State: Colorado

Study No. F243R-11

Title: Water Pollution Studies

Period Covered: July 1, 2003 to June 30, 2004

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 5ºC) water temperatures.

Accomplishments

Job A.1.

A method was tested to measure vitellogenin (Vtg), an estrogen-mediated protein. The method uses an enzyme linked immunosorbant assay kit (ELISA), and is capable of Vtg determination in whole body homogenates. Juvenile fathead minnows are currently being exposed to water collected from Fossil Creek which receives effluent from the Drake St. Waste Water Treatment Plant. Well water and reverse osmosis water, mixed to approximate the conductivity of Fossil Creek is the negative control. This mixture is also spiked with 25 ng estradiol/L which serves as a positive control. Fish were subsampled after one and two weeks of exposure. Post mitochondrial supernatants of whole body homogenates are currently stored at -70ºC for later analysis of Vtg. Additional fish will be subsampled after four and eight weeks of exposure. Surviving fish will be examined for abnormalities and gender ratio determined.

Job A.2.

Adult mottled sculpin are currently being maintained at the Colorado Division of Wildife Research Hatchery and the Native Aquatic Restoration Facility where attempts to induce spawning are ongoing. A manuscript on the toxicity of zinc to mottled sculpin was submitted to Environmental Toxicology and Chemistry.

Job A.3.

No activities during this segment.

Job A.3.

No activities during this segment.

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 B.1.

A colorimetric test was modified to determine low levels of rotenone in water samples. The method involves preconcentration using a C18 solid phase extraction cartridge followed by reaction with thymol reagent. The absorbance of the resulting colored solution is measured at 580 nm and compared to rotenone standards to determine concentration. Additional work will be conducted to establish optimum preconcentration parameters and reaction conditions. Performance of the method in waters with extreme quality characteristics (e.g. high dissolved solids, high organic matter) will also be determined.

Job B.2.

A considerable amount of time and effort was directed towards conducting tests to develop data on the toxicity of metals to brown and cutthroat trout. There are little data on the effects of metals to these species. Information on the toxicity of zinc and cadmium to trout are important to ensure water quality standards protect these species. Furthermore, this information can help set clean up goals in areas where aquatic species

have been adversely affected by historic mining activities. Recent work conducted by this project studied the effect of hardness on the toxicity to different life stages of brown trout (Davies et al. 2003). This line of research was extended to study effects of zinc on cutthroat trout and effects of cadmium of brown trout. The results of these studies are reported below. A study was also initiated to investigate acclimation of brown trout to a mixture of zinc and cadmium. The status of that study is also presented below.

Effect of Hardness on Cadmium Toxicity to Brown Trout (*Salmo trutta*) Embryos, Larvae and Fry.

ABSTRACT

Acute and chronic toxicity of cadmium to different life stages of brown trout were tested in 30, 75 and 150 mg/L water hardness. Increasing water hardness decreased cadmium toxicity. Cadmium was found to be very toxic to post swim-up brown trout fry but eggs and larvae were tolerant. Reduced survival or biomass at test termination was the most sensitive endpoint. Growth of swim-up fry was negatively affected by cadmium but at concentrations that were greater than those that reduced survival. Cadmium did not affect growth of brown trout early life stages (ELS). Median lethal concentrations (LC_{50}) after 96 hours for swim-up fry exposed to cadmium in 30, 75 and 150 mg/L water hardness were 1.23, 3.90 and 10.1 µg/L, respectively. Chronic values from the ELS tests were 3.52, 6.36 and 13.6 µg/L at 30, 75 and 150 water hardness, respectively. Chronic values from 30 day exposures conducted starting with swim-up fry were 1.02, 1.83 and 6.546 µg/L at 30, 75 and 150 water hardness, respectively. The large difference of chronic values between the ELS and swim-up fry is attributed to acclimation during egg and larval stages.

INTRODUCTION

An estimated 2080 km of streams in Colorado are impacted by metals (Water Quality Control Division 1988). Brown trout are an important component of Colorado ecosystems in many headwater streams, but their numbers are often reduced due to metal contamination in streams (Davies and Woodling 1980). Cadmium is a common metal contaminant associated with mining areas in Colorado. Data on the toxicity of cadmium to brown trout are extremely limited but indicate that brown trout are the most sensitive aquatic species to acute cadmium toxicity (USEPA 2001). The chronic value from a life cycle test with brown trout at a water hardness of 250 was 16.49 (Brown et al. 1994). An early life cycle test at a water hardness of 44 resulted in a chronic value of 6.67 for early eyed eggs (Eaton et al.1978). Acute toxicity of cadmium has been evaluated within a narrow range of water hardness. Median lethal concentrations (LC_{50}) after 96 hours are 1.4, 2.39 and 1.87 µg/L in water hardnesses of 43.5, 37.6 and 36.9 mg $CaCO_3$/L, respectively (Spehar 1984, Davies and Brinkman 1994). Curiously, hardness-adjusted LC_{50} values are much lower than chronic values derived from life cycle and early life stage tests. This phenomenon can arise from life cycle or early life stage tests where initial exposure occurs during a tolerant life stage. Acclimation while in the tolerant life stage results in reduced toxicity during a subsequent sensitive life stage (Sinley et al.

1974, Spehar 1976, Davies et al. 2002, Davies et al. 2003). Acute toxicity tests are usually conducted with unacclimated organisms during the most sensitive life stage.

The first objective of this study was to evaluate the acute and chronic toxicity of cadmium over an extended range of water hardness. Another objective was to compare cadmium toxicity between the embryo-larval life stage and post swim-up fry. These objectives were tested by conducting toxicity tests using both life stages at water hardnesses of 30, 75 and 150 mg/L.

MATERIAL AND METHODS

Organisms

Brown trout embryos were obtained as newly eyed eggs from the Colorado Division of Wildlife Research Hatchery in Bellevue Colorado. The source of the eggs was a Colorado Division of Wildlife spawning operation using feral brown trout in the North Delaney Butte Reservoir in Northern Colorado. Ten eggs were placed into each exposure chamber for the ELS tests. Additional eggs were set aside and maintained in glass aquaria for use in the fry toxicity tests. Eggs began hatching about 14 days after initiation of exposure. Brown trout embryos remained as sac fry for approximately 27 days before reaching swim-up stage. The ELS test exposure continued for an additional 14 days post swim-up when a water line break forced the tests to be terminated. Upon absorption of the yolk sac, fry were fed appropriately sized trout food (Silver Cup) four times daily (twice daily on weekends and holidays) at an estimated rate of 3% body weight /day. Trout food was supplemented with a concentrated suspension of <24 hr old brine shrimp naupalii (San Francisco brand).

The fry toxicity tests used 34 days post swim-up fry. Fry were not fed during the initial 96 hours of exposure, but subsequently were fed twice daily (once on weekends and holidays) at an estimated rate of 3% body weight/day. The fry toxicity tests lasted for 30 days.

Exposure Apparatus

Water from an on site well was diluted with either dechlorinated Fort Collins municipal tap water or reverse osmosis water to obtain nominal hardnesses of 30, 75 and 150 mg $CaCO_3$/L. Consistency of water hardness was maintained by using conductivity controllers (Eutech Instruments). Each water type supplied identical modified continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. The diluters 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/minute each. Exposure chambers consisted of polyethylene containers with a capacity of 2.8 liters. Test solutions overflowed from exposure chambers into water baths which were maintained at 12EC using temperature-controlled recirculators (VWR Scientific Products). Chemical stock solutions were prepared by dissolving a calculated amount of reagent grade Cadmium sulfate ($CdSO_4$) (Mallinkrodt) in deionized water. The chemical stock solutions were delivered to the diluters via peristaltic pumps (Cole-Palmer model C/L) at a rate of approximately 2.0 mls/minute. New stock solutions were prepared as needed during the

toxicity tests. Dim fluorescent lighting provided a 12 hour day/night photoperiod. Diluters and toxicant flow rates were monitored daily to ensure proper operation. Loading during the ELS was less than 0.63 g/L of tank volume and less than 0.01 g/L of flow per 24 hrs. During the fry tests, loading never exceeded 2.2 g/L of tank volume and less than 0.11 g/L of flow per 24 hrs. Loading was well below suggested maximum rates (ASTM 1993).

ELS Test Methods

The number of hatched eggs and mortality of eggs and fry were monitored and recorded daily. Dead fry were blotted dry with a paper towel and total length (to the nearest mm) and weight (to the nearest 0.001 g) measured and recorded. At the end of the tests, surviving fish from each exposure chamber were terminally anesthetized, blotted dry with a paper towel and total lengths and weights measured and recorded.

