Water Pollution Studies

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State: Colorado Study No. F243R

Title: Water Pollution Studies

Period Covered: July 1, 2012 to June 30, 2013

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: LABORATORY TOXICITY STUDIES

Brief Description: Conduct laboratory-based experiments to test effects of contaminants on aquatic organisms.

Job A.1. Reproductive Toxicity of Endocrine Disrupting Compounds

Job Objective: Determine whether exposure to hormonally active agents results in feminization of rainbow trout, fathead minnows and/or other aquatic organisms. Effects of feminization on reproduction and fecundity will be measured. Concentrations of endocrine disrupting compounds that result in significant feminization will be compared to concentrations observed in wastewater treatment plant effluents and in Colorado streams.

Job A.2. Reproductive Toxicity of Atrazine Herbicide

Job Objective: Measure fecundity and biomarkers of feminization of red shiners exposed to a range of atrazine. Relate concentrations that result in impairment in the laboratory with concentrations observed in Colorado eastern plains streams.

Job A.3. Toxicity of Metals to Fish

Job Objective: Measure 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 species. Results from these experiments will compare toxicity thresholds to USEPA metal criteria to ensure that these species are protected.

Job A.4. Effects of Dietary Exposure of Metals to Fish

Job Objective: Measure the effect of zinc, copper, cadmium and/or selenium from dietary sources on 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.5. Testing and Validation of the Biotic Ligand Model

Job Objective: Determine the ability of the Biotic Ligand Model to estimate acute and chronic toxicity effects of metals on aquatic organisms exposed under multiple water quality conditions.

STUDY PLAN B: TECHNICAL ASSISTANCE

Brief Description: Conducts toxicological experiments as requested from regulators to be incorporated into policy; conducts water chemistry analysis and training for Colorado Parks and Wildlife (CPW) and other agencies.

Job B.1. Water Quality Assistance to Parks & Wildlife Personnel and Other State and Federal Agencies

Job Objectives: To provide technical assistance and expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to CPW and other state and federal personnel as requested. Conduct short or long term experiments to produce toxicity data, or develop site-specific field studies, when such data in the literature are lacking or inadequate. Ultimately, these activities will assist regulatory agencies in the development, implementation, and enforcement of water quality standards needed to protect or enhance the aquatic resources of Colorado.

ACCOMPLISHMENTS

Job A.1. Reproductive Toxicity of Endocrine Disrupting Compounds

The project continued to provide equipment and support for onsite bioassays conducted by personnel at the University of Colorado and Unversity of Denver. The studies' objectives were to detect and quantify estrogenic activity in the city of Boulder wastewater treatment plant effluent after recent treatment plant process upgrades. Estrogenic activity was compared with tests conducted prior to the upgrades. Assistance was also provided to a Colorado State University study investigating effects of 17α-ethynylestradiol on fathead minnow reproduction in mesocosms.

Job A.2. Reproductive Toxicity of Atrazine Herbicide

No activities during this segment.

Job A.3. Toxicity of Metals to Fish

A series of toxicity tests were conducted to investigate acclimation of brown trout to copper alone, zinc alone and mixtures of zinc and copper and zinc and cadmium. Results are reported below.

Tests were conducted to investigate interaction of temperature and metal toxicity. Zinc and copper toxicity tests were conducted with brook trout at two different temperatures. Brook trout exposed to sublethal concentrations of the two metals were subjected to critical thermal maxima trials in order to evaluate effect of metal exposure on upper thermal tolerance. Water samples from the test are currently awaiting analysis. Results will be reported next segment.

Job A.4. Effects of Dietary Exposure of Metals to Fish

A test to investigate chronic effects of dietary and waterborne exposure to cadmium in cutthroat trout was conducted. A laboratory culture of the oligochaete, *Lumbriculus variegatus*, was exposed to waterborne cadmium. *Lumbriculus* cultured in cadmium-dosed and cadmium-free water were used as food for cutthroat trout fry. Fry were fed a cadmium-dosed and cadmium-free diet to determine effects of dietary cadmium on survival, growth and accumulation in different subcellular fractions of kidney, liver and intestine. Results of the study are reported below.

Job A.5. Testing and Validation of the Biotic Ligand Model

A fundamental assumption of the biotic ligand model is that the binding affinity and capacity of metals to gills is similar among different taxa. Thus, different tolerances of different species to metals such as zinc are due to different abilities to withstand different amounts of zinc on the gills, as measured by median lethal accumulation (LA50). Brook trout and brown trout fingerlings were exposed to a range of concentrations of the stable zinc isotope ^{67}Zn. Accumulation of the stable isotope by the gills was measured in low water hardness over a range of time intervals between 45 minutes and 72 hours. An acute toxicity test was conducted concurrently so that a median lethal accumulation value (LA50) could be calculated. The gill-binding affinity and capacity of brook trout and brown trout will be determined and compared to rainbow trout. Tissue and water samples are currently awaiting analysis by United States Geological Survey (USGS) and will be reported next segment.

Job B.1. Water Quality Assistance to Colorado Parks and Wildlife Personnel and Other State and Federal Agencies.

Evaluation of Rotenone Formulations

Rotenone is a piscicide that is an important tool for managing Colorado fisheries. CPW aquatic biologists have reported that a new formulation of rotenone, CFT Legumine, is more effective than older formulations. Experiments were conducted to compare the toxicity of CFT Legumine with Prenfish 5% in side-by-side tests using channel catfish. Toxicity test results with channel catfish were consistent with results reported last segment using rainbow trout, brook trout and fathead minnows in that no differences in the efficacy of both rotenone formulations were detected.

Effect of Incubation Temperature on Hatching Success of HXC Rainbow Trout Eggs

HXC rainbow trout eggs spawned at the CPW Poudre Fish Hatchery have recently experienced a high rate of pickoff. One possible cause offered for the high pickoff rate is the rapid transfer of freshly fertilized eggs from colder spawning temperatures to water harden at a warmer temperature. To test this, an experiment was conducted to evaluate the role of different temperature treatments on egg viability and hatching success. Treatments consisted of adjustment of temperature +5°F and +10°F immediately, and gradually over 3, 6 and 12 hours. Viability and hatching of eggs with no temperature adjustment served as a control.

A recently acquired high performance liquid chromatograph was installed in a pickup truck camper and used to provide real-time analysis of rotenone concentrations on site at two reclamation projects performed by CPW personnel at Wood's Lake and Paonia reservoir.

Acclimation and deacclimation of brown trout (*Salmo trutta*) to zinc and copper singly and zinc in combination with cadmium or copper.

ABSTRACT

Brown trout (*Salmo trutta*) were chronically exposed to low and high levels of zinc alone, copper alone, a zinc and copper mixture, and a zinc and cadmium mixture all starting with eyed eggs and continuing through to the fingerling stage. Exposure to the metals and metal mixtures resulted in acclimation as measured by higher median lethal concentrations (LC50s) relative to metal-naïve fry. The degree of acclimation was similar between the low and high exposures except for copper, where acclimation was observed at the high but not the low acclimation level. The increases in tolerance relative to metal-naïve controls were usually less than a factor of 2 and never exceeded a factor of 3. Acclimation exposures did not affect hatch or survival except for the high acclimation regime of zinc and copper. Acclimation came at an apparent metabolic cost since growth was reduced by most acclimation exposures. Deacclimation, as evidenced by a return of LC50 to naïve levels, occurred after 2-5 weeks in clean water.

INTRODUCTION

Discharges from mine tunnels and surface runoff from milling operations introduce metals, such as cadmium, copper, and zinc, into many lotic waters throughout western North America. Trout are often present in lotic waters downstream from metal contaminant sources, although densities and body condition may be reduced in comparison to adjacent uncontaminated areas (Davies and Woodling 1980; Albeke et al. 2001; Brinkman et al. 2006). Fish exposed to sublethal concentrations of toxicants can acclimate and withstand higher concentrations than fish that have not been exposed. Acclimation can take two forms. Increased tolerance refers to an ability to survive permanently in otherwise lethal concentrations whereas increased resistance refers to longer survival at concentrations that are ultimately toxic (Sprague 1985; Stubblefield et al. 1999). Acclimation of salmonids to metals is widely reported including cadmium (Pascoe and Beattie 1979; Yamamoto and Inoue 1985; Hollis et al. 1999; Stubblefield et al.1999); zinc (Bradley et al. 1985; Anadu et al. 1989; Stubblefield et al.1999), copper (Dixon and Sprague 1981; Buckley et al. 1982; McCarter and Roch 1983; Lauren and McDonald 1987; Taylor et al. 2000*),* and metal mixtures (Roch and McCarter 1984; Marr et al. 1995; Harper et al. 2008). Acclimation typically occurs within five to seven days of metal exposure (Dixon and Sprague 1981; Bradley et al. 1985) and is not permanent; it can be lost 7-10 days after return to metal-free water (Dixon and Sprague 1981; Bradley et al. 1985; Anadu et al. 1989).

Acclimation in the aforementioned laboratory studies was determined by conducting acute toxicity tests of naïve and pre-exposed fry for relatively short durations (7-30 days). In reality however, fish in metal-impacted stream reaches are most often chronically exposed to metals for long periods of time. As such, chronic exposures to sublethal metal concentrations could potentially sensitize fish to metals (Chapman 1985) and make them more susceptible to an acute pulse of metals. For example, an in situ study placed brown trout (*Salmo trutta*) in cages at a site with lethal concentrations of zinc and cadmium and found that trout chronically exposed to moderate levels of these metals had a lower resistance than trout from an adjacent site with lower

metal concentrations (Woodling 1993). In other words, acclimation to heavy metals under chronic conditions may incur other physiological costs that are not typically encountered in laboratory acclimation studies.

The objective of our study was to measure acclimation of brown trout chronically exposed to metals singly and in combinations. A second objective was to measure loss of acclimation following return to metal-free water. Brown trout embryos, larvae and fry were exposed for up to six months to two different levels of a single metal, zinc (Zn) or copper (Cu) or to the metal mixtures of zinc and cadmium (ZnCd) or zinc and copper (ZnCu). Fry were then challenged with acutely lethal concentrations. Acclimation and deacclimation was determined by comparing 96 h median lethal concentrations of the exposed groups to unexposed metal-naïve fry in a control group.

MATERIAL and METHODS

Overview

Four sets of acclimation experiments were performed over the course of four years, one each year. Minor changes in laboratory equipment and methodologies occurred over the years, which did not alter the over-all design but are noted as appropriate. The basic approach was to expose brown trout embryos, larvae and fry to a low and high level of metal or metal mixture to induce an acclimation response. Metal-exposed (acclimated) and unexposed (naïve) fry were then challenged with acute toxicity tests and 96 h median lethal concentrations (LC50s) for each group were derived. Significant differences among acclimation groups were assessed based on no overlap of 96h LC50 95% confidence intervals. Acclimation exposures were initiated during the embryo stage and were sufficiently long (67-126 days) to measure chronic effects on survival and growth and for potential sensitization to occur. Challenge tests were repeated for the metal mixtures ZnCd and ZnCu after 210-215 days of acclimation exposures. After the last acclimation challenge test, fry were transferred to clean water and acute toxicity tests were again conducted to measure deacclimation.

Test Organisms

Eyed brown trout eggs were obtained from the Colorado Division of Wildlife Bellvue Research Hatchery for the Zn, Cu, and ZnCu tests and from the Wyoming State Game and Fish Department at DuBois, Wyoming for the ZnCd tests. Eggs were held in dechlorinated Fort Collins, CO municipal tap water for 1-d to 14-d prior to initiating the acclimation exposures (Table 1). Hatching of eggs began from 3-d to 10-d after the acclimation exposures were initiated. Yolk sac absorption occurred approximately 22-d to 27-d after hatch (Table 1). Swimup fry were fed appropriately sized Silver Cup fish food (Piper et al. 1982) at approximately 3%/day. Initial feedings were supplemented with brine shrimp nauplii (San Francisco Bay brand) to stimulate feeding activity. Fry were fasted for 24 hours prior to and during the 96-h acute challenge tests.

Acclimation

Zn, ZnCd, and ZnCu acclimation exposure solutions were delivered to brown trout embryos and fry via an intermittent flow diluter (Mount and Brungs 1967) modified to deliver two levels of toxicant and a control. Two liters of dechlorinated Fort Collins, Colorado

municipal tap water were delivered approximately every five minutes to 90-liter glass aquaria. The Cu acclimation exposures utilized a continuous flow diluter (Benoit et al. 1982) and polypropylene exposure chambers, delivering two levels of toxicant and a control. Flows and water were similar to the other three exposure programs. Metals for the acclimation exposures and toxicity tests were added as reagent grade sulfate salts ($ZnSO_4 \bullet 7H_2O$, $CuSO_4 \bullet 5H_2O$, $CdSO_4$). Stock solutions for each acute test and the acclimation exposures were prepared as needed by dissolving a measured amount of dried chemicals in deionized water to achieve desired concentrations. Stock solutions were delivered to test diluters using a diaphragm pump (Cole-Parmer) for the intermittent flow diluters and a peristaltic pump (Cole-Parmer C/L) for the continuous flow diluters. Toxicant delivery and diluter performance were monitored daily. Target acclimation exposure concentrations were based on concentrations found in metal-impacted Colorado streams (Table 2).

