Water Pollution Studies

Federal Aid Project F-243-R23

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Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Parks & Wildlife

Aquatic Research Section

Fort Collins, Colorado

August 2016

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Table of Contents

State: Colorado Project No. F243R23

Project Title: Water Pollution Studies

Period Covered: July 1, 2015 to June 30, 2016

Project Objective: To develop quantitative chemical and toxicological data on the effects of pollutants and water quality on aquatic life, investigate water quality problems in the field, and provide expertise and method development in aquatic chemistry and aquatic ecotoxicology.

Job 1
Job title: Toxicology Research: experiments and biomonitoring to assess risk of emerging toxicants, derive new water quality standards and improve existing water quality standards.

Ultimate Objective: Gather quantitative data and conduct experiments to build water quality standards that are protective of sport fish and sport fish habitat.

Need:

Water Pollution Studies: Over seven million recognized chemicals exist and 80,000 are in common use (GAO 1994). However, Colorado limits surface water concentrations of only 63 organic and 15 inorganic chemicals (CDPHE 2013). Colorado's mining heritage has left a majority of watersheds in the Colorado Mineral Belt with elevated metal concentrations. Links between mining activity, metal pollution and degradation of aquatic communities in streams are well established in the literature (Clements et al. 2000). An estimated 20,000-50,000 mines in the western United States produce acid mine drainage (AMD) which seriously affects 5,000-10,000 miles of streams (USDA 1993) and has been described as the greatest water quality problem in the Rocky Mountain region (Mineral Policy Center 1997). Regulatory agencies such as the US Environmental Protection agency (EPA) and the Colorado Department of Public Health and Environment (CDPHE), including the Water Quality Control Commission, act as moderators when building or refining pollution standards. These agencies largely rely on research from external sources and alter standards after requests from industry or stakeholders. Colorado Parks and Wildlife is the primary stakeholder advocating for sustainable fisheries in Colorado by producing scientific evidence that ensures water quality standards are protective of fisheries.

Iron Toxicity: The current USEPA chronic criterion for protection of aquatic life is 1.0 mg/L (total recoverable) based largely on field observations not experiments (USEPA 1976). In

Colorado 1,454 miles of streams and rivers are considered heavily affected by Fe (Colorado State Department of Public Health and Environment 2008). There has been an increased interest in establishing new water quality standards for contaminants such as Fe and Al in Colorado and other states. Industry efforts to revise the 2007 standards for Al in New Mexico resulted in a markedly relaxed hardness-based criterion value (New Mexico State Surface Water Quality Bureau 2009), and the Colorado Mining Association recently suggested establishment of a dissolved standard for iron. Such a standard might result in reduced protection for aquatic life in pH-neutral waters in Colorado.

Emerging Toxicants of Concern: Endocrine disrupting chemical classes such as estradiols and statin drugs are believed to have the highest probability of having adverse effects on sport fish and the ecosystems that support sport fish. Statin drugs are marketed to control blood lipids by altering how the body stores and metabolizes fats. These drugs are often highly synergistic and are not removed in wastewater treatment. Fat regulation of fish largely affects fish survival and may be affected by exposure to statin pharmaceuticals. Increased demand from CPW researchers and managers to investigate these and other classes of toxicants warrants improving practices, knowledge and infrastructure to fulfill the CPW Aquatic Toxicology Laboratory's role in Colorado's fisheries management.

Works Cited:

Clements W.H., Carlisle D.M., Lazorchak J.M., Johnson P.C. (2000). Heavy metals structure benthic communities in Colorado mountain streams. Ecological Applications 10:626-38.

Colorado Department of Public Health and Environment - Water Quality Control Commission. (2013) 2013 Regulation No. 31. The Basic Standards and Methodologies For Surface Water (5 CCR 1002-31)

General Accounting Office (1994). Toxic Substances Control Act: Preliminary Observations on Legislative Changes to Make TSCA More Effective (Testimony, 07/13/94, GAO/T-RCED-94-263))

Mineral Policy Center (1997) Golden dreams, poisoned streams. Washington, D.C., USA

New Mexico State Surface Water Quality Bureau. (2010). 2010 Triennial Review Amendments to 20.6.4 NMAC Sections 7 through 15. Available from: ftp://ftp.nmenv.state.nm.us/www/swqb/Standards/ 2010/20.6.4NMAC-AmendedSections2010-11-01.pdf

Stephen C.E., Mount D.I., Hansen D.J., Gentile J.R., Chapman G.A., and Brungs W.A. (1985) Guidelines for Deriving Numerical National Water Quality Criteria for the Protection Of

Aquatic Organisms and Their Uses. US Environmental Protection Agency. Washington, DC USA. PB85-227049

USDA Forest Service. (1993). Acid Mine Drainage form Impact of Hard Rock Mining on the National Forests: A Management Challenge. Program Aid 1505.

USEPA. (1976). Quality Criteria for Water. Washington, DC USA PB263-943.

Job 1 Objectives:

1- Assess toxicity of emerging contaminants pertinent to Colorado surface waters by conducting toxicity trials on sport fish and food species important to sport fish populations.

2- Improve state and national water quality standards to ensure they are protective of sport fish important to Colorado. These standards include toxicants (e.g. Fe, Se, Cu, Cd, Zn, Al, Mn, benzene, petrochemicals) and Physical properties (e.g. total suspended solids, temperature, nutrients). This is done by conducting and publishing new toxicity trials for consideration when currently regulated toxicants or water quality parameters are reconsidered on a triannual basis by EPA and CDPHE. Improving standards requires the following improvements to traditional laboratory toxicity trials:

a) Continually assessing sensitivity of more species of sport fish as they become available from field and hatchery sources.

b) Exposing these strains for longer durations than previously studied and/or including sensitive life stages. (e.g. early life stages of rare strains of Cutthroat Trout, egg survival of walleye)

c) Considering ecologically relevant Sublethal endpoints as technology and infrastructure becomes available to CPW toxicology laboratory (e.g. predator avoidance, olfactory function, fecundity, thermal tolerance, apoptosis, protein carbonyl content)

d) Conduct toxicity trials that examine new routes of exposure (e.g. dietary exposure)

e) Conduct experiments that increase environmental realism by using natural habitat, natural assemblages, mesocosm communities, and food chains both in laboratory and field settings.

f) Consider multiple stressors simultaneously.

3- Create new aquatic life criteria ("regulatory standards") by conducting toxicity tests that meet the 1985 EPA requirements for standard derivation which include a minimum number of taxa, particular qualities and durations of trials and methods for calculating nominal values or hardness adjusted criteria. Present these findings to regulatory agencies.

Approach:

<u>Action #1.1 – Assessment of emerging pollutants: Statin like pharmaceuticals and pesticides</u>

<u>Action #1.1.1 – Assessment of statin drugs and statin like pharmaceuticals</u>

●*Level 1 Action Category: Data Collection and Analysis*

●*Level 2 Action Category: Research*
Expose fish to environmentally relevant levels and mixtures of statin drugs or other cholesterol lowering pharmaceuticals from larval to reproductive age. Control treatments will be compared to exposure treatments using the following endpoints: survival, mass, length, fat to protein ratio, blood chemistry, fecundity, metabolism.

Action #1.1.1 Accomplishments

Development of Biomarkers of Antilipidemic Drug Effects associated with exposure to the lipid-lowering drug gemfibrozil.

METHODS:

Study design: Fathead minnows (*Pimephales promelas)* were selected as a model species because of short life cycles. Fish were obtained from Aquatic Biosystems (Fort Collins, CO) and were acclimated for one week prior to the start of the study. During acclimation, fish were maintained in a static renewal system with dilution water in the same stainless steel testing chambers (hotel pans) that would be used for the study. Twenty five days after swim-up, 15 fish were randomly assigned to each experimental unit. After acclimation, fish were exposed daily to 0 mg gemfibrozil/L (control), 0.5 mg gemfibrozil/L (low), or 2.0 mg gemfibrozil/L (high) for 28 days. Partial (75%) renewal was done twice daily to ensure consistent exposure and water quality.

Mixing of solutions and dilution water: Dilution water (moderately hard water) was prepared by mixing dechlorinated tap water (Fort Collins, CO) and well water. The mixing was regulated with a conductivity meter and held at the desired testing temperature (25°C) in a 283 L tank. This water source was used for makeup water during the 25 d acclimation period and was used to prepare exposure solutions during the 28-day study period. Exposure solutions were prepared daily by mixing dilution water with stock solutions of gemfibrozil for 30 minutes prior to use. Because gemfibrozil stock solutions were prepared with dimethyl sulfoxide (DMSO), DMSO was added to dilution water and mixed for 15 minutes prior to use for the control exposures.

Feeding: *Artemia* (live cultured brine shrimp) were decapsulated twice daily and were fed to the fish for the duration of the acclimation and study period.

Holding and Renewal: Each 7.57 L stainless steel hotel pan held five fish in 4.0 liters of moderately hard water under static conditions. Hotel pans were held in a temperature controlled water bath that 25°C. Seventy-five percent of the solution in each pan was removed daily via siphoning and was replaced as described above. Aeration was provided with tubing connected to an aerator and Pasteur pipets. Timers for light exposure were set to a cycle of 14:10 (light:dark).

Termination: Fish were euthanized with tricaine methanesulfonate (TMS) at the completion of the 28-day study. Initial blot dry weight and length were determined on all fish and then immediately placed in cryogenic vials on dry ice. Fish were then either transferred to a -80°C chamber for storage until tissue analyses took place or desiccated on aluminum pans for dry weight determination.

Chemistries: Daily water quality measurements of the following were recorded: pH, dissolved oxygen, temperature, and conductivity. These were measured in new and old test solutions (prior to siphoning). Ammonia (mg NH3-N/L) was measured daily in old exposure solutions prior to water changes as well. Hardness and alkalinity were measured once daily. Water pH, dissolved oxygen, and conductivity were measured with calibrated probes and meters. Hardness and alkalinity were measured using standard titration methods. Temperatures in each exposure level were recorded daily using a NIST-traceable digital thermometer.

Analytical: To verify the concentration of gemfibrozil in the test solutions, high performance liquid chromatography was performed on samples from each exposure level that were collected and stored in the dark at 4°C.

Triglyceride content: Fish were homogenized with a Tissue-Tearor® homogenizer and deionized water then allowed to incubate at 100°C for 5 minutes with sodium citrate buffer added. Each sample was then placed on ice for 5 minutes then centrifuged for 5 minutes at 5,000 rpm. The soluble fraction was then used to determine triglyceride concentration. Triglyceride Reagent (Sigma T2449-10ml) which contains lipase and Free Glycerol Reagent (Sigma, F6428-40ml) which contains glycerol kinase were used and samples read at 540nm (adapted from Weber 2003 and Bennet 2007).

