Water Pollution Studies

Federal Aid Project F-243R-19

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Federal Aid in Fish and Wildlife Restoration

Job Progress Report

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Aquatic Wildlife Research Section

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

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State: Colorado

Study No. F243R

Title: Water Pollution Studies

Period Covered: July 1, 2011 to June 30, 2012

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

STUDY PLAN A: LABORATORY TOXICITY STUDIES

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

Job A.1. Reproductive Toxicity of Endocrine Disrupting Compounds

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

Job A.2. Reproductive Toxicity of Atrazine Herbicide

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

Job A.3. Toxicity of Metals to Fish

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

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

Job Objective: Measure the effect of zinc, copper, cadmium and/or selenium from dietary sources on survival and growth of fish in the laboratory. Evaluate the sensitivity of dietary-exposed organisms to waterborne exposure. Relate dietary levels that cause diminished performance in the laboratory with levels found in dietary sources in metal impacted areas such as the upper Arkansas River, Clear Creek and the Eagle River.

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

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

STUDY PLAN B: TECHNICAL ASSISTANCE

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

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

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

ACCOMPLISHMENTS

Job A.1. Reproductive toxicity of endocrine disrupting compounds

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

Job A.2. Reproductive toxicity of atrazine herbicide

No activities during this segment.

Job A.3. Toxicity of Metals to Fish

Results of aqueous cadmium acute and chronic early-life stage toxicity tests conducted with Rio Grande cutthroat trout are reported below.

A chronic 35d early-life stage iron toxicity test was conducted with boreal toad tadpoles. Results are reported below

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

A laboratory culture of the oligochaete, *Lumbriculus variegatus*, was exposed to waterborne cadmium. *Lumbriculus* cultured in cadmium-dosed and cadmium-free water were used as food for cutthroat trout fry. Fry were fed a cadmium-dosed and cadmium-free diet to determine effects of dietary cadmium on survival, growth and accumulation in different subcellular fractions of kidneys and liver. The subcellular fractions are currently being digested and prepared for analysis. The result will be reported next segment.

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

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

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

Evaluation of Rotenone Formulations: Rotenone is a piscicide that is an important tool for managing Colorado fisheries. CPW aquatic biologists have reported that a new formulation of rotenone, CTF Legumine, is more effective than older formulations. Experiments were conducted to compare the toxicity of CTF Legumine with Prenfish 5% in side-by-side tests using rainbow trout, brook trout, fathead minnows. An additional experiment compared rotenone detoxification of CTF Legumine and Prenfish by potassium permanganate. Results are reported below.

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

Acute and chronic toxicity of cadmium to early life stage Rio Grande cutthroat trout (*Oncorhyncus clarkii virginalis*)

INTRODUCTION

Cadmium concentrations are usually low and range 0.05-0.2µg/L in uncontaminated freshwater but become elevated from mining, minerals processing, and combustion of fossil fuel (Eisler 1985). Cadmium is among the most toxic of metals to aquatic life (Borgmann et al. 2005). Salmonids are particularly sensitive to cadmium toxicity and represent six of the seven most sensitive species (USEPA 2001). Salmonids are a significant component of ecosystems in many Colorado headwater streams and are also recreationally important. However, metal contamination can limit population density (Brinkman et al. 2006). Cadmium is commonly found in acid rock drainage resulting from Colorado's mining past. An estimated 2080 km of streams in Colorado are impacted by metals with 840 km impacted by cadmium (CDPHE 2010).

Rio Grande cutthroat trout *Oncorhynchus clarkii virginalis* historically occupied approximately 10,600 km of streams in New Mexico and Colorado but are currently restricted to about 1,300 km due to the introduction of non-native salmonids and habitat destruction (Behnke 2002). Recently, the subspecies was listed as a candidate for protection under the Endangered Species Act of 1973 (USFWS 2008). A search of published literature found no cadmium toxicity data for Rio Grande or any other subspecies of cutthroat trout. The objective of this study was to determine the acute and chronic toxicity of cadmium to early life-stage Rio Grande cutthroat trout.

MATERIALS and METHODS

Organisms

Rio Grande cutthroat trout eggs were collected from wild spawning adults. Cutthroat trout eggs were obtained from Rio Grande subspecies cutthroat trout *Oncorhynchus clarkii virginalis* from Haypress Lake CO. Eggs were stripped and fertilized in the field, placed in a cooler, and transported to the Colorado Parks and Wildlife Aquatic Toxicology Laboratory in Ft. Collins and then treated with 1600 ppm neutral buffered formalin for 15 minutes. Eggs were placed in egg incubation trays that received dechlorinated municipal tap water (200 mls/min). Unfertilized or fungused eggs were carefully removed daily. Fry were fed commercial soft moist trout chow starter supplemented with <24hr-old brine shrimp nauplii.

Exposure apparatus

Chilled dechlorinated Fort Collins municipal tap water supplied continuous-flow diluters (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components. The diluter delivered five exposure levels with a 50% dilution ratio and an exposure control. A flow splitter allocated each concentration equally among four replicate exposure chambers at a rate of 40 mLs/min each. Exposure chambers consisted of polypropylene containers with a capacity of 2.8 L. Test solutions overflowed from exposure chambers into a temperature-controlled water bath maintained at 12°C with a recirculating chiller. Semi-opaque lids covered the exposure

chambers and limited light exposure from dim fluorescent lighting (16-h/8-h photoperiod) and prevented organisms from escaping. Chemical stock solutions were prepared by dissolving calculated amounts of analytical reagent grade toxicant (CdSO4) in deionized water. Chemical stock solutions were delivered to the diluter via peristaltic pump at approximately 2.0 mLs/min. New stock solutions were prepared as needed during the toxicity tests. Diluters and toxicant flow rates were monitored daily to ensure proper operation.