Water quality characteristics of exposure water were measured weekly in all treatment levels within a replicate. Replicates were alternated each week. Hardness and alkalinity were determined according to Standard Methods (APHA 1985). A Thermo Orion 635 meter measured pH and conductivity. The meter was calibrated with 4.00 and 7.00 pH buffers and a conductivity standard prior to each use. Dissolved oxygen was measured using an Orion 1230 dissolved oxygen meter.

Water samples for cadmium analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 µm filter (Acrodisc), collected in disposable polystyrene tubes (Falcon), and immediately preserved with Ultrex7 triple distilled nitric acid (JT Baker) to pH <2. Water samples were analyzed using a SH4000 atomic absorption spectrometer with CTF 188 graphite furnace (Thermo Jarrell Ash) and using Smith-Hieftje background correction. Dibasic ammonium phosphate (0.1%) was used as a matrix modifier. 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 and spikes were collected and analyzed to verify analytical reproducibility and recovery. The cadmium detection limit was < 0.08 µg/L.

Fry Test Methods

Brown trout fry experiments utilized the same exposure apparatus as the ELS tests. Test methods were identical with the following exceptions. During the initial 96 hours of exposure, water quality characteristics were determined daily and cadmium analyzed three times. Fry were not fed during the initial 96 hours of exposure but were fed twice daily thereafter (once on weekends and holidays). Cadmium exposure lasted for a total of 30 days.

Statistical Analyses

Statistical analyses 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, fry and swim-up survival, biomass at the end of the test, mean time to hatch, and lengths and weights of surviving fish at test termination. 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 test, respectively (Weber et al., 1989). Treatment means were compared to the control using William's one-tailed test (Williams 1971, Williams 1972) or Dunnett's one-tailed test (Dunnett 1955, Dunnett 1964), both at $p<0.05$. For data that failed assumptions of normality or homogeneity of variance, Steel's Many-One Rank Test was used to compare treatment means (Weber et al. 1989). The highest cadmium 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 (IC_{20}), 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 (LC_{50}) were estimated by the Trimmed Spearman-Karber technique (Hamilton et al. 1977, 1978) using log transformed cadmium concentrations and 10% trim.

RESULTS

Hardness of the 30, 75 and 150 hardness ELS test waters were near target levels (Table 1). Standard deviations were generally low indicating that the water quality characteristics were consistent over the course of the experiments. Temperatures were near or slightly below the 12ºC target. Dissolved oxygen was near saturation.

Table 1. Mean of water quality characteristics of exposure water during ELS toxicity tests. Standard deviations are in parentheses.

Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	pH (S.U.)	Temperature (°C)	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)
30 Hardness					
30.6 (2.1)	22.9 (1.3)	7.72 (0.12)	11.6 (0.4)	52.9 (2.0)	8.49(0.58)
75 Hardness					
71.3 (2.7)	51.5 (1.6)	7.75 (0.14)	12.0 (0.3)	123.1 (4.8)	8.61(0.67)
150 Hardness					
149.2 (7.0)	106.9 (4.7)	7.83 (0.14)	11.8 (0.4)	255.2 (7.8)	8.32(0.64)

30 Hardness ELS

Mean time to hatch, hatching success and sac fry survival were not significantly affected by exposure concentrations used (Table 2). Hatching success was about 80% in all treatments. Little mortality occurred during the sac fry stage. Metal-related mortality occurred during the swim-up stage, after fry began exogenous feeding. Mortality

occurred at 2.54 and 4.87 µg/L, but only the higher exposure concentration was significantly different than the control at the p=0.05 level. Based on mortality, the no observed effect concentration (NOEC) was 2.54 µg/L and the lowest observed effect concentration was 4.87 µg/L. The chronic value for the 30 hardness ELS test was 3.52 µg/L. Cadmium exposure at the highest concentration tested appeared to have reduced growth, as measured by termination lengths and weights, however, this was not significant at the p<0.05 level (Table 3). Mean biomass at termination of the 30 hardness ELS test was significantly reduced at 4.87 (LOEC) but not at 2.54 µg/L (NOEC). The chronic value for the 30 hardness test based on biomass was 3.52 µg/L. The IC_{20} based on biomass at test termination was 2.22 µg/L. A summary of endpoints for all tests is presented in Table 15.

Table 2. Mean dissolved cadmium concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swim-up fry survival (%) of ELS brown trout exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.40 (0.04)	0.69 (0.05)	1.31 (0.08)	2.54 (0.22)	4.87 (0.56)
Time to Hatch (hrs)	361 (23)	374 (10)	382 (16)	387 (9)	381 (2)	380 (6)
Hatching Success (%)	82.5 (5.0)	82.5 (12.6)	72.5 (5.0)	70.0 (14.1)	87.5 (15.0)	70.0 (14.1)
Sac Fry Survival (%)	82.5 (5.0)	82.5 (12.6)	67.5 (9.6)	70.0 (14.1)	82.5 (9.6)	62.5 (9.6)
Swim-up Fry Survival (%)	80.0 (0.0)	82.5 (12.6)	67.5 (9.6)	67.5 (15.0)	65.0 (12.9)	15.0* (17.3)

*Significantly less than control (p<0.05)

Table 3. Mean dissolved cadmium concentrations (µg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout surviving 30 hardness test. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.40 (0.04)	0.69 (0.05)	1.31 (0.08)	2.54 (0.22)	4.87 (0.56)
Length (mm)	28.0 (0.7)	28.1 (0.6)	27.3 (1.5)	27.4 (0.6)	28.0 (0.3)	26.3 (1.0)
Weight (g)	0.170 (0.008)	0.167 (0.003)	0.163 (0.008)	0.167 (0.012)	0.168 (0.008)	0.148 (0.001)
Biomass (g)	1.360 (0.067)	1.378 (0.217)	1.101 (0.159)	1.111 (0.181)	1.087 (0.190)	0.222* (0.256)

*Significantly less than control (p<0.05)

IC_{20} (95% Confidence Interval) = 2.22 µg/L (0.61-2.75)

75 Hardness ELS

Cadmium concentrations were approximately a factor of two greater than the 30 Hardness test. Mean time to hatch, hatching success and sac fry survival were unaffected by cadmium exposure (Table 4). Significant mortality occurred following the sac fry stage in the highest cadmium concentration (8.64 µg/L) which served as the LOEC. The NOEC was 4.68 µg/L for a chronic value of 6.36 µg/L for ELS brown trout. Growth was unaffected but biomass was significantly reduced at the highest exposure concentration (Table 5) to provide the same LOEC and NOEC and chronic values as those based on reduced survival. The IC_{20} based on biomass at test termination was 4.71 µg/L.

Table 4. Mean dissolved cadmium concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swim-up fry survival (%) of ELS brown trout exposed in 75 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.60 (0.05)	1.13 (0.09)	2.46 (0.28)	4.68 (0.17)	8.64 (0.98)
Time to Hatch (hrs)	336 (6)	352 (10)	348 (11)	344 (11)	338 (16)	336 (5)
Hatching Success (%)	85.0 (10.0)	85.0 (5.8)	77.5 (12.6)	85.0 (12.9)	87.5 (15.0)	85.0 (10.0)
Sac Fry Survival (%)	82.5 (12.6)	80.0 (14.1)	72.5 (17.1)	80.0 (8.2)	82.5 (12.6)	85.0 (10.0)
Swim-up Fry Survival (%)	82.5 (12.6)	77.5 (18.9)	67.5 (12.6)	77.5 (9.6)	72.5 (20.6)	12.5* (12.6)

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

Table 5. Mean dissolved cadmium concentrations (µg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout surviving 75 hardness test. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.03)	0.60 (0.05)	1.13 (0.09)	2.46 (0.28)	4.68 (0.17)	8.64 (0.98)
Length (mm)	28.7 (1.1)	28.6 (0.5)	28.6 (0.7)	28.8 (1.1)	27.8 (0.7)	28.7 (0.6)
Weight (g)	0.183 (0.023)	0.172 (0.012)	0.177 (0.012)	0.183 (0.019)	0.167 (0.012)	0.189 (0.007)
Biomass (g)	1.499	1.349	1.190	1.403	1.207	0.233*

	(0.192)	(0.371)	(0.187)	(0.083)	(0.375)	(0.230)

*Significantly less than control ($p<0.05$)
IC_{20} (95% Confidence Interval) = 4.71 µg/L (0.95-5.46)

150 Hardness ELS

Cadmium exposure concentrations used in the 150 hardness ELS test were approximately double those of the 75 hardness and four times that of the 30 hardness test. As found in the 30 and 75 hardness tests, mean time to hatch, hatching success and sac fry mortality were not affected by cadmium (Table 6). Swim-up fry survival was significantly reduced at 19.1 but not 9.62 µg Cd/L (LOEC and NOEC, respectively). The chronic value was 13.56 µg Cd/L. Effects of cadmium on growth were not detected (Table 7). The LOEC, NOEC and chronic value based on a reduction in biomass were the same as those based on swim-up fry survival. The IC_{20} based on biomass at test termination was 13.6 µg/L.