Growth: Growth was determined by length (standard and total length measured with digital caliper) and mass (blot wet weight and dry weight). Also, DNA:RNA ratios and total protein concentrations are being determined in fish homogenate as additional measures of growth (adapted from Weber 2003). Fish homogenate for these endpoints are prepared with Tris-EDTA buffer, sodium dodecyl sulfate, and the tissue tearor, then centrifuged at 5,200 g and read with fluorescence spectrophotometry using two fluorescent dyes (Hoescht 33258 and ethidium bromide).

PRELIMINARY RESULTS:

Survival: No fish died during the acclimation or study period.

Behavior: No abnormal fish behavior was noted during the acclimation or exposure period.

Chemistries and analytical: Gemfibrozil concentrations did not lose more than 10% of the initial measured concentration. Gemfibrozil was within 9% of the nominal concentration. Testing

temperature was held within 1°C of the desired temperature. All measured water quality parameters were within expected normal limits.

Growth: Total and standard lengths were not statistically decreased as concentrations increased. Additionally, dry weight was not statistically significant. DNA:RNA ratios and total protein results are pending.

TGA: Triglyceride content is pending.

Weber L.P., Higgins P.S., Carlson R.I., Janz D.M. (2003). Development and validation methods for measuring multiple biochemical indices of condition in juvenile fishes. Journal of Fish Biology 63, 637-658.

Bennett P.M., Weber L.P., Janz D.M. (2007). Comparison of chloroform-methanol-extracted and solvent-free triglyceride determinations in four fish species. Journal of Aquatic Animal Health. 19, 179-185.

Personnel: Andrea Kingcade and Pete Cadmus

Action #1.1.2 – Assessment of relative importance of aqueous verse dietary exposure of insecticides on trout.

●*Level 1 Action Category: Data Collection and Analysis*

●*Level 2 Action Category: Research*

1-Trout, pteronarcyidae stone flies and leaf litter will be contained in mesocosms that simulate lotic flow and physical chemical characteristics observed in the Gunnison River watershed. An acute exposure of trout and trout food sources to Permethrin and/or carbaryl and/or the synergist piperonyl butoxide will be simulated.

2-Lethality and sublethal endpoints will be assessed.

Action #1.1.2 Accomplishments

Management practices at study sites changed to limit loss of aquatic insects and fish. Experimental design relied on field observations and assessments of leaf litter and aqueous concentrations of pollutants during application of insecticides. Data collection for Action #1.1.2 was not possible. Resources and attention were diverted to Action 1.1.1, 1.2 and 2.

Action # 1.2 – Toxicity experiments to improve existing water quality standards.

●*Level 1 Action Category: Data Collection and Analysis*

●*Level 2 Action Category: Research*

1-Method development from the 2014-2015 fiscal year has enabled the CPW Aquatic Toxicology Laboratory to include new sublethal toxicological endpoints and enabled trials to be conducted

on sensitive life stages previously unstudied. Method development in expanding tools and endpoints will continue.
2- Toxicity of contaminants regulated by Colorado (e.g.: Cu, Cd, Zn, Fe, Se, Mg, Al, Benzene) will be evaluated on egg, larval and early life stages of sport fish in the form of acute and chronic laboratory and field trials. When required, trials will include exposure of sport fish food such as aquatic insects.
3- These data will be presented in triannual recalculations of existing standards.

Action #1.2.1 Accomplishments

Development of sub-lethal endpoints for the study of sport fish and fish habitat

In an effort to expand the sublethal endpoints the Colorado Parks and Wildlife Aquatic Toxicology Laboratory can use during toxicity trials, considerable Research and Development of new skills and technologies is underway. Ecologically relevant responses on the behavioral, physiological and cellular level help better prescribe a safe concentration of pollutants for sport fish. Once complete, each of these endpoints can be used to evaluate differences between organisms that have been exposed to toxicants and control (unexposed) organisms. Below is a list of endpoints and their level of completion as it relates to use at the Colorado Parks and Wildlife Aquatic Toxicology Laboratory.

Respiration chambers – successful: Chambers have been developed to assess the respiration rates of fry and fingerling trout and smaller fish.

Fish motility – in progress: Infrastructure is in development for the assessment of fish motility. Once complete, this will assess how fish use three-dimensional space in standard (safe) conditions and when exposed to alarm and predator cues. This endpoint also has the potential to assess group motility and social interaction.

Response time and bolt speed – in progress: Infrastructure to assess speed of response to aversive stimulus is in development. This infrastructure will also assess bolt acceleration and velocity.

Sperm motility – in progress: Feasibility of using video analysis to assess sperm motility in salmonids is being investigated.

Olfactory assessment – in progress: Numerous methods of assessing behavioral response to olfactory food cues have been investigated. This could be extended to olfactory alarm or predator cues. Use of video analysis software, accelerometers and other techniques are being investigated for salmonids.

Erythrocyte (red blood cell) and leukocyte (white blood cell) counts – successful: This endpoint has been difficult to include in toxicity trials as small fish are both the most sensitive to toxicants and are difficult to draw blood from. Blood draw techniques were devised that are suitable for various size and life stage of fish. Appropriate staining and dilution of blood drawn for visualization cells and use of a hemocytometer to enumerate cells was established. This end point has been successful for all but the smallest fish, for which improved blood draw techniques are necessary to ensure no contamination by cerebrospinal or interstitial fluid.

White blood cell differentials – successful: Assessment of lymphocyte, neutrophil, monocyte and eosinophil ratio in salmonids is possible with newly developed infrastructure. At this time the expertise to perform this differential must be contracted to CSU veterinary diagnostic laboratory.

Packed cell volume (PCV) – in progress: Packed cell volume or hematocrit assessment may not be a feasible endpoint for fish due to concerns that changes in osmolality with handling stress prevent an accurate assessment. However, the Aquatic Toxicology Laboratory has developed the capability to assess PCV and must now evaluate its value as an endpoint.

Mean corpuscular volume (MCV) – in progress: MCV is the assessment of average erythrocyte volume calculated from hematocrit and red blood cell count. Development of this endpoint is complete assuming adequate blood can be drawn for hematocrit. However, as this is a calculation based on PCV (see above) so quality assurance efforts are underway.

Hemoglobin using a Hemocue® – in progress: Hemocue® provides real time hemoglobin level assessments. While we found hemoglobin level can be assessed on fish species, quality assurance studies are underway to determine if cross contamination of body fluids is a risk.

Histopathology – successful: Through collaboration with Colorado State University Veterinary Diagnostic Laboratory Staff many histopathological assessments of sport fish species will be available.

Hepatosomatic index and Viscerosomatic index – successful: A ratio of liver and whole viscera weight to somatic weight has been established for use in the CPW Aquatic Toxicology Laboratory.

Spleen somatic Index (SSI) – successful. A ratio of spleen to somatic weight can be assessed and may be an indicator of immunological changes.

Personnel: Pete Cadmus, Abbie Jefferson and Sam Duggan

Action #1.2.2 Accomplishments

Assessment of Rainbow Trout susceptibility to Benzene

Oil and gas extraction, agriculture, urbanization and industrialization are increasing in Colorado. A better understanding of sport fish susceptibility to hydrocarbons, insecticides, herbicides, industrial chemicals, pharmaceuticals and other carbon based (organic) pollution is needed. It is important to prepare Colorado to assess these new classes of stressors. As a means of developing new methods to assess potential organic toxicants, the effect of chronic benzene on Greenback Cutthroat Trout (*Oncorhynchus clarkii stomias*) and the Common Shiner (*Luxilus cornutus*) was investigated. The Piceance Basin on the western slope of Colorado hosts native Greenback Cutthroat Trout and many other sport fish. This area co-occurs with high density oil and gas extraction. The eastern plains, historic range for the expatriated Common Shiner and numerous sport fish, have also undergone changes as the result of oil and gas extraction. In March 2013 benzene concentrations in parachute creek reached 5300 ppb, the state acute water quality standard, after a natural gas pipeline valve leak. There is no chronic standard for benzene. Hydraulic fracturing fluid composition is varied and proprietary. However, benzene is a common component of fluid removed from fractured wells. Benzene was studied for broad applicability to water quality concerns in Colorado. Additionally, it is volatile and difficult to work with but not acutely toxic to humans. This allowed our laboratory to test infrastructure for assessment of many organic chemical classes.

METHODS:

Study design: A peristaltic pump delivered benzene stock solution to a PTFE and stainless steel diluter producing five treatment levels (776, 451, 244, 160, 100 and 0 µg/l) and a control (0 µg/l) from a stock solution and dechlorinated Fort Collins municipal water. Benzene was maintained in solution by a magnetic stir plate in a glass carboy. Each treatment level and the control were replicated four times for each species, resulting in a total of 48 experimental units. Treatments were randomly assigned to tanks. Flows were delivered by PTFE coated tubing at a rate of 30 ml/min. Eggs were cultured in stainless steel tea strainers in stainless steel tumblers; sac fry were raised in stainless steel hotel pans. As biomass of each unit increased, air was added via glass Pasteur pipettes to maintain adequate dissolved oxygen. Experimental apparatus were enclosed in a BioBubble® (Fort Collins, CO) negative pressure and ventilation system to ensure minimal exposure of surface water to volatilized benzene, and to protect workers.

Chemistries: Temperature, flows, dissolved oxygen, conductivity, pH, hardness and alkalinity were comparable between experimental units.

Analytical: Benzene levels were confirmed using isocratic high performance liquid chromatography. 200ml water samples were removed from exposure tanks and diluters and were passed through a Sep-Pak (Waters Corp., Milford, Massachusetts, United States) C18 solid phase extraction column. Samples were eluted in 80% methanol. 1000 µl of elutant was run through an Agilent 1220 isocratic HPLC using a mobile phase of 80% methanol and a Thermo Scientific Hypersil GOLD® c-18 column. This method appeared to be robust for laboratory samples. Benzene levels in splitter boxes of the diluter system held steady at 776 (255), 451 (66), 244 (60), 160 (28), 100 (24) and 0 µg/l (SE). Benzene levels in treatment tanks were significantly lower after the introduction of air stones immediately after swim up when samples averaged 151, 82, 88, 73, 50 and 0 µg/l. Benzene is very volatile and maintaining benzene levels while meeting dissolved oxygen needs of fish proved difficult.

