WATER POLLUTION STUDIES

Federal Aid Project #F-243R-6

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Federal Aid in Fish and Wildlife Restoration

Job Final Report

Colorado Division of Wildlife

Fish Research Section

Fort Collins, Colorado

August, 1999

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JOB PROGRESS REPORT

State: Colorado

Project No. F243R-6

Title: Water Pollution Studies

Period Covered: July 1, 1998 to June 30, 1999

Principal Investigator: Patrick H. Davies
Co-investigators: Stephen F. Brinkman, Matthew McIntyre

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: REGULATORY AND LEGAL ACTIVITIES

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

Job A1. Water quality standards for the protection of aquatic life in Colorado

Objectives:

1. To apply research results and toxicological information from literature toward the development, enactment, and implementation of water quality standards and appropriate aquatic life use classifications.

2. To provide technical information and/or expert testimony in aquatic toxicology and aquatic chemistry in agency meetings, regulatory hearings, and/or court litigations as needed to protect aquatic resources of Colorado.

3. To develop or compile toxicological and chemical data on toxicants for which state or federal governments have not developed a standard.

ACCOMPLISHMENT:

With the acquisition of a mobile aquatic toxicology laboratory, we initiated on-site toxicity tests on the Animas River below Silverton, CO. In cooperation with the Animas River Stakeholders Group, consisting of state, federal, municipal, and private parties, we initiated toxicity studies on assess clean-up levels in the Upper Animas River needed to maintain a reproducing trout fishery below Silverton. Acute/subchronic toxicity tests were conducted on Animas River water during spring run-off in June and July, 1999, using brown and brook trout exposed to zinc and copper. Additional testing will be conducted in October, 1999, with cutthroat trout to assess toxicity associated with fall water quality conditions. Tests will be repeated in March and April, 2000, with brown and brook trout to assess

toxicity during early spring run-off, the period of highest metal concentrations. Results from these experiments will be reported next year.

PLAN B: LABORATORY STUDIES

Objective: To research and develop information on, or analytical tools in, aquatic chemistry and aquatic toxicology to better assess toxic responses of pollutants to aquatic life in laboratory and natural waters, such as the Arkansas River.

Job B1. Chemical Equilibria and Kinetic Effects on the Bioavailability and Toxicity of Metals to Aquatic Life

Objective: To develop analytical methods using Ion Chromatography, ion separation and/or ultrafiltration to measure toxic fractions and effects of chemical kinetics on toxicity of zinc, copper, lead, cadmium and/or silver to *Ceriodaphnia dubia*, rainbow trout, brown trout and/or fathead minnows in waters of different complexing capacity. Concurrently, investigate effects of chemical kinetics on results obtained from toxicity tests.

Job B2. Use of Biochemical Methods to Measure Disruption of Ion Regulation and Stress in Aquatic Organisms Exposed to Metals

Objective: To develop biochemical methods to measure effects on enzyme systems using electrophoresis or other methods to assess stress in rainbow and brown trout exposed to zinc, copper, lead and/or cadmium.

Job B3. Investigations on the Toxicity of Silver to Aquatic Organisms in Waters Different Complexing Capacity

Objective: To develop acceptable toxicant concentrations of silver for rainbow trout, brown trout, and/or fathead minnows in hard, high alkaline, and soft, low alkaline waters.

Job B4. Effects of Calcium Hardness, Inorganic and Organic Ligands and Sediments on Toxicity of Metals to Aquatic Organisms

Objective: To determine antagonistic effects of calcium hardness in low alkaline waters and the effects of specific inorganic and organic ligands and sediments on acute and long-term toxicity of zinc, copper, lead, cadmium, and/or silver to rainbow trout, brown trout and/or fathead minnows.

Job B5. Investigations on Enhanced Toxicity of Unionized Ammonia to Fish at Cold Water Temperatures

Objective: To determine effects of temperature on toxicity of unionized ammonia to rainbow trout and fathead minnows or other warmwater species at optimal and less than 5°C water temperatures.

Job B6. Effects of Episodic Exposure on Toxicity and Sensitivity of Aquatic Life to Intermittent Exposure to Metals

Objective: To determine toxic effects and organism sensitivity to intermittent exposure of zinc, copper, lead, and/or cadmium to rainbow trout, brown trout and/or fathead minnows, and their ability to acquire and/or lose tolerance.

Job B7. Investigations on Enhanced Toxicity of Water-Borne Metals to Aquatic Life Exposed to Dietary Sources of Metals

Objective: To determine effects of water-borne zinc, copper, cadmium, lead and/or manganese on their toxicity to rainbow and brown trout following and/or concurrent with exposure to dietary metals.

Job B8. Investigations on Effects and Interactions of Multiple Metal Exposure on Toxicity to Aquatic Life

Objective: To determine effects of exposure of rainbow trout and/or brown trout to zinc, copper, cadmium, lead, and manganese at different combinations found in Colorado's mining areas. Will require an ability to measure bioavailable forms on metals as outlined in Job 1.

Job B9. Investigations of Analytical Methods to Measure Rotenone Concentrations in the Field and Antimycin Concentrations in the Laboratory

Objective: To develop a field method to measure rotenone and a laboratory method for measuring antimycin at concentrations less than 5 ppb using cation exchange chromatography.

ACCOMPLISHMENTS:

Toxicity of Zinc to Brown Trout under Conditions of Embryonic Exposure (Acclimated), Non-exposure (Unacclimated) and Following 2 and 3 Weeks Deacclimation

ABSTRACT

Seven hundred and fifty eyed brown trout eggs were acclimated to 0, 200, and 400 µg/liter of zinc (Zn) for eighty days. Egg and fry mortality during this period were 18, 23, and 25 percent, respectively, suggesting a possible effect on survival at the 200 and 400 levels. Each of the three exposure groups were chronically exposed to nominal Zn concentrations of 3200, 1600, 800, 400, 200, 100, 50, and 0 µg/liter. Chronic values (i.e. geometric mean of the effect, no-effect concentrations) for the 0, 200, and 400 pre-exposure groups were 303, 1329, and 1329 µg/liter, respectively. These results clearly demonstrate that fish embryonically exposed to Zn attain an increased tolerance four times that of non-exposed brown trout.

Fish from the 200 and 400 Zn pre-exposure groups were transferred to control water with no added Zn and allowed to deacclimate for a period of 2 and 3 weeks. Sub-chronic tests of 18 and 13 days,

respectively, for the 2 and 3 week deacclimated fish were performed to assess the rate and magnitude at which fish lose their acquired tolerance. The chronic value for the unacclimated was 194 µg/liter. For 200 acclimated and 400 acclimated groups, chronic values were 360, and 360 µg/liter, respectively for fish deacclimated for 2 weeks, and 342 and 342 µg Zn/liter for the 3 week deacclimated brown trout. This shows that brown trout quickly lose any increased tolerance from having been embryonically exposed to Zn. This finding also suggests that setting elevated water quality standards for trout in mining polluted streams based on embryonic acclimation may not be appropriate. In Colorado mountain streams below mining areas, we typically see elevated concentrations of Zn. However, during spring run-off, dissolved Zn concentrations frequently decrease substantially for periods of 4 weeks or longer, due to high dilution flows from snow melt. As a result, increased tolerance to Zn may be lost during this time. In such instances, higher water quality standards based on acclimation would not be protective. Chronic toxicity data from these experiments strongly support a zinc standard for brown trout that should not exceed 200 µg Zn/liter in a water hardness of 50 mg/liter (Table 3).

Acute data were collected during each of the acute phase, i.e., first 96 hours, of the chronic and sub-chronic tests. Ninety-six hour LC50, Zn concentrations killing 50 percent of the fish, show the same acclimation, deacclimation relationships determined from the chronic experiments.

INTRODUCTION

Little information is available on the chronic toxicity of zinc (Zn) to brown trout. A series of experiments were conducted to assess the toxicity of Zn to unacclimated brown trout (no previous exposure to Zn) and to brown trout acclimated to 200 and 400 µg Zn/liter. We also assessed effects of two and three week deacclimation, in water containing no Zn, on acute and chronic toxicity.

METHODS

Pre-exposure

Newly eyed brown trout eggs were received from CDOW's Bellvue Research Hatchery. Eggs were initially obtained from Delany Buttte Reservoir wild trout spawn. Acclimation to temperature and test water conditions, feeding and maintenance of test organisms were performed according to ASTM guidelines. Laboratory dilution/control water consisted of dechlorinated Fort Collins tap water with a water hardness of 50 mg/liter as $CaCO_3$. Three groups of 750 eggs each were placed on hatching trays submersed in 110 liter aquaria with an estimated water volume of 40 liters. Water was delivered to the aquaria from modified Mount and Brungs diluter (1967) at a rate of four liters every 5 minutes at a temperature of 12°C. Zinc concentrations (as reagent grade $ZnSO_4 \cdot 7H_2O$) delivered by the diluters were 0.0 µg/liter (unacclimated, no Zn added), 200 µg Zn/liter(acclimated) and 400 µg/liter (acclimated). After hatching and yolk-sac absorption, swim-up fry were fed Bio-Kiowa starter diet and as fish grew, different amounts and sizes of trout chow according to Piper et al. 1982. Dead eggs and fry were recorded daily. Pre-exposure of unacclimated and acclimated fish continued for 80 days prior to initiating acute and chronic toxicity tests.

Acute and Chronic Tests on Pre-exposed Brown Trout

Acute and chronic tests were performed on each of the three pre-exposure groups to determine difference in toxicity of zinc between fish that had been embryonically exposed (acclimated) to 200 and 400 µg Zn/liter compared to fish not previously exposed (unacclimated). After 80 days pre-exposure of unacclimated and both 200 and 400 acclimated groups, juvenile brown trout were exposed to Zn concentrations ranging from 3200 to 50 µg/liter, at a 50% dilution ratio and corresponding control tanks

using two continuous flow diluters (Benoit et al. 1982), giving two replicates for each of seven levels of exposure and a control. Fifteen fish were randomly placed into each of 2.8 liter polypropylene tanks receiving a flow rate of 40 mls/min. Effects of zinc exposure on acute survival were determined during the first 96-hr of chronic test. Mortality was monitored and recorded daily. Fish were feed trout chow diet as per Piper et al. 1982. All three chronic tests were terminated after 31 days.

Acute and Sub-chronic Tests following 2 and 3 Week Deacclimation

Toxicant flows were shut down to the 200 and 400 pre-exposed (acclimated) brown trout tanks and flushed with control water with no added Zn to allow an assessment of the magnitude and rate of loss of acclimation. Following a period of two weeks deacclimation, fish from the unacclimated and both 200 and 400 acclimated groups were used in the deacclimation tests. Testing conditions, nominal zinc concentrations, and fish numbers were identical to the previous acute/chronic tests. One of the two replicates was terminated after eight days to initiate similar tests on the 200 and 400 acclimated groups following 3 weeks (22 days) deacclimation in control water. The sub-chronic phase of the one remaining replicate of the 2 week deacclimation experiments was terminated after 18 days. The 3 week deacclimation acute tests on the 200 and 400 acclimated groups were conducted as in the previous acute test except it was not replicated. The sub-chronic phase of these tests were terminated after 13 days.

Water Quality and Zinc Analyses

Hardness, alkalinity, pH, temperature, dissolved oxygen, and conductance were measured weekly in alternate replicates during each of the acute and chronic tests according to *Standard Methods* (APHA 1985).

Water samples for zinc analysis were collected daily as 5 ml aliquots of a seven day composite sample for each of the unacclimated, 200 acclimated and 400 acclimated pre-exposure tanks. During the acute and chronic tests, alternating replicates of each exposure level were collected weekly as grab samples. Samples were preserved with *Ultrex* ®, triple distilled, nitric acid to pH <2. Water samples were analyzed for Zn using an Instrumentation Laboratory Video 22, flame, atomic absorption spectrophotometer (AAS).

Statistical Analyses

Analysis of survival and growth data using hypothesis testing was performed using Toxstat version 3.5 (West and Gulley 1996). Survival data were transformed by arcsine square root prior to analysis (Snedecor and Cochran 1980). Body weight in each replicate was determined as the mean wet (blotted dry) weight per surviving fish. Normality and homogeneity assumptions of survival and weight were evaluated by the Chi Square test and Cochran's test, respectively ($\rho \leq 0.10$). All data satisfied Analysis of variance (ANOVA), followed by one-tailed William's multiple comparison test was used to compare ($\rho \leq 0.05$) organism performance in the experimental treatments with that observed in the control. Response means for each test treatment were compared to that in the concurrent control groups for a given study. Groups in which a statistically significant decrease in survival occurred were first identified. The lowest Zn concentration associated with a significant effect was designated the *lowest observed effect concentration* (LOEC). The highest Zn concentration associated with no significant effect was designated the *no observed effect concentration* (NOEC). Median lethal concentrations were estimated using the Trimmed Spearman Karber technique (Hamilton et al. 1977, Hamilton et al. 1978).