Table 6. Mean dissolved cadmium concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swim-up fry survival (%) of ELS brown trout exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.08)	1.30 (0.14)	2.95 (0.32)	5.47 (0.40)	9.62 (0.79)	19.1 (2.3)
Time to Hatch (hrs)	357 (7)	362 (10)	361 (7)	353 (14)	359 (14)	356 (8)
Hatching Success (%)	85.0 (12.9)	95.0 (5.8)	85.0 (5.8)	90.0 (8.2)	92.5 (5.0)	85.0 (10.0)
Sac Fry Survival (%)	80.0 (11.5)	90.0 (8.2)	80.0 (8.2)	85.0 (5.8)	92.5 (5.0)	77.5 (18.9)
Swim-up Fry Survival (%)	80.0 (11.5)	90.0 (8.2)	80.0 (8.2)	85.0 (5.8)	90.0 (8.2)	57.5* (17.1)

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

Table 7. Mean dissolved cadmium concentrations (µg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of ELS brown trout surviving 150 hardness test. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.1 (0.08)	1.30 (0.14)	2.95 (0.32)	5.47 (0.40)	9.62 (0.79)	19.1 (2.3)
Length (mm)	28.2 (0.2)	27.7 (0.9)	27.4 (0.5)	27.7 (0.6)	27.1 (0.5)	27.4 (0.2)

Weight (g)	0.168 (0.008)	0.168 (0.005)	0.161 (0.011)	0.165 (0.011)	0.158 (0.008)	0.154 (0.007)
Biomass (g)	1.35 (0.26)	1.52 (0.16)	1.20 (0.08)	1.40 (0.10)	1.42 (0.18)	0.88* (0.25)

*Significantly less than control ($p<0.05$)
IC_{20} (95% Confidence Interval) = 13.6 µg/L (10.8-17.3)

Measured water hardnesses of the brown trout fry tests were near target levels except for the 75 hardness test which was only 67.6 ppm (Table 8). Temperatures were within 1ºC of the 12 ºC target value and dissolved oxygen was near saturation. Standard deviations indicate water quality conditions were constant through the duration of the fry tests. In general, water quality characteristics are very similar between the ELS and fry tests (Tables 1 and 8).

30 Hardness FRY

There was complete survival in the control and lowest exposure concentration after acute exposures of 96 hours (Table 9). Mortality increased with increasing cadmium concentration resulting in complete mortality at 5.64 µg/L, the highest concentration. The 96 hour median lethal concentration (LC_{50}) for the 30 hardness test was 1.23 µg/L. After the initial 96 hours, low levels of mortality occurred in the 0.42 and 0.74 µg/L concentrations. The dose-response relationship indicate that the mortality that occurred in these lower concentrations are likely metal-related. However, interpretation of statistical results in not unambiguous. Growth, measured by length and weight at test termination was decreased in the single fish surviving at 2.72 µg/L (Table 10). Biomass was significantly reduced at 1.40 (LOEC) but not 0.74 µg/L (NOEC). The chronic value was 1.02 µg/L based on biomass. The IC_{20} based on biomass at test termination was 0.87 µg/L.

Table 8. Mean of water quality characteristics of exposure water during fry toxicity tests. Standard deviations are in parentheses.

Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	pH (S.U.)	Temperature (EC)	Conductivity (µS/cm)	Dissolved Oxygen (mg/L)
30 Hardness					
29.2 (0.9)	21.7 (0.8)	7.54 (0.13)	11.7 (0.1)	51.5 (0.5)	8.61 (0.22)
75 Hardness					
67.6 (1.5)	47.9 (1.1)	7.60 (0.10)	11.4 (0.2)	115.1 (2.1)	8.88 (0.17)
150 Hardness					
151.4 (2.2)	107.1 (1.8)	7.51 (0.12)	11.8 (0.4)	259.9 (2.0)	8.58 (0.14)

Table 9. Mean dissolved cadmium concentrations (µg/L) and associated acute and 30 day survival (%) of brown trout fry exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.04)	0.42 (0.05)	0.74 (0.08)	1.40 (0.14)	2.72 (0.23)	5.64 (0.12)
96 hr Survival (%)	100 (0)	100 (0)	97.5 (5.0)	32.5 (15.0)	2.5 (5.0)	0 (0)
30 day Survival (%)	100 (0)	90.0 (8.2)	87.5 (9.6)	32.5* (15.0)	2.5* (5.0)	0* (0)

96 hour LC_{50} (95% C.I.) = 1.23 µg Cd/L (1.09-1.38)
*Significantly less than control ($p<0.05$)

Table 10. Mean dissolved cadmium concentrations (µg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of brown trout fry surviving 30 hardness test. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.04)	0.42 (0.05)	0.74 (0.08)	1.40 (0.14)	2.72 (0.23)	5.64 (0.12)
Length (mm)	39.1 (0.9)	39.8 (0.8)	40.1 (1.0)	40.4 (1.7)	34* --[1]	--
Weight (g)	0.584 (0.019)	0.611 (0.031)	0.612 (0.043)	0.637 (0.088)	0.320* --[1]	--
Biomass (g)	5.84 (0.19)	5.51 (0.57)	5.32 (0.25)	2.08* (0.98)	0.08* (0.16)	0.00* (0.00)

*Significantly less than control ($p<0.05$)
[1]Single surviving fish
IC_{20} (95% Confidence Interval) = 0.87 µg/L (0.82-0.93)

75 Hardness FRY

Exposure to 8.86 µg/L for 96 hours resulted in near complete mortality (Table 11). During the initial 96 hours, there was no mortality of fry exposed to cadmium concentrations 1.30 µg/L, although some mortality occured by 30 days. The 96 hour LC50 was 3.90 µg/L. The LOEC was 2.58 µg/L which resulted in 30% mortality in 30 days, though most of the mortality happened during the first 96 hours. The NOEC based on mortality was 1.30 µg/L for a chronic value of 1.83 µg/L. Weights and lengths at test termination were significantly reduced at 4.49 and 8.86 µg/L, respectively (Table 12). Reduction of growth, while detected in the 75 hardness test, was not as sensitive an endpoint as mortality or biomass. The LOEC and NOEC based on biomass was the same

as those based on mortality for a chronic value of 1.83 µg/L. The IC_{20} based on biomass at test termination was 2.18 µg/L.

Table 11. Mean dissolved cadmium concentrations (µg/L) and associated acute and 75 day survival (%) of brown trout fry exposed in 75 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.04)	0.69 (0.09)	1.30 (0.16)	2.58 (0.24)	4.49 (0.32)	8.86 (0.75)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	80.0 (14.1)	35.0 (12.9)	2.5 (5.0)
30 day Survival (%)	92.5 (9.6)	95.0 (5.8)	97.5 (5.0)	70.0* (11.5)	35.0* (12.9)	2.5* (5.0)

96 hour LC_{50} (95% C.I.) = 3.90 µg Cd/L (3.39-4.48)
*Significantly less than control ($p<0.05$)

Table 12. Mean dissolved cadmium concentrations (Φg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of brown trout fry surviving 75 hardness test. Standard deviations are in parentheses.

Dissolved Cd (Φg/L)	<0.08 (0.04)	0.69 (0.09)	1.30 (0.16)	2.58 (0.24)	4.49 (0.32)	8.86 (0.75)
Length (mm)	41.0 (1.2)	40.2 (0.8)	40.0 (0.4)	40.6 (0.5)	38.9 (1.3)	38* --[1]
Weight (g)	0.654 (0.066)	0.614 (0.034)	0.602 (0.010)	0.610 (0.023)	0.544* (0.046)	0.490* --[1]
Biomass (g)	6.01 (0.39)	5.82 (0.14)	5.87 (0.26)	4.27* (0.71)	1.94* (0.86)	0.12* (0.24)

*Significantly less than control ($p<0.05$)
[1]Single surviving fish
IC_{20} (95% Confidence Interval) = 2.18 µg/L (1.85-2.70)

150 Hardness FRY

All trout exposed for 96 hours to cadmium concentrations as high as 4.81 µg/L survived, whereas fish exposed to 8.88 and 16.4 µg/L had survival rates of 62.5 and 10%, respectively (Table 13). The 96 hour LC50 at 150 hardness was 10.1 µg/L. Mortality after the initial 96 hours was low. After 30 days, survival of trout exposed to 8.88 µg/L was significantly lower than the control (LOEC) but was unaffected at 4.81 µg/L (NOEC). The chronic value based on mortality was 6.54 µg/L. Effects of cadmium exposure on growth was not detected even at the highest concentration which experienced near complete mortality (Table 14). Biomass was significantly reduced at the

highest two exposure concentrations primarily as a result of significant mortality. The LOEC, NOEC and chronic value based on biomass was the same as those based on mortality. The IC_{20} based on biomass at test termination was 6.62 µg/L.

