

Water Pollution Studies

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State: Colorado

Study No. F-243R-15

Title: Water Pollution Studies

Period Covered: July 1, 2007 to June 30, 2008

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 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 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 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 other warmwater species at optimal and very cold (less than 5°C) 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 subpiscidal 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 continued to provide equipment and support for an onsite bioassays conducted by the University of Colorado. The studies' objectives were to detect and quantify estrogenic activity in wastewater treatment plant effluents from the cities of Boulder and Vail.

An experiment was conducted designed to determine the effect of exposure of rainbow trout embryo, larvae and fry to the estrogenic compound nonylphenol. Results of the exposure are presented below.

Graduate work on estrogenic effects of estradiol on male red shiners was completed by MS student Michelle McGree, conducted through Dr. Dana Winkelman in the Cooperative Unit, Department of Fish, Wildlife and Conservation Biology. The abstract of the thesis that resulted from completion of this work is attached in Appendix A. The thesis is archived with the Division of Wildlife Library, Fort Collins, CO and also with the Colorado State University library, Fort Collins, CO.

Job A.2.

Mountain whitefish eggs were collected in the field and brought to the Aquatic Toxicology Laboratory. Chronic and acute tests were conducted to determine toxicity of cadmium, copper, and zinc to whitefish embryos, larvae, and fry. 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. Analyses of samples and data are ongoing, and 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 copper and a copper-zinc mixture to the mayfly, *Drunella grandis*. Graduate student Peter Cadmus prepared a draft Masters research proposal to investigate the interactive effects of dietary and aqueous exposure of metals on the scraping mayfly *Rhithrogena* sp.

Job B.3.

DOW participated as Party Status in several Water Quality Control Commission Rulemaking and Administrative Action Hearings, including the Rulemaking Hearing for the 303(d) listings (February 2008) and the Rulemaking Hearing for the Colorado and North Platte River basins (June 2008). 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 Student Chapter of the American Fishery Society at Colorado State University (January 2008). DOW developed a research program to explore avenues to reduce mercury burdens in walleye, a popular sportfish in Colorado Reservoirs. A postdoctoral fellow was hired in March 08 to begin this project. Future updates on this project will be provided under a new DJ Study number, starting in July 08.

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. 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.

Effect of nonylphenol on toxicity of copper and zinc to rainbow trout

INTRODUCTION

Nonylphenol is a breakdown product of nonylphenol ethoxylates, a widely used group of nonionic detergents. A survey of organic wastewater contaminants in 139 streams found that nonylphenol was detected more frequently and measured at higher concentrations than most of the other contaminants (Kolpin et al. 2002). Nonylphenol is classified as a xenoestrogen, based on its ability to induce vitellogenin in aquatic organisms (Sonnenschein and Soto 1998, Servos 1999). Endocrine disruption is known to have adverse effects on fish populations. For example, a 21 day exposure to nonylphenol can result in a delay of smoltification of atlantic salmon (*Salmo salar*) for up to a year after exposure (McCormack et al. 2005, Lerner et al. 2007). Decreased activity of sodium-potassium-activated adenosine triphosphatase (Na,K ATPase) in the gills was identified as the mechanism for delayed smoltification. Copper disrupts sodium pathways of fish and other aquatic animals, and this is believed to be the primary mechanism of toxicity (Lauren and MacDonald 1986, Wood 1992). As such, interference of Na,K ATPase activity by nonylphenol may increase toxicity of copper. The objective of this study was to determine whether exposure to nonylphenol altered the toxicity of copper and zinc compared to unexposed fry.

MATERIALS and METHODS

Nonylphenol pre-exposure

A flow-through toxicant delivery system was constructed using materials believed to be inert to nonylphenol. A float valve, head tank, needle valves, and fish tanks were constructed of stainless steel. Teflon tubing delivered the nonylphenol stock solution to the pumps and fish tanks. Wetted parts of the toxicant delivery pumps were made of stainless steel and ceramic. Stock solutions were prepared and stored in glass ehrlenmeyer flasks and prepared daily to minimize losses and breakdown of the active ingredient. 4-n-nonylphenol (Riedel-de Haen) was dissolved in HPLC-grade methanol and stored at -7°C. Prior to the start of the fish exposures, a 10X nonylphenol stock solution was delivered to the pre-exposure tank for four days prior to the start of the exposure in an attempt to saturate sorption sites on tubing, tanks, etc. The system was then flushed for 24 hours prior to introduction of fish. Nonylphenol stock solutions were prepared daily by mixing 200µL of the methanol stock solution with 2 liters of deionized water. The stock solutions were pumped at 1.25 mls/min and mixed with 100 mls/min dilution water for a final concentration of 10 µg/L nonylphenol. Stock solutions of methanol without nonylphenol were similarly prepared and delivered to act as a solvent control. Final concentration of methanol delivered to fish was 0.0001%. Water samples for nonylphenol analysis were collected on day 38 and 55 in pre-cleaned amber glass bottles (VWR TraceClean) and analyzed by Dr. Thomas Borch (Colorado State University).

Organisms

Rainbow trout eggs (eyed) from Ennis National Fish Hatchery (Lot RBT-ERD-07-ENN) were picked to remove blanks or dead eggs and then randomly divided into two 14 L stainless steel tanks. Each tank received a mixture of reverse osmosis water and onsite well water at a rate of 100 mls/minute. One tank received nonylphenol at a calculated total concentration 10 µg/L in 0.0001% methanol while the other tank received methanol only (NP control). Exposure solutions overflowed from the tanks into a water bath maintained at 12°C by a recirculating chiller. Solutions were passed through an activated carbon filter prior to discharge. Eggs started hatching approximately seven days after start of exposure. Swim-up occurred approximately 27 days after start of exposure. Fry were fed starter trout chow at an estimated 2.5% /day.

Copper and Zinc Challenge

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 water hardness near 45 mg/L. A continuous-flow serial diluter (Benoit et al. 1982) delivered the 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 ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ JT Baker, $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ Mallincrodt). Stock solutions were delivered to the diluter via a peristaltic pump at a rate of 2.0 mls/min. The diluter delivered five concentrations with a 50% dilution ratio and a control. Target concentrations for the zinc test were 1000, 500, 250, 125, 62, and 0 µg/L. Target concentrations for the copper test were 400, 200, 100, 50, 25, 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. At the start of the test, ten trout fry were randomly allocated to each exposure chamber. Trout fry that were pre-exposed to nonylphenol were placed in two of the four chambers. Solvent control fry were placed in the other two chambers.

Water quality parameters were measured daily from two randomly selected tanks. 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 for dissolved metal analysis daily from each exposure level within a replicate. Replicates were alternated at each sampling event. Treatments with no surviving organisms were not sampled. Exposure water was passed through a 0.45µm filter and immediately preserved with high purity nitric acid to pH <2. 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.

Ninety six hour median lethal concentrations (LC_{50}) of zinc and copper to nonylphenol and solvent control fry were estimated using the Trimmed Spearman-Kärber technique with automatic trim (Hamilton et al. 1977, 1978).

RESULTS and DISCUSSION

Water quality characteristics were consistent during pre-exposure to nonylphenol and during the metal challenges (Table 1). Dissolved oxygen was maintained near saturation levels. Alkalinity, pH, and conductivity were slightly lower in the copper and zinc challenges compared to the nonylphenol pre-exposure, while temperature was slightly higher.

Nonylphenol in water samples collected from the exposure tanks were not detected (D.L. $5\mu/L$) but were near expected values in the stock solution ($1.03mg/L$). Embryo survival was 64% and 63% for nonylphenol-pre-exposure treatment and solvent control, respectively. Once hatched, fry survival was 90% for both groups. The generally poor survival may be due to small egg size (640/oz.) and young brood stock age (2 years). Mean weights of fry were similar between nonylphenol and solvent control groups (0.123 g and 0.120 g, respectively).

High levels of mortality at all copper exposure levels prevented an estimation of the LC_{50} . Mortality at the lowest copper exposure was 30% and 15% for the nonylphenol and solvent control group, respectively. Toxicity of zinc was similar between nonylphenol and solvent control groups. The 96 hour median lethal zinc concentrations (95% confidence interval) were $354\mu g/L$ (297-422) and $332\mu g/L$ (276-400) for the nonylphenol and solvent control groups, respectively.

Precautions were taken to ensure delivery of nonylphenol to the exposure tanks including use of inert materials (stainless steel, glass, Teflon), pumping a concentrated nonylphenol solution prior to the test to saturate and passivate sorption surfaces, and daily preparation of stock solutions. Despite these precautions, nonylphenol was not detected in the exposure tanks. Nonylphenol sorbs on to virtually any material including glass (Howard Ramsdell, Colorado State University, personal communication). The nonylphenol pre-exposure group was exposed to much lower than target levels and perhaps not at all. Consequently, we are unable to draw any conclusions on the effect of nonylphenol on toxicity of copper or zinc.

Table 1. Mean, standard deviation and range of water quality characteristics of exposure water during nonylphenol pre-exposure and copper and zinc challenges. Standard deviations are in parentheses.

Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (°C)	Conductivity (μ S/cm)	Dissolved Oxygen (mg O ₂ /L)
Nonylphenol Pre-exposure					
38.5 (0.5)	36.8 (1.5)	7.41 (0.17)	11.5 (0.4)	89.6 (1.9)	8.33 (0.07)
Copper Challenge					
35.1 (0.7)	28.8 (0.6)	7.25 (0.04)	12.5 (0.1)	74.4 (0.8)	8.42 (0.13)
Zinc Challenge					
34.2 (0.4)	28.9 (0.5)	7.13 (0.04)	12.3 (0.1)	72.3 (1.1)	8.72 (0.07)

Table 2. Dissolved copper concentrations (μ g/L) and associated survival (%) of nonlyphenol and solvent control rainbow trout. Standard deviations are in parentheses.

Target [Cu]	0	25	50	100	200	400
Dissolved [Cu]	<10 (4)	31 (1)	56 (1)	108 (2)	212 (14)	416 (16)
Nonylphenol Pre-exposure	100 (0)	30 (0)	0 (0)	0 (0)	0 (0)	0 (0)
Solvent Control	100 (0)	15 (7)	0 (0)	0 (0)	0 (0)	0 (0)

Table 3. Dissolved zinc concentrations (μ g/L) and associated survival (%) of nonlyphenol and solvent control rainbow trout. Standard deviations are in parentheses.

Target [Zn]	0	62	125	250	500	1000
Dissolved [Zn]	<10 (2)	81 (4)	151 (3)	285 (5)	532 (10)	994 (9)
Nonylphenol Pre-exposure	100 (0)	100 (0)	100 (0)	65 (7)	20 (0)	0 (0)
Solvent Control	100 (0)	100 (0)	100 (0)	50 (14)	25 (7)	0 (0)

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Toxicity of cadmium, copper and zinc to early life stages of mountain whitefish

INTRODUCTION

Site-specific water quality standards for heavy metals rely on knowledge of toxicity to resident species. In recent years, state and federal regulatory authorities have solicited data on metals toxicity to native fishes in Colorado. To this end, we conducted chronic and acute tests to determine toxicity of cadmium, copper, and zinc to whitefish embryos, larvae, and fry. Whitefish are one of the few native salmonids, along with the three sub-species of cutthroat trout, still present in Colorado waters. Little is known about the water quality needs for protection of this fish species, although its absence in metals-laden water suggests that it may be particularly sensitive.

MATERIALS and METHODS

Organisms

Adult mountain whitefish in spawning condition were collected from Mad Creek, Colorado on October 16, 2007. Eggs were stripped and fertilized in the field, placed in a cooler, and transported to the Colorado Division of Wildlife Aquatic Toxicology Laboratory in Ft. Collins. Eggs were placed in egg baskets that received 7.5°C dechlorinated municipal tap water (200 mls/min). Unfertilized eggs and those with fungus were carefully removed daily. Eggs were treated weekly with 1600 ppm neutral buffered formalin for 15 minutes to control fungus (Piper et al. 1982). Eggs reached the eyed stage after 19 days. After eye-up, twenty eggs were randomly allocated to incubation cups. Incubation cups were constructed from 2.54 cm² X 75 mm acrylic material with 1000 mesh nylon screen affixed to the end with aquarium-grade silicone adhesive. Incubation cups were suspended in the exposure chambers and received 40 mLs/min flow of exposure water from the diluter. Eggs were monitored daily to measure hatching success. The first ten eggs to successfully hatch were removed from the incubation cup and placed in the exposure chamber. Eggs remaining in the incubation cup were monitored for hatching and removed once hatching was completed. Thus, hatching success is based on twenty organisms in each incubation cup, while fry survival and growth is based on ten organisms transferred to the exposure chamber.

Exposure apparatus

Dechlorinated Fort Collins municipal tap water supplied three identical continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. Each diluter delivered five exposures with a 50% dilution ratio, and an exposure control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mLs/min each. Exposure chambers consisted of polypropylene containers with a capacity of 2.8 L. Test solutions overflowed from exposure chambers into water baths which were maintained near 10°C using temperature-controlled recirculators. Semi-opaque lids limited light

exposure from dim fluorescent lighting (16-h/8-h photoperiod) and prevented organisms from escaping. Chemical stock solutions were prepared by dissolving calculated amounts of analytical reagent grade toxicant ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, CdSO_4) in deionized water. Chemical stock solutions were delivered to the diluters via peristaltic pumps at approximately 2.0 mLs/min. New stock solutions were prepared as needed during the toxicity tests. Diluters and toxicant flow rates were monitored daily to ensure proper operation.

ELS test methods

Test methods followed guidance provided by ASTM method E1241, *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes* (ASTM 1997) with the exception that temperature was measured weekly in each exposure level. Guidance specifies that temperature must be recorded hourly in at least one test chamber or minimum and maximum temperatures must be measured daily.

The number of hatched eggs and mortality of eggs and fry were monitored and recorded daily. Median time from eyed egg stage to hatch was about 30 days. The yolk sac was resorbed and exogenous feeding started at approximately six days post-hatch. Fry were fed brine shrimp naupaliii at an initial rate of 5% DW/g body weight/day. Mass of brine shrimp was increased by 10% per week to accommodate growth of fry. The amount of brine shrimp fed to each exposure chamber was adjusted weekly based on survival rates of each chamber. Exposure chambers were cleaned as needed to remove feces and uneaten food. Fry were exposed for 60 days post-hatch. At the end of the tests, surviving fish from each exposure chamber were terminally anesthetized with MS-222, blotted dry with a paper towel and total weights measured and recorded.

Water quality characteristics of exposure water were measured weekly in two randomly selected exposure chambers. Alkalinity was determined according to Standard Methods (APHA 1998). A Thermo Orion 635 meter was used to measure pH. Conductivity was measured with an YSI model 35 conductance meter. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter. The pH and dissolved oxygen meters were 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), immediately preserved with Ultrex7 triple distilled nitric acid (JT Baker) to $\text{pH} < 2$, and stored in the dark at 4°C until analysis. 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. Copper and cadmium concentrations were measured using a Thermo Jarrell Ash ICP (IRIS) with ultrasonic nebulization. Sodium and potassium concentrations were measured using atomic absorption spectrometry with 0.1% cesium chloride added to control ionization. Calcium and magnesium were measured using ICP. The spectrometers were calibrated prior to each use and the calibration verified using a NIST traceable QAQC standard (High Purity Standards, Charleston SC). 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 to verify analytical reproducibility and recovery. Additional water samples were gravity-filtered through pre-combusted 47 mm glass fiber filters (1.0 μ M size particle retention) (Gelman Sciences Inc., Ann Arbor, MI, USA) using a stainless steel filter holder into pre-cleaned amber glass bottles (VWR Trace Clean) and submitted to a commercial laboratory (ACZ Laboratories, Steamboat CO) for dissolved organic carbon (DOC) analyses.

Fry test methods

Acute toxicity tests with whitefish fry were conducted following the conclusion of the ELS tests. Fry used in this experiment were not previously exposed to metals at the egg stage (to avoid the possibility of acclimation from early life-stage exposures). Exposure apparatus and test methods were identical to the ELS tests with the following exceptions. Water quality characteristics were determined daily and samples for metals, major cations and anions were measured three times during the initial 96 h and weekly thereafter. Fry were not fed during the initial 96 h of exposure but were fed twice daily thereafter (once daily on weekends). Fry tests were terminated after 30 days. After termination of the fry tests, an additional 96 hour acute copper test was conducted to confirm the high copper sensitivity of whitefish observed in the ELS and fry tests.

Statistical analysis

Statistical analyses of data were conducted using Toxstat version 3.5 software (West, Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included hatching success, swim-up survival, mean weights of surviving fry, and biomass of surviving fish at test termination. Hatching success and survival data were arcsine square root transformed prior to ANOVA. Normality and homogeneity of variances were tested using Chi-square and Bartlett's test, respectively. Treatment means were compared to the control using Williams's one-tailed test (Williams 1971, Williams 1972) at $p < 0.05$. Test data that did not meet assumptions of normality or homogeneity of variance were analyzed using Steel's Many-One Rank Test ($p < 0.05$). The highest metal 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 cadmium 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. The inhibition concentration (IC₂₀), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving organisms from each treatment (biomass or standing crop). Ninety six hour median lethal concentrations (LC₅₀) were estimated by the Trimmed Spearman-Kärber technique (Hamilton et al. 1977, Hamilton 1978) using automatic trim. Acute to chronic ratios (ACR) were calculated by dividing the 96 hr LC₅₀ from the fry test by the IC₂₀ from the ELS test.

RESULTS

Water quality parameters were consistent over the duration of the 90 day ELS and the 30 day fry exposures (Table 4). Dissolved oxygen was near saturation (Ft. Collins elevation = 1520 ft above sea level). Dissolved organic matter was near 2 mg/L. Temperature was consistent within each test but varied somewhat among the different metals. Overall, water quality parameters were within the range expected for mountain streams where mountain whitefish are found.

Cadmium

Hatch success varied between 80% and 94% and was unaffected by cadmium exposure up to 13.5 µg/L, the highest concentration tested (Table 5). Survival of fry after 60 days post-hatch was significantly reduced at cadmium concentrations ≥ 3.4 µg/L but were not affected at cadmium concentrations ≤ 1.71 µg/L. Weight and biomass were significantly reduced at cadmium concentrations ≥ 1.71 µg/L but were not affected at cadmium concentrations ≤ 0.92 µg/L. The IC20 of the cadmium ELS test was 1.29 µg/L.