RESULTS

Pre-exposure

Mean Zn concentrations and mortality results during the 80 day pre-exposure period are reported in Table 1. In each of the pre-exposures, i.e. unacclimated, 200 acclimated and 400 acclimated tanks, complete hatching of eggs occurred within 11 days after initiating the pre-exposure period. Egg mortality in the pre-exposure tanks were 79, 79, and 92 deaths, respectively. After 80 days pre-exposure to approximately 200 and 400 µg Zn/liter in the two acclimation tanks, mortalities of 23.3% and 25% may be significant higher than the unacclimated control tank. But since the pre-exposure tanks were not replicated, we cannot say that the difference in mortality were statistically significant.
Water quality characteristics for hardness, alkalinity, pH, dissolved oxygen, temperature and conductance in each of the pre-exposure, acclimation tanks are reported in Table 5.

Chronic Tests on Acclimated Brown Trout

Embryonic exposure of brown trout to 200 and 400 µg/liter significantly increased their tolerance to Zn. Chronic values obtained, during the 31 day chronic exposure period, were 1329 µg/liter for the 200 and 400 Zn acclimation fish compared to a chronic value of 303 µg/liter for the unacclimated brown trout (Tables 2 and 3). This increase in tolerance by a factor of greater than four over unacclimated fish (not previously exposed to Zn) is the same as that reported for rainbow trout (Sinley et al. 1976). Effect concentrations were based on mortality in the unacclimated and 200 and 400 acclimated tests. No statistical difference in growth was found between the three pre-exposure groups (Table 4). This is not surprising since each of the experiments was initiated with juvenile where difference in growth can be masked due to increase variance in size associated with older fish as compared early life stage (ELS) toxicity tests. Water quality characteristics the chronic tests in the unacclimated, 200 acclimated and 400 acclimated brown trout tests were the same (Table 5.)

Sub-chronic Tests following 2 and 3 Week Deacclimation

Brown trout pre-exposed to 200 and 400 µg/liter of Zn were deacclimated in control water containing no added Zn for a period two and three weeks. Following each of the deacclimation periods, sub-chronic tests of 18 and 13 days, respectively, were conducted on fish from the unacclimated group and both the 200 and 400 acclimation groups. These experiments were performed to determine the magnitude and rate at which the acclimated fish would loss their tolerance to Zn afforded by their embryonic pre-exposure. Sub-chronic data derived from these tests show that the acquired tolerance to Zn was substantially reduced within the two week deacclimation period. (Table 2 and 3). Chronic values derived from the 2 and 3 week deacclimation periods for both the 200 and 400 Zn acclimated fish were virtually identical corresponding to 360 and 360 µg/liter of Zn compared to 342 and 342 µg/liter, respectively. The chronic value of the unacclimated control fish was 194 µg Zn/liter. There was no significant difference in mean length and weight of brown trout from the three pre-exposure groups in the sub-chronic experiments on either the 2 week and 3 week deacclimation tests (Table 6). Water quality characteristics during the sub-chronic deacclimation experiments are reported in Table 5.

Acute Toxicity Tests

Acute toxicity tests were performed on brown trout from each of the three pre-exposure groups during the acute phase (first 96 hours) of the chronic and sub-chronic tests. The 96-hr LC50 for the brown trout from the unacclimated group was significantly lower than the 200 and 400 acclimation groups which were not significantly different from each other (Table 7 and 8). After the two week deacclimation period, the 96-hr LC50's for the 200 and 400 Zn acclimated brown trout were virtually identical, i.e., 561

and 506 µg/liter, respectively. These values are about two-thirds the 96-hr LC50's obtained from the 200 and 400 Zn acclimated fish but still significantly higher than the 392 µg/liter LC50 determined for the unacclimated fish.. This suggests that the increased tolerance gained during embryonic exposure to Zn has not been completely lost. However, after three weeks deacclimation, all three LC50's for the unacclimated, 200 acclimated, and 400 acclimated groups are statistically the same.

DISCUSSION

The chronic values derived for unacclimated brown trout (i.e., no previous exposure to Zn) were 303 and 194 µg/liter. Compared to the chronic values of 1329 µg/liter obtained from brown trout embryonically acclimated to 200 and 400 µg Zn/liter, the acclimated fish acquired an increased tolerance to Zn between 4 and 5 times that of unacclimated fish. Transferring the two groups of acclimated brown trout to control water containing no added Zn caused a rapid loss of this increased tolerance. Chronic values for both the 200 and 400 acclimated groups were 360 and 342 µg/liter for fish following a deacclimation period of 2 and 3 weeks, respectively. This has important implications for the way Colorado establishes water quality standards for Zn in streams impacted by metal mining. Because of the ability of fish to acclimate to higher Zn concentrations when embryonically exposed to Zn and some other metals, Colorado has set water quality standards in many metal polluted streams at these higher levels based on this acclimation. This new research, which shows a rapid loss in tolerance when returned to clean water, raises serious questions regarding establishment of water quality standards based on acclimation. During spring run-off, dissolved Zn concentrations decrease substantially for periods of two to four weeks due to high dilutions flows from snow melt. Therefore, where increased tolerance to Zn may have occurred during lower flow periods, that increase in tolerance could be lost quickly substantially increasing fish sensitivity to Zn well below levels still permitted by an elevated Zn standard which now could become highly toxic. Chronic toxicity data from these experiments strongly support a zinc standard for brown trout that should not exceed 200 µg/liter at a water hardness around 50 mg/liter.

Table 1. Mean, minimum, and maximum zinc concentrations, and percent mortality during eighty days pre-exposure of brown trout to 0 µg/liter (Unacclimated), 200 µg/liter (Acclimated), and 400 µg/liter (Acclimated) of zinc). Acclimation initiated as eyed eggs. Standard Deviation in parentheses.

	0 UNACCLIMATED	**200 ACCLIMATED**	**400 ACCLIMATED**
Mean Zn (µg/liter) Minimum - Maximum	7 (3.1) 3 - 14	192 (7.7) 183 -204	416 (17.0) 392 -447
# Dead/Initial #.	135/750	174/750	191/750
% Mortality	18%	23.2%	25.0%

Table 2. Comparison of maximum acceptable toxicant concentrations (µg/liter total Zn), expressed as a chronic value (C.V.), for brown trout with no pre-exposure to zinc (i.e. unacclimated), and pre-exposed to 200 and 400 µg/liter of zinc (i.e. acclimated). Sub-chronic values are also reported for 200 and 400 acclimated brown trout following a 2 and 3 week deacclimation period in water containing no zinc.

Pre-Exposure	**NOEC** [a] µg/liter	**LOEC** [b] µg/liter	**Chronic Value** [c] µg/liter
Chronic Toxicity of Brown Trout Acclimated to 0, 200, and 400 µg Zn/L			
UNACCLIMATED	210	438	**303**
200 ACCLIMATED	976	1811	**1329**
400 ACCLIMATED	976	1811	**1329**
Sub-chronic [d] Toxicity of Brown Trout Following 2 Week Deacclimation			
UNACCLIMATED	148	255	**194**
200 ACCLIMATED	255	507	**360**
400 ACCLIMATED	255	507	**360**
Sub-chronic [d] Toxicity of Brown Trout Following 3 Week Deacclimation			
UNACCLIMATED	Same as Two Week Deacclimation Test		
200 ACCLIMATED	226	518	**342**[d]
400 ACCLIMATED	132	226	**342**[d]

[a] NOEC - *no observable effect concentration*
[b] LOEC - *lowest observable effect concentration*
[c] CV - *geometric mean of NOEC and LOEC*
[d] Sub-chronic test periods for 2 and 3 week deacclimation experiments were 18 and 13 days, respectively. Sub-chronic mortality in 3 wk deacclimation test same as acute test, Table 8.

Table 3. Mean zinc concentrations, and percent mortality of brown trout from chronic toxicity tests with non-exposed (i.e. unacclimated), and pre-exposed brown trout (i.e. acclimated to 200 and 400 µg/liter of zinc). Standard Deviation in parentheses.

Nominal	3200	1600	800	400	200	100	50	0.0
	Chronic Toxicity of Brown Trout Acclimated to <10, 200, and 400 µg Zn/L							
Zn (µg/liter)	3356 (34.3)	1811 (74.8)	976 (86.7)	438 (47.6)	210 (39.0)	101 (24.0)	49.5 (17.1)	<10
% Mortality **Unacclimated**	100 (0.0)	100 (0.0)	43.4 (16.6)	**20.0** [a] (6.7)	**10.0** [b] (3.3)	0.0	3.4 (3.4)	3.4 (3.4)
% Mortality **200 Acclimated**	100 (0.0)	**93.4** [a] (6.6)	**0.0** [b]	0.0	0.0	0.0	0.0	0.0
% Mortality **400 Acclimated**	100 (0.0)	**73.4** [a] (6.6)	**0.0** [b]	0.0	0.0	0.0	0.0	0.0
Chronic Values [c]: UNACCL.= 303 µg/l **200 ACCL.=1329** µg/l **400 ACCL.= 1329** µg/l								
	Sub-chronic Toxicity of Brown Trout following 2 Week Deacclimation							
Zn (µg/liter)	3306 (99.4)	1671 (49.7)	912 (49.1)	507 (19.8)	255 (38.8)	148 (22.6)	88 (24.0)	<0
% Mortality Unacclimated	100 (0.0)	100 (0.0)	93.4 (6.6)	63.4 (3.4)	**33.3** [a] (0.0)	**0.0** [b]	0.0	0.0
% Mortality 200 Acclimated	100 (0.0)	100 (0.0)	93.4 (6.6)	**36.6** [a] (16.6)	**0.0** [b]	0.0	0.0	0.0
% Mortality 400 Acclimated	100 (0.0)	96.6 (3.4)	96.6 (3.4)	**43.3** [a] (10)	**10.0** [b] (10)	0.0	0.0	0.0
Chronic Values [c]: UNACCL.= 194 µg/l **200 ACCL.=360** µg/l **400 ACCL.=194** µg/l								

[a] NOEC - *no observable effect concentration*
[b] LOEC - *lowest observable effect concentration*
[c] CV - *geometric mean of NOEC and LOEC*

Table 4. Means and (standard deviations) of lengths and weights (g) for chronic toxicity tests with unacclimated, acclimated, and two and three week deacclimated brown trout exposed to zinc.

Zn Exposure (μg/liter)	**Length (mm)**	**Weight (g)**
Chronic Toxicity Test of Unacclimated Brown Trout Pre-exposed to 0.0 μg Zn/L		
985	47.1 (0.57)	1.018 (0.028)
429	49.0 (1.06)	1.118 (0.124)
210	48.8 (1.13)	1.110 (0.131)
97	49.1 (1.41)	1.156 (0.076)
47	47.8 (0.64)	1.031 (0.000)
0.0	50.3 (0.57)	1.232 (0.043)
Chronic Zinc Toxicity Test of Brown Trout Acclimated to 200 μg Zn/L		
985	49.4 (1.06)	1.142 (0.070)
429	48.2 (0.64)	1.079 (0.052)
210	49.7 (0.71)	1.184 (0.012)
97	49.0 (1.84)	1.112 (0.125)
47	49.4 (0.71)	1.176 (0.006)
0.0	49.0 (0.07)	1.138 (0.011)
Chronic Zinc Toxicity Test of Brown Trout Acclimated to 400 μg Zn/L		
1826	45.9 (2.97)	0.963 (0.163)
985	49.0 (0.07)	1.144 (0.026)
429	48.2 (0.21)	1.080 (0.028)
210	47.6 (0.92)	1.101 (0.088)
97	48.2 (0.21)	1.062 (0.042)
47	48.2 (0.21)	1.062 (0.016)
0.0	49.6 (0.07)	1.194 (0.023)

Table 5. Mean water quality characteristic for acute and chronic toxicity tests with unacclimated, acclimated, and two and three week deacclimated brown trout exposed to zinc. Standard deviation in parentheses.