Table 13. Mean dissolved cadmium concentrations (µg/L) and associated acute and 75 day survival (%) of brown trout fry exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.05)	1.01 (0.07)	2.44 (0.18)	4.81 (0.36)	8.88 (0.52)	16.4 (1.5)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	62.5 (5.0)	10.0 (8.2)
30 day Survival (%)	97.5 (5.0)	97.5 (5.0)	97.5 (5.0)	97.5 (5.0)	55.0* (5.8)	7.5* (5.0)

96 hour LC_{50} (95% C.I.) = 10.1 µg Cd/L (8.95-11.4)
*Significantly less than control ($p<0.05$)

Table 14. Mean dissolved cadmium concentrations (µg/L) and associated mean lengths (mm) and weights (g) and biomass (g) of brown trout fry surviving 150 hardness test. Standard deviations are in parentheses.

Dissolved Cd (µg/L)	<0.08 (0.05)	1.01 (0.07)	2.44 (0.18)	4.81 (0.36)	8.88 (0.52)	16.4 (1.5)
Length (mm)	39.6 (0.7)	40.4 (0.7)	39.9 (1.5)	40.2 (0.8)	40.5 (1.3)	40.3 (0.6)
Weight (g)	0.614 (0.012)	0.621 (0.042)	0.607 (0.033)	0.605 (0.026)	0.617 (0.072)	0.596 (0.013)
Biomass (g)	5.99 (0.41)	6.04 (0.16)	5.91 (0.10)	5.89 (0.18)	3.37* (0.30)	0.447* (0.30)

*Significantly less than control ($p<0.05$)
IC_{20} (95% Confidence Interval) = 6.62 µg/L (6.32-6.92)

Table 15. Endpoints and associated chronic values (µg/L) of cadmium toxicity tests conducted with brown trout ELS and fry in 30, 75 and 150 mg/L water hardness.

	30 Hardness		75 Hardness		150 Hardness	
	ELS	Fry	ELS	Fry	ELS	Fry
Time to Hatch	>4.87	--	>8.64	--	>19.1	--
Hatch Success	>4.87	--	>8.64	--	>19.1	--
Sac Fry Survival	>4.87	--	>8.64	--	>19.1	--
Swim-up Fry Survival	3.52	1.02	6.36	1.83	13.6	6.54
Length	>4.87	1.95	>8.64	6.31	>19.1	>16.4
Weight	>4.87	1.95	>8.64	3.40	>19.1	>16.4
Biomass	3.52	1.02	6.36	1.83	13.6	6.54
IC_{20}	2.22	0.87	4.01	2.18	13.6	6.62
LC_{50}	--	1.23	--	3.90	--	10.1

DISCUSSION

A break in the water line leading to the Colorado Division of Wildlife Aquatic Toxicology Laboratory caused the ELS tests to be terminated prematurely. Exposure of the fry was only 41 days post hatch. ELS tests should be conducted for 60 days post hatch (USEPA 1985) or at least 30 days post swim-up (ASTM 1993). Typically, the majority of mortality occurs shortly after swim-up and it is unlikely that significant additional mortality would have taken place. In fact, it is recommended that ELS test results should not be used if mortality occurs near the end of the test (USEPA 1985). However, reduced growth could be expected if the tests were extended resulting in lower IC_{20}s or chronic values based on biomass.

Mean time to hatch was unaffected by cadmium exposure. This result contrasts with zinc which increased time to hatch of brown trout eggs at relatively low concentrations (Davies et al. 2002, Davies et al. 2003). Hatching success and sac fry survival were unaffected by the cadmium concentrations used in the ELS tests. Egg and sac fry life stages of salmonids are generally more tolerant to metal exposure than the subsequent swim-up fry stage (Chapman 1978, Van Leeuwen et al. 1985). Metal-related mortality in the ELS tests occurred after brown trout embryos reached swim-up stage and began exogenous feeding. No effect of cadmium exposure on growth was detected in any of the ELS tests. Reduced growth, observed in the 30 and 75 fry tests, occurred at higher concentrations than reduced survival. Survival through the swim-up stage and biomass at

test termination were equally sensitive at detecting effects of cadmium. Over all, the most sensitive endpoint was the IC_{20}. The inhibitory concentration (IC) is interpolated from a dose-response relationship and provides an estimate of a reduction of biological performance, in this case a reduction of 20% biomass. This approach integrates effects of exposure on both survival and weight at test termination. Chronic values based on NOEC and LOEC are determined using hypothesis testing and can be influenced by selection of exposure concentrations and variability of the data set. Furthermore, chronic values provide almost no information on the magnitude of the effect at the LOEC. For fry but not ELS tests, the IC_{20} and the chronic value based on biomass were in close agreement. In contrast, chronic values from the 30 and 75 hardness ELS tests were considerably greater than the corresponding IC_{20} values. High variability inherent to ELS tests may decrease statistical power to detect reduced survival or biomass.

Chronic endpoints of the ELS tests are consistently greater than the fry and actually exceeded 96 hour LC_{50} values. Low variability of the fry tests relative to ELS tests do not fully explain this finding since the IC_{20} estimates are likewise lower in the fry tests. More likely, exposure of test organisms during cadmium-tolerant egg and larval stages resulted in acclimation. Consequently, exposed organisms were more tolerant to lethal effects during the subsequent sensitive fry stage (Sinley et al. 1974, Spehar 1976, Davies et al. 2003).

Chronic water quality criteria are derived from life-cycle, partial life cycle or ELS tests (USEPA 1985). Chronic criteria may not be protective if based on tests where acclimation occurred. Results from this study found acutely lethal cadmium concentrations to swim-up fry were well below chronic values derived from ELS tests. This finding parallels USEPA's latest cadmium criteria document which reports a Species Mean Acute Value of 1.613 µg/L and a much higher Species Mean Chronic Value of 5.004 µg/L (USEPA 2001). The chronic value was derived from a life cycle test where exposure was initiated with sexually mature adults followed by fertilized eggs, both of which are tolerant life stages where acclimation could occur. Guidelines for deriving water quality criteria require that all life stages of an organism should be protected by criteria. However, chronic criteria derived from tests where acclimation occurred may not protect sensitive life stages. Fortunately, the document recognizes that an acute-chronic ratio (ACR) less than 2 is probably due to acclimation during the chronic test. In such cases, an ACR of 2 is assumed because acclimation and continuous exposure in field situations can not be assured. Based on data from this study, this approach would protect the sensitive brown trout swim-up fry stage.

LC_{50} values from this study and previously reported values (Spehar 1984, Davies and Brinkman 1994) appear consistent and demonstrate decreasing toxicity with increasing hardness (Figure 1). Assuming a log-log relationship between hardness and toxicity (USEPA 1985), the relationship between LC_{50} and hardness has a correlation coefficient of 0.95. Dividing a hardness-adjusted LC_{50} by a factor of 2 should protect brown trout from acute exposures to cadmium and is often called the Criteria Maximum Concentration (CMC) (USEPA 1985). The one hour average concentration should not exceed the CMC. The equation for the brown trout cadmium CMC is

