Water Pollution Studies

Federal Aid Project F-243-R26

Pete Cadmus
Aquatic Research Scientist

Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Parks and Wildlife

Aquatic Research Section

Fort Collins, Colorado

August 2019

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AQUATIC RESEARCH STAFF

George J. Schisler, Aquatic Research Leader
Kelly Carlson, Aquatic Research Program Assistant
Pete Cadmus, Aquatic Research Scientist/Toxicologist, Water Pollution Studies
Eric R. Fetherman, Aquatic Research Scientist, Salmonid Disease Studies
Ryan Fitzpatrick, Aquatic Research Scientist, Eastern Plains Native Fishes
Eric E. Richer, Aquatic Research Scientist/Hydrologist, Stream Habitat Restoration
Matthew C. Kondratieff, Aquatic Research Scientist, Stream Habitat Restoration
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Tracy Davis, Hatchery Technician, Fish Research Hatchery
Andrew Perkins, Hatchery Technician, Fish Research Hatchery

Jim Guthrie, Federal Aid Coordinator
Alexandria Austermann, Librarian

Prepared by: _____
Pete Cadmus, Aquatic Research Scientist

Approved by: _____
George J. Schisler, Aquatic Wildlife Research Chief

Date: 9/17/19

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State: Colorado **Project No. F-243-R26**

Project Title: Water Pollution Studies

Period Covered: July 1, 2018 – June 30, 2019

Project Objective: To develop quantitative chemical and toxicological data characterizing 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 No. 1 Toxicology Research: Experiments and biomonitoring to assess the risk of emerging toxicants, derive new water quality standards and improve existing water quality standards and policy.

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

Need

Over seven million recognized chemicals exist and 80,000 are in common use (GAO 1994). However, Colorado regulates 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).

Downstream of urban, industrial or agricultural land uses, organic (carbon based) pollutants have become the predominant and perhaps least studied form of pollution in Colorado (Daughton 2004). Only a minority of insecticides or herbicides are regulated by standards for aquatic life. Endocrine disrupting chemical classes such as estradiols and pharmaceuticals are known to have an adverse effect on fish populations but the effects of most of these chemicals are unstudied. In example, 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 altered by exposure to statin pharmaceuticals. Rates of hydrocarbon extraction have increased in Colorado over the last 10 years. This presents new risks from extraction and transport processes. Uptake and trophic transfer of hydrocarbons from benthos to fish in both acute and chronic (Lytle and Peckarsky 2001) exposure regimes is well documented (Neff 1979; Giesy et al. 1983; Lamoureux and Clements et al. 1994; Brownawell 1999; Schuler et al. 2003). Increased susceptibility to disease

is often correlated with polycyclic aromatic hydrocarbon (PAH) exposure (Damasio et al. 2007; Bravo et al. 2011). Safe concentrations of these chemicals are unknown.

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.

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Objectives

1- Assess toxicity of emerging contaminants pertinent to Colorado surface waters by conducting toxicity trials on sport fish and forage 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, pharmaceuticals) and physical properties (*e.g.* total suspended solids, temperature, nutrients). Improved standards rely on improved experimentation that is published in a timely manner and is designed to inform triannual reevaluation of toxicant standards by EPA and CDPHE. Experiments should:

a) Include rare or sensitive sport fish species or forage species underrepresented in the literature.

b) When possible expose rare or sensitive taxa not laboratory cultured organisms. Expose for long environmentally relevant durations not the standardized 96 hour and 30 day trials.

Expose organisms during sensitive life stages (*e.g.* early life stages, egg survival, drift of sac fry, mating, winter survival).

c) Consider 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, histopathology, blood chemistry)

d) Examine all routes of exposure and all toxic pathways (*e.g.* dietary vs. aqueous exposure, indirect vs. direct toxicity)

e) Increase environmental realism by using natural habitat, natural assemblages, mesocosms, communities, and food chains both in laboratory and field settings.

f) Consider multiple stressors simultaneously, not limited to interactions between numerous toxicants, interactions between toxicants and temperature or interactions between toxicants and disease (*e.g.* whirling disease).

3- Use original research and published research to characterize risk to Colorado's sport fish and forage species. When possible, derive new acute and chronic values for consideration as aquatic life criteria (also known as 'standards' or 'standards for aquatic life'). Employ new techniques to ensure aquatic life standards and management policies are protective of Colorado's sport fish and forage species. Present these findings to regulatory agencies through professional society meetings and peer reviewed publications.

Approach

Action #1.1: Assessment of emerging pollutants: Statin like pharmaceuticals and/or pesticides and/or petroleum hydrocarbons

Sub-Action #1.1.1: Assessment of statin drugs and statin like pharmaceuticals on fish.

- *Level 1 Action Category: Data Collection and Analysis*
- *Level 2 Action Strategy:* Research

Expose fish to environmentally relevant levels and mixtures of statin drugs or other cholesterol lowering pharmaceuticals from embryonic development 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, embryo development and tissue pathology.

Action #1.1.1 Accomplishments:

Use of Zebrafish (*Danio rerio*) and Fathead Minnow (*Pimephales promelas*) to triage risk of antilipidemic pharmaceutical mixtures in Colorado surface waters.

Personnel: Andrea Kingcade, Abbie Lee Jefferson, Paula Shafer, Deborah Garrity, Howard S. Ramsdell and Pete Cadmus

Introduction

Municipal waste water treatment plants have limited ability to remove pharmaceuticals from municipal sewage. Antilipidemics are commonly detected in waterways downstream of municipal wastewater treatment plants. Antilipidemics include some of the most prescribed pharmaceuticals in North America (Medscape 2014) and use has increased substantially since the early 2000s (Gu et al. 2014). River and stream flows downstream of the Colorado urban corridor are often reduced as water is diverted for municipal and agricultural uses. The dilution provided by these flow regimes is likely to be reduced as Colorado's human population continues to grow and climate change alters precipitation levels and snow pack. These factors coupled with increased prescription of antilipidemic drugs suggests concentrations in water ways will increase significantly beyond current levels.

Fibrates and statins are both classes of antilipidemics that are considered highly synergistic in humans. Fibrates increase activation of peroxisome proliferator activated receptors (PPAR) leading to fatty acid catabolism. Statins inhibit the rate-limiting enzyme of cholesterol synthesis. Cholesterol synthesis pathway is highly conserved in metazoan taxa (Santos 2016) and fish rely primarily on triglycerides as their primary energy storage (Bennett 2007). Most fish have two to six times higher levels of cholesterol compared to mammals (Larsson 1977; Babin 1989) and plaque buildup in fish coronary arteries (atherosclerosis) was observed in *Salmo salar* (Atlantic salmon; Saunders 1992). For these reason, fish species may be highly susceptible to antilipidemics and potential for synergism exists.

Fat storage is needed for egg production, growth and winter survival of Colorado fish species. Antilipidemics alter cholesterol and tri-glycerides which might manifest into reduced growth, survival and fecundity. Significant reductions of cholesterol were observed in fish exposed up to 200 μg/L bezafibrate which led to spermatogenesis defects (Velasco-Santamaria 2011). After 30 day dietary exposure of adult Zebrafish to gemfibrozil, atorvastatin, or a mixture of both (equivalent to human dosages of each drug) cholesterol and triglycerides were significantly altered (Al-Habsi 2016). This response was sex dependent suggesting population level sex ratios may be altered. Environmentally relevant concentrations of antilipidemics have been shown to have sublethal effects in goldfish (Mimeault et al. 2005). Omega-3 fatty acids were reduced when juvenile female Rainbow Trout (*Onchorynchus mykis*s) were injected with 100 mg/L gemfibrozil five times over 15 days (Prindiville 2011).

Seven common statins (atorvastatin, fluvastatin, lovastatin, pitavastatin, pravastatin, rosuvastatin, and simvastatin) and two fibrates (fenofibrate and gemfibrozil) are currently sold in the USA and eight of nine of these drugs have previously been measured in surface water or effluent. As a continuation of the work described in the Federal Aid Project Report F-243-R25 (Cadmus 2018), this research aimed to characterize how these nine drugs impact early (embryonic) fish development. These nine drugs were evaluated individually with Zebrafish

embryos (See Cadmus 2018). Within this report we address effects of mixtures of antilipidemics on Fathead Minnows and Zebrafish.

Methods

Dilution water, exposure solution preparation, and experimental design involving heterozygous transgenic Zebrafish have been previously described (Cadmus 2018). The concentration of dimethyl sulfoxide did not exceed 0.01% in any exposure solution and was used as a solvent control in both mixture studies. To prepare the stock solution for each study, each of the nine drugs were combined into a single volumetric flask and solubilized with dimethyl sulfoxide which was then diluted further with dimethyl sulfoxide. These stock solutions prepared in 100% dimethyl sulfoxide were mixed with dilution water to achieve the target exposure concentrations (Table 1.1.1-1). Dilution water for the Fathead Minnow study was prepared with Type 1 ultra-pure laboratory water with four salts (KCl, $NaCO_3$, $MgSO_4$, and $CaSO_4$) to achieve a moderate hardness level of 95 mg/L $CaCO_3$ and alkalinity of 60 mg/L $CaCO_3$, and was aerated.

