Water Pollution Studies

Federal Aid Project F-243-R25

Pete Cadmus
Aquatic Research Scientist

Federal Aid in Fish and Wildlife Restoration

Job Progress Report

Colorado Parks & Wildlife

Aquatic Research Section

Fort Collins, Colorado

November 2018

STATE OF COLORADO

John W. Hickenlooper, Governor

COLORADO DEPARTMENT OF NATURAL RESOURCES

Bob Randall, Executive Director

COLORADO PARKS & WILDLIFE

Bob Broscheid, Director

WILDLIFE COMMISSION

John V. Howard, Chairman
Michelle Zimmerman, Vice-Chair
James Vigil, Secretary
Robert William Bray
Marie Haskett
VACANT
Carrie Besnette Hauser
Marvin McDaniel
Dale E. Pizel
Robert "Dean" Wingfield
Alexander Zipp

Ex Officio/Non-Voting Members: Don Brown, Bob Randall and Bob Broscheid

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
Dan Kowalski, Aquatic Research Scientist, Stream & River Ecology
Adam G. Hansen, Aquatic Research Scientist, Coldwater Lakes and Reservoirs
Kevin B. Rogers, Aquatic Research Scientist, Colorado Cutthroat Studies
Kevin G. Thompson, Aquatic Research Scientist, 3-Species and Boreal Toad Studies
Andrew J. Treble, Aquatic Research Scientist, Aquatic Data Management and Analysis
Brad Neuschwanger, Hatchery Manager, Fish Research Hatchery
Tracy Davis, Hatchery Technician, Fish Research Hatchery
David Karr, 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: 11/12/18

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

Table of Contents

State: Colorado **Project No. F-243-R25**

Project Title: Water Pollution Studies

Period Covered: July 1, 2016 – June 30, 2017

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.

Bravo, C. F., L. R. Curtis, M. S. Myers, J. P. Meador, L. L. Johnson, J. Buzitis, T. K. Collier, J. D. Morrow, C. A. Laetz, F. J. Loge and M. R. Arkoosh. 2011. Biomarker responses and disease susceptibility in juvenile rainbow trout Oncorhynchus mykiss fed a high molecular weight PAH mixture. Environmental Toxicology and Chemistry 30:704-714.

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

Clements, W. H., J. T. Oris, and T. E. Wissing. 1994. Accumulation and food-chain transfer of floranthene and benzo[a]pyrene in Chironomus riparius and Lepomis macrochirus. Archives of Environmental Contamination and Toxicology 26:261-266.

Colorado Department of Public Health and Environment - Water Quality Control Commission. 2013 Regulation No. 31. The basic standards and methodologies for surface water (5 CCR 1002-31)

Damasio, J. B., C. Barata, A. Munne, A. Ginebreda, H. Guasch, S. Sabater, J. Caixach, and C. Porte. 2007. Comparing the response of biochemical indicators (biomarkers) and biological indices to diagnose the ecological impact of an oil spillage in a Mediterranean river (NE Catalunya, Spain). Chemosphere 66:1206-1216.

Daughton, C. G. 2004. Non-regulated water contaminants: emerging research. Environmental Impact Assessment Review 24:711-732.

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)

Giesy, J. P., S. M. Bartell, P. F. Landrum, G. J. Leversee, and J. W. Bowling. 1983. Fates and biological effects of polycyclic aromatic hydrocarbons in aquatic systems. United States Environmental Protection Agency, Athens, Georgia, USA. EPA 600-S3-83-053.

Lamoureux, E. M., and B. J. Brownawell. 1999. Chemical and biological availability of sediment-sorbed hydrophobic organic contaminants. Environmental Toxicology and Chemistry 18:1733-1741.

Lytle, D. A., and B. L. Peckarsky. 2001. Spatial and temporal impacts of a diesel fuel spill on stream invertebrates. Freshwater Biology 46:693-704.

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

Neff, J. M. 1979. Polycyclic aromatic hydrocarbons in the aquatic environment: sources, fates, and biological effects. Applied Science Publishers Ltd, London, UK.

Schuler, L. J., M. Wheeler, A. J. Bailer, and M. J. Lydy. 2003. Toxicokinetics of sediment-sorbed benzo[a]pyrene and hexachlorobiphenyl using the freshwater invertebrates Hyalella azteca, Chironomus tentans, and Lumbriculus variegates. Environmental Toxicology and Chemistry 22:439-449.

Stephen, C. E., D. I. Mount, D. J. Hansen, J. R. Gentile, G. A. Chapman, and W. A. Brungs. 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.

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 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- Create new aquatic life criteria (also known as 'standards' or 'standards for aquatic life') for toxicants pertinent to Colorado by conducting toxicity tests that meet the minimal requirements for derivation. Then 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 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:

Use of Zebrafish (*Danio rerio*) to triage risk of antilipidemic pharmaceuticals in Colorado surface waters.

Personnel: Andrea Kingcade, Abbie Lee Jefferson and Pete Cadmus(P.I.)

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 to 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 rate-limiting enzyme of cholesterol synthesis. As the cholesterol synthesis pathway is highly conserved in metazoan taxa (Santos 2016) and fish rely primarily on triglycerides as their primary energy storage (Bennett 2007), we might expect synergistic effects in fish. Most fish have 2- to 6-fold 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 this reason, they may be additionally susceptible to antilipidemics.

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, reduced survival and reduced 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).