$$\text{Brown Trout Cd CMC} = e^{(1.258*(\ln(\text{hardness}))-4.692)}$$

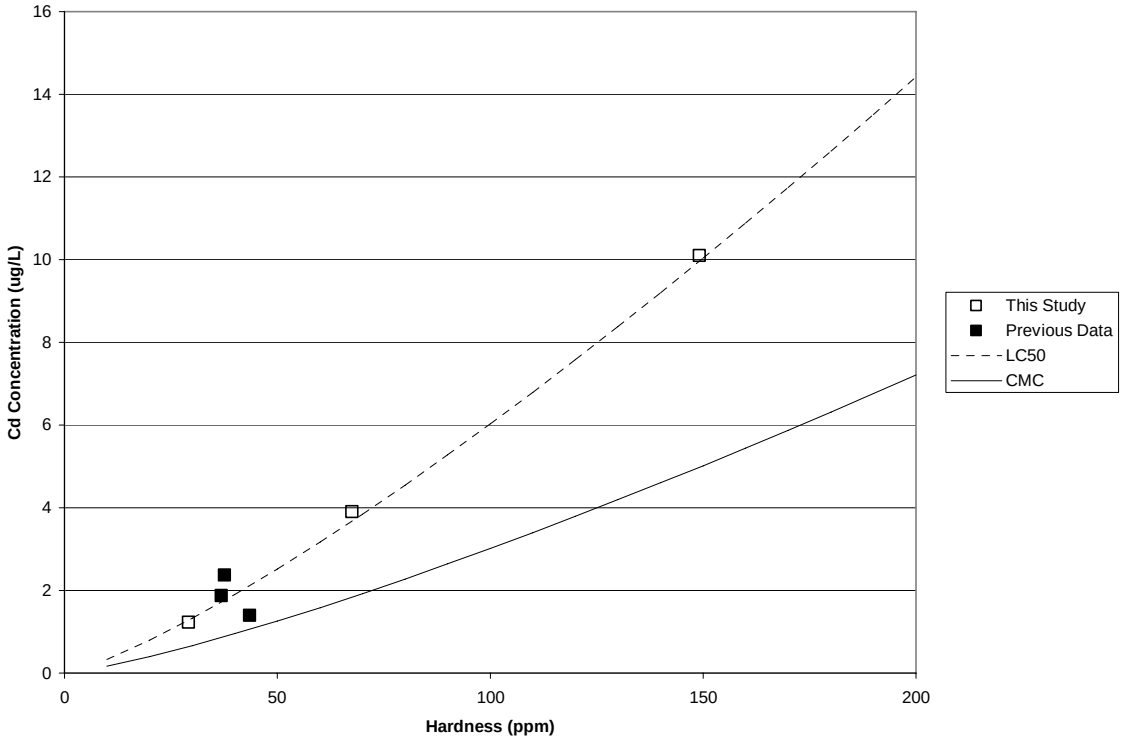

Figure 1. Brown trout LC_{50} values and CMC versus water hardness.

Chronic values from this study and previously reported values (Eaton et al. 1978, Brown et al. 1994) show decreasing chronic toxicity with increasing hardness (Figure 2). The log-log regression of the ELS chronic values including an ELS test at 44 mg/L (Eaton et al. 1978) and life-cycle test at 250 mg/L hardness (Brown et al. 1994) is a reasonable fit with a correlation coefficient of 0.97. The equation describing the regression line for the ELS tests is

$$\text{Brown Trout ELS Chronic Cd} = e^{(0.7033*(\ln(\text{hardness}))-1.017)}$$

Cadmium concentrations predicted from this equation could be expected to protect brown trout in instances where exposure is constant. Brown trout from clean tributaries or upstream of a cadmium source would not be protected if they migrate into or are washed into contaminated reaches. Loss of acclimation has been shown to occur once exposure to metals is removed (Gasser 1998, Davies and Brinkman 1999, Davies et al. 2002). 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 can also occur during spring runoff when dilution from spring snowmelt substantially reduces metal concentrations in streams.

Chronic values from tests conducted with swim-up fry are clearly lower than chronic values from ELS and life-cycle tests (Figure 2). The equation describing the regression for the fry tests (correlation coefficient=0.97) is

$$\text{Brown Trout Fry Chronic Cd} = e^{(1.093*(\ln(\text{hardness}))-3.734)}$$

Cadmium concentrations predicted by this equation can be expected to protect unacclimated brown trout.

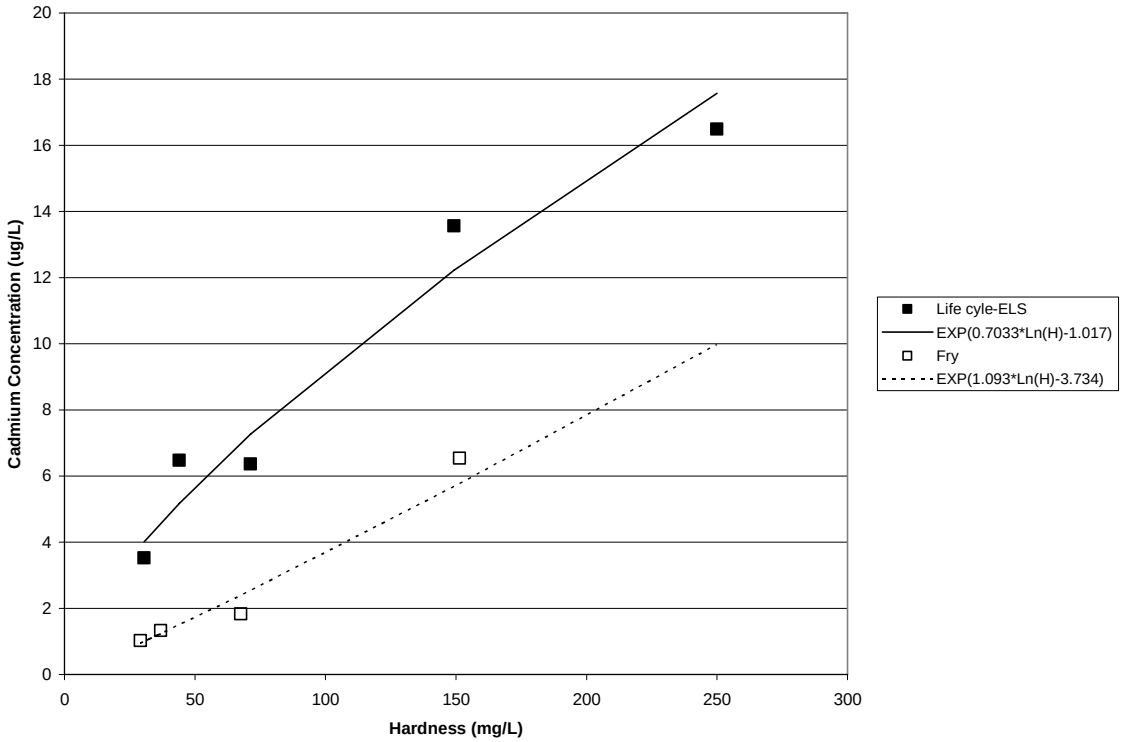

Figure 2. Brown trout chronic values versus water hardness.

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Effect of Hardness on Zinc Toxicity to Colorado River Cutthroat (*Onchorhynchus clarkii pleuriticus)* and Rainbow Trout (*Oncorhynchus mykiss)* Embryos and Fry.

ABSTRACT

This study investigated the acute and chronic toxicity of zinc to cutthroat and rainbow trout. The toxicity to early life stages (ELS) and 42 day post swim up fry were compared. Tests were conducted in low (30 mg/L) and high (150 mg/L) water hardness. Increasing water hardness by a factor of five decreased zinc toxicity by 2.2-10.3 times. Post swim up rainbow trout fry were much more sensitive than ELS. Cutthroat trout were slightly more tolerant to zinc than rainbow trout. Growth and hatching success was generally unaffected by zinc exposure. Fry survival and biomass at test termination were the most sensitive endpoints for the post swim up fry. Time to hatch was increased by zinc exposure and was the most sensitive endpoint for ELS tests. However, the increase was modest and may not represent an adverse effect.

INTRODUCTION

Zinc is a common contaminant in areas impacted by past mining activities. Many areas impacted by zinc in Colorado occur in headwater streams where cutthroat trout (*Onchorhynchus clarkii pleuriticus*) may reside. However, data on the toxicity of zinc to cutthroat trout are very limited. Cutthroat trout are not included in calculations that determine ambient water quality criteria for zinc (USEPA 1987, USEPA 1995). The purpose of these studies was to measure the acute and chronic toxicity of zinc to cutthroat trout during early life stages (ELS) and as post swim up fry. Toxicity tests were conducted at 30 and 150 mg/L water hardness to determine the effect of hardness on zinc toxicity. Identical tests were conducted using rainbow trout (*Oncorhynchus mykiss*) for comparison and to determine if water quality standards that protect rainbow trout from zinc toxicity would also protect cutthroat trout.