Eggs were randomly assigned to 90 L aquaria on hatching trays and incubated at a temperature near 12° C. Mortality was monitored and recorded daily. Aquaria were cleaned using a siphon to remove uneaten food and feces as needed. Aliquots of water from each aquarium were collected daily and combined into a weekly composite sample for metal analysis. Metals samples were acidified with high purity nitric acid (Ultrex JT Baker) and refrigerated until analyzed. Water quality analyses were conducted weekly in all aquaria during the acclimation phase of each exposure. Hardness and alkalinity were determined according to Standard Methods (APHA 2005). The pH was determined using an Orion Research pH meter 811 calibrated prior to each use with pH 4.0 and pH 7.0 buffers. Conductivity was measured using a YSI model 35 conductance meter. Dissolved oxygen was measured using a YSI model 58 dissolved oxygen meter calibrated prior to each use.

Acclimation Challenge Tests

Acclimation was assessed by challenging acclimated and metal-naïve fry with 96hr acute toxicity tests. Water quality characteristic were the same as during the acclimation. Metal mixtures used in the acute challenges were in the same ratio as the acclimation exposures. Acute challenge tests were conducted after 80-d and 67-d of acclimation for the Zn and Cu acclimations, respectively. For the metal mixtures, acute challenge tests were conducted at two time periods. Acute challenge tests were conducted after 116-d and 215-d of acclimation to the ZnCd mixture. Tests were conducted after 126-d and 210-d of acclimation for the ZnCu mixture. Continuous flow serial diluters (Benoit et al. 1982) with a 50% dilution ratio were used to deliver 5 exposures and a control for the Zn, Cu, and ZnCu challenge tests. Intermittent flow diluters (Mount and Brungs 1967) were used in the ZnCd challenge tests, delivering 6 exposures and a control. Challenge tests were replicated except the ZnCd tests. Ten fish were randomly distributed to each aquarium. Fish loadings and flow of exposure water were within established guidelines (ASTM 1997).

Tanks were checked hourly for mortality. Dead and moribund organisms were removed, measured for total length (mm), blotted dry with a paper towel and weighed (0.001g). Fish surviving the 96-h challenge tests were terminally anesthetized with MS222 and measured for length and weight. Estimates of lengths and weights of brown trout at the end of each acclimation period were based on fish utilized in the acute challenge tests. Insufficient numbers of fish survived the high ZnCu acclimation to perform any challenge tests, so lengths and

weights for this treatment were based on ten fry that survived acclimation.

Water quality characteristics were determined two to five times from each aquarium using the procedures outlined in the preceding section. Water samples for metal analyses were collected at least three times during each test. Water samples were filtered through a 0.4µm filter into 60 ml high-density polyethylene bottles, immediately preserved with high purity nitric acid (JT Baker) and stored at 4ºC until analysis**.**

Deacclimation Challenge Tests

All remaining pre-exposed trout not used in the acclimation challenge tests were returned to clean, undosed 90-L aquaria to assess loss of acclimation. Following deacclimation time periods, 96-h acute challenge toxicity tests were conducted using the same procedures used to assess acclimation. Fry from the ZnCd acclimation exposure groups were tested after 7 and 14 days of deacclimation. The Zn acclimated fry were tested after 14 and 21 days being returned to clean water. The ZnCu and Cu deacclimation tests had to be restarted with fresh organisms due to diluter failures so deacclimation was assessed after 35d.

Metal Analyses

Zinc concentrations were determined using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame. Cadmium and copper concentrations were determined using a Thermo-Jarrell Ash SH 40000 spectrometer with a CTF 188 graphite furnace atomizer. Both spectrometers utilized Smith-Hieftje background correction. The spectrometers were calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard from an outside source.

Statistical Analyses

Statistical analyses were conducted using Toxstat version 3.5 software (West Inc. 1996). Analysis of variance (ANOVA) was used to test for differences of hatching success, fry and swim-up survival, and lengths and weights. Hatching success and survival data were arcsine square root transformed prior to ANOVA (Snedecor and Cochran 1980). Normality and homogeneity of variances were tested using Chi-square and Levene's tests, respectively. Mean values from acclimation groups were compared to their respective acclimation control using Dunnett's one-tailed test ($p<0.05$) (Dunnett 1955, Dunnett 1964). Median lethal concentrations (LC50s) were estimated using the trimmed Spearman-Karber method (Hamilton et al. 1997, 1998). Differences of LC50 values were based on nonoverlap of estimated 95% confidence intervals.

RESULTS

Water Quality

Water quality characteristics were consistent within each acclimation exposure and were comparable among tests through the entire period of acclimation and deacclimation testing. The mean hardness varied from a low of 49.1 mg/L $CaCO_3$ in the Cu test to a high of 54.1 mg/L $CaCO_3$ in the Zn test. Dissolved oxygen was usually near saturation and did not fall below 5.5 mg/L while mean pH ranged between 7.3 and 7.5. Temperature varied between 10.3ºC to a high of 14.6ºC. Mean metal concentrations were near target concentrations except for the Cu

acclimation, which were about 75 % of nominal (Table 2).

Acclimation

Hatching success was not altered by acclimation exposures except for the high ZnCu (400 µg/L zinc and 16 µg/L) exposure, which resulted in a significantly lower hatch (80.4%) compared to the corresponding control fish (89.2%) (Table 3). Hatching success in the Zn, Cu, and ZnCd acclimations exceeded 85.8%. Sac fry survival and the swim up fry survival was reduced significantly in the high ZnCu acclimation exposure compared to corresponding control fish. Survival through the sac fry stage and swim up fry stage was not affected by the Zn, Cu, and ZnCd acclimation exposures. Relatively high mortality occurred in the control fish and both acclimation levels of the ZnCu test at the swim up stage as a result of failure to adapt well to exogenous food. An insufficient number of brown trout survived the high ZnCu exposure to perform challenge tests.

Growth was significantly reduced at the end of all acclimation exposures with the exception of the Zn acclimation exposures (Table 4). Lengths and weights of fry acclimated to ZnCu and Cu were significantly less than metal-naïve fry. Lengths and weights of fry acclimated to ZnCd were significantly reduced after 215 days but not after 116 days of acclimation.

Acclimation Challenge Tests

Water quality characteristics during the acclimation challenge tests were similar to the acclimation exposures. Measured metal concentrations were consistent for the duration of each acute challenge test.

Acclimation occurred for each of the metals and metal mixtures but was not observed at all acclimation levels or at all acclimation durations (Figure 1). LC50s of low and high acclimation exposures to Zn were not significantly different from each other but both were significantly greater than the naïve LC50 after 80 d of acclimation. The zinc LC50s of low and high acclimated fry were 1.6 and 1.8 times greater, respectively, than the LC50 of the naïve fry, which was 871 µg/L. The pattern of acclimation to the ZnCd mixture was similar to Zn. After 116d and 215d of acclimation, the LC50s of the low and high ZnCd acclimated fry were both significantly greater than the naïve LC50s but not significantly different from each other. The LC50s of ZnCd mixture to naïve fry were 725 µg/Zn/L + 2.01µg Cd/L after 116 days and 412 µg Zn/L+1.17 µg Cd/L after 215 days. The low and high ZnCd acclimated fry were 1.6 and 1.8 times more tolerant than naïve fry after 116 days of acclimation, respectively. After 215 days of acclimation, low and high ZnCd acclimated fry were 2.6 and 3.0 times more tolerant than naïve fry, respectively. After 67 days of acclimation to Cu, the high but not the low acclimation group was more tolerant than naive fry. The LC50 of the naïve fry was 30.2 µg Cu/L. The high acclimated fry were 1.5 times more tolerant to copper alone than naïve fry. The low acclimated fry were slightly less tolerant than naïve fry (0.9x) but the difference was not significant. An acclimation challenge test could not be conducted with high ZnCu acclimated fry due to high mortality. The low ZnCu acclimated fry were significantly more tolerant (1.9x) than naïve fry after 126 d of acclimation, but were not significantly more tolerant after 210 d of acclimation (1.2x). The LC50s of ZnCu mixture to naïve fry were 571 µg/Zn/L + 20.2 µg Cu/L after 126 days and 523 µg Zn/L+19.7 µg Cu/L after 210 days.

Deacclimation Challenge Tests

Water quality characteristics were consistent during the deacclimation tests and were similar to the acclimation exposures and acclimation challenge tests. Measured metal concentrations were consistent for the duration of all tests.

Deacclimation was observed for each of the metals and mixtures (Figure 2). The Zn LC50 of metal-naïve fry was 392 µg/L. After 14 days of deacclimation, the LC50 of the low acclimated fry remained significantly greater than naive fry LC50 (1.4x), but the high level Zn acclimated fry were similar (1.3X). After 21 days of deacclimation, the LC50s of high and low Zn-acclimated groups were similar to naïve fry (1.0x and 1.1x, respectively). ZnCd-acclimated fry did not deacclimate after 7 days but did deacclimate after 14 days. The LC50 of the ZnCd mixture to naïve fry was 511µg/L Zn+1.11 µg/L Cd and 632 µg/L Zn+1.31 µg/L Cd after 7 and 14 days. The low ZnCd-acclimated fry were 1.4x and 1.1x more tolerant than naïve fry after 7 and 14 days of deacclimation, respectively. A 95% confidence interval for the LC50 after 7 days deacclimation could not be calculated but after 14 days deacclimation the confidence interval overlapped with those of naïve fry. The high ZnCd-acclimated fry were 2.2x and 1.2x more tolerant than naïve fry after 7 and 14 days of deacclimation, respectively. The high ZnCd-acclimated remained significantly more tolerant than naïve fry after 7 days of deacclimation but were not significantly more tolerant after 14 days of deacclimation. The LC50 of Cu to naïve fry from the deacclimation test was 39.4 µg/L Cu. After 35 days of deacclimation the LC50s of the low and high Cu-acclimated fry were 0.9x and 1.2x of the naïve fry, respectively. The tolerance of the low Cu acclimated fry remained similar to naïve fry. The high Cu-acclimated fry deacclimated after 35 days. Insufficient numbers of fry survived the high ZnCu acclimation exposure to conduct acclimation and deacclimation challenge tests. The LC50 of the low ZnCu acclimated group was 1.1x the LC50 of naïve group which was 412 µg/L Zn+15.4µg/L Cu. The low ZnCu acclimated group were not more tolerant than naïve group after 210 d of deacclimation so it was expected that the LC50s would be similar after 35 d of deacclimation.

DISCUSSION

Brown trout are an important component of many Rocky Mountain streams but population densities are reduced downstream of sources of metal contamination (Davies and Woodling 1980; Brinkman et al. 2006; Clements et al. 2010). The sensitivity of brown trout to metals such as copper, cadmium and zinc has not been as well studied as it has for other salmonids, such as rainbow trout. In general, the toxicity values derived from naïve (control) brown trout in this study are consistent with limited available toxicity data. The 96-h zinc LC50s for control fish in the current study ranged from a high of 871 µg/L to a low of 392 µg/L after 80-d acclimation and 21-d deacclimation, respectively. A zinc 9-day LC50 of 640 µg/L for brown trout was reported by Nehring and Goettl (1974). A series of studies using water of similar characteristics as the current tests reported 96-h brown trout LC50s ranged from 382 µg/L zinc to 1,033 µg/L zinc (Brinkman et al. 2006)). The 96-h copper LC50s for naïve (control) brown trout were 30.2 µg/L and 39.4 µg/L, similar to brown trout values of 35.8 µg/L and 29.4µg/L reported by Davies and Brinkman (2002). Cadmium LC50 values in this study were 1.17 µg/L and 1.31 µg/L, similar to 1.4 µg/L reported by Spehar and Carlson (1984) and 1.23 µg/L reported by Brinkman and Hansen (2007).

The present tests were designed to investigate commonalities of acclimation responses by brown trout when they are exposed to metals individually and in combination. Results of the tests demonstrated not only some commonalities but also differences in brown trout response to different acclimation regimes. These differences confound any attempt to precisely characterize acclimation of brown trout to metal exposure either singly or in combination.