Organisms: Greenback Cutthroat Trout: Greenback Cutthroat Trout were spawned at 8.6°C at the Poudre River Hatchery on June 15th 2016 in raceway water spiked with the same benzene concentrations in which the fry would be raised. Each experimental unit was composed of milt from three males and eggs from one female. After hardening, fertilized eggs were transported to the Colorado Parks and Wildlife Aquatic Toxicology Laboratory where they were allowed to warm to the temperature of the experimental set up (12°C). Prior to introduction to tea strainers, eggs were treated with iodine per hatchery protocol to prevent fungus growth.

Organisms: Common Shiner: Five female and three male Common Shiners were collected from West Plum Creek on June 15th 2016 and held in the Colorado Parks and Wildlife Aquatic Toxicology Laboratory in dechlorinated Fort Collins municipal water at until June 18th, when they were spawned in 13°C. Fertilized eggs were sorted from unfertilized by sight. Twenty-five were delivered into experimental units, and hardened in the concentration at which they would be exposed for the duration of the experiment. Unfortunately, Common Shiner fertilization failed. By July 2nd 2015, no eggs remained.

Feeding: After swim up, trout fry were offered decapsulated brine shrimp (*Artemia salina*) and then Rangen trout chow twice daily totaling 3% of body weight. By September 8th, fish were large enough to consume chopped *Lumbriculus variegatus* at a rate of 2% fish mass /day. Lumbriculus had been cultured at the same concentration as the fish to which they were fed in hotel pans fed by the same diluter. Apparatus to culture algae, *Psuedokirschneriella subcapitata*, in each concentration of benzene were developed to feed *Ceriodaphnia dubia*, also cultured in each concentration of benzene were developed for trout feeding, but were unable to reach a biomass from which feeding would be sustainable.

Termination and assessment: On august 1st 2015 fish tank populations were reduced to 26 fish to prevent population effects. On September 10th 2015, fish were fish were euthanized and preserved for histopathological assessment performed by Dr Paula Schaffer, Colorado State University. Blood draws were performed by cervical dislocation and, after dilution and staining

with Natt & Herricks solution, a hemocytometer was used to assess total erythrocyte (RBC) and leukocyte (white blood cell - WBC) counts. Hematocrits were attempted, but inadequate blood could be drawn from the fish due to size, thus packed cell volume (PCV) and mean corpuscular volume (MCV) could not be assessed. Critical thermal maximum (CTmax) and dissolved oxygen minimum (DOmin) tolerances were assessed.

RESULTS:

RBC and WBCs counts showed no significant difference between treatments and controls, nor did CTmax or DOmin. Preliminary assessments (30 day post swim up) of histopathology indicated a possible difference in sexual development of high treatments compared to controls. A small subset of controls analyzed had ovarian tissue primarily composed of perinuclear oocytes; while high treatment specimens had ovarian tissue composed of primary oogonia. This was surprising given the low levels of benzene in treatment tanks. For this reason the experiment was extended from a ~30 day duration to a three month duration to ensure sufficient maturation of sex cells. After this time fish were euthanized and preserved for pathology. With the larger sample sizes associated with complete assessments of each replicate developmental issues were found not to be different across groups.

While the data from the research was insignificant, the same infrastructure and endpoints can now be used to assess the affect of organic pollutants on other organisms where previously the laboratory could only assess metal toxicants.

Personnel: Abbie Jefferson, Pete Cadmus and Paula Schaffer

Action #1.2.3 Accomplishments

Assessment of persistent aromatic hydrocarbons and effects on trout species.

Oil development has expanded dramatically in Colorado over the last decade as the state has become the 9th leading producer of oil in the United States. Associated with the rapid expansion has been a significant increase in the number of accidental releases and spills of petroleum hydrocarbons into the environment. In 2014 over 300 spills of petroleum products were reported to the Colorado Department of Health.

Quantifying effects of petroleum spills on aquatic ecosystems is challenging, in part because the persistence and toxicity of these compounds are highly variable and differ significantly among classes (e.g., gasoline vs. diesel). In addition, most research on impacts of petroleum spills has been conducted in warmwater and marine systems. Known effects on coldwater streams are, comparatively, insufficient for preparation and response to spill events by resource managers. Research is also needed to identify an appropriate suite of bioassessment techniques that can be

utilized by resource managers attempting to characterize the well-being of a petroleum spill impacted coldwater stream.

Objective:

As part of ongoing efforts to determine potential effects of petroleum spills, as well as develop bioassessment techniques that can be rapidly employed at spill sites in Colorado, salmonids and other coldwater organisms will be exposed to environmentally relevant concentrations of diesel in simulated spill scenarios under laboratory conditions. This research is intended to identify likely adverse effects and recovery potential of resident coldwater organisms while also identifying an appropriate suite of bioassessment techniques that can be used to quantify biological effects of petroleum hydrocarbons on coldwater stream ecosystems. Another goal is to use these results to inform future spill related biomonitoring methods and improve evidence based approaches for holding responsible parties accountable for damages.

Experiment 1: Exposure of periphyton to diesel.

METHODS:

Sport fish populations depend on primary productivity of ecosystems. Effects of petroleum on periphyton productivity were assessed. Natural periphyton communities were colonized on 2'' x 2'' porcelain tiles for two weeks in an artificial stream facility receiving natural light. Water was supplied from Horsetooth Reservoir, a deep mesotrophic reservoir with similar physiochemical characteristics as typical coldwater streams in Colorado. Following the two week colonization period, periphyton tiles were transplanted to CPW's Aquatic Toxicology Laboratory where they were simultaneously exposed to wide spectrum UV-A/B, visible light and a pulse of diesel fuel.

It is important to note that the addition of UV light is critical for the environmental relevance of toxicity tests involving petroleum products as UV light from the sun plays an important role in activating the toxic compounds found in petroleum. It is also important to note that UV light is rarely used in laboratory toxicity tests.Therefore much of the available literature regarding petroleum toxicity lacks environmental realism.

Five treatment levels (0, 20, 200, 2000 and 20000 parts-per-million diesel in water), in triplicate, each containing six colonized tiles were examined for 84-hours. Treatments were dosed with diesel initially and 50% daily water changes were used to reduce the diesel concentration over time, thereby simulating diminishing exposure concentrations as would occur in an actual stream diesel spill. Using a Benthotorch, a highly convenient and field portable handheld fluorometer, and a Pulse Amplitude Modulated (PAM) fluorometer, periphyton biomass and physiological endpoints, respectively, were quantified at three time-points throughout the experiment.

RESULTS:

Periphyton biomass mass diminished in a concentration dependent manner during the 84-hour experiment with respect to diesel exposure concentration (see figure 1). Biomass in controls (0 ppm diesel) were 145% (+/- 24 SEM) higher after the 84-hour exposure period indicating growth during the experiment. In contrast, after 84-hours of exposure, periphyton biomass for the 2,000 ppm and 20,000 ppm diesel treatments were 34% (+/- 11 SEM) and 20% (+/- 12 SEM), respectively, of their biomass directly preceding exposure. Physiological results from the PAM fluorometer are still being analyzed.

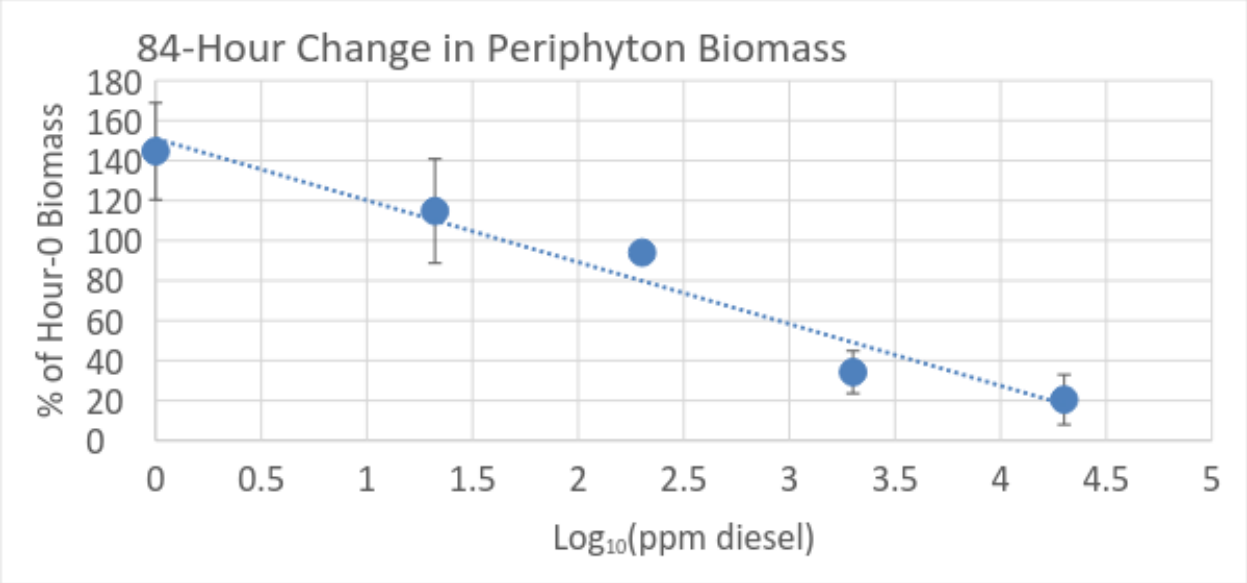

Figure 1: Effects of diesel exposure (0, 20, 200, 2000 and 20000 ppm diesel in water) on periphyton biomass after 84-hours of exposure.

Experiment 2: Exposure of salmonids to diesel.

METHODS:

Several iterations of experimentation were conducted on salmonids obtained from the CPW hatchery system. All salmonids were exposed to a simulated diesel spill utilizing similar exposure scenarios. Salmonids (n=4-6) were added to experimental tanks containing control water and 750g of biologically active sediment collected from the headwaters of the Cache la Poudre River. Salmonids were acclimated to wide spectrum UV-A/B and visible light for a minimum of 48-hours before a pulse of diesel fuel began the exposure. The diesel pulse ranged in volume resulting in concentrations ranging from 0 ppm to 2,000 ppm. Following 6-hours of exposure, a 25% water change dramatically reduced the diesel exposure concentration to reflect how a petroleum spill would behave in a flowing stream. Subsequent daily 25% water changes further reduced the diesel concentrations within the experimental treatments. Throughout the experiment, behavior and mortality were observed; at end of the experiment (96-hours post-

exposure) physiological tests (e.g. dissolved oxygen minimum, oxygen consumption, and critical thermal maximum) were conducted and samples were collected for hematology and/or histology.