The chronic early life-stage test method followed guidance provided by ASTM method E1241, *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes* (ASTM 1997). Cadmium exposure was started within 24 hours after embryos reached the eyed egg stage. Twenty eggs were randomly distributed to incubation cups constructed from 53 mm I.D. X 75 mm PVC pipe and 1000 micron mesh nylon screen affixed to the PVC pipe with aquarium-grade silicone adhesive. Incubation cups were suspended in the exposure chamber and received 40 mLs/min flow of exposure water from the diluter. Eggs were monitored daily for mortality and hatching. The first ten eggs to successfully hatch were removed from the incubation cup and placed in the exposure chamber. Remaining eggs were monitored for hatching and removed after hatching. Thus, hatching success is based on twenty organisms in each incubation cup, while fry survival and growth is based on ten organisms transferred to the exposure chamber. At swimup, fry were fed commercial trout chow four times a day with an automatic feeder (Fish Mate, Aquatic Ecosystems, FL) and supplemented with <24 hr brine shrimp nauplii. Initial feed rate was an estimated 5% body weight/day and increased 10% per week to accommodate growth of fry. Feedings for each tank were adjusted weekly based on number of surviving fry. Exposure chambers were cleaned with a siphon to remove feces and uneaten food as needed. Cutthroat trout eyed embryos were exposed for 11 days before hatch, followed by 12 days before resorption of the yolk sac and onset of exogenous feeding. Exposure continued for an additional 30 days post-swimup. At the end of the test, surviving fish from each exposure chamber were terminally anesthetized with MS-222, blotted dry with a paper towel and the weights measured and recorded.

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

Acute toxicity tests were conducted after the conclusion of the ELS test. Acute test methodology following established guidelines (ASTM 2001). The acute test used fry that were 0.26 g and 37 days post-swimup. Exposure apparatus and test methods were identical to the ELS tests except that water quality characteristics, metals, major cations and anions were measured at 0, 48 and 96 hours. At the end of the test, surviving fish from each exposure chamber were terminally anesthetized with MS-222, blotted dry with a paper towel and the weights measured and recorded. Fry were fasted for 24 hours before the start of the acute test and were not fed during the exposure.

Statistical analyses

Test data were analyzed using Toxstat version 3.5 software (West, Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included hatching success, swim-up survival, mean weights of surviving fry, and biomass of surviving fish at test termination. Hatching success and survival data were arcsine square root transformed prior to ANOVA. Normality and homogeneity of variances were tested using Shapiro-Wilk's and Bartlett's test, respectively. Treatment means were compared to the control using Dunnett's one-tailed test at $p<0.05$. The highest metal concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest concentration of cadmium associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC. The inhibition concentration (IC20), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving organisms from each treatment (biomass or standing crop). Ninety six hour median lethal concentrations (LC50) were estimated using the Trimmed Spearman-Karber technique (Hamilton et al. 1977, Hamilton 1978) with automatic trim. Acute to chronic ratios (ACR) were calculated by dividing the 96 hr LC50 from the fry test by the IC20 from the ELS test.

RESULTS

Water quality parameters were consistent over the duration of the test (Table 1). Dissolved oxygen concentrations were suitable for salmonids and near saturation (Ft. Collins elevation = 1520 m above sea level). Mean hatch rate ranged from 64% to 80% and was not significantly affected by cadmium exposure (Table 2). Survival of fry ranged between 95% in the control to 0% at 8.03 µg/L. Fry survival was significantly lower at Cd concentrations ≥3.37 µg/L (LOEC) but no effect was detected at concentrations ≤1.48 µg/L (NOEC). Mean fry weight and biomass at test termination was similarly significantly lower at Cd concentration ≥3.37 µg/L but unaffected at concentrations ≤1.48 µg/L. The chronic value based on fry survival, weight and biomass was 2.23 µg/L. The IC20 was 1.82 µg/L. The 96 hour LC50 of cadmium to post-swimup fry was 2.40 µg/L with a 95% confidence interval between 2.24 and 2.57 µg/L (Table 3). The acute to chronic ratio (ACR) was 1.31.

DISCUSSION

Hatch rate of Rio Grande cutthroat trout eggs was not affected by cadmium exposures used in this test (Table 2). Salmonid eggs are generally tolerant to metals compared to other life-stages and are typically not a sensitive endpoint (Chapman 1978, Van Leeuwen 1985, Davies et al. 2002, Brinkman and Hansen 2004, Brinkman and Hansen 2007, Brinkman and Vieira 2008). Survival and growth were adversely affected by long term exposures to cadmium at concentrations as low as 3.37 µg/L. The 96-h LC50 was 2.40 µg/L. Results of the study demonstrate that Rio Grande cutthroat trout are sensitive to cadmium. In order to place the sensitivity of Rio Grande cutthroat trout fry in context, toxicity values from this study were normalized to a hardness of 50 mg/L and compared with values for other salmonids and to Colorado water quality standards (Table 4). Toxicity values for Rio Grande cutthroat trout are similar to brown trout, bull trout, brook trout and rainbow trout which are the four most acutely sensitive species to cadmium (USEPA 2001).

Acknowledgements - Audrey Crockett and Patricia Forsberg assisted with fish care and toxicity tests.

Table 1. Water quality characteristics and major ions of exposure water used for mountain whitefish and Rio Grande cutthroat trout cadmium toxicity tests.

Hardness (mg/L as $CaCO_3$)*	44.9 (2.6)
Alkalinity (mg/L)	35.3 (0.8)
pH (S.U.)	7.19 (0.13)
Temperature (°C)	12.7 (0.2)
Dissolved Oxygen (mg/L)	8.88 (0.24)
Conductivity (µS/cm)	93.8 (3.7)
Calcium (mg/L)	15.4 (0.9)
Magnesium (mg/L)	1.6 (0.2)
Sodium (mg/L)	2.3 (0.9)
Potassium (mg/L)	0.7 (0.1)
Chloride (mg/L)	3.3 (0.2)
Sulfate (mg/L)	10.2 (0.3)
DOC (mg/L)	1.2 (0.2)

*Calculated from calcium and magnesium concentrations

Table 2. Mean dissolved cadmium concentrations (µg/L) and associated mean hatching rate (%), fry survival (%), mean weight at test termination (g) and biomass at test termination (g) of early life-stage cutthroat trout exposed to cadmium. Standard deviations are in parentheses. Treatments means with asterisks are significantly less than control ($p<0.05$).

Target Cd Concentration (µg Cd/L)	0	0.5	1.0	2.0	4.0	8.0
Measured Cd concentration (µg Cd/L)	<0.10 (0.07)	0.26 (0.04)	0.64 (0.06)	1.48 (0.10)	3.37 (0.16)	8.03 (0.28)
Hatch Success (%)	77.5 (8.7)	67.5 (6.5)	70.0 (12.2)	80 (4.1)	72.5 (6.5)	63.8 (14.4)
Fry Survival (%)	95.0 (5.8)	95.0 (10.0)	92.5 (9.6)	82.5 (12.6)	57.5* (20.6)	0.0* (0.0)
Weight at Termination (g)	0.430 (0.018)	0.389 (0.032)	0.400 (0.011)	0.430 (0.029)	0.360* (0.040)	--
Biomass at Termination (g)	4.09 (0.32)	3.67 (0.20)	3.70 (0.312)	3.53 (0.37)	2.12* (0.91)	0.00* (0.00)

IC20 = 1.82 µg Cd/L

Table 3. Mean dissolved cadmium concentrations (µg/L) and associated 96 hour survival of Rio Grande cutthroat trout fry. Standard deviations are in parentheses.