MATERIAL AND METHODS

Organisms

Cutthroat trout embryos were obtained as newly eyed eggs from the Colorado Division of Wildlife State hatchery in Glenwood Springs, Colorado. Rainbow trout embryos were obtained as newly eyed egg from Ennis National Fish Hatchery in Ennis, Montana. Ten eggs were placed into each exposure chamber for the ELS tests. Remaining eggs were divided into two lots and placed in 5 gallon plexi-glass aquaria supplied with the same waters used in the 30 and 150 hardness ELS tests. After hatching, the acclimated fry were later used for the corresponding fry toxicity tests. Rainbow and cutthroat trout eggs started to hatch approximately 7 days after start of exposure. Cutthroat trout embryos in both hardnesses remained as sac fry for approximately 15

days before reaching swim up while the rainbow embryos reached swim up in 26 days. The ELS tests continued for an additional 30 days post swim up for a total exposure time of 54 days for the cutthroat and 63 days for the rainbows. Swim up fry were fed appropriately sized trout food (Silver Cup) four times daily (twice daily on weekends and holidays) at an estimated rate of 3% body weight/day. Swim up fry in the ELS tests were fed the trout food diet supplemented with a concentrated suspension of brine shrimp naupalii (INVE brand). The fry toxicity tests were initiated using approximately 42 days post swim up fry. Fry were not fed during the initial 96 hrs of exposure but were fed there after appropriately sized trout food (Silver Cup) twice daily (once daily on weekends and holidays) at an estimated rate of 3% body weight/day.

Exposure Apparatus

Source water for the 30 µg/L hardness tests consisted of a mixture of de-chlorinated Fort Collins municipal tap water and reverse osmosis water. The 150 mg/L hardness water was a mixture of well water and de-chlorinated Fort Collins municipal tap water. These waters supplied two modified continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. The chemical stock solutions were prepared by dissolving a calculated amount of reagent grade zinc sulfate heptahydrate ($ZnSO4 \cdot 7H2O$)(Mallinkrodt) in de-ionized water. Stock solutions were delivered to the diluters via peristaltic pumps (Cole Parmer model C/L) at a rate of approximately 2.0 mls/minute. New stock solutions were prepared as needed throughout the testing period. The diluters delivered five exposures with a 50% dilution ratio and an exposure control. A flow splitter allocated each concentration equally among four replicated exposure chambers at a rate of 30 mls/minute each. Exposure chambers consisted of polyethylene containers with a capacity of 2.8 liters. Test solutions overflowed from the exposure chambers into water baths which were maintained at 12 º C using temperature controlled re-circulators (VWR Scientific Products). Dim fluorescent lighting provided a 12 hour day/night photoperiod. The diluters and toxicant flow rates were monitored daily to ensure proper operation.

ELS Test Methods

The target zinc exposure concentrations for the 30 hardness tests were 800, 400, 200, 100, 50 and 0 µg/L. Target exposures for the 150 hardness tests were 1600, 800, 400, 200, 100 and 0 µg/L. The number of hatched eggs and mortality of eggs and fry were monitored and recorded daily. Dead fry were blotted with a paper towel and total length (to the nearest mm) and weight (to the nearest 0.001 g) were measured and recorded. At the end of the tests, surviving fish from each exposure chamber were terminally anesthetized, blotted dry with a paper towel and measured for lengths and weights. Water quality characteristics of exposure water were measured weekly in all treatment levels within a replicate. Replicates sampled were alternated weekly. Hardness and alkalinity were determined according to Standard Methods (APHA 1985). A Thermo Orion model 635 meter was used to measure temperature, pH and conductivity. The meter was calibrated using 4.00 and 7.00 pH buffers and a conductivity standard of 1413. A Thermo Orion model 1230 meter was used to measure dissolved oxygen.

Water samples for dissolved zinc analyses were collected weekly from each exposure level with surviving fry. Exposure water was passed through a 0.45 µm filter (Acrodisc), collected in disposable polystyrene tubes (Falcon), and immediately preserved with Ultrex® triple distilled nitric acid to a pH < 2. Analysis of samples occurred within 24 hours of collection. Analyses were performed using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with an 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 and spikes were collected and analyzed to verify analytical reproducibility and recovery. The zinc detection limit was < 10 µg/L.

Fry Test Methods

The fry experiments utilized the same exposure apparatus as in the ELS tests. Test methods were identical with the following exceptions: The target zinc exposure concentrations in the 30 hardness tests were 400, 200, 100, 50, 25 and 0 µg/L for the cutthroat trout and 800, 400, 200, 100, 50 and 0 µg/L for the rainbow trout test. For the 150 hardness tests, the target concentrations for the rainbow trout were 1600, 800, 400, 200, 100 and 0 µg/L and, initially, 800, 400, 200, 100, 50 and 0 µg/L for the cutthroat, however, an additional high level concentration of 1600 µg/L was added after the acute 96 hour period as a high concentration of 800 µg/L was insufficient to cause significant mortality. Samples for water quality characteristics and zinc analysis were collected daily during the first 96 hours, then weekly thereafter. Zinc exposure lasted for a total of 30 days. Ten rainbow trout were placed in each exposure chamber. For the cutthroat trout tests, only eight fry per chamber were used.

Statistical Analysis

Statistical analyses 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, fry and swim up survival, biomass at the end of the test, mean time to hatch and lengths and weights of surviving fish at test termination. Hatching success and survival data were transformed using the arcsine square root prior to ANOVA (Snedecor and Cochran 1980). Normality and homogeneity of variance were tested using Shipiro-Wilk's test. Treatment means were compared to the control using William's one-tailed test (Williams 1971, Williams 1972) or Dunnett's one-tailed test (Dunnett 1955, Dunnett 1964), both at $p<0.05$. The highest zinc 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 zinc that was 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. Ninety six hour median lethal concentrations (LC50) were estimated by the Trimmed Spearman-Karber technique (Hamilton et al. 1977, 1978) using log transformed zinc concentrations and 10% trim.

RESULTS

Zinc analyses exhibited a high level of accuracy and precision. Mean recovery of external QAQC standard was 98.5% (range 96.6-103%). Spiked sample recoveries averaged 104% (range 97.3-109%). Relative percent difference of split sample analyses averaged 1.1% with a range of 0-4.3%. Zinc detection limit was 6 µg/L.

Hardnesses of exposure water were in general, very close to target values (Table 16). Temperatures were maintained near 12°C. Alkalinity, pH and conductivity were similar within hardness groups.

Table 16. Mean water quality characteristics of rainbow and cutthroat trout ELS and fry tests. Standard deviations are in parentheses.

Hardness (ppm)	Alkalinity (ppm)	pH (S.U.)	Temperature (°C)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
30 Hardness ELS Rainbow Trout					
33.3 (1.6)	23.2 (1.6)	7.50 (0.15)	11.8 (0.3)	60.9 (3.5)	8.30 (0.31)
150 Hardness ELS Rainbow Trout					
150.9 (24.7)	104.6 (19.6)	7.59 (0.09)	11.8 (0.3)	274 (38.8)	8.50 (0.27)
30 Hardness Rainbow Trout Fry					
33.2 (2.1)	19.5 (0.8)	7.35 (0.2)	12.2 (0.2)	65.5 (3.7)	7.47 (0.74)
150 Hardness Rainbow Trout Fry					
145.4 (13.1)	95.8 (7.9)	7.54 (0.07)	12.1 (0.3)	265 (21.3)	8.22 (0.83)
30 Hardness Cutthroat Trout Fry					
31.1 (5.4)	23.5 (3.5)	7.24 (0.13)	12.1 (0.1)	59.5 (9.7)	7.46 (0.64)
150 Hardness Cutthroat Trout Fry					
149.4 (10.1)	104.5 (9.4)	7.53 (0.09)	12.0 (0.2)	262 (17.1)	7.54 (1.41)

Cutthroat trout ELS

Hatching success and survival of cutthroat larvae were generally poor in all treatments, including controls. Consequently results of cutthroat ELS tests are not reported.

Rainbow trout ELS – 30 Hardness

Hatching success was not affected by zinc and was ≥ 95% in all exposures of the 30 hardness tests (Table 17). In contrast, time to hatch increased as a result of zinc exposure and was significantly greater at 112 µg/L. Most of the zinc-related mortality occurred during the sac fry stage. Survival of rainbow trout in 30 hardness was significantly decreased (LOEC) at 220 and 422 µg/L for sac fry and swim up fry, respectively. Chronic values for the 30 hardness ELS test are 81, 157, and 305 based on time to hatch, sac fry survival, and swim up fry survival, respectively. There appears to be a slight decreasing trend of surviving lengths and weights with zinc exposure concentrations though this was not significant (Table 18). Biomass was significantly reduced at 220 µg/L (LOEC) but not at 112 µg/L (NOEC) for a chronic value of 157 µg/L.