Experiment 2.1: Diesel tolerance range for Rainbow Trout

METHODS:

Four Rainbow Trout (*Oncorhynchus mykiss*) fingerlings per tank, in triplicate, were exposed to 0, 50, 200, 500, or 2000 ppm diesel in water as described above. When found dead, moribund, or at the end of the 96-hour experiment, blood samples were collected for a blood film, hematological analysis and were preserved for histopathology whenever possible. Water and sediment chemistry samples were also collected at several time points.

RESULTS:

Rainbow Trout mortality increased with increasing concentrations of diesel and lethality occurred more quickly in higher diesel concentrations than lower (see figures 2 and 3). A 96-hour LC50 of 1,042 ppm diesel was calculated for Rainbow Trout fingerlings exposed to diesel during this simulated spill scenario. Blood film, hematological and histopathology as well as chemistry samples have not been analyzed yet.

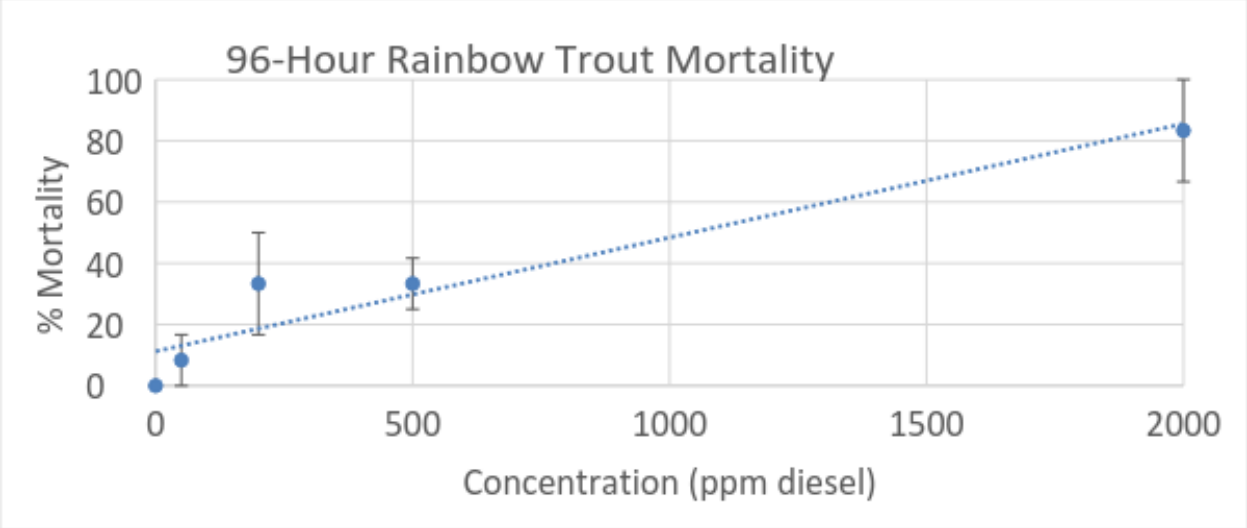

Figure 2: Effects of simulated diesel spills of various sizes (0, 50, 200, 500 and 2000 ppm) on mortality of Rainbow Trout fingerlings. Error bars are standard errors of mean mortality for three replicates containing four fish each.

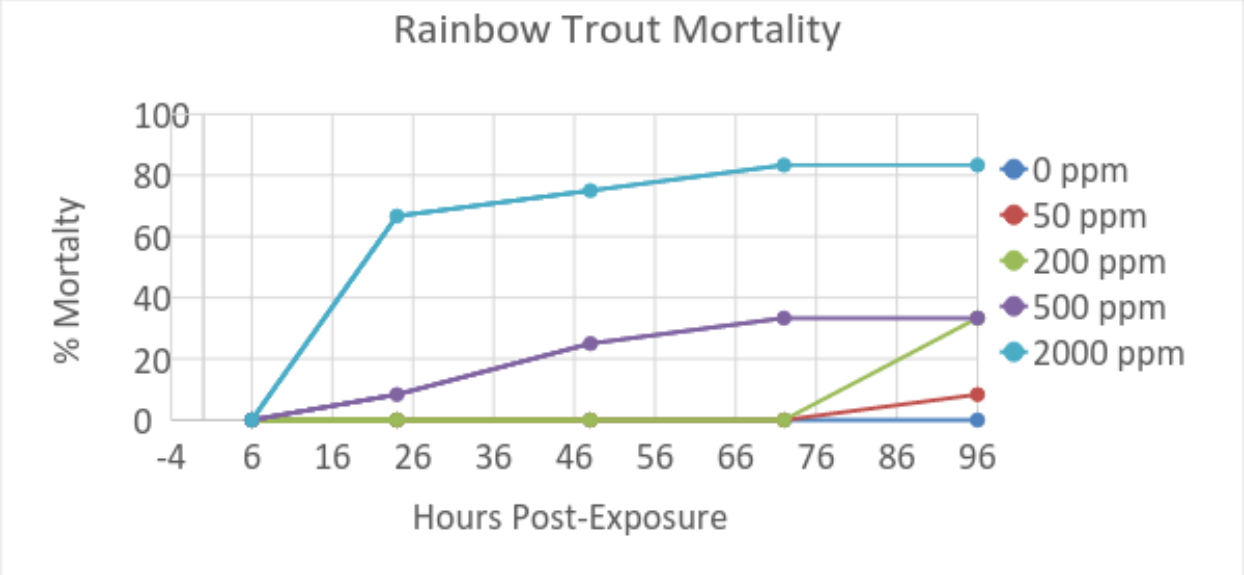

Figure 3: Effects of simulated diesel spills of various sizes (0, 50, 200, 500 and 2000 ppm) on mortality of Rainbow Trout fingerlings over the duration of time exposed.

Experiment 2.2: Diesel tolerance range for Greenback Cutthroat Trout

METHODS:

Six Greenback Cutthroat Trout (*Oncorhynchus clarkii* stomias) fry per tank, in triplicate, were exposed to 0, 100, 500, or 1000 ppm diesel in water as described above. When found dead, moribund, or at the end of the 96-hour experiment, samples were collected for histopathology whenever possible. Water and sediment chemistry samples were also collected at several time points. After the 96-hour experiment surviving fish were analyzed for their physiological tolerance to minimal dissolved oxygen as an indicator of sublethal injury. Oxygen consumption was also measured in a respirometer.

RESULTS:

No fish from the 0 and 100 ppm diesel treatments died during the experiment; 17 of 18 died from the 500 ppm treatments and all fish died from the 1000 ppm treatment (see figures 4 and 5). A 96-hour LC50 of 412 ppm diesel was calculated for Greenback Cutthroat fry exposed to diesel during this simulated spill scenario. While not statistically significant, DO minimum and oxygen consumption test demonstrated nominal trends (see figures 6 and 7). Histopathology results have not been analyzed yet.

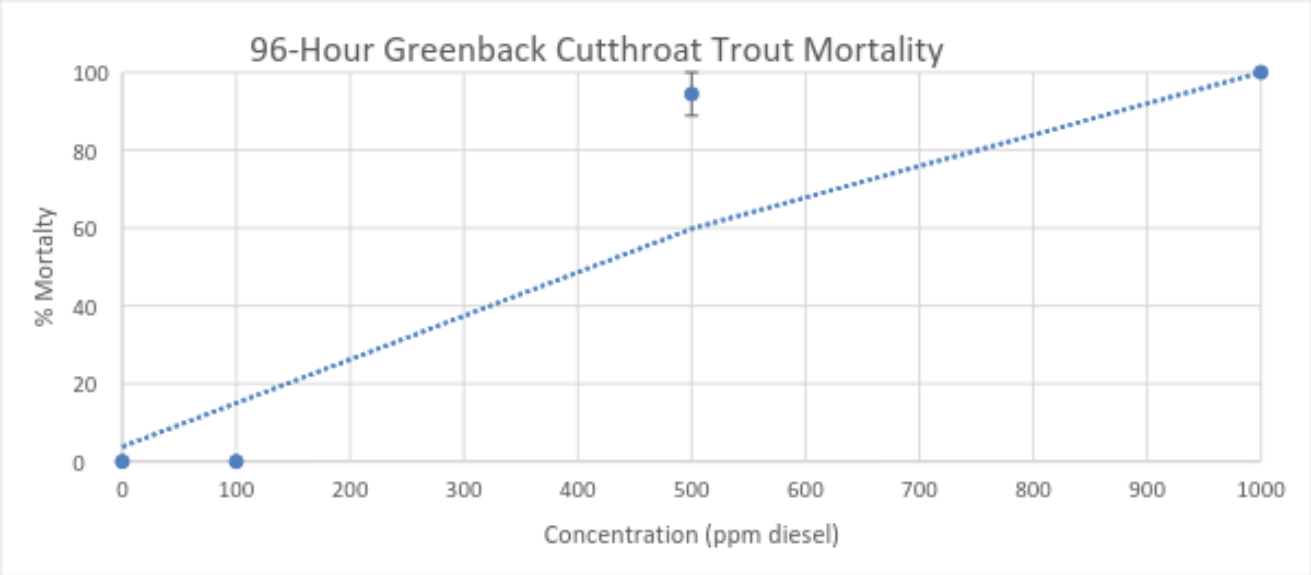

Figure 4: Effects of simulated diesel spills of various sizes (0, 100, 500, and 1000 ppm) on mortality of Greenback Cutthroat fry. Error bars are standard errors of mean mortality for three replicates containing 6 fish each.