All metals and metal combinations resulted in acclimation except for the low Cu exposure. Brown trout exposed to the low Cu acclimation regime of 5.8 μg/L Cu (19% of the median 96 h LC50 determined for naïve control brown trout) did not acclimate, whereas exposure to 15.0 μg/L Cu (50% of the median 96 h LC50 for control brown trout) resulted in 50% increased tolerance. Dixon and Sprague (1981) observed a similar response with rainbow trout where a low-level acclimation exposure to 18% of the copper LC50 failed to elicit an acclimation response while exposure to 29%, 40% and 59% of the LC50 resulted in acclimation. As such, increased brown trout tolerance cannot be assumed to be an automatic acclimation response of brown trout to sub lethal copper exposure.

Brown trout acclimated to all other individual metal concentrations or different combination of metals in this study. The degree of acclimation was rather modest and never resulted in a doubling of tolerance except for the high ZnCd acclimation exposure after 215d where the acclimation factor was 2.6x and 3.0x for the low and high acclimation exposures, respectively (Figure 1). However, a decrease in the LC50 of naïve controls that occurred from 116d to 215d accounts for the higher acclimation factors and not an increase in the LC50 of the acclimated fish. The acclimation factors for the other tests ranged from 1.47x in the high Cu acclimation to 1.9x for the zinc in the low ZnCu exposure (Figure 1). Brown trout acclimation factors for copper and metal mixtures observed in the present study are similar to those reported for other salmonids. Acclimation factors for copper have been reported as <2.0x for rainbow trout (Dixon and Sprague 1981; Taylor et al. 2000) and 2.5x for Coho salmon (*Onchorhynchus kisutch*) (McCarter and Roch 1983). Acclimation factors for rainbow trout exposed to a mixture of zinc, copper and cadmium was ≤1.5 (Roch and McCarter 1984). Reported acclimation factors for zinc tend to be somewhat higher and include 2.5x (Bradley et al. 1985), 2.3x – 4.7x (Anadu et al. 1989), and 2.9x (Stubblefield et al. 1999), all with rainbow trout. The relatively low acclimation factors found in our studies suggest that acclimation provides minimal protection for wild brown trout populations in stream reaches with high metal concentrations.

Deacclimation occurred 14d to 35d following return to clean water for all metals and combinations tested. Increased tolerance to zinc remained after 14d but was lost by 21d (Figure 2). For the ZNCD mixture, brown trout were deacclimated after 14d but not 7d (Figure 2). Time to deacclimation in the present study is somewhat longer than 7-14d that has been reported previously (Dixon and Sprague 1981; Anadu et al. 1989; Bradley et al. 1985; Marr et al. 1995). A notable factor that may have contributed to longer deacclimation times in our study is longer acclimation periods (67d-215d) that started with embryos. Dixon and Sprague (1981), Anadu et al. (1989), Bradley et al. (1985) and Marr et al. (1995) reported more rapid deacclimation but utilized shorter acclimation exposures (21-42d) and started with juveniles.

Two acute challenges were conducted for the metal mixtures ZnCd and ZnCu to evaluate

whether longer acclimation exposures increased the degree of acclimation. Results were different for the two metal mixtures. The acclimation factors for our ZnCu exposures were higher after 215d of exposure than after 116d of exposure (Figure 1), though this effect was due a decrease in the LC50 of the naïve controls over that time period. The absolute values of the LC50s the ZNCD acclimated fry were similar between the exposure periods. For the ZnCu acclimation tests, the increased tolerance detected at 126d was not observed after 210d. In the literature, the effects of extending the duration of acclimation exposure are similarly contradictory. Acclimation increased with increasing duration of exposure to a metal mixture from 3 to 5 weeks (Marr et al 1995) though Anadu et al. (1989) did not find increased acclimation as duration of exposure increased from 1 week to 3 weeks. Loss of acclimation with constant exposure has been observed studies with fathead minnows exposed to zinc (Hobson and Birge 1989) and coho salmon exposed to copper (McCarter and Roch 1983).

Acclimation was associated with an apparent metabolic cost, which was manifested by decreased growth observed in fry exposed to the ZnCd (215d), ZnCu and the high copper exposure regimes. Reduced growth was detected in the ZnCd acclimation after 215d but not after 116d. Reduced growth was not detected in the Zn acclimation. Reduced growth associated with acclimation has been reported for copper (Dixon and Sprague 1981; Collvin 1984; Sprague 1985), zinc (Hobson and Birge 1989) and a metal mixture (Marr et al. 1995) in the laboratory. Wild brown trout ≤2 years weighed less than brown trout from adjacent reference stream reaches in Colorado streams contaminated by the metals studied in this paper (Albeke et al. 2001). Responses to long-term metal exposures such as protective and detoxification mechanisms and repair of cell damage increase energy expenditures that would otherwise be available for energy stores and growth (Hogstrand et al. 1995; Marr et al. 1995).

Acclimation levels used in these studies were intended to be sub-lethal so that loss of metal-sensitive individuals would not occur. Survival was not affected by acclimation exposures except in the high ZnCu acclimation. Thus, we believe that higher LC50 values of acclimated treatments in our tests were due to physiological responses and not due to selection against sensitive individuals during the acclimation exposures. Natural populations of brown trout experience multiple selection pressures. Loss of metal-sensitive individuals at metal-contaminated areas will contribute to a loss of genetic diversity and may decrease the fitness of the population as a whole.

The CuZn acclimation exposure was much more toxic to brown trout embryos and fry than the same metals singly. Hatch rate was significantly decreased and few fry survived the high CuZn mixture acclimation (405 μg/L zinc and 16 μg/L copper) whereas no adverse effect on survival was observed during the Zn acclimation (416 μg/L) or the Cu acclimation (11.3 μg/L) (Table 3). Harper et al. (2008) posited additive toxicity for cutthroat trout attributable to cadmium and zinc in water of a similar hardness to the current study. Our study design did not include and analysis of cadmium alone so we cannot address the issue of additive zinc and cadmium interactions to brown trout. Our results and the results of Harper et al. (2008) both indicate that multiple metals can act in an additive fashion. Existing US EPA criteria used to establish stream standards are based on an analysis of metals acting singly, not in combination. This current US EPA concept of determining stream standards based on toxicity of metals acting singly may not result in protective standards due to the additive nature of metals such as copper

and zinc.

Water quality criteria guidelines for the United States of America preclude the use of toxicity test data where organisms have been previously exposed to test material. Some may argue that using toxicity data from naïve fish and ignoring the ability to acclimate results in water quality criteria that are overly conservative. However, metal acclimation is a temporary response that is lost after extended residence in uncontaminated water. Migration into a clean tributary could lead to a loss of acclimation, followed by toxicity on return to a contaminated stream reach. Loss of acclimation also could occur during spring runoff, when dilution from spring snowmelt substantially reduces metal concentrations in streams.

In summary, brown trout fry exposed to sub lethal levels of metals acclimated and developed greater tolerance (as measured by higher LC50s) than metal-naïve fry. The acclimation response appeared to have a concentration threshold, below which, acclimation did not occur. The acclimation response did not appear to increase significantly with increasing magnitude of exposure if the exposure is above the threshold. Nor did the acclimation response increase with increasing duration of exposure. Loss of acclimation occurred within a few weeks after transfer to clean water. The acclimation response was associated with an apparent metabolic cost manifested as decreased growth. Acclimation may well increase the probability of survival of metal pulses compared to unacclimated fry, however reduced growth will increase risk of predation, limit access to habitat with faster flowing water and decrease energy stores that enable overwinter survival. Ability of fish to acclimate should not be utilized as justification to allow more relaxed metal criteria while the toxicity of metals in combination is an indication that the current USEPA criteria development process may not provide adequate protection for wild populations of brown trout.

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Table 1. Brown trout development and hatching information.

Test	Days egg tempered Prior to acclimation	Days to hatch during acclimation	Yolk absorption days post-hatch
Zinc tests	7	3	26
Zinc and cadmium	1	10	27
Zinc and copper	4	6	24
Copper	14	3	22

Table 2. Nominal and average measured acclimation concentrations (µg/L) for the 1996 (zinc), 1998 (zinc and cadmium), 1999 (zinc and copper) and 2000 (copper) toxicity tests. Standard deviations are in parentheses.

Test		Control	Low	High
Zn	Nominal	<10	200	400
	Actual	<10 (3.1)	192 (7.7)	416 (17)
ZnCd	Nominal	<10/0.1	200/0.5	400/1
	Actual	<10(8))/<0.1(0.01)	195(18))/0.44(0.07)	436(29)/1.01(0.08)
ZnCu	Nominal	<10/1.0	200/7.5	400/15
	Actual	<10(4.5)/<1.0(0.3)	210(20)/8.7(0.7)	405(12.5)/16(1.3)
Cu	Nominal	<1.0	7.5	15
	Actual	<1.0(0.8)	5.5 (2.1)	10.4 (1.6)

Table 3. Hatching success, survival through sac fry and survival through swim up fry stage (%) of brown trout pre-exposed to zinc (Zn), zinc and cadmium in combination (ZnCd), zinc and copper in combination (Zn/Cu) and copper (Cu). Standard deviations are in parentheses. Zinc acclimation not replicated. *Significantly less than control ($p<0.05$)

Test	Control	Low	High
Hatching success			
Zn	89.5	89.6	87.7
ZnCd	95.7 (0)	94.3 (0.5)	95.2 (0.7)
ZnCu	89.2 (1.1)	85.8 (2.1)	80.4 (1.5)*
Cu	98.8 (1.0)	97.8 (2.1)	99.1 (0.6)
Survival through sac fry			
Zn	82.1	79.1	76.4
ZnCd	91 (0.9)	93 (0.5)	91.4 (0.9)
ZnCu	80.2 (1.2)	75.2 (3.4)	28.4 (0.1)*
Cu	95.9 (2.1)	95.3 (1.9)	98.8 (1.0)
Survival through swim up			
Zn	82.0	77.1	74.5
ZnCd	81.6 (6.4)	87.6 (0.8)	83.8 (2.6)
ZnCu	54.2 (1.6)	48.6 (2.5)	1.5 (2.5)*
Cu	89.1 (3.1)	80.6 (7.8)	90.3 (3.3)

Table 4. Mean lengths (mm) and weights (g) of brown trout pre-exposed to metals and metal mixtures for the different lengths of time. Standard deviations are in parentheses. *Significantly less than control ($p<0.05$).

Acclimation	Duration (days)	Control	Low	High
Length mm				
Zn	80	46.2 (7.9)	47.7 (8.2)	47.9 (7.9)
ZnCd	116	50.6 (4.9)	51.8 (5.9)	52 (5.8)
	215	83.1 (9.8)	78.4 (1.2)*	76.6 (10.2)*
Zn/u	126	47.3 (5.3)	42.7 (4.5)*	31.1 (6.0)*
	210	79.8 (9.8)	74 (9.2)*	--
Cu	67	31.6(0.3)	30.8 (0.4)	29.7 (0.6)*
Weight				
Zn	80	0.99 (0.58)	1.1 (0.66)	1.1 (0.59)
ZnCd	116	1.16 (0.34)	1.28 (0.43)	1.3 (0.44)
	215	5.9 (2.26)	4.88 (1.95)*	4.42 (1.95)*
ZnCu	126	0.91 (0.34)	0.64 (0.22)*	0.25 (0.19)*
	210	4.83 (1.96)	3.85 (1.62)*	--
Cu	67	0.23 (0.01)	0.21 (0.02)	0.19 (0.02)*