WATER QUALITY CHARACTERISTICS					
Hardness mg/L	**Alkalinity** mg/L	**pH**	**Diss. Oxygen** mg/L	**Temperature** °C	**Conductivity** µS/cm
Pre-Exposure of Embryonic Brown Trout - Acclimated to 400, 200, and 0 µg Zn/L					
52.5 (1.43)	35.4 (1.37)	7.50 (0.18)	9.06 (0.45)	11.8 (1.80)	89.1 (3.22)
Acute Toxicity Tests of Brown Trout Acclimated to 400, 200, and 0 µg Zn/L					
51.9 (2.16)	35.2 (1.02)	7.67 (0.12)	9.10 (0.62)	12.6 (0.6)	88.8 (2.73)
Chronic Toxicity Tests of Brown Trout Acclimated to 400, 200, and 0 µg Zn/L					
50.9 (1.67)	33.1 (1.11)	7.58 (0.17)	8.78 (0.77)	12.9 (0.42)	88.4 (2.38)
Acute Zinc Toxicity Tests of Brown Trout Deacclimated for 2 Weeks					
51.8 (1.75)	35.5 (0.93)	7.87 (2.77)	8.06 (1.28)	12.5 (0.46)	89.7 (1.28)
Sub-chronic Zinc Toxicity Tests of Brown Trout Deacclimated for 2 Weeks					
52.0 (1.75)	35.6 (0.98)	7.83 (2.63)	7.89 (1.34)	12.6 (0.55)	90.0 (2.83)
Acute and Sub-chronic Zinc Toxicity Tests of Brown Trout Deacclimated for 3 Weeks					
53.7 (0.83)	36.3 (0.53)	7.61 (0.04)	7.23 (0.68)	13.3 (0.13)	91.9 (0.88)

Table 6. Means and (standard deviations) of lengths (mm) and weights (g) for acute and chronic toxicity tests with unacclimated, acclimated, and two and three week deacclimated brown trout exposed to zinc.

Pre-exposure	Length (mm)	Weight (g)
Acute Toxicity Tests of Brown Trout Acclimated to 0, 200, and, 400 µg Zn/L		
Unacclimated	39.6 (3.6)	0.526 (0.159)
200 Acclimated	40.1 (4.1)	0.550 (0.200)
400 Acclimated	40.1 (4.0)	0.558 (0.175)
Acute Zinc Toxicity Tests of Brown Trout Deacclimated for 2 Weeks		
Unacclimated	52.3 (5.5)	1.419 (0.483)
200 Acclimated	50.7 (5.6)	1.254 (0.474)
400 Acclimated	50.9 (5.1)	1.275 (0.416)
Chronic Zinc Toxicity Tests of Brown Trout Deacclimated for 2 Weeks		
Unacclimated	51.8 (7.0)	1.420 (0.659)
200 Acclimated	51.6 (6.4)	1.368 (0.591)
400 Acclimated	50.8 (6.7)	1.323 (0.605)
Acute and Sub-chronic Zinc Toxicity Tests of Brown Trout Deacclimated for 3 Weeks		
200 Acclimated	57.1 (5.9)	1.907 (0.661)
400 Acclimated	55.7 (6.0)	1.738 (0.562)

Table 7. Comparison of 96-hr LC50 concentrations and 95% confidence intervals (C.I.) from acute toxicity tests with non-exposed (i.e. unacclimated), and pre-exposed (i.e. acclimated to 200 and 400 µg/liter of zinc) brown trout.

Pre-Exposure	**96-Hr LC50** µg Zn/L	**95% Confidence Interval** µg Zn/L
Acute Toxicity of Brown Trout Acclimated to <10, 200, and 400 µg Zn/L		
Unacclimated	**871**	729 to 1041
200 Acclimated	**1397**	1321 to 1477
400 Acclimated	**1578**	1430 to 1742
Acute Toxicity of Brown Trout with 2 Week Deacclimation from Pre-exposure to <10, 200, and 400 µg Zn/L		
Unacclimated	**392**	332 to 464
200 Acclimated	**561**	495 to 635
400 Acclimated	**506**	437 to 585
Acute Toxicity of Brown Trout with 3 Week Deacclimation from Pre-exposure to 200 and 400 µg Zn/L		
Unacclimated	**Same as 2 Week Deacclimation**	
200 Acclimated	**438**	366 to 524
400 Acclimated	**384**	305 to 483

Table 8. Mean zinc concentrations, and percent mortality of brown trout from acute toxicity tests with non-exposed (i.e. unacclimated), and pre-exposed brown trout (i.e. acclimated to 200 and 400 µg/liter of zinc). Standard Deviation in parentheses.

Nominal	3200	1600	800	400	200	100	50	0.0
	Acute Toxicity of Brown Trout Acclimated to 0, 200, and 400 µg Zn/L							
Zn (µg/liter)	3345 (211)	1826 (79.2)	985 (103)	429 (50.6)	210 (41.3)	97 (18.8)	47 (11.2)	<10
% Mortality **Unacclimated**	100 (0.0)	100 (0.0)	40.0 (13.3)	16.6 (3.4)	3.4 (3.4)	0.0	0.0	3.4 ()
% Mortality **200 Acclimated**	100 (0.0)	93.4 (6.6)	0.0	0.0	0.0	0.0	0.0	0.0
% Mortality **400 Acclimated**	100 (0.0)	73.4 (6.6)	0.0	0.0	0.0	0.0	0.0	0.0
Unaccl. LC50=871 µg/l	**200 Accl. LC50=1397 µg/l**		**400 Accl. LC50=1578 µg/l**					
	Acute Toxicity of Brown Trout following 2 Week Deacclimation							
Zn (µg/liter)	3306 (99.4)	1672 (52.3)	916 (49.9)	508 (20.8)	254 (40.4)	148 (23.8)	88 (25.2)	<10
% Mortality **Unacclimated**	100 (0.0)	100 (0.0)	90.0 (10)	63.4 (3.4)	30.0 (3.3)	0.0	0.0	0.0
% Mortality **200 Acclimated**	100 (0.0)	100 (0.0)	93.4 (6.6)	36.6 (16.6)	0.0	0.0	0.0	0.0
% Mortality **400 Acclimated**	100 (0.0)	96.6 (3.4)	96.6 (3.4)	43.3 (10)	10.0 (10)	0.0	0.0	0.0
Unaccl. LC50=392 µg/l	**200 Accl. LC50=561 µg/l**		**400 Accl. LC50=506 µg/l**					
	Acute Toxicity of Brown Trout following 3 Week Deacclimation							
Zn (µg/liter)	---	1716 (11.7)	989 (8.66)	518 (11.5)	226 (8.1)	132 (6.0)	68 (6.5)	<10
Unacclimated	Not tested same as 2 week deacclimation							
% Mortality **200 Acclimated**	---	100	100	**66.7** [a]	**0.0** [b]	0.0	0.0	0.0
% Mortality **400 Acclimated**	---	100	100	66.7	**13.3** [a]	**6.7** [b]	0.0	0.0
200 Acclimation - LC50 =438	**400 Acclimation - LC50 =384**							

[a] NOEC - *no observable effect concentration*
[b] LOEC - *lowest observable effect concentration*

[c] CV - *geometric mean of NOEC and LOEC*

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Chronic Toxicity of Zinc to Early Life Stage brown trout (*Salmo trutta*)

ABSTRACT

An Early Life Stage (ELS) toxicity test was conducted to evaluate the toxicity of zinc to brown trout. Newly eyed brown trout eggs were exposed to 2000, 1500, 750, 375 188 and 0 µg Zn/L, four replicates each, with a water hardness of 50 mg $CaCO_3$/L. Exposure continued for a total of sixty eight days. Survival was not affected by zinc exposure as high as 2000 µg Zn/L. However, hatching success, length and weight were reduced by zinc exposure. The lowest observed effect level (LOEC) based on those three endpoints was 698 µg Zn/L. The no observed effect level (NOEC) was 280 µg Zn/L. The chronic value calculated as the geometric mean of the LOEC and NOEC was 381 µg Zn/L. The chronic value agrees well with the IC25 based on mean weight per original organism which estimated at 401 µg Zn/L.

INTRODUCTION

Data regarding the toxicity of zinc to brown trout (*Salmo trutta*) are scarce. An Early Life Stage (ELS) toxicity test was conducted in order to develop some data regarding the toxicity of zinc to brown trout and to supplement data regarding acclimation and deacclimation of brown trout to zinc.

MATERIAL AND METHODS

Organisms

Brown trout were acquired as "eyed" eggs from the Colorado Division of Wildlife Bellevue-Watson Research Hatchery. The source of the eggs were wild brown trout from North Delaney Butte reservoir near Walden Colorado. Eggs were placed on submerged egg trays and acclimated to the diluter source water for forty eight hours prior to initiation of the early-life stage tests. After hatching and yolk-sac absorption, hatched organisms were fed four times daily (twice a day on week-ends). Fish received 0.5 ml per chamber of concentrated suspension of brine shrimp nauplii (San Francisco Bay Brand) supplemented with Biokyowa 400 µm fish food at each feeding. As the test progressed, the amount of food supplied to each chamber was increased. All test chambers received an equal of food regardless of the number of surviving fish. Test aquaria were routinely cleaned using a siphon to remove excess feed and fish feces.

Toxicant

Zinc was added as reagent grade zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$) (Mallincrodt). Chemical stocks for toxicity test were prepared by dissolving a calculated amount of $ZnSO_4 \cdot 7H_2O$ in deionized water to achieve the nominal stock solution concentration. The resulting stock solution was delivered to the test diluters as described below. New stock solutions were prepared as needed during the tests.

Test Methods

A continuous-flow diluter (Benoit et al. 1982) was used to deliver the exposure solutions. The source water for the diluter consisted of dechlorinated Fort Collins municipal. The diluter was constructed of polyethylene and polypropylene plastic components with silicone stoppers and Nalgene FDA food grade tubing. A zinc stock solution was delivered to the diluter via a peristaltic pump (Cole-Palmer model C/L) at a rate of 2.0 mls/minute. The diluter delivered five concentrations of zinc and control.

Nominal zinc concentrations for the test were 2000, 1500, 750, 375, 188, and 0 µg Zn/L. A flow splitter allocated each concentration equally among four replicate exposure chambers. Flow rate of test solution to each exposure chamber was 40 mls/minute. Exposure chambers consisted of polyethylene containers with a capacity of 2.8 liters. Test solutions overflowed from the exposure chambers into a water bath. The temperature of the water bath was maintained at 12 °C using a temperature-controlled recirculator (VWR Scientific Products Recirculator).

Water quality parameters were measured weekly in alternating replicates Hardness and alkalinity were determined according to Standard Methods (APHA 1985). pH was measured using an Orion Research pH meter 811 calibrated prior to each use with pH 7.00 and pH 4.00 buffers. Conductivity was determined using a YSI Model 35 conductance meter. Dissolved oxygen was measured using a YSI Model 58 dissolved oxygen meter.

Twenty eyed brown trout eggs were randomly placed in each exposure chamber. Mortality was monitored and recorded daily. Exposure continued for sixty eight days at which time surviving fish were anaesthetized with MS222, blotted dry with a paper towel and lengths and weights measured.

Zinc Analysis

Water samples for zinc analysis were collected weekly from all treatments within a replicate. Replicates were alternated each week. Total (acid soluble) samples were collected in 60 ml high density polyethylene bottles and preserved with Ultrex®, triple distilled, nitric acid to pH <2 immediately. Samples were analyzed for zinc using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration curve verified using a NIST QAQC standard from an outside source.

Statistical Analyses

Survival data were transformed by arcsine squareroot prior to analysis (Snedecor and Cochran 1980). Hatching success, terminal length and weight and transformed survival data satisfied assumptions of normality and homogeneity of variance as evaluated by the Shapiro-Wilk's test and Bartlett's test, respectively ($\rho \leq 0.10$). Analysis of variance (ANOVA), followed by William's one-tailed multiple comparison test was used to compare means in the experimental treatments with that in the control. The highest zinc concentration not associated with a treatment effect (e.g. increased mortality, 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 (IC_{25} value), the concentration estimated to cause a 25% reduction in organism performance compared with the control, was also calculated (USEPA 1993) using terminal weight data. Mean treatment weights were determined as the combined weight of all surviving organisms divided by the total number of viable eggs at the start of the experiments. Thus, if an organism died, a weight value of zero was assigned.