The 96 hour LC50 of the toxicity tests initiated with post-swimup fry was 4.70 µg/L with a 95% confidence interval between 4.37 and 5.05 µg/L. There was no mortality after the initial 96 hour acute period except at 4.32 µg/L (Table 6). Survival after 30 days was significantly reduced at cadmium concentrations ≥ 4.32 µg/L but was unaffected at concentrations ≤ 2.41 µg/L. Likewise, mean weight of surviving fry and biomass were significantly reduced at cadmium concentrations ≥ 4.32 µg/L but was unaffected at concentrations ≤ 2.41 µg/L. The IC20 of the cadmium fry test was 3.02 µg/L. The acute to chronic ratio (ACR) was 3.64.

Copper

Hatch success varied between 82 and 92% and was not affected at any of the copper concentration tested (Table 7). Survival of fry after 60 days post-hatch was significantly reduced at copper concentrations ≥ 21.1 µg/L but were not affected at copper concentrations ≤ 8.3 µg/L. Weight of surviving fry was significantly reduced at 8.3 µg Cu/L but not at 3.9 µg Cu/L. Biomass at test termination of the ELS tests was significantly reduced at copper concentrations ≥ 22.1 µg/L. The IC20 of the ELS test was 6.7 µg Cu/L.

The 96 hour LC50 of the test initiated with post-swimup fry was 6.0 µg/L with a 95% confidence interval of 5.6-6.4 µg/L. A single mortality occurred in a control treatment between the end of the acute 96 hour phase of the test and test termination at 30 days. Survival of fry after 30 days was significantly reduced at copper concentrations ≥ 8.7 µg/L but not at 4.0 µg/L. Weight of surviving fry were significantly reduced at 8.7 µg/L but not at 4.0 µg/L. Biomass at test termination was reduced at copper concentrations ≥ 8.7 µg/L. The IC20 of the 30 day fry test was 4.8 µg Cu/L.

A second acute test was conducted after termination of the 30 day fry test to confirm the high sensitivity observed in the ELS and fry tests. The 96 hour LC50 of the second acute copper test was 5.0 µg/L with a 95% confidence interval of 4.2-6.1 µg/L. The geometric mean of the two copper LC50s was used to calculate the ACR which was 0.82.

Zinc

Hatch success was around 90% for all exposure levels except the high zinc concentration, 1507µg/L, which at 61% was significantly lower than the control (Table 10). Survival of fry after 60 days post-hatch, weight of fry, and biomass at test termination were significantly reduced at zinc concentrations ≥ 744 µg/L. The IC20 of the zinc ELS test based on biomass at test termination was 422 µg/L.

Survival in the zinc test initiated with fry was 100% at zinc concentrations ≤ 208 µg/L (Table 11). The 96 hour LC50 (95% confidence limits) was 471 µg/L (425-521). No additional mortality occurred after the initial 96 hours of exposure except for a single additional mortality at 422 µg/L. Survival and biomass at test termination were significantly reduced at zinc concentrations ≥ 422 µg/L. Effects of zinc exposure on weight at test termination were not detected. The IC20 of the zinc test initiated with fry was 309 µg/L. The ACR was 1.12.

DISCUSSION

Hatching success of mountain whitefish eggs was not affected except at the highest zinc exposure of 1507 µg/L. Compared to other endpoints, hatching success was not a sensitive endpoint, as has been reported for other salmonids (Chapman 1978, Van Leeuwen 1985, Davies et al. 2003, Brinkman and Hansen 2004, Brinkman and Hansen 2007). In general, the most sensitive chronic endpoints were the IC20s (Table 12). Other chronic endpoints were derived using hypothesis testing which has two notable disadvantages. First, the NOEC and LOEC are limited to test concentrations. Second, the power to detect effects is often weakened if data are non-normal. Non-normality is a problem in tests that have treatment levels with 0 or 100% survival. Growth, as measured by weight of fry at test termination, tended to be a sensitive endpoint because normal data allowed for use of parametric tests for comparison with controls.

For copper and zinc, tests initiated with fry were more sensitive than longer term ELS tests. In fact, the copper and zinc LC50 of the fry tests were near or below ELS chronic values. This paradox may be because in ELS tests, acclimation during metal-tolerant embryo and larval life stages may result in tolerance during the sensitive swim-up fry stage (Sinley et al. 1974, Spehar et al. 1976, Brinkman and Hansen 2007). Acute tests start with a sensitive life stage with unacclimated organisms. The acclimation effect was not observed with whitefish exposed to cadmium.

Toxicity values for whitefish from this study were normalized to a hardness of 50 mg/L and compared with values for other salmonids and to Colorado water quality standards (Table 13). Salmonids are acutely sensitive to cadmium toxicity and represent 3 of the 4 most sensitive

genera (USEPA 2001). Compared to other salmonids, whitefish were tolerant of acute cadmium toxicity but would still rank as the 5th most sensitive genus. However, whitefish were chronically more sensitive than most other salmonids. Colorado acute and chronic water quality standards for cadmium are below toxicity thresholds for mountain whitefish. By contrast, mountain whitefish were very sensitive to copper. Acute and chronic toxicity values were less than other salmonids. The Colorado acute water quality standard is greater than the whitefish LC50 and would not likely protect whitefish from copper toxicity. The Colorado chronic copper standard is less than the whitefish chronic value but may not be protective when compared to the whitefish LC50. Mountain whitefish were intermediate in their sensitivity to zinc relative to other salmonids. Colorado acute and chronic water quality standards are well below levels that are a concern for whitefish.

Overall, Colorado water quality standards are protective of mountain whitefish except for the acute copper standard. Mountain whitefish are sensitive to cadmium, copper and zinc and will need to be included in derivations of site-specific water quality standards. Additional research should be conducted to confirm the extreme copper sensitivity of mountain whitefish observed in this study.

Table 4. Water quality characteristics and major ions of exposure water used for mountain whitefish ELS and fry tests.

Hardness (mg/L)*	47.8 (6.2)
Alkalinity (mg/L)	35.9 (2.8)
pH (S.U.)	6.81 (0.18)
Cadmium test temperature (°C)	9.5 (0.3)
Copper test temperature (°C)	9.6 (0.2)
Zinc test temperature (°C)	9.0 (0.2)
DO (mg/L)	9.2 (0.6)
Conductivity (µS/cm)	77.0 (4.4)
Calcium (mg/L)	16.2 (2.1)
Magnesium (mg/L)	1.8 (0.4)
Sodium (mg/L)	3.6 (0.2)
Potassium (mg/L)	1.0 (0.1)
Chloride (mg/L)	3.1 (0.1)
Sulfate (mg/L)	10.5 (0.7)
DOC (mg/L)	1.9 (1.1)

*Calculated from calcium and magnesium concentrations

Table 5. Mean dissolved cadmium concentrations ($\mu\text{g/L}$) and associated mean hatching success and fry survival of mountain whitefish. Standard deviations are in parentheses.

Cadmium Concentration ($\mu\text{g Cd/L}$)	<0.20 (0.02)	0.92 (0.19)	1.71 (0.21)	3.40 (0.24)	6.69 (0.26)	13.5 (0.59)
Hatch Success (%)	87.5 (5.0)	81.2 (7.5)	90.0 (7.1)	93.8 (2.5)	91.2 (2.5)	93.8 (6.3)
Fry Survival (%)	77.5 (9.6)	75.0 (12.9)	65.0 (12.9)	52.5* (17.1)	0* (0)	0* (0)
Weight at Termination (g)	0.155 (0.018)	0.141 (0.026)	0.129* (0.010)	0.102* (0.018)	--	--
Biomass at Termination (g)	1.19 (0.03)	1.05 (0.22)	0.84* (0.16)	0.54* (0.22)	0* (0)	0* (0)

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

Table 6. Mean dissolved cadmium concentrations ($\mu\text{g/L}$) and associated 96 hour and 30 day survival of mountain whitefish. Standard deviations are in parentheses.

Cadmium Concentration ($\mu\text{g Cd/L}$)	0.22 (0.17)	1.48 (0.13)	2.41 (0.08)	4.32 (0.11)	6.74 (0.26)	13.5 (0.28)
Fry Survival – 96 hours (%)	97.5 (5.0)	100 (0)	100 (0)	72.5 (25.0)	0 (0)	0 (0)
Fry Survival – 30 days (%)	97.5 (5.0)	100 (0)	100 (0)	45.0* (0)	0* (0)	0* (0)
Weight at Termination (g)	0.195 (0.010)	0.197 (0.012)	0.194 (0.003)	0.154* (0.022)	--	--
Biomass at Termination (g)	1.90 (0.16)	1.97 (0.12)	1.94 (0.03)	0.72* (0.34)	0* (0)	0* (0)

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

Table 7. Mean dissolved copper concentrations ($\mu\text{g/L}$) and associated mean hatching success and fry survival of mountain whitefish. Standard deviations are in parentheses.