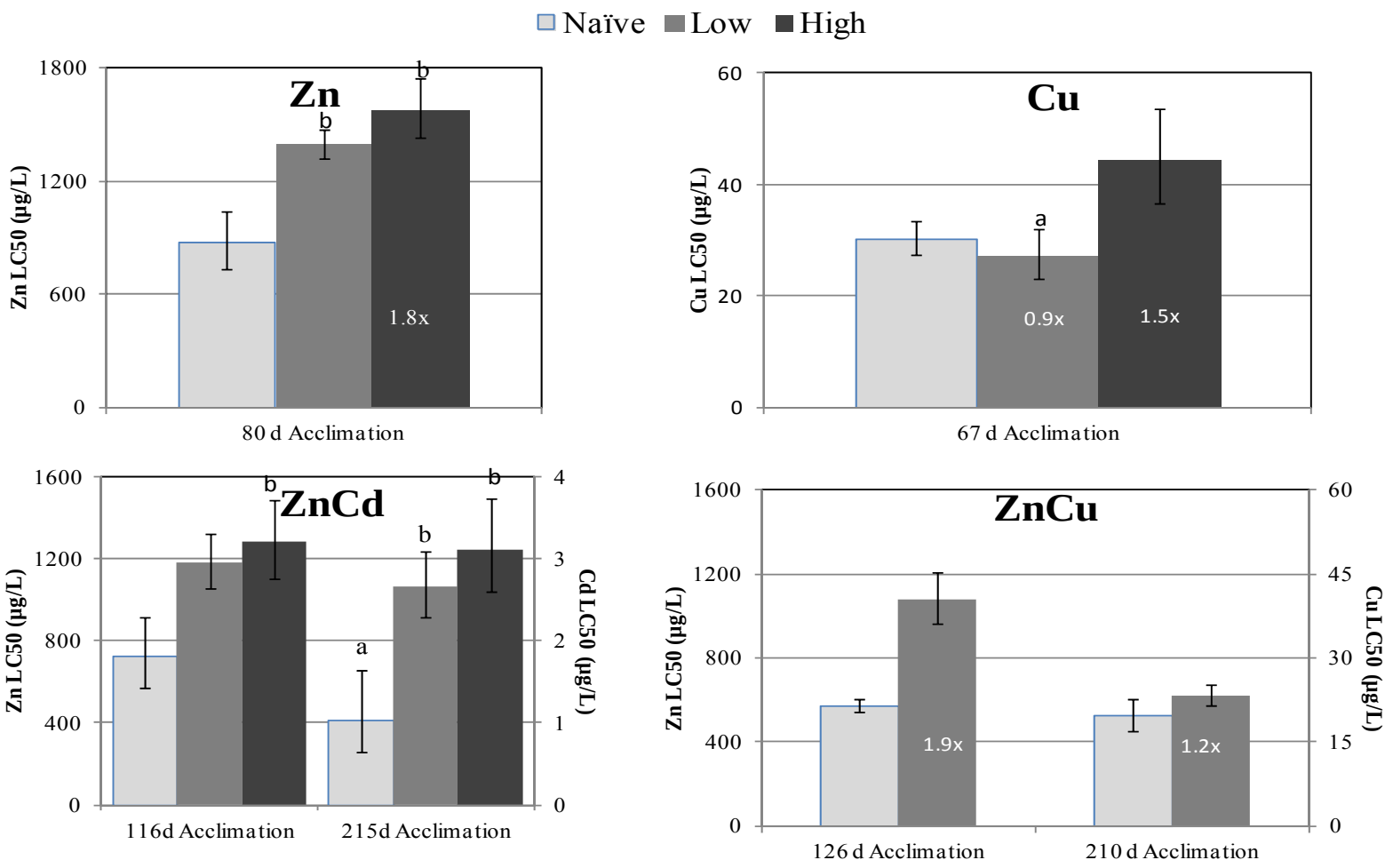

Figure 1. 96 median lethal concentrations (LC50s) of zinc, copper, and metal mixtures to naïve (light grey bars), low (dark grey bars) and high (black bars) acclimated brown trout after various periods of acclimation. Error bars represent 95% confidence intervals. Different letters denote significantly different LC50s as determined by nonoverlap of confidence intervals. Numbers within bars indicate acclimation factor i.e. LC50 relative to naïve LC50.

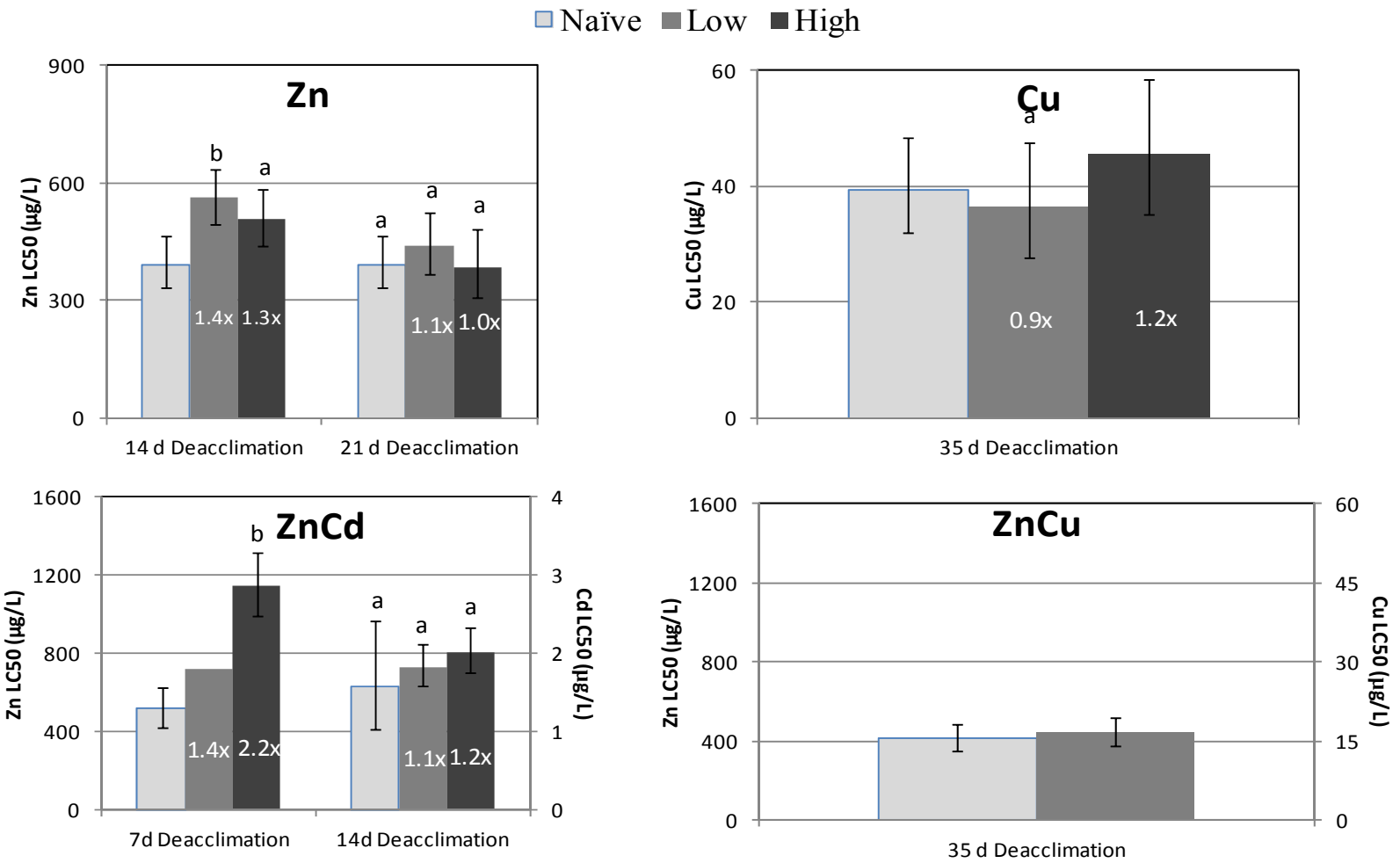

Figure 2. 96 median lethal concentrations (LC50s) of zinc, copper, and metal mixtures to naïve (light grey bars), low (dark grey bars) and high (black bars) acclimated brown trout after various periods of deacclimation. Error bars represent 95% confidence intervals. Different letters denote significantly different LC50s as determined by nonoverlap of confidence intervals. Numbers within bars indicate acclimation factor i.e. LC50 relative to naïve LC50.

Survival, growth and cadmium accumulation of cutthroat trout exposed to dietary and aqueous cadmium

INTRODUCTION

With few exceptions (e.g. Hg, Se), water quality standards and criteria for protection of aquatic life are based on laboratory experiments using aqueous exposure. Historically, dietary exposure has been ignored because early experiments that used commercial fish feed spiked with metal salts suggested that bioaccumulation of metals was low relative to aqueous exposures and that dietary toxicity of metals was minimal. Earlier investigations conducted by this project revealed that exposure of rainbow trout to inorganic salts of zinc, lead, and molybdenum in food pellets was not lethal (Goettl et al. 1975, Goettl et al. 1976). However, assimilation of dietary metals is influenced by the form of the metal and how the metal is distributed within different cellular compartments (Meyer et al. 2005). Thus, metals allowed to incorporate in to live prey or food may have higher or lower assimilation than commercial food spiked with chemical salts. Indeed, several studies in which invertebrates collected from contaminated and uncontaminated sites and then fed to fish suggest that dietborne metals are toxic, reducing survival and growth as well as causing and other measures of sublethal toxicity (Woodward et al.1995, Farag et al. 1999). To the extent that dietborne metals are toxic, water quality criteria may be underprotective. For this reason, dietborne exposures have recently received considerable attention.

The objective of the following study was to investigate effects of dietary exposure of cadmium to cutthroat trout. Cadmium is among the most toxic of metals to aquatic life (Borgmann et al. 2005). Salmonids are particularly sensitive to cadmium toxicity and represent six of the seven most sensitive species (USEPA 2001). Salmonids are a significant component of ecosystems in many Colorado headwater streams and are also recreationally important. However, metal contamination can limit density and biomass of salmonids (Brinkman et al. 2006). Cadmium is commonly found in acid rock drainage resulting from Colorado's mining past. An estimated 2080 km of streams in Colorado are impacted by metals with 840 km impacted by cadmium (CDPHE 2010). Cutthroat trout are a popular salmonid in Rocky Mountain streams and are particularly sensitive to cadmium (Brinkman 2012). As a nonesstential metal, cadmium is regulated to a lesser degree than essential metals such as copper and zinc. As a result, cadmium is accumulated and retained in important organs such as the liver and kidney of fishes. A live diet of *Lumbriculus variegatus* was selected due to its nutritional sufficiency and ease of culture (Mount et al. 1994).

MATERIAL and METHODS

Lumbriculus variegatus was cultured in 38L glass aquaria which received 50 mls/min dechlorinated Ft Collins municipal tap water. A peristaltic pump delivered a stock solution prepared by dissolving a calculated amount of cadmium chloride to achieve a target concentration of 1.0 µg/l in one aquarium and 2.0 µg/L in another aquarium. *Lumbriculus* in a third aquarium did not receive any cadmium and served as the control diet. Substrate consisted of washed silica sand. Each culture was fed a slurry of starter trout chow (Rangens) daily. The *Lumbriculus* cultures were maintained for 3 months prior to the start of the feeding experiment in

order to create a sufficient mass for the experiment and to ensure that subcellular distribution of cadmium was stable.

Cutthroat trout eggs from the Colorado Division of Parks and Wildlife Poudre River Rearing Unit were raised onsite at the Aquatic Toxicology Laboratory. Eggs and fry were raised in dechlorinated Ft Collins municipal tap water. Upon swimup, fry were fed trout chow soft-moist starter diet supplemented with newly hatched brine shrimp nauplii (*Artemia*) to stimulate feeding. After three weeks on the commercial trout chow diet, fry were fed unexposed *Lumbriculus* for seven days in order to accustom the fry to the new diet.

At the start of the experiment, fry were transferred one at a time to twenty one 9L glass aquaria and the process repeated until a total of three fry were in each aquarium. There were a total of seven treatments with three replicates each, where fry were exposed to a low and high level of cadmium via aqueous alone, diet alone or both. Specifically, the treatments were 1.0 µg/L aqueous exposure with control diet, 2.0 µg/L aqueous exposure with control diet, no aqueous exposure with 1.0 µg/L contaminated diet, no aqueous exposure with 2.0 µg/L contaminated diet, 1.0 µg/L aqueous exposure with 1.0 µg/L contaminated diet, 2.0 µg/L aqueous exposure with control diet. No aqueous exposure and uncontaminated diet served as the control. Treatment designations are shown below:

0 aq+ 0 diet	Exposed to control water and fed *Lumbriculus* cultured in control water
0 aq + 1 diet	Exposed to control water and fed *Lumbriculus* cultured in 1.0 µg/L Cd
0 aq + 2 diet	Exposed to control water and fed *Lumbriculus* cultured in 2.0 µg/L Cd
1 aq + 0 diet	Exposed to 1.0 µg/L Cd water and fed *Lumbriculus* cultured in control water
2 aq + 0 diet	Exposed to 2.0 µg/L Cd water and fed *Lumbriculus* cultured in control water
1 aq + 1 diet	Exposed to 1.0 µg/L Cd water and fed *Lumbriculus* cultured in 1.0 µg/L Cd
2 aq + 2 diet	Exposed to 2.0 µg/L Cd water and fed *Lumbriculus* cultured in 2.0 µg/L Cd

Aqueous exposure was achieved using a modified continuos flow diluter (Benoit et al. 1982) constructed of polyethylene, polypropylene and teflon components which delivered exposure water at a rate of 50 mls/min to each aquarium via food-grade vinyl tubing.