RESULTS

Water quality parameters were stable throughout the course of the experiment (Table 9). All viable eggs hatched by 21 days after start of the experiment. Measured concentrations of zinc and associated hatching success are shown in Table 10. Hatching success in control treatments was very good. Hatching success in zinc exposures of 698 µg Zn/L or greater were reduced relative to the control (Table 10). The lowest observed effect concentration (LOEC) and no observed effect concentration (NOEC) were 698 and 280 µg Zn/L respectively. The chronic value based on hatching success was 381 µg Zn/L. In

general, survival was poor in all treatments including the control. Survival in zinc exposures of 1470 µg Zn/L and lower were all in the range of 50-65%. The highest zinc exposure concentration, 2139 µg Zn/L, had a somewhat lower survival rate of 34%. Survival was not significantly different than the control for any of the zinc exposures tested ($p>0.05$). A chronic value based on survival could not be determined. There was a considerable effect of zinc exposure on growth of brown trout. The LOEC and NOEC based on length or weight were 698 and 208 µg Zn/L, respectively. The chronic value based on length or weight is 381 µg Zn/L. The chronic values based on hatching success, length and weight are in good agreement with the estimated IC25 of 409 µg Zn/L.

DISCUSSION

The brown trout in this experiment were exposed to zinc for 21 days as eggs, 18 days as sac fry and 29 days as swimup fry. This first stage is generally regarded as tolerant while the latter stage as more sensitive stage for fish exposed to metal toxicants. It is therefore curious that we observed a reduction of hatching success due to zinc exposure but did not see an effect on overall survival (Table 10). This may be due to several factors. First of all, the variability within treatments of hatching success was rather low while the variability of survival was quite high. This decreased our ability to statistically detect an effect. Secondly, after hatch, mortality was between 20 and 30% in all the treatments including the control. It is likely that zinc exposure led to the death of more sensitive individuals leaving more tolerant ones. Finally, zinc exposure during the tolerant egg and sac fry stages may have provided an opportunity for the brown trout to acclimate to zinc. This phenomena has been widely reported for fish exposed to zinc (Roch and McCarter 1984, Bradley et al. 1985). This acclimation comes at an apparent energetic cost as the growth of trout in this experiment was greatly reduced at higher exposure levels. While not directly lethal, deceased growth would substantially increase risk of predation by larger and older fish.

The chronic value derived from this ELS test was 381 µg Zn/L based on hatching success, length and weight. The IC25 was estimated at 401 µg Zn/L. These values are similar other chronic values from unacclimated brown trout which ranged between 194 and 303 µg Zn/L (Table 2 page 8, this report) and 187 µg Zn/L for brown trout exposed in 50 mg $CaCO_3$/L hardness and 37 mg $CaCO_3$/L alkalinity (Table 15 page 27,also this report). The similarity of these numbers derived from several experiments serve to increase the confidence of the results and conclusions of each.

Table 9. Water quality parameters of Early Life Stage zinc toxicity test conducted with brown trout.

	pH (S.U.)	Temperature (°C)	Hardness (mg $CaCO_3$/L)	Alkalinity (mg $CaCO_3$/L)	Conductivity (µS/cm)	Dissolved Oxygen (mg O_2/L)
Mean	7.52	12.0	54.1	35.3	92.1	9.09
Std. Dev.	0.20	0.2	2.0	1.2	5.3	0.35

Table 10. Nominal and measured zinc concentrations and associated survival (%), length (mm) and weight (g) of brown trout in early life stage toxicity test. Standard deviations in parentheses.

Nominal Zinc Concentration (µg/L)	0	188	375	750	1500	2000
Measured Zinc Concentration (µg/L)	<10 (0)	66 (24)	208 (55)	698 (86)	1470 (152)	2139 (245)
Hatching Success (%)	91 (8)	81 (5)	84 (2)	72 (10)*	80 (8)*	64 (18)*
Survival (%)	65 (34)	59 (19)	60 (24)	52 (15)	51 (19)	34 (18)
Length (mm)	28.7 (1.3)	27.0 (1.2)	28.8 (1.6)	26.0* (1.5)	26.0* (0.5)	24.8* (0.8)
Weight (g)	0.184 (0.026)	0.147 (0.027)	0.180 (0.033)	0.105* (0.037)	0.113* (0.011)	0.101* (0.012)

* Indicates significantly less than control ($p<0.05$)

Chronic Value based on hatching success, length or weight = 381 µg Zn/L

IC25 = 409 µg Zn/L

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Effect of Hardness and Alkalinity on the Toxicity of Zinc to Brown Trout (*Salmo trutta*)

ABSTRACT

A series of toxicity experiments were conducted in order to determine the effect of elevated hardness alone (High Hardness-Low Alkalinity), elevated alkalinity alone (Low Hardness-High Alkalinity) , and both elevated hardness and alkalinity (High Hardness-High Alkalinity) on the toxicity of zinc to brown trout. Ninety six hour median lethal concentrations (LC50) and chronic values were compared to unenhanced water (Low Hardness-Low Alkalinity). The exposures lasted for a total of thirty days. Low Hardness-High Alkalinity had little or no effect on the acute or chronic toxicity of zinc when compared to the Low Hardness-Low Alkalinity experiment. The LC50 (95% C.I.) of the two tests were 690 µg Zn/L (565-816) and 1033 µg Zn/L (694-1372), respectively. The chronic values of the two were 219 and 187 µg Zn/L, respectively. The High Hardness-Low Alkalinity provided protection against the toxic effects of zinc when compared to the Low Hardness-Low Alkalinity test. The LC50 (95% C.I.) for the High Hardness-Low Alkalinity was 2267 µg Zn/L (1929-2604) and the chronic value was1009 µg Zn/L; about a 3-4 times reduction in toxicity. The acute and chronic toxicity of zinc in the High Hardness-High Alkalinity test could not be determined due to insufficient mortality. Growth (as measured by length and weight of surviving organisms) was not affected by zinc exposure in any of the water quality types.

INTRODUCTION

It is well recognized that increasing hardness reduces toxicity of most metals to aquatic organisms. Alkalinity is also thought to play an important role in the toxicity of metals as well. However, hardness and alkalinity are often correlated; in general as hardness increases alkalinity also increases. This has made it difficult to study the effects of hardness and alkalinity independent of each other. In this experiment, we seek to study the mitigating roles of hardness on toxicity of zinc independent of alkalinity and visa versa.

MATERIAL AND METHODS

Four experiments were conducted in order to assess the relative effects of hardness and alkalinity on the toxicity of zinc to brown trout. The experiments tested the toxicity of zinc to brown trout in four different water qualities. The water qualities tested consisted of 1) Low Hardness-Low Alkalinity, 2) High Hardness-Low Alkalinity, 3) Low Hardness-High Alkalinity and 4) High Hardness-High Alkalinity. The experimental parameters of each water quality type are described below.

Low Hardness-Low Alkalinity

This experiment was conducted using a modified Mount and Brungs intermittent flow diluter (Mount and Brungs 1967) supplied with Ft Collins city tap water which was cooled to approximately 13 degrees centigrade. The diluter delivered six concentrations of zinc and control. Nominal concentrations for this experiment were 800, 600, 450, 225, 112, 66 and 0 µg Zn /L added as zinc sulfate heptahydrate (Mallincrodt). Each diluter cycle delivered two liters of each concentration which was then split prior to flowing to 16 liter randomized glass aquaria so that each exposure concentration had two replicates. The diluter cycled every ten minutes. Brown trout used in the tests were received as eyed eggs from the Colorado Division of Wildlife Research Hatchery which obtained them from spawning operations conducted in North Delaney Butte Reservoir, Colorado. Brown trout were hatched and reared in dechlorinated Fort Collins City tap water until the start of the test. The trout were fed appropriately sized

Silver Cup trout food. Ten brown trout were randomly assigned to each aquarium. Mortalities were removed from the tanks, blotted dry with a paper towel and the length (mm) and weight (g) recorded. Water quality parameters were measured in each exposure three times during the first 96 hour acute period and weekly thereafter until the end of the test, thirty days after start of exposure. Hardness and alkalinity were determined according to Standard Methods (APHA). pH was determined using an Orion Research pH meter 811 calibrated daily using pH 7.00 and 4.00 pH buffers. Conductivity was determined using a YSI Model 35 conductance meter. Water samples for zinc analysis were collected daily during the first 96 hours of the test. Thereafter, water samples were collected weekly in each exposure level from alternating replicates. Samples were stored in 2 oz. high density polyethylene bottles and preserved by acidification to pH<2 using Ultrex nitric acid. Zinc exposure concentrations were measured using an Instrumentation Laboratory Video 22 atomic absorption spectrometer with air-acetylene flame using Smith-Hieftje background correction. The spectrometer was calibrated each day that analyses were performed.

High Hardness-Low Alkalinity

This experiment was conducted concurrently with the above experiment and followed the same procedures with the following exceptions. The water delivered to the Mount and Brungs diluter was modified by the addition of a stock solution of calcium chloride ($Ca(Cl)_2 \cdot 2H_2O$, Mallincrodt) so that the nominal final hardness was 200 mg $CaCO_3$/L. The hardness of the source water was measured daily to ensure proper operation of the system. The alkalinity would remain unchanged and near the Low Hardness-Low Alkalinity test at 37 mg $CaCO_3$/L. Because of the mitigating effects of hardness on zinc toxicity, the nominal zinc concentrations were increased to 2500, 1875, 406, 203, 102, 51 and 0 µg Zn /L, also added as zinc sulfate heptahydrate (Mallincrodt). The brown trout used in this experiment were acclimated to this water quality for seven days prior to the start of the experiment.

Low Hardness-High Alkalinity

This experiment followed the procedures of the High Hardness-Low Alkalinity experiment except that the water delivered to the diluter was modified using sodium bicarbonate (Mallincrodt). The alkalinity of the source water was measured daily to ensure the system was operating properly. The hardness would remain unchanged; near the Low Hardness-Low Alkalinity test at 54 mg $CaCO_3$/L. Nominal zinc exposure concentrations were the same as the High Hardness-Low Alkalinity experiment.

High Hardness-High Alkalinity

Dechlorinated Ft Collins city tap water was mixed with onsite well water to achieve a nominal hardness of 200 mg CaCO3/L and alkalinity of 140 mg CaCO3/L. Nominal zinc exposure concentrations were the same as the High Hardness-Low Alkalinity and Low Hardness-High Alkalinity experiments. This test was not replicated. Thirteen fish were placed in each aquarium.

Statistical Analyses

The 96 hour median lethal concentration (LC50) was obtained by probit analysis using Toxstat version 3.5 (West Inc and Gulley 1996). Mortality and growth were compared using ANOVA. Mortality data were transformed with arcsine square root (Snedecor and Cochran 1980). Normality and homogeneity of variance of data were verified using chi-square and Cochrans tests respectively. Means were compared using Williams test. The highest zinc concentration not associated with a treatment effect (e.g. increased mortality, 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.

RESULTS

Water Quality

Water quality parameters for the four experiments are presented in Table 11. Parameters in all cases were consistent throughout the 30 days of the tests. The final measured hardness of the High Hardness-High Alkalinity and High Hardness-Low Alkalinity tests were quite similar and near their target level of 200 mg $CaCO_3$/L. Likewise, the measured alkalinity of the High Hardness-High Alkalinity and Low Hardness-High Alkalinity tests were also nearly equal to each other. Addition of calcium chloride to adjust the hardness of the High Hardness-Low Alkalinity test did not appreciably alter any of the other measured water quality characteristics. However, this was not true of the addition of sodium bicarbonate to adust alkalinity in the Low Hardness-High Alkalinity test. Addition of sodium bicarbonate increased the pH of the water to about 8.4, all other water quality characteristic measured remained unchanged.

Acute Toxicity

Measured zinc concentrations and associated mortality during the first 96 hours of the test are presented in Table 12 for the four water quality types tested. Zinc concentrations were stable throughout the test. With the exception of the Low Hardness-High Alkalinity, none of the tests achieved 100% mortality. The estimated 96 hour LC50 are shown in Table 13. The LC50s of the Low Hardness-Low Alkalinity and Low Hardness-High Alkalinity water quality types were not significantly different. The High Hardness-Low Alkalinity was significantly greater than both the Low Hardness-Low Alkalinity and Low Hardness-high Alkalinity experiments. A LC50 for High Hardness-High Alkalinity test could not be calculated due insufficient mortality.