In a preliminary laboratory study, growth and length were evaluated at the end of a 28-day exposure in 60-day old fathead minnows exposed to 0.5 or 2.2 mg/L gemfibrozil. We observed no significant effects on growth when we exposed larval fathead minnows for 28 days to gemfibrozil. The lack of response might be an artifact of the short exposure duration but could

also be related to the age class of the organisms. Most development of organs and systems is complete at this age class. Early stages of development are more likely to be sensitive to pharmaceuticals. Proper embryonic development is essential for species survival. In example, gastrulation may be sensitive to xenobiotic exposures whereas older age classes may be able to compensate against toxic stress.

Muscle-related genes and structures are highly conserved within mammals (Smith 2013) and may be altered with antilipidemic exposures. Sirvent et al. (2005) reported myopathy and hepatotoxicity in simvastatin-treated humans as a result of a calcium signaling disruption. Rhabdomyolysis was also detected in humans prescribed cerivastatin in combination with gemfibrozil (SoRelle 2001). In vitro studies found any one statin (atorvastatin, cerivastatin, fluvastatin, pravastatin, or simvastatin) synergistically triggered rhabdomyolysis when present with one fibrate (bezafibrate; Matzno 2003). Myotoxicity was also observed in vitro in human skeletal cells with 72-hour exposures to atorvastatin, fluvastatin, pravastatin, or simvastatin. Simvastatin had the highest potential to cause myotoxicity followed by fluvastatin, pravastatin, then atorvastatin (Skottheim 2008).

Zebrafish vasculogenesis and angiogenesis effects were observed in wildtype Zebrafish exposed to simvastatin or atorvastatin. Exposed for 48 hours beginning at 6 hours post fertilization (hpf), intracranial hemorrhages were identified in Zebrafish exposed to simvastatin (2 – 42 µg/L) or atorvastatin (28 – 559 µg/L) in a dose-dependent manner with 100% hemorrhaged at the highest tested concentrations (Gjini 2011). Additionally, time-lapse imaging in 1-day-old Zebrafish exposed to atorvastatin showed 90% vessel rupture during vasculogenesis of the central arteries from the hindbrain and 10% of vessels ruptured in the forebrain or midbrain. Atorvastatin (0.5 mg/L) was found to induce misshapen and dilated cerebral vessels of the forebrain and midbrain at 1-2 hpf and cerebral hemorrhaging in 43% of organisms at 33 hpf (Eisa-Beygi 2013). The same study found cerebral hemorrhaging in a majority of organisms (33 hpf) after exposure to cerivastatin.

Even without pathological injury, embryo growth (size), development and behavior has been found to be affected by antilipidemics. Insufficient yolk absorption resulted in under sized Zebrafish larvae when they were exposed to 5 mg/L gemfibrozil or clofibrate (0.75 mg/L; Raldua 2008). This study suggests that mechanical strain placed on the embryonic axis likely resulted in delayed swim bladder inflation and digestive system formation, slowed locomotion, and delayed feeding of exogenous food. Vasculogenesis and organization and striation of muscle fibers were also disrupted with clofibrate exposure. Pericardial edema, and abnormal contractility and morphology, including atrium position and chamber length, were recorded at 3-4 dpf (days post fertilization) after exposure to 0.75 mg/L clofibrate (Raldua 2008).

To triage the risk of antilipidemics to Colorado sport fish we assessed seven statin drugs and two fibrate drugs currently prescribed to human populations (Table 1.1.1-1). To address concerns of synergisms we examined the effects of a mixture of all nine drugs.

A common fish species in developmental biology is Zebrafish (*Danio rerio*) because they are transparent in the earliest stages, have large clutches, and are easy to culture. Zebrafish embryos develop faster than sport fish allowing for a more rapid triage of toxicants. Zebrafish were judged an appropriate model for this research because pathways affected by antilipidemics are well conserved across all fish taxa. During exposure the following endpoints were measured; survival, successful completion of gastrulation, maximum swim speed (velocity), apoptosis, anterior-posterior axis angle, yolk area, arrangement of muscle fibers, presence of edemas (yolk or pericardial) and hemorrhaging, and angiogenesis of subintestinal and intersegmental vessels. Some of these assessments are observed in live unpreserved samples using specialized microscopy and techniques. To improve the usefulness of Zebrafish embryo studies to experiments using salmonids and other sport fish we coupled many assessments with traditional pathology techniques using preserved samples.

METHODS

Stock solutions of toxicants

Stock solutions of antilipidemics were prepared once within 24 hours of each test initiation and used to prepare exposure solutions. All stock solutions were stored at 4°C in the dark. When needed, solutions were warmed to 28°C and shaken. Dimethylsulfoxide (DMSO) was used as a solvent to ensure pharmaceuticals fully dissolved. All experiments contained a solvent control and true control for this reason.

Exposure solutions (make-up water)

Exposure solutions were mixed daily with buffered culture water and the appropriate amount of dimethylsulfoxide (DMSO)-dissolved drug. Buffered culture water maintained sufficient osmotic balance for developing Zebrafish embryos. The culture water (E3) was prepared from a mixture of five salts: NaCl, KCl, $CaCl_2$, $NaHCO_3$, and $MgSO_4$ and adjusted to a pH of approximately 7 (Laboratory protocol, unpublished). Solutions were renewed daily to ensure water chemistry values were not confounding, and to prevent protists from feeding on debris (dead embryos, empty chorions, etc). All petri dishes were pre-rinsed three times with the appropriate exposure solution prior to filling with the aqueous exposure solution in which the embryos were placed.