Table 1.1.1-1. - Concentrations of Pharmaceuticals					
Study:	Drug:	Nominal Testing Concentrations:			
		Ultra Low µM	Low µM	Medium µM	High µM
ZF Mixture	Fenofibrate (FENO)	--	0.005 (1.8 µg/L)	0.05 (18 µg/L)	0.5 (181 µg/L)
	Gemfibrozil (GEM)	--	0.005 (1.2 µg/L)	0.05 (12 µg/L)	0.5 (125 µg/L)
	Atorvastatin (ATO)	--	0.005 (2.8 µg/L)	0.05 (28 µg/L)	0.5 (280 µg/L)
	Fluvastatin (FLUV)	--	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (206 µg/L)
	Lovastatin (LOV)	--	0.005 (2.0 µg/L)	0.05 (20 µg/L)	0.5 (202 µg/L)
	Pitavastatin (PIT)	--	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (211 µg/L)
	Pravastatin (PRAV)	--	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (213 µg/L)
	Rosuvastatin (ROS)	--	0.005 (2.4 µg/L)	0.05 (24 µg/L)	0.5 (241 µg/L)
	Simvastatin (SIM)	--	0.005 (2.1 µg/L)	0.05 (21 µg/L)	0.5 (210 µg/L)
FHM Mixture	Fenofibrate (FENO)	0.0005 (0.18 µg/L)	0.005 (1.8 µg/L)	0.05 (18 µg/L)	--
	Gemfibrozil (GEM)	0.0005 (0.12 µg/L)	0.005 (1.2 µg/L)	0.05 (12 µg/L)	--
	Atorvastatin (ATO)	0.0005 (0.28 µg/L)	0.005 (2.8 µg/L)	0.05 (28 µg/L)	--
	Fluvastatin (FLUV)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	--
	Lovastatin (LOV)	0.0005 (0.20 µg/L)	0.005 (2.0 µg/L)	0.05 (20 µg/L)	--
	Pitavastatin (PIT)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	--
	Pravastatin (PRAV)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	--
	Rosuvastatin (ROS)	0.0005 (0.24 µg/L)	0.005 (2.4 µg/L)	0.05 (24 µg/L)	--
	Simvastatin (SIM)	0.0005 (0.21 µg/L)	0.005 (2.1 µg/L)	0.05 (21 µg/L)	--
Each of the nine individual drugs at the displayed nominal concentrations which were mixed together, solubilized, and diluted prior to each mixture study. All exposure concentrations had a standardized dimethyl sulfoxide (DMSO) concentration of 0.01%. ZF = Zebrafish, FHM = Fathead Minnow					

Adult Fathead Minnow were cultured at the Colorado Parks and Wildlife Aquatic Toxicology Laboratory (Fort Collins, CO, USA) in flow through conditions with aerated, moderately hard water. Well water and dechlorinated tap water were mixed using conductivity controllers to maintain moderate hardness and heated to 25 °C. Unlike Zebrafish, the Fathead Minnows did not have transgenes inserted into their genomes. Thus endpoints requiring translucent tissues could not be evaluated in the Fathead Minnow mixture study. Fathead Minnow embryos were collected from breeding tanks and transported 0.4 km to Colorado State University's Biology Department (Fort Collins, CO, USA). Twenty blastulating embryos up to six hours post fertilization with normal cleavage pattern were exposed to 10 milliliters of exposure solution in glass petri dishes and incubated at 23-25 °C for six days. Exposure solutions were renewed approximately every 24 hours in both mixture studies after allowing stock solutions to warm from 4 °C to room temperature. Neither Fathead Minnow nor Zebrafish embryos were fed during the mixture study.

Four categories of endpoints were evaluated in the mixture studies; developmental defects, muscle abnormalities, yolk abnormalities and cardiovascular abnormalities. Timing of observations were slightly altered to accommodate the slower developmental rate of Fathead Minnows.

1. **Developmental Defects** (Table 1.1.1-2): cumulative mortality, developmental delay, and dechorionation

In both mixture studies, mortality was defined as observing a developed heart that was not beating or observing undeveloped heart that exhibited opaque cells with unclear cell membranes. Developmental delay was recorded on day one in the Zebrafish or on day two in Fathead Minnow study. The criteria for developmental delay was one or more of the following; unestablished anterior-posterior axis, absence of somites, immature eye development. Early dechorionation was recorded on day two in the Zebrafish study prior to manual dechorionation of the remaining embryos. The number of Fathead Minnow embryos that had naturally dechorionated on day six (test termination) was also recorded.

2. **Muscle Abnormalities** (Table 1.1.1-3): maximum achievable velocity and abnormal muscle fiber patterning

Per Cadmus et al. (2018), maximum swimming speed or maximum velocity was measured only in the non-transgenic-expressing Zebrafish embryos on day two with a high-speed video captured after a touch stimulus. If a minimum of four non-transgenic embryos were not present in each replicate, transgenic-expressing Zebrafish embryos were randomly selected for this evaluation. This was not evaluated in Fathead Minnow embryos due to their restlessness and frequent movements. Transgenic-expressing Zebrafish embryos and dechorionated Fathead Minnow embryos were assed for abnormal muscle fiber pattern (birefringence). Muscle fiber

pattern was deemed abnormal if muscle fibers appeared non-parallel or opaque under a polarized lens.

3. **Yolk Abnormalities** (Table 1.1.1-4): yolk absorption and abnormal anterior-posterior axis.

The amount of yolk remaining at test termination and the angle of the anterior-posterior axis in both mixture studies (transgenic-expressing Zebrafish embryos and dechorionated Fathead Minnow embryos specifically) were evaluated. Yolk absorption was determined by measuring the area of the remaining yolk and the yolk extension. If the anterior-posterior axis was any other angle besides 180 degrees, this was recorded as abnormal.

4. **Cardiovascular Abnormalities** (Table 1.1.1-5 and 1.1.1-6): abnormal vessel development, presence of edemas (pericardial and yolk), presence of hemorrhages, and measurement of heart rate.

Two types of vessel systems (intersegmental vessels and subintestinal veins) were evaluated in the Zebrafish mixture study due to the presence of the green fluorescent protein transgene (transgenic-expressing embryos). If intersegmental vessels were underdeveloped or missing, this was recorded as abnormal. Subintestinal vein (SIV) abnormalities were divided into three subcategories (underdeveloped, overdeveloped, and missing) and the sum of these categories were evaluated (SIV-total). The presence of yolk and pericardial edemas and hemorrhages were recorded in both mixture studies. The heart rates of five randomly-selected embryos from each of the four replicates in the Fathead Minnow mixture study (chorionated or dechorionated) were recorded.

Fathead Minnow embryos were anesthetized with tricaine methanesulfonate (MS-222) after heart rates were measured. Embryos from both mixture studies were terminated at test termination with MS-222 and preserved in Bouin's solution for histopathological evaluation (Colorado State University Veterinary Diagnostic Laboratory, Fort Collins, CO). The planned analysis evaluating apoptosis using acridine orange was abandoned and not pursued further for individuals of these mixture studies after inconclusive method development. Attempts to evaluate apoptosis using an antibody specifically for a protein expressed further in the apoptotic pathway (caspase 3) is pending.

Preliminary results

Preliminary results are shown in Tables 1.1.1-2 to 1.1.1-6. Representative histopathological images from each fish species is provided in Table 1.1.1-7. Statistical analyses are pending.

Developmental Defects (Table 1.1.1-2)

Complete mortality was observed in Zebrafish embryos exposed in the two highest concentration groups (0.05 and 0.5 µM). Because of this mortality, lower concentrations were selected for the Fathead Minnow mixture study to achieve sublethal observations. Approximately 50% mortality was observed in Fathead Minnow embryos exposed to the highest concentration (0.05 µM). Zebrafish and Fathead Minnow embryos exposed to the 0.05 µM group exhibited almost 45% developmental delay. Zebrafish embryos exposed to 0.5 µM exhibited 87% developmental delay. Embryos reach a developmental stage and naturally dechorionate or hatch from their protective chorion. By day two, 23% and 39% percent of Zebrafish embryos had dechorionated in the control and low treatment level respectively. Aside from the highest treatment level, 91-99% of Fathead Minnow embryos naturally dechorionated by day six. Forty-five percent of the Fathead Minnow embryos exposed to the highest concentration (0.05 µM) exhibited natural dechorionation on day six of the study.

Table 1.1.1-2. - Developmental Toxicity (Preliminary Results)

Study:	Nominal Mixture Concentration:	Percent Cumulative Mortality (%)	Developmental Delay (%)	Dechorionation (%)
ZF Mixture	DMSO (0.01%)	1 ± 3	1 ± 3	23 ± 14
	0.005 µM	3 ± 3	1 ± 3	39 ± 26
	0.05 µM	100 ± 0	43 ± 20	--
	0.5 µM	100 ± 0	87 ± 9	--
FHM Mixture	DMSO (0.01%)	1 ± 3	1 ± 3	91 ± 10
	0.0005 µM	5 ± 4	5 ± 4	99 ± 3
	0.005 µM	0 ± 0	0 ± 0	97 ± 3
	0.05 µM	51 ± 6	44 ± 6	45 ± 20

Mean ± one standard deviation is displayed for each endpoint. Cumulative mortality was calculated at test termination. Developmental delay and dechorionation in the ZF mixture study included both transgenic and non-transgenic embryos. Early dechorionation was recorded in the ZF mixture study and natural dechorionation at test termination in the FHM mixture study is displayed.
ZF = Zebrafish, FHM = Fathead Minnow

Muscle Abnormalities (Table 1.1.1-3)

Zebrafish embryos exposed to 0.005 µM exhibited a reduced maximum swim speed relative to controls. At 0.005 µM, Fathead Minnow embryos exhibited more muscle fiber abnormalities than Zebrafish embryos. Twenty percent of Fathead Minnows exposed to the highest exposure concentration (0.05 µM) did not exhibit abnormal muscle fiber patterning.

Table 1.1.1-3. - Muscle Defects (Preliminary Results)			
Study:	Nominal Mixture Concentration:	Maximum Velocity (cm/s)	Abnormal Muscle Fiber Pattern (%)
ZF Mixture	DMSO (0.01%) 0.005 µM 0.05 µM 0.5 µM	4.7 ± 1.0 3.5 ± 1.6 -- --	2 ± 3 29 ± 11 -- --
FHM Mixture	DMSO (0.01%) 0.0005 µM 0.005 µM 0.05 µM	NM	4 ± 5 3 ± 5 45 ± 22 80 ± 14
Mean ± one standard deviation is displayed for each endpoint. Maximum achieved velocity was only determined in the non-transgenic-expressing embryos (including randomly selected transgenic-expressing embryos to evaluate at least four embryos per replicate) in the ZF mixture study. Muscle fiber patterns were evaluated only in dechorionated FHM embryos. ZF = Zebrafish, FHM = Fathead Minnow, NM = not measured			

Yolk Abnormalities (Table 1.1.1-4)

The amount of yolk remaining at test termination in the Zebrafish embryos increased slightly between embryos in the unexposed group compared to Zebrafish embryos exposed to the 0.005 µM group. Fathead Minnow embryos exposed to the highest exposure level (0.05 µM) exhibited 12% more yolk remaining at test termination (4.8×10^5 µm^2). Zebrafish embryos in the 0.005 µM exposure group experienced more anterior-posterior axis abnormalities (27%) than Fathead Minnow embryos exposed to the same level (5%). Approximately 50% of Fathead Minnow embryos exposed at the 0.05 µM level experienced abnormal anterior-posterior body axis.