Chronic Toxicity

Mortality of brown trout exposed to zinc for thirty days in each of the different water qualities are shown in Table 14. Comparison of Table 14 with Table 12 shows that the large majority of mortality occurred within the initial 96 hours of exposure. Zinc exposure concentrations continued to remain consistent. The No observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) range for the Low Hardness-Low Alkalinity test was 134-261 µg Zn/L. This is nearly identical to the NOEC-LOEC range for the Low Hardness-High Alkalinity test which was 162-297 µg Zn/L The chronic values for the Low Hardness-Low alkalinity and Low Hardness-High Alkalinity were 187 and 219 µg Zn/L, respectively. The NOEC-LOEC range for the High Hardness-Low Alkalinity test was considerably greater at 743-1370 µg Zn/L with a chronic value of 1009. The LOEC-NOEC could not be calculated for the High Hardness-High Alkalinity test. The NOEC, LOEC and chronic value of the tests are summarized in Table 15.

The weights in grams of brown trout surviving the zinc exposures are presented in Table 16. Weight of brown trout were not affected by zinc exposure in any of the water quality types ($p>0.05$). Lengths were similarly unaffected (Table 17).

DISCUSSION

While data are not clear cut, increased alkalinity clearly did not substantially affect toxicity of zinc on either an acute or chronic basis. The 96 hour median lethal concentration (LC50) of the Low Hardness-Low Alkalinity was 1033 µg Zn/L while the Low Hardness-High Alkalinity test was lower at 690 µg Zn/L (Table 13). The 95% confidence of the two intervals overlap indicating no statistical difference. The Lowest Observed Effect Concentration (LOEC) and No Observed Effect Concentration (NOEC) of the two tests were similar as well. This is in spite of the fact that the alkalinity of the Low Hardness-High Alkalinity was over 100 mg /L greater than the Low Hardness-Low alkalinity test; a 3.7 fold increase.

Increasing the hardness provided much greater protection against zinc toxicity. The LC50 of the High Hardness-Low Alkalinity was 2267 µg Zn/L; double the LC50 of the Low Hardness-Low Alkalinity test (Table 13). The LOECs of the High Hardness-Low Alkalinity test and Low Hardness-Low Alkalinity test were 1370 and 261 respectively (Table 14). Since all other measured water quality characteristics between the two tests were the same (Table 11), the increased hardness must account for the decrease in zinc toxicity.

Of the four water quality types tested, the High Hardness-High Alkalinity seemed to be the least toxic. A LC50 for this water quality type could not be calculated and the test was not replicated so that LOEC and NOEC could not be determined. The acute and chronic mortality in the High Hardness-High Alkalinity was a little lower than the High Hardness-Low Alkalinity test at similar concentrations, though this difference in percent mortality is sometimes rather small (Table 13). The increased pH of the High Hardness-High Alkalinity test may account for some of this difference (Table 11). However, an even greater pH in the Low Hardness-High Alkalinity test (caused by the addition of sodium bicarbonate) failed to provide any noticeable protection against zinc toxicity.

Reduced growth is frequently affected by exposure to zinc, often at concentrations lower than those causing reduced survival. Neither weight nor length was significantly affected by zinc exposure in any of the four water quality types tested. The most likely reason for this is the duration of zinc exposure (30 days) was insufficient to cause an observable effect.

Table 11. Water quality characteristics of zinc toxicity tests for the different experiments.

	Low Hardness-Low Alkalinity	High Hardness-Low Alkalinity	Low Hardness-High Alkalinity	High Hardness-High Alkalinity
Hardness (mg $CaCO_3$/L)	54.4 (2.1)	206.7 (9.5)	54.0 (1.9)	207.2 (2.5)
Alkalinity (mg $CaCO_3$/L)	37.4 (1.6)	37.5 (1.9)	139.6 (5.0)	141.4 (1.8)
pH (S.U.)	7.54 (0.37)	7.69 (0.34)	8.41 (0.13)	7.92 (0.10)
Temperature (C)	13.5 (0.7)	13.6 (0.7)	13.6 (0.6)	14.5 (0.7)
Conductivity (S/cm)	97.6 (6.2)	371.2 (12.6)	233.8 (9.8)	360.0 (0.6)
Dissolved Oxygen (mg/L)	9.29 (0.36)	9.18 (0.57)	9.32 (0.35)	8.96 (0.39)

Table 12. Zinc exposure concentrations (µg/L) and associated brown trout mortality (%) during the initial 96 hours of exposure. Standard deviations in parentheses.

LOW HARDNESS LOW ALKALINITY							
Zn Conc.	<10 (0)	78 (7)	136 (16)	260 (16)	465 (8)	647 (11)	846 (44)
Mortality	0 (0)	0 (0)	5 (7)	10 (0)	20 (14)	20 (0)	35 (7)
LOW HARDNESS HIGH ALKALINITY							
Zn Conc	<10 (0)	176 (81)	311 (70)	779 (108)	1452 (57)	2137 (171)	2473 (177)
Mortality	0 (0)	5 (7)	10 (0)	60 (14)	100 (0)	100 (0)	100 (0)
HIGH HARDNESS LOW ALKALINITY							
Zn Conc	<10 (0)	52 (26)	360 (11)	714 (70)	1321 (87)	1746 (146)	2337 (60)
Mortality	0 (0)	0 (0)	0 (0)	0 (0)	20 (14)	15 (7)	55 (21)
HIGH HARDNESS HIGH ALKALINITY							
Zn Conc	<10 (0)	246 (27)	443 (7)	783 (14)	1380 (21)	2000 (98)	2660 (201)
Mortality	0	0	0	0	8	8	15

Table 13. Ninety six hour median lethal concentrations and 95% confidence intervals of zinc to brown trout for the different water quality types tested.

	LC50 (µg Zn/L)	95 % Confidence Interval
Low Hardness-Low Alkalinity	1033	694-1372
Low Hardness-High Alkalinity	690	565-816
High Hardness-Low Alkalinity	2267	1929-2604
High Hardness-High Alkalinity	Unable to calculate, >2660	--

Table 14. Zinc exposure concentrations (µg/L) and associated brown trout mortality (%) following 30 days exposure in the different water qualities. Standard deviations in parentheses.

LOW HARDNESS LOW ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	77 (5)	134 (13)	261 (11)	472 (11)	655 (130	841 (32)
Mortality (%)	0 (0)	0 (0)	5 (7)	15 (7)*	21 (12)*	28 (8)*	50 (0)*
LOW HARDNESS HIGH ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	162 (60)	297 (53)	748 (87)	1493 (70)	2164 (129)	2532 (150)
Mortality (%)	0 (0)	6 (8)	11 (1)*	63 (10)*	100 (0)*	100 (0)*	100 (0)*
HIGH HARDNESS LOW ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	45 (22)	370 (17)	743 (63)	1370 (91)	1778 (113)	2356 (51)
Mortality (%)	0 (0)	0 (0)	0 (0)	0 (0)	22 (11)*	17 (7)*	65 (7)*
HIGH HARDNESS HIGH ALKALINITY†							
Zn Conc. (µg/L)	<10 (0)	243 (20)	451 (13)	791 (15)	1392 (22)	2000 (70)	2598 (166)
Mortality (%)	0	0	0	0	8	8	15

*Indicates mortality greater than control ($p<0.05$)

†Unable to statistically compare mortality due to lack of replication.

Table 15. No Observed Effect Concentrations (NOEC), Lowest Observed Effect Concentrations (LOEC) and chronic values (µg Zn/L) based on reduced survival.

	NOEC	LOEC	Chronic Value
Low Hardness-Low Alkalinity	134	261	187
Low Hardness-High Alkalinity	162	297	219
High Hardness-Low Alkalinity	743	1370	1009
High Hardness-High Alkalinity	Unable to Calculate		

Table 16. Zinc exposure concentrations (µg/L) and associated weights (g) of surviving brown trout following 30 days exposure in the different water qualities. Standard deviations in parentheses.

LOW HARDNESS LOW ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	77 (5)	134 (13)	261 (11)	472 (11)	655 (130	841 (32)
Weight (g)	2.394 (0.218)	2.598 (0.071)	2.511 (0.075)	2.888 (0.264)	2.770 (0.002)	3.110 (0.332)	3.117 (0.027)
LOW HARDNESS HIGH ALKALINITY							
Zn Conc (µg/L)	<10 (0)	162 (60)	297 (53)	748 (87)	1493 (70)	2164 (129)	2532 (150)
Weight (g)	2.617 (0.081)	3.504 (0.392)	3.136 (0.286)	3.656 (0.578)	--	--	--
HIGH HARDNESS LOW ALKALINITY							
Zn Conc (µg/L)	<10 (0)	45 (22)	370 (17)	743 (63)	1370 (91)	1778 (113)	2356 (51)
Weight (g)	2.788 (0.066)	2.742 (0.292)	2.938 (0.011)	2.651 (0.297)	3.358 (0.0255)	3.052 (0.376)	3.298 (0.168)
HIGH HARDNESS HIGH ALKALINITY							
Zn Conc (µg/L)	<10 (0)	243 (20)	451 (13)	791 (15)	1392 (22)	2000 (70)	2598 (166)
Weight (g)	2.936	3.157	3.079	2.752	3.032	3.008	2.800

Table 17. Zinc exposure concentrations (µg/L) and associated lengths (mm) of surviving brown trout following 30 days exposure in the different water qualities. Standard deviations in parentheses.

LOW HARDNESS LOW ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	77 (5)	134 (13)	261 (11)	472 (11)	655 (130	841 (32)
Length (mm)	61.0 (1.3)	62.9 (0.0)	61.8 (0.1)	64.8 (2.2)	64.3 (0.4)	65.3 (4.0)	66.3 (0.4)
LOW HARDNESS HIGH ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	162 (60)	297 (53)	748 (87)	1493 (70)	2164 (129)	2532 (150)
Length (mm)	62.7 (0.6)	68.1 (4.7)	67.1 (1.3)	67.6 (3.0)	--	--	--
HIGH HARDNESS LOW ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	45 (22)	370 (17)	743 (63)	1370 (91)	1778 (113)	2356 (51)
Length (mm)	63.6 (0.7)	63.8 (1.9)	63.9 (1.1)	62.5 (2.0)	66.8 (0.8)	65.4 (2.6)	64.8 (1.6)
HIGH HARDNESS HIGH ALKALINITY							
Zn Conc. (µg/L)	<10 (0)	243 (20)	451 (13)	791 (15)	1392 (22)	2000 (70)	2598 (166)
Length (mm)	66.2	67.2	66.7	65.2	66.9	66.4	64.3

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Acute Toxicity of Silver to Brook trout (*Salvelinus fontinalis*) in soft water

MATERIAL AND METHODS

Silver (added as silver nitrate) was delivered to 90 liter aquaria using a Mount and Brungs diluter (1967) modified to deliver six concentrations and control. Diluter delivered 2 liters per cycle at a rate of one cycle every five minutes. Dilution water consisted of dechlorinated Fort Collins municipal tap water. Nominal exposure concentrations were 20.0, 15.0, 11.25, 7.60, 3.60, 2.00 and 0 µg Ag/L. Ten brook trout were randomly placed into each aquarium and were not fed during the course of the acute exposure. Mean length and weight of brook trout was 147 mm and 26.722 grams respectively. The experiment was conducted under dim artificial lighting with a photoperiod that was controlled by an outdoor photocell. Mortality was monitored every 2 hours during the day. Fatalities were recorded, blotted dry with a paper towel then measured for length and weight. Exposure continued for 168 hours whereupon surviving brook trout were terminally anesthestized with MS-222, blotted dry with a paper towel and measured for length and weight. Water samples for silver analysis were collected three times during the first 96 hours of exposure and analyzed immediately. Silver concentrations were measured using a Thermo Jarrell Ash SH4000 atomic absorption spectrometer with CTF180 graphite furnace. Background was corrected using Smith-Hieftje. Ammonium phosphate (0.1%) was added as a matrix modifier. Water quality parameters were measured daily from a randomly selected aquarium. Hardness and alkalinity were measured using Standard methods (APHA). pH was determined using an Orion Research pH meter 811 calibrated prior to each use with pH 7.00 and 4.00 pH buffers. Conductivity was determined using a YSI Model 35 conductance meter. Median lethal concentration of silver was calculated using the Spearman-Karber technique (Hamilton et al. 1977, Hamilton et al. 1978).

