflows, minimum flows, flows during the metals season and baseflows; and the quality and quantity of the prey base (number of young of year fish, and benthic invertebrate densities and richness). We also investigated how these variables interact to influence trout metrics.

METHODS

CDOW, through a partnership with CDPHE and EPA has monitored fish populations, macroinvertebrate communities and water quality in the Eagle River near the Eagle Mine Superfund Site since 1990. Monitoring efforts, which were funded in part by CBS-Viacom International, sought to evaluate the progress of biological recovery in the Eagle River during and after mine remediation efforts. Another objective of monitoring activities was to provide a biological data set that would support the development of biologically-based water quality standards for the Eagle Mine Superfund Site. Positive benefits from major remediation efforts began to take effect starting in the year 1997. CDOW has also monitored water quality and fish population changes in the upper Arkansas River Superfund Site since 1994, in conjunction with the Bureau of Reclamation (BOR), CDPHE, EPA, and GEI Consultants, Inc, Chadwick Ecological Division (contracted by Resurrection Mine Co.). Chadwick Division and researchers from Colorado State University monitored changes in benthic invertebrate communities during this time period. A number of remediation activities upstream of the CDOW monitoring sites already took place before 1994, and as such, our monitoring was targeted at documenting the long-term effects of those efforts and of ongoing activities in California Gulch.

Eagle River Data Summary

Physical, chemical, and biological data have been collected since 1990 in the Eagle River above and below the mine site by CDOW, CBS-Viacom, and other parties such as USGS. CDOW collected brown trout data and semi-quantitative invertebrate samples at eight sites during the spring (pre-runoff) between the years 1994-2005. Viacom collected macroinvertebrate and water quality data for some sites. One of the purposes of the biological monitoring activities was to provide data to support the development of biologically-based water quality standards. The "Biological Approach" included comparing brown trout metrics (total density, relative weight) at mine sites to metrics at reference sites. For site attainment, spring densities of brown trout at mine sites were to be equal to or greater than 95 percent of the mean of the population densities at the reference sites for three consecutive years. Attainment of this 95 percent criterion at mine sites was considered to indicate no statistically significant difference between the mine sites and the reference sites. The Biological Approach was designed such that the biological results at each sampling location would be compared against the water quality data for that location and if metric attainment had been achieved, then the water quality data for that sampling location could be used in the calculation of the new water quality standards.

Monitoring sampling methods and data collection details for the Eagle River mine site are reported elsewhere (Woodling and Rollings 2004, 2005; CDPHE 2005). We used summary data provided in these studies for brown trout metrics, water quality and benthic invertebrates. To minimize data gaps (missing data), we used data from the reference station ER1.9 and from the mine sites ER2.2, ER 2.9, ER3, ER4.2 and ER5 (see **Figure IV.1** from CDPHE 2005). We only analyzed data from 1997 to 2005 because these years represented a "post-remediation" time period during which time biocriteria would represent a viable option for determining water quality standards. Stream flow data was derived from USGS stream gage stations on the Eagle River near Redcliff, Minturn, and Avon. Flow was extrapolated from these gages to sample sites

that lie between them. The following flow variables were calculated: 1) base flow, 2) peak flow, 3) minimum flow and 4) average "May and June" flow during the metals loading season. Base flow values were determined by calculating the average cfs during November, December and the following year January and February flows.

We selected the following brown trout metrics for analysis: 1) fish density in the spring (#/ha for fish > 13 cm in length), 2) average brown trout weight in the spring (g), and 3) average relative weight in the spring. Young of year fish were excluded from analysis because sampling did not specifically target this size of fish, and collections of these fish can vary considerably with the flow conditions and timing of sampling (Vieira et al 2005). This is especially true during spring sampling, which can be difficult to time with the onset of snowmelt and the rising limb of runoff.

Dissolved metals concentrations were translated into "toxicity units" based on the following CDOW's hardness-based equations for brown trout (Davies et al. 2003; Brinkman and Hansen 2004):

Cd Toxicity Unit = in-stream Cd concentration/ ($e^{1.258*\ln(\text{hardness})-3.999}$)
Cu Toxicity Unit = in-stream Cd concentration/ ($e^{0.9422*\ln(\text{hardness})-0.2022}$)
Zn Toxicity Unit = in-stream Cd concentration/ ($e^{1.062*\ln(\text{hardness})+2.151}$)
Total Toxicity Unit = Sum of Cd, Cu and Zn toxicity units.

These equations have since been revised and updated resulting in minor changes. However, these revisions were not available during the data collection period for the Eagle River. We used semi-quantitative measures of invertebrate richness and abundance (CDOW data) for the spring of each year to represent trout prey base. The number of young of year trout was also included as a potential prey item.

Arkansas River Data Summary

CDOW has also monitored water quality and fish population changes in the upper Arkansas River Superfund Site since 1994 (e.g. Davies et al. 1997, Davies et al. 2000, Davies et al. 2002, Brinkman et al. 2006), in conjunction with the Bureau of Reclamation (BOR), CDPHE, EPA, and GEI Consultants, Inc, Chadwick Ecological Division (contracted by Resurrection Mine Co.). Fish sampling largely occurred in the late summer to fall (August-October), with the exception of sampling in 1998 and 2000 which occurred in the spring (pre-runoff). For year to year consistency, only late summer and fall data were used in this analysis to compare brown trout metrics. Chadwick Ecological Division (2006) and researchers from Colorado State University (e.g. Clements 1994; Clements and Kiffney 1995; Clements 2004) also monitored changes in benthic invertebrate communities during this time period. Unfortunately, water quality data, fish data and benthic invertebrate data were not collected every year and at the same locations each year, resulting in many data gaps for our analysis. We chose sampling stations AR1 as the reference station, and sites AR3a, AR4 and AR5 as the mine-impacted reaches because these stations had the fewest data gaps (see **Figure IV.2**). Flow data was obtained for the USGS gage station at AR1 and was extrapolated to downstream sites using patchy data from California Gulch, AR4, a gage near AR5 and a gage on Halfmoon Creek. In general, peakflows

at AR3a were considered to only be approximately 10 cfs greater than peakflows at AR1, while those at AR4 and AR5 were estimated to be 2-2.5 times greater. Baseflows and minimum flows at these downstream stations were estimated to be 3-4 times greater. Once again, data gaps existed for flow in certain years.

We chose the following brown trout metrics, which different slightly from those chosen for the Eagle River: brown trout density in the fall (#/acre of fish >10 cm in length), brown trout biomass (lbs per acre) in the fall, and average relative weight. Young of year fish were excluded from analysis because sampling did not specifically target this size of fish, and collections of these fish can vary considerably with the flow conditions and timing of sampling (Vieira et al 2005; Chadwick Ecological Division 2006). Dissolved metals concentrations were translated into "toxicity units" based on the CDOW's hardness-based equations for brown trout presented above.

Statistical Methodology

Response variables (brown trout density, brown trout biomass, brown trout relative weight) and predictor variables (e.g. metals, flow, preybase, etc.) for statistical analyses were selected *before* relationships between these variables were explored with an Information-Theoretic model selection approach. Once model selection analyses were complete, additional "data mining" techniques were employed to determine whether our *a priori* selected variables and modeling efforts were worthwhile.

Information-Theoretic Approach to Model Selection

To determine the relative influence of predictor variables on a response, model selection techniques such as forward, backward, and stepwise regression are typically used. These techniques are useful when the number of predictors relative to the sample size is low, and for data mining in larger datasets. However, such methods of model selection 1) do not include *a priori* scientific knowledge, 2) have a strong chance of including meaningless predictors in a model due to spurious correlations, and 3) run the risk of over-fitting, where precision of parameter estimates are over-estimated. An alternative approach to model selection is the Information-Theoretic Approach. This approach employs criteria such as Akaike's Information Criterion (AIC) that operate on the principle of parsimony, where both bias and variance are simultaneously minimized (Burnham and Anderson 2002). In other words, this technique seeks to balance the problem of over-fitting models (more predictors than necessary are included in a model, and some may be spurious) and under-fitting models (too few predictors are included in the model). Furthermore, this technique allows several models, rather than a single model, to be identified as important, and parameter estimates can be derived by averaging across these models. *A priori* models compared with the AIC model selection method are listed for the Eagle River in **Table IV.1a** and for the upper Arkansas River in **Table IV.1b**. The following variables appear in these models:

Response variables:

Biomass = brown trout biomass for Eagle River (in average g of weight) or in pounds per acre for the Arkansas River. Fish <10 cm in length were not included in biomass estimates for the Arkansas R. *All* fish were considered in the Eagle R. metric.

Rel WT = relative weight of brown trout based on standard length vs. weight regressions

Density = estimates of # fish >13 cm per hectare for the Eagle River, and the # fish >10 cm per acre for the Arkansas River

Independent variables:

ZnTU, CdTU, and CuTU = acute toxicity units for zinc, cadmium and copper based on a comparison of *current* year metals levels in-stream versus laboratory 96-hour toxicity test LC50 values for brown trout

PZnTU, PCdTU, and PCuTU = acute toxicity units for zinc, cadmium and copper based on a comparison of *previous* year metals levels in-stream versus laboratory 96-hour toxicity test LC50 values for brown trout

TU_all and PTU_all = total toxicity units for the current year or previous year, calculated by adding ZnTU+CdTU+CuTU or PZnTU+PCdTU+PCuTU

PEAKFL or PPEAKFL = peakflow (cfs) in the current year, or previous year, respectively as calculated from the nearest USGS stream gages

#YOY = # of young of year trout collected at sample sites (<13 cm in Eagle River and <10 cm in Arkansas River)

#PREY = mean # of benthic invertebrates per sample (Eagle River) or per square meter (Arkansas River)

RICH = mean richness of benthic invertebrates per sample (Eagle River) or per square meter (Arkansas River)

AIC calculation options are selected based on: a) whether the sample size is small (i.e., if the ratio of sample size to the # of estimated parameters is < 40), and b) whether the data are over-dispersed (estimated with Chi-squared goodness of fit statistic divided by the degrees of freedom for the global largest model). For our analysis, we used the AICc equation for smaller sample sizes:

$$AICc = -2\log(MLE) + 2K + (2K(K+1)/n-K-1)$$

MLE= maximum likelihood estimator
K = # of parameters to estimate, n = sample size

Residual sums of squares (SS) from linear regression modeling were used to approximate the MLE in a "least squares" case of AICc:

$$AICc = n*\log(RSS/n) + 2K + (2K(K+1)/n-K-1)$$

RSS = residual sums of squares from regression model output
K = # of regression parameters +1 for the intercept and +1 for the residual variance
n = sample size

Models in the candidate set are compared by calculating Δ AICc for each model "*i*" relative to the model with lowest AICc value to rank models:

$$\Delta\ AICc_i = AICc_i - AICc_{minimum}$$

And then calculating Akaike weights to determine the relative likelihood of a model given the data and given the set of candidate models:

$$W_i = (\exp(-0.5\ \Delta\ AICc_i))/(\Sigma\ \exp(-0.5\ \Delta\ AICc) \text{ over all models})$$

In addition to the AIC model selection steps outlined above, adjusted R-squared values of the regression models were examined and compared to determine how much total variance in the response was explained by each model. Assumptions of linear models and statistical outliers were checked with diagnostic tests (Neter et al. 1990) available in SAS statistical software (PROC UNIVARIATE PLOT, PROC PLOT with student residuals, VIF and COOK's D), and necessary data transformations were performed (SAS 1998).

After we ran our *a priori* models, we conducted a data mining exercise with stepwise regression model selection (PROC REG/STEPWISE, both with rules for variable entry/exit into and out of models of p = 0.10). Data mining models were used to evaluate whether our suite of *a priori* models did, in fact, include variables that were most strongly correlated with the response variables. The same variables considered in the *a priori* models were considered here. Additional variables included annual minimum flows, baseflows in the fall, and mean stream flows in the peak metals season (early spring). Assumptions were checked with diagnostic tests mentioned above (SAS 1998).

RESULTS

Tables IV.2a -2b show descriptive statistics for the data compiled for mine-impacted reaches for the Eagle River and the upper Arkansas River. These statistics demonstrate substantial variability in response variables and independent variables during the post-remediation years of interest in both rivers. Such variability in response variables is desired for development of sensitive ("useful") biocriteria metrics. Independent variables also ranged widely, including metals levels and flow conditions, which allowed us to determine whether brown trout metrics responded to both anthropogenic and natural changes in environmental conditions. Between the years 1997-2005, zinc levels were higher than predicted acute values for brown trout (ZnTU>1) at upper sites in the Eagle River while the other metals remained below toxic levels. Toxicity units for all three metals ranged at least two-fold during this time period. Zinc and cadmium levels in the Arkansas River were substantially greater than levels predicted to be protective of brown trout in certain years between 1995-2006. Toxicity units for all three metals ranged more widely for this river than for the Eagle River. Brown trout density and biomass ranged two- to three-fold during 1997-2005 at Eagle River sites, and ranged up to five-fold in Arkansas River sites. Peakflow and invertebrate biomass in both rivers ranged up to an order of magnitude between years, while relative weights, young of year counts and invertebrate richness showed the least variability between years.

AIC Model Selection

Tables IV.3a-3b present the P-value, the adjusted R-squared value, the AIC_c value, the delta AIC_c value, and the Akaike weights (w_i) for the Eagle River and upper Arkansas River models, respectively. Models with a delta AIC of 2 or less are considered to be the most parsimonious, and thus the "best" models (Burnham and Anderson 2002). For the most part, AIC selected the models within the *a priori* set list which had the highest explanatory power (R_{adj}^2). An exception to this trend is the model for brown trout density in the Arkansas River, where a simple but less explanatory model was selected over the more complex models with the higher R_{adj}^2. This phenomenon is discussed later when the *a posteriori* models are presented. The best density model for the Eagle River explained 61% of the variability in the data. All models for relative weight had weak explanatory power, while models for brown trout biomass ranged from 47-51% (Eagle River) to 78% (Arkansas River) explanatory power. In general, the "best" models (AIC < 2) were clearly distinguished, with few competing models.

Variables and parameter estimates for the best AIC models are presented in **Tables IV.4a-4b**. For brown trout biomass, models for the two rivers were similar in that they included significant, positive interactions with benthic invertebrate densities. The number of young of year, which represents another potential prey item for cannibalistic brown trout, showed a negative relationship with biomass in the Eagle River. This was likely due to the fact that in years where the mean weight of fish was lower, more of the smaller-sized fish were collected and thus contributed to this lower mean weight. Recall that unlike the density metric, biomass metrics provided for the Eagle River did not remove fish which were less than 13 cm in length. Invertebrate prey taxa richness was positively related to biomass in the Arkansas River sites.

Best models for biomass also included peakflow (Arkansas R.) or previous year peakflow (Eagle R.), but slopes for this variable were not significantly different from zero in the models. Changes in metals toxicity units were not included in the best models, suggesting that biomass metrics in the years of study were not sensitive to moderate fluctuations in water quality after remediation efforts. By contrast, zinc and cadmium were negatively related to brown trout density in the Eagle River, and zinc and copper interacted to further reduce brown trout densities. For the Arkansas River, reductions in brown trout densities appeared to be more influenced by high peakflows.

Because models were so weak for the relative weight metric, we will not discuss this metric further. Tables 2a-2b demonstrate that this metric showed little variability across years compared to the other two metrics. In comparison to the best AIC-selected models presented in Tables 4a-4b, *post hoc* models were run with other flow variables (minimum flow, baseflow and mean metals seasons flows). This *post hoc* analysis revealed that the same models were selected as the "best" with AIC, but the explanatory power was reduced when these flow variables were substituted for peakflow. As such, only the models with peakflow are presented and interpreted.