Table 1.1.1-4. - Yolk Abnormalities (Preliminary Results)			
Study:	Nominal Mixture Concentration:	Yolk Absorption (µm^2)	Abnormal Anterior-Posterior Axis (%)
ZF Mixture	DMSO (0.01%) 0.005 µM 0.05 µM 0.5 µM	$2.6 \times 10^5 \pm 3.8 \times 10^4$ $2.7 \times 10^5 \pm 5.9 \times 10^4$ -- --	3 ± 6 27 ± 8 -- --
FHM Mixture	DMSO (0.01%) 0.0005 µM 0.005 µM 0.05 µM	$4.4 \times 10^5 \pm 7.4 \times 10^4$ $4.4 \times 10^5 \pm 7.8 \times 10^4$ $4.3 \times 10^5 \pm 1.3 \times 10^5$ $4.8 \times 10^5 \pm 8.4 \times 10^4$	4 ± 5 0 ± 0 5 ± 8 55 ± 34
Mean ± one standard deviation is displayed for each endpoint. Both yolk abnormalities were only determined in the ZF transgenic-expressing embryos and in dechorionated FHM embryos. ZF = Zebrafish, FHM = Fathead Minnow			

Cardiovascular Abnormalities (Table 1.1.1-5 and Table 1.1.1-6)

Abnormal vessel development was evaluated in Zebrafish on day three (test termination) in the embryos expressing green fluorescent protein (transgenic-expressing). Underdeveloped or missing intersegmental vessels were observed in 13% of Zebrafish embryos exposed at the 0.005 µM level compared to 2% abnormalities in unexposed Zebrafish embryos. Twenty-two percent of exposed Zebrafish embryos exhibited abnormal subintestinal vein development compared to 11% in unexposed Zebrafish embryos. Of the three subcategories of subintestinal vein abnormalities that were evaluated, underdeveloped subintestinal veins were the most prevalent (≥10%) compared to the other two subcategories (≤5%).

Table 1.1.1-5. - Cardiovascular Abnormalities – Part 1 (Preliminary Results)

Study:	Nominal Mixture Concentration:	Abnormal Intersegmental Vessel Development (%)	Abnormal Subintestinal Vein Development - Under (%)	Abnormal Subintestinal Vein Development - Over (%)	Abnormal Subintestinal Vein Development - Missing (%)	Abnormal Subintestinal Vein Development - Total (%)
ZF Mixture	DMSO (0.01%)	2 ± 3	10 ± 4	0 ± 0	2 ± 3	11 ± 6
	0.005 µM	13 ± 0	14 ± 9	3 ± 4	5 ± 6	22 ± 10
	0.05 µM	--	--	--	--	--
	0.5 µM	--	--	--	--	--
FHM Mixture	DMSO (0.01%)	NM	NM	NM	NM	NM
	0.0005 µM					
	0.005 µM					
	0.05 µM					

Mean ± one standard deviation is displayed for each endpoint. Cardiovascular abnormalities were only determined in the transgenic-expressing embryos in the ZF mixture study.
ZF = Zebrafish, FHM = Fathead Minnow, Under = underdeveloped, Over = overdeveloped, NM = not measured

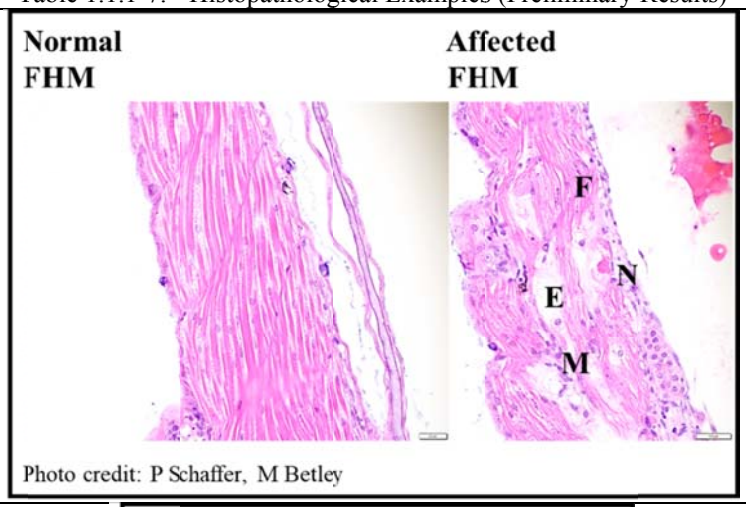
The presence of yolk or pericardial edema and hemorrhage was evaluated in Zebrafish and Fathead Minnow mixture studies. After exposure to the 0.005 µM mixture, 21%, 8% and 40% of Zebrafish embryos exhibited the presence of pericardial edema, yolk edema, and hemorrhage, respectively. At the same concentration, 52%, 43% and 67% of Fathead Minnow embryos exhibited the presence of these cardiovascular abnormalities, respectively. Heart rate decreased 31% from controls across the gradient of toxicants.

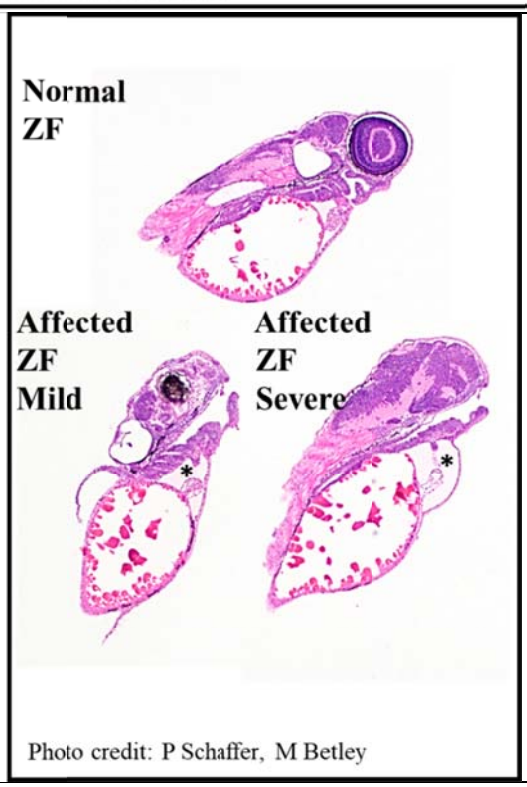
Table 1.1.1-6. - Cardiovascular Abnormalities – Part 2 (Preliminary Results)					
Study:	Nominal Mixture Concentration:	Presence of Pericardial Edema (%)	Presence of Yolk Edema (%)	Presence of Hemorrhage (%)	Heart Rate (beats per minute)
ZF Mixture	DMSO (0.01%) 0.005 µM 0.05 µM 0.5 µM	2 ± 3 21 ± 10 -- --	0 ± 0 8 ± 8 -- --	14 ± 8 40 ± 20 -- --	NM
FHM Mixture	DMSO (0.01%) 0.0005 µM 0.005 µM 0.05 µM	3 ± 3 0 ± 0 52 ± 18 98 ± 5	0 ± 0 0 ± 0 43 ± 23 53 ± 45	21 ± 10 24 ± 14 67 ± 18 98 ± 5	167 ± 8 166 ± 16 127 ± 10 115 ± 11
Mean ± one standard deviation is displayed for each endpoint. Cardiovascular abnormalities were only determined in the transgenic-expressing embryos in the ZF mixture study. Presence of pericardial edema and hemorrhage was evaluated in both chorionated and dechorionated FHM embryos. Presence of yolk edema was evaluated only in dechorionated FHM embryos. ZF = Zebrafish, FHM = Fathead Minnow, NM = not measured					

Histopathology (Table 1.1.1-7)

All surviving embryos from both mixture studies were preserved for histopathological evaluation. Embryos were assessed for signs of muscle damage and edema. Muscle damage was observed in exposed Fathead Minnow embryos and pericardial edema (mild versus severe) was observed in Zebrafish and Fathead Minnow embryos. Additional signs of toxicity including coelomic effusion and cranial edema were observed in Fathead Minnow embryos.

Table 1.1.1-7. - Histopathological Examples (Preliminary Results)





Histopathological evaluation was performed with embryos from both mixture studies. Hematoxylin and eosin staining was used. Upper panel: signs of muscle damage = necrosis-"N," edema-"E," more macrophages-"M," and disrupted muscle fiber pattern-"F") is shown at 400x. Lower panel: mild and severe examples of pericardial edema ("*") are shown at 400x.
ZF = Zebrafish, FHM = Fathead Minnow

Developmental delay and muscle, yolk, and cardiovascular abnormalities were observed in Zebrafish and Fathead Minnow embryos exposed to a mixture of nine antilipidemic drugs. Appearance of hemorrhage, yolk edema, abnormal muscle fiber patterning, and pericardial edema in affected Fathead Minnow embryos were more severe compared to Zebrafish embryos. However, these and all other endpoints (besides yolk absorption and maximum velocity) were scored dichotomously. This difference in species response to toxicants may have been due to gestation time or composition of chorion. For FHM, the exposure duration was increased and timing of assessments was delayed relative to ZF. This was done to match the slower developmental rate of FHM. These subtle differences might also have contributed to differences between taxa.

Field collected concentrations of antilipidemic drugs are generally much lower than what were evaluated in these studies. However, the experimental exposure was of very limited duration relative to nature and no dietary exposure was considered. As antilipidemic prescription rates and human population increases and as agricultural and municipal demands for water reduce surface water flows, concentrations of pharmaceuticals are likely to increase in nature. If a fish embryo does not properly develop, the organism will be less likely to survive and reproduce. These studies identified lethality and sublethal endpoints of concern. Maximum swimming speed represents ability to respond to environment including ability to escape predation. Correspondingly, if muscle fibers fail to properly develop, a fish embryo may have a decreased likelihood of survival to adulthood.

Zebrafish are a common model organism for salmonids and other Colorado fish species. Endpoints considered in this study would not be possible without transgenic phenotypes. Zebrafish develop rapidly relative to Colorado sport fish. This facilitated many observations in a short period of time. Fathead Minnow embryos offer the advantage of evaluating a native Colorado fish species and may serve as an ideal model species for threatened and endangered fish native to the eastern plains.

Future work would include field exposure and collection of Fathead Minnow embryos to compare to laboratory findings. Molecular events that may be more sensitive compared to the morphological characteristics screened in these studies and warrant further investigation. Furthermore, RNA sequencing may elucidate additional endpoints effected by antilipidemic drugs. Transgenerational or full life cycle testing would include a more environmentally relevant exposure duration.

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Action #1.1.2: Assessment of hydrocarbon pollution on trout species and sport fish food sources.