Copper Concentration ($\mu\text{g Cu/L}$)	<2.0 (0.7)	3.9 (0.8)	8.3 (1.1)	21.1 (2.8)	39.4 (3.8)	76.1 (6.0)
Hatch Success (%)	87.5 (6.4)	92.5 (5.0)	86.2 (4.8)	82.5 (6.4)	90.0 (7.1)	82.5 (2.9)
Fry Survival (%)	82.5 (9.6)	85.0 (12.9)	67.5 (32.0)	0* (0)	0* (0)	0* (0)
Weight at Termination (g)	0.146 (0.010)	0.159 (0.009)	0.129* (0.013)	--	--	--
Biomass at Termination (g)	1.20 (0.18)	1.35 (0.15)	0.88 (0.44)	0* (0)	0* (0)	0* (0)

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

Table 8. Mean dissolved copper concentrations ($\mu\text{g/L}$) and associated 96 hour and 30 day survival of mountain whitefish. Standard deviations are in parentheses.

Copper Concentration ($\mu\text{g Cu/L}$)	<2.0 (0.2)	4.0 (0.4)	8.7 (0.7)	22.3 (5.1)	44.3 (0.6)	88.3 (3.8)
Fry Survival – 96 hours (%)	100 (0)	97.5 (5.0)	5.0* (10)	0* (0)	0* (0)	0* (0)
Fry Survival – 30 days (%)	97.5 (5.0)	97.5 (5.0)	5.0* (10.0)	0* (0)	0* (0)	0* (0)
Weight at Termination (g)	0.152 (0.006)	0.145 (0.014)	0.123* (**)	--	--	-
Biomass at Termination (g)	1.48 (0.04)	1.41 (0.07)	0.06* (0.12)	0* (0)	0* (0)	0* (0)

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

**Measurement from a single treatment

Table 9. Mean dissolved copper concentrations ($\mu\text{g/L}$) and associated 96 hour survival of mountain whitefish from acute test conducted after termination of initial fry test. Standard deviations are in parentheses.

Copper Concentration ($\mu\text{g Cu/L}$)	<2.0 (0.3)	1.6 (0.8)	4.1 (0.6)	8.2 (0.5)	19.0 (3.4)	40.0 (0)
Fry Survival – 96 hours (%)	100 (0)	97.5 (5.0)	55.0 (19.1)	25.0 (25.1)	7.5 (9.6)	2.5 (5.0)

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

**Single measurement

Table 10. Mean dissolved zinc concentrations ($\mu\text{g/L}$) and associated mean hatching success and fry survival of mountain whitefish. Standard deviations are in parentheses.

Zinc Concentration ($\mu\text{g Zn/L}$)	<10 (3)	95 (16)	193 (27)	380 (49)	744 (92)	1507 (59)
Hatch Success (%)	91.2 (4.8)	91.2 (4.8)	90.0 (4.1)	88.8 (4.8)	90.0 (7.1)	61.2* (10.3)
Fry Survival (%)	92.5 (10.0)	87.5 (10.0)	82.5 (5.0)	75.0 (17.3)	47.5* (5.0)	0* (0)
Weight at Termination (g)	0.163 (0.016)	0.166 (0.017)	0.158 (0.006)	0.170 (0.009)	0.140* (0.027)	--
Biomass at Termination (g)	1.494 (0.023)	1.449 (0.154)	1.307 (0.126)	1.286 (0.347)	0.449* (0.161)	0* (0)

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

Table 11. Mean dissolved zinc concentrations ($\mu\text{g/L}$) and associated 96 hour and 30 day survival of mountain whitefish. Standard deviations are in parentheses.

Zinc Concentration ($\mu\text{g Zn/L}$)	<10 (1)	102 (5)	208 (8)	422 (14)	821 (5)	1574 (**)
Fry Survival – 96 hours (%)	100 (0)	100 (0)	100 (0)	67.5 (20.6)	0 (0)	0 (0)
Fry Survival – 30 days (%)	100 (0)	100 (0)	100 (0)	65.0* (23.8)	0* (0)	0* (0)
Weight at Termination (g)	0.141 (0.006)	0.136 (0.010)	0.139 (0.008)	0.127 (0.010)	--	--
Biomass at Termination (g)	1.41 (0.06)	1.36 (0.10)	1.39 (0.08)	0.84* (0.36)	0* (0)	0* (0)

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

**Single measurement

Table 12. Summary of toxicity endpoints. Chronic values, LC50s, and IC20s of cadmium, copper, and zinc Early-Life Stage (ELS) and 30 day fry tests conducted with mountain whitefish. No observed effect concentrations and lowest observed effect concentrations are in parentheses.

Endpoint	Cadmium		Copper		Zinc	
	ELS	Fry	ELS	Fry	ELS	Fry
Hatch Success	>13.5	--	>76.1	--	1059 (744,1507)	--
Survival	2.41 (1.71,3.40)	3.23 (2.41,4.32)	13.2 (8.3,21.1)	5.9 (4.0;8.7)	532 (380,744)	296 (208,422)
Weight	1.25 (0.92,1.71)	3.23 (2.41,4.32)	5.7 (3.9,8.3)	5.9 (4.0,8.7)	532 (380,744)	>422
Biomass	1.25 (0.92,1.71)	3.23 (2.41,4.32)	13.2 (8.3,21.1)	5.9 (4.0,8.7)	532 (380,744)	296 (208,422)
IC20	1.29	3.02	6.7	4.8	422	309
96 hr LC50	--	4.70	--	6.0 (5.0*)	--	471

*LC50 from a second acute copper toxicity test.

Table 13. Comparison of mountain whitefish toxicity values with species mean acute values (SMAV) and species mean chronic values (SMCV) of other salmonids. All values normalized to 50 mg/L water hardness. Salmonid toxicity values from USEPA water quality criteria unless otherwise noted (USEPA 1984, USEPA 1987, USEPA 2001).

	Cadmium		Copper		Zinc	
	SMAV	SMCV	SMAV	SMCV	SMAV	SMCV
Mountain Whitefish	4.92	1.33	5.5	6.7	504	451
Colorado Standard	0.90	0.25	6.7	4.8	76	66
Brown Trout	2.52*	2.52*	32.9**	7.7**	316***	216***
Bull Trout	2.152					
Brook Trout	<1.791	2.643	110.4	14.20	2100	899.4
Rainbow Trout	2.108	1.308	42.25		689.3	443.6
Chinook Salmon	4.305	2.612	42.26	<9.448	446.4	635.3
Coho Salmon	6.221	4.265	70.25		1628	
Atlantic Salmon		7.922	196.6		2176	
Lake trout		8.088				
Sockeye Salmon			233.8		1502	>247.3
Cutthroat Trout			66.26		256****	

* Brinkman and Hansen 2007

** Davies et al. 2002

*** Brinkman et al. 2006

**** Brinkman et al. (in prep)

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Acute toxicity of aqueous copper and a copper-zinc mixture to *Drunella grandis* and *Rhithrogena sp* nymphs

INTRODUCTION

The sensitivity of native stream invertebrates to heavy metals pollution remains unresolved. On the one hand, single species 96-hour acute tests with single metals shows that these organisms are considerably more tolerant than fish species. On the other hand, field observations and microcosm experiments with whole benthic insect communities suggest that certain mayfly species are significantly more sensitive than resident salmonids. There are a number of hypotheses regarding the reasons behind this discrepancy, including the following: the role of dietary metals exposure and the role of metals avoidance through drift (neither of which are tested in traditional laboratory studies); the interactive effects of metals mixtures in field and microcosm studies; and differences in water quality parameters between studies (e.g. calcium and magnesium, temperature, and dissolved organic carbon). The goal of this study was to compare toxicity of zinc and a zinc-copper mixture to two mayfly species, one of which is known to be sensitive to heavy metals pollution in the field. For comparison, a simultaneous study was conducted by Peter Cadmus and Dr. Will Clements in experimental microcosms at the Ecotoxicology greenhouse laboratory, Colorado State University (CSU). Results from this study are in preparation. Future studies will be conducted at CSU to explore the role of dietary exposures on these organisms.

MATERIALS AND METHODS

Collection and Handling

Nymphs were collected in shallow riffles from the Michigan River (Jackson County, CO, USA) on 09/14/2007 and 10/13/2007 for the for the copper test and copper-zinc combination test, respectively. *Drunella grandis* nymphs were collected on both occasions and *Rhithrogena sp.* nymphs were collected for the copper-zinc combination test. Nymphs were transported from the collection site to the testing laboratory in Ft. Collins CO in aerated plastic containers placed in a cooler and chilled during transport with an Iceprobe (Coolworks). Upon arrival at the laboratory, the containers were placed in an incubator at 8°C (the temperature of the stream) and aerated with a gentle stream of air. Water was gradually replaced (50% per day) with test dilution water and the incubator temperature was gradually increased (2°C per day) to test temperature (11-12°C). Nymphs were maintained in the incubator for three days prior to the start of the copper test and two days prior to the start of the copper-zinc combination test.

Test Methods

Source water consisted of a mixture of onsite well water and reverse osmosis water. A conductivity controller maintained the diluent source water hardness near 36 mg/L in order to approximate the water quality conditions of the microcosm experiments being conducted by

CSU (Horsetooth Reservoir water, Larimer County, CO, USA). 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 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. The air-lift maintained dissolved oxygen levels at saturation levels and provided continuous, circular flow in the exposure chamber. Nitex screen (1000 micron mesh) was placed in the exposure chambers as substrate. This exposure system has been used successfully with several benthic invertebrates (Brinkman and Vieira 2007, Brinkman and Johnston 2008).