Cadmium Concentration (µg Cd/L)	0	0.5	1.0	2.0	4.0	8.0
Measured Cd concentration (µg Cd/L)	<0.10 (0.04)	0.23 (0.06)	0.64 (0.08)	1.49 (0.05)	3.40 (0.08)	8.03 (0.14)
Fry Survival – 96 hours (%)	100 (0)	100 (0)	100 (0)	100 (0)	92.5 (9.6)	0 (0)

LC50 (95% confidence interval) = 2.40 µg Cd/L (2.24-2.57 µg Cd/L)

Table 4. Comparison of Rio Grande cutthroat trout cadmium toxicity values with species mean acute values (SMAV) and species mean chronic values (SMCV) of other salmonids (all as µg Cd/L). All values normalized to 50 mg/L water hardness. Toxicity values from USEPA water quality criteria (USEPA 2001) unless otherwise noted.

	SMAV	SMCV
Rio Grande cutthroat trout	2.68	1.97
Colorado Standard	0.90	0.25
Brown trout	2.52*	1.72*
Bull trout	2.152	
Brook trout	<1.791	2.643
Rainbow trout	2.108	1.308
Chinook salmon	4.305	2.612
Coho salmon	6.221	4.265
Atlantic salmon		7.922
Lake trout		8.088
Mountain whitefish	4.92**	1.33**

*Brinkman and Hansen 2007
**Brinkman and Vieira 2008

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Chronic toxicity of iron hydroxide to boreal toad tadpoles (*Bufo boreas*)

INTRODUCTION

Over 955 miles of streams and rivers in Colorado are adversely affected by iron (Fe) (CDPHE 2010). Iron leaches into streams from natural sources as well as from mining activities in the Mineral Belt of the Rocky Mountains. Iron is not soluble in oxygenated, neutral pH waters precipitating out of solution as ferric hydroxide and ferric oxide precipitates. As such, Fe is generally considered less toxic than soluble metals such as cadmium, copper and zinc. Though the chemical toxicity of iron precipitates may be low to target organs such as fish gills, the precipitates can otherwise adversely affect aquatic life through increased turbidity, reduced primary production and of interstitial space in the benthic zones and smothering of bottom-dwelling invertebrates, plants and incubating fish eggs (USEPA 1976, Goettl and Davies 1977, DeNicola et al. 2002, McKnight and Feder 1984, Vuori 1995, Linton et al. 2007). Precipitated iron also physically clogs fish gills and leads to gill damage that may cause respiratory impairment (Peuranen et al. 1994, Dalzell and McFarlane 1999). The Colorado chronic table value standard for iron is 1.0 mg/L (total recoverable). The value is primarily based on field observations that determined that trout and other fishes were not present in an iron-polluted Colorado stream until dilution or loss of iron from the water column resulted in a concentration less than 1.0 mg/L (USEPA 1976). A search of the literature found very few chronic toxicity tests have been conducted. Toxicity data are insufficient and from a range of taxa too limited to derive a water quality criterion using established guidelines (USEPA 1985). The objective of this study is to measure chronic toxicity of iron to boreal toad tadpoles in order to derive chronic toxicity data to support the existing chronic iron standard and to be able to derive a standard and criteria using established guidelines.

MATERIALS and METHODS

Test methods followed guidance provided by ASTM method E1241, *Standard Guide for Conducting Early Life-Stage Toxicity Tests with Fishes* (ASTM 1997). A continuous-flow diluter (Benoit et al. 1982) constructed of teflon, polyethylene and polypropylene components delivered five exposure levels of iron hydroxide and an exposure control. Target iron concentrations were 8000, 4000, 2000, 1000, 500, and 0 µg/L. Source water was dechlorinated Ft. Collins municipal tap water. Iron stock solution was prepared by dissolving ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$, Mallinkrodt analytical reagent grade) with sufficient sodium hydroxide (NaOH BDH analytical reagent grade) (1:3 stoichiometry) to neutralize acid caused by precipitation of ferric hydroxide. The stock solution was pumped to the diluter with a peristaltic pump at a rate of 2 mLs/min. A flow splitter allocated each iron concentration equally at 40 mLs/min to each of four replicate 2.8 L polypropylene tanks via food-grade vinyl tubing. Exposure solutions overflowed from the tanks through a screen and into a temperature-controlled water bath maintained at 20°C. Five tadpoles (c.a. stage 18; Gosner 1960) were carefully distributed into each tank using a pipette. Tadpoles were fed an excess of mixture of Mazuri amphibian feed and powdered algae wafers (1:1) and a processed slurry of kale, mustard greens and squash. Tanks were cleaned to remove feces and excess food every 2 days. Tanks were

monitored daily for mortality. The test was terminated 35 days after the start of exposure. Tadpoles were terminally anesthetized with MS222 and lengths (mm), weights (g) and developmental stage (Gosner 1960) measured for each tadpole.

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

Water samples for iron analyses were collected weekly from each exposure level. Grab samples for total iron were collected in 2 oz HDPE bottles (Nalgene), immediately preserved with high purity nitric acid (JT Baker) to pH <2. Filtered samples for dissolved iron analyses were passed through a 0.45 µm filter (Acrodisc) and stored in trace metal grade 15 mL centrifuge tubes and preserved with high purity nitric acid (JT Baker) to pH <2. Iron concentrations were measured using an Instrumentation Laboratory Video 22 (Allied Analytical Systems, Franklin, MA) atomic absorption spectrometer with air-acetylene flame and Smith-Hieftje background correction. The spectrometer was calibrated prior to each use and the calibration curve verified through analyses of external quality assurance samples (High Purity). Sample splits and spikes were collected at each sampling event to verify analytical reproducibility and recovery.