- *Level 1 Action Category: Data Collection and Analysis*
- *Level 2 Action Category: Research*

Develop techniques to conduct experiments and field studies examining various classes of hydrocarbons. Expose fish to environmentally relevant levels and mixtures of hydrocarbons in laboratory studies or field studies. Control treatments will be compared to exposure treatments using the following endpoints: survival, behavior (e.g. drift, bolt speed, olfactory function) mass, length, fat to protein ratio, blood chemistry, fecundity, and metabolism. Data from laboratory trials will be used to design field experiments and observational studies. These studies will be deployed during spill events to better study and document loss and recovery of sport fish.

Action #1.1.2 Accomplishments: List of Activities
Personnel: Pete Cadmus

●Results from studies exposing trout to petrochemicals published in previous reports were analyzed and prepared for publication. West Creek petroleum spill field data are being incorporated into these publications.

●HPLC techniques for numerous pesticides and herbicides were conducted. Solid phase extraction efficiency was assessed for six insecticides and three herbicides common on the eastern plains. Mobile phase alternatives for chlorpyrifos were investigated.

●Stainless steel, glass and PTFE exposure systems were fabricated to allow experiments using organic/hydrocarbon toxicants.

Action #1.2: Conduct novel research to inform and refine water quality standards and policy to ensure Colorado fisheries are protected

Sub-Action #1.2.1: Laboratory toxicity experiments to inform water quality standards and policy.

- *Level 1 Action Category: Data Collection and Analysis*
- *Level 2 Action Strategy: Research*

1-Method development (see 2014 to 2017 progress reports) 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. When possible, method development in new tools and endpoints will continue.
2- Toxicity of contaminants common in Colorado (e.g.: Cu, Cd, Zn, Fe, Se, Mg, Al, Benzene, PAHs, agrochemicals and pharmaceuticals) will be evaluated using egg, larval and early life stages of sport fish in the form of acute and chronic laboratory trials and field experiments. Results from such studies will be analyzed and published.

3- These data will be published for inclusion in triannual recalculations of existing standards or will be used to inform creation of new policies to ensure policies and standards are protective of Colorado's sport fisheries.

Action #1.2.1 Accomplishments: List of Activities
Personnel: Pete Cadmus

●Results from experiments that investigated interactions between temperature and metal toxicity reported in previous federal aid reports were compiled and edited for publication to ensure results are included in triannual review of Cu, Zn and temperature standards.

●Previously reported results from experiments exposing aquatic organisms to ferric Iron were published and were presented to stakeholders and regulatory agencies as a model for an experimentally derived iron standard.

●Previously reported results from experiments exposing aquatic organisms to Zinc were prepared for peer review and were presented to stakeholders and regulatory agencies.

●Laboratory plumbing and infrastructure was modified to enable long term experiments previously not possible.

●*Tanitarus sp.* cultures were established. A subsample was shipped to the EPA research laboratory in Cincinnati, OH.

●Emergence nets over 150 gallon mesocosm were constructed. A modified gravity fed serial diluter was raised above the 24 mesocosm tanks to provide a gradient of metal exposure. This system will serve as a platform for future metal research examining emergence and population dynamics as endpoints. Chronic (>30d), multigenerational (~9 month), mixed stressor, and episodic exposures will assessed in these systems in the years to come.

●Early instar results described in "Experimental evidence of size dependent sensitivity to aqueous metals and the implications to risk assessment and policy making" by Cadmus (Colorado Parks and Wildlife Water Pollution Study 2018, Federal Aid Project Report F-243-R26) are being adapted for *Tanitarus sp.*

●"Experimental evidence of size dependent sensitivity to aqueous metals and the implications to risk assessment and policy making" and two other manuscripts describing metal toxicity experiments are in peer review at scientific journals.

Sub-Action #1.2.2: Field experiments, laboratory experiments and biomonitoring projects that investigate direct and indirect effects of pollution and interactions with other stressors.

- *Level 1 Action Category: Data Collection and Analysis*
- *Level 2 Action Strategy:* Research

1- In the last decade CPW's Aquatic Toxicology Laboratory has completed single species experiments on sport fish species not limited to Brown Trout, Salmon, Mountain Whitefish and numerous strains of Cutthroat Trout and Rainbow Trout. These trials examined direct and indirect effects of 12 pollutants with and without interactions of other toxicants, water quality (e.g. temperature, flow regimes) and disease (e.g. whirling disease). These experiments are being published to inform recalculations of water quality standards on the state and national level. Additional experiments will be conducted to compliment these studies when needed.
2- CPW, in collaboration with Colorado State University and Colorado School of Mines, conducted numerous experiments looking at effects of aqueous and particulate metals on aquatic ecosystems. This information will be analyzed and compared to the chronic criterion value based on single species trials to ensure policy is protective of sport fish and sport fish habitat. Field and laboratory acquired data will be used to a) test existing models of toxicity and bioavailability b) build models to predict subcellular accumulation of metals c) inform and predict reestablishment of fish and insect (primary food source of trout species) populations after mine reclamation efforts.

Action #1.2.2 Accomplishments: List of activities.
Personnel: Pete Cadmus

●Conducted aquatic insect and fish sampling at abandoned mine sites at the North Fork of Clear Creek near Blackhawk Colorado. These data will be used by National Institutes of Health, Colorado State University, Colorado School of Mines, and other agencies to document the return of aquatic life after mine restoration. Numerous federal and state water quality and land management agencies will use these data to prioritize mine restorations to maximize fish habitat. Studies are ongoing.

●Conducted data analysis of previous years' results that included fish cages and in-stream serial dilution systems to predict what fish species and age classes should be stocked in the North Fork of Clear Creek. Prepared these methods for publication in adaptive resource management journals. Presented these methods and findings at the Society of Environmental Toxicology and Chemistry annual meeting as a cost effective way to study metal mixtures.

Job No. 2 Water Quality Technical Assistance to Colorado Parks and Wildlife Personnel and Other State and Federal Agencies

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

Need

Water quality characteristics and pollution affect fish health and the viability of fisheries. Water chemistry and aquatic ecotoxicology demand specialized skill sets and unique instrumentation/infrastructure. Fisheries managers faced with chronic pollution issues, acute (accidental) spill events, fish kill 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:

- *Level 1 Action Category: Data Collection and Analysis*
- *Level 2 Action Category: Research*

As requested, CPW's Aquatic Toxicology Laboratory will aid in the following:

1. Collect and analyze water samples for rotenone (and other piscicides) as part of reclamation projects as requested.

2. Provide technical support in assessing effects of chemical stressors on trout populations in the Animas River as requested. Additionally, help design field experiments, monitoring protocol and prioritize restoration efforts with federal agencies as needed.
3. Continue to collect and analyze water samples and consult on biomonitoring and experimental design at mine restoration sites in Colorado including long term monitoring sites on the Arkansas River near Leadville, Animas River near Silverton and North Clear Creek near Blackhawk as needed.
4. Provide experimental design support in investigations examining indirect or physical toxicity of Fe, Al and/or Mn in mine impacted watersheds as requested during mine restoration efforts and/or increases in mine pollution.
5. Collect, analyze and interpret water samples and biotic samples as part of fish kill investigations, pollution investigations and restoration efforts as requested.
6. Conduct biological monitoring and field experiments as part of reclamation projects that allow managers to better predict effects of rotenone on target and non-target fish as well as the insects and algal species that support sport fish populations as requested.
7. Provide the fisheries managers of Colorado and other states with milt extender as requested.
8. Develop fish kill kits and train CPW staff on water chemistry sampling techniques.
9. Provide analysis of chlorophyll and algal community composition to CPW researchers and managers working to improve sport fish production in Colorado reservoirs by altering algal community composition and productivity as requested.
10. Provide managers and cooperating regulatory agencies with toxicology experiments using species and water quality characteristics pertinent to Colorado in an effort to better inform changes to water quality standards or site specific derivations to water quality standards as requested.
And / or
11. Provide ecotoxicological support and expertise to CPW managers, Colorado universities and fellow natural resource management agencies as requested. .

Action #2 Accomplishments: List of Activities
Personnel: Pete Cadmus

● Conducted onsite assessment of rotenone during chemical reclamation projects to restore cut-throat trout habitat. Not limited to: George Creek Reclamation Project, Rock Creek Reclamation project, Hermosa Creek Reclamation Project, Toponas Creek Reclamation Project.

● Produced milt extender for federal and state natural resource management agencies across the country.

● Provided advising and analytical support for Colorado River Watch, a non-profit that provides Colorado Parks and Wildlife and other state and federal agencies free water quality monitoring data across Colorado. CPW's Aquatic Toxicology Laboratory staff examined laboratory policies and procedures and laid the ground work for improving good laboratory practice and sample storage. We consulted in reorganizing scientific staff and contractors and equipment use and rental. We began reviewing QAQC policies to improve results, turn-around time of results,

reporting information. We researched and advised on equipment options for replacing an ICP-OES with an ICP-MS. Lastly, we explored cost saving measures.

● Collaborated with Colorado Department of Public Health and Environment to investigate effects of Selenium on fish (White Sucker and Brown Trout). Fish were spawned and eggs were reared. Tissue concentrations were digested and assessed for Selenium levels. Biomonitoring studies were conducted to determine what risk elevated selenium levels have on sport fish reproduction.

● Assisted area wildlife biologists with potential spills and water quality issues by consulting on sampling techniques, providing analytical support, and supplying equipment and sample vessels.

● Provided biomonitoring services of algae and insects at mine restoration efforts in the North Fork of Clear Creek near Blackhawk, Colorado. This drainage was once void of life and now has aquatic insects and fish occupying reaches downstream of adits.

● Provide ecotoxicological support and expertise to CPW managers, Colorado universities and fellow natural resource management agencies as requested.

Action #2 Accomplishments: Methodologies for onsite analysis of rotenone concentrations associated with fish removal operations/piscicide application

Personnel: Pete Cadmus, Stephen F. Brinkman and Ken Kehmeier

Introduction

Rotenone is a natural pesticide derived from the roots of tropical plants found in Australia, Southeast Asia, Africa and South America. It has been used for centuries to capture fish for human consumption and has been used for 150 years as a garden insecticide. Fishery managers have used rotenone since the 1930s to sample fish populations, control undesirable fish, control fish diseases, eradicate invasive fish and as a primary tool for restoration of threatened and endangered fish species.