Lumbriculus were harvested from the aquaria and separated from the sandy substrate, transferred to a Buchner funnel, rinsed with deionized water and air-dried under vacuum for 10 seconds. Fry were fed their respective diets at a rate of 10% body weight five times per week. Bulk weights of fry in each aquarium were measured each week and feeding rates adjusted. Aqueous and dietary exposure continued for a total of 59 days. Instantaneous growth rates (% BW/d) were determined from exponential regressions of mean weights of fry plotted versus time.

At the end of the test, differential centrifugation was used to measure subcellular distribution of cadmium in liver, kidney and intestine tissues. Cadmium was measured in three fractions consisting of cell fragments and organelles, cytosolic heat-labile proteins and bound to metallothionein-like proteins. Tissues of fry within each aquarium were removed and pooled. Each tissue was homogenized (TissueTearor) in 1.7 mls of ice-cold HEPES buffer (pH 7.4). Aliquots of 1.5 mls of homogenate were transferred to a 1.5 ml centrifuge tube and centrifuged

at 15,000 g for 10 minutes at 4°C. Cadmium present in the resulting pellet was bound to organelles and cellular debris. The supernatant was carefully transferred to a new 1.5 ml microcentrifuge tube, heated at 100°C for 10 minutes followed by immersion in an ice-bath for 10 minutes, followed centrifugation at 15,000 g for 10 minutes at 4°C. Cadmium present in the resulting pellet from this step was associated with cytosolic heat-labile proteins. The supernatant from the final step was transferred to a separate microcentrifuge tube. Cadmium present in the final supernatant was bound to metallothionein-like proteins. Microcentrifuge tubes were heated in aluminum hot blocks at 100°C until dry. Contents of the tubes were digested with 0.1 mls of high purity nitric acid for four hours at 100C followed by an additional four hours at 100C with 0.1 ml of high purity hydrogen peroxide. Digests were diluted to 1.5 mls with 1% nitric acid and analyzed for cadmium by atomic absorption spectrometry.

Water quality characteristics of exposure waters were measured weekly. Alkalinity was determined titrimetrically according to Standard Methods (APHA 1998). Dissolved oxygen, conductivity and pH were measured using electronic meters calibrated prior to each use. Water samples for metal analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 μm filter (Acrodisc), collected in 2 oz HDPE bottles (Nalgene) and immediately preserved with high purity nitric acid (JT Baker) to pH <2. Cadmium, sodium, potassium, calcium and magnesium concentrations were measured using a Thermo Jarrell Ash ICP (IRIS) spectrometer calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard (High Purity Standards, Charleston SC). Water samples for chloride and sulfate analyses were collected weekly and analyzed with a Flow Injection Analyzer (QuikChem 8000, Lachat Instruments, Loveland, CO, USA) using EPA methods 325.1 and 375.4, respectively. Sample splits and spikes were collected at each sampling event. Water samples for dissolved organic carbon (DOC) were gravity-filtered through pre-combusted 47 mm glass fiber filters (1.0 μm size particle retention) (Gelman Sciences Inc., Ann Arbor, MI, USA) using a stainless steel filter holder into pre-cleaned 500 ml amber glass bottles (VWR Trace Clean) and submitted to a commercial laboratory for analysis.

RESULTS

Water quality parameters were constant over the course of the experiment (Table 5). Measured concentrations of cadmium were slightly less than target concentrations for both the *Lumbriculus* culture and cutthroat trout aqueous exposures (Table 6).

Fry survival in most treatments was 100% (Figure 3). A single fry died in the 0 aq + 1 diet and the 0 aq + 2 diet treatments and two fry died in the 1 aq + 0 diet treatment. No effect of cadmium exposure on survival was detected. Fry grew exponentially (Figure 4) with growth rates between 0.019 g/g/day to 0.021 g/g/d. Growth rates were not significantly affected by cadmium exposure (Figure 3).

Kidney, liver and intestine tissue accumulated significant amounts of cadmium in fry exposed to cadmium from aqueous and dietary exposure (Figures 5, 6 and 7). Accumulation was in a concentration or dose-related manner. The internal organs, kidney and liver, accumulated cadmium from aqueous but not dietary exposures (Figures 6 and 7). In contrast, intestine accumulated cadmium from dietary but not aqueous exposure (Figure 7). Accumulation of cadmium in the tested organs followed the order kidney>intestine>liver. A large majority (>70%) of cadmium in each of the tissues was bound to metallothionein-like proteins. Low levels of cadmium were associated with the cell fragments or cytosolic protein fractions.

DISCUSSION

Salmonids are particularly sensitive to cadmium toxicity and represent six of the seven most sensitive species tested (USEPA 2001). Survival and growth of Rio Grande cutthroat trout fry were affected by chronic cadmium aqueous exposures as low as 3.37 µg/l with a 96 hr median lethal concentration of 2.40 µg/L (Brinkman 2012). At the test water hardness of 47.7 mg/L, the acute and chronic USEPA water quality criteria for cadmium is 0.98 and 0.15 µg/L, respectively (USEPA 2001). Cadmium acute and chronic water quality standards for the state of Colorado are 0.89 and 0.24 µg/L, respectively. In the present test, no mortality or adverse effects on growth were detected after 59d aqueous exposures of 1.77 µg/L. Addition of a diet cultured in .62 µg/L Cd also did not affect growth or survival. These results suggest that Colorado's water quality standards for cadmium are protective of cutthroat trout from aqueous and dietary exposure.

Accumulation of cadmium in the kidney, liver and intestine was consistent with previous reports (Olsson and Hogstrand 1987, Brinkman 2008, Hollis et al. 2001). The role of metallothioneie-like proteins in binding a large majority of cadmium in tissues is consistent with its purported role in cadmium sequestration and protection against toxicity.

Table 5. Water quality characteristics and major ions of exposure water used for cutthroat trout dietary and aqueous cadmium exposure. Standard deviations are in parentheses.

Hardness (mg/L as $CaCO_3$)*	47.7 (0.6)
Alkalinity (mg/L)	33.7 (0.6)
pH (S.U.)	7.37 (0.16)
Temperature (°C)	13.0 (0.2)
Dissolved Oxygen (mg/L)	8.68 (0.25)
Conductivity (µS/cm)	86.1 (1.5)
Calcium (mg/L)	14.4 (0.2)
Magnesium (mg/L)	1.1 (0.0)
Sodium (mg/L)	1.9 (0.1)
Potassium (mg/L)	0.53 (0.02)
Chloride (mg/L)	2.7 (0.1)
Sulfate (mg/L)	11.2 (0.2)
DOC (mg/L)	1.7 (0.3)

*Calculated from calcium and magnesium concentrations

Table 6. Mean measured cadmium concentration (µg/L) of *Lumbriculus* cultures and aqueous exposure of cutthroat trout. Standard deviations are in parentheses.

	Control	1.0	2.0
Measured Cd concentration Lumbriculus culture	<0.2 (0.1)	0.96 (0.20)	1.62 (0.38)
Measured Cd concentration aqueous exposure	<0.2 (0.1)	0.89 (0.04)	1.77 (0.13)

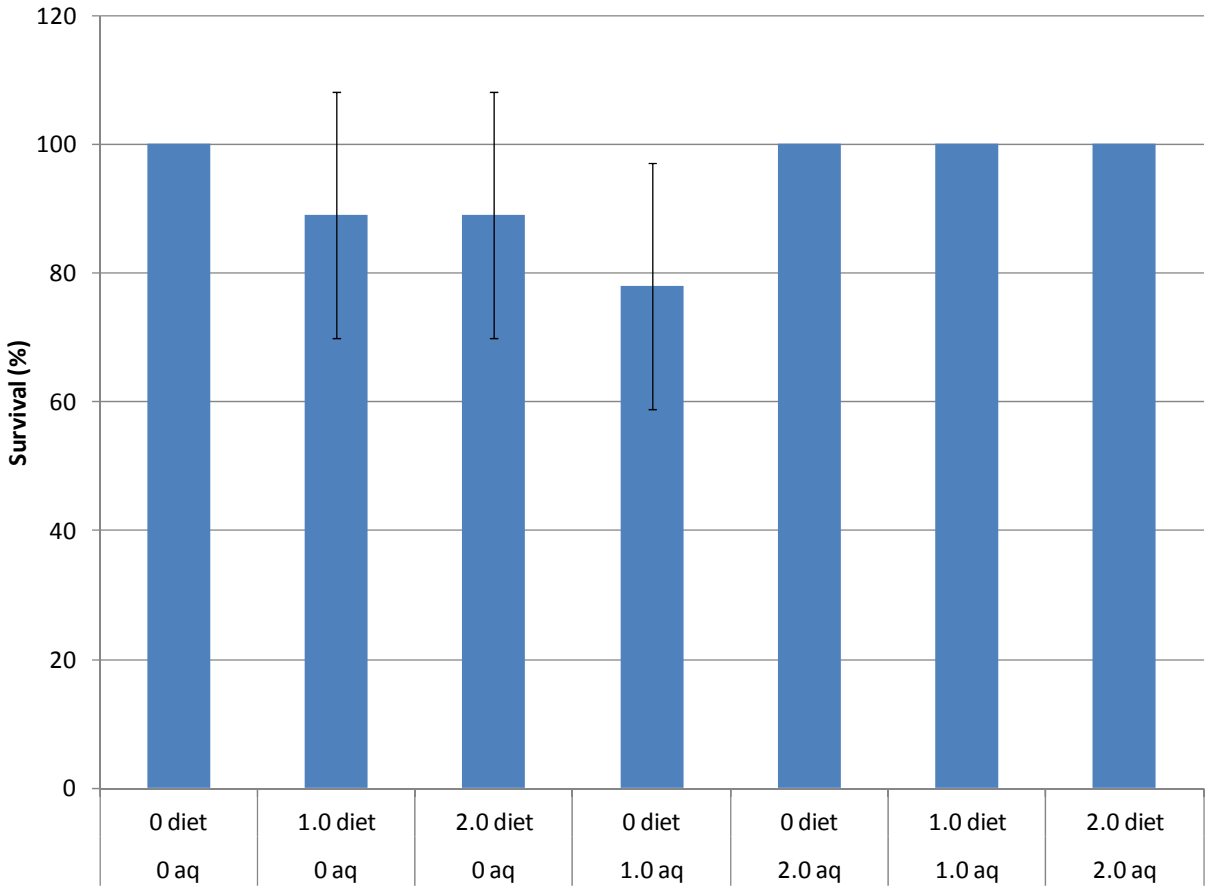

Figure 3. Survival (%) of cutthroat trout exposed to dietary and or aqueous cadmium for 59 days. Error bars represent standard deviation.

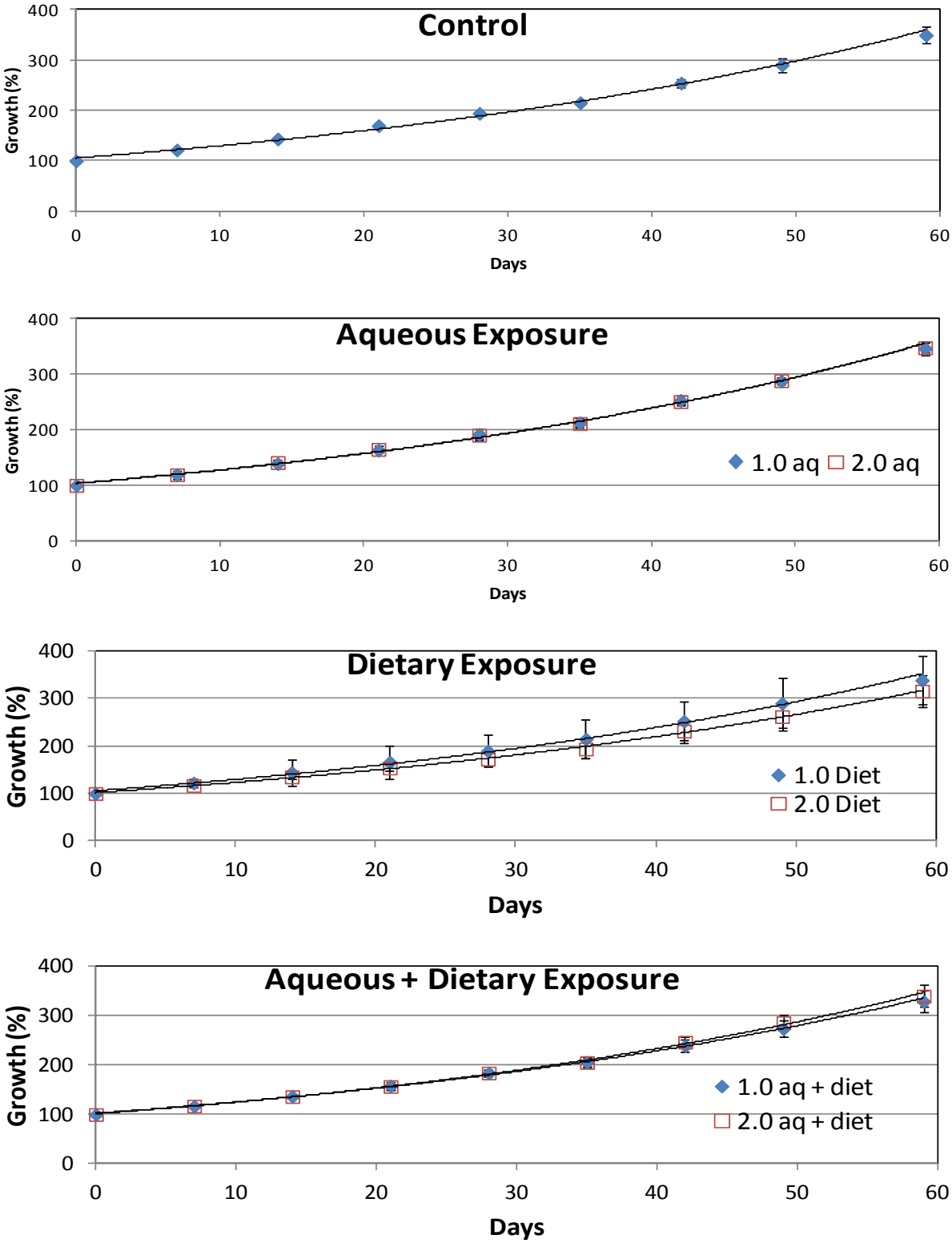

Figure 4. Growth rates of cutthroat trout exposed to aqueous and/or dietary cadmium exposure for 59 days. Error bars are standard deviation.