Data Mining with Stepwise Model Selection

Results of *a posteriori* modeling are found in **Tables IV.5a-5b**. We also included variables that were not considered in the *a priori* regression models (such as minimum flow and other flow variables) to see if we "missed" any important variables. We did not include interaction terms to reduce the possibility that collinearity would result in spurious results. That is, we allowed models to choose the most influential "raw" variables without highly correlated interactions "masking" these relationships.

Stepwise regression generally supported the "best" models selected with AIC, confirming that our *a priori* suite of models were meaningful. Invertebrate density was still the most important predictor for brown trout biomass in both rivers. For the Eagle River, zinc was still the most important factor influencing brown trout densities (with a negative relationship). Minimum flow was added as an important variable, where lower flow years resulted in higher trout densities. By contrast, the stepwise selection model for brown trout densities in the Arkansas River was substantially different than the best AIC model. Peakflow was still negatively related to densities, but prey base variables (especially invertebrate density) and metals toxicity (copper and cadmium) were added to the model. This discrepancy between the best AIC and stepwise models was likely due to the fact that sample sizes differed between models (e.g., invertebrate data was only available through 2004 while flow data was available through 2006). AIC modeling penalized the models with invertebrate prey items because of this lower sample size, and sought a more parsimonious model with fewer variables. Once stepwise selection determined the most explanatory models, we added interaction terms to look for further improvement. Since we did not find that these interactions were significant, these results are not presented or interpreted.

DISCUSSION

Previous comparisons between mine-impacted stream reaches and reference sites in post-remediation years in the Eagle River (1997-2005) and the upper Arkansas River (1995-2005) have revealed notable improvements in brown trout population measures, including density and biomass (Vieira et al. 2005; Brinkman et al, 2006; Chadwick Ecological Division 2006). However, in a biocriteria context, these metrics have yielded inconsistent results, where biological conditions at some contaminated sites do not meet reference site conditions in multiple, consecutive years. **Tables IV.6a-6b** show how brown trout metrics at reference sites compared to metrics at mine sites using a simple calculation of attainment, where attainment is achieved when mine site metrics are equal to or greater than 100% of the reference site values. This analysis is overly simplistic, but can be used to demonstrate how brown trout metrics, especially densities, are not consistently meeting reference conditions despite substantial improvements in metals levels. Our analyses revealed a likely reason for this inconsistency: brown trout metrics have responded to environmental conditions other than metals toxicity during post-remediation years, including positive responses to increased prey base and negative responses to peakflow. Others have found that brown trout populations respond to flow, where recruitment success was inversely related to spring runoff discharge in Colorado streams (Nehring and Anderson 1985, 1993). In the Arkansas River, brown trout density and growth have been shown to be negatively related to stream discharge, albeit at sites well below AR5 (Nehring 1986, Anderson and Krieger 1994).

Our study suggests that brown trout biomass, relative weight, and densities do not meet our criteria for useful and reliable biocriteria metrics. First, these metrics did not respond well to moderate changes in metals levels, even though toxicity was reduced from levels above brown trout LC50 levels to concentrations well below these values (see **Appendix IV.A.1**). In addition, temporal patterns in these metrics were confounded by other environmental conditions not related to mining activities (e.g., peakflows and prey base; see **Appendix IV.A.1**). Interestingly, this pattern held true in two rivers which differ in hydrological and geomorphological conditions. The Eagle River through the Superfund area is a larger, wider 4th order river on the western slope and the upper Arkansas through the Superfund area is a smaller, colder 3rd order stream which drains to eastern Colorado. An important similarity between the two rivers is that streamflow responded by an order of magnitude to climate-induced changes in precipitation during the period of study. Both studies included record drought years in 2002, and in general, flows were often below average. Our study results also suggest that if reference sites are not hydrologically comparable to mine-impacted stream segments, these fluctuations may confound the ability of trout metrics to indicate biological responses to metals. For the most part, reference sites for the Eagle and Arkansas River sites were located upstream of the mining areas and were not hydrologically similar to the lower mine-impacted segments, where flows increase due to the addition of tributaries.

Prey base, especially benthic invertebrate density, was an important factor driving brown trout metrics between years in both rivers, especially brown trout biomass. One could argue that fluctuations in prey base may reflect an indirect effect of metals contamination, where high metals years were associated with lower invertebrate biomass. While this has been observed at some sites in the Arkansas River (e.g. Clements 2004), interactions between metals and these

natural conditions were explored and were not found to influence brown trout metrics. Differences in brown trout metrics were more likely driven by substantial differences in site quality (e.g., substrate composition, embeddedness, % pool and riffle, channel configuration, etc.), where higher quality habitats supported a more robust prey base. In addition to habitat quality, there are other variables that influence trout survival and growth, but for which we did not have adequate data for statistical testing. For instance, stream temperature strongly influences fish growth by driving consumption and metabolic rates. Such relationships may override more subtle impacts of metals contamination, especially for trout biomass metrics, thereby reducing our ability to measure water quality improvements after remediation activities.

Of the three fish metrics we tested, brown trout density appeared to be the best candidate for use as a biocriteria metric. Density of juvenile and adult fish (excluding young of year) was the most sensitive metric to metals levels in both rivers. For the Eagle River, this pattern is clear using all of the sites combined. For the Arkansas River, this pattern is more evident when the site most affected by metals loading from California Gulch (AR3) is isolated for analysis (Brinkman et al. 2006; Chadwick Ecological Consultants 2006). Snow-pack and timing of snowmelt were not considered in this study but also likely influence trout densities because the timing of metals delivery can impact development of sensitive (early) life-stages. Trout densities will decrease in years where low recruitment occurs as a result of sensitive life stages being exposed to metals prior to the ameliorating effects of increased hardness during peakflow. A drawback to using trout density metrics to determine water quality criteria for metals is that density may artificially increase in years with lower flows, regardless of the metals loads for that year. This increase is partly due to the fact that fish density in low flow years is calculated using lower water volume, and also because fish may be easier to collect due to fewer deep pools. This problem is less pronounced if sampling occurs during baseflow, such as the later summer sampling schedule used for the Arkansas River. If trout density metrics are inflated in low flow years due to an artifact of sampling, and metals loads are correspondingly higher in these years, metrics may appear to be in attainment and high metals levels may be inappropriately applied to determine "protective" existing water quality for brown trout populations.

Alternative approaches to determining protective water quality standards, such as laboratory toxicity tests for brown trout or use of EPA's recalculation procedure (also based on acute and chronic laboratory tests), will likely result in metals standards which are more protective than those determined from biocriteria. This is certainly true for both the Eagle and Arkansas Rivers, and is a primary reason why the recalculation procedure is being pursued in favor of using fish biometrics (WQCC 2005, 2007). Since long-term impacts of metals exposure to trout are unknown, it is critical to err on the side of caution and to develop standards which are as protective as possible within the scope of technological or economic feasibility. Fish communities in Colorado's mountain streams are not diverse, leaving few options for fish metrics. For instance, community composition and diversity are fairly meaningless when only a handful of species are expected under the best environmental conditions. Fish-based biocriteria may be more useful for assessing water quality impacts in warm water fisheries of Colorado, which are far more species rich. Benthic invertebrate communities in mountains streams are also quite diverse and a plethora of metrics have been identified for biocriteria purposes at mine sites. Several of these metrics, especially the number of Heptageniidae mayflies, appear to be far more sensitive to metals pollution than fish metrics (Clements 2004; Chadwick Ecological Division

2006; Brinkman and Johnston 2007). Benthic invertebrate community metrics were not evaluated in this analysis due to lack of data availability, but may prove to be useful and reliable tools for setting metals standards in mine-impacted streams, and for ground-truthing standards developed from laboratory-based toxicity tests.

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Table IV.1a. *A priori* linear regression models tested with AIC model selection for brown trout metrics in the Eagle River. Sample sizes reflect data from mine-impacted reaches between the years 1997-2005. Metals toxicity units and flow variables represent conditions from the previous year to best relate to impacts observed in early spring fish sampling. Variable codes are explained in the text.

				A PRIORI MODELS FOR AIC MODEL SELECTION								
				Predictors and Interesting Interactions								
Model	**Response**	**N**	**K**	***Metals***			***Peakflow***	***Prey base measures and interaction terms***				
BM1	BIOMASS	44	3	PZNTU								
BM2	BIOMASS	44	3				PPEAKFL					
BM3	BIOMASS	44	5	PZNTU			PPEAKFL	PPEAKFL*PZNTU				
BM4	BIOMASS	44	5	PZNTU	PCDTU				PZNTU*PCDTU			
BM5	BIOMASS	44	7	PZNTU	PCDTU		PPEAKFL	PPEAKFL*PZNTU	PZNTU*PCDTU			
BM6	BIOMASS	44	3	PTU_ALL								
BM7	BIOMASS	44	5	PTU_ALL			PPEAKFL	PPEAKFL*PTU_ALL				
BM8	BIOMASS	44	7	PZNTU	PCUTU	PCDTU			PZNTU*PCUTU	PZNTU*PCDTU		
BM9	BIOMASS	44	5					#PREY	RICH	#YOY		
BM10	BIOMASS	44	7	PZNTU				#PREY	RICH	#YOY	#PREY*PZNTU	
BM11	BIOMASS	44	7				PPEAKFL	#PREY	RICH	#YOY	#PREY*PPEAKFL	
RW1	REL WT	44	3	PZNTU								
RW2	REL WT	44	3				PPEAKFL					
RW3	REL WT	44	5	PZNTU			PPEAKFL	PPEAKFL*PZNTU				
RW4	REL WT	44	5	PZNTU	PCDTU				PZNTU*PCDTU			
RW5	REL WT	44	7	PZNTU	PCDTU		PPEAKFL	PPEAKFL*PZNTU	PZNTU*PCDTU			
RW6	REL WT	44	3	PTU_ALL								
RW7	REL WT	44	5	PTU_ALL			PPEAKFL	PPEAKFL*PTU_ALL				
RW8	REL WT	44	7	PZNTU	PCUTU	PCDTU			PZNTU*PCUTU	PZNTU*PCDTU		
RW9	REL WT	44	5					#PREY	RICH	#YOY	#PREY*PZNTU	
RW10	REL WT	44	7	PZNTU				#PREY	RICH	#YOY		
RW11	REL WT	44	7				PPEAKFL	#PREY	RICH	#YOY	#PREY*PPEAKFL	
DN1	DENSITY	45	3	PZNTU								
DN2	DENSITY	45	3				PPEAKFL					
DN3	DENSITY	45	5	PZNTU			PPEAKFL	PPEAKFL*PZNTU				
DN4	DENSITY	45	5	PZNTU	PCDTU				PZNTU*PCDTU			
DN5	DENSITY	45	7	PZNTU	PCDTU		PPEAKFL	PPEAKFL*PZNTU	PZNTU*PCDTU			
DN6	DENSITY	45	3	PTU_ALL								
DN7	DENSITY	45	5	PTU_ALL			PPEAKFL	PPEAKFL*PTU_ALL				
DN8	DENSITY	45	7	PZNTU	PCUTU	PCDTU			PZNTU*PCUTU	PZNTU*PCDTU		
DN9	DENSITY	45	5					#PREY	RICH	#YOY	#PREY*PZNTU	
DN10	DENSITY	45	7	PZNTU				#PREY	RICH	#YOY		
DN11	DENSITY	45	7				PPEAKFL	#PREY	RICH	#YOY	#PREY*PPEAKFL	

Table IV.1b. *A priori* linear regression models tested with AIC model selection for brown trout metrics in the upper Arkansas River. Sample sizes reflect data from mine-impacted reaches between the years 1995-2006. Metals toxicity units and flow variables represent conditions from the current year to best relate to impacts observed in fall fish sampling. See text for variable codes.

				A PRIORI MODELS FOR AIC MODEL SELECTION									
				Predictors and Interesting Interactions									
Model	**Response**	**N**	**K**	***Metals***				***Peakflow***	***Prey base measures and interaction terms***				
BM1	BIOMASS	29	3		ZNTU								
BM2	BIOMASS	30	3					PEAKFL					
BM3	BIOMASS	29	5		ZNTU			PEAKFL	PEAKFL*ZNTU				
BM4	BIOMASS	29	5		ZNTU	CDTU				ZNTU*CDTU			
BM5	BIOMASS	29	7		ZNTU	CDTU		PEAKFL	PEAKFL*ZNTU	ZNTU*CDTU			
BM6	BIOMASS	29	3	TU_ALL									
BM7	BIOMASS	29	5	TU_ALL				PEAKFL	PEAKFL*TU_ALL				
BM8	BIOMASS	29	7		ZNTU	CUTU	CDTU			ZNTU*CUTU	ZNTU*CDTU		
BM9	BIOMASS	23	5						#PREY	RICH	#YOY	#PREY*ZNTU	
BM10	BIOMASS	22	7		ZNTU				#PREY	RICH	#YOY		
BM11	BIOMASS	23	7					PEAKFL	#PREY	RICH	#YOY	#PREY*PEAKFL	
RW1	REL WT	25	3		ZNTU								
RW2	REL WT	26	3					PEAKFL					
RW3	REL WT	25	5		ZNTU			PEAKFL	PEAKFL*ZNTU				
RW4	REL WT	25	5		ZNTU	CDTU				ZNTU*CDTU			
RW5	REL WT	25	7		ZNTU	CDTU		PEAKFL	PEAKFL*ZNTU	ZNTU*CDTU			
RW6	REL WT	25	3	TU_ALL									
RW7	REL WT	25	5	TU_ALL				PEAKFL	PEAKFL*TU_ALL				
RW8	REL WT	25	7		ZNTU	CUTU	CDTU			ZNTU*CUTU	ZNTU*CDTU		
RW9	REL WT	22	5						#PREY	RICH	#YOY	#PREY*ZNTU	
RW10	REL WT	21	7		ZNTU				#PREY	RICH	#YOY		
RW11	REL WT	22	7					PEAKFL	#PREY	RICH	#YOY	#PREY*PEAKFL	
DN1	DENSITY	29	3		ZNTU								
DN2	DENSITY	30	3					PEAKFL					
DN3	DENSITY	29	5		ZNTU			PEAKFL	PEAKFL*ZNTU				
DN4	DENSITY	29	5		ZNTU	CDTU				ZNTU*CDTU			
DN5	DENSITY	29	7		ZNTU	CDTU		PEAKFL	PEAKFL*ZNTU	ZNTU*CDTU			
DN6	DENSITY	29	3	TU_ALL									
DN7	DENSITY	29	5	TU_ALL				PEAKFL	PEAKFL*TU_ALL				
DN8	DENSITY	29	7		ZNTU	CUTU	CDTU			ZNTU*CUTU	ZNTU*CDTU		
DN9	DENSITY	23	5						#PREY	RICH	#YOY		
DN10	DENSITY	22	7		ZNTU				#PREY	RICH	#YOY	#PREY*ZNTU	
DN11	DENSITY	23	7					PEAKFL	#PREY	RICH	#YOY	#PREY*PEAKFL	

Table IV. 2a. Descriptive statistics for brown trout metrics and predictor variables for the Eagle River from mine-impacted stream reaches. Data reflects the years 1997-2005.