In Colorado, rotenone has been a critical tool for restoring populations of the native Greenback cutthroat trout (*Oncorhynchus clarkii*) by eliminating non-native fish species that would outcompete or hybridize with native cutthroats. Use of rotenone in fish reclamation projects requires significant amounts of preparation, site characterization, cost and personnel. In the past (1987 to 2003), Colorado Parks and Wildlife had rotenone concentrations analyzed by high performance liquid chromatography (HPLC) in our analytical chemistry laboratory. However, restoration sites are often remote and transport of rotenone samples to the laboratory required several hours. Consequently, results were too late to be of use to fishery managers. Furthermore, rotenone rapidly degrades and is oxidized in natural waters even when shipped at low temperatures. Measured concentrations hours after sample collection were often suspect. This is especially true for samples collected downstream of potassium permanganate treatment stations because the piscicide was likely degraded in the sample container shortly after the sampling event.

In 2003, our agency developed a field based method to analyze rotenone. Timely on-sight assessment of rotenone has ensured that applied rotenone concentrations were at effective levels for appropriate durations. Potassium permanganate ($KMnO_4$) is often used to oxidize rotenone to safe levels downstream of the project area. Timely feedback of rotenone concentrations above and below $KMnO_4$ drip stations ensures detoxification by potassium permanganate is sufficient but not excessive. In the decade that followed, we arrived at a methodology, mobile laboratory and system that embodied the following requirements:

1. Mobile- personnel, equipment and supplies must be transported long distances over highways and rugged roads potentially requiring high clearance vehicle with four wheel drive.
2. Rugged and Robust- equipment must tolerate shaking and vibration over long stretches of steep, rocky and washboard roads and function in temperature and weather extremes using a poorly regulated power supply from solar panels, batteries or generators.
3. Sensitive- the method must be able to measure concentrations below piscicidal levels and drinking water standards.
4. Accurate and precise- results must be reliable because significant amounts of resources are invested in reclamation projects, the importance of success and consequences of decisions based on incorrect information required accurate and precise results that were repeatable in a wide variety of water characteristics.

5. Rapid results- turnaround time had to be less than twenty minutes providing results in a timeframe that was useful to rotenone project supervisors.

The on-site rotenone analysis methodologies presented here within were based on methods reported by Dawson et al. (1983), a methodology CPW has utilized in the laboratory since 1987. In the three decades that followed CPW made modifications of the method and conducted several important quality assurance experiments.
-The method was modified for use in the field using unconventional power and equipment. (see 'Methods' and "Discussion" sections below)
-A sodium thiosulfate preservative was employed and shown to not affect results. (See 'Sodium Thiosulfate Preservative' section below)
-Use of pH buffer was found unnecessary. (See 'pH Buffers' section below)
Additionally, this method has aided managers in comparing piscicides, has helped managers confirm target concentrations for invasive species, and helped determine timing and effectiveness of detoxification by $KMnO_4$ (See Brinkman 2012 for examples and results).

Methods

Equipment

- **High Performance Liquid Chromatograph (HPLC)**. Our agency has used the following systems with success:
  -Dionex GP-2 pump with Dionex VDM-2 detector (Dionex Corporation. Sunnyvale, CA, USA).
  -Dionex GP40 pump with Dionex AD20 detector (Dionex Corporation. Sunnyvale, CA, USA) and an HP 3395B integrator (Hewlett Packard. later acquired by Agilent Technologies Inc., Santa Clara, CA, USA)
  -Agilent 1220 infinity LC with UV detector (Agilent Technologies Inc., Santa Clara CA, USA)

  All systems used stainless steel capillaries and valves. The UV-Vis (Ultra-violet and visible light) detectors were set to 295 nm wavelength. All machines had auto-sampler systems removed because manual injection allowed flexible prioritization of samples. Ability to heat the column and mobile phase was helpful but not needed.
  Note: HPLCs are versatile and come with many features. Modular systems allow you to remove unwanted features. Old technology, deemed obsolete by some laboratories, is often ideal for field work because of the build quality, simplicity, and ability to handle derisory electrical power. We advise working with your agency water quality and chemistry experts to find an affordable system that meets the needs of your agency (detection limits, correct sensors, power needs, etc.) This method needs only an isocratic pump (only one mobile phase). Gradient pumps can be programmed to run as isocratic pumps.
- **Laptop computer, printer and graphing software** to graph trends in sample results for the managers in real time.
- **100µl stainless loop** compatible with your HPLC's valve fittings. (100µl was used in this method however bringing additional loop sizes into the field is advised)

□ **Several HPLC injection syringes**. (These must be compatible with the injection port on the valve on the HPLC. Each model has its own requirements).
□ **Several HPLC columns with C18 selectivity**. We found Hypersil GOLD C18 selectivity HPLC column (55M 250x4.6. Catalog number: 25005254630, Fisher Scientific, Waltham, Massachusetts, US) to be an ideal column for this methodology and has been successful with every water chemistry we have observed in Colorado. However, it is advised that mobile laboratories carry several different columns in different sizes to accommodate odd waters or chemistries in the field. Prior to adopting sodium thiosulfate preservative in every sample, we observed $KMnO_4$ in samples degrading HPLC columns even after filtering with the Sep-Pak. Having extra columns is always advised.

Reagents

□ **HPLC grade methanol:** This comprises the eluent for solid phase extraction and is the primary ingredient for mobile phase. It is also used to clean glassware and prime Sep-Paks. Methanol is saturated with helium prior to departing for field site.
□ **Deionized water (DI water)**: >18 microphms using a Bronsted Nanopure Dionizer. Low dissolved organic carbon water saturated with helium prior to departing for field site.
□ **Mobile phase reagent**: 75%:25% (v/v) HPLC grade methanol: low DOC water. This is saturated with helium prior to departing for field site to degas oxygen.
□ **Rotenone Standard**: 0.01030 g rotenone brought to 50 ml of methanol in a volumetric flask (+/- 0.05ml). Heat, using a warm water bath, is sufficient to dissolve the powered but small amounts of acetone can be used without interference of the chromatograph.
□ **pH 4 Buffer**: Sample buffer is prepared by dissolving 1.915 g sodium acetate trihydrate, mixing with 0.343 ml glacial acetic acid and diluted to 200 ml with DI water. (see 'pH Buffer' section below)
□ **Sodium thiosulfate preservative**: 0.37 g sodium thiosulfate is individually packaged in disposable coin envelopes prior to trips. This mass should be more than adequate to react with $KMnO_4$ in samples even below $KMnO_4$ drip stations (also known as 'detox stations'). In the field this granular solid is coarsely measured volumetrically to 0.25 ml in a graduated centrifuge tube.
□ **Municipal tap water:** Used for cleaning glass ware.
□ **Helium gas cylinder with regulator and aeration stone:** This is used to aerate mobile phase and methanol with helium gas to reduce dissolved oxygen levels in methanol and water (for safety reasons we no longer transport compressed air cylinders on rough off-highway trails. We now saturate mobile phase and methanol with Helium gas prior to each project to reduce the amount of dissolved oxygen. In theory this reduces oxidation of sample however in emergencies we have neglected this step without ill effects.

Analytical Supplies:

□ **Sep-Pak® C18 solid phase extraction cartridges**: (Part number: WAT051810. Waters Corporation. Milford, MA, USA)
□ **250 ml glass amber sample bottles with PTFE lined caps**.

□ **Label tape**: VWR brand General-Purpose Laboratory Labeling Tape has good retention of writing in wet environments (VWR. Radnor, PA, USA)
□ **Markers:** Fine and extra fine permanent laboratory/industrial markers
□ **100µl pipette**: calibrated gravimetrically prior to leaving for the field.
□ **2000µl pipette**: (or 1000 if not available) calibrated gravimetrically prior to leaving for the field.
□ **5000µl pipette**: (calibrated to 3 ml for buffer) calibrated gravimetrically prior to leaving for the field.
□ **Transfer pipettes**: These assist in measuring sample volume in graduated cylinder to 200ml. (see Figure 2.7)
□ **250ml Graduated cylinders**: for measuring 200 ml of sample.
□ **1000ml graduated cylinder**: for preparing or modifying mobile phase.
□ **Solvent resistant labels**: we use CILS International (West Sussex, UK) polyester labels (product number: AL8-9100HT-5)
□ **3ml syringes**: for prepping and eluting Sep-Paks with water and methanol. (BD 3ml Syringe. Product number: 309656. BD, Franklin Lakes, NJ, USA)
□ **Norm-ject 50 ml (60ml) Luer lock syringes**: (Catalog number: 48500030000) or other large volume rubber free syringe.
□ **Glass vials**: 3.8 ml borosilicate glass vials with PTFE lined caps. 14.7 x 45mm (QORPAK. Catalog number: glc-00980) and appropriate test tube racks.
□ **Eye protection**
□ **Nitrile or latex gloves** for lab work.
□ **Coin Envelopes:** (part number 50160. Quality Park Products. St. Paul, MN USA)

Field laboratory supplies and equipment

□ **Buckets**: Many buckets are used for hazardous waste, mobile phase eluent, sample bottle cleaning station and seats.
□**Hoses, sprayers or water squirters** for rinsing contaminated bottles and surfaces.
□ **Mild detergent** or dilute soap.
□ **Large cooler** and ice to hold samples prior to analysis.
□ **Small coolers** with ice packs for transporting sample bottles.
□ **Stopwatches and timers** to aid in timing HPLC runs when using a manual injector.
□ **Syringe pump** or battery powered caulk gun.
□ **Lawn chairs**
□ **Many tarps** to cover work space, supplies and generator.
□ **Collapsible tables**
□ Multiple large gauge electric **extension cords**
□ **Generator** and jerry cans of fuel.
□ **PPE:** Large waterproof trapper gloves, PPE and waders for sampling.
□ **Optional filtering or centrifuging equipment** if working in turbid waters.
□ **Wrenches** for columns and fittings.
□ **Headlamps** and large battery powered **work lamps** for processing samples late into the night.
□ **Van Buren water sampler** if working in lentic habitat.
□ **Closed cell camping pads** and **bubble wrap** to insulate fragile equipment from shock.
□ **Camping equipment**

□ **Off-road travel equipment**
□ **Tools** for repairing equipment and vehicles.
□ **Non-rotenone water sampling equipment** including a colorimeter capable of assessing free chlorine (Used to estimate $KMnO_4$ concentration), pH meters, DO meters and other assessment tools that are sometimes needed by managers problem solving at rotenone projects.
□ **Volt and amp meter**
□ **Electrical wire and fittings**
□ **HPLC repair supplies**: capillary tubes, fittings, syringes of various sizes, replacement parts and other materials needed to fix systems and prime pumps on the HPLC.