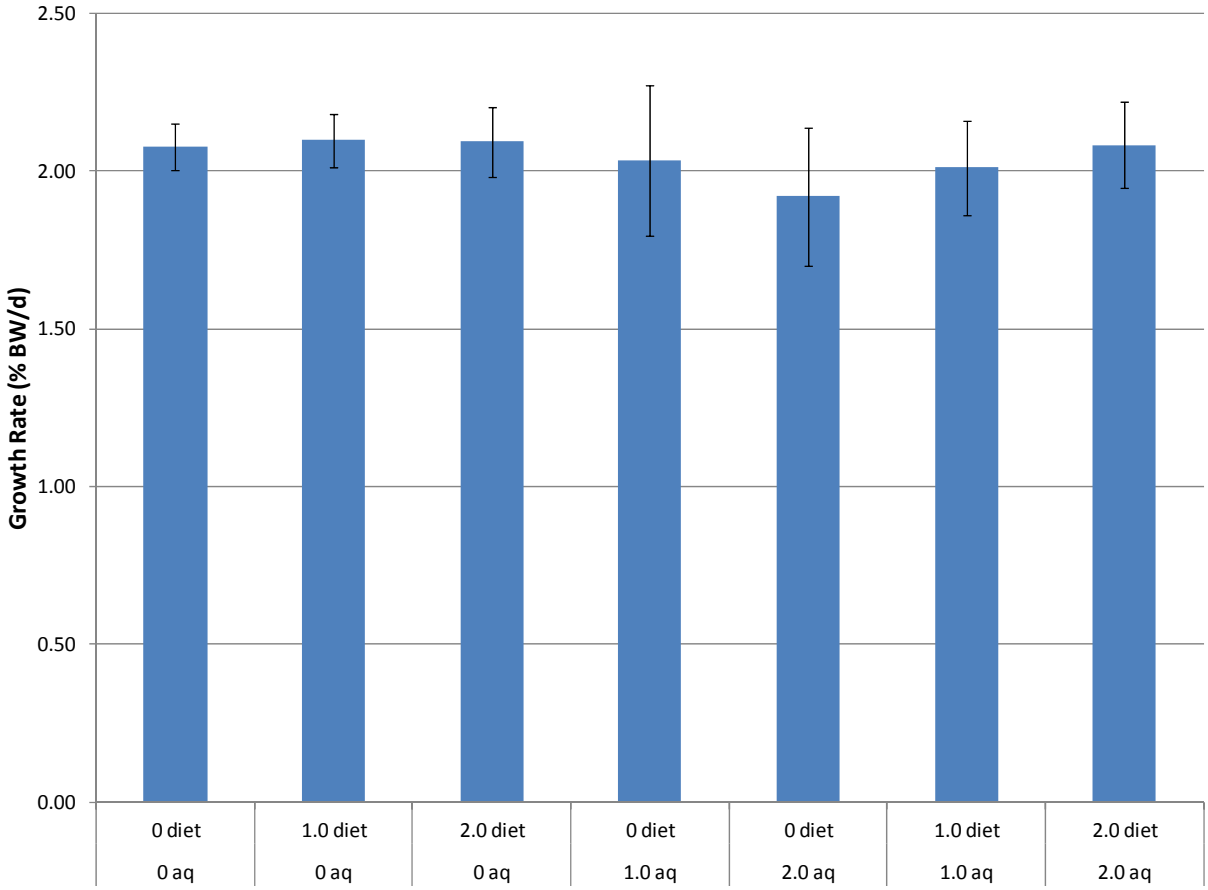

Figure 5. Instantaneous growth rates (%BW/d) of cutthroat trout exposed to dietary and or aqueous cadmium for 59 days. Error bars represent standard deviation.

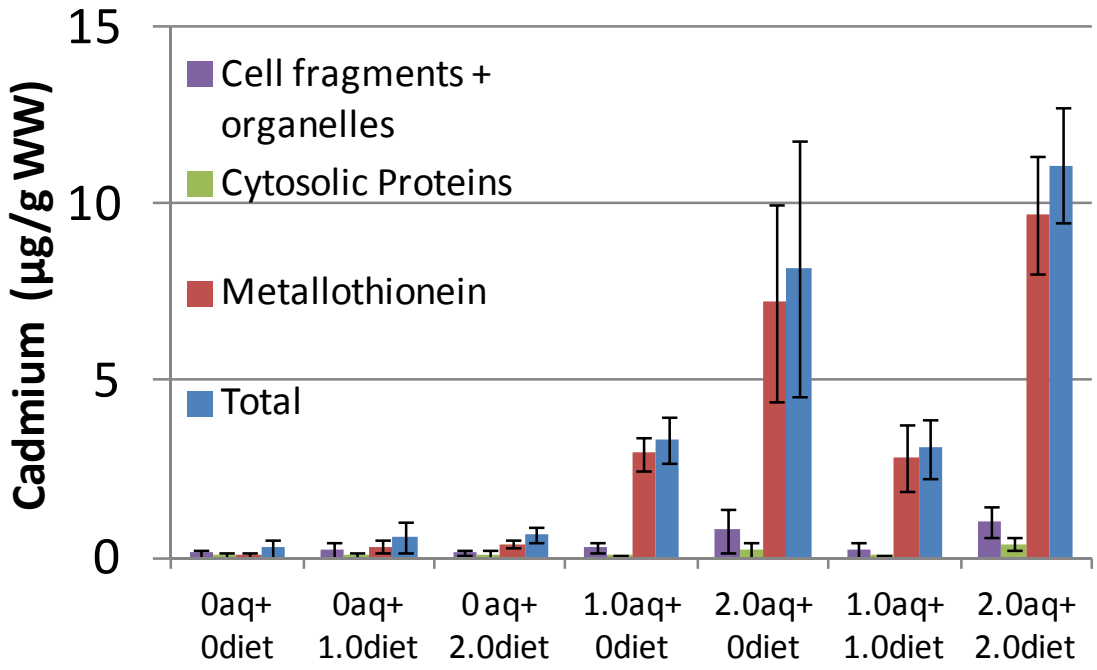

Figure 6. Total cadmium content (µg/g wet weight) and cadmium in cell fragment, cytosolic proteins, bound to metallothionein-like proteins of the kidney of cutthroat trout fry exposed to aqueous and/or dietary cadmium for 59 days. Error bars are standard deviation of the mean.

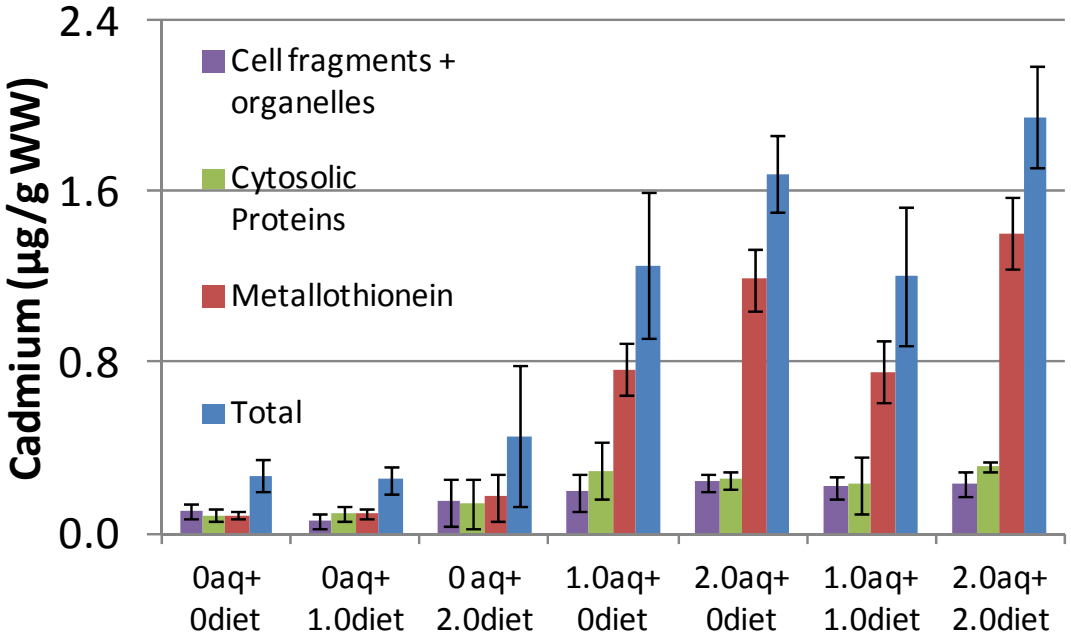

Figure 7. Total cadmium content (µg/g wet weight) and cadmium in cell fragment, cytosolic proteins, bound to metallothionein-like proteins of the liver of cutthroat trout fry exposed to aqueous and/or dietary cadmium for 59 days. Error bars are standard deviation of the mean.

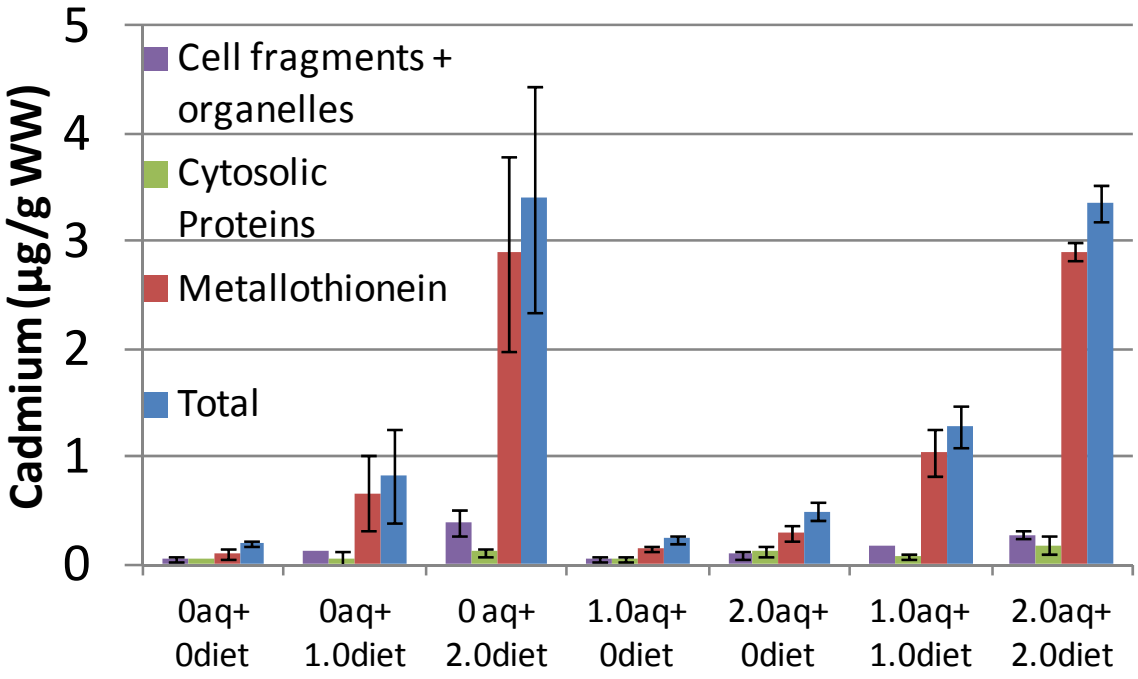

Figure 8. Total cadmium content (µg/g wet weight) and cadmium in cell fragment, cytosolic proteins, bound to metallothionein-like proteins of the intestine of cutthroat trout fry exposed to aqueous and/or dietary cadmium for 59 days. Error bars are standard deviation of the mean.