Statistical analyses of data were conducted using Toxstat version 3.5 software (West, Inc. 1996). Analysis of variance (ANOVA) was used to test toxicity endpoints which included survival, lengths weights, development (Gosner 1960) and biomass of surviving tadpoles at test termination. Survival data were arcsine square root transformed prior to ANOVA. Normality and homogeneity of variances were tested using Shapiro-Wilk's test and Bartlett's test, respectively. Treatment means were compared to the control using Dunnett's one-tailed test. The highest measured iron concentration not associated with a treatment effect (e.g. decreased survival, decreased body weight) was designated as the no-observed-effect concentration (NOEC). The lowest measured iron concentration of cadmium associated with a treatment effect was designated as the lowest-observed-effect concentration (LOEC). Chronic values were calculated as the geometric mean of the LOEC and NOEC. The inhibition concentration (IC20), the concentration estimated to cause a 20% reduction in organism performance compared with the control (USEPA 1993), was calculated using the combined weight of surviving organisms (biomass or standing crop) from each treatment.

RESULTS

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

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

Mixing a stock solution of ferric chloride with dilution water from the diluter would alter pH and alkalinity and confound interpretation of toxicity results. To prevent changes in pH and alkalinity, sodium hydroxide was added to the stock solution in a 3:1 stoichiometric ratio in order to neutralize the acid formed by the precipitation of ferric hydroxide. As a result, measured

alkalinity and pH were similar among the iron exposure levels (Table 5). Neutralization with sodium hydroxide resulted in a slight increase in conductivity associated with iron exposure levels from 109 µS/cm in the control exposure to 148 µS/cm in the highest iron concentration.

Tadpole development and growth, as measured by length and weight at test termination, were significantly reduced at 8115 µg/L but no difference was detected at ≤3831 µg/L (Table 6, Figure 1). Survival was 100% in the control exposure and ≥95% at iron concentrations ≤ 2044 µg/L. Survival and biomass was significantly decreased at iron concentrations ≥3831 µg/L. The LOEC and NOEC based on survival and biomass are 3831 µg/L and 2044 µg/L, respectively. The iron chronic value for boreal toad tadpoles was 2798 µg/L. The IC20 was 2772 µg/L

DISCUSSION

Elevated iron concentrations from acid mine drainage are strongly associated with low pH, a phenomenon known as acid rock drainage (ARD) which has been called the greatest water quality issue in the Western United States (Mineral Policy Center 1997). Adding soluble iron to the lab dilution water would lower the pH and alkalinity of the exposure water in direct association with the iron concentration. While these conditions would have been a more environmentally realistic exposure scenario, it was decided in the present test to neutralize the stock solution to isolate the toxic effects of iron from effects of lowered pH and any possible interaction.

The iron chronic value for boreal toad tadpoles was 2798 µg/L. In good agreement with the chronic value was the IC20 which was 2772 µg/L. A previous study conducted by Porter and Hakanson (1976) found that boreal toad tadpoles tolerated iron concentrations as high as 20 mg/L. The difference in toxicity between the two studies is probably explained by the use of a ferrous salt by Porter and Hakanson and the use of a ferric salt used in the present study. The most sensitive endpoint for ferric hydroxide toxicity was lethality though sublethal effects of decreased growth and development were also detected. Decreased growth and development of boreal toad tadpoles occur with cadmium, copper manganese and zinc exposure (Davies et al. 1997, Davies and Brinkman 1999). A delay in development could lead to recruitment failure if tadpoles fail to metaphase before onset of winter or before temporary ponds dry up. Rapid development is particularly important for boreal toad tadpoles, which at 8,000 ft have a short summer in which to metamorphose and disperse. Pre-metamorphed tadpoles have been observed in late fall under the ice in ponds (John Goettl Jr., personal communication).

Boreal toad tadpoles are intermediate in sensitivity to chronic iron exposure. Chronic values of more sensitive species include 935 µg/L for mountain whitefish (Brinkman and Vieira 2011), 569 and 2000 µg/L for fathead minnows (Birge et al. 1985, Smith et al. 1973), 1483 µg/L for rainbow trout (Davies and Goettl 1977) and 1952 µg/L for coho salmon (Smith and Sykora 1976). More tolerant species include brown trout (<5149 µg/L, Brinkman and Vieira 2011) and brook trout (10,224 µg/L, Smith and Sykora 1976).

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Table 5. Water quality characteristic of chronic boreal toad tadpole toxicity tests in each of the iron exposure levels. Standard deviations are in parentheses.

Target Iron Concentration	0	500	1000	2000	4000	8000
Acid Soluble Fe concentration (µg/L)	<50 (14)	654 (103)	1073 (62)	2044 (100)	3831 (244)	8115 (784)
Dissolved Fe Concentration (µg/L)	<50 (16)	<50 (41)	<50 (35)	<50 (21)	<50 (18)	<50 (13)
Alkalinity (mg/L)	34.9 (1.1)	33.2 (0.7)	33.9 (1.4)	34.5 (2.1)	35.4 (2.7)	33.4 (1.4)
pH (SU)	7.10 (0.12)	7.00 (0.08)	7.15 (0.14)	7.13 (0.16)	7.13 (0.15)	7.08 (0.16)
Conductivity (µS/cm)	109 (5)	110 (4)	113 (5)	121 (6)	131 (7)	148 (15)
Dissolved Oxygen (mg/L)	7.19 (0.37)	7.49 (1.0)	7.60 (0.50)	7.36 (0.58)	7.45 (0.50)	7.65 (0.13)

Table 6. Mean measured total iron concentrations (µg/L) and associated survival (%), lengths (mm), weight (g), Gosner developmental stage and biomass of boreal toad tadpoles exposed for 35 days. Standard deviations are in parentheses. Asterisk denote treatment means significantly less than control ($p<0.05$).