Site	Variable	N	Mean	St. Dev	Max	Min
ER2.2	*Biomass (mean wt. in g)*	9	127	25	155	83
	Relative weight (index)	9	100	5	107	92
	Density (#/ha)	9	436	192	673	72
	Cu toxicity unit (index)	9	0.18	0.05	0.24	0.11
	Zn toxicity unit (index)	9	0.41	0.25	1.04	0.23
	Cd toxicity unit (index)	9	0.20	0.07	0.36	0.13
	Baseflow (cfs)	9	86	27	141	59
	Peakflow (cfs)	9	806	437	1429	208
	Minimum flow (cfs)	9	30	8	44	18
	Metals season flow (cfs)	9	387	179	692	125
	Young of year count (#/station)	9	31	15	49	2
	Insect abundance (#/ insects/sample)	9	365	149	722	237
	insect richness (# taxa/sample)	9	27	5	36	20
ER2.9	*Biomass (mean wt. in g)*	9	116	25	153	68
	Relative weight (index)	9	102	4	109	96
	Density (#/ha)	9	178	36	249	129
	Cu toxicity unit (index)	9	0.17	0.05	0.21	0.10
	Zn toxicity unit (index)	9	0.56	0.26	1.19	0.32
	Cd toxicity unit (index)	9	0.24	0.08	0.35	0.14
	Baseflow (cfs)	9	95	30	155	65
	Peakflow (cfs)	9	885	481	1570	229
	Minimum flow (cfs)	9	34	8	49	20
	Metals season flow (cfs)	9	425	196	761	138
	Young of year count (#/station)	9	37	32	101	3
	Insect abundance (#/ insects/sample)	9	394	149	644	239
	insect richness (# taxa/sample)	9	27	3	32	21
ER3	*Biomass (mean wt. in g)*	9	85	13	101	58
	Relative weight (index)	9	95	7	104	83
	Density (#/ha)	9	275	82	371	163
	Cu toxicity unit (index)	9	0.09	0.05	0.16	0.04
	Zn toxicity unit (index)	9	0.42	0.24	0.83	0.17
	Cd toxicity unit (index)	9	0.16	0.11	0.42	0.07
	Baseflow (cfs)	9	95	30	155	65
	Peakflow (cfs)	9	1150	702	2100	229
	Minimum flow (cfs)	9	34	8	49	20
	Metals season flow (cfs)	9	425	196	761	138
	Young of year count (#/station)	9	53	34	101	12
	Insect abundance (#/ insects/sample)	9	82	30	125	38
	insect richness (# taxa/sample)	9	20	5	25	12

Site	Variable	N	Mean	St. Dev	Max	Min
ER4.2	*Biomass (mean wt. in g)*	9	95	16	123	66
	Relative weight (index)	9	95	10	105	74
	Density (#/ha)	9	321	103	452	173
	Cu toxicity unit (index)	9	0.07	0.04	0.16	0.03
	Zn toxicity unit (index)	9	0.33	0.16	0.62	0.17
	Cd toxicity unit (index)	9	0.12	0.08	0.32	0.05
	Baseflow (cfs)	9	100	30	160	70
	Peakflow (cfs)	9	1112	481	1797	456
	Minimum flow (cfs)	9	39	8	54	25
	Metals season flow (cfs)	9	652	196	988	365
	Young of year count (#/station)	9	42	22	77	12
	Insect abundance (#/ insects/sample)	9	138	100	347	31
	insect richness (# taxa/sample)	9	21	7	29	7
ER5	*Biomass (mean wt. in g)*	9	107	9	124	96
	Relative weight (index)	9	97	11	105	69
	Density (#/ha)	9	238	79	381	130
	Cu toxicity unit (index)	9	0.05	0.02	0.08	0.03
	Zn toxicity unit (index)	9	0.27	0.12	0.53	0.16
	Cd toxicity unit (index)	9	0.09	0.03	0.15	0.04
	Baseflow (cfs)	9	100	30	160	70
	Peakflow (cfs)	9	1112	481	1797	456
	Minimum flow (cfs)	9	39	8	54	25
	Metals season flow (cfs)	9	652	196	988	365
	Young of year count (#/station)	9	45	27	94	7
	Insect abundance (#/ insects/sample)	9	383	146	610	206
	insect richness (# taxa/sample)	9	28	5	34	21

Table IV.2b. Descriptive statistics for brown trout metrics and predictor variables for the upper Arkansas River mine-impacted stream reaches. Data reflects the years 1995-2006.

Site	Variable	N	Mean	St. Dev	Max	Min
AR3	*Biomass (lbs/acre)*	10	81	59	167	13
	Relative weight (index)	9	96	4	101	88
	Density (#/acre)	10	349	276	808	51
	Cu toxicity unit (index)	12	0.08	0.18	0.65	0.01
	Zn toxicity unit (index)	12	0.81	0.83	3.29	0.15
	Cd toxicity unit (index)	12	0.75	1.35	4.99	0.07
	Baseflow (cfs)	12	21	3	26	18
	Peakflow (cfs)	12	568	303	1130	138
	Minimum flow (cfs)	12	14	3	19	10
	Metals season flow (cfs)	12	120	45	238	71
	Young of year count (#/station)	10	29	32	110	2
	Insect abundance (#/m2)	10	5840	6046	19414	522
	insect richness (#/m2)	10	40	11	60	27
AR4	*Biomass (lbs/acre)*	10	142	73	242	44
	Relative weight (index)	9	92	2	96	89
	Density (#/acre)	10	362	180	700	107
	Cu toxicity unit (index)	11	0.16	0.15	0.51	0.01
	Zn toxicity unit (index)	11	0.38	0.18	0.62	0.17
	Cd toxicity unit (index)	11	0.69	1.26	4.43	0.07
	Baseflow (cfs)	12	102	13	125	85
	Peakflow (cfs)	12	1103	624	2240	256
	Minimum flow (cfs)	12	66	17	99	44
	Metals season flow (cfs)	12	276	104	548	163
	Young of year count (#/station)	10	168	162	444	23
	Insect abundance (#/m2)	10	18825	8025	27945	6625
	insect richness (#/m2)	10	45	8	58	38
AR5	*Biomass (lbs/acre)*	10	77	28	130	40
	Relative weight (index)	8	95	4	102	90
	Density (#/acre)	10	237	125	457	113
	Cu toxicity unit (index)	12	0.15	0.18	0.60	0.01
	Zn toxicity unit (index)	12	0.41	0.46	1.78	0.09
	Cd toxicity unit (index)	12	0.44	0.73	2.70	0.07
	Baseflow (cfs)	12	102	13	125	85
	Peakflow (cfs)	12	1103	624	2240	256
	Minimum flow (cfs)	12	66	17	99	44
	Metals season flow (cfs)	12	276	104	548	163
	Young of year count (#/station)	9	15	16	49	0
	Insect abundance (#/m2)	10	8101	3313	14943	4231
	insect richness (#/m2)	10	43	8	54	30

Table IV.3a. Results of AIC model selection for brown trout metrics for the Eagle River using data from mine sites between 1997-2005. Models with a delta AICc < 2 are "the best" based on the principle of parsimony (see **boldface type).** P-values and Adjusted R-squared values are given for each model. Refer to Table 1a for a list of parameters in each model.

Model	Pmodel	Adj-R2	AICc	Δ AICc	wi
BM1	0.6669	0.00	-126.8	26.1	0.00
BM2	0.5227	0.00	-127.1	25.8	0.00
BM3	0.6306	0.00	-123.6	29.3	0.00
BM4	0.5987	0.00	-123.8	29.1	0.00
BM5	0.5453	0.00	-120.7	32.2	0.00
BM6	0.3962	0.00	-127.5	25.4	0.00
BM7	0.3240	0.00	-125.5	27.4	0.00
BM8	0.2993	0.03	-123.0	29.9	0.00
BM9	**0.0001**	**0.47**	**-152.8**	**0.1**	**0.44**
BM10	0.0001	0.49	-150.7	2.2	0.16
BM11	**0.0001**	**0.51**	**-152.6**	**0.3**	**0.40**
RW1	0.8416	0.00	187.9	8.2	0.01
RW2	0.5585	0.00	187.6	7.8	0.02
RW3	0.3130	0.02	189.0	9.3	0.01
RW4	0.1449	0.06	187.0	7.3	0.02
RW5	0.0457	0.15	185.8	6.1	0.04
RW6	0.5995	0.00	187.6	7.9	0.01
RW7	0.4312	0.00	189.9	10.2	0.00
RW8	**0.0050**	**0.26**	**179.7**	**0.0**	**0.77**
RW9	0.0743	0.09	185.4	5.7	0.05
RW10	0.0282	0.18	184.5	4.7	0.07
RW11	0.1877	0.06	190.1	10.4	0.00
DN1	0.0001	0.37	-158.4	15.2	0.00
DN2	0.0728	0.05	-139.8	33.9	0.00
DN3	0.0001	0.39	-156.7	16.9	0.00
DN4	0.0001	0.44	-161.1	12.5	0.00
DN5	0.0001	0.46	-159.5	14.1	0.00
DN6	0.0005	0.23	-149.3	24.4	0.00
DN7	0.0032	0.23	-146.3	27.4	0.00
DN8	**0.0001**	**0.61**	**-173.7**	**0.0**	**1.00**
DN9	0.1416	0.06	-137.2	36.4	0.00
DN10	0.0012	0.32	-148.4	25.3	0.00
DN11	0.1153	0.09	-135.7	37.9	0.00

Table IV.3b. Results of AIC model selection for brown trout metrics for the upper Arkansas River using data from mine sites between 1995-2006. Models with a delta AICc < 2 are "the best" based on the principle of parsimony (see **boldface type).** P-values and Adjusted R-squared values are given for each model. Refer to Table 1b for a list of parameters in each model.

Model	Pmodel	Adj-R2	AICc	Δ AICc	wi
BM1	0.4845	0.00	-11.4	22.2	0.00
BM2	0.1083	0.06	-14.9	18.7	0.00
BM3	0.2990	0.00	-9.4	24.2	0.00
BM4	0.1083	0.11	-12.0	21.6	0.00
BM5	0.1217	0.15	-9.0	24.6	0.00
BM6	0.4745	0.00	-11.4	22.2	0.00
BM7	0.3519	0.00	-8.9	24.7	0.00
BM8	0.2173	0.09	-6.7	26.9	0.00
BM9	0.0001	0.63	-27.3	6.3	0.04
BM10	0.0002	0.67	-21.1	12.5	0.00
BM11	**0.0001**	**0.78**	**-33.6**	**0.0**	**0.95**
RW1	0.1056	0.07	69.1	2.8	0.12
RW2	0.0546	0.11	70.9	4.6	0.05
RW3	0.1778	0.09	72.3	6.0	0.02
RW4	0.2046	0.08	72.6	6.3	0.02
RW5	0.2737	0.07	77.6	11.3	0.00
RW6	0.2754	0.01	70.6	4.3	0.05
RW7	0.3411	0.02	74.1	7.8	0.01
RW8	0.2666	0.08	77.5	11.2	0.00
RW9	**0.2088**	**0.09**	**66.3**	**0.0**	**0.47**
RW10	**0.1968**	**0.15**	**68.4**	**2.1**	**0.17**
RW11	0.1612	0.17	70.0	3.7	0.08
DN1	0.5166	0.00	-60.3	8.4	0.01
DN2	**0.0122**	**0.18**	**-68.7**	**0.0**	**0.86**
DN3	0.0811	0.14	-61.9	6.8	0.03
DN4	0.1360	0.10	-60.6	8.1	0.02
DN5	0.0243	0.28	-62.9	5.8	0.05
DN6	0.7354	0.00	-60.0	8.7	0.01
DN7	0.1543	0.09	-60.3	8.5	0.01
DN8	0.1448	0.13	-57.2	11.5	0.00
DN9	0.0041	0.41	-54.0	14.7	0.00
DN10	0.0063	0.48	-47.8	20.9	0.00
DN11	0.0003	0.64	-59.5	9.2	0.01

Table IV.4a. Means (ESTIMATE), standard errors (SE) and statistical significance (P) of parameters in the "best models" from AIC model selection for the Eagle River. Direction of association between brown trout metric responses and parameters is also shown in the sign of the estimate, where a negative sign indicates that the variable contributes to reductions in brown trout metrics. **Significant P-values are in boldface type.**

BEST MODELS	*Parameters*	Parameter Code	Estimate	SE	P
Ln(Biomass)					
BM9	*Intercept*	Bo	3.948	0.177	**0.0001**
	Insect abundance	Ln(#PREY)	0.484	0.115	**0.0001**
(Adj. R2= 0.47)	*Insect richness*	RICH	-0.013	0.007	0.0834
n=44	*# of young of year*	#YOY	-0.003	0.001	**0.0061**
BM11	*Intercept*	Bo	2.153	1.738	0.2230
	Insect abundance	Ln(#PREY)	1.486	0.731	**0.0491**
(Adj. R2= 0.51)	*Insect richness*	RICH	-0.011	0.007	0.1158
n=44	*# of young of year*	#YOY	-0.004	0.001	**0.0022**
	Peakflow previous year	Log10(PPEAKFL)	0.627	0.571	0.2787
	Interaction 1	Ln(#PREY)*PPEAKFL	-0.349	0.244	0.1607
Relative Weight					
RW8	*Intercept*	Bo	102.800	4.350	**0.0001**
	Zn toxicity units previous year	PZNTU	-29.882	13.767	**0.0363**
(Adj. R2 = 0.26)	*Cu toxicity units previous year*	PCUTU	-49.369	88.725	0.5812
n=44	*Cd toxicity units previous year*	PCDTU	24.976	62.445	0.6914
	Interaction 1	PZNTU*PCDTU	-96.930	123.713	0.4382
	Interaction 2	PZNTU*PCUTU	308.440	183.053	0.1000
Log10(Density)					
DN8	*Intercept*	Bo	2.590	0.082	**0.0001**
	Zn toxicity units previous year	PZNTU	-0.980	0.257	**0.0005**
(Adj. R2= 0.61)	*Cu toxicity units previous year*	PCUTU	7.350	1.683	**0.0001**
n=45	*Cd toxicity units previous year*	PCDTU	-3.730	1.184	**0.0031**
	Interaction 1	PZNTU*PCDTU	9.240	2.333	**0.0003**
	Interaction 2	PZNTU*PCUTU	-14.300	3.465	**0.0002**

Table IV.4b. Means (ESTIMATE), standard errors (SE) and statistical significance (P) of parameters in the "best models" from AIC model selection for the upper Arkansas River. Direction of association between brown trout metric responses and parameters is also shown in the sign of the estimate. **Significant P-values are in boldface type.**

BEST MODELS	*Parameters*	Parameter Code	Estimate	SE	P
Ln(Biomass)					
BM11	*Intercept*	Bo	-1.007	1.670	0.5417
	Insect abundance	Ln(#PREY)	1.161	0.383	**0.0076**
(Adj. R2= 0.78)	*Insect richness*	RICH	0.029	0.009	**0.0089**
n=23	*# of young of year*	#YOY	0.001	0.001	0.6447
	Peakflow current year	Log10(PEAKFL)	-0.001	0.001	0.8118
	Interaction 1	Ln(#PREY)*PEAKFL	-0.001	0.001	0.9448
Relative Weight					
RW9	*Intercept*	Bo	108.439	7.803	**0.0001**
	Insect abundance	Ln(#PREY)	-2.854	2.137	0.1982
(Adj. R2 = 0.09)	*Insect richness*	RICH	-0.072	0.091	0.4373
n=22	*# of young of year*	#YOY	-0.003	0.008	0.7603
RW10	*Intercept*	Bo	95.281	18.767	**0.0001**
	Insect abundance	Ln(#PREY)	0.572	5.041	0.9111
(Adj. R2 = 0.15)	*Insect richness*	RICH	-0.126	0.092	0.1912
n=21	*# of young of year*	#YOY	-0.004	0.009	0.6432
	Zn toxicity units current year	ZNTU	15.212	27.259	0.5849
	Interaction 1	Ln(#PREY)*ZNTU	-2.530	7.054	0.7252
Log10(Density)					
DN2	*Intercept*	Bo	4.069	0.506	**0.0001**
(Adj. R2 = 0.26)	*Peakflow current year*	Log10(PEAKFL)	-0.587	0.177	**0.0026**
n=30					