Organisms

Adult transgenic Zebrafish were raised and maintained in cultures in the Garrity Laboratory located at Colorado State University (Fort Collins, CO, USA). These transgenic fish had two transgenes inserted into the genome: green fluorescent protein (GFP) and red fluorescent protein (RFP). Heterozygous parents were mated and embryos were collected. Only a proportion of embryos expressed the transgenes (heterozygous). The GFP transgene was expressed in endothelial cells (utilizing the fli promoter specifically expressed in this cell type) and the RFP

transgene was expressed in red blood cells (utilizing the gata promoter specifically expressed in this cell type).

Zebrafish embryos up to four hours post fertilization (hpf) were used in drug exposures. After collection from breeding tanks, embryos were maintained in 10 milliliters of exposure solution in glass petri dishes at 28°C. Each replicate per concentration contained 20 embryos per petri dish. Dimethylsulfoxide (DMSO) was included as a solvent control because each (or all nine) drug(s) was dissolved in this solvent. The concentration of DMSO did not exceed 0.025% in any exposure solution.

Embryos were not fed for the duration of each study (72 hours) and exposure solutions (Table 1.1.1-1) were renewed after each endpoint was evaluated (at 24 and 48 hpf). Endpoints were generally observed within a four-hour window of the time listed below and survival was recorded daily (Table 1.1.1-2). Embryos were euthanized at the completion of each study.

Endpoints

Numerous endpoints were evaluated in each experiment within each study:

- Mortality (Table 1.1.1-2)
- Completion of gastrulation and gastrulation defects (Table 1.1.1-3)
- Early dechorionation (Table 1.1.1-4)
- Birefringence defects (Table 1.1.1-5)
- Presence of hemorrhages (Table 1.1.1-6)
- Presence of pericardial edemas (Table 1.1.1-7)
- Presence of yolk edemas (Table 1.1.1-8)
- Abnormal anterior-posterior axis (Table 1.1.1-9)
- Abnormal subintestinal vessels (SIV) development (Table 1.1.1-10)
- Abnormal intersegmental vessel (ISV) development (Table 1.1.1-11)
- Bolt speed and reaction time (results pending)
- Yolk area of embryos (results pending)

Completion of gastrulation (Table 1.1.1-3) was determined at 24 hpf by visualization under a dissecting microscope. The proportion of embryos that reached 90% epiboly (the morphogenetic movement in fish), that failed to complete gastrulation, or that did not survive gastrulation was recorded. Each embryo was observed by being transferred to a petri dish with a small volume of exposure solution. Each embryo was then transferred to a second petri dish with the appropriate exposure solution. Heterozygosity resulted in a fraction of each clutch of embryos not expressing the transgenes. As a result, after gastrulation was assessed, the expression of green fluorescent protein (GFP) was also assessed. The non-transgenic embryos were placed into an additional glass petri dish with the appropriate volume and drug concentration.

Muscle defects (adapted from Smith 2013) were determined with a "touch assay" that measured reaction to a stimulus and birefringence. At approximately 48 hpf, the touch assay was performed after all embryos were dechorionated and renewal of the exposure solution occurred. Dechorionating involved gentle removal of the chorion using two dissecting probes under a dissecting microscope. Generally, at least four embryos from each non-transgenic replicate participated in the touch assay. Each embryo was placed into a petri dish with the appropriate exposure solution and given up to a 60-second acclimation period. Each embryo was then encouraged to move with a gentle touch of a dissecting probe and the maximum velocity (cm/s) was recorded. In some studies, four animals per non-transgenic replicate were not available. In this case transgenic-expressing embryos from the same replicate were randomly selected to afford a minimum of four touch assay assessments. Birefringence (Table 1.1.1-5) was blindly (exposure level is hidden from scientist) evaluated at 72 hpf in transgenic embryos. Birefringence is a property where light passes through polarized light filters and will rotate as it then passes through muscle sarcomeres. Patterns were evaluated per Smith (2013). The proportion of those expressing abnormal phenotypes was recorded.

Cardiovascular defects (hemorrhage, angiogenesis, and pericardial edema) were blindly evaluated at approximately 72 hpf in transgenic embryos. The presence or absence of hemorrhages and pericardial edemas were observed and representative images were captured. Angiogenesis was scored based on normal or abnormal phenotypes of two categories of vessels: intersegmental vessels (ISVs) and sub-intestinal vessels (SIVs, Serbedzija 1999). Representative images were captured with QImaging Ocular software.

Yolk defects were evaluated at 72 hpf in transgenic embryos. The proportion of embryos with yolk edemas were recorded and a random number generator was used to capture three embryos per replicate for photo analysis. The area of the yolk including the yolk extension was measured with ImageJ software and analyzed. Deviation of the angle of the anterior-posterior axis (180 degrees) in all transgenic fish was also assessed at this time.

Florescence of acridine orange (AO) stain was employed to visualize presence of dead cells in non-transgenic embryos at ages up to 48 hpf. One microliter of AO stock (5 mg/ml) was added to every milliliter of exposure solution. After 30 min embryos were transferred to culture water free of dye. Organisms were assessed under a dissecting scope with the green fluorescent protein filter. ImageJ count nuclear foci software (Duke University) was used to evaluate the acridine orange staining. Increased amounts of dead cells can manifest into poor embryonic development.