RESULTS

Silver exposure concentrations and associated mortality are shown in Table 18. Measured concentrations of silver were constant throughout the duration of the test. Exposure to the highest concentration of silver used, 18.1 µg/L, resulted in complete mortality. Exposure to 14.68 and 10.98 µg/L resulted in 80 and 30 percent mortality respectively. There was no mortality at concentrations lower than 7.6 µg/L. No additional mortality occurred between 96 hours and 168 hours. The median lethal concentration was 11.74 µg Ag/L with a 95% confidence interval of 10.22-13.49 µg Ag/L. Water quality parameters were constant throughout the duration of the test (Table 19).

DISCUSSION

The median lethal concentration derived from this experiment was 11.74 µg Ag/L. This value is considerably greater than the Colorado state standard for the protection of aquatic life which is 0.6 µg Ag/L for a hardness of 50 mg $CaCO_3$/L. However, the size of fish used in this experiment were fairly large at 147 mm. Younger fish are generally regarded as more sensitive to the effects of metals. Additional testing using a more sensitive life stage would provide more useful information regarding the acute toxicity of silver to brook trout.

Table 18. Silver concentrations (µg/L) and associated mortality of brook trout after 96 hours of exposure. Standard deviations in parentheses.

Nominal Concentration (µg/L)	20.0	15.0	11.25	7.60	3.60	2.00	0
Measured Concentration (µg/L)	18.10 (0.91)	14.68 (0.82)	10.98 (0.35)	6.26 (0.37)	3.52 (0.11)	1.84 (0.10)	<0.20 (0.00)
Mortality (%)	100	80	30	0	0	0	0

Table 19. Water quality parameters measured during the acute silver toxicity experiment. Standard deviations in parentheses.

Hardness (mg $CaCO_3$/L) 50.0 (1.1)
Alkalinity (mg $CaCO_3$/L) 37.0 (0.8)
pH (S.U.) 7.55 (0.26)
Dissolved Oxygen (mg/L) 9.6 (0.6)
Temperature (°C) 11.7 (0.1)
Conductivity (µS/cm) 91.6 (12.7)

APHA. 1985. *Standard Methods for the Examination of Water and Wastewater,* 16th edn. American Public Health Association, American Water Works Association, and Water Pollution Control Federation. Washington, D.C.

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PLAN C: TECHNICAL ASSISTANCE

Objective: To provide expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Division of Wildlife personnel, and other state and federal agencies.

Job C1. Water Quality Assistance to Other Personnel

Objectives:

1. To oversee the training and evaluation of metal analysis by laboratory technicians.

2. To assist Division and other state and federal personnel in the analysis and toxicological assessment of water quality data.

3. To develop and maintain a quality assurance program to evaluate the quality of analytical results for metals.

4. To collect and analyze metals concentrations in samples from the Arkansas River.

Water quality characteristics and or metal analyses were performed for the following persons and agencies:

Barb Horn, Colorado Division of Wildlife (CDOW)
John Woodling, CDOW
Mark Jones, CDOW
Phil Schler, CDOW
Mike Japhet, CDOW
Bill Emblad, CDOW
Dan Brauch, CDOW
Mike Pruel, Colorado state University (CSU)
Elizabeth Harrahy, CSU
Lisa Courtney, CSU
Will Clements, CSU
Cathy Bedwell, CSU
Del Nimmo, U. S. Geological Survey (USGS)

ACCOMPLISHMENTS:

Two toxicity experiments were conducted to assist with investigations into potential water quality related causes for the decline of boreal toads in Colorado and are reported below.

Toxicity of manganese to Boreal toad tadpoles (*Bufo boreas*)

ABSTRACT

Boreal toad eggs were exposed to 80, 40, 20, 10, 5.0, 2.5, 1.25 and 0 mg Mn/L. Exposure continued until just prior to metamorphosis into toadlets. Exposed organisms were monitored for effects on mortality, development, growth and whole body accumulation of manganese. The median lethal concentration (LC50) of manganese during the first 96 hours of exposure was 16.7 mg Mn/L with a 95%

confidence interval of 13.8-20.2. The no observed effect concentration (NOEC) based on mortality after 2 weeks of exposure was 2.16 mg Mn/L. The lowest observed effect level (LOEC) was 3.41mg Mn/L. The NOEC-LOEC range remained unchanged after 6 weeks. Development of boreal toad tadpoles was affected by manganese at the same NOEC-LOEC range as mortality after 4 and 6 weeks but was unaffected after 2 weeks of exposure. Weight, total length and snout-vent length were also affected at the same NOEC-LOEC range after 2 and 4 weeks exposure. Weight was not affected after 6 weeks of exposure. This was due to decreased development of manganese tadpoles relative to the controls. Weight loss associated with metamorphosis occurred in control exposures prior to the manganese exposures. This masked the growth differences of the controls relative to the manganese exposed tadpoles. Whole body accumulation did not increase proportionally to exposure concentrations but rather reached a plateau. Duration of exposure and developmental stage may play a role in whole body manganese content.

INTRODUCTION

Boreal toad (*Bufo boreas*) numbers have declined in Colorado over the last quarter century. They have been listed by the state of Colorado as endangered since November 1993 and federally listed as "warranted but precluded" since March 1995 (Goettl [eds.] and the Boreal Toad Recovery Team 1997). The Colorado Division of Wildlife Aquatic Toxicology Laboratory is assisting with investigations into possible causes of this decline by evaluating water quality characteristics that may limit survival and distribution of boreal toad tadpoles. These efforts include analysis of water samples collected from current and historic breeding ponds, developing techniques to measure effects of toxicants (heavy metals, pesticides, deicing compounds) to tadpoles, and conducting experiments to determine toxicity of selected metals to boreal toad tadpoles. The goals of the toxicity experiments are to develop background toxicity data for boreal toads as well as identify important endpoints and techniques for toxicity testing with amphibians. The toxicity of manganese to boreal toad tadpoles was previously investigated using both static and flow-through techniques (Davies and Brinkman 1998). However the duration of exposure was limited to ten days. Toxicity experiments conducted with cadmium and copper have demonstrated that longer term exposures can cause a range of sublethal effects such as reduced growth and development (Davies and Brinkman 1997). This experiment investigates the effects of longer exposures of manganese on survival, growth, and development of boreal toad tadpoles.

MATERIAL AND METHODS

A serial diluter (Benoit et al. 1982) delivered seven concentrations of manganese (as manganese sulfate monohydrate) and control to completely randomized 2.2 liter glass exposure chambers at a rate of twenty mls/minute. The experiment was replicated four times with nominal manganese exposure concentrations of 80.0, 40.0, 20.0, 10.0, 5.0, 2.5, 1.25 and 0 mg Mn/L. Source water consisted of dechlorinated Fort Collins tap water. The exposure chambers were placed in a water bath maintained near 20°C using a Remcor water recirculating heater/cooler. Two fluorescent blacklights and two florescent full spectrum (Repta Sun) bulbs suspended approximately 12 inches above the exposure chambers provided artificial lighting with 16 hour light and 8 hour dark photoperiod. Twenty boreal toad tadpoles about Gosner stage 16-17 (Gosner 1960) were randomly placed in each exposure chamber. Tadpoles were fed a mixture of Mazuri, Silver Cup trout food and frozen romaine lettuce *ad libitum*. Exposure chambers were checked daily for mortality.

Four tadpoles were subsampled from each treatment after 2, 4, and 6 weeks in order to assess effects of manganese exposure on development, growth, and whole body manganese content. Tadpoles were sampled only from treatments with sufficient survival. Total length and snout-vent length were not determined at 6 weeks because the tadpoles started metamorphosis into toadlets and were resorbing their tail. After anesthesia with MS-222, tadpole development was measured using a rating system developed by Gosner (1960). Growth was assesed by measuring weight (g), total length and snout-vent length

(mm). Tadpoles were rinsed with deionized water and placed in polypropylene centrifuge tubes and dried to constant weight at 80°C. The tadpoles were digested with trace metal grade nitric acid and heated for four hours in a dry bath at 100°C. Trace metal grade hydrogen peroxide was added and the tubes heated an additional four hours. The digests were diluted to volume with deionized water and analyzed for manganese as described below.

Water quality parameters were measured in each exposure weekly. Hardness and alkalinity were determined according to Standard Methods (APHA). pH was determined using an Orion Research pH meter 811 calibrated daily using pH 7.00 and 4.00 pH buffers. Conductivity was determined using a YSI Model 35 conductance meter. Hardness and conductivity were measured only in control treatments due to interference of manganese on these measurements.

Water samples for manganese analysis were collected twice during the first 96 hours of the test. Thereafter, water samples were collected weekly in each exposure level from alternating replicates. Samples were stored in 2 oz. high density polyethylene bottles and preserved by acidification to pH<2 using Ultrex nitric acid. Manganese concentrations in water samples and tissue digests were measured using an Instrumentation Laboratory Video 22 atomic absorption spectrometer with air-acetylene flame with Smith-Hieftje background correction.

Statistics

The 96-hour median lethal concentration (LC50) was determined using Spearman-Karber (Hamilton et al. 1977, Hamilton et al. 1978). Analysis of variance (ANOVA) was performed on mortality, development and growth (weight, total length and snout-vent length) data using SAS computer software (SAS 1989). Mortality data were transformed by arcsine squareroot prior to analysis (Snedecor and Cochran 1980). Assumptions of normality and homogeneity of variance were evaluated using Shapiro Wilk's test and Bartlett's test respectively ($p<0.10$). Treatment means were compared using one-tailed Dunnett's multiple comparison test ($p<0.05$) to evaluate differences from control.

RESULTS

Water quality parameters were constant throughout the test (Table 20). Mean hardness and alkalinity were approximately 50 and 35 mg $CaCO_3$ /L respectively. Temperature was near 20°C, conductivity near 100 S/cm and pH was slightly above neutral at 7.4. Dissolved oxygen was more than adequate to sustain the tadpoles and indicate that flow rates were sufficient given the number of test organisms and food present in the exposure chambers. Manganese exposure concentrations were likewise consistant throughout the acute and six week exposure periods (Tables 21 and 22).

Mortality

Acute mortality from the first ninetysix hours of exposure is shown in Table 21. The 96 hour median lethal concentration (LC50) was 16.7 mg/L with a ninetyfive percent confidence interval between 13.8 and 20.2 mg /L. Mortality after 2, 4, and 6 weeks exposure are shown in Table 22. Between ninetysix hours and two weeks, mortality rates continued to increase in manganese exposure concentrations above 3 mg Mn/L. After two weeks, mortalities leveled off and did not increase significantly over the next 4 weeks. Survival in the control treatments was 90% or greater. The lowest observed effect concentration (LOEC) resulting in increased mortality compared to the control was 3.41 mg/L at 2, 4, and 6 weeks. The no observed effect concentration (NOEC) based on mortality was 2.16 mg/L.

Development

Gosner stage of development sampled after 2, 4, and 6 weeks are shown in Table 23. Development was not affected after 2 weeks of exposure. After 4 and 6 weeks of exposure, manganese had a LOEC of 3.41 mg Mn/L. At concentrations of 2.16 mg/L (the NOEC) and below there were no statistical differences from the control.

Growth

Mean weight of tadpoles sampled after 2, 4, and 6 weeks of exposure to manganese are shown in Table 24. Tadpoles exposed to 3.41 mg Mn/L for 2 and 4 weeks weighed significantly less than the control tadpoles. Tadpoles exposed to this concentration of manganese weighed 73 and 48% of the controls after 2 and 4 weeks respectively. There were no significant differences in tadpole weight at 6 weeks. Between 4 and 6 weeks the tadpoles began to metamorphose into toadlets. During metamorphosis, tadpoles lost a lot of weight (Table 24). Tadpoles in the control exposure lost substantially more weight between 4 and 6 weeks than the other manganese exposure groups. In fact, the highest manganese exposure group sampled actually gained weight between the 4th and 6th week.

Total length of tadpoles was also affected by manganese exposure (Table 25). Tadpoles exposed to 3.41 mg/L were significantly shorter than control tadpoles after 2 and 4 weeks. Snout-vent lengths of tadpoles were affected at similar concentrations as total length (Table 26).

Whole Body Manganese Content

Boreal toad tadpoles stongly accumulated manganese though not in a dose dependent manner (Figure 1.). All exposure concentrations resulted in significantly greater manganese content than controls. Whole body manganese content increased sharply between control and 1.28 mg Mn/L but leveled off at higher manganese exposure concentrations. For all exposure levels, whole body manganese content increased between 2 and 4 weeks of exposure. However, after 6 weeks of exposure, manganese content decreased to levels below those at 2 weeks.