Table IV. 5a. Regression models from stepwise selection for the Eagle River using data from mine sites between the years 1997-2005.
Means (ESTIMATE), standard errors (SE) and statistical significance (P) of parameters are given. Direction of association between responses and parameters is also shown in the sign of the estimate, where a negative sign indicates that the variable contributes to reductions in the brown trout metric. Partial R-squared values show the relative contributions of each variable. **P-values are in boldface type.**

BEST MODELS	*Parameters*	Parameter Code	Estimate	SE	P	Partial R2
Biomass	*Overall model significance*				**0.0001**	
	Intercept	Bo	4.063	0.185	**0.0001**	
(Adj. R2 = 0.49)	*Invertebrate abundance*	Ln(# PREY)	0.490	0.111	**0.0001**	0.30
n= 44	*Young of year count*	#YOY	-0.004	0.001	**0.0008**	0.17
	Invertebrate richness	RICH	-0.013	0.007	0.0834	0.04
	Zn toxicity units previous year	PZNTU	-0.212	0.122	0.0914	0.04
Relative Weight	*Overall model significance*				**0.0129**	
	Intercept	Bo	78.693	7.433	**0.0001**	
(Adj. R2 = 0.13)	*Invertebrate abundance*	Ln(# PREY)	8.242	3.174	**0.0129**	0.13
n=44						
Density	*Overall model significance*				**0.0001**	
	Intercept	Bo	2.878	0.113	**0.0001**	
(Adj. R2 = 0.54)	*Minimum flow previous year*	PMINFL	-0.010	0.003	**0.0008**	0.15
n= 44	*Zd toxicity units previous year*	PZNTU	-0.724	0.114	**0.0001**	0.39
	Cu toxicity units previous year	PCUTU	0.813	0.419	0.0592	0.04

Table IV.5b. Regression models from stepwise selection for the upper Arkansas River using data from mine sites between the years 1994-2006. Means (ESTIMATE), standard errors (SE) and statistical significance (P) of parameters are given. Direction of association between responses and parameters is also shown in the sign of the estimate, where a negative sign indicates that the variable contributes to reductions in the brown trout metric. Partial R-squared values show the relative contributions of each variable. NA = not given in model results. **P-values are in boldface type.**

BEST MODELS	*Parameters*	Parameter Code	Estimate	SE	P	Partial R2
Biomass	*Overall model significance*				**0.0001**	
	Intercept	Bo	2.068	0.894	**0.0327**	
(Adj. R2 = 0.80)	*Invertebrate abundance*	Ln(# PREY)	1.230	0.212	**0.0004**	0.47
n= 22	*Peakflow current year*	Log10(PEAKFL)	-0.537	0.110	**0.0001**	0.30
	Invertebrate richness	RICH	0.021	0..010	**0.0277**	0.05
Relative Weight	*Overall model significance*				**0.0258**	
	Intercept	Bo	98.000	3.551	**0.0001**	
(Adj. R2 = 0.26)	*Zn toxicity units current year*	ZNTU	6.285	2.722	0.0503	0.19
n=21	*Invertebrate richness*	RICH	-0.149	0.075	0.0614	0.14
Density	*Overall model significance*				**0.0001**	
	Intercept	Bo	0.385	0.388	0.3335	
(Adj. R2 = 0.71)	*Invertebrate abundance*	Ln(# PREY)	0.462	0.106	**0.0004**	0.27
n= 22	*Peakflow current year*	Log10(PEAKFL)	-0.645	0.1323	**0.0253**	0.16
	Invertebrate richness	RICH	0.012	0.005	**0.0042**	0.34
	Overall model significance				**0.0001**	
(Adj. R2 = 0.74)	*Intercept*	Bo	1.430	0.535	**0.0161**	
n= 23	*Invertebrate abundance*	Ln(# PREY)	0.503	0.105	**0.0002**	na
	Peakflow current year	Log10(PEAKFL)	-0.468	0.151	**0.0065**	na
	Invertebrate richness	RICH	0.006	0.004	0.2016	na
	Cd toxicity units current year	CDTU	0.388	0.230	0.1102	na
	Cu toxicity units current year	CUTU	-1.184	0.676	0.0977	na

Table IV.6a. Percent difference in metrics at mine sites compared to reference site ER1.9 in the Eagle River.

Site	Year	Density	Attain?	Biomass	Attain?
ER2.2	1997	24	N	119	Y
	1998	152	Y	54	N
	1999	295	Y	54	N
	2000	181	Y	75	N
	2001	162	Y	80	N
	2002	209	Y	107	Y
	2003	95	N	na	--
	2004	108	Y	110	Y
	2005	111	Y	106	Y
ER2.9	1997	44	N	106	Y
	1998	60	N	80	N
	1999	86	N	85	N
	2000	93	N	74	N
	2001	60	N	77	N
	2002	101	Y	47	N
	2003	27	N	na	--
	2004	44	N	93	N
	2005	77	N	70	N
ER3	1997	62	N	74	N
	1998	56	N	66	N
	1999	120	Y	51	N
	2000	114	Y	48	N
	2001	90	N	52	N
	2002	124	Y	66	N
	2003	70	N	na	--
	2004	78	N	64	N
	2005	103	Y	66	N
ER4.2	1997	64	N	55	N
	1998	87	N	65	N
	1999	112	Y	62	N
	2000	130	Y	59	N
	2001	116	Y	56	N
	2002	98	N	61	N
	2003	70	N	na	--
	2004	75	N	68	N
	2005	68	N	87	N
ER5	1997	46	N	80	N
	1998	53	N	81	N
	1999	102	Y	62	N
	2000	88	N	65	N
	2001	67	N	73	N
	2002	76	N	68	N
	2003	55	N	na	--
	2004	68	N	84	N
	2005	54	N	73	N

Table IV.6b. Percent difference in metrics at mine sites compared to reference site AR1 in the Arkansas River.

Site	Year	Density	Attain?	Biomass	Attain?
AR3	1994	na	--	na	--
	1995	na	--	na	--
	1996	26	N	33	N
	1997	14	N	11	N
	1998	na	--	na	--
	1999	52	N	44	N
	2000	na	--	na	--
	2001	63	N	83	N
	2002	96	N	111	Y
	2003	139	Y	164	Y
	2004	80	N	126	Y
	2005	116	Y	145	Y
	2006	91	N	123	Y
AR4	1994	na	--	na	--
	1995	na	--	na	--
	1996	33	N	68	N
	1997	40	N	40	N
	1998	na	--	na	--
	1999	56	N	104	Y
	2000	na	--	na	--
	2001	79	N	195	Y
	2002	83	N	184	Y
	2003	76	N	173	Y
	2004	84	N	198	Y
	2005	71	N	185	Y
	2006	111	Y	257	Y
AR5	1994	na	--	na	--
	1995	na	--	na	--
	1996	na	--	na	--
	1997	29	N	46	N
	1998	na	--	na	--
	1999	36	N	85	N
	2000	na	--	na	--
	2001	56	N	101	Y
	2002	54	N	52	N
	2003	55	N	92	N
	2004	60	N	130	Y
	2005	67	N	82	N
	2006	72	N	148	Y

na = brown trout not sampled at reference site AR1 during these years, or not sampled at all sites during the fall season (e.g. sites sampled during spring in 1998 and 2000)