Nominal Concentrations	0	500	1000	2000	4000	8000
Measured Concentrations	<50 (14)	654 (103)	1073 (62)	2044 (100)	3831 (244)	8115 (784)
Survival (%)	100 (0)	100 (0)	95 (10)	100 (0)	60 (16)*	35 (10)*
Mean length (mm)	55 (2)	56 (2)	55 (2)	55 (1)	52 (1)	36 (2)*
Mean weight (g)	1.384 (0.088)	1.529 (0.136)	1.491 (0.115)	1.493 (0.087)	1.241 (0.100)	0.414 (0.061)*
Gosner Stage	39.4 (0.2)	39.4 (0.2)	39.7 (0.1)	39.6 (0.6)	39.0 (0.8)	35.8 (0.3)*
Biomass (g)	6.112 (0.440)	7.452 (0.680)	7.057 (0.682)	7.464 (0.436)	3.737 (1.047)*	0.728 (0.251)*

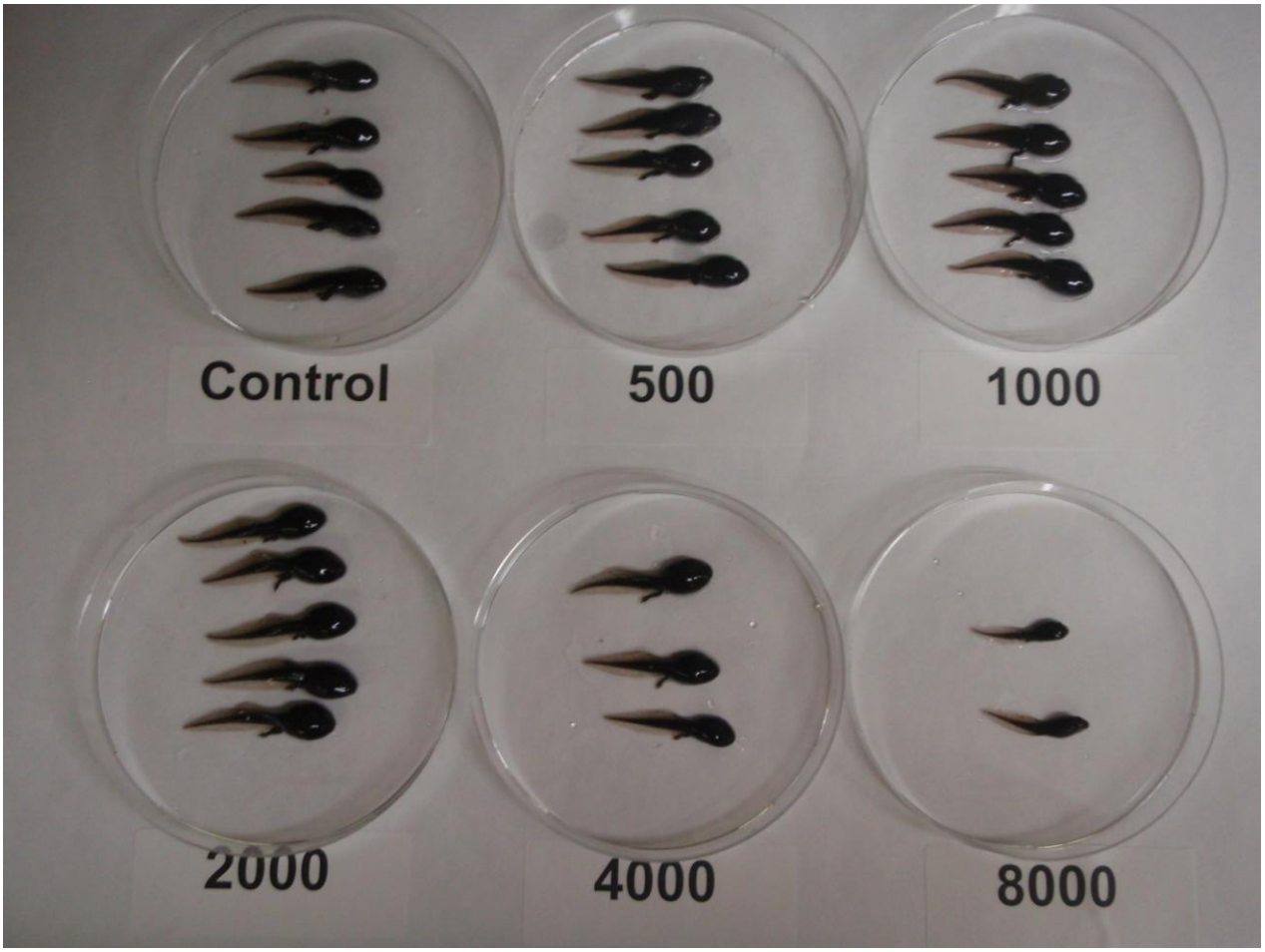

Figure 1. Boreal toad tadpoles exposed to 8000, 4000, 2000, 1000, 500 µg/L iron at termination of 35 day toxicity test. Tadpoles exposed to 8000 µg/L were significantly smaller and less developed than controls ($p<0.05$).

Comparison of the toxicity and detoxification of two rotenone formulations

INTRODUCTION

Rotenone is a piscicide that is an important tool for managing Colorado fisheries. CTF Legumine is a new formulation of rotenone that has recently come to market. Colorado aquatic biologists have reported that CTF Legumine has greater effectiveness than older formulations. This study had multiple objectives. The first was to compare the toxicity of CTF Legumine with Prenfish 5% in side-by-side tests using rainbow trout, brook trout, fathead minnows and channel catfish. The second objective was to compare the rate of rotenone detoxification of CTF Legumine and Prenfish formulations by potassium permanganate. A third objective was to evaluate the potential of hydrogen peroxide and potassium persulfate for detoxification of rotenone.

METHODS

Toxicity of CTF Legumine and Prenfish

Brook trout were received as freshly fertilized eggs collected from wild adults at Trapper's Lake Colorado. Eggs from ripe females were stripped and mixed with the milt from two males. Fertilization rates were enhanced by maintaining anhydrous conditions during the process, with conception occurring in the ovarian fluid bath. Eggs were then hardened for an additional 30 min in water from the brood source before making the 6 h journey to the toxicology laboratory in 4 L coolers where they were then treated with 1600 mg/L formalin to control fungus (Piper et al. 1982). Eyed rainbow trout eggs (Hofer strain) were obtained from the Colorado Parks and Wildlife Research hatchery. Two month-old fathead minnow juveniles were received from Aquatic Biosystems (Ft. Collins CO). Brook and rainbow trout were incubated and raised in Ft. Collins dechlorinated municipal tap water until the toxicity tests. Fathead minnow juveniles were acclimated to Ft. Collins dechlorinated municipal tap water for seven days prior to the test.