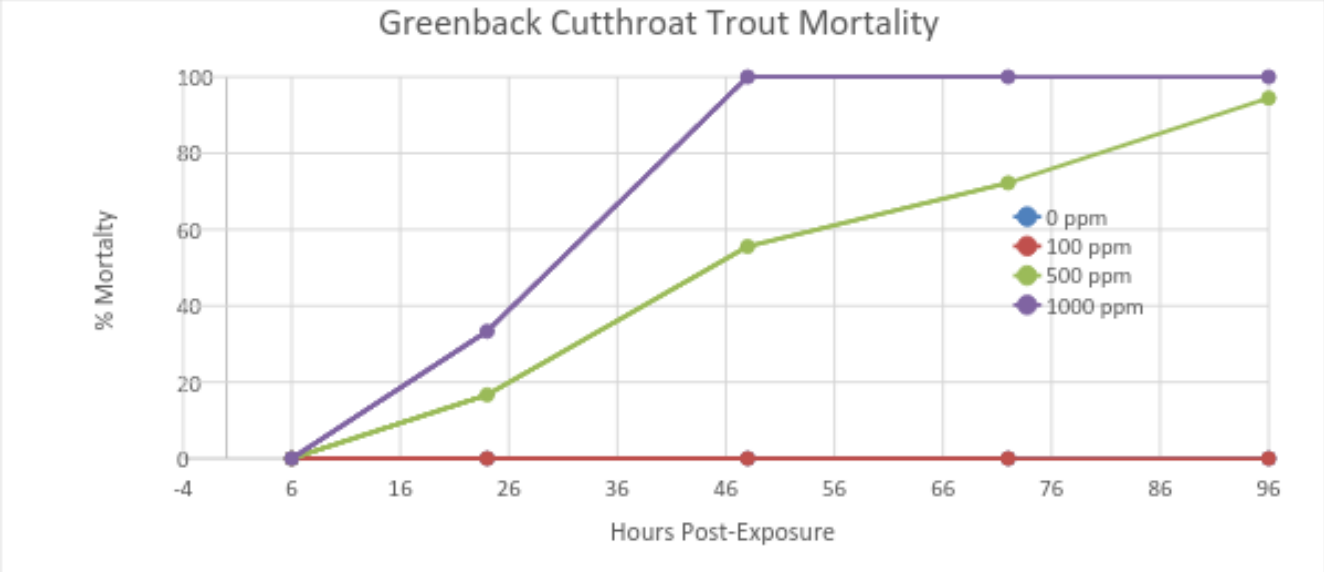

Figure 5: Effects of simulated diesel spills of various sizes (0, 100, 500, and 1000 ppm) on mortality of Greenback Cutthroat fry over the duration of time exposed.

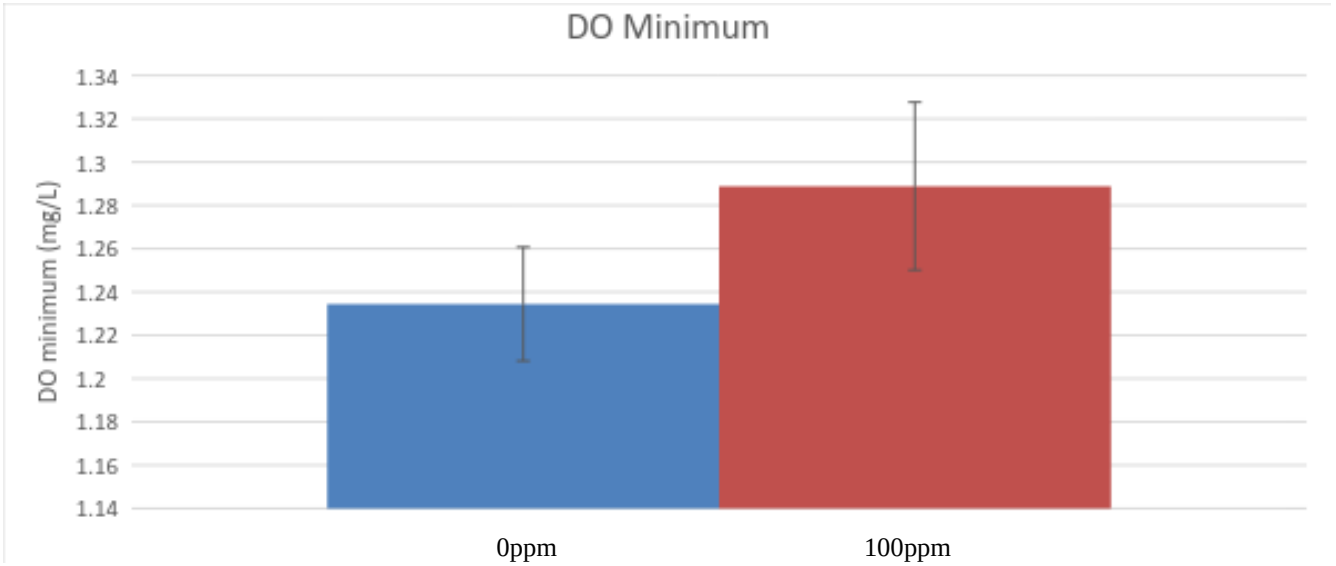

Figure 6: Effects of 0 and 100 ppm diesel on the minimum dissolved oxygen where equilibrium was lost by treated trout. Error bars are standard errors of mean DO minimum for three replicates containing three fish each. 500 and 1000 ppm treatments were omitted from this analysis because of insufficient fish survival.

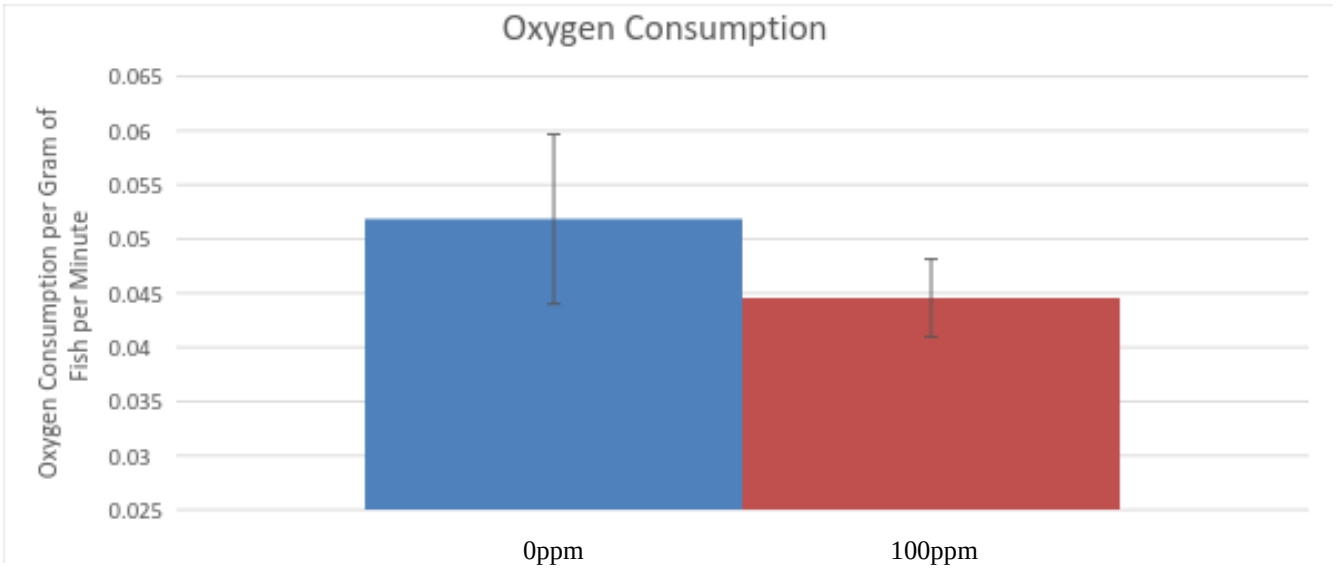

Figure 7: Effects of 0 and 100 ppm diesel on oxygen consumption. Error bars are standard errors of mean DO minimum for three replicates containing three fish each. 500 and 1000 ppm treatments were omitted from this analysis because of insufficient fish survival.

Experiment 2.3: Rainbow Trout diesel exposures with and without UV light:

METHODS:

Four Rainbow Trout fingerlings per treatment tank, in triplicate, were exposed to 0 and 500 ppm diesel in water using a factorial design with and without supplemental UV light. This resulted in four treatments: 1) 0 ppm with visible light only 2) 0 ppm with visible and wide spectrum UV-A/B light 3) 500 ppm with visible light only 4) 500 ppm with visible and wide spectrum UV-A/B light. All other details of the exposure scenario were the same as described above. When found dead, moribund, or at the end of the 96-hour experiment, blood samples were collected for a blood film, and samples were preserved for histopathology. After the 96-hour experiment surviving fish were analyzed for their physiological tolerance to minimal dissolved oxygen and critical thermal maximum as an indicator of sublethal injury.

RESULTS:

No fish died from any treatment except treatment #4 (500 ppm diesel with visible and wide spectrum UV-A/B light) where 25% mortality occurred. No significant differences were observed from critical thermal maximums (see figure 8). However, for dissolved oxygen minimums, treatment #4 (500 ppm with visible and wide spectrum UV-A/B light) was significantly higher than all other treatments ($p=0.0243$) indicating a reduced tolerance to low dissolved oxygen conditions (see figure 9). Histopathology results have not been analyzed yet.

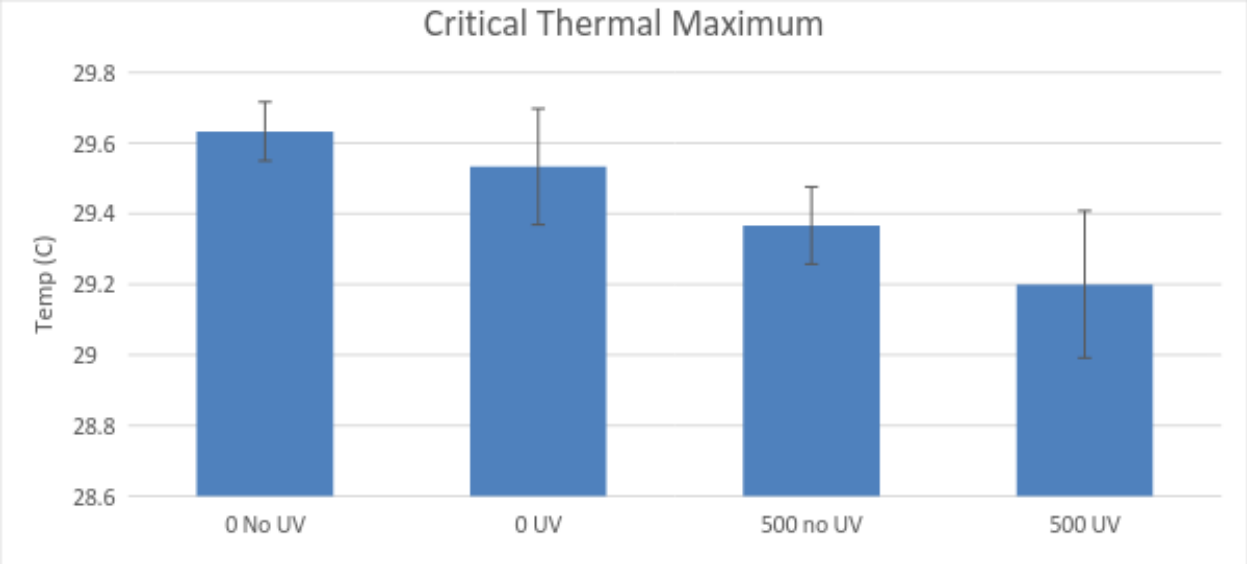

Figure 8: Effects of 0 and 500 ppm diesel interacting with and without UV-A/B light on the critical thermal maximum where equilibrium was lost by treated trout. Error bars are standard errors of mean DO minimum for three replicates containing two fish each. 500 and 1000 ppm treatments were omitted from this analysis because of insufficient fish survival.