Five *D. grandis* nymphs were randomly assigned to each exposure chamber. Ten *Rhithrogena* nymphs were allocated for the copper-zinc combination test. Each exposure level was replicated four times for the copper test and twice for each of the two taxa in the copper-zinc combination test. Nymphs were not fed during the experiments. Mortality, defined as failure to respond to repeated prodding, was recorded daily. Mortalities were preserved in ethyl alcohol for species identification. In the copper test, one organism was identified as *Drunella doddsi* and was excluded from mortality calculations. *Rhithrogena* was found to be composed of two species. The large majority of test organisms were *Rhithrogena hageni*, however, zero to one organism per treatment (0-10%) was identified as *Rhithrogena robusta*.

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.

At the end of 168 hours of exposure, surviving mayflies were analyzed for tissue concentrations of copper, zinc, sodium, and potassium. Nymphs were soaked for 60 seconds in 0.01 M EDTA (magnesium salt), transferred to filter paper on buchner funnel, rinsed with DI, and aspirated dry. Mayflies were dried to constant weight at 80°C in a drying oven and weighed. Whole bodies were digested with 0.1 ml HNO₃ at 100°C for 4 hours followed by 0.1 ml H₂O₂ at 100°C for 4 hours. Metals were analyzed by atomic absorption spectrometry. Sodium and potassium were analyzed with 1000 mg/L cesium to control ionization. Copper and zinc

concentrations in water samples and digests were analyzed with no modifiers 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. Water samples for metals analyses included sample splits and spikes collected during each sampling event to verify reproducibility and to quantify analytical recovery.

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

RESULTS and DISCUSSION

Water quality parameters were consistent during the toxicity tests (Table 14). Dissolved oxygen was near saturation (Fort Collins, CO elevation 1520 m). Although dissolved organic carbon was not measured, previous analyses of the source waters were low and near 1 mg/L. Exposure concentrations were consistent during the tests and near target concentrations (Tables 15 and 16).

The 96-hr LC_{50} (95% confidence interval) for *Drunella grandis* exposed to copper was 201 $\mu\text{g/L}$ (136-297). Survival in the lowest copper concentration was high after 96 hours but declined as exposure continued through to test termination at 168 hrs (Figure 1). All organisms in the control treatment survived. Previously reported copper toxicity studies with mayflies vary in test hardness and duration but are in reasonably good agreement with this study. A 14-day copper TL_{50} for *Drunella grandis* at a hardness 30-70 mg/L was 180-200 $\mu\text{g/L}$ (Goettl et al. 1972). A 48-hr TL_{50} for *Ephemerella subvaria* was 320 $\mu\text{g Cu/L}$ at a hardness of 40 mg/L (Warnick and Bell 1969). A 96-hr LC_{50} for *Rhithrogena hageni* was 137 $\mu\text{g/L}$ at 44 mg/L hardness (Brinkman and Johnston 2008). Copper 48-hr LC_{50} s for *Isonychia bicolor* and *Stenonema sp.* were 223 and 453, respectively (hardness = 100-120 mg/L) (Dobbs et al. 1994).

Drunella grandis was more tolerant to a mixture of copper and zinc than to copper alone. *Drunella grandis* survival after 96 hours was $\geq 80\%$ in all concentrations and calculation of a LC_{50} was not possible (Table 16). After 96 hours, mortality began to occur in the higher exposures (Figure 2). The reason for the decreased sensitivity of the copper-zinc mixture compared to copper alone is unclear. A previous laboratory experiment found *Drunella doddsi* to be tolerant of high levels of zinc (Brinkman and Johnston 2007) but it is unlikely that zinc would be so dramatically antagonistic to copper toxicity. Organisms used in the copper-zinc mixture test were collected a month later than the copper alone test and were consequently somewhat larger. Larger organisms have been found to be more tolerant to metals (Kiffney and Clements 1996, Clark and Clements 2006).

Rhithrogena sp. was much more sensitive to a copper-zinc mixture than *Drunella grandis* (Table 16). The 96-hour LC_{50} for *Rhithrogena sp.* was a mixture of 69.0 $\mu\text{g Cu/L}$ and 1352 μg

Zn/L. Previously reported LC50 for *Rhithrogena hageni* are 137µg/L and 50,500 µg/L for copper and zinc respectively at 44 mg/L hardness (Brinkman and Johnston 2008).

D. grandis and *Rhithrogena sp.* exhibited very different metal uptake characteristics. Copper accumulation was much greater for *D. grandis* than *Rhithrogena sp.* exposed to similar concentrations (Figure 4). A similar difference in uptake characteristics was found for zinc (Figure 5). Buchwalter and Luoma (2005) found similar differences in 4 hour uptake rate of radiolabeled zinc and cadmium in *D. grandis* and *Rhithrogena morrisoni*. It is interesting that laboratory experiments have found that *Rhithrogena sp.* are more sensitive than *D. grandis*, and yet they accumulate less copper and zinc.

Copper disrupts sodium regulation by aquatic animals and this is believed to be the primary mechanism of toxicity (Lauren and MacDonald 1986, Wood 1992, Wood et al. 1997). Whole body sodium content of *Drunella grandis* surviving exposure to aqueous copper and a copper-zinc mixture decreased significantly at all exposure concentrations (Figure 6). *Drunella* exposed to copper alone lost more sodium and were more sensitive than *Drunella* exposed to a copper-zinc mixture. Potassium content was unaffected by copper or copper-zinc exposure (Figure 7). Curiously, sodium content of *Rhithrogena sp.* nymphs was not decreased by copper exposure (Figure 8).

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Table 14. Water quality characteristics of dilution water for toxicity test with copper alone and copper and zinc mixture. Standard deviations are in parentheses.

Hardness (mg/L)	36.4 (0.6)
Alkalinity (mg/L)	29.3 (0.4)
pH (SU)	7.38 (0.11)
Temperature (°C)	13.4 (0.2)
Conductivity (µS/cm)	76.1 (2.0)
DO (mg/L)	8.39 (0.07)
Ca	10.34 (0.17)
Mg	3.00 (0.06)
Na	6.43 (0.2)
K	0.21 (0.01)
Sulfate (mg/L)	10.7
Chloride (mg/L)	2.88

Table 15. Target and mean measured dissolved copper concentrations (µg/L) and associated 96h and 168h survival (%) of *Drunella grandis*. Standard deviations are in parentheses.

Target Cu	0	31	62	125	250	500
Dissolved Cu	<10 (3)	35 (3)	63 (4)	121 (4)	234 (3)	458 (8)
Survival at 96 h (%)	100 (0)	95.0 (10)	95.0 (10)	65.0 (25.2)	45.0 (10.0)	25.0 (10.0)
Survival at 168 h (%)	100 (0)	83.8 (11.1)	55.0 (19.1)	30.0 (11.5)	10.0 (11.5)	15.0 (19.1)

Table 16. Target and mean measured dissolved copper and zinc concentrations (µg/L), cumulative criterion units (CCU) and associated 96h and 168h survival (%) of *Drunella grandis* and *Rhithrogena* nymphs. Standard deviations are in parentheses.

Target Cu	0	16	31	62	125	250
Dissolved Cu	<10 (1)	19 (2)	30 (7)	62 (3)	121 (6)	233 (14)
Target Zn	0	312	625	1250	2500	5000
Dissolved Zn	<10 (5)	329 (34)	616 (52)	1188 (84)	2401 (95)	4721 (193)
CCU	0	10	18	36	72	141
<i>Drunella</i> (96 h)	100 (0)	100 (0)	100 (0)	90 (14)	80 (0)	80 (0)
<i>Drunella</i> (168 h)	90 (14)	100 (0)	80 (28)	50 (14)	70 (14)	30 (14)
<i>Rhithrogena</i> (96 h)	100 (0)	95 (7)	65 (7)	65 (21)	35 (35)	35 (35)
<i>Rhithrogena</i> (168h)	100 (0)	90 (14)	20 (7)	5 (7)	10 (14)	0 (0)