Mobile Laboratory

Colorado Parks and Wildlife (CPW) has conducted on-site assessment of Rotenone using laboratory equipment in a tent (1997-2012), and a trailer and a slide-in popup camper on the bed of a high clearance 4x4 pickup truck (2012 to present; Figure 2.1). The popup camper provided protection from rain and sun, storage for equipment, tools and supplies during transport and analyses, space for sample preparation and doubles as sleeping quarters for analytical personnel at night. A 2000-watt portable generator (EU 200i Honda. Model:EAAJ-1393388) provided sufficient electricity to the camper and analytical equipment. Solar panels and marine deep cycle batteries with inverters/regulators had potential to supply all electrical needs. Laboratory preparation space typically consisted of tarps over collapsible tables (Figure 2.2). Laboratory space was best located in level areas that received shade in the afternoon when summer temperatures can alter retention time of samples in HPLC columns. Laboratory space should be located far from sleeping and eating areas and very far from rotenone preparation or equipment decontamination areas. Traffic cones, flagging and signs were eventually adopted to prevent non-laboratory staff wearing dirty waders from contaminating sample preparation sites. Sampling equipment (Figures 2.3 and 2.4) and sample bottle wash stations (Figure 2.5) should be available to field staff but a deterring distance from sample preparation activities.

Sample Collection

Amber glass bottles (250ml) with labels, markers, and envelopes of sodium thiosulfate were made available to workers responsible for sampling (Figures 2.3 and 2.4). If using expensive verified clean vials rinsing was not needed. If reusing (acid-washed and methanol rinsed) vials we encouraged samplers to rinse the bottles with river or lake water from the site prior to taking a sample from the water column (not surface or hyporheic zone). A small amount of sample was decanted to allow only enough head space to accommodate the addition of sodium thiosulfate crystals. Bottles were inverted numerous times until crystals had dissolved. Samples were shielded from sunlight and transported on ice to the sample decontamination station (Figure 2.5) where each sample was rinsed and scrubbed with a dilute soap, rinsed with water and placed on ice. These procedures minimized rotenone contamination of laboratory preparation space.

Sample Preparation

Using a graduated cylinder, 200 ml of sample was measured (Figures 2.6 and 2.7) and transferred to a 250 ml glass sample bottle (a new bottle was preferred but reusing the original sample bottle was also acceptable). Using a calibrated pipette, 4 ml of sample buffer was added to the 200 ml of sample. Sample was capped and inverted numerous times to homogenize. Rotenone in the water sample was concentrated using solid phase extraction using a Sep-Pak C_{18} disposable cartridge (Waters, Milford, CT, USA; Figure 2.8). Each Sep-Pak was preconditioned by attaching a 3 ml syringe to the Sep-Pak and pushing (term for passing a liquid through a column or cartridge using a syringe) ~3 ml of methanol through, followed by ~3 ml of deionized water. Because 200 ml syringes are not available, the extraction of rotenone from the sample required multiple pushes using a 60ml syringe. The piston was removed from the syringe, the Sep-Pak was attached, 50ml of the sample was loaded into the back of the syringe (Figure 2.9), the piston was reinserted and the sample was pushed through the Sep-Pak (Figure 2.10). This was repeated until the sample was completely passed through the Sep-Pak at a flow rate that did not exceed 40 ml/min. A crude but effective way to verify the flow rate does not exceed 40 ml/min was to visually monitor that the sample leaving the Sep-Pak does so in drops and not as a stream. Syringe pumps did not stand up to field conditions, while battery powered caulk guns (Figure 2.11) have proven useful in automating extraction. After the entire sample has been pushed through the Sep-Pak, a syringe full of air (~60ml) is pushed through to remove as much retained water as possible. All liquids pushed through the Sep-Pak during the preparation and extraction processes were dropped into a hazardous waste bucket.

The rotenone was then eluted form the Sep-Pak. A 3ml syringe with piston removed was attached to the Sep-Pak. A calibrated 2000µl pipette (or two aliquots using a 1000µl) was used to load the syringe with 2.0ml methanol. The methanol was slowly pushed through the Sep-Pak and all eluate (term for eluent and targeted analyte) was retained in a 3.8ml borosilicate sample vial with PTFE-lined lid (Qorpak, Clinton, PA, USA). To completely remove the sample the syringe was also used to pass ~10ml of air. Ethanol proof labels were used to label borosilicate vials. The sample eluate was inverted multiple times to ensure complete mixture.
The sample eluate of the solid phase extraction is then loaded onto the HPLC loop (Figure 2.12). Good laboratory practices suggest the eluate should be the same percentage solvent and water as the mobile phase. Both proved acceptable results. Mobile phase ratios were sometimes altered during peak separation efforts. Using one hundred percent methanol did not widen the rotenone peak, did not alter retention time and only added a small amount of noise to instrument blanks (a term for a type of QAQC sample, see below). This noise (a small peak) elutes from the HPLC column at ~2 minutes. This is well before the rotenone peak is observed on the chromatograph. Additionally, we felt the 100% methanol elution ensured 100% removal of rotenone from the Sep-Pak.

We have found rotenone in 100% methanol is extremely stable relative to rotenone in water. After solid phase extraction the urgency or running samples was not driven by the decay of rotenone in water but by the need of results. Additionally, when on-sight HPLC services were not available our agency's staff has sent the eluate of the sample rather than the water sample to the analytical laboratory. This improved accuracy but required the correct hazardous material placards for shipping boxes.

Sample Analysis

A Dionex GP40-AD20 (Dionex Corporation. Sunnyvale, CA, USA) and later an Agilent 1220 infinity (Technologies Inc., model 1220, Santa Clara CA; Figure 2.13) high performance liquid chromatograph (HPLC) analyzed rotenone using an isocratic 75%:25% methanol:water mobile phase pumped at 1 mL/min through an analytical column (Hypersil GOLD C18 selectivity HPLC column. 55M 250x4.6. Catalog number: 25005254630, Fisher Scientific, Waltham, MA, US) producing a pressure near 150 bar (+/-40). A UV-Vis detector graphed absorbance at 295 nm which routinely observed dyes used by fish biologists, algae, and other water chemistries but rarely at the 6-8 min retention time of the rotenone. Integration was set to end at 8 to 9 minutes after injecting the sample but 10 to 15 minutes was typically needed between sample injections to clear all residue from the column.

Sample extraction, purification and preparation techniques in the field are limited. But on-site alteration of column temperature, methanol:water ratio, flow rate and pressure, column size and material, monochromator/detector wave length and loop size (volume of sample eluat used) can be altered by the HPLC technician in the field to separate peaks. These techniques are beyond the scope of this report but will be common knowledge to any chemist experienced with liquid chromatography. The HPLC column was maintained at a constant temperature using a metal pipe wrapped with heat tape (Figure 2.14) powered through a relay (Crydom D2425; Figure 2.15) plugged into a temperature controller (Love Controls Model 16B; Figure 2.16). This system proved efficient when power was limited to solar panels, batteries and generators. Temperature was adjusted daily to exceed the forecasted high temperature of the day or to aid in separation of peaks present at each site. Commercial ovens for mobile phase and column are available for most HPLC. These proved unreliable in the field setting where temperature fluctuations from below freezing to 43°C are often observed. Additionally, peaks were easily separated by adjusting the methanol to water ratio. Adjusting the ratio 'on-the-fly' was aided by using a mobile phase container that was graduated with volume increments.

Calibration curves were developed using standards prepared from commercially available purified rotenone powder (>97% pure, Aldrich Chemical Company; St. Louis, MO, USA) that were prepared fresh daily and treated and extracted using the procedure for water samples described above. In addition to one blank (used as 0 in the calibration curve), additional spikes (standard plus DI water) were produced at 100 and 200 µg/l rotenone by adding 100 and 200µl of the standard described above. Field operations in Colorado have never required calibration of standards above 1ppm total formula of CTF Legumine® (which is 0.05 ppm rotenone), but during method development calibration curves were linear to over 5 times this value (r^2=0.98 to 0.999). Retention time of 7 minutes (+/- 1 min) depended on column, temperature, methanol:water ratio and other factors.

pH Buffers:

Dawson (1983) encouraged use of a pH 4 buffer and presented evidence that the lower pH had superior retention than 7. During numerous field analysis projects CPW ran out of sodium acetate buffer (pH 4). We saw no change in results despite the pH of the waters being

neutral. Laboratory quality assurance experiments revealed that modern Waters Sep-Paks (C18) had no significant differences in recovery when 4, 7 and 10 pH buffers were employed during solid phase extraction. After three point calibration of the HPLC nine samples of deionized water were assigned 4ml of one of three buffers at pH 4, 7 and 10. Each sample was spiked with the same concentrations of rotenone. Preparation and analysis was ordinated in a Latin squared design to reduce differences in rotenone decay time. Dawson reported a pH of 4 being optimal while the manufacturer of the SEP-Pak (waters) advises a pH between 2-8. We found no statistically significant difference in recovery between the 4, 7 and 10 pH buffers (ANOVA: F=0.83 p=0.46; Figure 2.17). Given suitable fish habitat is typically within the pH range of 4 to 10, the method presented herewithin is likely tolerant to pH difference across sites with or without the use of a buffer. It is speculated that Dawson's results might have been due to systematic decay of rotenone during laboratory preparation or that solid phase extraction resin has improved.

Sodium Thiosulfate Preservative

Frequently managers wanted to measure rotenone concentrations below the $KMnO_4$ drip station (AKA 'detox') used to oxidize rotenone in lotic reclamation projects. This was troublesome because $KMnO_4$ would continue to degrade samples while in transit to the mobile laboratory. We then began adding excessive amounts of sodium thiosulfate to quench the oxidizer. $KMnO_4$ preferentially attacked the sodium thiosulfate preservative. In laboratory studies, 8 samples were spiked with equal amounts of rotenone and a stoichiometric equivalent amount of $KMnO_4$. Four of the bottles were also quenched with sodium thiosulfate ($Na_2S_2O_3$) preservative as described in the methods. Samples were randomly processed 0, 10, 20 and 30 minutes afterwards to examine if sodium thiosulfate reduced oxidation of rotenone (Figure 2.18). Without sodium thiosulfate nearly all rotenone had been degraded at each observation time but bottles preserved with sodium thiosulfate largely avoided rotenone loss. To ensure sodium thiosulfate did not interfere with rotenone recovery in environmental samples, 20 duplicate (two bottles taken at the same time and location) samples at five field projects (providing diverse environmental matrices) were used to examine if excessive amounts of sodium thiosulfate interfered with rotenone recovery. Excessive amounts (3 ml or ~4.4g) of sodium thiosulfate crystals were added to one of each duplicate sample. Rotenone peak area of the sample was compared to area of the duplicate receiving $Na_2S_2O_3$. Recovery of rotenone was not affected. This has allowed a concentration of rotenone downstream of detox stations to be characterized such that it was representative of the exact time of collection. Subsequently, sodium thiosulfate (~0.25 ml of solid crystals) preservative has been added to every sample, even samples upstream of detox stations, to maintain a consistent method.