The toxicity tests used static exposure methodology based on established guidelines (ASTM 2001). Exposures were conducted in glass tanks (18 x 9 x 12 cm) containing 2 L of Ft. Collins dechlorinated municipal tap water (hardness=45 mg/L as CaCO3, alkalinity = 32 mg/L as CaCO3, pH=7.2). Exposure tanks were aerated to assist with mixing and to maintain adequate dissolved oxygen. Temperature of the exposure solutions were held constant by a temperature-controlled water bath maintained at 12°C for brook and rainbow trout and at 18°C for fathead minnows. Tests for each species used six exposure concentrations and a control, with each exposure level replicated three times. Stock solutions of CTF Legumine and Prenfish (Envincio LLC) were prepared immediately before spiking the exposure solutions. Target concentrations for the brook and rainbow trout tests were 0.150, 0.125, 0.100, 0.050, 0.025, and 0 mg/L, as formulation. Target concentrations for the fathead minnow test were 0.60, 0.50, 0.40, 0.30, 0.20, 0.10 and 0 mg/L, as formulation. Ten brook trout (0.16g), eight rainbow trout (0.24g) and eight fathead minnows (0.31g) were distributed randomly to each exposure tank. Mortality, defined as failure to respond to repeated prodding with a dip net and lack of visible gilling, was measured at 3, 6, 12, and 24 hours of exposure. Dead fry were removed with a dip net, blotted with a paper towel and weighed. Overhead lights were turned off except to check for mortality. Water samples

for rotenone analyses (200 mLs) were collected prior to the introduction of test organisms and stored in a pre-cleaned amber glass bottle. Water samples were analyzed by high performance liquid chromatography based on the method by Dawson et al. (1983). Water samples were adjusted to pH 5.0 with 4 mls of buffer. Rotenone was extracted via vacuum filtration through a preconditioned Sep-Pak C_{18} disposable cartridge (Waters, Milford CT) and eluted with 2 mLs of methanol. Extracted samples were analyzed using an Agilent 1220 HPLC with an isocratic 75%:25% methanol:water eluant pumped at 1 mL/min through a Hypersil Gold HPLC analytical column 250 x 4 mm (Thermo Scientific) and detected at 295 nm. Calibration curves were developed using standards prepared from commercially available purified rotenone (97%, Aldrich Chemical Company) that were prepared fresh daily and treated and extracted as the exposure solutions.

Potassium permanganate degradation of CTF Legumine and Prenfish

Solutions of CTF Legumine and Prenfish, both at 1.0 mg/L, were prepared and placed in the dark in an incubator maintained at 12˚C. Both rotenone solutions were spiked with potassium permanganate to provide a final concentration of 1.0 mg/L. Water samples for rotenone analyses were subsampled immediately (0 minutes) and at 10,20, 30, and 40 minutes after addition of potassium permanganate. The potassium permanganate-rotenone reaction of subsamples was immediately quenched by the addition of sodium thiosulfate to a final concentration of 4 mg/L. Water samples were then treated and analyzed for rotenone as described above. Measured rotenone concentrations of each formulation were plotted against time of reaction and an exponential function fitted to the curve. The first-order rate constant was estimated from the regression equation.

Detoxification of CFT Legumine with hydrogen peroxide and potassium persulfate

Hydrogen peroxide and potassium persulfate were evaluated as possible alternatives to potassium permanganate for detoxification of rotenone. Four 2.0 mg/L solutions of CTF Legumine were prepared in dechlorinated Ft. Collins municipal tap water at 12°C. Each solution of CTF Legumine received either 2.0 mg/L potassium permanganate, 2.0 mg/L hydrogen peroxide, 2.0 mg/L potassium persulfate, or a mixture of 2.0 mg/L each hydrogen peroxide and potassium persulfate. Solutions were mixed and allowed to react for 10 minutes after which the reaction was quenched by the addition of 4.0 mg/L sodium thiosulfate. Solutions were treated and analyzed for rotenone as described above.

RESULTS and DISCUSSION

Toxicity of CTF Legumine and Prenfish

The concentrations of rotenone used in the tests did not cause sufficient mortality after 3h to calculate reliable median lethal concentrations (LC50s). Mortality was sufficient to calculate LC50s after 6h, 12h and 24h for rainbow trout and brook trout. Median lethal concentrations for fathead minnows could only be calculated for 12h and 24h. Toxicity of CTF Legumine and Prenfish rotenone formulations were very similar to each at each time interval and for each of the

species tested (Table 7, Figure 2). The 95% confidence intervals of the two formulations had considerable overlap at each duration of exposure and for each of the species tested to date. Data from this study do not support the hypothesis that CTF Legumine and Prenfish differ in their toxicity. Brook trout were more sensitive than rainbow trout after 6h but sensitivity was similar at 12h and 24h. Both salmonids were about 5X more sensitive than fathead minnows. Toxicity values for the three species tested were similar to published values (Table 8). Comparisons of rotenone toxicity values with earlier studies is often problematic due to differing methodologies, fish sizes and a failure to verify exposure concentrations by chemical analysis (Finlayson et al. 2010). Nonetheless, toxicity values among studies are in generally good agreement. As might be expected, the general trend is for LC50s to decrease sharply for the first 24 hours of exposure before leveling off.

Two other studies have compared toxicity of different rotenone formulations. Marking and Bills (1976) found that the synergized formulation of rotenone, Pro-Noxfish (2.5%), was more toxic to rainbow trout relative to Noxfish (5%) and powered (33%) formulations when expressed as active ingredient. They found that the powdered and Noxfish formulations were similar in toxicity. However, Finlayson et al. (2010) found that rainbow trout were equally sensitive to the synergized and non synergized formulations, though they determined that benthic insects were generally more sensitive to the synergized formulation. A direct comparison of toxicity to rainbow trout by Finlayson et al. (2010) was somewhat confounded because the average weight of fry used for the synergized rotenone formulation test (1.25g) were about 5 times larger than fry used for the CTF Legumine formulation test (0.22g).

Potassium permanganate degradation of CTF Legumine and Prenfish

Potassium permanganate degraded the rotenone active ingredient in CTF Legumine and Prenfish at similar rates (Figure 3). Several conclusions can be derived from the data used to generate the figure. The degradation curves for CTF Legumine and Prenfish were accurately described by exponential function (coefficient of determination 0.996 and 0.997, respectively) which is characteristic of a first order reaction. The first order reaction rate constant is described by the coefficient of the exponent. The rate constants for degradation of CTF Legumine and Prenfish by potassium permanganate were 0.108 and 0.107 respectively. No difference was detected between the degradation rate constants for the two formulations. Engstrom-Heg (1972) reported a rate constant for degradation of rotenone by 1.0 mg/L potassium permanganate as 0.067, which can be considered to be in relatively good agreement considering that his study estimated rotenone concentrations using a bioassay.