Water Quality

Daily measurement of pH occurred in all studies. Temperature and dissolved oxygen were recorded. Due to the limited volume of Petri dishes (exposure tanks) these were measured in new and old test solutions (prior to test renewal). Water pH and dissolved oxygen were measured

with calibrated probes and meters. Temperatures in each exposure level were recorded daily using a NIST-traceable digital thermometer.

To verify toxicant concentrations in the exposure solutions a method was developed using a Waters Xevo UPLC-MS/MS (triple quadrupole). A gradient mobile phase with acetonitrile and water with 2% ammonium acetate was used to separate all nine tested drugs. Standards or samples were either analyzed within 24 hours of preparation or were collected and stored in the dark at 4°C. Method development at Colorado State University's Central Instrument Facility (Fort Collins, CO) is ongoing.

Analysis

Data from each endpoint were transformed (arcsine square root) or normalized to a proportion of the control. Normality (Shapiro-Wilks test), heteroscedasticity (Bartlett's test, Levene's), and analyses of variance and/or multiple comparison studies (Dunnett's, Tukeys, Kruskal-Wallis, RegWQ, Fisher's) was conducted in R.

PRELIMINARY RESULTS and CONCLUSIONS

Method Development Studies

Method development to ensure adequacy of testing systems is ongoing. We examined the effects of DMSO at high concentrations and found the maximum concentration of our solvent control (0.025% DMSO) was not eliciting a toxic response. Standardized methodologies for Zebrafish regularly use of plastic containers. We compared toxic effects of several antilipidemics in glass and plastic Petri dishes. Plastic glassware has a significant protective effect. This is likely because organic pharmaceuticals like antilipidemics are hydrophobic and quickly bind to the surface of plastic glassware more than glass. For this reason glass was the preferred vessel for all components of this experiment. Standard methods for Zebrafish culture contain fish in a highly concentrated solution of NaCl, KCl, $CaCl_2$, $NaHCO_3$, and $MgSO_4$ (E3). We found these salts provide a strong protective effect against antilipidemics compared to dechlorinated municipal water (hardness ~35 mg/L). These inert salts likely compete with the toxicant for tissue binding sites or these salts bind to the toxicant preventing absorption into the organisms. Colorado surface waters are oligotrophic and often have low hardness and alkalinity values. Toxicity trials conducted in E3 may overestimate a safe threshold for Colorado sport fish. For this reason toxicant concentrations here within were prescribed at levels higher levels than observed in nature. Formalized results from these studies are pending and will be published in subsequent reports.

Mixture Studies

Two mixture studies (all nine drugs mixed together each with a nominal concentration of 100 µg/L) were performed. Complete mortality was observed by 48 hpf when declorinated municipal water was used as in place of E3. Complete mortality was also observed by 48 hpf when E3 was used. A repeat mixture study with all 9 drugs will be repeated. Formalized results from these studies are pending and will be published in subsequent reports.

Individual Studies

Preliminary results from experiments that evaluated each of the nine individual drugs are shown in Tables 1.1.1-2 to 1.1.1-7. Two endpoints (maximum velocity and yolk area) are not shown and results are pending. The proportion of embryos that exited their chorions before technicians manually removed them at 48 hpf (early dechorionation) is included in Table 1.1.1-4. Finally, Table 1.1.1-10 displays subcategories of subintestinal values were not measured for gemfibrozil. The inclusion of subcategories began after gemfibrozil was tested (first of the drugs studied). This individual drug study will be repeated to include these measurements.

Statistical interpretation is still pending on all studies and will be reported in subsequent reports. Complete mortality was observed at 48 hpf for only lovastatin and simvastatin. These are the only drugs prescribed to humans in their lactone forms. These two lactone drugs also experience the highest proportion of embryos with gastrulation defects at 24 hpf at the highest exposure levels. We also observed an increase in AP axis deformities, premature dechorionated, birefringence, hemorrhages, pericardial and yolk edemas, abnormal SIV development in embryos exposed to fluvastatin and pitavastatin.

Other trends noted that occurred in the highest exposure concentration are the following:

- Abnormal ISV development with fluvastatin
- Abnormal SIV development with fenofibrate and gemfibrozil (total SIV)
- Abnormal SIV development (missing) with fenofibrate
- Abnormal AP axis formation with gemfibrozil
- Presence of hemorrhages with atorvastatin
- Presence of pericardial and yolk edemas with fenofibrate and gemfibrozil

These trends observed in the highest concentration are important to note because the highest concentration compares the potential impact of each drug based on the number of molecules instead of the concentration based on weight. Therefore, each exposure at the highest concentration had the same number of molecules in solution (nominally). Our results show that individual drugs exhibit different potencies with potential for synergistic and antagonistic interactions. All of these interactions warrant further investigation and consideration.