Table 17. Mean dissolved zinc concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swim up fry survival (%) of rainbow trout ELS exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	58 (5)	112 (5)	220 (9)	422 (20)	903 (50)
Time to Hatch (hrs)	195 (8)	206 (20)	280* (31)	287* (34)	317* (42)	273* (23)
Hatching Success (%)	97.5 (5.0)	97.5 (5.0)	95.0 (5.8)	97.5 (5.0)	95.0 (5.8)	100 (0)
Sac Fry Survival (%)	97.5 (5.0)	87.5 (15.0)	90.0 (8.2)	57.5* (22.2)	57.5* (39.5)	47.5* (35.9)
Swim up Fry Survival (%)	90.0 (8.2)	82.5 (15.0)	90.0 (8.2)	57.5 (22.2)	52.5* (45.0)	42.5* (33.0)

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

Table 18. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of rainbow trout surviving 30 hardness ELS test. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	58 (5)	112 (5)	220 (9)	422 (20)	903 (50)
Mean Length (mm)	34.5 (0.3)	34.6 (1.3)	34.1 (0.9)	33.8 (1.7)	32.9 (0.2)	32.5 (3.7)
Mean Weight (g)	0.340 (0.012)	0.366 (0.048)	0.328 (0.013)	0.337 (0.056)	0.301 (0.010)	0.310 (0.088)
Mean Biomass (g)	3.05 (0.20)	2.97 (0.16)	2.94 (0.17)	1.93* (0.80)	2.08* (0.99)	1.28* (0.92)

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

Rainbow trout ELS – 150 Hardness

Results of the ELS rainbow trout test conducted in 150 water hardness parallel those of the 30 hardness. Hatching success was high in all treatments and was not affected by zinc exposures at the concentrations used in this study (Table 19). Time to hatch was again the most sensitive endpoint with a LOEC of 437 µg/L, a NOEC of 224 µg/L and a chronic value of 313 µg/L. Sac fry and swim-up fry survival were both affected at the highest exposure concentration of 1579 µg/L for a chronic value of 1145 µg/L. In contrast to the 30 hardness ELS test, significant effects on growth were detected (Table 20). Mean weight and biomass, but not mean length, of surviving fry was significantly lower at a zinc exposure of 1579 µg/L. Chronic values based on weight and biomass are 1145 µg/L. A summary of chronic values and endpoints are presented in Table 26.

Table 19. Mean dissolved zinc concentrations (µg/L) and associated time to hatch (hrs), hatching success, sac fry and swim up fry survival (%) of rainbow trout ELS exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (4)	112 (5)	224 (12)	437 (18)	831 (29)	1579 (79)
Time to Hatch (hrs)	186 (10)	195 (12)	202 (12)	214* (7)	252* (34)	249* (11)
Hatching Success (%)	97.5 (5)	95 (5.8)	100 (0)	97.5 (5)	100 (0)	100 (0)
Sac Fry Survival (%)	95.0 (10.0)	90.0 (11.5)	92.5 (9.6)	92.5 (15.0)	97.5 (5.0)	65.0* (43.6)
Swim up Fry Survival (%)	90.0 (14.1)	90.0 (11.5)	77.5 (15.0)	85.0 (12.9)	90.0 (14.1)	60.0* (38.3)

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

Table 20. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of rainbow trout surviving 150 hardness ELS test. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (4)	112 (5)	224 (12)	437 (18)	831 (29)	1579 (79)
Mean Length (mm)	34.6 (1.0)	34.6 (1.6)	34.7 (0.8)	33.7 (1.1)	33.1 (1.4)	32.9 (0.9)
Mean Weight (g)	0.336 (0.032)	0.349 (0.043)	0.361 (0.033)	0.327 (0.031)	0.317 (0.033)	0.290* (0.026)
Mean Biomass (g)	2.99 (0.24)	3.10 (0.05)	2.76 (0.34)	2.76 (0.24)	2.82 (0.26)	1.80* (1.17)

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

Rainbow Trout Fry-30 Hardness

Complete mortality occurred at zinc concentrations ≥407 µg/L in the test conducted at 30 mg/L hardness (Table 21). Survival of fry after 96 hours was only 20% at 204 µg/L and 62.5% at 1047 µg/L. The 96 hour LC_{50} at 30 hardness was 125 µg/L. The LOEC and NOEC after 30 days of exposure were 104 and 52 µg Zn/L, respectively, resulting in a chronic value equal to 74 µg/L in 30 water hardness. Exposure to zinc in 30 mg/L hardness did not decrease growth of rainbow trout (Table 22). Biomass was significantly reduced at a zinc concentration of 204 µg/L (LOEC) due to reduced survival at this concentration. The chronic value based on biomass was 125 µg/L.

Table 21. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of rainbow trout fry exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	52 (10)	104 (10)	204 (11)	407 (25)	822 (28)
96 hr Survival (%)	92.5 (15.0)	95.0 (5.8)	62.5 (15.0)	20.0 (27.1)	0 (0)	0 (0)
30 day Survival (%)	85.0 (17.3)	92.5 (9.6)	60.0* (14.1)	15.0* (17.3)	0* (0)	0* (0)

LC_{50} (95% C.I.)=125 µg Zn/L (108-144)
*Significantly less than control ($p<0.05$).

Table 22. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of surviving rainbow trout fry exposed in 30 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	52 (10)	104 (10)	204 (11)	407 (25)	822 (28)
Mean Length (mm)	44.9 (2.5)	43.5 (1.2)	47.1 (1.0)	49.8 (2.8)	--	--
Mean Weight (g)	0.926 (0.178)	0.819 (0.067)	1.073 (0.060)	1.252 (0.230)	--	--
Mean Biomass (g)	7.65 (0.36)	7.52 (0.22)	6.45 (1.56)	2.28* (1.59)	0* (0)	0* (0)

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

Rainbow Trout Fry-150 Hardness

The 96 hr LC_{50} was 588 µg Zn/L (Table 23). The LOEC after 30 days based on survival was 440 µg/L and the NOEC was 240 µg/L resulting in a chronic value of 325 µg Zn/L. No significant effect on mean lengths and weights of surviving organisms was detected (Table 24). Based on biomass, the LOEC, NOEC and chronic value were the same as 30 day survival.

Table 23. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of rainbow trout fry exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	135 (5)	240 (7)	440 (15)	851 (28)	1585 (103)
96 hr Survival (%)	100 (0)	97.5 (5.0)	95.0 (10.0)	62.5 (5.0)	30.0 (8.2)	22.5 (9.6)
30 day Survival (%)	87.5 (9.6)	85.0 (19.1)	87.5 (12.6)	55.0* (5.8)	25.0* (5.8)	15.0* (5.8)

96 hour LC_{50} (95% C.I.)=588 µg Zn/L (464-743)
*Significantly less than control ($p<0.05$)

Table 24. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of rainbow trout fry exposed in 150 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (2)	135 (5)	240 (7)	440 (15)	851 (28)	1585 (103)
Mean Length (mm)	44.0 (1.8)	44.1 (2.1)	43.8 (1.6)	45.2 (1.2)	42.8 (4.9)	42.8 (5.3)
Mean Weight (g)	0.875 (0.136)	0.857 (0.157)	0.852 (0.124)	0.952 (0.089)	0.810 (0.244)	0.823 (0.340)
Mean Biomass (g)	7.67 (0.52)	7.07 (0.75)	7.35 (0.29)	5.23* (0.74)	2.08* (0.90)	1.16* (0.44)

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

Cutthroat Trout Fry – 30 Hardness

The majority of mortality in the 30 hardness fry test occurred within the initial 96 hours of exposure, reaching 100% in the high zinc exposure of 416 µg/L and 87.5% in the second concentration of 190 µg/L (Table 25). Little mortality occurred throughout the remaining exposure. The 96 hour LC_{50} was 140 µg/L. The chronic value based on survival after 30 days of exposure was 134 µg/L. Mean lengths and weights of surviving cutthroat trout were slightly reduced at 190 µg/L, though the reduction was not statistically significant. Reduction of biomass resulted in a LOEC of 190 µg/L and a NOEC of 95 µg/L, giving a chronic value of 134 µg/L (Table 26).