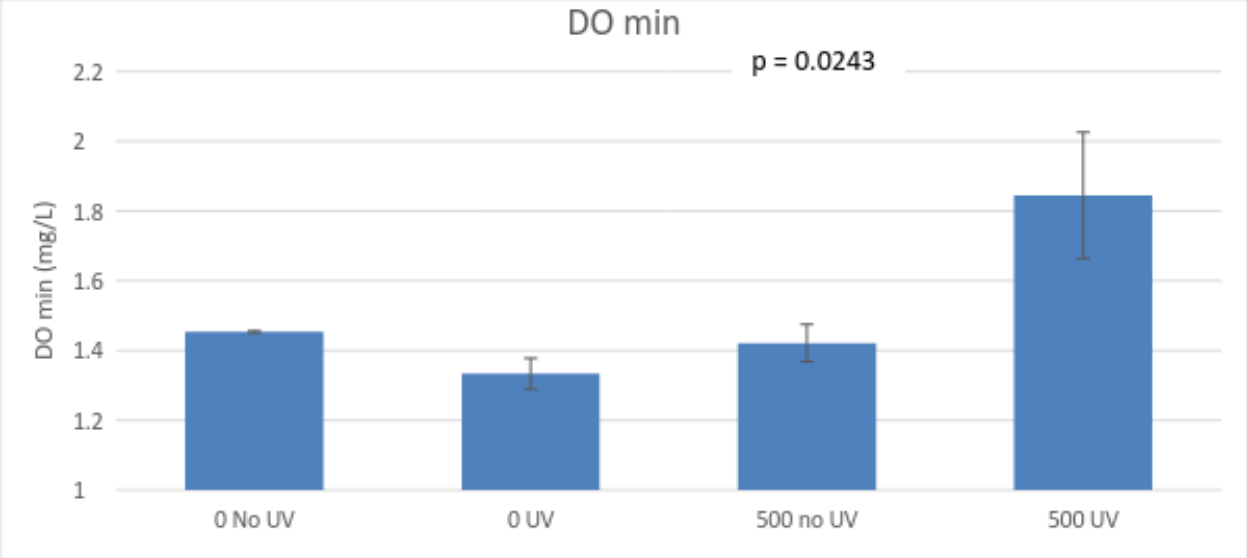

Figure 9: Effects of 0 and 500 ppm diesel interacting with and without UV-A/B light on the minimum dissolved oxygen where equilibrium was lost by treated trout. Error bars are standard errors of mean DO minimum for three replicates containing two fish each. 1000 ppm treatments were omitted from this analysis because of insufficient fish survival.

Personnel: Pete Cadmus and Sam Duggan

Action #1.3: Calculation of a Ferric Fe standard for Colorado

●*Level 1 Action Category: Data Collection and Analysis*

●*Level 2 Action Category: Research*

1- We will complete the remaining ferric Fe toxicity trials needed to meet the minimum species requirements defined by the EPA (Stephans et. al 1985).

2- CPW studies will be published.

3- Results from chronic Fe trials conducted at neutral pH will be complied. These include trials conducted by CPW researchers and studies published in literature. A species sensitivity

distribution will be created and aquatic life criterion value (AKA "Water Quality Standard) will be derived per EPA protocol (Stephan 1985).

4- The new calculation will be published and compared to field data and mesocosm research conducted by CPW and CSU as evidence that this standard is ecologically appropriate. These findings will be presented to CDPHE and the Water Quality Control Commission as a proposed new standard for Colorado.

Accomplishments Action #1.3: Calculation of a Ferric Fe standard for Colorado

Chronic toxicity of ferric iron and a derivation of a chronic water quality criterion

Iron is abundant in the earth's crust and occurs naturally in the aquatic environment. Iron concentrations can be elevated due to human activities such as mining activities that expose pyrite and other sulfidic minerals to air and water leading to oxidation and release of iron and sulfuric acid in a process known as acid mine drainage (AMD). An estimated 20,000 to 50,000 mines in the western United States produce AMD which "seriously" affects 8,000-15,000 km of streams (USDA 1993) and has been described as the greatest water quality problem in the Rocky Mountain region (Mineral Policy Center 1997). In the eastern United States, acid drainage from coal mines affect more than 7000 km of streams (Kim 1982). Despite the widespread and harmful effects of iron, fewer than half of states have adopted a numeric chronic iron standard to protect aquatic life, and several states have deleted existing iron standards. The current USEPA chronic iron criterion of 1,000 µg/L (total recoverable) for protection of aquatic life was adopted in 1976 and is largely based on field observations of an iron-polluted Colorado stream in which trout and other fishes were absent at iron concentrations < 1,000 µg/L (USEPA 1976). A field study conducted in Kentucky supported the 1,000 µg/L criterion (Birge et al. 1985). Nevertheless, the basis for the chronic iron criterion is generally regarded to be poor (Thurston et al. 1979, Ohio EPA 1998). A more scientifically rigorous iron criterion is hindered by iron's complex speciation which is influenced by redox, dissolved oxygen, light, pH, and organic matter (Vuori 1995) and by a lack of chronic toxicity data. In the aqueous environment, iron exists in two oxidation states: as reduced ferrous ion (Fe II) and as the oxidized ferric ion (Fe III). In oxygenated waters, soluble ferrous ions (Fe II) oxidize to ferric ions (Fe III) (Hem 1985). In circumneutral waters (pH 6.5-8.5) ferric ions are insoluble and precipitate as hydroxides and oxyhydroxides in a matter of seconds to minutes (Hem 1985, Kimball et al. 2008). So while iron speciation is indeed complex, ferric precipitates are the overwhelmingly predominant form in waters capable of supporting aquatic life (i.e. oxygenated and circumneutral pH). Thus, the ferric precipitates should be the most appropriate basis for a water quality chronic criterion for the protection of aquatic life.

U.S. water quality criteria are usually derived using methodology outlined by Stephan et al. (1985). Briefly, a final chronic criterion is intended to be protective of 95% of genera estimated

from a dataset of toxicity values consisting of minimum of eight families that includes Salmonidae, another fish family in class Osteichthyes, a third family in Chordata, a planktonic crustacean, a benthic crustacean, an insect, a family in a phylum other than Arthropoda or Chordata, and finally a family in any order of insect or phylum not already represented. A literature review was conducted to identify chronic iron toxicity tests that met the following criteria:

1. The species of test organisms used must exist in freshwater systems in North America,

2. The duration of the test was sufficiently long to detect sublethal effects (≥25 days or ≥21 days for Daphnids),

3. Ferrous iron was used as the toxicant. This third criterion was selected because ferrous iron and its precipitates are the overwhelming predominant form of iron in circumneutral oxygenated waters,

4. Toxicity tests were conducted at pH between 6.5 and 9.0 in order to minimize confounding effects of pH on toxicity results (see e.g. Radford 1997).

The literature review detected few experiments that met the above criteria. Suitable tests were conducted with genera from Salmonidae (Oncorhynchus, Salvelinus), another fish from class Osteichthyes (Pimephales), a planktonic crustacean (Daphnia) and a benthic crustacean (Orconectes) and an insect (Chironomus) (Table 1). With the addition of unpublished toxicity tests conducted by Colorado Parks and Wildlife on a Chordate (Bufo), an insect (Hexagenia), a non-arthropod invertebrate family (Lumbriculus), additional members of Salmonidae (Salmo, Prosopium) and a family in another insect order or a phylum not otherwise represented (Dugesia) a sufficient amount of toxicity data with a diverse array of organisms enables calculation of a scientifically-based chronic iron criterion.

METHODS:

Literature searches of EPA's ECOTOX database (USEPA 2015b) and science literature databases were used to identify iron toxicity tests that were of sufficient duration (≥25d, Daphnids ≥7d) to detect sublethal effects such as reduced growth or reproduction. Only studies of circumneutral pH (6.5 to 9.0) and that used the ferric form of iron were used. If sufficient data were reported, regression analysis was used to calculate chronic values. USEPA's Toxicity Relationship Analysis Program version 1.30a (TRAP), was used to estimate concentrations of total iron concentrations that caused a 20 percent reduction in response (EC20), a low level of effect that would not be expected to cause chronic impacts at the population level and a threshold used by USEPA to develop water quality criteria for protection of aquatic life. If insufficient partial effects were observed to produce a reliable estimate or if insufficient data were reported to run TRAP, MATCs or other effect concentrations reported by the authors were used.

Chronic values of toxicity tests that met the screening requirements are reported in Table 1. Details on TRAP results and chronic values are reported in Supplementary materials. Species Mean Chronic Values (SMCV) were calculated as the geometric mean of chronic values in the limited instances where multiple chronic values were available for the same species. Genus Mean Chronic Values (GMCV) were calculated as the geometric mean of relevant SMCVs. GMCVs were ranked and a final chronic value (FCV) was calculated using methods described by Stephan et al. (1985).

RESULTS:

Of the 14 genera from acceptable toxicity tests, Pimephales (688 µg/L total iron GMCV), Lumbriculus (870 µg/L total iron GMCV), Prosopium (1318 µg/L total iron GMCV), and Daphnia (2,048 µg/L total iron GMCV) were the four most sensitive genera (Fig. 1, Table 1). Per Stephan et al. (1985), we derived a final chronic value (FCV) of 534 µg/L total (unfiltered) Fe or total recoverable Fe (Table 2). In the experiments that drove this final chronic value, Fe hydroxide precipitates were readily soluble after adding the minimal acid needed for preservation of samples for analysis (approximately one drop of concentrated nitric acid per 5 ml of sample). Thus, sample preparation by total recoverable was not warranted and would have produced the same values.