Concentrations in this study were higher than observed in nature. When triaging the risk of pharmaceuticals it would be inappropriate to base risk on an acute exposure in a laboratory setting at the low levels found in nature. Doing so would risk a Type II error in which no effect

of a toxicant was observed when indeed there was an effect. We identified many concerns with testing infrastructure and model organisms that encouraged us to use a high concentration of toxicant, not limited to the following:

- Static renewal studies often loose toxicants relative to nominal concentrations as toxicants bind to vessels and become denatured.
- Dietary exposure was not included in this study and is the likely source of most antilipidemics.
- Duration of this study is limited to 3 days where as in nature organisms are exposed for an entire life cycle.
- Eggs were naive to the toxicant whereas parental body burden may contribute to tissue concentrations in nature.
- E3 solution may reduce toxicity or bioavailability of pharmaceuticals.
- Synergistic interactions with other toxicants in nature may be present as combinations of drugs are understudied in fish species.
- Zebrafish may be more tolerant to toxicants than sport fish of concern to Colorado.
- If a toxicant has sublethal ailments undetectable by our methods the stress and demands of nature may limit fish survival and recruitment and viability of a local population. These functions are not limited to reproduction, feeding, predator avoidance, immigration (colonization), emigration (drift and migration).

Use of Zebrafish embryos and the methods developed here proved valuable in triaging effects of pharmaceuticals in short term tests. Expanding these studies to include fish native to Colorado holds potential to refine water quality standards. Even if methodologies are limited to Zebrafish, the ability to rapidly characterize potential risk to fish helps focus future research. Risks identified here within should be further studies using environmentally relevant exposure pathways, concentrations, durations and Colorado species.

Table 1.1.1-1. Concentrations of pharmaceuticals				
Class:	**Drug:**	**ERC µg/L:**	**Nominal Testing Concentrations:**	
			Low µM:	**High µM:**
Fibrate	Fenofibrate	2.8E-4[1] (0.1 µg/L)	2.8E-3 (100 µg/L)	1 (361 µg/L)
	Gemfibrozil	3.2E-3[2] (0.8 µg/L)	3.2E-2 (800 µg/L)	1 (250 µg/L)
Statin	Atorvastatin	1.4E-4[3] (0.8 µg/L)	1.4E-3 (80 µg/L)	1 (559 µg/L)
	Fluvastatin	2.4E-5[4] (0.01 µg/L)	2.4E-4 (10 µg/L)	1 (411 µg/L)
	Lovastatin	2.5E-5[1] (0.01 µg/L)	2.5E-4 (10 µg/L)	1 (404 µg/L)
	Pitavastatin	Not measured	2.4E-4 (10 µg/L)	1 (421 µg/L)
	Pravastatin	1.4E-4[1] (0.06 µg/L)	1.4E-3 (60 µg/L)	1 (425 µg/L)
	Rosuvastatin	6.2E-4[3] (0.3 µg/L)	6.2E-3 (300 µg/L)	1 (482 µg/L)
	Simvastatin	2.4E-6[1] (0.001 µg/L)	2.4E-5 (1 µg/L)	1 (419 µg/L)

Environmentally relevant concentrations (ERCs) from effluent are listed and were the foundation for determination of the ERC level. Ten times the ERC was tested for the low concentration. The high concentration was standardized for all drug concentrations based on the number of molecules. Pitavastatin values were selected based on the similar molecular weight to fluvastatin and lovastatin.

1: Hernando 2007
2: Deo 2014
3. Lee 2009
4. Gros 2012

Table 1.1.1-2. Percent (%) cumulative mortality (# dead/total alive) in individual drug studies (Preliminary Results)

STUDY	DRUG	24 HPF	48 HPF	72 HPF
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	11 (9/80) 6 (5/79) 14 (11/80)	13 (10/76) 6 (5/79) 14 (11/79)	14 (11/76) 13 (10/79) 27 (21/79)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	13 (10/80) 11 (9/80) 6 (5/80)	14 (11/80) 11 (9/80) 33 (26/80)	15 (11/73) 11 (9/80) 35 (28/80)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	4 (3/80) 10 (8/80) 5 (4/80)	4 (3/79) 11 (9/80) 5 (4/80)	4 (3/79) 11 (9/80) 8 (6/80)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	1 (1/80) 1 (1/78) 0 (0/79)	1 (1/79) 1 (1/77) 0 (0/78)	1 (1/79) 1 (1/77) 0 (0/78)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	3 (2/80) 4 (3/79) 4 (3/80)	5 (4/80) 4 (3/79) 100 (80/80)	6 (5/80) 4 (3/79) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	1 (1/80) 1 (1/80) 5 (4/80)	1 (1/80) 1 (1/80) 5 (4/80)	1 (1/80) 1 (1/80) 6 (5/80)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	1 (1/80) 3 (2/80) 1 (1/80)	1 (1/79) 3 (2/80) 1 (1/80)	1 (1/79) 3 (2/80) 1 (1/80)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	0 (0/80) 0 (0/79) 3 (2/80)	0 (0/80) 0 (0/78) 3 (2/79)	0 (0/80) 0 (0/78) 3 (2/79)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	13 (10/80) 7 (6/81) 9 (7/80)	15 (12/80) 8 (6/77) 100 (80/80)	18 (14/79) 9 (7/77) ----

Cumulative mortality (%) from four replicates (each beginning with 20 embryos in each dish). Mortality was added to the mortality of the previous day and divided by the number of embryos alive in each study. Mortality not recorded or caused by technician error were removed from each calculation. The number of animals dead divided by the number of animals alive that day is shown in parentheses. Concentrations are nominal.