Discussion

Quality Controls and Quality Assurance (QAQC)

Falsely low or falsely high estimates of rotenone do more harm than good when informing on-the-ground decision making at reclamation projects. For this reason, analytical chemists employ blanks, spikes, duplicates, splits and external standards. Each of these can be

used at any step in the collection, preparation and analysis of rotenone. If blanks show rotenone, a contamination issue has occurred. If spikes show a reduced amount of rotenone other samples in that batch may have been falsely low. External quality controls ensure accuracy, while splitting samples or taking duplicate samples characterizes the precision. Use of these QAQC samples is beyond the scope of this paper but is important to mention. Fish biologists should work with their agency's water quality and analytical chemistry experts to devise a schedule for quality assurance samples at each stage of the sample preparation and instrumentation. QAQC samples are typically more numerous if the results are intended to be legally defensible than if simply informing managers of approximate trends over time.

Limitations and advantages of the field setting

Rotenone is routinely analyzed using HPLC in the laboratory, where environmental conditions are stable. HPLC instrumentation and analyses are generally considered to be sensitive to changing temperature and electrical supply fluctuations. Nevertheless, we have analyzed rotenone on-site at elevations from 693m to 3500m (MSL) and in temperatures below freezing to temperatures above 43° C. The system has worked in rain, sun and snow, powered off a small generator as an electrical power source. Colorado's headwaters are considerably oligotrophic relative to lowlands and locations with more dissolved carbon and pollution. Thus some regions may need to make many modifications to separate peaks. However, this method has provided analytical results from beaver dams and erosional areas that were high in turbidity and dissolved organic carbon. The methods discussed here have been proven robust to not only the elements but to the limitations of working in remote locations with limited supplies. These methods require ultrapure deionized water, but when our deionizer failed we successfully made all reagents with generic distilled water from a local grocery store. Electric caulk guns ($40) do not work better than syringe pumps ($1200-2000) however syringe pumps often lasted less than 5 days in the field. Proper HPLC ovens exist but were not as steady as the use of a Love temperature controller, a hunk of steel pipe and some heat tape (Figures 2.14-2.16). Solid phase extraction with Sep-Pak cartridges concentrated the sample (which improves detection limits) and also filtered impurities avoiding the need for a guard column. Those accustomed to the luxury of laboratory settings might see the methods described herewithin as unprofessional, not limited to: failing to continuously degas reagents with helium, calibrating pipets a couple days prior to use, eluting with a ratio not identical to the mobile phase, measuring $Na_2S_2O_3$ crystals volumetrically. In the field we have limited space, limited time and limited resources. Laboratory equipment is expensive and is not robust enough for rain storms. Surely, slight precision and accuracy could be gained with refinement of these techniques. However the detection limits of these methods (on either of the machines listed) were well below the drinking water standards recommended by the USEPA, which are below the levels that are toxic to fish. So, method improvements might be of little value especially if such refinements slow the turn-around time of priority samples.

High performance liquid chromatography does not need to be high priced liquid chromatography. Old retired HPLC equipment is perfectly acceptable for this methodology and can be found at very reasonable prices. Although we do not reuse bottles, vials, syringes and

Sep-Paks such practices are not unacceptable if proper cleaning and quality assurance practices are adopted.

How HPLC informs the use of rotenone

Each reclamation project and each manager is different. Some managers requested HPLC analysis exclusively to monitor rotenone concentrations above and 30 minutes below detox every half hour from start to finish. (Note: 30 minutes below was found to be the time in which all $KMnO_4$ is depleted, see Brinkman (2012). This has been confirmed with field observations in oligotrophic waters). This process ensured detox was functioning. Many managers used HPLC analysis intensely throughout the application areas on the first day to ensure all drippers and spraying crews were meeting concentration targets for the appropriate durations. The subsequent day's activities were then fine-tuned by adding drippers and fine-tuning sprayer crews. HPLC assessments are a valuable tool when problem solving. In some chemistries or physical anomalies (***e.g.*** beaver dams, water falls, algal mats) mortality was lower than predicted and the HPLC has allowed managers to determine if rotenone is being lost. Use of HPLC during bioassays prior to project dates helped ensure the product had not decayed and that the fish would respond at the appropriate levels. Chiefly, the HPLC gives piece of mind. Managers sleep sound knowing targets were met at the correct duration, knowing that no rotenone made it past detox stations, and knowing they have a tool that lets them determine rotenone levels if problems occur.

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Figure 2.1 – Mobile Laboratory. Our analytical service is often located next to detox (the MnO4 drip station used in lotic reclamation projects) because this is typically where the majority of samples are taken. Some of the detox stations in Colorado were assembled using pack mules or helicopters. Trailers were often difficult to maneuver on Colorado's rough 'Jeep trails.' Sample preparation was done outside and space was limited, However, this system allowed off-road ability, a dry location for the instrument.

Figure 2.2 – Typical makeshift sample preparation area.

Figure 2.3 – Field collection equipment station. Every morning bottles, envelopes of preservative, small coolers (bucket) and PPE were made available to field staff responsible for collecting samples.

Figure 2.4 – Sodium thiosulfate preservative. A graduated centrifuge tube was used to measure ~0.25ml of Sodium thiosulfate which was sealed in a disposable coin envelope. This substance prevented the decay of rotenone in samples. The mass of this solid is in excess of what is needed to neutralize $KMnO_4$, thus precision was not needed.

Figure 2.5- Sample decontamination station. Decontamination of sample bottles and keeping wader clad field staff from the preparation area reduced contamination issues in sample preparation and analysis. The wash station included buckets with rinse water and mild detergent (Simple Green), scrub brushes, water squirters or pressurized sprayers, personal protective equipment and an ice chest.

Figure 2.6 – Samples were measuring to 200ml in a graduated cylinder.

Figure 2.7 – Transfer pipettes were used to remove excess sample.

Figure 2.8- Equipment used in extractions, sample preparation, and making a standard curve. Left to right: 100µl pipette, 3 sample bottles (250ml) used to create a calibration curve, 1 glass amber (125ml) containing the rotenone standard, 250ml graduated cylinders, 1-5ml adjustable pipette, 5 Sep-Pak cartridges and 3ml syringes, squirt bottles of methanol and DI water.

Figure 2.9 – The 60ml syringe was loaded from the back end.

Figure 2.10 – Samples were “pushed” through a Sep-Pak at 40 ml/minute over a hazardous waste bucket.

Figure 2.11- Battery powered caulk gun. These caulk guns ($40) were far more reliable in the field than laboratory syringe pumps ($1200-3000).

Figure 2.12 – HPLC, laptop and printer. Photo from inside the mobile laboratory showing the Agilent 1220.

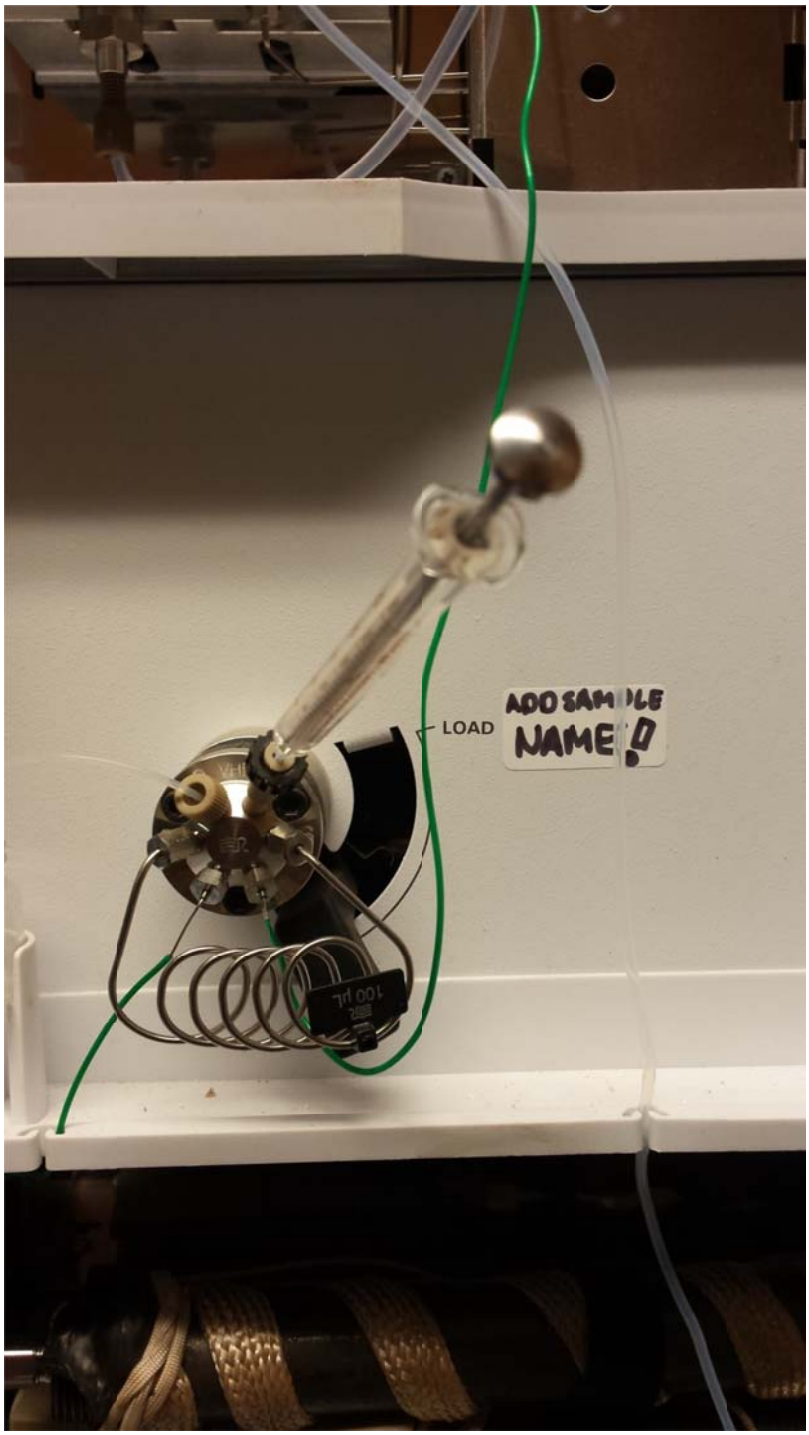


Figure 2.13 – HPLC valve, 100µl injection loop and syringe.