DISCUSSION

The 96 hour median lethal concentration (LC50) of manganese to boreal toad tadpoles has been previously reported as 42.3 mg/L (Davies and Brinkman 1998). Although conducted with similar water quality characterics, the 96 hour LC50 derived from this experiment was somewhat lower at 16.7 mg/L. This difference may result from using flow-through methodologies instead of static renewal and also utilized a slightly less developed stage of tadpole. Metals in static renewal tests may sorb on to food and container walls reducing bioavailability when compared to flow-through tests. Earlier stages of development of *Bufo arenarum* embryos have been found to be more sensitive to cadmium (Ferrari et al 1993, Herkovits and Perez-Coll 1993). After the first 96 hours of exposure, mortality rates in manganese exposure above 2.16 mg/L increased until 2 weeks of exposure. At 2 weeks, the NOEC and LOEC based on mortality were 2.16 and 3.41 mg/L respectively. Mortality rates after 2 weeks were esentially the same and did not affect the NOEC-LOEC range (Table 22). Metal-related mortality may have ceased after the first 2 weeks for several reasons. First, sensitive individuals within a treatment may have died shortly after exposure to manganese leaving more tolerant individuals remaining. Second, tadpoles may acclimate to manganese after the initial week or so. Finally, the initial 2 weeks of the experiment may have occurred during a more sensitive life stage which the tadpoles quickly developed out of. All three of these factors probably combined to give the observed mortality patterns which have also been observed with boreal toad tadpoles exposed to copper (Davies and Brinkman 1977) and zinc (this report). Additional research is necessary to evaluate relative sensitivity of different life-stages of boreal toad tadpoles. If it is found that some life stages are much more sensitive than others, then the timing of metal inputs to breeding ponds may be more important to the survival of tadpoles than the magnitude of that input.

Exposure to manganese resulted in delayed development (Table 23) and decreased growth (Tables 24-26). These sublethal effects occurred at manganese concentrations that also reduced survival. This is unlike previous toxicity experiments conducted with cadmium, copper (Davies and Brinkman 1997) and zinc (this report) where growth and development were affected at much lower concentrations than those that affected survival. These sublethal effects are a common response of boreal toad tadpoles to other metals such as cadmium, copper (Davies and Brinkman 1977), and zinc (this report). Sublethal effects may impact survival of tadpoles in natural breeding ponds and reduce recruitment to adults. For example,

reduced growth of tadpoles may result in increased risk of predation from dytisid beetle larvae (L. Livo, personal communication) and and lead to decreased overwinter survival of toadlets. A delay in development could be lethal to tadpoles inhabiting temporary ponds. Rate of development is particularly important for boreal toad tadpoles which are usually found above an elevation of 8,000 feet. Short summers at such elevations require that tadpoles quickly develop prior to onset of winter. Failure of tadpoles to develop into toadlets and disperse before the first big snowfall is likely to be fatal. Use of length or wet weight to evaluate growth of tadpoles may not be appropriate in situations where development is also affected. During metamorphosis, length and weight both decrease. This can lead to instances where a more developed tadpole has a shorter length and weighs less than a less developed tadpole. A more appropriate approach may be to normalize growth to development by measuring length and weight at a particular

Whole body accumulation of manganese by boreal toad tadpoles was poorly related to exposure concentrations. Tadpoles exposed to 1.28 mg Mn/L accumulated similar levels of manganese as tadpoles exposed to much higher levels. Possible explanations for this phenomenon are that excretion of manganese increases to compensate for greater uptake at higher concentrations. Alternately, rate of uptake may be actively regulated by the tadpoles or pathways of uptake may become saturated at high enough concentrations of manganese. Another interesting aspect of manganese accumulation is the effect of time. Manganese accumulation increased between 2 and 4 weeks but declined to much lower levels between the 4th and 6th week of exposure. A similar pattern was observed with accumulation of cadmium by boreal toad tadpoles (Davies and Brinkman 1997). The most likely explanation for the dramatic loss of manganese during this time is that the process of metamorphosis results in substantial loss or excretion of manganese, cadmium and perhaps other metals as well. Whole body manganese content in boreal toad tadpoles would make a poor bioindicator of exposure. Accumulation of manganese is poorly related to exposure concentrations above about 1 mg/L. Developmental stages also play an important role. Combined, these two factors would make interpretation of manganese content difficult.

Table 20. Mean, standard deviation and range of water quality parameters of exposure water during manganese toxicity test.

	Mean	Standard Deviation	Range
Hardness (mg $CaCO_3$/l)	51.7	1.2	50.4-53.7
Alkalinity (mg $CaCO_3$/l)	35.2	1.3	32.4-38.6
pH (S.U.)	7.38	0.18	7.14-7.76
Dissolved Oxygen (mg/L)	8.3	0.4	7.5-9.0
Temperature (°C)	19.4	1.2	17.5-21.7
Conductivity (µS/cm)	101.5	3.5	97.0-106.

Table 21. Manganese concentrations (mg/l) and associated acute mortality (%) of boreal toad tadpoles during the first 96 hours of the flow-through toxicity test. Standard deviations in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Mortality (%)
0	<0.02 (0)	2.5 (2.9)
1.2	1.33 (0.02)	0 (0)
2.5	2.50 (0.05)	3.8 (4.8)
5.0	4.06 (0.08)	3.8 (2.5)
10.0	8.44 (0.00)	33.8 (20.6)
20.0	19.4 (0.4)	33.8 (34.2)
40.0	35.8 (0.1)	91.2 (11.8)
80.0	92.2 (0.8)	100 (0)

LC50 = 16.7 mg Mn/L (13.8-20.2)

Table 22. Manganese concentrations (mg/L) and associated mortality (%) after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Mortality (%)		
		2 Weeks	4 Weeks	6 Weeks
0	<0.02 (0)	3.8 (4.8)	6.5 (4.7)	6.5 (4.7)
1.25	1.28 (0.07)	10.0 (10.0)	10.0 (10.0)	10.0 (10.0)
2.50	2.16 (0.3)	6.2 (4.8)	13.0 (10.4)	13.0 (10.4)
5.00	3.41 (0.5)	40 (5.8) *	40.0 (5.8) *	40.0 (5.8) *
10.0	7.45 (0.7)	97.5 (2.9) *	97.5 (2.9) *	97.5 (2.9) *
20.0	19.4 (0.4)	100 (0) *	100 (0) *	100 (0) *
40.0	35.8 (0.1)	100 (0) *	100 (0) *	100 (0) *
80.0	92.2 (0.8)	100 (0) *	100 (0) *	100 (0) *

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

Table 23. Manganese concentrations (mg/L) and associated Gosner stage of development after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Gosner Stage		
		2 Weeks	4 Weeks	6 Weeks
0	<0.02 (0)	29.6 (0.9)	36.9 (0.4)	39.4 (1.2)
1.25	1.28 (0.07)	30.1 (0.8)	37.1 (0.1)	39.5 (0.3)
2.50	2.16 (0.3)	29.8 (0.7)	36.4 (1.2)	39.0 (1.3)
5.00	3.41 (0.5)	29.2 (0.7)	32.9 (2.3)*	37.5 (0.8)*

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

Table 24. Manganese concentrations (mg/L) and associated weight (g) after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Weight (g)		
		2 Weeks	4 Weeks	6 Weeks
0	<0.02 (0)	0.306 (0.026)	0.923 (0.058)	0.828 (0.098)
1.25	1.28 (0.07)	0.295 (0.148)	0.868 (0.086)	0.899 (0.066)
2.50	2.16 (0.3)	0.262 (0.052)	0.900 (0.188)	0.819 (0.199)
5.00	3.41 (0.5)	0.222 (0.077)*	0.443 (0.226)*	0.711 (0.254)

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

Table 25. Manganese concentrations (mg/L) and associated total length (mm) after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Total Length (mm)	
		2 Weeks	4 Weeks
0	<0.02 (0)	28.6 (0.8)	46.9 (1.2)
1.25	1.28 (0.07)	28.0 (0.3)	46.6 (0.4)
2.50	2.16 (0.3)	26.6 (1.5)	44.9 (3.8)
5.00	3.41 (0.5)	25.1 (2.7)*	33.9 (6.5)*

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

Table 26. Manganese concentrations (mg/L) and associated snout-vent length (mm) after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (mg/L)	Measured Concentration (mg/L)	Snout-vent Length (mm)	
		2 Weeks	4 Weeks
0	<0.02 (0)	11.7 (0.4)	17.3 (0.4)
1.25	1.28 (0.07)	11.4 (0.1)	17.1 (0.4)
2.50	2.16 (0.3)	10.6 (0.7)	17.1 (1.3)
5.00	3.41 (0.5)	10.1 (1.1)*	14.2 (1.8)*

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

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Toxicity of zinc to Boreal toad tadpoles (*Bufo boreas*)

ABSTRACT

Boreal toad eggs were exposed to 4000, 2000, 1000, 500, 250, 125, 68 and 0 µg Zn/L. Exposure continued until just prior to metamorphosis into toadlets. Exposed organisms were monitored for effects on mortality, development, growth and whole body accumulation of zinc. The median lethal concentration (LC50) of zinc during the first 96 hours of exposure was 840 µg Zn/L with a 95% confidence interval of 760-929. The no observed effect concentration (NOEC) based on mortality after 2 weeks of exposure was 404 µg Zn/L. The lowest observed effect level (LOEC) was 922 µg Zn/L. The NOEC-LOEC range remained unchanged after 6 weeks. Development of boreal toad tadpoles was unaffected after 2 weeks of exposure to zinc but after 4 and 6 weeks, the NOEC-LOEC range was 172-404 µg Zn/L. Weight was significantly reduced at all zinc exposure concentrations after 2 weeks but this effect was gone by 4 weeks. After 2 weeks of exposure, total length was reduced at 404 µg Zn/L but not at 172 µg Zn/L. As with weight, total length recovered and their was no statistical effect after 4 weeks. Snout-vent length was not affected. Whole body accumulation of zinc increased proportionally to exposure concentrations but were unaffected by the duration of exposure.

INTRODUCTION

Boreal toad (*Bufo boreas*) numbers have declined in Colorado over the last quarter century. They have been listed by the state of Colorado as endangered since November 1993 and federally listed as "warranted but precluded" since March 1995 (Goettl [eds.] and the Boreal Toad Recovery Team 1997). The Colorado Division of Wildlife Aquatic Toxicology Laboratory is assisting with investigations into possible causes of this decline by evaluating water quality characteristics that may limit survival and distribution of boreal toad tadpoles. These efforts include analysis of water samples collected from current and historic breeding ponds, developing techniques to measure effects of toxicants (heavy metals, pesticides, deicing compounds) to tadpoles, and conducting experiments to determine toxicity of selected metals to boreal toad tadpoles. The goals of the toxicity experiments are to develop background toxicity data for boreal toads as well as identify important endpoints and techniques for toxicity testing with amphibians. The toxicity of zinc to boreal toad tadpoles was previously investigated using both static and flow-through techniques (Davies and Brinkman 1998). However the duration of exposure was limited to ten days. Toxicity experiments conducted with cadmium and copper have demonstrated that longer term exposures can cause a range of sublethal effects such as reduced growth and development (Davies and Brinkman 1997). This experiment investigates the effects of longer exposures of zinc on survival, growth, and development of boreal toad tadpoles.

MATERIAL AND METHODS

A serial diluter (Benoit et al. 1982) delivered seven concentrations of zinc (as zinc sulfate heptahydrate) and control to completely randomized 2.2 liter glass exposure chambers at a rate of twenty mls/minute. The experiment was replicated four times with nominal zinc exposure concentrations of 4000, 2000, 1000, 500, 250, 125, 62, and 0 µg Zn/L. Source water consisted of dechlorinated Fort Collins tap water. The exposure chambers were placed in a water bath maintained near 20°C using a Remcor water recirculating heater/cooler. Two fluorescent blacklights and two florescent full spectrum (Repta Sun) bulbs suspended approximately 12 inches above the exposure chambers provided artificial lighting with 16 hour light and 8 hour dark photoperiod. Twenty boreal toad tadpoles about Gosner stage 16-17 (Gosner 1960) were randomly placed in each exposure chamber. Tadpoles were fed a mixture of Mazuri, Silver Cup trout food and frozen romaine lettuce *ad libitum*. Exposure chambers were checked daily for mortality.