Table 1.1.1-3. Gastrulation defects (%) in individual drug studies (Preliminary Results)

STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	13 (10) 6 (3) 14 (5)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	13 (3) 11 (3) 31 (47)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	4 (5) 11 (13) 6 (9)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	1 (3) 1 (3) 0 (0)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	3 (3) 4 (3) 100 (0)
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	1 (3) 1 (3) 5 (4)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	1 (3) 3 (3) 1 (3)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	0 (0) 0 (0) 3 (5)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	15 (9) 7 (6) 100 (0)

Percent of all embryos (NT and T) that exhibited gastrulation defects. One standard deviation is shown in parentheses. Concentrations are nominal.

Table 1.1.1-4. Early dechorionation (%) in individual drug studies (Preliminary Results)

STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	8 (6) 15 (8) 12 (13)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	26 (13) 25 (10) 39 (14)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	0 (0) 6 (7) 0 (0)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	11 (19) 3 (3) 32 (28)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	17 (10) 25 (14) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	1 (3) 5 (4) 40 (24)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	4 (5) 4 (5) 0 (0)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	3 (5) 8 (7) 8 (3)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	12 (9) 7 (7) ----

Percent of all embryos (NT and T) that dechorionated on their own prior to manual dechorionation. One standard deviation is shown in parentheses. Concentrations are nominal.

Table 1.1.1-5. Birefringence defects (%) in individual drug studies (Preliminary Results)

STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	0 (0) 0 (0) 11 (3)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	10 (7) 9 (3) 19 (4)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	9 (7) 7 (9) 11 (6)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	3 (4) 3 (4) 29 (9)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	0 (0) 2 (3) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	0 (0) 0 (0) 15 (7)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	0 (0) 3 (4) 1 (3)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	2 (4) 3 (4) 4 (5)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	6 (4) 4 (4) ----

Percent of embryos (T only) that displayed abnormal muscle fiber arrangement (birefringence). One standard deviation is shown in parentheses. Concentrations are nominal.

Table 1.1.1-6. Presence of Hemorrhages (%) in individual drug studies (Preliminary Results)

STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	2 (4) 0 (0) 8 (11)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	9 (11) 5 (3) 13 (4)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	2 (3) 9 (13) 34 (20)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	11 (11) 10 (8) 67 (17)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	4 (7) 8 (10) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	11 (8) 14 (11) 72 (8)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	3 (4) 6 (5) 0 (0)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	4 (7) 5 (6) 9 (10)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	7 (9) 4 (7) ----

Percent of embryos (T only) that had hemorrhages present. One standard deviation is shown in parentheses. Concentrations are nominal.

Table 1.1.1-7. Presence of pericardial edemas (%) in individual drug studies (Preliminary Results)		
STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	0 (0) 2 (4) 39 (14)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	6 (9) 8 (12) 21 (11)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	3 (7) 2 (5) 0 (0)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	0 (0) 0 (0) 47 (9)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	2 (4) 5 (6) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	0 (0) 0 (0) 22 (4)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	0 (0) 0 (0) 3 (4)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	2 (3) 2 (4) 2 (4)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	6 (9) 5 (7) ----
Percent of embryos (T only) that had pericardial edema present. One standard deviation is shown in parentheses. Concentrations are nominal.		

Table 1.1.1-8. Presence of yolk ydemas (%) in individual drug studies (Preliminary Results)		
STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	0 (0) 2 (4) 26 (14)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	2 (3) 5 (6) 17 (5)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	2 (3) 4 (5) 0 (0)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	0 (0) 0 (0) 19 (15)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	0 (0) 0 (0) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	0 (0) 0 (0) 14 (1)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	0 (0) 2 (3) 2 (3)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	0 (0) 0 (0) 2 (4)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	0 (0) 2 (4) ----
Percent of embryos (T only) that had yolk edema present. One standard deviation is shown in parentheses. Concentrations are nominal.		

Table 1.1.1-9. Abnormal anterior-posterior axis (%) in individual drug studies (Preliminary Results)		
STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	0 (0) 0 (0) 2 (4)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	0 (0) 0 (0) 13 (4)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	15 (6) 4 (5) 6 (4)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	2 (3) 2 (3) 66 (13)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	2 (3) 0 (0) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	0 (0) 0 (0) 16 (14)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	0 (0) 2 (3) 1 (3)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	2 (3) 2 (4) 0 (0)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	4 (5) 0 (0) ----
Percent of embryos (T only) who's anterior to posterior axis was not 180 degrees. One standard deviation is shown in parentheses. Concentrations are nominal.		

Table 1.1.1-10. Abnormal SIV development (%) in individual drug studies (Preliminary Results)					
STUDY	DRUG	UNDERDEV	OVERDEV	MISSING	TOTAL
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	13 (6) 15 (5) 16 (5)	2 (4) 2 (4) 3 (5)	2 (4) 2 (4) 18 (3)	16 (6) 19 (7) 36 (7)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	NM	NM	NM	32 (5) 28 (12) 46 (13)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	22 (16) 20 (3) 17 (12)	4 (5) 4 (8) 0 (0)	0 (0) 4 (5) 0 (0)	26 (20) 28 (10) 17 (12)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	3 (4) 5 (3) 13 (5)	2 (3) 0 (0) 2 (3)	2 (4) 0 (0) 16 (9)	6 (0.4) 5 (3) 31 (10)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	19 (7) 17 (9) ----	0 (0) 2 (3) ----	0 (0) 0 (0) ----	19 (7) 18 (11) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	8 (6) 9 (8) 13 (5)	2 (3) 0 (0) 0 (0)	0 (0) 0 (0) 13 (5)	10 (9) 9 (8) 27 (8)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	10 (6) 13 (5) 11 (11)	0 (0) 3 (4) 0 (0)	0 (0) 0 (0) 1 (3)	10 (6) 16 (9) 13 (9)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	11 (11) 6 (9) 14 (10)	0 (0) 0 (0) 0 (0)	0 (0) 2 (4) 0 (0)	11 (11) 8 (8) 14 (10)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	11 (12) 7 (6) ----	0 (0) 0 (0) ----	4 (5) 5 (4) ----	15 (16) 13 (10) ----
Percent of embryos (T only) that exhibited abnormal subintestinal vessel (SIV) development. The abnormalities were divided into three categories (underdeveloped (UNDERDEV), overdeveloped (OVERDEV), or missing SIVs). The sum of all three categories were also analyzed (TOTAL). One standard deviation is shown in parentheses. Concentrations are nominal.					