CONCLUSIONS:

Using published CPW iron toxicity values and results of toxicity tests reported here, a chronic criterion of 584 µg/L total iron was derived using methodology outlined by Stephan et al. (1985). This value is intended to be protective of an estimated 95% of taxa. Our derivation is considerably lower than the current U.S. criterion of 1,000 µg/L total recoverable Fe. An assessment of field data suggests that iron concentrations as low as 210 µg/L may be necessary to protect sensitive species (Linton et al. 2007). Some field studies observed that aquatic life appear unaffected at iron concentrations that exceed the water quality criterion of 1,000 µg/L (Ohio EPA 1998, Loeffelman et al. 1985). However, it is important to note that water quality criteria are intended to protect 95% of species, and as such, may be overly protective (Stephan et al. 1985) in circumstances where more tolerant organisms are present. Field studies that fail to detect adverse effects to aquatic life at concentrations above a criterion value should not necessarily be construed as demonstrating an overprotective criterion.

Reasonable arguments can be made about the criteria used to select toxicity tests used in our derivation of a chronic iron criterion. Specifically, deriving a criterion based on tests that used ferric as opposed to ferrous iron. The reduced form of iron ions (Fe II, ferrous) is more acutely toxic than the oxidized form (Fe III, ferric) to aquatic life (Loeffelman et al. 1986). However, indirect effects of ferric iron precipitates are more detrimental to aquatic life than direct effects of dissolved ferrous iron (Vuori 1995). Ferric precipitates increase turbidity, reduce primary

production, clog interstitia and affect physical characteristic in benthic zones and smother bottom-dwelling invertebrates, plants and incubating fish eggs (USEPA 1976, Goettl and Davies 1977, DeNicol et al. 2002, McKnight et al. 1984, Vuori 1995, Linton et al. 2007, Hayer et al. 2013). Also, limiting results from tests conducted at circumneutral pH ignores the potential of acidic conditions to increase toxicity of iron. However, tests conducted at low or toxic pH values confound interpretation of iron toxicity results. Unpublished CPW tests and those of others buffered acid using sodium hydroxide (CPW unpublished data) or lime (Smith papers) or were conducted in alkaline water capable of buffering large pH changes (Goettl and Davies 1977). We did not find any toxicity tests that systematically studied the interaction of pH and iron concentrations in a manner that would enable us to adjust toxicity of iron based on pH. This is obviously an area for additional study.

Of the states in the U.S. that have numeric chronic iron standards, most have adopted USEPA's recommended criterion of 1000 μg/L, and none have standards sufficiently protective based on the iron criterion of 584 μg/L derived from these studies with the exception of West Virginia's chronic iron standard of 500 μg/L for trout streams. Further research that studies chronic effects of iron on a diverse array of aquatic organisms and life stages is needed to further refine the total iron criterion and ensure it is fully protective of aquatic organisms. Unlike aqueous toxicants, Fe precipitates can accumulate over time. Future studies should not only target organisms affected by embedded benthic habitat but should consider durations that allow deposition as observed in nature.

Personnel: Pete Cadmus and Steve Brinkman

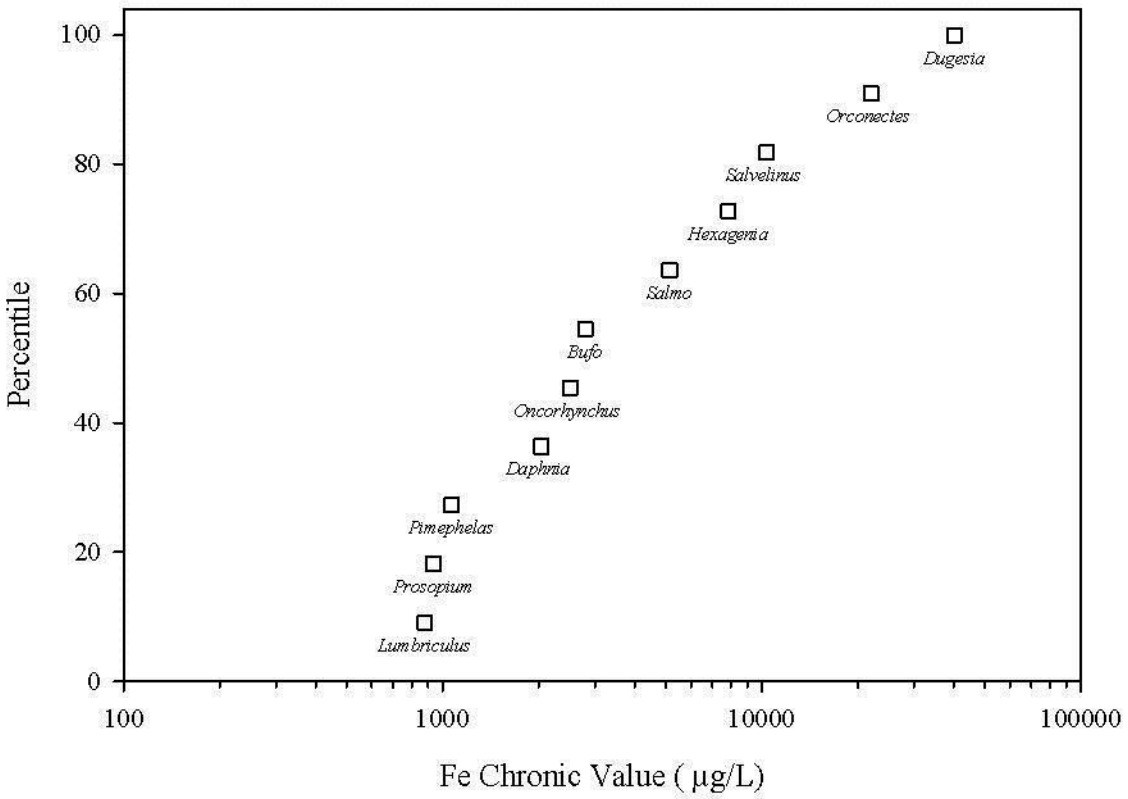

Figure 1: Sensitivity distribution of genera to iron.

Table 1: Chronic Values from experiments included in calculation of Species Mean Chronic Values (SMCV) and Genus Mean Chronic Values (GMCV). Fe concentrations in µg/L total or total recoverable Fe.

Rank	Scientific name	Common name	Chronic Value (µg/L)	SMCV (µg/L)	GMCV (µg/L)	Reference
14	Dugesia dorotocephala	Planarian	>40134	40134	40134	CPW unpublished data
13	Cheumatopsyche	Caddisfly nymph	33288	33288	33288	Sykora et al. 1972
12	Orconectes limosus	Crayfish	22000	22000	22000	Boutet and Chaisemartin 1973
11	Chironomus riparius	Midge	19811	19811	19811	Radford 1997
10	Salvelinus fontinalis	Brook trout	9237	9237	9237	Sykora et al. 1975
9	Hexagenia limbata	Mayfly	>7863	7863	7863	CPW unpublished data
8	Salmo trutta	Brown trout	>5146	5146	5146	CPW unpublished data
7	Gamarus minus	Freshwater shrimp	<4120	4120	4120	Sykora et al. 1972
6	Oncorhynchus kisutch	Coho salmon	4870	4009	3657	Smith and Sykora 1976
	Oncorhynchus kisutch	Coho salmon	>3300			Brenner and Cooper 1978
	Oncorhynchus mykiss	Rainbow trout	1483	3335		Goettl and Davies 1977
	Oncorhynchus mykiss	Rainbow trout	>7500			Steffens et al. 1993
5	Bufo boreas	Boreal toad (tadpoles)	3145	3145	3145	CPW unpublished data
4	Daphnia magna	Cladoceran	4380	4380	2048	Biesinger and Christensen 1972
	Daphnia pulex	Cladoceran	958	958		Birge et al. 1985
3	Prosopium williamsoni	Mountain whitefish	1318	1318	1318	CPW unpublished data

2	Lumbriculus variegatus	Worm	870	870	870	CPW unpublished data
1	Pimephelas promelas	Fathead minnow	910a	688	688	Birge et al. 1985
	Pimephalas promelas	Fathead minnow	520			Smith et al. 1973

a Apparent outlier not included in calculations

Table 2: Fe concentrations in µg/L total or total recoverable Fe.

Scientific name	Common name	NOEC	LOEC	MATC	EC20	Reference
Dugesia dorotocephala	Planarian	>40134		>40134	>40134	CPW unpublished data
Cheumatopsyche	Caddisfly nymph	22800	48600	33288	28184a	Sykora et al. 1972
Orconectes limosus	Crayfish			22000c	b	Boutet and Chaisemartin 1973
Chironomus riparius	Midge	15000	30000	21213	19818	Radford 1997
Salvelinus fontinalis	Brook trout	7800	13420	10231	9237	Sykora et al. 1975
Hexagenia limbata	Mayfly	>7863		>7863	>7863	CPW unpublished data
Salmo trutta	Brown trout	>5146		>5146	>5146	CPW unpublished data
Gamarus minus	Freshwater shrimp		<4120	<4120	9269a	Sykora et al. 1972
Oncorhynchus kisutch	Coho salmon	2830	4635	3621	4870	Smith and Sykora 1976
Oncorhynchus kisutch	Coho salmon	>3300		>3300	>3300	Brenner and Cooper 1978
Oncorhynchus mykiss	Rainbow trout	1000	2200	1483	b	Goettl and Davies 1977
Oncorhynchus mykiss	Rainbow trout	>7500		>7500	>7500	Steffens et al. 1993
Bufo boreas	Boreal toad (tadpoles)	2044	3831	2798	3145	CPW unpublished data
Daphnia magna	Cladoceran			4380d	b	Biesinger and Christensen 1972
Daphnia pulex	Cladoceran	700	1310	958	979a	Birge et al. 1985
Prosopium williamsoni	Mountain whitefish	658	1329	935	1318	CPW unpublished data

Lumbriculus variegatus	Worm	593	1145	880	870	CPW unpublished data
Pimephelas promelas	Fathead minnow	316	1008	569	910	Birge et al. 1985
Pimephalas promelas	Fathead minnow		<2000	<2000	520	Smith et al. 1973

a Insufficient partial effects for reliable estimate of EC20. Value reported for exploratory purposes only.
b Insufficient data reported to run TRAP.
c LC50 reported by authors used as MATC.
d EC16 reported by authors used as MATC.

Biesinger, K. E., & Christensen, G. M. (1972). Effects of various metals on survival, growth, reproduction and metabolism of Daphnia magna. Journal of the Fisheries Research Board of Canada 29: 1691-1700.

Birge, W. J., Black, J. A., Westerman, A. G., Short T. M., Taylor S. B., Bruser, D. M. & Wallingford, E. D.(1985). Recommendations on numerical values for regulating iron and chloride concentrations for the purpose of protecting warmwater species of aquatic life in the commonwealth of Kentucky. Memorandum of Agreement No. 5429.