Four tadpoles were subsampled from each treatment after 2, 4, and 6 weeks in order to assess effects of zinc exposure on development, growth, and whole body manganese content. Tadpoles were sampled only from treatments with sufficient survival. Total length and snout-vent length were not determined at 6 weeks because the tadpoles started metamorphosis into toadlets and were resorbing their tail. After anesthesia with MS-222, tadpole development was measured using a rating system developed by Gosner (1960). Growth was assesed by measuring weight (g), total length and snout-vent length (mm). Tadpoles were rinsed with deionized water and placed in polypropylene centrifuge tubes and dried to constant weight at 80°C. The tadpoles were digested with trace metal grade nitric acid and heated for four hours in a dry bath at 100°C. Trace metal grade hydrogen peroxide was added and the tubes heated an additional four hours. The digests were diluted to volume with deionized water and analyzed for zinc as described below.

Water quality parameters were measured in each exposure weekly. Hardness and alkalinity were determined according to Standard Methods (APHA). pH was determined using an Orion Research pH meter 811 calibrated daily using pH 7.00 and 4.00 pH buffers. Conductivity was determined using a YSI Model 35 conductance meter. Hardness and conductivity were measured only in control treatments due to interference of manganese on these measurements.

Water samples for zinc analysis were collected twice during the first 96 hours of the test. Thereafter, water samples were collected weekly in each exposure level from alternating replicates. Samples were stored in 2 oz. high density polyethylene bottles and preserved by acidification to pH<2 using Ultrex nitric acid. Zinc concentrations in water samples and tissue digests were measured using an Instrumentation Laboratory Video 22 atomic absorption spectrometer with air-acetylene flame with Smith-Hieftje background correction.

Statistics

The 96-hour median lethal concentration (LC50) was determined using Spearman-Karber (Hamilton et al. 1977, Hamilton et al. 1978). Analysis of variance (ANOVA) was performed on mortality, development and growth (weight, total length and snout-vent length) data using SAS computer software (SAS 1989). Mortality data were transformed by arcsine squareroot prior to analysis (Snedecor and Cochran 1980). Assumptions of normality and homogeneity of variance were evaluated using Shapiro Wilk's test and Bartlett's test respectively ($p<0.10$). Treatment means were compared using one-tailed Dunnett's multiple comparison test ($p<0.05$) to evaluate differences from control.

RESULTS

Water quality parameters were constant throughout the test (Table 27). Mean hardness and alkalinity were approximately 57 and 36 mg $CaCO_3$ /L respectively. Temperature was near 20°C, conductivity near 100 S/cm and pH was slightly above neutral at 7.2. Dissolved oxygen was more than adequate to sustain the tadpoles and indicate that flow rates were sufficient given the number of test organisms and food present in the exposure chambers. Zinc exposure concentrations were likewise consistant throughout the acute and six week exposure periods (Tables 28 and 29).

Mortality

Acute mortality from the first ninetysix hours of exposure is shown in Table 28. The 96 hour median lethal concentration (LC50) was 840 µg/L with a ninetyfive percent confidence interval between 760 and 929 µg/L. Mortality rates after 2, 4, and 6 weeks of exposure are shown in Table 29. Survival of tadpoles exposed to less than 404 µg/L was generally good throughout the duration of the test. Mortality of tadpoles exposed to 922 µg/L gradually increased from 65% after 96 hours to 98% after 6 weeks. The no observed effect concentration (NOEC) after 2 weeks of zinc exposure was 404 µg/L. The lowest observed effect concentration (LOEC) based on mortality was 922 µg/L. The NOEC-LOEC range did not change as duration of exposure increased from 2 to 6 weeks.

Development

Gosner stage of development sampled after 2, 4, and 6 weeks are shown in Table 30. Development was not affected after 2 weeks of exposure. There was a general trend of decreased development with increasing zinc concentration after 4 and 6 weeks of exposure but only the 404 µg/L zinc concentration was significantly less than the control. Development of tadpoles exposed to 172 µg Zn/L or lower was not significantly affected.

Growth

Mean weight of tadpoles sampled after 2, 4, and 6 weeks of exposure to zinc are shown in Table 31. After 2 weeks, mean weight of tadpoles in all zinc exposure concentrations were significantly lower than control. By 4 weeks, the effect of zinc exposure on mean tadpole weights diminished. Zinc exposure did not significantly reduce weight at 4 and 6 weeks. Weight of tadpoles exposed to 404 µg/L for 4 and 6 weeks were 72 and 81% of control weights respectively but this difference was not significant ($p>0.05$). Mean body weight of tadpoles decreased between the 4th and 6th week due to metamorphosis.

Total length of tadpoles was mildly affected by exposure to zinc (Table 32). After 2 weeks of exposure to 404 µg/L zinc, tadpoles were significantly shorter than controls. Tadpoles were not statistically affected at lower concentrations nor were they affected after 4 weeks of exposure. Snout-vent length of tadpoles was not significantly affected by exposure to zinc at any time period or concentration (Table 33).

Whole Body Zinc Content

Whole body accumulation of zinc by boreal toad tadpoles was dose-related (Figure 2.). All exposure concentrations resulted in significantly greater whole body zinc content compared to controls. Accumulation was roughly linearly correlated to zinc exposure concentration. Increasing duration of exposure had no effect on accumulation.

DISCUSSION

The 96 hour median lethal concentration (LC50) of zinc to boreal toad tadpoles has been previously reported as 1326 µg /L (Davies and Brinkman 1998). The LC50 from this test is similar at 840 µg /L. As with manganese, the lower value reported from this experiment may be due to the use of earlier life stage of tadpole compared to previously (this report). Earlier life stages of toad embryos may be more sensitive to the effects of some metals (Ferrari et al 1993, Herkovits and Perez-Coll 1993). After 2 weeks of exposure, the LOEC based on survivial was 922 µg /L. The NOEC after 2 weeks was 404 µg /L. Mortality of toads exposed 922 µg /L continued to increase with the duration of exposure however this did not affect the NOEC-LOEC range.

The concentration of zinc that caused reduced development (404 µg /L) was lower than that which caused decreased survival (922 µg /L) (Table 30). Delayed development of boreal toad tadpoles also occurs when exposed to cadmium and copper (Davies and Brinkman 1977) and manganese (this report). In the cases of copper and cadmium, reduced development occurs at concentrations much lower than those that cause lethality. Rate of development is particularly important for boreal toad tadpoles in Colorado since they are found above an elevation of 8,000 feet where the summers are typically short. It is vital that tadpoles develop into toadlets and disperse prior to the onset of winter. Temporary ponds that form in the Spring of year may dry up near the end of Summer. Under such conditions any delay in development could lead to death and recruitment failure.

There is a strong trend towards decreasing snout-vent length with increasing exposure to zinc though this was not significant using ANOVA (Table 33). Total length exhibited a similar trend and was significantly reduced at 404 µg/L after two but not four weeks (Table 32). Wet weight was reduced at all zinc exposures after two weeks but was not significantly reduced after weeks (Table 31). Reduced growth of tadpoles may result in increased risk of predation from dytisid beetle larvae (L. Livo, personal

communication) and lead to decreased overwinter survival of toadlets. Use of length or wet weight to evaluate growth of tadpoles may not be appropriate in situations where development is also affected. During metamorphosis, length and weight both decrease. This can lead to instances where a more developed tadpole has a shorter length and weighs less than a less developed tadpole. A more appropriate approach may be to normalize growth to development by measuring length and weight at a particular

Whole body accumulation of zinc by boreal toad tadpoles was approximately linearly related to exposure concentration (Figure 2). However, duration of exposure had little effect on whole body content of zinc indicating that zinc content is well regulated by tadpoles. This pattern of accumulation is similar to copper, another essential metal. Zinc accumulation could be used as a bioindicator of exposure to zinc since it does not seem to be affected by development stage.

Table 27. Mean, standard deviation and range of water quality parameters of exposure water during zinc toxicity test.

	Mean	Standard Deviation	Range
Hardness (mg $CaCO_3$/l)	57.0	7.6	49.2-68.8
Alkalinity (mg $CaCO_3$/l)	35.9	1.9	30.6-40.8
pH (S.U.)	7.22	0.22	6.59-7.59
Dissolved Oxygen (mg/L)	8.6	0.5	7.7-9.0
Temperature (°C)	19.4	1.3	17.3-22.2
Conductivity (µS/cm)	103.0	5.9	94.0-109

Table 28. Zinc concentrations (µg/L) and associated acute mortality (%) of boreal toad tadpoles during the first 96 hours of the flow-through toxicity test. Standard deviations in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Mortality (%)
0	<10 (0)	1.2 (2.5)
62.5	55 (1)	0 (0)
125	83 (1)	0 (0)
250	198 (2)	1.2 (2.5)
500	440 (1)	0 (0)
1000	964 (11)	65 (13.5)
2000	1915 (21)	100 (0)
4000	4550 (0)	100 (0)

LC50 (95% C.I.)= 840 µg Zn/L (760-929)

Table 29. Zinc concentrations (µg/L) and associated mortality (%) after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Mortality (%)		
		2 Weeks	4 Weeks	6 Weeks
0	<10	6.7 (5.8)	6.7 (5.8)	6.7 (5.8)
62.5	42 (10)	11.2 (13.2)	11.2 (13.2)	13.5 (12.5)
125	66 (12)	11.7 (10.4)	11.7 (10.4)	11.7 (10.4)
250	172 (19)	6.2 (9.5)	6.2 (9.5)	12.0 (8.5)
500	404 (25)	0.0 (0)	4.8 (9.5)	8.0 (16.0)
1000	922 (44)	67.5 (8.7)*	96.2 (4.8)*	98.8 (2.5)*
2000	1915 (21)	100 (0)*	100 (0)*	100 (0)*
4000	4550 (0)	100 (0)*	100 (0)*	100 (0)*

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

Table 30. Zinc concentrations (µg/L) and associated Gosner development after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Gosner Stage		
		2 Weeks	4 Weeks	6 Weeks
0	<10	28.9 (1.2)	37.1 (0.4)	39.8 (0.2)
62.5	42 (10)	29.3 (0.9)	36.9 (1.2)	39.1 (2.3)
125	66 (12)	29.1 (1.0)	36.3 (0.1)	39.4 (0.6)
250	172 (19)	29.2 (0.9)	36.1 (1.0)	38.3 (1.3)
500	404 (25)	29.1 (1.3)	34.1 (1.9)*	36.7 (1.1)*

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

Table 31. Zinc concentrations (µg/L) and associated wet weight (g) of tadpoles after 2, 4, and 6 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Wet Weight (g)		
		2 Weeks	4 Weeks	6 Weeks
0	<10	0.311 (0.027)	1.228 (0.264)	0.856 (0.060)
62.5	42 (10)	0.254 (0.012)*	1.275 (0.166)	0.855 (0.336)
125	66 (12)	0.257 (0.033)*	1.117 (0.115)	0.945 (0.027)
250	172 (19)	0.266 (0.028)*	1.293 (0.158)	0.965 (0.085)
500	404 (25)	0.175 (0.039)*	0.882 (0.288)	0.694 (0.183)

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

Table 32. Zinc concentrations (µg/L) and associated total length (mm) of tadpoles after 2, and 4 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Total Length (mm)	
		2 Weeks	4 Weeks
0	<10	28.6 (1.6)	46.3 (2.6)
62.5	42 (10)	27.1 (0.8)	46.9 (2.0)
125	66 (12)	27.4 (1.2)	45.2 (1.0)
250	172 (19)	27.3 (1.7)	46.2 (2.6)
500	404 (25)	24.4 (2.0)*	40.4 (5.2)

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

Table 33. Zinc concentrations (µg/L) and associated snout-vent length (mm) of tadpoles after 2, and 4 weeks of exposure. Standard deviation in parentheses.

Nominal Concentration (µg/L)	Measured Concentration (µg/L)	Snout-Vent Length (mm)	
		2 Weeks	4 Weeks
0	<10	11.6 (0.9)	17.5 (1.4)
62.5	42 (10)	11.1 (0.2)	17.3 (0.7)
125	66 (12)	10.3 (1.8)	17.2 (0.6)
250	172 (19)	10.8 (0.4)	17.6 (1.0)
500	404 (25)	9.8 (1.1)	15.9 (2.5)

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