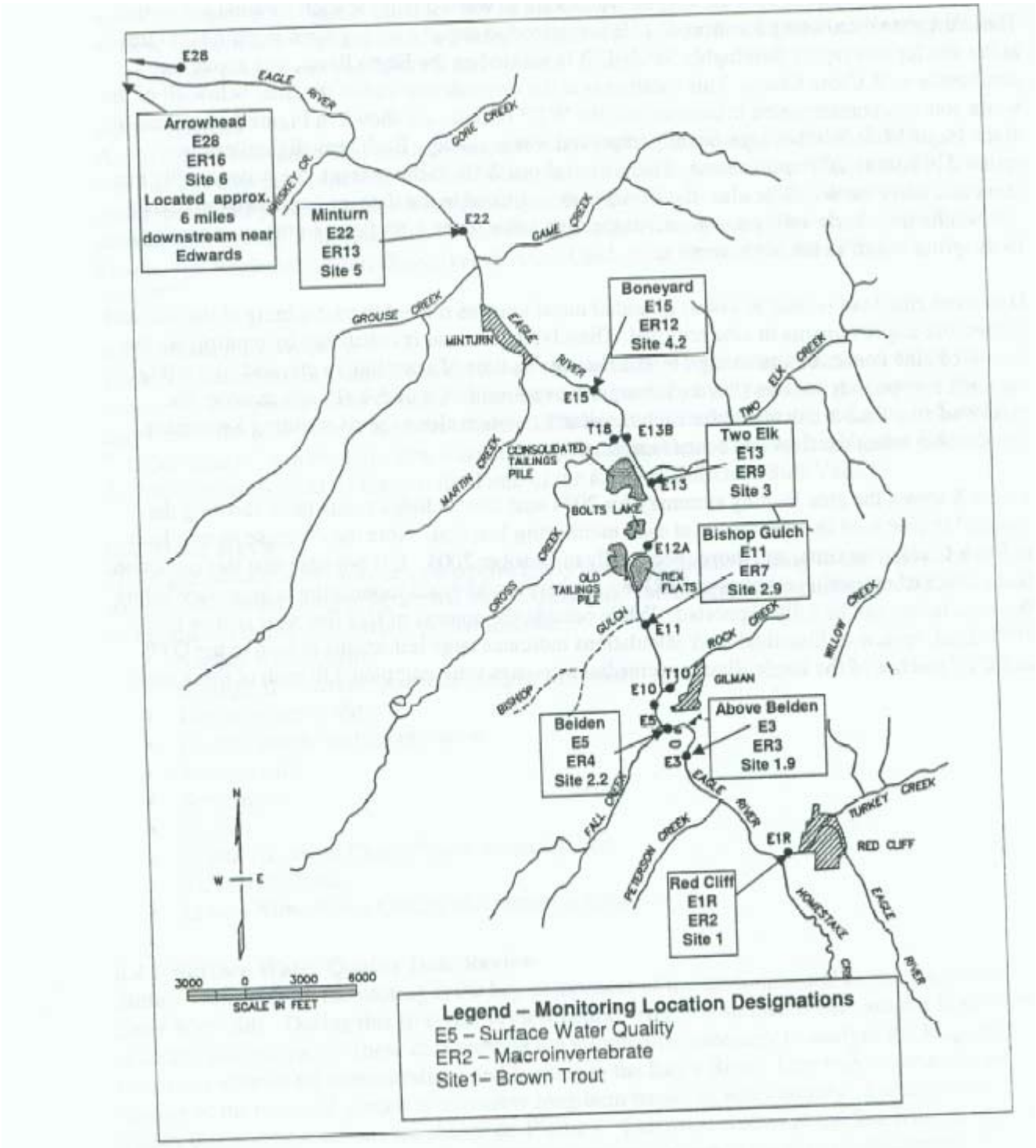


Figure IV.1. Map of Eagle River sites

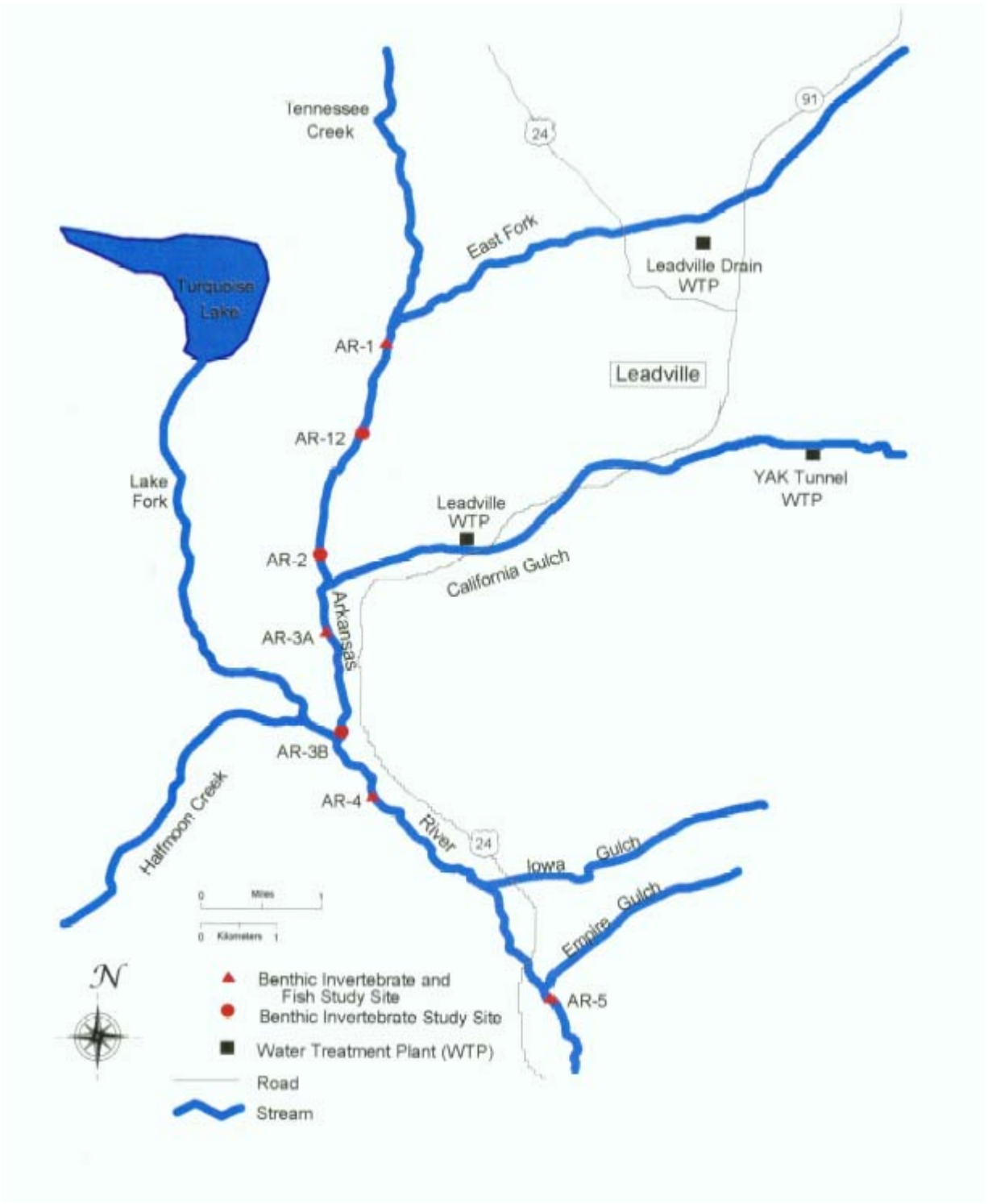
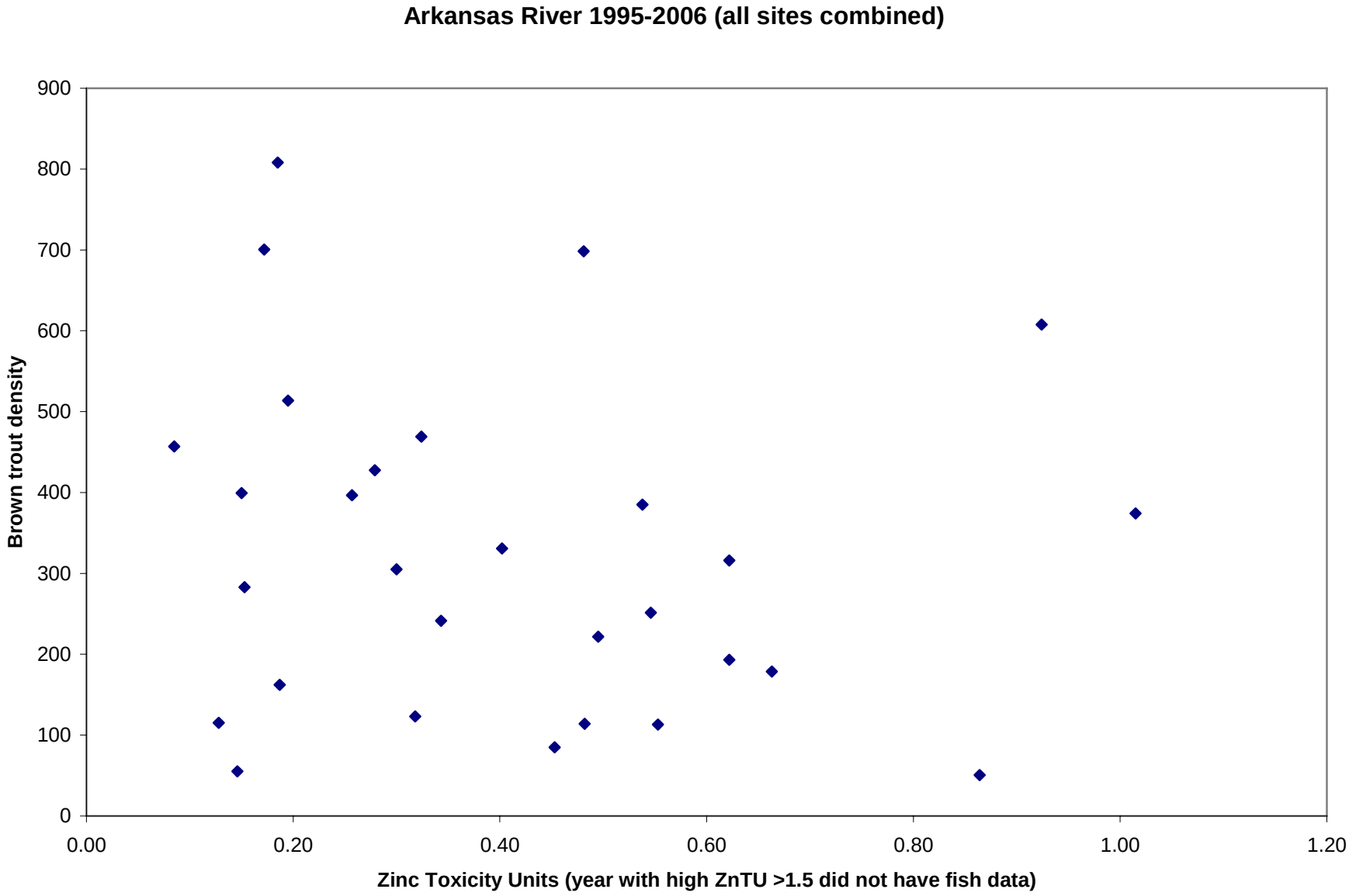
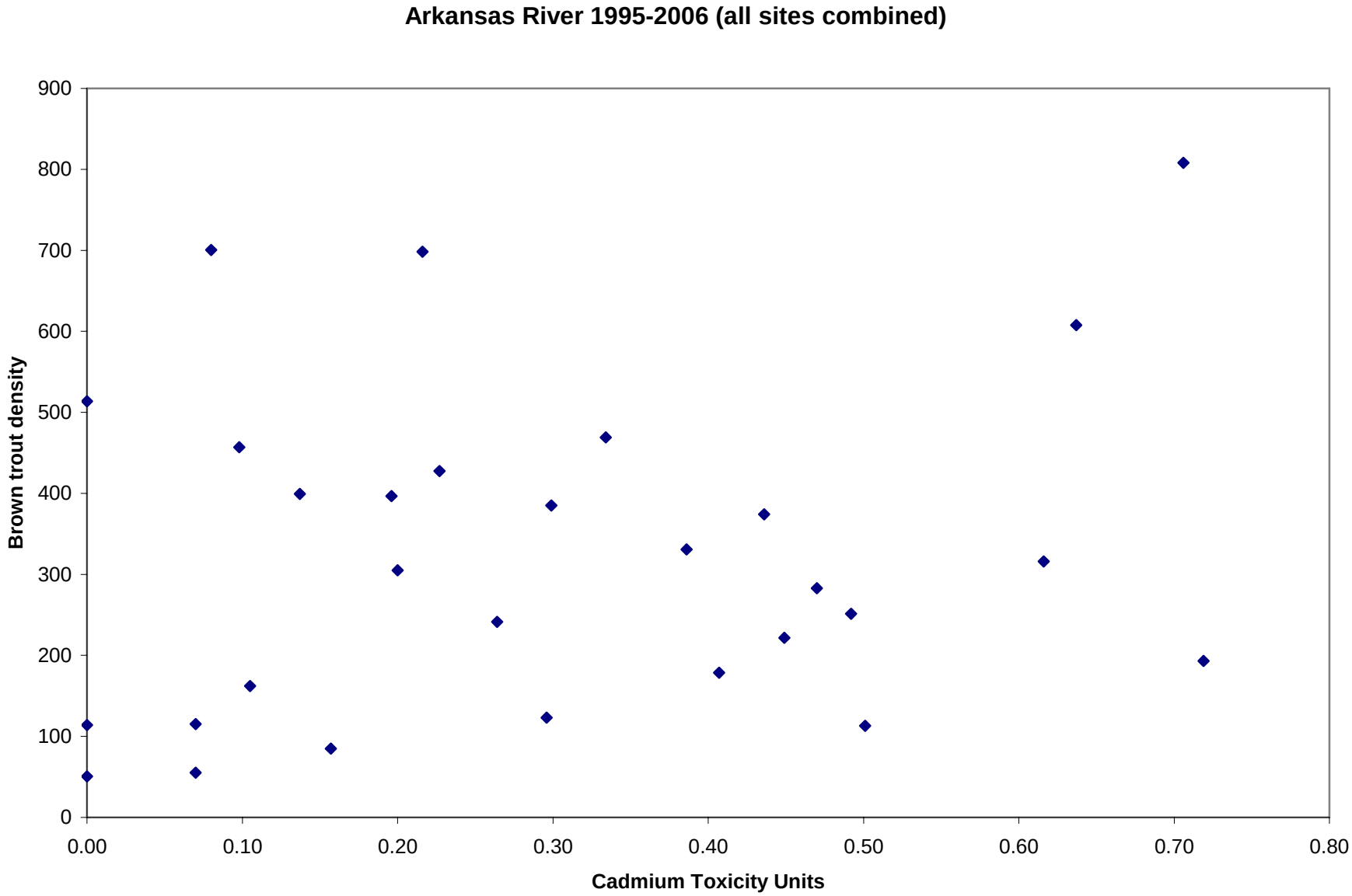


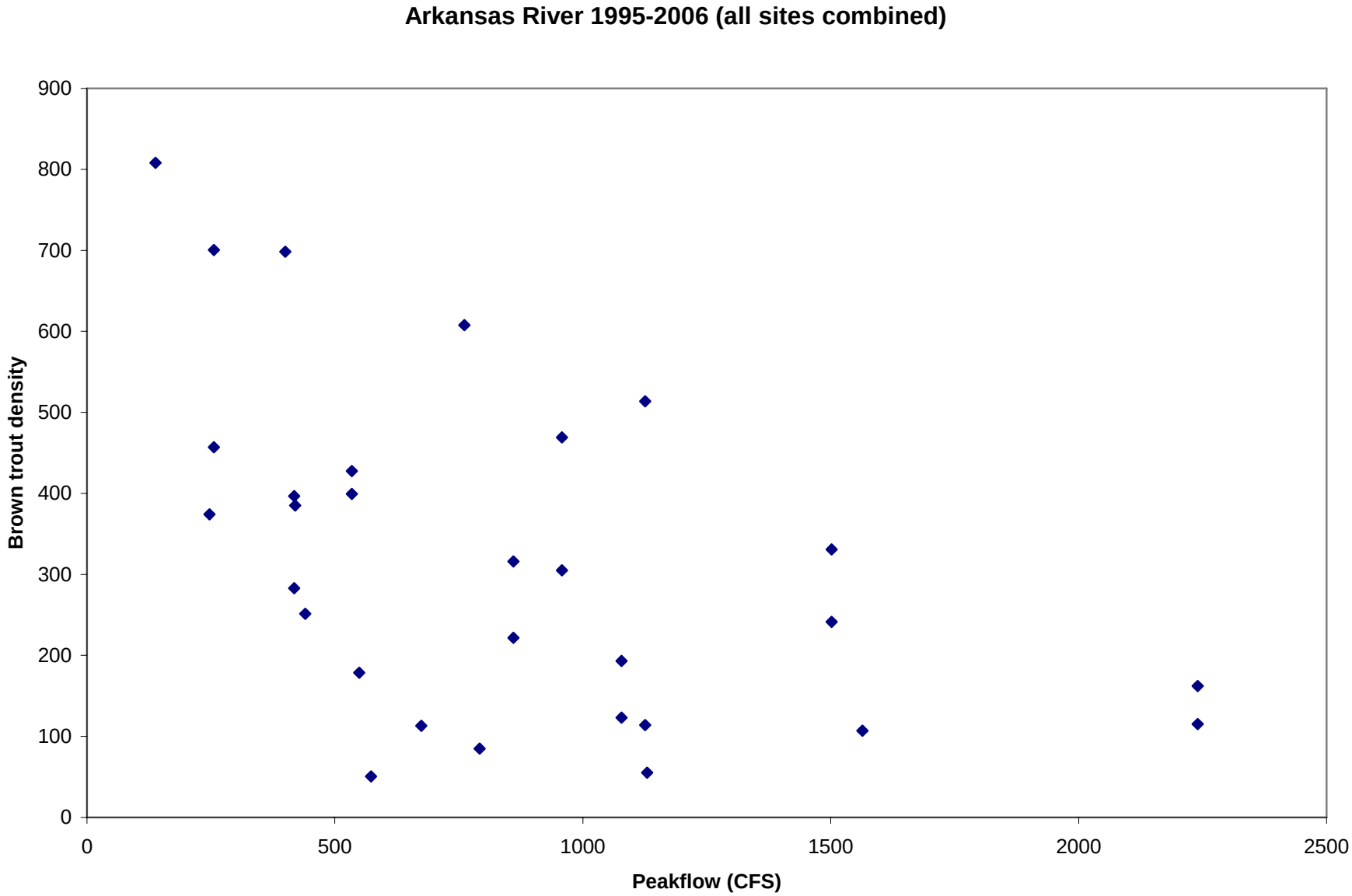
Figure IV.2. Map of upper Arkansas River sites.

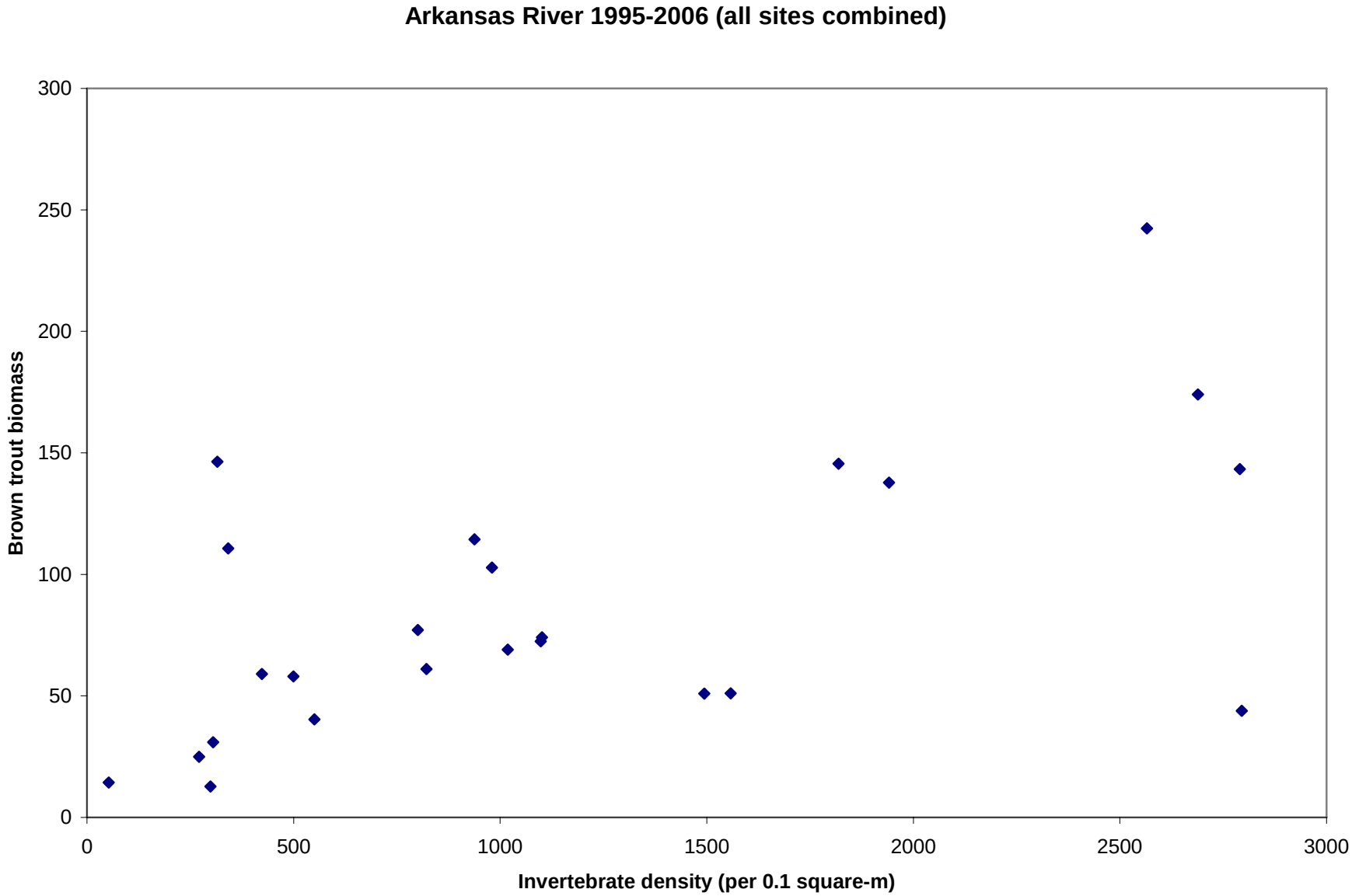
APPENDIX IV. A. 1.