Finally, the degradation of rotenone was relatively slow. A 1.0 mg/L rotenone treatment detoxified with 1.0 mg/L potassium permanganate would require 20 minutes to degrade to the 24 hr LC50 for rainbow trout and 25 minutes to detoxify to a concentration that does not cause rainbow trout mortality (Figure 3). Increasing the concentration of potassium permanganate accelerates the rate degradation of rotenone but may also become a source of toxicity if the concentration is too high (Engstrom-Heg 1972). This delay in full detoxification is an important consideration for rotenone treatment of streams. Biologists should be aware that there may remain a zone of rotenone toxicity for up to 20 minutes downstream from a detoxification station. Live cars used to monitor detoxification of rotenone should be located in such a way to allow full contact time for detoxification to occur. Placement of a live car too close to a

detoxification station could lead to the incorrect conclusion that insufficient potassium permanganate has been added.

Detoxification of CFT Legumine with hydrogen peroxide and potassium persulfate

Hydrogen peroxide and potassium persulfate are strong oxidizers with an oxidizing potential similar to potassium permanganate which is traditionally used to detoxify rotenone. Unlike potassium permanganate, hydrogen eroxide and potassium persulfate are relatively nontoxic to fish and the breakdown products are not visible and relatively harmless. Hydrogen peroxide breaks down to water and dissolved oxygen whereas persulfate breaks down to sulfate. As such, hydrogen peroxide and/or potassium persulfate would be a preferred alternate for rotenone detoxification that provides a greater margin of safety and does not result in the unsightly brown manganese dioxide precipitate that results from the use of potassium permanganate. However, hydrogen peroxide, potassium persulfate and their mixture degraded a 1.0 mg/L solution of CTF Legumine by less than 6% after 10 minutes compared to 57% degradation by potassium permanganate. Hydrogen peroxide and potassium persulfate would not be useful for detoxification of rotenone.

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Table 7. Median lethal concentrations (LC50s) of Prenfish and CTF Legumine rotenone formulations to rainbow trout, brook trout and fathead minnow after 6h, 12h and 24 h of exposure. Concentrations are µg/L of active ingredient. 95% confidence intervals are in parentheses. Insufficient fathead minnow mortality occurred at 6h to calculate a LC50.

Species	Formulation	6h	12h	24h
Rainbow trout	Prenfish 5%	6.6 (6.1-7.2)	5.2 (4.8-5.8)	4.5 (4.1-4.9)
	CTF Legumine 5%	6.6 (6.0-7.3)	5.1 (4.7-5.5)	4.6 (4.3-4.8)
Brook trout	Prenfish 5%	5.4 (5.2-5.6)	5.0 (4.7-5.3)	5.0 (4.7-5.3)
	CTF Legumine 5%	5.0 (4.8-5.3)	4.8 (4.5-5.0)	4.8 (4.6-5.0)
Fathead minnow	Prenfish 5%	NA	25.5 (22.0-29.5)	21.0 (18.5-24.0)
	CTF Legumine 5%	NA	26.0 (23.5-29.5)	21.0 (18.5-23.0)

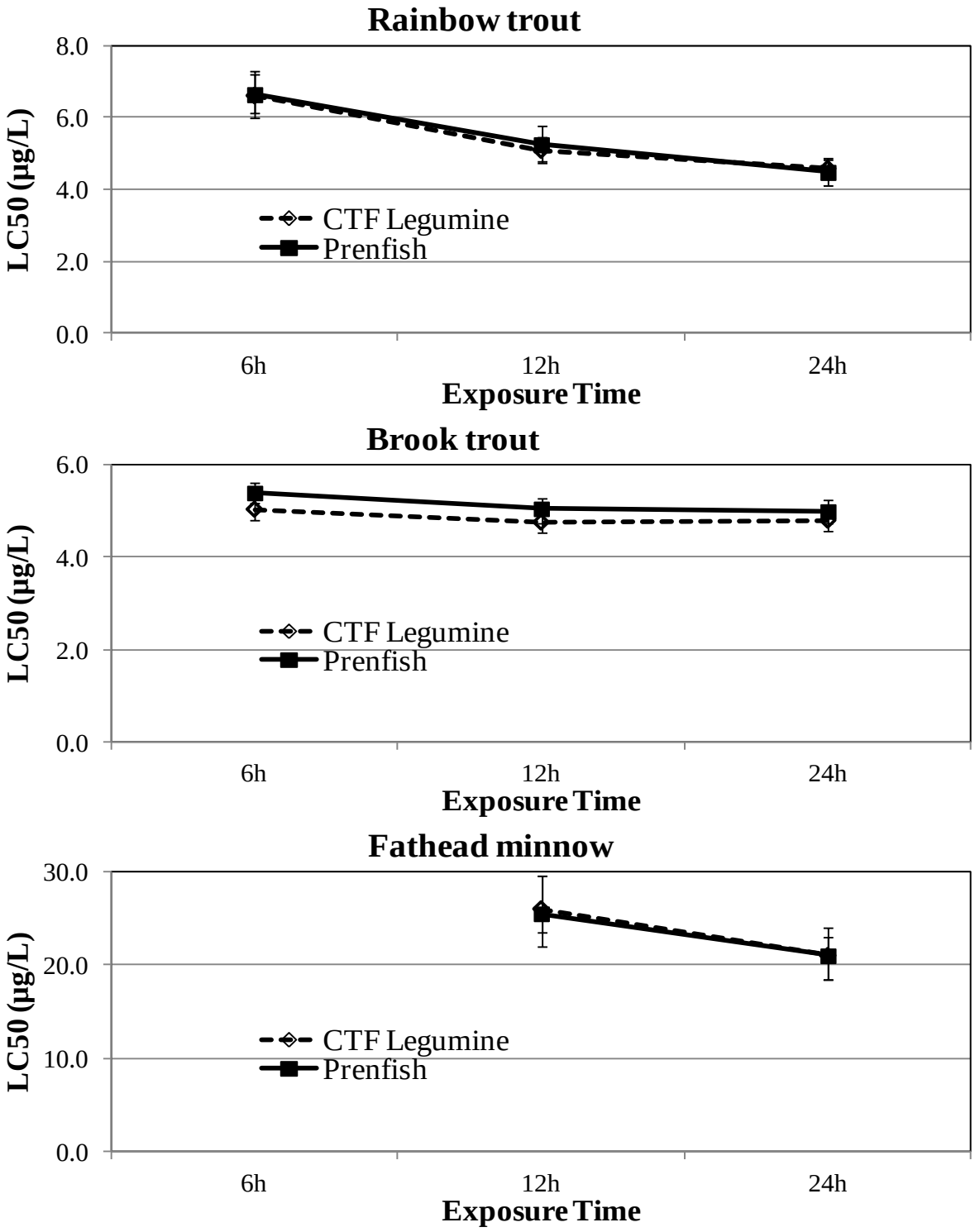

Figure 2. Median lethal concentrations (LC50) of CTF Legumine and Prenfish rotenone formulations for rainbow trout (top,), brook trout (middle) and fathead minnow (bottom) after 6h, 12h and 24 h of exposure. Concentrations expressed as µg/L active ingredient. Error bars represent 95% confidence intervals. Insufficient fathead minnow mortality occurred at 6h to calculate a LC50.