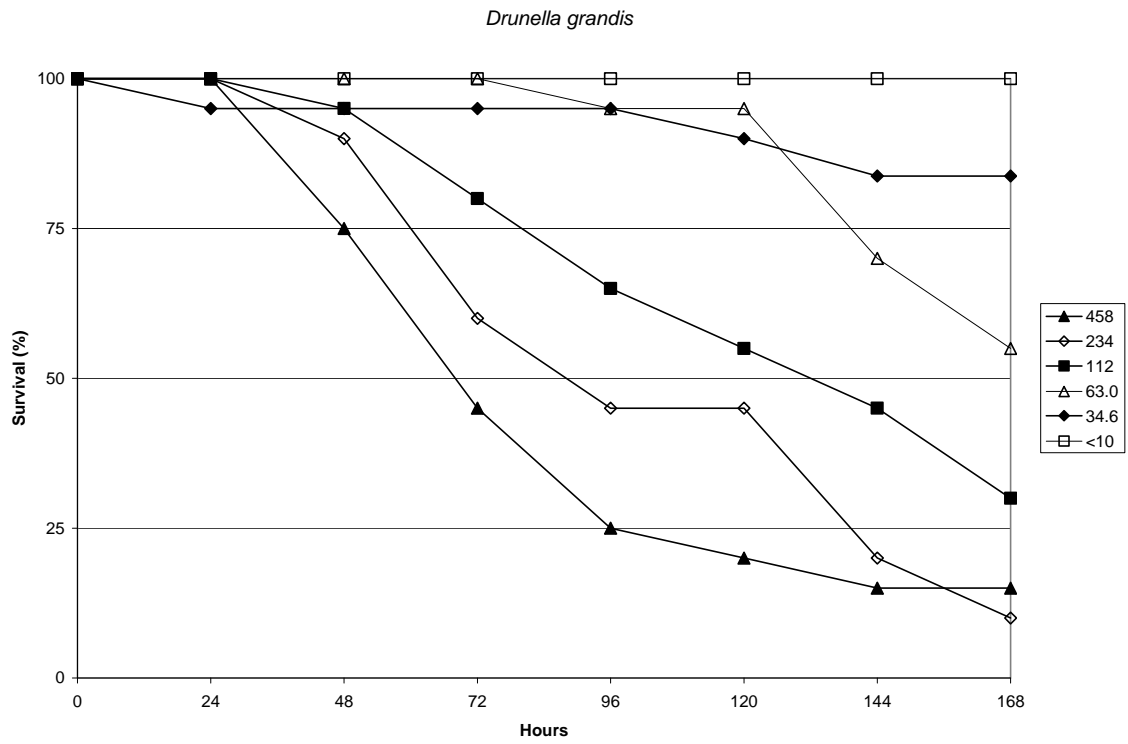


Figure 1. *Drunella grandis* nymph survival over time at different concentrations of copper ($\mu\text{g/L}$).

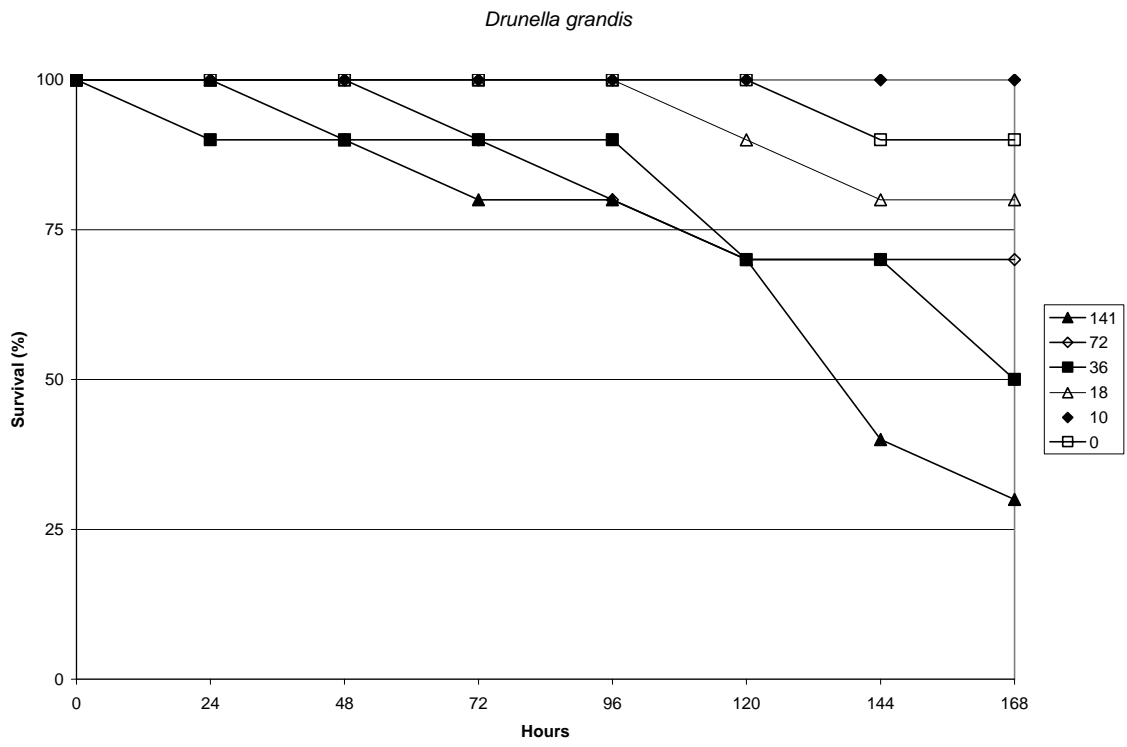


Figure 2. *Drunella grandis* nymph survival over time at different levels of exposure to a copper and zinc mixture expressed in CCUs.

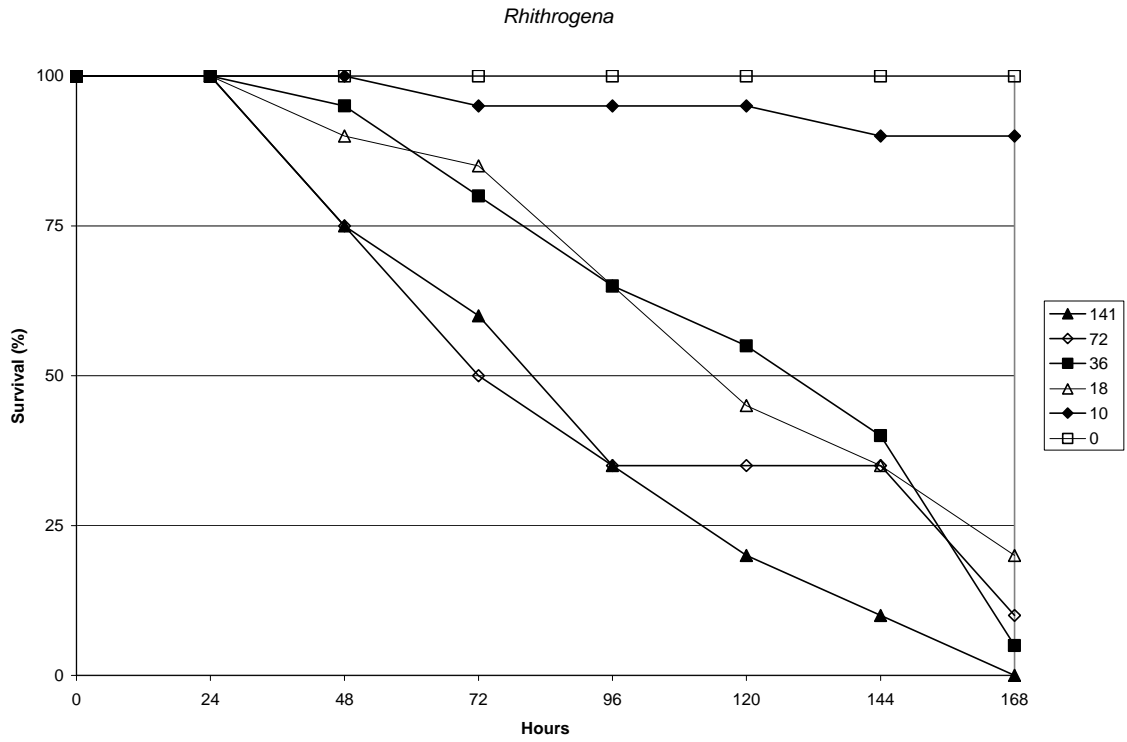


Figure 3. *Rhithrogena* nymph survival over time at different levels of exposure to a copper and zinc mixture (CCU).