Table 1.1.1-11. Abnormal intersegmental vessel (ISV) development (%) in individual drug studies (Preliminary Results)

STUDY	DRUG	%
1	DMSO (0.01%) ERCx10 (Feno) Feno (1uM)	0 (0) 0 (0) 0 (0)
2	DMSO (0.01%) ERCx10 (Gem) Gem (1uM)	5 (6) 14 (11) 19 (8)
3	DMSO (0.01%) ERCx10 (Ato) Ato (1uM)	0 (0) 2 (5) 0 (0)
4	DMSO (0.01%) ERCx10 (Fluv) Fluv (1uM)	3 (4) 0 (0) 11 (6)
5	DMSO (0.01%) ERCx10 (Lov) Lov (1uM)	0 (0) 0 (0) ----
6	DMSO (0.01%) ERCx10 (Pit) Pit (1uM)	0 (0) 0 (0) 5 (6)
7	DMSO (0.01%) ERCx10 (Ros) Prav (1uM)	0 (0) 0 (0) 0 (0)
8	DMSO (0.01%) ERCx10 (Ros) Ros (1uM)	0 (0) 2 (4) 0 (0)
9	DMSO (0.01%) ERCx10 (Ros) Sim (1uM)	2 (4) 0 (0) ----

Percent of embryos (T only) that displayed abnormal intersegmental vessel (ISV) development. One standard deviation is shown in parentheses. Concentrations are nominal.

Table 1.1.1-12 - list of common abbreviations for antilipidemic studies.

AP - anterior-posterior axis
Ato - Atorvastatin
DMSO - dimethyl sulfoxide
ERC - environmentally relevant concentration
Feno - Fenofibrate
Fluv - Fluvastatin
Gem - Gemfibrozil
GFP - green fluorescent protein
Hpf - hours post fertilization
ISV - intersegmental vessels
Lov - Lovastatin
NM - not measured
NT - non transgenic
Pit - Pitavastatin
Prav - Pravastatin
Ros - Rosuvastatin
Sim - Simvastatin
SIV - subintestinal vessels
T - transgenic (green fluorescent protein (GFP) expressed)

Al-Habsi, A., A. Massarsky, and T. W. Moon. 2016. Exposure to gemfibrozil and atorvastatin affects cholesterol metabolism and steroid production in Zebrafish (*Danio rerio*). Comparative Biochemistry and Physiology- Part B 199:87-96.

Babin, P. J., and J. M. Vernier. 1989. Plasma lipoproteins in fish. The Journal of Lipid Research 30:467–490.

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

Deo, R. 2014. Pharmaceuticals in the Surface Water of the USA: A Review. Current Environmental Health Reports 1:113-122.

Eisa-Beygi, S., G. Hatch, S. Noble, M. Ekker, and T. W. Moon. 2013. The 3-hydroxy-3methylglutaryl-CoA reductase (HMGCR) pathway regulates developmental cerebral–vascular stability via prenylation-dependent signalling pathway. Developmental Biology 373:258–266.

Gjini, E., L. H. Hekking, A. Kuchler, P. Saharinen, E. Wienholds, J. A. Post, K. Alitalo, and S. Schulte-Merker. 2011. Zebrafish Tie-2 shares a redundant role with Tie-1 in heart development and regulates vessel integrity. Disease Models Mechanisms 4:57–66.

Gros, M., S. Rodriguez-Mozaz, and D. Barcelo. 2012. Fast and comprehensive multi-residue analysis of a broad range of human and veterinary pharmaceuticals and some of their metabolites in surface and treated waters by ultra-high-performance liquid chromatography coupled to quadrupole-linear ion trap tandem mass spectrometry. Journal of Chromatography A 1248: 104-121.

Gu, Q., R. Paulose-Ram, V. L. Burt, and B. K. Kit. 2014. Prescription cholesterol-lowering medication use in adults aged 40 and over: United States, 2003-2012. NCHS data brief, no 177. Hyattsville, MD.

Hernando, M. D., A. Aguera, and A. R. Fernandez-Alba. 2007. LC-MS analysis and environmental risk of lipid regulators. Analytical and Bioanalytical Chemistry 387:1269–1285.

Larsson, A., and R. Fange. 1977. Cholesterol and free fatty acids (FFA) in the blood of marine fish. Comparative Biochemisty and Physiology Part B 57:191–196.

Lee, H. B., T. Peart, M. L. Svoboda, and S. Backus. 2009. Occurrence and fate of rosuvastatin, rosuvastatin lactone, and atorvastatin in Canadian sewage and surface water samples. Chemosphere. 77:1285–1291.