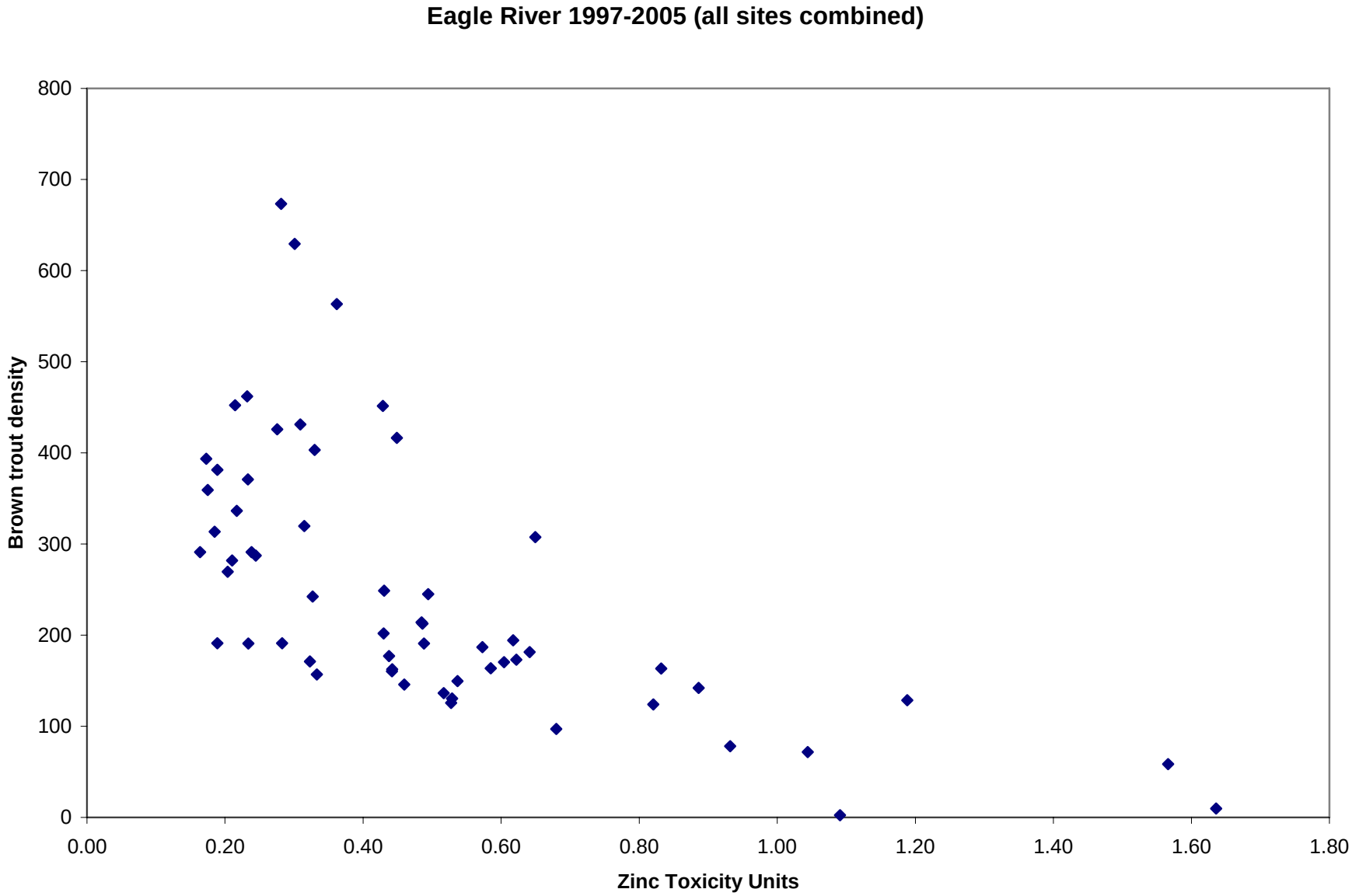
This appendix provides figures for aid in interpretation of statistical models presented in the Biocriteria Report. Variables and the relationships between the variables are provided in the report.

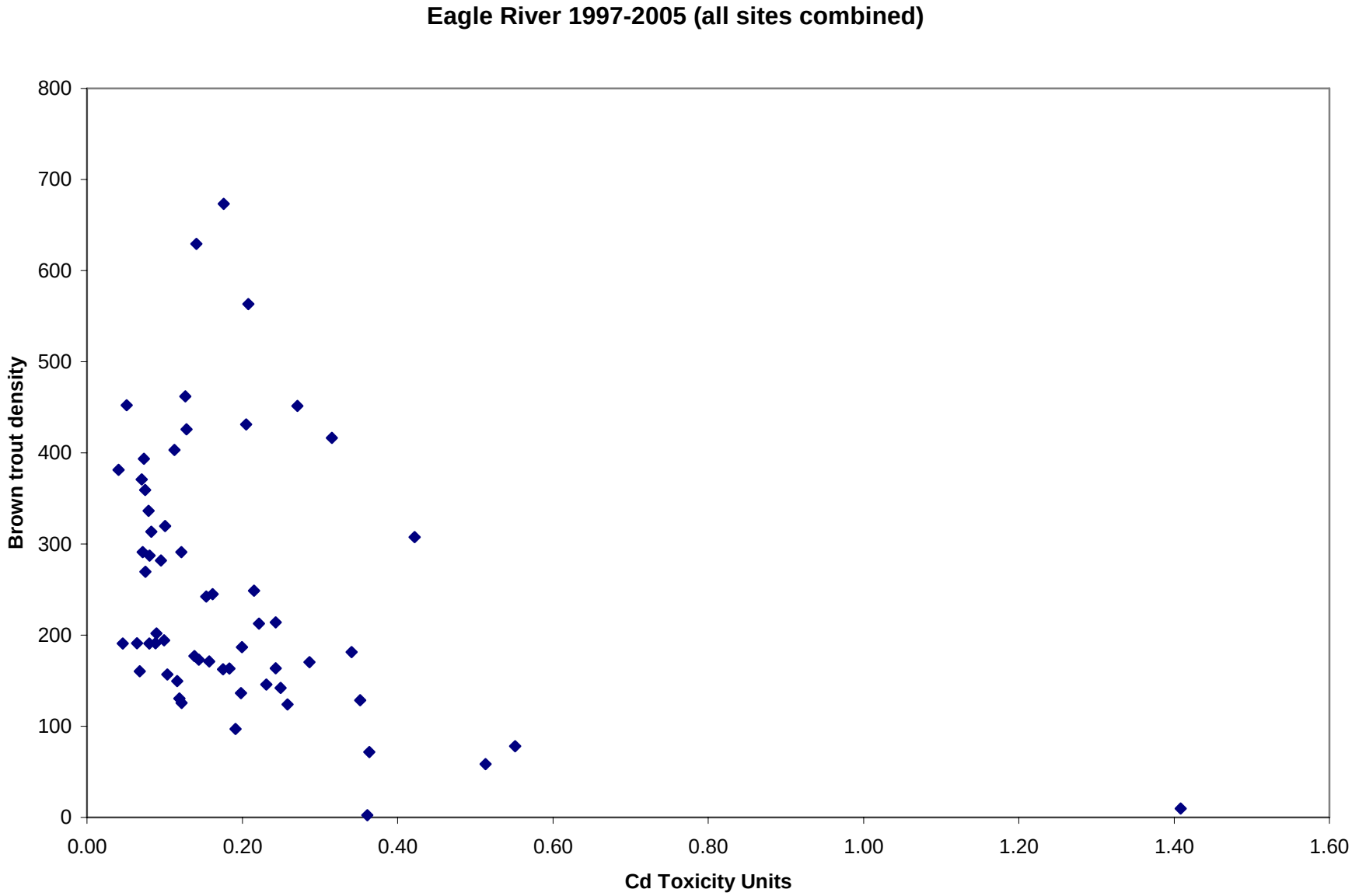

Arkansas River 1995-2006 (all sites combined)
Brown trout density
900
800
700
600
500
400
300
200
100
0
0.00
0.20
0.40
0.60
0.80
1.00
1.20
Zinc Toxicity Units (year with high ZnTU >1.5 did not have fish data)

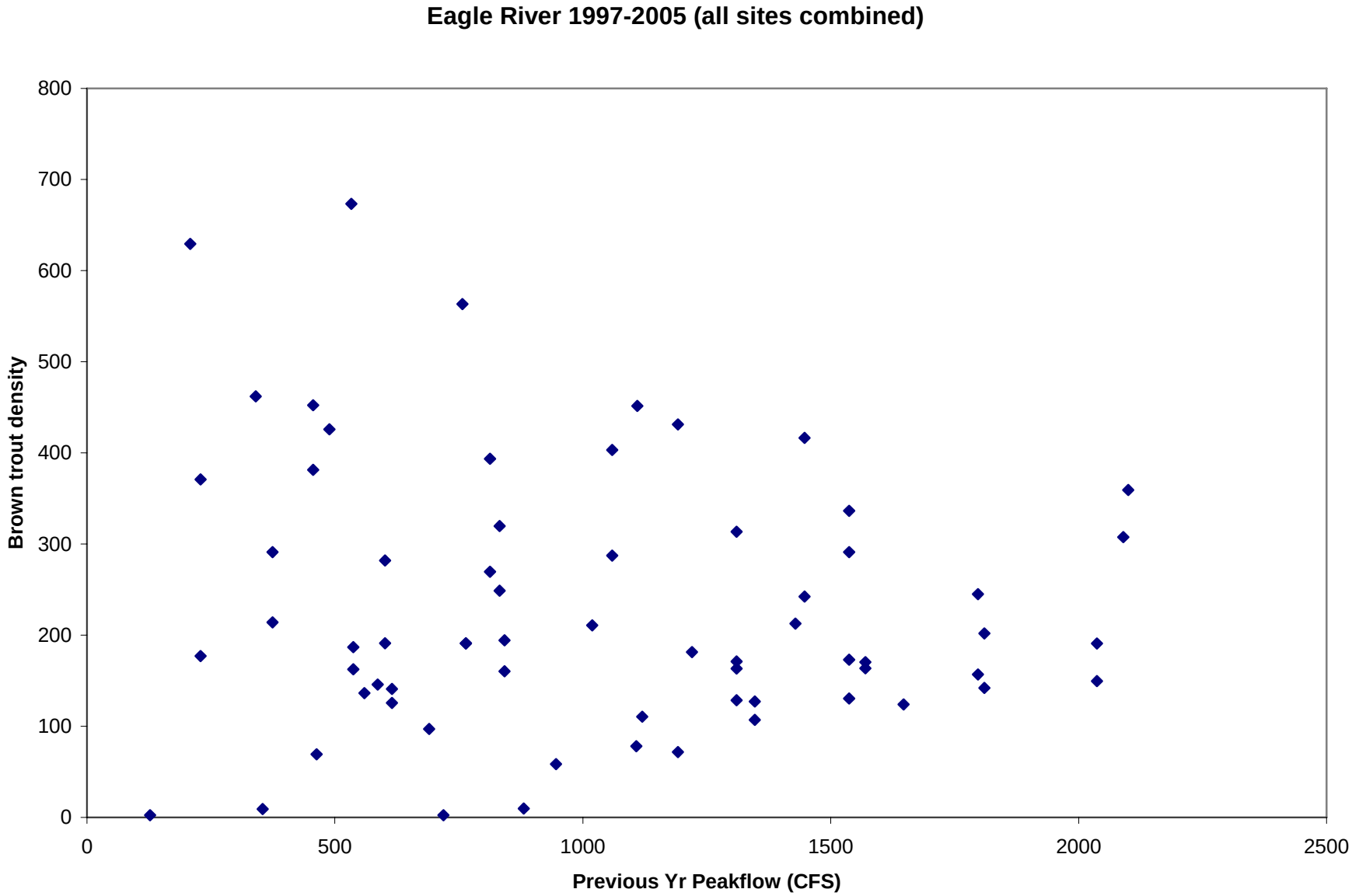

Arkansas River 1995-2006 (all sites combined)
Brown trout density
900
800
700
600
500
400
300
200
100
0
0.00
0.10
0.20
0.30
0.40
0.50
0.60
0.70
0.80
Cadmium Toxicity Units

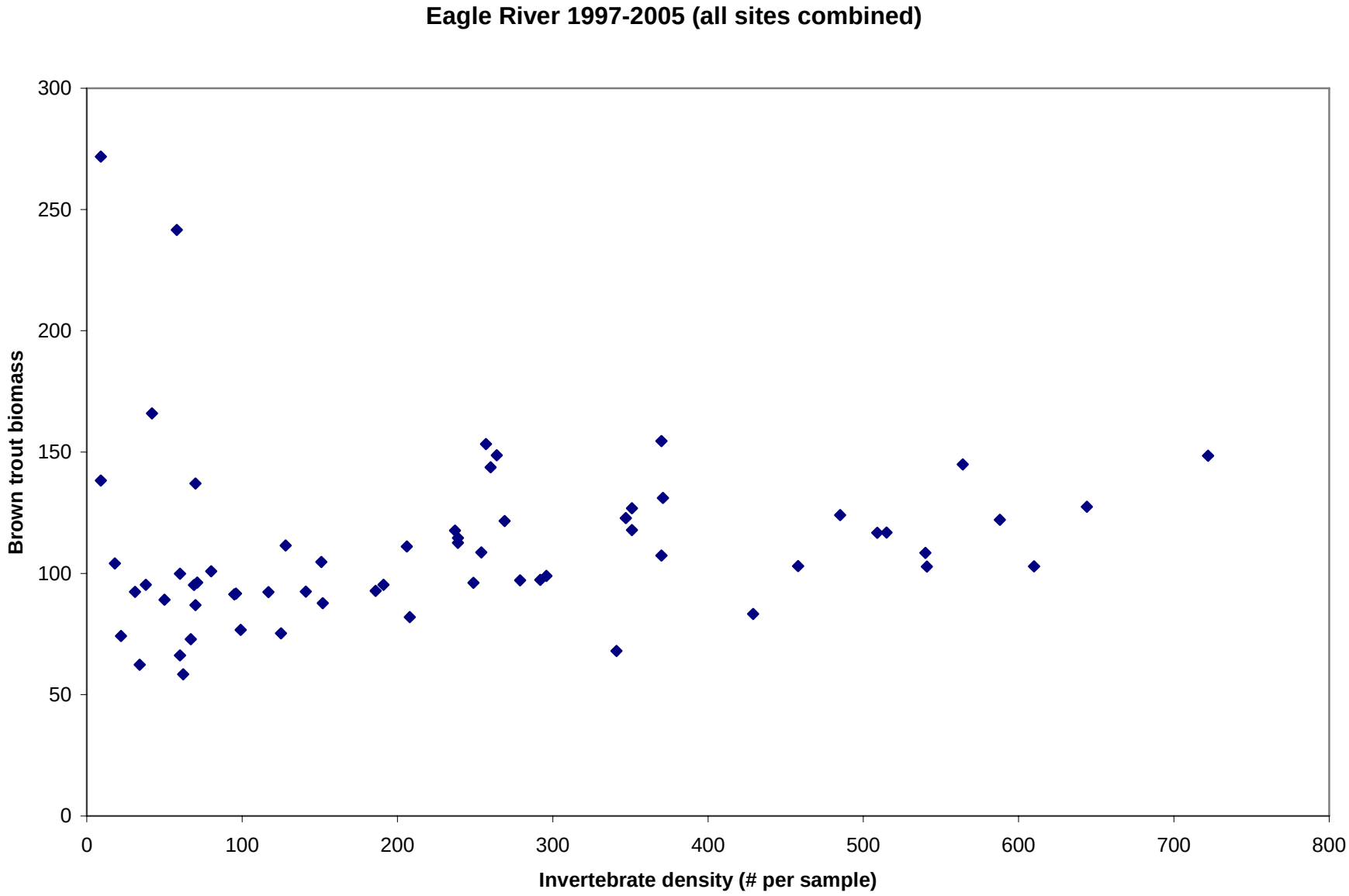

Arkansas River 1995-2006 (all sites combined)
Brown trout density
900
800
700
600
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Peakflow (CFS)


Arkansas River 1995-2006 (all sites combined)
Brown trout biomass
300
250
200
150
100
50
0
0
500
1000
1500
2000
2500
3000
Invertebrate density (per 0.1 square-m)


Eagle River 1997-2005 (all sites combined)
Brown trout density
800
700
600
500
400
300
200
100
0
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
1.80
Zinc Toxicity Units


Eagle River 1997-2005 (all sites combined)
Brown trout density
800
700
600
500
400
300
200
100
0
0.00
0.20
0.40
0.60
0.80
1.00
1.20
1.40
1.60
Cd Toxicity Units


Eagle River 1997-2005 (all sites combined)
Brown trout density
800
700
600
500
400
300
200
100
0
0
500
1000
1500
2000
2500
Previous Yr Peakflow (CFS)


Eagle River 1997-2005 (all sites combined)
Brown trout biomass
300
250
200
150
100
50
0
0
100
200
300
400
500
600
700
800
Invertebrate density (# per sample)

Toxicity of Zinc and Cadmium to Mottled Sculpin (*Cottus bairdi*)

By Stephen Brinkman, Katharine Mitchell and Nicole Vieira

INTRODUCTION

Sculpin are widely distributed in North America. Previous investigations have determined that mottled sculpin (*Cottus bairdi*) are among the most sensitive species of fish to the toxicity of zinc (Woodling et al. 2002, Brinkman and Woodling 2005). This finding supports numerous field observations of negative impacts to this species (McCormick et al. 1994, Maret and MacCoy 2002, Farag et al. 2003) and has been confirmed in other laboratory tests (USEPA 2006). Brinkman and Woodling (2005) related water hardness and toxicity of zinc to young of year mottled sculpin to determine hardness-based toxicity equations for use in water quality standards. In an attempt to further refine the hardness-toxicity relationship, an additional zinc toxicity tests was conducted using an intermediate water hardness level. Toxicity of cadmium to young of year mottled sculpin was also investigated.