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Acute toxicity of ammonia to suckermouth minnows

Lindsy Ciepiela, Stephen F. Brinkman and Ryan Fitzpatrick.

INTRODUCTION

Suckermouth minnows (*Phenacobius mirabilis*) inhabit riffle areas in prairie streams of the Mississippi River basin from Ohio and West Virginia in the east to Wyoming, Colorado, and New Mexico in the west and from Minnesota in the north to the Gulf Coast in the south (Page and Burr 1991). Like many other fishes of the plains of Colorado, suckermouth minnows are in decline. In 1998, the Colorado Wildlife Commission listed the suckermouth minnow as endangered. Cause(s) of the decline have not been specifically identified but may include habitat alteration, nonnative species interactions and water quality. Prairie streams in eastern Colorado such as the South Platte River are dominated by wastewater treatment plant effluent during much of the year. Ammonia is a common contaminant of WWTP effluent. Additional sources of ammonia include concentrated animal feedlots and surface runoff of agricultural areas which are also features of the South Platte River in eastern Colorado. Ammonia differs from many other toxicants in that ammonia is produced endogenously by fish which often rely on diffusion for excretion. Elevated concentrations of ammonia in the surrounding water may reduce or prevent ammonia excretion leading to a buildup in the plasma of fish leading to death. A toxicity test was conducted to determine toxicity of ammonia to suckermouth minnows. Ammonia concentrations collected in the South Platte were compared to toxicity values to evaluate risk of suckermouth minnows to adverse effects of ammonia.

METHODS

A modified continuous-flow serial diluter (Benoit et al. 1982) constructed of Teflon, polyethylene, and polypropylene components delivered exposure concentrations via food-grade vinyl tubing (Nalge Nunc International Corporation, Rochester NY) to 9 L glass aquaria. Test solutions overflowed from aquaria into a water bath maintained at 17°C using a recirculating chiller. An ammonia stock solution was prepared by dissolving a calculated amount of ammonium chloride (Mallincrodt Analytical Reagent grade) in deionized water. The ammonia stock solution was delivered to the diluter via a peristaltic pump at a rate of 2.0 mls/min. The diluter delivered five concentrations of ammonia with a 50% dilution ratio and a control. Target total ammonia concentrations were 200, 100, 50, 25, 12.5 and 0 mg NH_3-N/L. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 ml/min for each chamber. Fluorescent lights suspended 1.3 m above the exposure chambers provided a 16-h/8-h light-dark photoperiod. Dark lids placed over part of the chambers served to screen some of the light. Diluter and toxicant flow rates were monitored daily to ensure proper operation. Suckermouth minnows used in the test were received from the Colorado Parks and Wildlife Native Aquatic Species Restoration Facility. They were acclimated to dilution water for three months prior to the start of the ammonia test. At the start the test, fry (mean weight 0.731

g) were distributed one at a time to each tank and the process repeated until a total of 12 fry were added to each tank.

Water quality parameters were measured 0, 48, and 96 hours. Hardness and alkalinity were determined titrimetrically 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 for ammonia analysis were collected weekly in all exposure containers within a replicate. Different replicates were selected each week for sampling. Water samples for ammonia determinations were preserved with 0.25% H_2SO_4 and refrigerated at 4°C until analysis. Ammonia concentrations were measured using a Flow Injection Analyzer (QuikChem 8000, Lachat Instruments, Loveland CO) using EPA method 350.1. Sample splits, spikes, and blanks were collected. Detection limit for ammonia analyses was 0.03 mg NH_3-N/L.

RESULTS and DISCUSSION

Water quality parameters were constant throughout the experiment (Table 7). Mean pH was 6.99. Measured ammonia concentrations were about 10% greater than target concentrations but were constant throughout the experiment (Table 8). Ammonia was observed in the control exposures due to excretion of ammonia by the test organisms. No mortality occurred at ammonia concentrations ≤15.8 mg/L. At 112 mg/L, survival was 86% after 96 hours of exposure and 82% after 144 hours of exposure. No suckermouth minnow fry survived exposure at 220 mg/L. Median lethal ammonia concentration after 96 hours was 143.5 mg N/L (95% CI; 132.0-154.9). After 144 hours, the median lethal concentration decreased slightly to 138 mg N/L (95% CI; 126.8-151.0). Toxicity of ammonia is greatly affected by pH. As pH increases, ammonia toxicity increases. To enable comparison of toxicity tests results conducted at different pH, toxicity values can be normalized to a pH = 8 using the following equation (USEPA 1999):

$$AV_{pH}=(AV_8)[(0.0489/(1+10^{7.204-pH}))+(6.95/(1+10^{pH-7.204}))]$$

Where AV_{pH} is the LC50 at test pH, AV_8 is LC50 normalized to a pH=8, and pH is test pH. Normalized to a pH of 8, the 96 hour median lethal concentrations for suckermouth minnows was 33.12 mg/L.

Ammonia discharged from domestic and industrial treatment facilities is regulated throughout the United States of America based on periodically updated criteria developed by the United States Environmental Protection Agency (USEPA) (USEPA 1985, 1998, 1999). The LC50 for suckermouth minnow would be ranked 13th out of 34 genera that were used to calculate ammonia criteria (USEPA 1999). The pH-normalized LC50 value for suckermouth minnow is similar to genus mean acute values of other cyprinids including 25.60 mg-N/L for *Notropis*, 26.97 mg-N/L for *Campostoma*, 34.4 mg-N/L for *Ictalurus*, 38.11 mg-N/L for *Catostomus* and 43.55 mg-N/L for *Pimephales* (USEPA 1999).

Measured ammonia concentrations in the South Platte River downstream from Front Range WWTP effluents are well below the acutely lethal value of suckermouth minnows. In the South Platte River at Kersey in 1994-1996, ammonia concentrations normalized to pH = 8.0 were all below 4.5 mg-N/L (Figure 9). Ammonia concentrations exhibited an annual pattern that illustrates the influence of WWTP effluent on the South Platte River on the eastern plains of Colorado. Ammonia concentrations in all three years were highest in the winter months when WWTP effluent dominates river flows and are lowest during spring and summer when spring runoff significantly dilutes WWTP discharges.

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Table 7. Mean, standard deviation and range of water quality characteristics of 96h acute ammonia toxicity test exposure water conducted with suckermouth minnows.

	Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (ºC)	DO (mg O_2/L)
Mean	743.7	34.9	6.99	15.9	7.95
Std. dev.	2.0	1.3	0.08	0.3	0.42
Range	38.8-48.0	31.6-37.2	6.90-7.17	15.3-16.4	7.24-8.65

Table 8. Mean ammonia concentrations (standard deviations in parentheses) and associated mean survival of suckermouth minnow fry.

Target concentration (mg N/L)	0	12.5	25	50	100	200
Measured concentration (mg N/L)	7.25 (1.27)	15.1 (0.05)	30.8 (0.15)	58.7 (0.42)	112 (2.08)	220 (1.53)
Measured concentration (normalized to pH 8) (mg N/L)	1.67 (0.29)	3.49 (0.01)	7.11 (0.04)	13.5 (0.10)	25.9 (0.48)	50.8 (0.35)
96h survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	86 (5)	0 (0)
144h survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	82 (5)	0 (0)

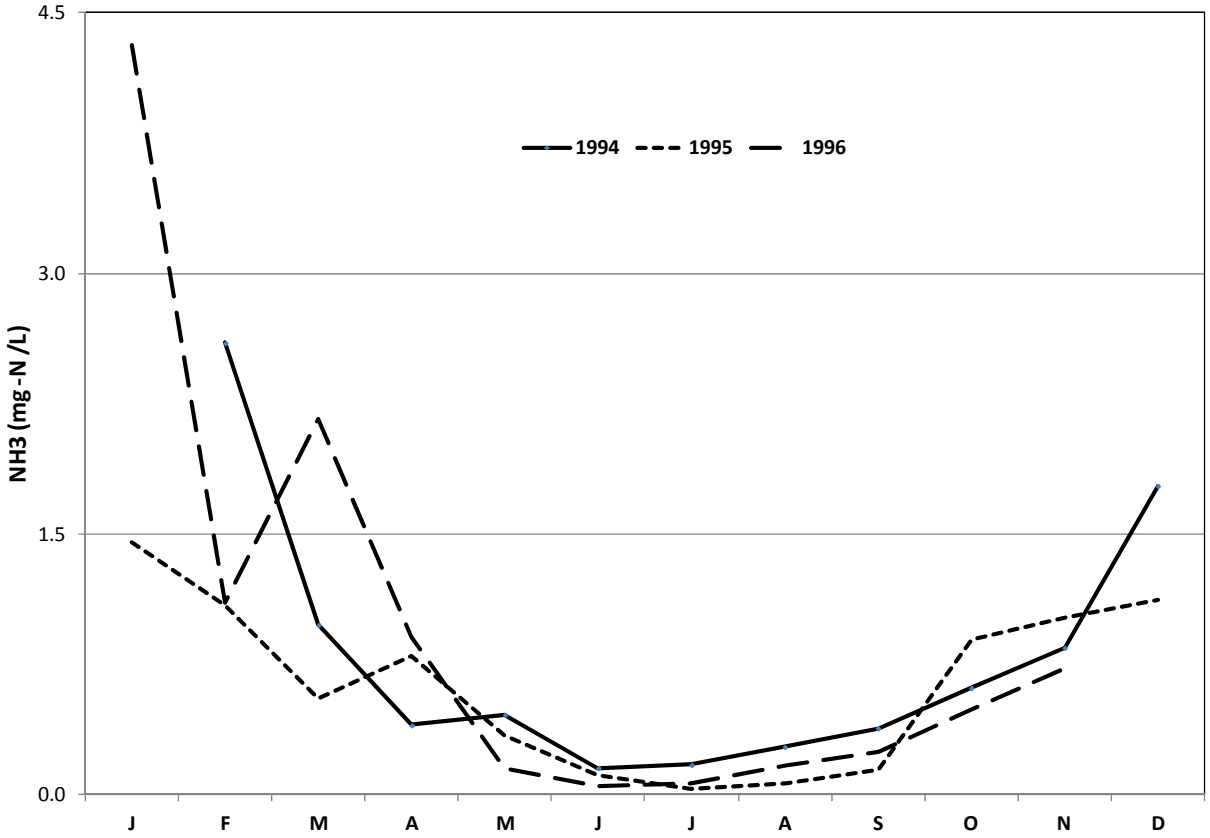

Figure 9. Measured ammonia concentrations in the South Platte River near Kersey during different months of the year in 1994-1996. Ammonia concentrations normalized to pH=8 according to USEPA criteria document (1999).

Chronic toxicity of ferric precipitates to the oligochaete, *Lumbriculus variegatus*

INTRODUCTION

Acid mine drainage (AMD) is a significant source of iron (Fe) for many streams in the western United States. AMD occurs when the mining process exposes pyrite and other sulfidic minerals to air and water, thereby leading to oxidation and the release of iron and sulfuric acid. There are an estimated 20,000-50,000 mines that produce AMD which seriously affects 5,000-10,000 miles of streams (USDA 1993). In Colorado alone, over 955 miles of streams are affected by iron (CDPHE 2010). In oxygenated, circumneutral pH waters, iron is insoluble and precipitates out of solution as ferric hydroxide. As such, Fe is generally considered less toxic to aquatic life than soluble metals such as cadmium, copper and zinc. In general, results of short-term acute toxicity tests using standard laboratory organisms generally support this notion. Though the chemical toxicity of iron precipitates may be low to target organs such as fish gills, the precipitates can otherwise adversely affect aquatic life through increased turbidity, reduced primary production and of interstitial space in the benthic zones and smothering of bottom-dwelling invertebrates, plants and incubating fish eggs (USEPA 1976, Davies and Goettl 1977, McKnight and Feder 1984, Vuori 1995, Linton et al. 2007). The current USEPA criterion and Colorado chronic iron standard for protection of aquatic life is 1.0 mg/L (total recoverable). This value is largely based on field observations which concluded that trout and other fishes were not present in an iron-polluted Colorado stream until dilution or loss of iron from the water column resulted in a concentration less than 1.0 mg/L (USEPA 1976). Few chronic laboratory toxicity tests with iron have been conducted from a limited range of taxa to derive a water quality criterion using established guidelines (USEPA 1985). This project has in the recent past conducted chronic iron toxicity tests to better understand the effects of chronic exposures to iron hydroxide precipitates on early life stages of brown trout (*Salmo trutta*), mountain whitefish (*Prosopium williamsoni*) and boreal toad tadpoles (*Bufo boreas*) (Brinkman and Vieira 2011, Brinkman 2012). In order to broaden our understanding of the effects of iron on aquatic life, a toxicity test was conducted on the oligochaete *Lumbriculus variegatus*.