Boutet, C., & Chasemartin, C. (1973). Proprietes toxiques specifiques des sels metalliques chez Austropotamobius pallipes pallipes et Orconectes limosus. Comptes Rendus des Seances de la Societe de Biologie et de ses Filiales 167(12): 1933-1938.

Brenner, F.J., & Cooper, W. L., (1978). Effect of suspended iron hydroxide on the hatchability and embryonic development of Coho salmon. Ohio Journal of Science 78(1):34-38.

Brinkman, S. F., Crockett, H. J., & Rogers, K. B. (2013). Upper thermal tolerance of mountain whitefish eggs and fry. Transactions of the American Fisheries Society 142:834-832.

CDPHE (2010) Colorado Department of Public Health and Environment. Status of water quality in Colorado – 2010. The update to the 2008 305(b) report.

Dave, G. (1984). Effects of waterborne iron on growth, reproduction, survival and haemoglobin in Daphnia magna. Comparative Biochemistry and Physiology 78C:433-438.

Davies, P.H., Brinkman, S F. & McIntyre, M.W. (1997). Effects of chronic exposure of boreal toad tadpoles (Bufo boreas) to cadmium. Water pollution studies, federal aid in fish and wildlife restoration project F-243-R4 pp 3-18. Fort Collins: Colorado Parks and Wildlife.

Goettl J.P. and P.H. Davies. 1977. Water pollution studies, federal aid in fish and wildlife restoration project F-33-R12. Fort Collins: Colorado Parks and Wildlife.

Hayer, C.A., Holcomb, B. M. & Chipps, S. R. (2013). Associations between iron concentration and productivity in montane streams of the Black Hills, South Dakota. Prairie Naturalist 45, 68-76.

Kim, A.G., Heisey, B., Kleinmann, R., & Duel, M. (1982). Acid Mine Drainage: Control and Abatement Research. U.S. DOI, Bureau of Mines IC 8905, p.22.

Kimball, B.A., Walton-Day, K., & Runkel, R. L. (2008) Quantification of metal loading by tracer injection and synoptic sampling 1996-2000. In Church, S. E., von Guerard, P. & Finger, S. E. (Eds) Integrated Investigations of Environmental Effects of Historical

Mining in the Animas River Watershed, San Juan County, Colorado. Professional Paper 1615. United States Geological Society.

Linton, T.K., Pachego, M.A.W., McIntyre, D.O., Clement, W.H., & Goodrich-Mahoney, J. (2007). Development of bioassessment-based benchmarks for iron. Environmental Toxicology and Chemistry 26:1291-1298.

Loeffelman, P.H., Van Hassel, J.H., Arnold, T.E., & Hendricks, J.C. (1986). A new approach for regulating iron in water quality standards. In Bahner R. C., (Ed) Aquatic Toxicology and Hazard Assessment, Eighth Symposium. STP 891-EB. American Society for Testing and Materials, Philadelphia, PA. pp 137–152.

McKnight, D.M., & Feder, G.L. (1984). The ecologicial effect of acid conditions and precipitation of hydrous metal oxides in a Rocky Mountain stream. Hydrobiologia 119:129-138.

Mineral Policy Center (1997). Golden dreams, poisoned streams. Washington, D.C., USA

Ohio Environmental Protection Agency. 1998. Empirically derived guidelines for determining water quality standards and NPDES discharge limits for iron protective of aquatic life in Ohio rivers and streams. Technical Bulletin MAS/1998-9-1. Division of Surface Water, Columbus, OH, USA.

Radford, N.P. (1997). Ecotoxicological impact of iron III sulphate dosing on chironomid cultures and profundal reservoir communities. Doctoral Thesis. Department of Zoology, University of Leicester

Randall, S., Harper, D., & Brierley, B. (1999). Ecological and ecophysiological impacts of ferric dosing in reservoirs. Hydrobiologia 395/396: 355-364.

Stephan, C.E., D.I. Mount, D.J. Hansen, J.R. Gentile, G.A. Chapman and W.A. Brungs. 1985. Guidelines for deriving numerical standards for the protection of aquatic organisms and their uses. USEPA PB85-227049. Washington, DC USA.

Steffens, W., Mattheis, T.H., & Riedel, M. (1993). Field observations on the production of Rainbow Trout (Oncorhynchus mykiss) under high concentrations of water-borne iron. Aquatic Sciences 55: 173-178.

Sykora, J.L., Smith, E.J., Synak, M., & Shapiro, M.A. (1975). Some observations on spawning brook trout (Salvalinus fontinalis Mitchell)in lime neutralized iron hydroxide suspensions. Water Research 9: 451-458.

Sykora, J.L., Synak, M., & Smith, E.J. (1972). Effect of lime neutralized iron hydroxide suspensions on juvenile brook trout (Salvelinus fontinalis, Mitchill). Water Research 6:935

Thurston, R.V., Russo, R.C., Fetterolf Jr, C.M., Edsall, T.A., & Barber Jr., Y.M. (1979). A Review of the EPA Red Book: Quality Criteria for Water. American Fisheries Society, Bethesda, MD, USA.

USDA Forest Service (1993) Acid mine drainage from mines on the National Forests: A management challenge. Program Aid 1505.

USEPA 1976. Quality Criteria for Water. PB263-943.

U.S.EPA. 2015a. Toxicity Relationship Analysis Program (TRAP Version 1.20). NHERL. Duluth, MN.

USEPA. 2015b. ECOTOX User Guide: ECOTOXicology Database System. Version 4.0. Available: http:/www.epa.gov/ecotox/

Van Dam, R.A., Barry, M.J., Ahokas, J.T., & Holdway, D.A. (1998). Effects of water-borne iron and calcium on the toxicity of diethylenetriamine pentaacetic (DTPA) to Daphnia carinata. Aquatic Toxicology 42:49-66.

Vuori, K.M. (1995). Direct and indirect effects of iron on river ecosystems. Annals of Zoology Fennici 32:317-329.

West Inc. 1996. Toxstat Version 3.5. Western Ecosystems Technology, Cheyenne WY.

Job 2
Job Title: Water Quality Technical Assistance to Colorado Parks and Wildlife Personnel and Other State and Federal Agencies.

Ultimate Objective: Provide technical support to CPW managers and other agencies.

Need:
Water quality characteristics and pollution affect fish health and viability of fisheries. Water chemistry and aquatic ecotoxicology demands specialized skill sets and unique instrumentation/infrastructure. Fisheries managers faced with chronic pollution issues, acute (accidental) spill events, fish kills events and other anthropogenic disturbances often need assistance with analysis of samples and characterization of toxicant effects before, during and

after toxicological disturbance. Site specific and state wide water quality alterations risk compromising fisheries health in Colorado. Decision makers need to be informed of risks to Colorado's fisheries. Efforts to restore Colorado surface waters often require precise use of piscicides, all of which are difficult to assess in the field. However, the unique analytical capabilities of the CPW Aquatic Toxicology Laboratory have recently been employed to provide this information on short turnaround using a mobile laboratory. Collaborators at state agencies and universities frequently approach research topics that complement the goals of CPW including those listed in Job A of this narrative. Providing these researchers with expertise and sharing equipment/infrastructure often produces better data that is useful to CPW.

Objectives:
To provide technical assistance and expertise, consultation, evaluation and training in aquatic toxicology and aquatic chemistry to Colorado Parks and Wildlife and other state and federal personnel as requested. Assist in the investigation of fish kills. 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. Collect and analyze water and/or fish tissues to assess water quality problems as requested. Analyze rotenone (and other piscicides) in water samples as part of Colorado Parks and Wildlife reclamation projects. Publish results of experiments and water quality investigations in peer-reviewed journals for consideration in policy making by other agencies.

Approach:
Action # 2.1
●*Level 1 Action Category: Data Collection and Analysis*
●*Level 2 Action Category: Research*

As requested the following aid and support was provided by the Colorado Parks and Wildlife Aquatic Toxicology Laboratory:

- Collected and analyze water samples for rotenone as part of reclamation projects for Hemosa Creek (Aug 3-7th), Haypress Lake (Aug 17-21), Dry Creek (Sept 28-Oct 4).
- Provided laboratory space and expertise to managers for a bioassay that assessed potency of Antimycin for a lentic reclamation project.
- Provided technical support in assessing effects of chemical stressors on trout populations, aquatic macroinvertebrates and functional measures (primary productivity, decomposition rates) algae in the Animas River before the Gold King Mine incident.
- Responded to the Gold King Mine spill on August 7 – 12th of 2015. Prepared and deployed fish cages in the Gold Medal reaches prior to plume, provided toxicological expertise to managers, obtained and analyzed water quality observations before and during the first days of exposure. Provided ongoing expertise to managers and politicians

in the months that followed. Advocated for improved sampling methods in the post spill biomonitoring projects. Aided Colorado River Watch in processing samples.

- Provided oversight of annual biomonitoring efforts of North Fork of Clear Creek, in Gilpin and Clear Creek counties, CO.
- Conducted in-stream experiments and feasibility studies that will inform introduction of sport fish species to North Fork of Clear Creek after the installation of a mine effluent treatment plant scheduled for 2017. These studies included algae and insect assessments as well as fish cage experiments with young Greenback Cutthroat Trout.
- Analyzed and interpreted water samples from fish kills and spills throughout Colorado. This included the Animas River Gold King Mine incident (Aug 7th 2015), the Big Thompson River fish kill (March 9 2015) incident and other small incidents throughout the state.
- Produced milt extender for Colorado and cooperating states.
- Provided managers and other section of CPW new tools for analysis of algal communities in lakes and rivers threatened by eutrophication or water chemistry imbalances.
- Created methods for analysis of gemfibrozil by high performance liquid chromatography.
- Provided Colorado State University with laboratory space and infrastructure that supported investigation of endocrine disrupting toxicants on fish populations.
- Provided Colorado State University with laboratory space and infrastructure that supported investigation of *Mysis* susceptibility to Rotenone and feasibility studies to remove Mysis from mountain lakes in which Lake Trout co-occur with Kokanee Salmon.
- Collaborated with Colorado Department of Public Health and Environment to investigate effects of Selenium on fish (Walleye & Brown trout). Fish were spawned and eggs were reared. Tissue concentrations were digested and assessed for Selenium levels. Biomonitoring studies were planned to determine what risk elevated selenium levels have on sport fish reproduction.
- Provided chemical analysis of water samples from fish biologists and hatchery staff through the year as needed.
  Provide ecotoxicological support and expertise to CPW managers, Colorado universities and fellow natural resource management agencies as requested.

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