Table 8. Published median lethal concentrations (LC50s) of rotenone to various fish species at different durations of exposure. Concentrations are reported as µg/L of active ingredient. N.R. = not reported.

Species	Size	Formulation	3h	4h	6h	8h	12h	24h	48h	96h	Reference
Rainbow trout	0.24 g	CTF Legumine 5%			6.6		5.1	4.6			This study
	0.24 g	Prenfish 5%			6.6		5.2	4.5			This study
	N.R.	Chem Fish Regular 5%							2.85	2.85	Howland 1969
	81 mm	Derris Powder 6.5%						1.6			Rowe-Rowe 1971
	1-1.5g	Noxfish 5% Noxfish 5% Pro-Noxfish 2.5% Powdered 33%	8.7 8.7 4.5 8.1		4.3 5.5 3.0 6.6			3.4 3.2 1.8 3.8		2.3 3.0 1.0 3.2	Marking and Bills 1976
	0.8-1.2g	Noxfish							2.0		Waller et. al 1993
	0.22g	CTF Legumine 5%		7.4		5.3					Finlayson et. al 2010
	1.25g	Nusyn-Noxfish 2.5%		7.7		6.2					Finlayson et. al 2010
Brook trout	0.16g	CTF Legumine 5%			5.0		4.8	4.8			This study
	0.16g	Prenfish 5%			5.4		5.0	5.0			This study
	1-1.5g	Noxfish 5%	7.0		4.0			2.4		2.2	Marking and Bills 1976
Fathead minnow	0.31g	CTF Legumine 5%					26.0	21.0			This study
	0.31g	Prenfish 5%					25.5	21.0			This study
	1-1.5g	Noxfish 5%			59.5			20.0		7.1	Marking and Bills 1976

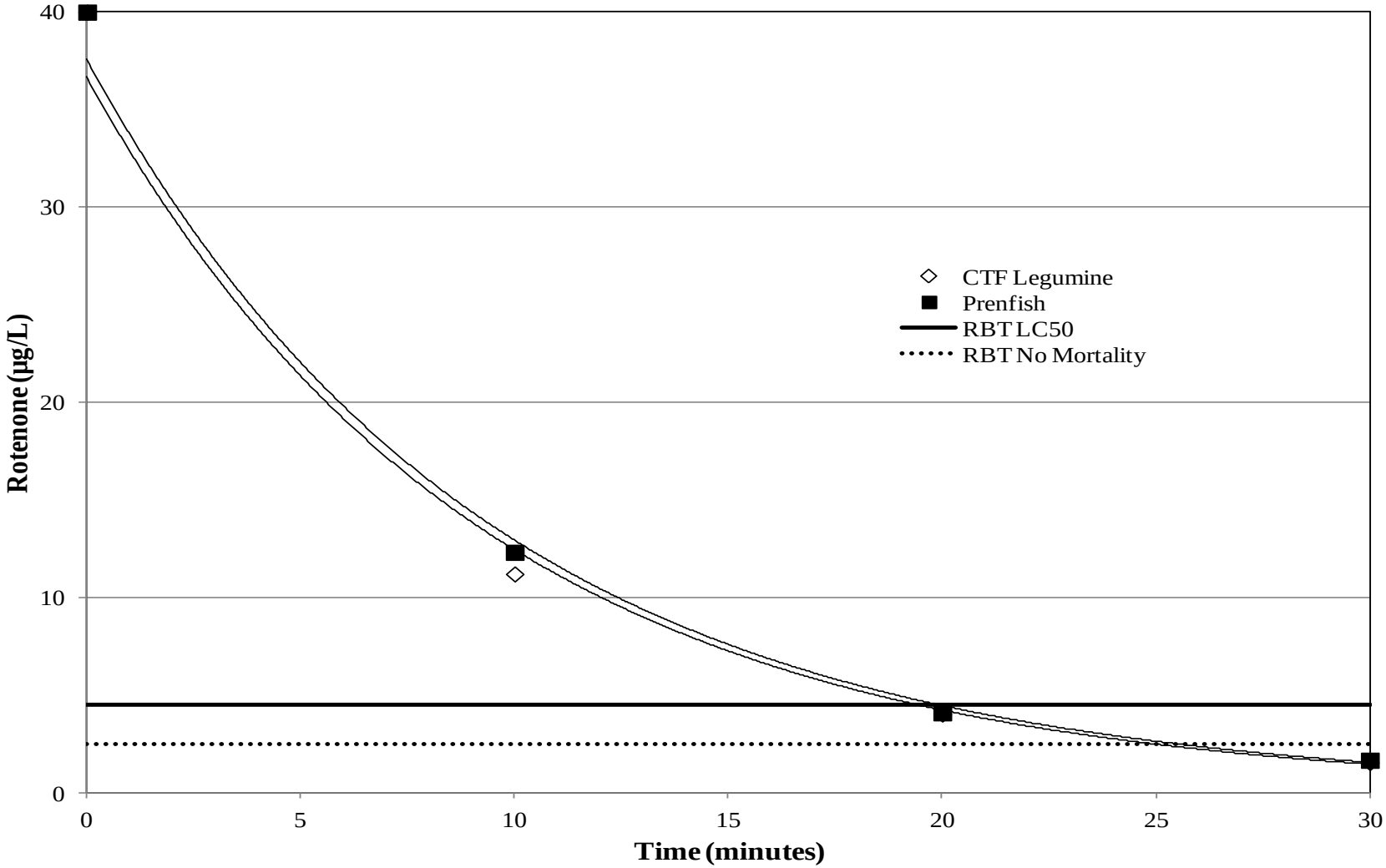

Figure 3. Degradation of CFT Legumine and Prenfish rotenone formulations by potassium permanganate. The solid horizontal line denotes a concentration of rotenone lethal to 50% of rainbow trout fry. The dashed horizontal line denotes a concentration in which no mortality occurs.