MATERIAL and METHODS

Organisms

Young-of-year *C. bairdi* were collected using a backpack electrofishing unit with pulsed DC current. Sculpin for the zinc toxicity test were collected from the Dolores River near Lizard Head Pass in August 2006. Sculpin for the cadmium test were collected in October, 2006 from the White River near Meeker, Colorado. The fish were transported to the Colorado Division of Wildlife Toxicology Laboratory in Fort Collins, Colorado, USA in an aerated cooler. Temperature during transport was maintained using ice packs and a 12 V Chiller (Coolworks Inc, San Rafael, CA). Upon arrival, fish were immersed in a 3% sodium chloride solution for 3 minutes to remove ectoparasites, and then were placed in an aquarium supplied with a mixture of reverse osmosis water and onsite well water at a ratio that approximated the hardness and temperature of the collection site water. Hardness was slowly adjusted to test hardness levels at a rate of 5-10 mg/L per day. Temperature was adjusted to 12°C at rate of 1°C per day. Sculpin were acclimated to test water quality for 12 days prior to the start of the cadmium tests and 14 days before the start of the zinc test.

Sculpin were fed starter trout chow (Rangen Inc., Buhl ID) supplemented with <24 hr brine shrimp naupalii (GSL Brine Shrimp, Ogden UT) twice daily (once daily on weekends and holidays). Fry mortality during the holding period was minimal (7%) and appeared to be due to injuries from electroshocking. The weights of sculpin fry from the Dolores River increased from 0.13 g to 0.45 g in the 63 days between collection and termination of the zinc test. Sculpin fry from the White River increased in weight from 0.40 g to 0.60 g in the 33 days between collection and termination of the cadmium test.

Test Methods

Source water for the test consisted of a mixture of onsite well water and reverse osmosis water. Conductivity controllers (Oakton) maintained a constant mixture with a water hardness level near 45 mg/L. A continuous-flow serial diluter (Benoit et al. 1982) delivered exposure concentrations. The diluter was constructed of Teflon, polyethylene, and polypropylene components. Nalgene food-grade vinyl tubing delivered test solutions to exposure chambers. Test solutions overflowed from the exposure chambers into a water bath maintained at 12°C using a recirculating chiller (VWR model 1175MD). A metal stock solution was prepared by dissolving a calculated amount of metal sulfate salts in deionized water ($CdSO_4$ JT Baker, $ZnSO_4 \cdot 7H_2O$ Mallincrodt). New stock solutions were prepared as needed during the exposure period. Stock solutions were delivered to the diluter via a peristaltic pump at a rate of 2.0 ml/min. The diluter delivered five concentrations with a 50% dilution ratio and a control. Target concentrations for the zinc test were 800, 400, 200, 100, 50, and 0 µg/L. Target concentrations for the cadmium test were 32, 16, 8, 4, 2, and 0 µg/L. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 ml/min for each chamber.

Exposure chambers consisted of 2.8 L polypropylene containers. Dim fluorescent lighting provided a 16-h/8-h light-dark photoperiod. Diluters and toxicant flow rates were monitored daily to ensure proper operation. Eight sculpin were randomly allocated to each exposure chamber. High mortality occurred in the cadmium test. As a result, an additional treatment was added with a cadmium concentration about 1.0 µg/L. Due to limited numbers of sculpin, only four were placed in each chamber at the lower Cd concentration. Sculpin were not fed during the initial 96 hours of the toxicity tests.

During the initial 96 hours of exposure, water quality parameters were measured daily in all treatment levels within a replicate. Later, water quality parameters were measured weekly in all treatment levels within a replicate. Different replicates were selected at each sampling event. Hardness and alkalinity were determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH and conductivity. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. The conductivity, pH and dissolved oxygen meters were calibrated prior to each use.

Water samples were collected daily for dissolved metal analysis during the first 96 hours of the test. Exposure water was passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH <2. Chambers with no survivors remaining were not sampled. Zinc and copper were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source (High Purity Standards, Charleston SC). Sample splits were collected and spikes prepared at each sampling event to verify reproducibility and analytical recovery. Cadmium concentrations were measured with a Thermo Jarrell Ash Iris optical emission ICP spectrometer.

Ninety six hour median lethal concentrations (LC_{50}) were estimated using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978). Thirty day survival of fry of the zinc test were analyzed using Steel's Many-One Rank Test. Lengths and weights of surviving fry of the zinc test at test termination were analyzed with one-way ANOVA. The assumptions of normal error distribution and homogeneous group variances were tested using Shipiro-Wilk's test and Levene's test, respectively.

RESULTS

Water quality during the toxicity tests was constant with in each test, as evidenced by relatively low standard deviations (**Table V.1**). Hardness in the zinc test (99.5 ppm) was near the 100 ppm target level. Dissolved oxygen was consistent and exceeded 8.3 and 8.6 mg/L at all times in the zinc and cadmium tests, respectively.

Zinc exposure concentrations were consistent over the duration of the test and near target concentrations (**Table V.2**). After 96 hours of exposure, all fish exposed to 796 µg/L of zinc had died, partial mortality was observed at 210 and 426 µg/L, and all fish survived ≤105 µg/L. The median lethal zinc concentration (LC_{50}) after 96 hours was 331 µg/L. Mortality continued to occur after the initial 96 hours and reached 69 and 100% at 210 and 426 µg/L, respectively. No mortality occurred after 10 days. At test termination, fry mortality was significantly greater than control at zinc concentrations ≥210 µg/L (LOEC). No mortality was observed at zinc concentrations ≤105 µg/L (NOEC). The geometric mean of the LOEC and NOEC is 148 µg/L. No effect on growth, as measured by lengths and weight of surviving fry was detected.

Cadmium exposure concentrations were consistent over the duration of the test and near target concentrations (**Table V.3**). All fry exposed to ≥8.50 µg/L died, and 71.9 and 96.6% mortality was observed at 2.31 and 4.47 µg/L, respectively. All fry in the control survived. Due to the high mortality rate, an additional exposure level was created at a lower Cd concentration. All fry exposed to 1.01 µg/L of zinc survived. The 96 hour cadmium LC_{50} was 1.92 µg/L.

DISCUSSION

Mottled sculpin are widely distributed in North America. Previous toxicity tests have demonstrated that mottled sculpin are highly sensitive to zinc (Woodling et al. 2002, Brinkman and Woodling 2005). The results of the studies indicate that mottled sculpin may not be protected by current USEPA zinc criteria at lower water hardness. As a result, the Colorado Water Quality Control Commission adopted chronic zinc standards for the protection of mottled sculpin in the Dolores, Gunnison, and San Juan Rivers. The present zinc toxicity test was conducted in order to confirm and refine the hardness-toxicity relationship for mottled sculpin. Combining the results of zinc acute toxicity studies with Colorado mottled sculpin demonstrates a strong relationship between water hardness and 96 hour LC_{50}s (**Figure V.1**). The LC_{50} can be predicted based on hardness using the equation ($r^2 = 0.98$):

96 hour LC_{50} = EXP[1.020*ln(Hardness) + 1.100]

Chronic endpoints for zinc follow a similar hardness relationship (**Figure V.2**). The regression equation describing the line is ($r^2 = 0.98$):

Chronic Value =EXP[2.220*ln(Hardness) – 5.478]

It can be seen from **Figure V.2** that the revised chronic equation for sculpin which includes the new data point is very similar to the equation previously calculated. It is also apparent that Colorado chronic zinc table value standard is lower than the sculpin regression at higher hardness values. Current water quality standards for zinc are calculated for sculpin when hardness levels are below 113 mg/L. Similarly, our revised equation shows that at water hardness ≤106 mg/L, Colorado mottled sculpin are not protected by the table value standard.

Besser et al. (2006) recently reported the results of cadmium, copper and zinc toxicity tests conducted with multiple life stages of mottled sculpin populations from Minnesota and Missouri. The results confirmed the sensitivity of mottled sculpin to zinc as well as a high sensitivity to cadmium and copper. Sensitivity of different life stages to zinc increased in the following order: newly hatched = swimup>juvenile>adults. The young of year sculpin used in our tests were 0.4 – 0.6 g and would be described as juvenile. The Minnesota and Missouri populations differed significantly in their responses to metals, leading the authors to conclude that site-specific adjustments may be necessary to characterize impacts to local sculpin populations in metal impacted areas. Colorado mottled sculpin are intermediate in zinc sensitivity between Minnesota and Missouri sculpin for acute (**Figure V.3**) and chronic (**Figure V.4)** responses.

The cadmium LC_{50} for Colorado mottled sculpin was 1.92 µg/L at a water hardness of 48.7 mg/L. Colorado mottled sculpin are slightly more tolerant of cadmium than brown and brook trout, the most acutely sensitive species reported in the updated cadmium EPA criteria document (USEPA 2001). The LC_{50} for Colorado mottled sculpin is in the range of LC_{50}s found for Minnesota and Missouri mottled sculpin determined by Besser et al. (2006) when adjusted for water hardness (**Table V.4**).

Acknowledgements: We wish to thank Colorado Division of Wildlife Aquatic Researcher Barry Nehring and his crew for collection of the young of the year sculpin.

Table V.1. Mean (standard deviation) of water quality characteristics of exposure water.

Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Zinc					
99.5 (5.7)	73.8 (3.6)	7.50 (0.08)	12.2 (0.5)	176 (7)	9.0 (0.4)
Cadmium					
48.7 (1.9)	37.6 (2.5)	7.32 (0.10)	10.8 (0.3)	74.4 (4.1)	9.0 (0.2)

Table V.2. Mean (standard deviation) of zinc concentrations and associated mortality, lengths, and weights of mottled sculpin. *=significantly different than control.

Dissolved Zn (µg/L)	<10 (3)	55 (5)	105 (8)	210 (13)	426 (5)	792 (16)
96 h mortality (%)	0 (0)	0 (0)	0 (0)	3 (6)	84 (12)	100 (0)
30 d mortality (%)	0 (0)	0 (0)	0 (0)	69 (12)*	100 (0)*	100 (0)*
Surviving lengths (mm)	34.7 (0.5)	34.1 (1.4)	35.2 (1.0)	34.6 (2.3)	--	--
Surviving weights (g)	0.438 (0.013)	0.423 (0.067)	0.466 (0.072)	0.450 (0.088)	--	--

Table V.3. Mean (standard deviation) of cadmium concentrations and associated mortality of mottled sculpin.

Dissolved Cd (µg/L)	<0.15 (0.05)	1.01 (0.04)	2.31 (0.10)	4.47 (0.26)	8.50 (0.47)	15.5 (1.0)	30.8 (2.2)
96 h mortality (%)	0 (0)	0 (0)	71.9 (6.2)	96.9 (6.2)	100 (0)	100 (0)	100 (0)

Table V.4. 96 hour LC_{50}s, test water hardness and hardness-adjusted LC_{50}s for Colorado, Minnesota, and Missouri mottled sculpin.

Strain	Life stage	LC_{50}	Test Hardness	LC_{50} adjusted to 50 ppm hardness
Colorado	Juvenile	1.92	48.7	1.97
Minnesota[1]	Swim up	7.9	101	3.87
Minnesota[1]	Swim up	3.6	101	1.76
Minnesota[1]	Juvenile	17	101	8.32
Minnesota[1]	Juvenile	23	101	11.2
Minnesota[1]	Adult	>67	101	>32.8
Missouri[1]	Newly Hatched	2.9	101	1.42
Missouri[1]	Swim up	5.6	101	2.74

[1] From Besser et al. 2006

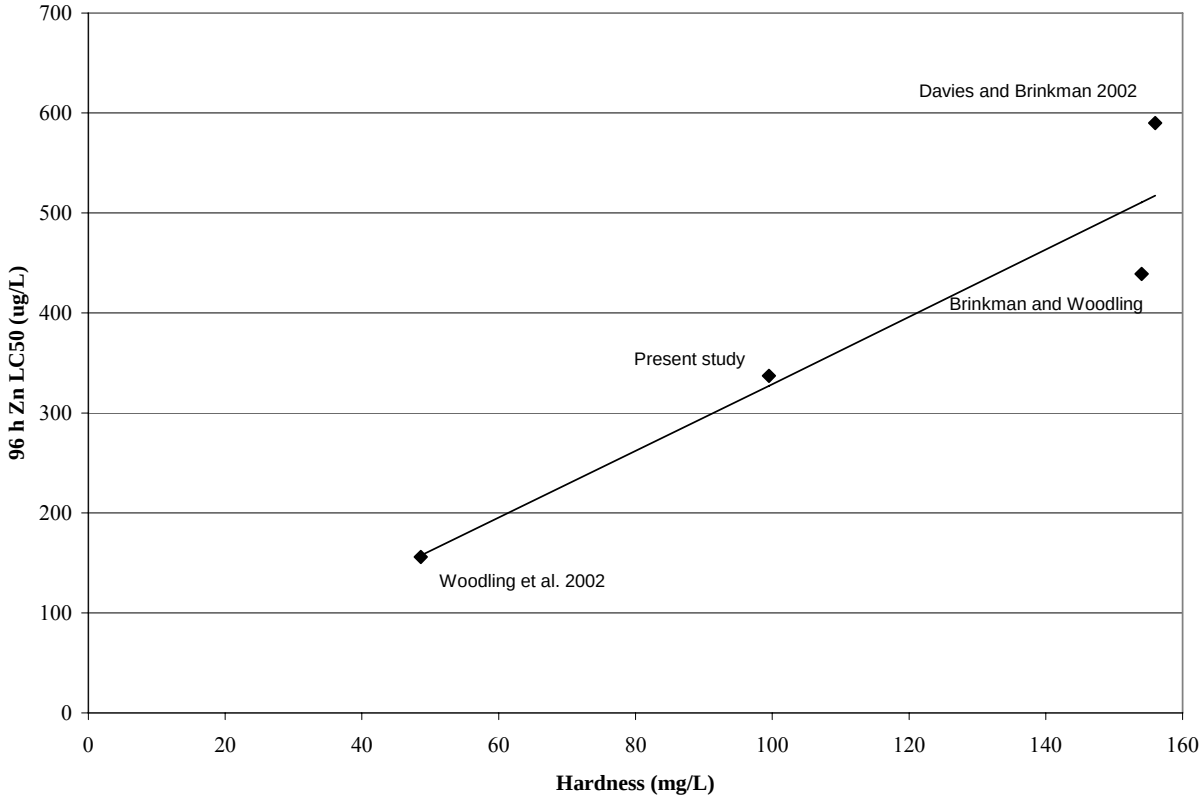


Figure V.1. Colorado mottled sculpin 96 hour zinc LC_{50} as a function of water hardness.