MATERIAL and METHODS

A culture of *Lumbriculus variegates,* originally founded from individuals obtained from the USEPA laboratory in Duluth MN, was maintained in a 39L glass aquarium with washed coarse sand as a substrate and fed a slurry of trout starter feed. At the start of the experiment, 15 individuals were weighed and placed into each 2.7L polypropylene exposure chamber containing 150 mls of coarse washed sand. A continuous-flow diluter (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components delivered four exposure levels of iron hydroxide and an exposure control. Target iron concentrations were 8000, 4000, 2000, 1000, and 0 µg/L. Each exposure level was replicated five times. Source water was dechlorinated Ft. Collins municipal tap water. Iron stock solution was prepared by dissolving ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Mallinkrodt analytical reagent grade) with sufficient NaOH (1:3 stoichiometry) to neutralize acidic conditions caused by precipitation of ferric hydroxide. The stock solution was pumped to the diluter with a peristaltic pump at a rate of 2 mLs/min. A flow splitter allocated each iron concentration equally at 40 mLs/min to each of five replicate 2.7 L polypropylene tanks via food-grade vinyl tubing. Exposure solutions overflowed from the tanks

through a screen and into a temperature-controlled water bath maintained at 21°C. After 35 days of exposure, individuals in each tank were counted and weighed.

Water samples for iron analyses were collected weekly from each exposure level. Grab samples for total iron were collected in 2 oz HDPE bottles (Nalgene), immediately preserved with high purity nitric acid (JT Baker) to pH <2. Iron 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 curve verified through analyses of external quality assurance samples (High Purity). Sample splits and spikes were collected at each sampling event to verify analytical reproducibility and recovery.

Water quality characteristics were measured weekly in all aquariums within a replicate. A different replicate was selected each week. Alkalinity was determined according to Standard Methods (APHA 1998). Dissolved oxygen and pH measured with electronic meter (Oakton Model 300) calibrated prior to each use. Conductivity was measured with an YSI model 35 conductance meter.

Test data were analyzed using Toxstat version 3.5 software (West, Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included number of organisms and biomass at the end of the test. Normality and homogeneity of variances of test data were tested using Shapiro-Wilk's and Bartlett's test, respectively. Treatment means were compared to the control using Dunnett's one-tailed test at $p<0.05$. The highest iron concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest concentration of iron associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC.

RESULTS

Dissolution of ferric chloride and the subsequent precipitation of ferric hydroxide release acidic protons according to reaction:

$$FeCl_3 + 6H_2O \rightarrow Fe(OH)_3 + 3H^+ + Cl^-$$

Mixing a stock solution of ferric chloride with dilution water from the diluter would alter pH and alkalinity and confound interpretation of toxicity results. To prevent changes in pH and alkalinity, sodium hydroxide was added to the stock solution in a 3:1 stoichiometric ratio in order to neutralize the acid formed by the precipitation of ferric hydroxide. As a result, measured alkalinity and pH were similar among the iron exposure levels (Table 9). Neutralization of the stock solution with sodium hydroxide resulted in a slight increase in conductivity associated with iron exposure levels from 109 µS/cm in the control exposure to 148 µS/cm in the highest iron concentration. Water quality parameters for the three tests were constant over the course of the exposures and generally similar among the tests. Alkalinity and pH did not vary significantly among the different iron exposure levels demonstrating the neutralization of acid produced by precipitation of ferric hydroxide. Measured target concentrations of iron were lower than target

concentrations most likely due to precipitated iron falling out of the water column and depositing onto the substrate.

Number of *Lumbriculus* and biomass at the end of the test were both significantly reduced by exposure to iron precipitates (Figures 10 and 11). The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) based on number of *Lumbriculus* was 593 µg/L and 1145 µg/L total iron, respectively. The chronic value based on number of *Lumbriculus* was 880 µg/L total iron. Biomass was significantly reduced at 3087 µg/L (LOEC) but no significant reduction was detected at 1145 µg/L (NOEC). The chronic value based on biomass was 2110 µg/L total iron.

DISCUSSION

Elevated iron concentrations from acid mine drainage are strongly associated with low pH, a phenomenon known as acid rock drainage (ARD) which has been called the greatest water quality issue in the Western United States (Mineral Policy Center 1997). Adding soluble iron to the lab dilution water would lower the pH and alkalinity of the exposure water in direct association with the iron concentration. While these conditions would have been a more environmentally realistic exposure scenario, it was decided in the present test to neutralize the stock solution to isolate the toxic effects of iron from effects of lowered pH and any possible interaction. This approach of neutralizing low pH induced by precipitation of ferric hydroxide in toxicity experiments has been utilized in other chronic iron toxicity experiments with fathead minnows, coho salmon, brook trout, brown trout, mountain whitefish and boreal toad tadpoles (Smith et al. 1973, Smith and Skorka 1976, Brinkman and Vieira 2011, Brinkman 2012).

The chronic value based on number of *Lumbriculus* derived from this study was 880 µg/L total iron. Most likely, *Lumbriculus* were adversely affected by iron precipitates that dropped out of the water column and accumulated on and in the substrate. This is consistent with the notion that iron is a physical stressor that negatively impacts organisms indirectly rather through direct toxicity due to ion activity (McKnight and Feder 1984, Vuori 1994). The Colorado water quality chronic standard for iron is 1000 µg/L. These results suggest that Colorado's water quality standard for iron may not protect benthic invertebrates from adverse effects of iron. Further research that studies chronic effects of iron on a diverse array of aquatic organisms is needed in order to develop more appropriate iron water quality standards.

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Table 9. Mean measured water quality characteristics in each of the iron exposure levels. Standard deviations are in parentheses.

Target Iron Concentration	0	1000	2000	4000	8000
Acid Soluble Fe concentration (µg/L)	<100 (14)	593 (93)	1145 (171)	3087 (345)	7592 (519)
Hardness (mg/L)	44.6 (2.0)	44.5 (1.7)	44.1 (1.2)	44.4 (1.1)	44.2 (1.3)
Alkalinity (mg/L)	35.4 (2.4)	34.6 (1.6)	34.4 (1.4)	34.6 (2.0)	34.0 (2.9)
pH (SU)	7.86 (0.11)	7.91 (0.12)	7.88 (0.14)	7.88 (0.14)	7.85 (0.09)
Temperature (°C)	22.1 (0.1)	22.1 (0.1)	22.0 (0.3)	22.2 (0.1)	22.1 (0.1)
Conductivity (µS/cm)	144.0 (4.1)	116.4 (6.4)	121.2 (6.9)	134.2 (5.3)	165.7 (5.0)
Dissolved Oxygen (mg/L)	7.55 (0.79)	7.51 (0.86)	7.57 (0.86)	7.54 (0.85)	7.62 (0.82)

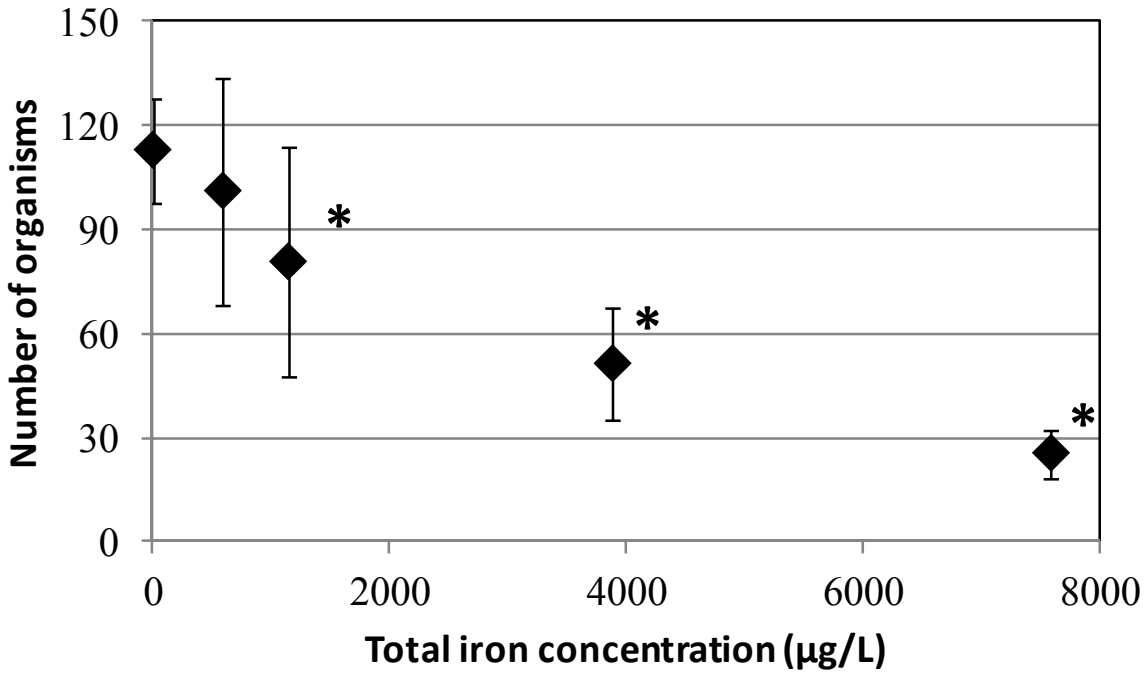

Figure 10. Number of *Lumbriculus variegatus* after 35 days exposure to iron. Error bars represent standard deviation. Asterisks indicate treatment mean is less than control ($p<0.05$).

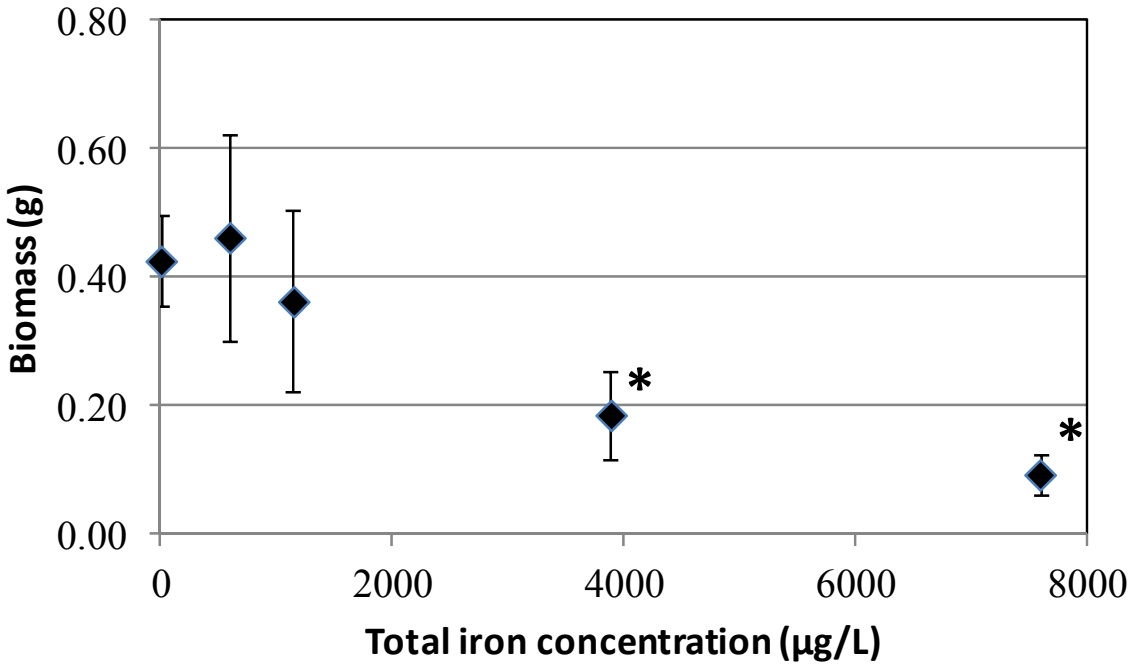

Figure 11. Biomass (g) of *Lumbriculus variegatus* after 35 days exposure to iron. Error bars represent standard deviation. Asterisks indicate treatment mean is less than control ($p<0.05$).