Table 25. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of cutthroat trout fry exposed in 30 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	23 (3)	48 (3)	95 (5)	190 (9)	416 (13)
96 hr Survival (%)	100 (0)	96.9 (6.3)	100 (0)	96.9 (6.3)	12.5 (14.4)	0 (0)
30 day Survival (%)	100 (0)	96.9 (6.3)	96.9 (6.3)	96.9 (6.3)	12.5* (14.4)	0* (0)

LC_{50} (95% C.I.)=140 µg Zn/L (125-156)
*Significantly less than control ($p<0.05$)

Table 26. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of surviving cutthroat trout fry exposed in 30 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (3)	23 (3)	48 (3)	95 (5)	190 (9)	416 (13)
Mean Length (mm)	53.4 (0.9)	54.6 (0.8)	52.6 (1.1)	54.1 (0.8)	52.0 (3.5)	0 (0)
Mean Weight (g)	1.332 (0.098)	1.409 (0.080)	1.320 (0.46)	1.394 (0.069)	1.259 (0.243)	0 (0)
Mean Biomass (g)	10.65 (0.79)	10.89 (0.46)	10.22 (0.46)	10.78 (0.52)	5.66* (0.06)	0* (0)

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

Cutthroat Trout Fry – 150 Hardness

The 150 hardness cutthroat trout fry test consisted of two acute phases. Due to a lack of acute mortality, an additional high concentration of approximately 2000 µg/L was added after the initial 96 hours. After 96 hours, only 34.4% survived at 1978 µg Zn/L (Table 27). The 96 hour LC_{50} was 1645 µg/L. Reliable 95% confidence intervals could not be calculated using the Spearman-Karber technique. Mortality continued to occur in the highest exposure after the 96 hour acute phase, however this did not affect the LOEC (1978 µg/L) or the NOEC (912 µg/L). The chronic value based on 30 day survival was 1343 µg/L. No effect of zinc exposure on surviving lengths and weights was detected

(Table 28). Biomass was reduced at 1978 µg/L, primarily due to lower survival at that treatment.

Table 27. Mean dissolved zinc concentrations (µg/L) and associated acute and 30 day survival (%) of cutthroat trout fry exposed in 150 mg/L water hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (5)	62 (8)	101 (16)	210 (10)	426 (16)	912 (24)	1978 (26)
96 hr Survival (%)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)	34.4 (27.7)
30 day Survival (%)	97.5 (5.0)	--	100 (0)	100 (0)	100 (0)	100 (0)	9.4* (18.8)

96 hour LC_{50} =1645 µg Zn/L
*Significantly less than control ($p<0.05$)

Table 28. Mean measured dissolved zinc concentrations (µg/L) and associated mean lengths (mm) and weights (g) of surviving cutthroat trout fry exposed in 150 mg/L hardness. Standard deviations are in parentheses.

Dissolved Zn (µg/L)	<10 (5)	101 (16)	210 (10)	426 (16)	912 (24)	1978 (26)
Mean Length (mm)	52.5 (0.6)	53.1 (0.8)	52.5 (1.3)	53.5 (1.6)	52.4 (1.7)	53.0 (0)
Mean Weight (g)	1.327 (0.049)	1.338 (0.052)	1.307 (0.064)	1.315 (0.141)	1.314 (0.080)	1.363 (0)
Mean Biomass (g)	10.28 (0.75)	10.71 (0.41)	10.46 (0.52)	10.52 (1.13)	10.51 (0.65)	4.09* (0)

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

Table 29. Summary of chronic values (µg/L) and endpoints for zinc toxicity tests conducted with rainbow cutthroat trout ELS and fry in 30 and 150 mg/L water hardness.

Endpoint	Rainbow trout				Cutthroat Trout	
	30 Hardness		150 Hardness		30 Hardness	150 Hardness
	ELS	Fry	ELS	Fry	Fry	Fry
Time to Hatch	81	--	313	--	--	--
Hatch Success	>903	--	>1579	--	--	--
Sac Fry Survival	157	--	1145	--	--	--
Swim up Fry Survival	305	74	1145	325	134	1343
Length	>903	>204	>1585	>1585	>190	>1978
Weight	>903	>204	1145	>1585	>190	>1978
Biomass	157	146	1145	325	134	1343
LC_{50}	--	125	--	588	140	1645

DISCUSSION

A summary of chronic values for the zinc toxicity tests reveals a number of trends (Table 29). The most obvious trend confirms the well established relationship between hardness and reduced metal toxicity (Sprague 1985). As hardness increased from 30 to 150 mg/L, chronic values increased by factors of 2.2 - 10.3. Hardness appears to provide a greater protective effect for cutthroat than rainbow trout, though the reason for this is unknown. Hatching success was not a sensitive measure of zinc toxicity. However, time to hatch was a very sensitive measure of zinc exposure. Increased time to hatch is a common effect of zinc exposure on salmonid eggs (Davies et al. 2002, Davies et al. 2003). The relative increase in time to hatch is rather modest and may not represent an adverse effect. Growth, as measured by length and weight of surviving organisms, was generally unaffected by zinc exposure. Growth has not been found to be greatly affected by exposures to contaminants (Woltering 1984). Reduced biomass and fry survival were about equally sensitive at detecting adverse effects of zinc exposure.

Data on the toxicity of zinc to cutthroat trout are somewhat limited. Previously reported values are consistent with the 96 LC50 derived from the 30 hardness test. Rabe and Sappington (1979) reported a 90 µg Zn/L 96 hour LC_{50} value, though they did not provide the hardness of the water they used. Other reported values include 130, 411 and

1749 µg/L in 41, 51, and 211 mg/L water hardness, respectively (Davies et al. 2000). Overall, cutthroat trout appear to be slightly more tolerant to zinc than rainbow trout. Zinc stream standards that protect rainbow trout should also protect cutthroat trout.

Chronic endpoints of the ELS tests are consistently greater than the fry and actually exceeded 96 hour LC_{50} values. While counterintuitive, similar results were found with zinc studies using rainbow trout (Sinley et al. 1974), brown trout (Davies et al. 2003) and cadmium studies with brown trout (this report). The likely cause is acclimation that occurs during metal tolerant life stages during the ELS exposure. Organisms exposed during metal tolerant life stages become tolerant than organisms previously unexposed (Sinley et al. 1974, Spehar 1976, Davies et al. 2003).

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Toxicity of a zinc and cadmium mixture to acclimated and unacclimated brown trout

In late May 2004, a series of toxicity tests will be conducted to determine the toxicity of a zinc and cadmium mixture to brown trout fry. The toxicity tests are a cooperative effort of the Colorado Division of Wildlife Aquatic Toxicology Laboratory and the Environmental Protection Agency. The primary purpose of these tests is to compare cadmium and zinc toxicity between Arkansas River and laboratory water. The secondary purpose is to determine the effect of acclimation to sublethal levels of zinc and cadmium on subsequent toxicity. The goal is to determine concentrations of zinc and cadmium that would maintain a healthy brown trout fishery in the Arkansas River. California Gulch, a tributary to the Arkansas River near Leadville, is a major source of zinc and cadmium and has historically reduced brown trout density for several miles downstream. Recent remediation work has reduced zinc and cadmium loading and improved water quality in the Upper Arkansas River. Results from these experiments will form the basis of water quality standards for the Upper Arkansas and determine whether additional remediation efforts are necessary in the Leadville and California Gulch superfund site area.

Brown trout eggs were collected in October 2003 as part of Colorado Division of Wildlife spawning operations at North Delaney Butte Reservoir. After eye-up, eggs were divided into two groups. One group of eggs was exposed to sublethal concentrations of zinc and cadmium in order to induce acclimation to these metals. Nominal acclimation conditions are 290 µg Zn/L, 0.89 µg Cd/L and 130 mg CaCO3/L hardness. These levels are characteristic of the Arkansas River downstream from California Gulch during the winter. The other group has been maintained in metal-free water and will act as an acclimation control. Both groups were maintained at low water temperatures (1-5º C) to delay development.

During spring runoff, the EPA will conduct onsite toxicity tests using water drawn from the Arkansas River and California Gulch. The Aquatic Toxicology Laboratory tests will simulate the zinc-cadmium ratio and water quality characteristics of the Arkansas River water used in the EPA study. Results of the two tests will be compared in order to ascertain how well laboratory tests predict toxicity in natural waters. Test results on acclimated and unacclimated brown trout will be compared to determine how sublethal metal exposure affects subsequent toxicity.

Job B.3.

Assistance was provided to Vicky Peters of the Colorado Attorney Generals Office to assess damages to the East Fork of the Arkansas River as a result of discharge from the Leadville Mine Drainage Tunnel. Interpretation of water quality and fish population estimates was also provided for Vicky Peters. Stephen Brinkman also reviewed and provided comments to Lee Pivonka of the Colorado Department of Health on a report titled “Characterization of risks to aquatic receptors from mining-related contaminants in the upper Arkansas River California Gulch superfund site, operable unit 12”. Stephen Brinkman is reviewing proposed water quality standards on Mosquito Creek and will prepare comments to be submitted to the Water Quality Control Commission. Analytical services were provided to Natural Resource Management Institute for water samples

collected from Colorado Gulch and the Lake Fork of the Arkansas River. Metal content in bryophytes was provided for Del Nimmo and Scott Hermann of Colorado State University - Pueblo as part of bioaccumulation investigations on the Arkansas River. Analytical and laboratory equipment and services were provided to various Colorado State University graduate students from the Department of Fishery and Wildlife Biology.