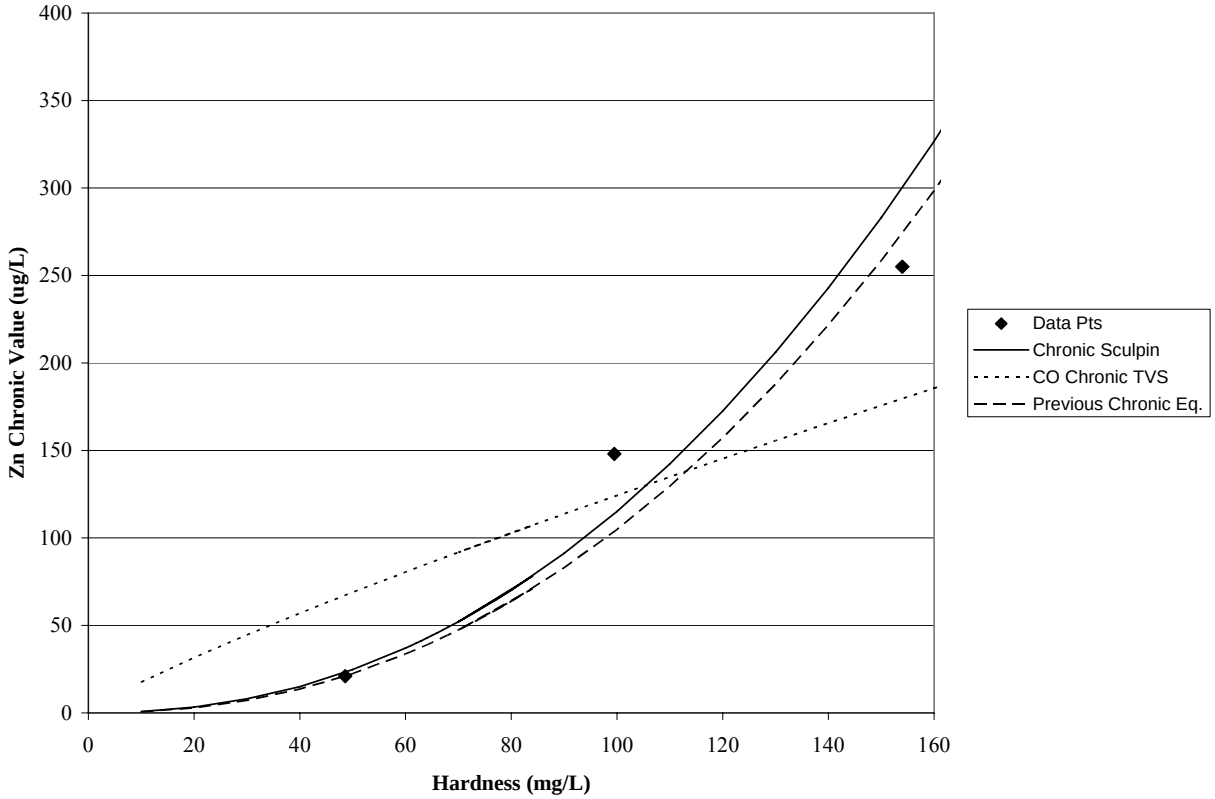


Figure V.2. Colorado mottled sculpin zinc chronic values and Colorado Table Value Standards (CO TVS) as a function of water hardness.

NEW Hardness based Equation for mottled sculpin: EXP[2.220*ln(Hardness) – 5.478]

OLD Hardness based Equation for mottled sculpin: EXP[2.227*ln(Hardness) – 5.604]

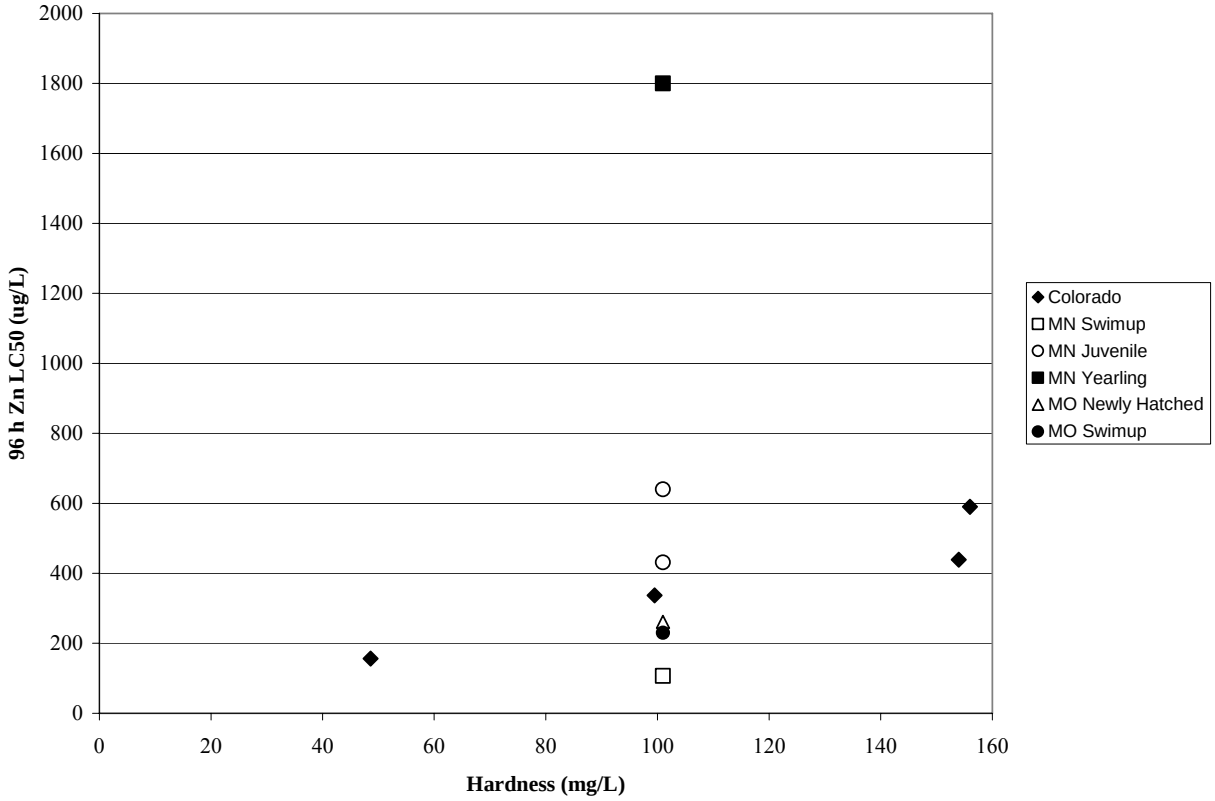


Figure V.3. 96 hour zinc LC50s for Colorado, Minnesota, and Missouri mottled sculpin.

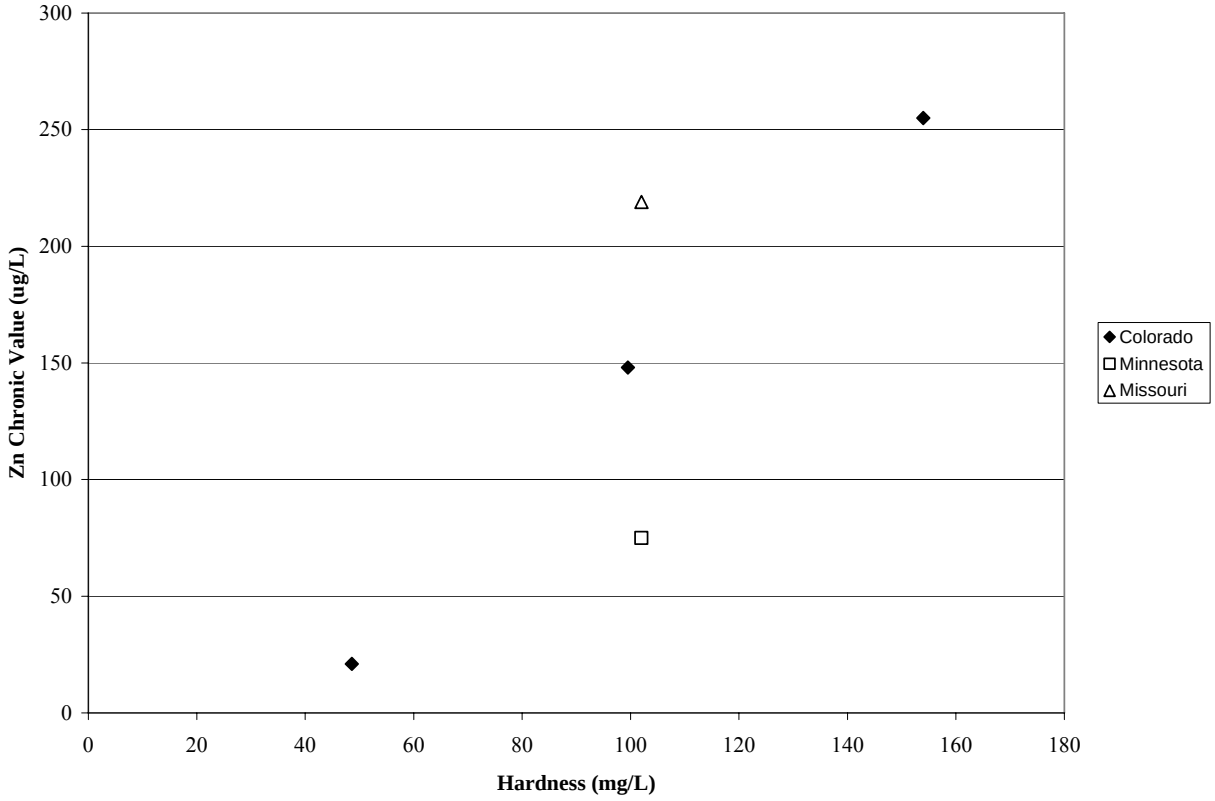


Figure V.4. Zinc chronic values for Colorado, Minnesota, and Missouri mottled sculpin.

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Toxicity of Zinc to Longnose Dace (*Rhinichthys cataractae*)

By Stephen Brinkman and Katharine Mitchell

INTRODUCTION

Toxicity of metals to trout species has been extensively studied and is well understood. However, there are essentially no data on the sensitivity of small cold water, littoral fishes to metals. Recent experiments by this project have found mottled sculpin to be among the most sensitive freshwater fish species to the toxic effects of zinc and they may not be protected by current USEPA zinc criteria (Woodling et al. 2002, Davies et al. 2002, Brinkman and Woodling 2006). There is a critical need for acute and chronic toxicity data for small fishes which provide forage for economically important species such as trout. A zinc toxicity test was conducted with young of the year longnose dace (*Rhinichthys cataractae*) for use in refining water quality criteria and to assist with development of site-specific zinc standards in Colorado.

MATERIAL and METHODS

Organisms

Young-of-year longnose dace were collected using a backpack electrofishing unit with pulsed DC current. Dace were collected from the Cache la Poudre in October 2006. Organisms were identified in the field and transported to the Colorado Division of Wildlife Toxicology Laboratory in Fort Collins, Colorado, USA in an aerated cooler. Temperature during transport was maintained using a 12 V Chiller (Coolworks Inc, San Rafael, CA). Upon arrival, fish were immersed in a 3% sodium chloride solution for 3 minutes to remove ectoparasites, and then were placed in an aquarium supplied with dechlorinated Fort Collins municipal tap water. No tempering or adjustment of water quality characteristics was necessary because of the similarity of Cache la Poudre River water and Fort Collins municipal tap water in terms of hardness, alkalinity, pH and temperature. Dace were fed starter trout chow (Rangen Inc., Buhl ID) supplemented with <24 hr brine shrimp naupalii (GSL Brine Shrimp, Ogden UT) twice daily (once daily on weekends and holidays). Fry were acclimated to laboratory conditions for 18 days prior to the start of the toxicity test. During the 18 day holding period, a single mortality occurred out of 325 fry collected.

Test Methods

Source water for the test consisted of dechlorinated Fort Collins municipal tap water. A continuous-flow serial diluter (Benoit et al. 1982) delivered exposure concentrations. The diluter was constructed of Teflon, polyethylene, and polypropylene components. Nalgene food-grade vinyl tubing delivered test solutions to exposure chambers. Test solutions overflowed from the exposure chambers into a water bath maintained at 12°C using a recirculating chiller (VWR model 1175MD). A metal stock solution was prepared by dissolving a calculated amount of zinc

sulfate in deionized water. New stock solutions were prepared as needed during the exposure period. Stock solutions were delivered to the diluter via a peristaltic pump at a rate of 2.0 ml/min. The diluter delivered five concentrations with a 50% dilution ratio and a control. Target concentrations for the zinc test were 8000, 4000, 2000, 1000, 500, and 0 µg/L. A flow splitter allocated each concentration equally among three replicate exposure chambers at a rate of 50 ml/min for each chamber.

Exposure chambers consisted of 20 L glass aquaria. Dim fluorescent lighting provided a 16-h/8-h light-dark photoperiod. Diluter and toxicant flow rates were monitored daily to ensure proper operation. Seventeen longnose dace were randomly allocated to each aquaria. Dace were not fed during the test.

During the initial 96 hours of exposure, water quality parameters were measured daily in two randomly selected treatment levels within a replicate. Different replicates were selected each sampling event. Hardness and alkalinity were determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH and conductivity. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. The conductivity, pH and dissolved oxygen meters were calibrated prior to each use.

Water samples were collected daily for dissolved metal analysis during the first 96 hours of the test. Exposure water was passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH <2. Chambers with no survivors remaining were not sampled. Zinc concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source (High Purity Standards, Charleston SC). Sample splits were collected and spikes prepared at each sampling event to verify reproducibility and analytical recovery.

Ninety six hour median lethal concentrations (LC_{50}) were estimated using the Trimmed Spearman-Karber technique with automatic trim (Hamilton et al. 1977, 1978).

RESULTS and DISCUSSION

Water quality characteristics were constant through the duration of the test (**Table VI.1**). Water quality characteristic during the test were similar to Cache la Poudre (hardness = 48.0, alkalinity = 32.8, pH = 7.41, conductivity = 110, temperature = 11.3°C).

Zinc concentrations were consistent and within of 10% target levels (**Table VI.2**). All dace exposed to ≥ 3920 µg/L died within 96 hours of exposure whereas all dace exposed to ≤531 µg/L for 7 days survived. At 96 hours, partial mortality of 9.8% and 51.0% occurred at intermediate concentrations 1068 and 2052 µg/L, respectively. No mortality occurred after the initial 96 hours except at 2052 µg/L which increased slightly to 54.9% mortality. The 96 hour LC_{50} was 1905 µg/L. The LC_{50} value for longnose dace is approximately in the middle of the species

distribution reported in the EPA zinc criteria document (USEPA 1987) and will minimally affect derivation of site-specific water quality standards.

Acknowledgements: We wish to thank Colorado Division of Wildlife aquatic biologist Kenny Kehmeier and his crew for collection of the young of the year longnose dace

Table VI.1. Mean (standard deviation) of water quality characteristics of exposure water of zinc toxicity test with longnose dace.

Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
49.9 (1.3)	40.5 (1.0)	7.48 (0.15)	11.0 (0.1)	87.7 (11.5)	8.9 (0.2)

Table VI.2. Mean (standard deviation) of zinc concentrations and associated mortality of longnose dace.

Dissolved Zn (µg/L)	<10 (3)	531 (8)	1068 (23)	2052 (59)	3920 (26)	7335 (92)
96 h mortality (%)	0 (0)	0 (0)	9.8 (3.4)	51.0 (9.0)	100 (0)	100 (0)
7 d mortality (%)	0 (0)	0 (0)	9.8 (3.4)	54.9 (12.2)	100 (0)	100 (0)