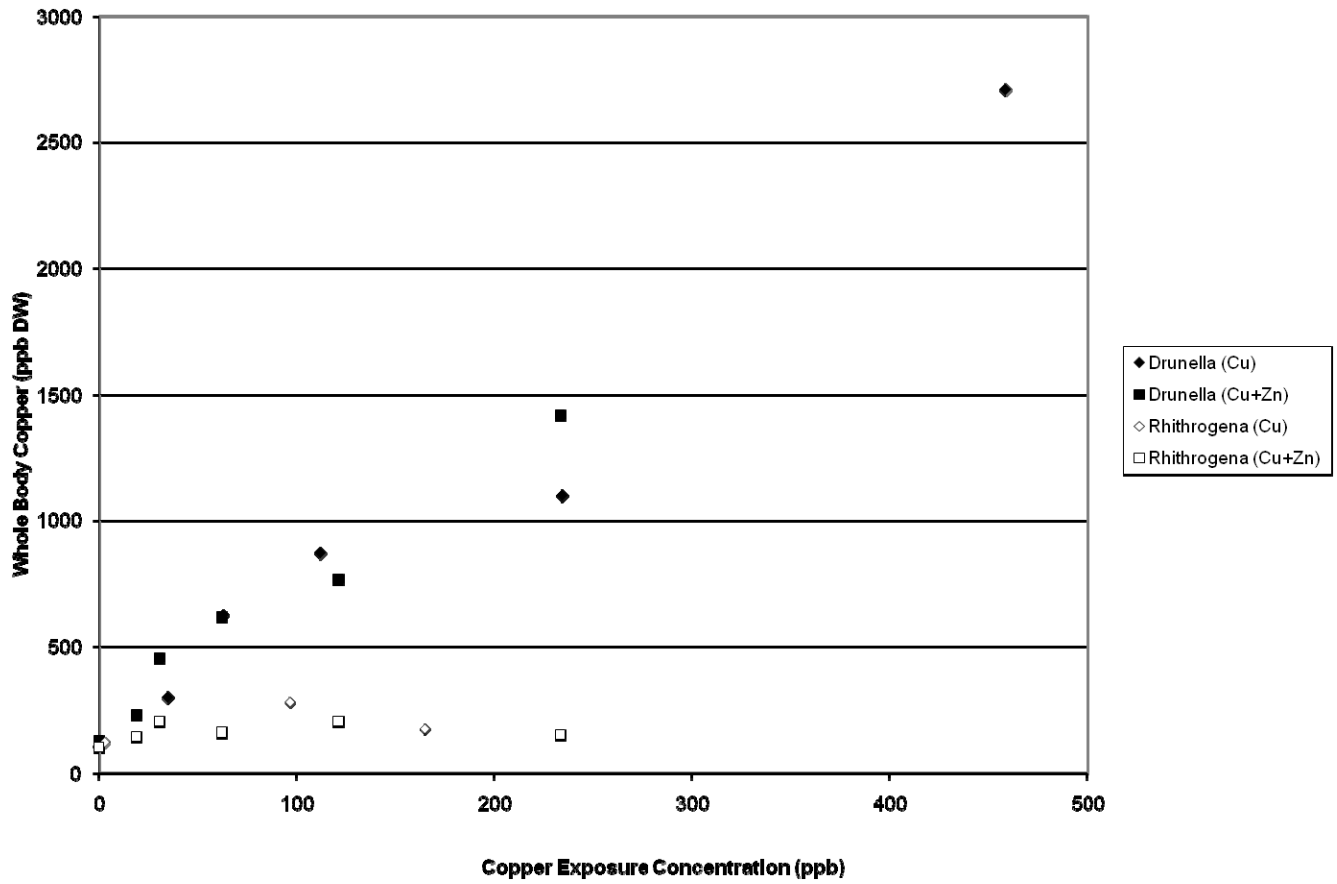


Figure 4. Whole body copper content of *Drunella grandis* and *Rhithrogena* exposed to different concentrations of aqueous copper.

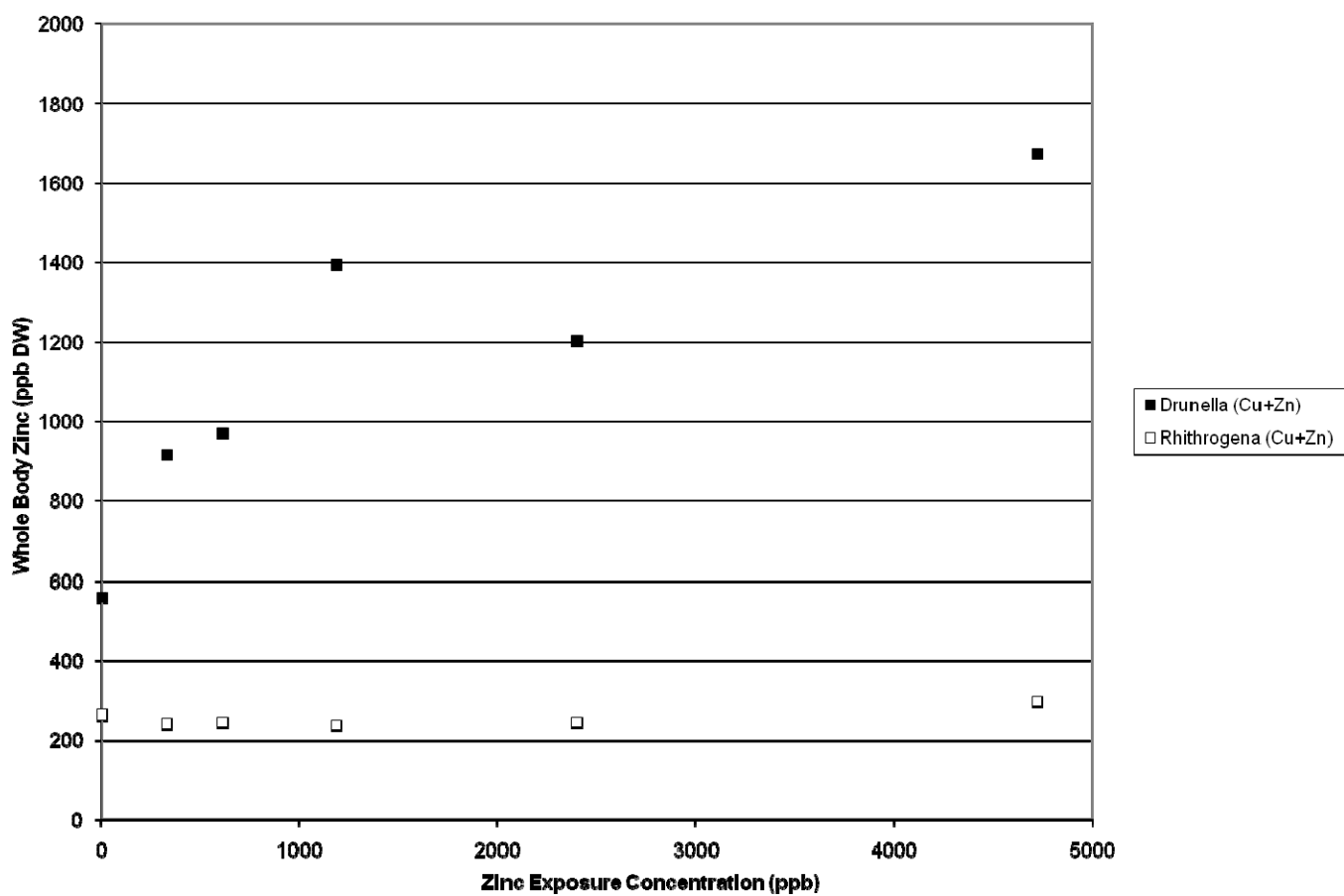


Figure 5. Whole body zinc content of *Drunella grandis* and *Rhithrogena* exposed to different concentrations of aqueous zinc.

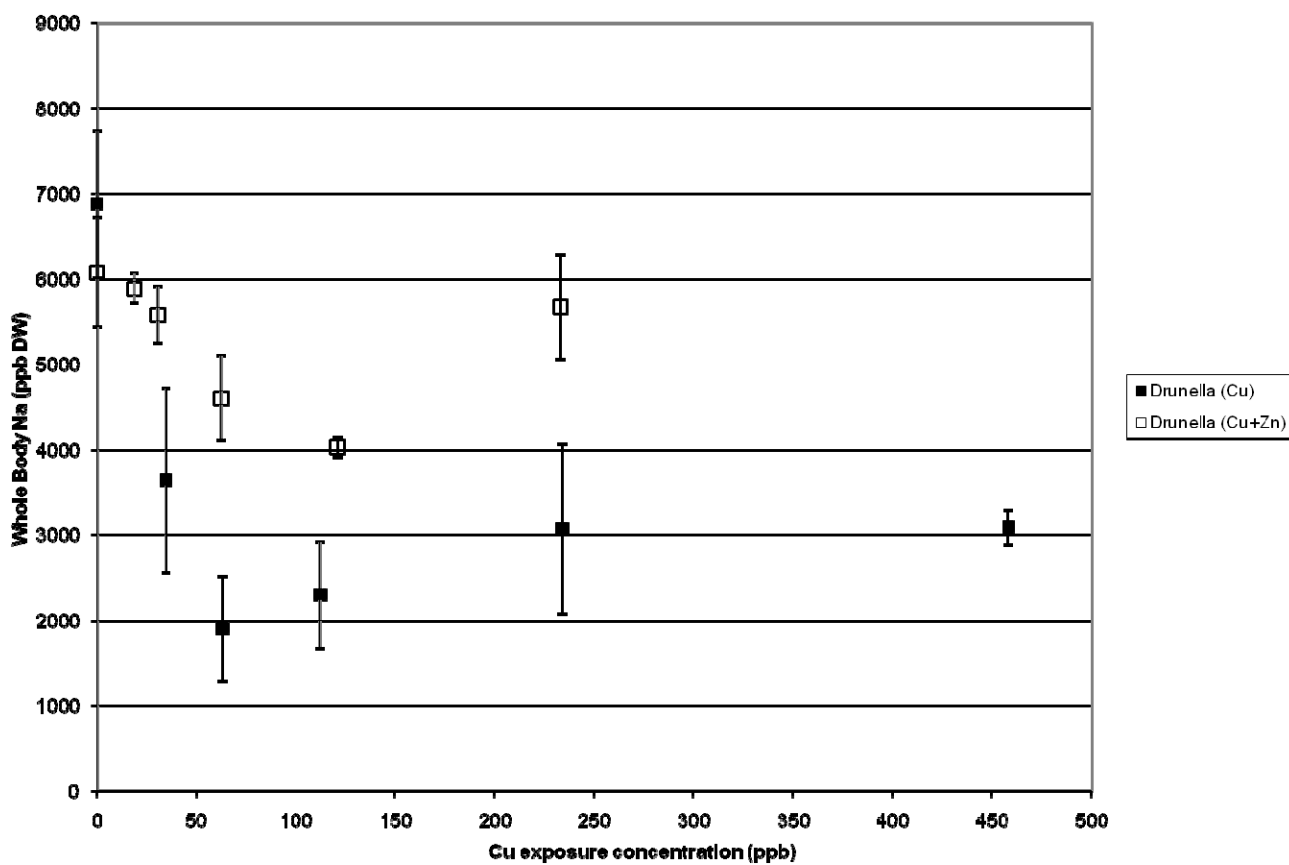


Figure 6. Whole body sodium content of surviving *Drunella grandis* exposed to aqueous copper for 168 hours.