Figure 2.13 – Agilent 1220 HPLC with column hidden in a heating system consisting of a 6 inch piece of steel pipe, pipe heat tape, and a temperature controller.

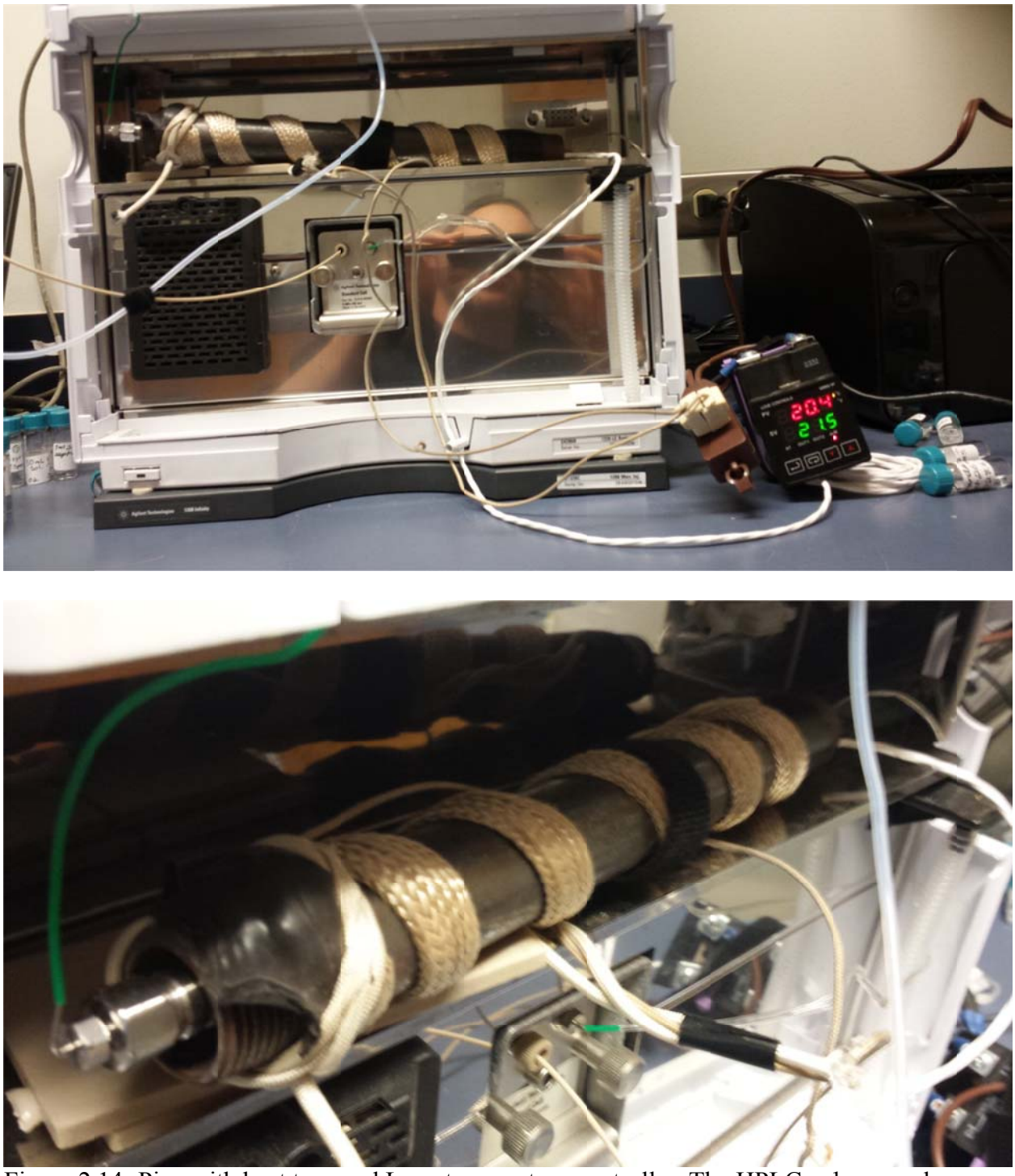
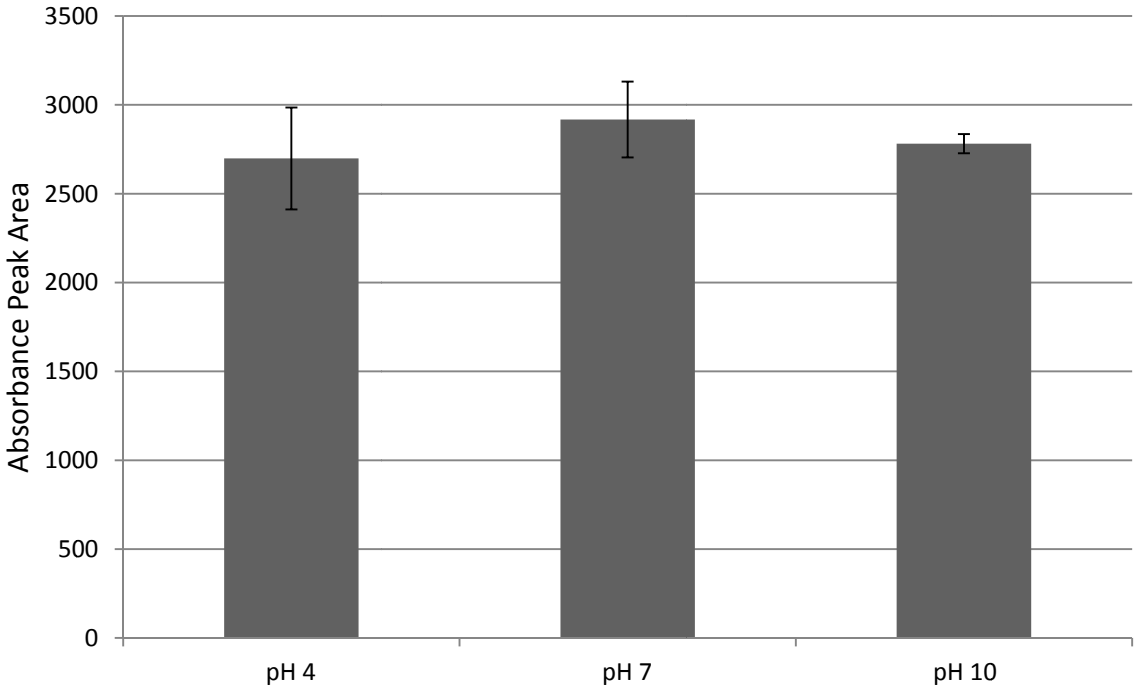


Figure 2.14- Pipe with heat tape and Love temperature controller. The HPLC column and thermometer for the temperature controller were placed in the steel pipe.

Figure 2.15- Crydom D2425 relay controlled by the Love brand temperature controller.

Figure 2.16- Love 16B temperature controller.



Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
pH 4 Buffer	3	8095	2698.333	82372.33
pH 7 Buffer	3	8753	2917.667	45650.33
pH 10 Buffer	3	8345	2781.667	4790.333

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	73547.56	2	36773.78	0.830652	0.480337	5.143253
Within Groups	265626	6	44271			
Total	339173.6	8				

Figure 2.17- Comparison of rotenone recovery in pH 4, 7 and 10 buffers. No significant difference was observed suggesting buffer is not needed.

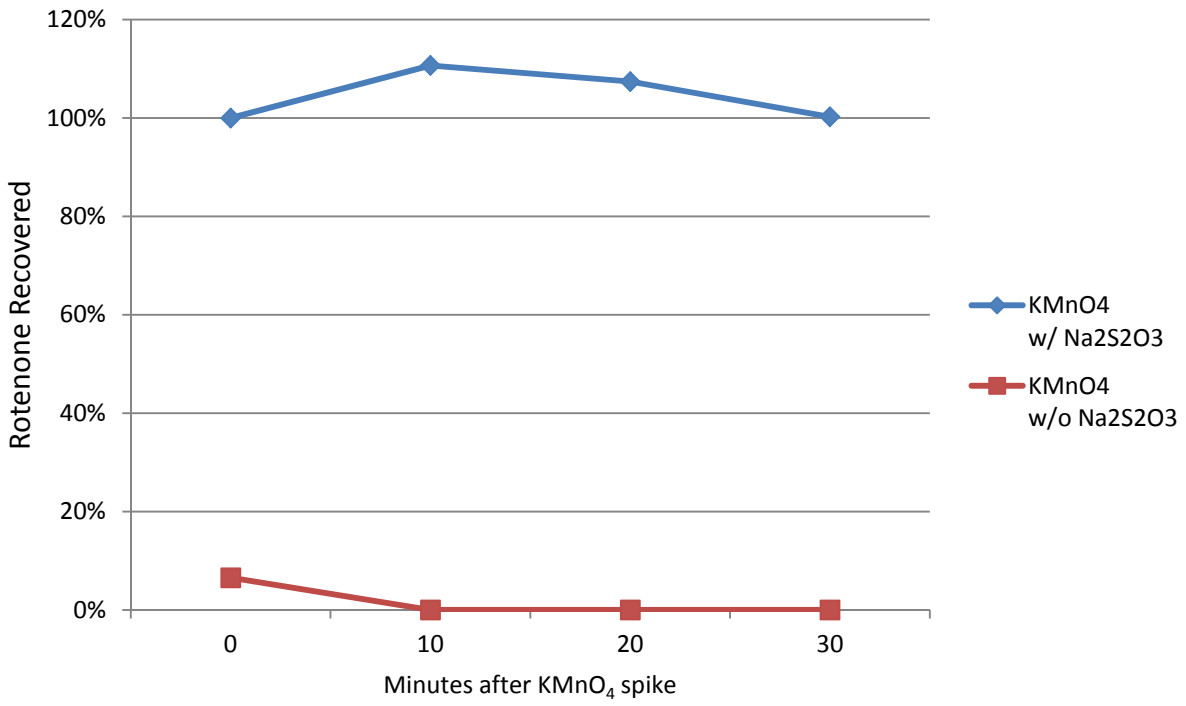


Figure 2.18- Importance of $Na_2S_2O_3$ preservative. Rotenone recovery was substantially greater for samples containing sodium thiosulfate ($Na_2S_2O_3$) at 0, 10, 20 and 30 minutes after the addition of the oxidizer $KMnO_4$.