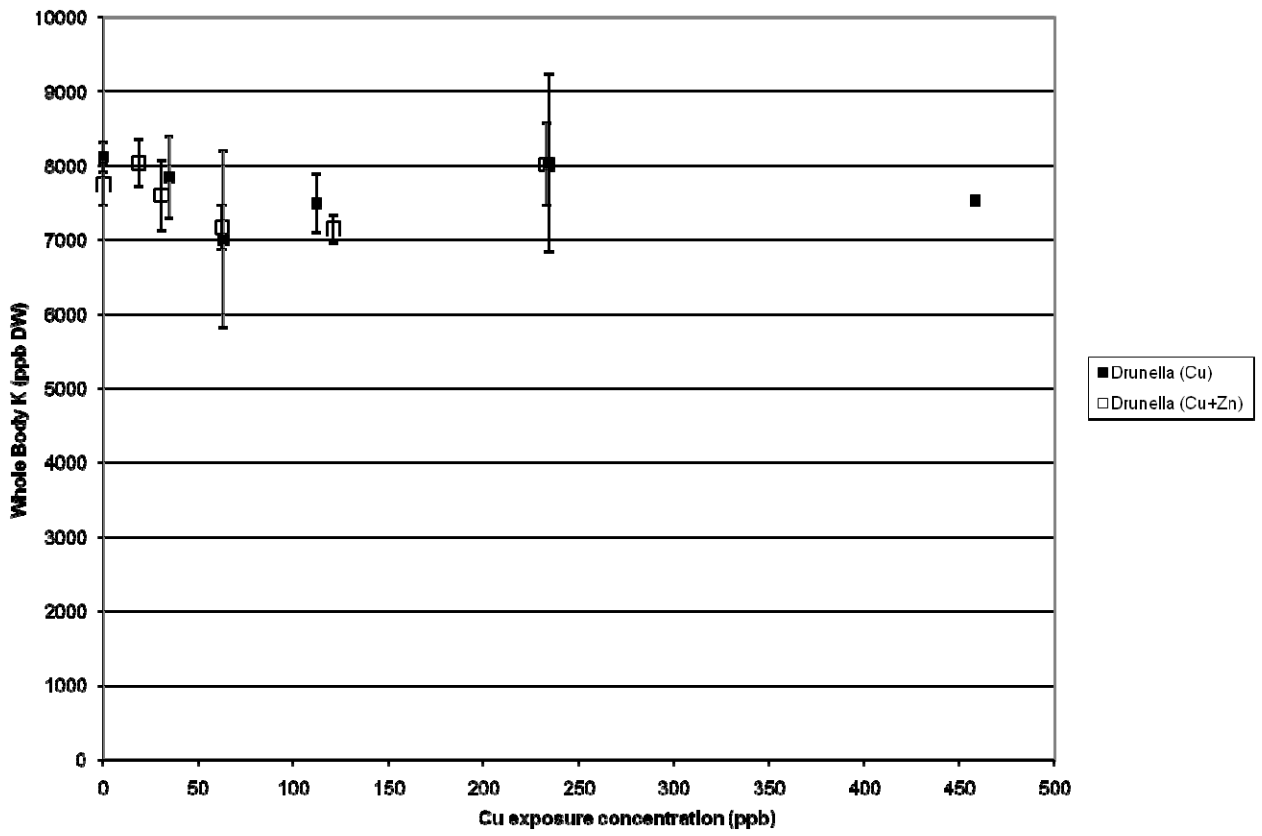


Figure 7. Whole body potassium content of surviving *Drunella grandis* exposed to aqueous copper for 168 hours.

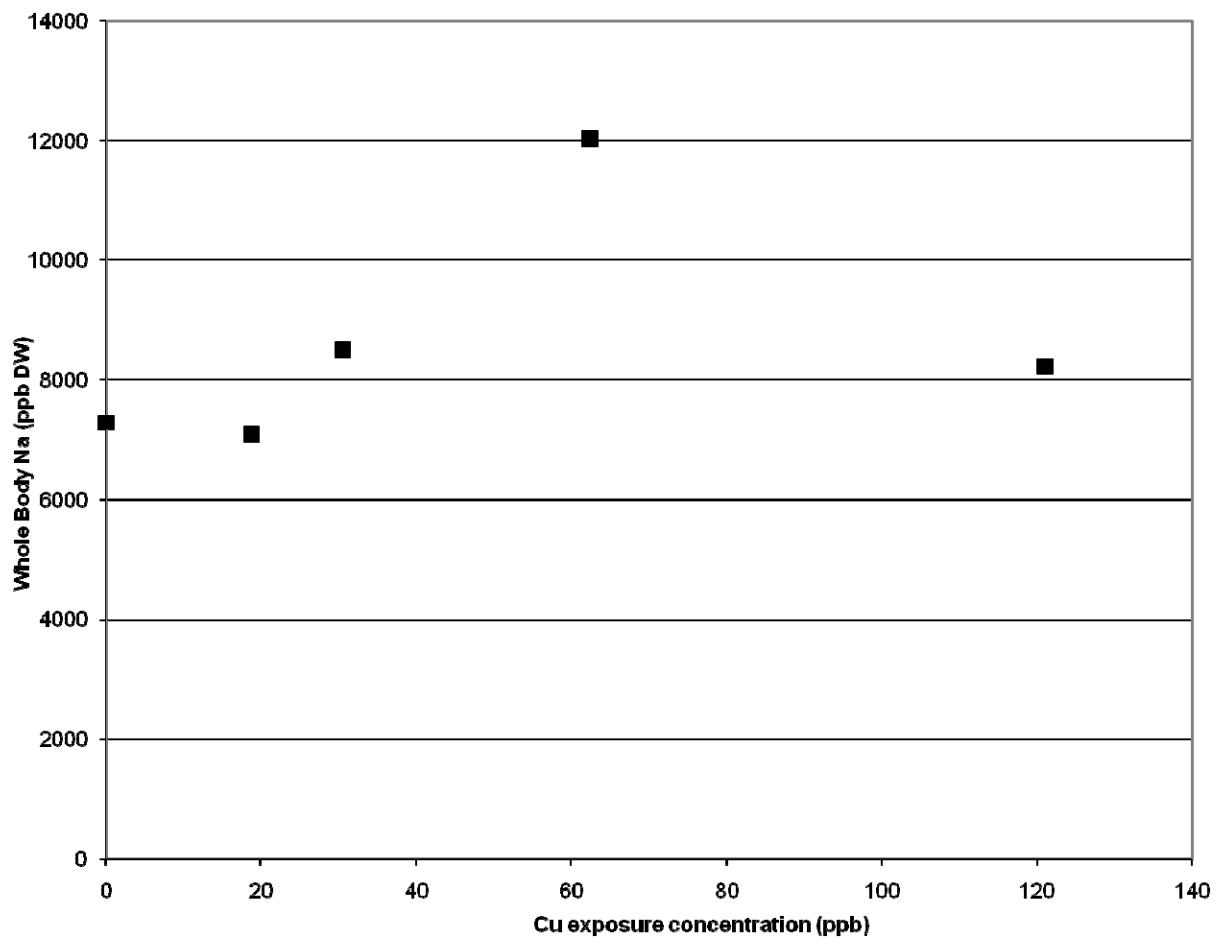


Figure 8. Whole body sodium content of surviving *Rhithrogena sp.* nymphs exposed to aqueous copper for 168 hours.

APPENDIX A.

ABSTRACT OF THESIS: EXPOSURE TO 17 β -ESTRADIOL ALTERS REPRODUCTION OF THE ADULT RED SHINER (*CYPRINELLA LUTRENSIS*)

Endocrine disrupting compounds (EDCs) are prevalent in aquatic ecosystems worldwide and can lead to developmental and reproductive problems in fishes. Concern exists regarding how exposure to EDCs may be contributing to declines in Great Plains fishes in eastern Colorado. We conducted a study using the red shiner as a model organism to examine how estrogenic EDCs might adversely affect Plains fish populations. Male red shiners were exposed to 17 β -estradiol (estradiol), a natural estrogen found in wastewater effluent. Our objectives were to characterize the effects of estradiol exposure on morphometric and behavioral reproductive traits of males, to investigate changes in female mate choice, and to determine whether estradiol exposure reduces fecundity. We also measured reversibility in these reproductive responses when exposures were discontinued. For this purpose, adult males were exposed to nominal concentrations of 120 ngL⁻¹ estradiol, 2.4 ngL⁻¹ estradiol, a solvent control, or a water control for at least one month. Exposures to the highest estradiol concentration resulted in alterations in plasma vitellogenin concentrations, changes in gonadal tissues, and inhibition of mating coloration and tubercles. Furthermore, mating behaviors were altered and reproductive success was reduced; exposed males fertilized fewer eggs and produced no viable progeny. All reproductive endpoints improved when males were removed from the estradiol treatment and allowed to mate in control water. Estradiol had significant adverse effects on adult male red shiners, indicating that wild populations may have low reproductive success if they are chronically exposed to estrogenic compounds in the field.

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