Matzno, S., K. Tazuya-Murayama, H. Tanaka, S. Yasuda, M. Mishima, T. Uchida, T. Nakabayashi, and K. Matsuyama.2003. Evaluation of the synergistic adverse effects of concomitant therapy with statins and fibrates on rhabdomyolysis. Journal of Pharmacy and Pharmacology 55:795-802.

Medscape.com. Top 100 Most Prescribed, Top Selling Drugs. Published May 13, 2014. Accessed July 30, 2017. http://www.medscape.com/viewarticle/825053.

Mimeault, C., A. J. Woodhouse, X. S. Miao, C. D. Metcalfe, T. W. Moon, and V. L. Trudeau. 2005. The human lipid regulator, gemfibrozil bioconcentrates and reduces testoterone in the goldfish, *Carassius auratus*. Aquatic Toxicology 73:44–54.

Prindiville, J. S., J. A. Mennigen, J. M. Zamora, T. W. Moon, and J. M. Weber. 2011. The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout. Toxicology and Applied Pharmacology 251:201-208.

Raldua, D., M. Andre, and P. J. Babin. 2008. Clofibrate and gemfibrozil induce an embryonic malabsorption syndrome in Zebrafish. Toxicology and Applied Pharmacology228:301-314.

Santos, M. M., R. Ruivo, M. Lopes-Marques, T. Torres, C. B. de los Santos, L. F. Castro, and T. Neuparth. 2016. Statins: An undesirable class of aquatic contaminants? Aquatic Toxicology 174:1-9.

Saunders, R. L., A. P. Farrell, and D. E. Knox. 1992. Progression of coronary arterial lesions in Atlantic salmon (*Salmo salar*) as a function of growth rate. Canadian Journal of Fisheries and Aquatic Sciences 49:878–884.

Sirvent, P., J. Mercier, G. Vassort, and A. Lacampagne. 2005. Simvastatin triggers mitochondria-induced Ca2+ signaling, alteration in skeletal muscle. Biochemical and Biophysical Research Communications 329:1067–1075.

Skottheim, I. B., A. Gedde-Dahl, S. Hejazifar, K. Hoel, and A. Asberg. 2008. Statin induced myotoxicity: the lactone forms are more potent than the acid forms in human skeletal muscle cells in vitro. European Journal of Pharmaceutical Sciences 33:317–325.

Smith, L. L., A. H. Beggs, and V.A. Gupta. 2013. Analysis of skeletal muscle defects in larval Zebrafish by birefringence and touch-evoke escape response assays. Journal of Visualized Experiments. [online serial] 82, e50925. DOI: 10.3791/50925.

SoRelle, R. 2001. Baycol withdrawn from market. Circulation 104:E9015–E9016.

Action #1.1.2:

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

Expose fish to environmentally relevant levels and mixtures of hydrocarbons in laboratory 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, 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.

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 presented 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 will be presented to stakeholders and regulatory agencies as a model for an experimentally derived iron standard.

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

●Laboratory space was modified to enable isolation of organisms with disease from the main experimental area.

Action #1.2.1 Accomplishments:

Experimental evidence for size dependent sensitivity to aqueous metals and the implications to risk assessment and policy making.

Personnel: Christopher Kotalik, Abbie Lee Jefferson and Pete Cadmus (P.I.)

Aquatic macroinvertebrates are the primary food source for Colorado's trout populations. Laboratory assessments of trace metal toxicity generally find aquatic insects to be tolerant to aqueous metals However, mesocosm experiments and field biomonitoring results suggest effects at relatively low metals concentrations. Several hypotheses have been proposed to reconcile these discrepancies, yet minimal experimental research has addressed how the developmental size progression of organisms, particularly invertebrates, influences their responses to metals exposure. Naturally collected benthic communities were exposed to trace metals using mesocosm experiments, and a novel single species test system was used to expose early, mid, and late instar invertebrates. These experimental approaches tested the hypothesis that small invertebrate size classes are more sensitive compared to large, mature individuals. Importantly, mesocosm exposures utilized naturally colonized benthic communities which contained diverse size assemblage structure among and between dominant taxa groups, enabling size-response comparisons among metals treatments. Field collected early to late instar Baetis were exposed using single species testing methods. Mesocosm results suggest size-dependent responses to metals based on body mass and head capsule widths among the dominant aquatic insect orders examined, with smaller organisms generally displaying disproportionate trends in mortality compared to large, mature individuals. Size-dependent responses of *Baetis* mayflies were confirmed in all four mesocosm experiments, as well as in our single species test system. Zinc LC_{50} values for early instar Baetis were less than 5% of the previously documented LC_{50} value for late instar Baetis. Together, these results suggest that aquatic insect body size is a strong predictor of susceptibility to aqueous metals. Toxicity models which incorporate the natural size progression of organisms have the potential to improve our accuracy in predicting effects in the field. The toxicity testing methodologies which researchers use, such as the single species test system introduced in this study, as well as mesocosm testing, should be "acceptable" testing procedures to obtain these data.

Background

A majority of watersheds in the Colorado Mineral Belt have elevated metals concentrations due to historic hardrock mining. Links between metal pollution and degradation of aquatic communities in streams are well established in the literature (Clements 2004; Clements et al. 2000). Laboratory results often suggest that aquatic insects are tolerant to metal pollution (Brix et al. 2005; Brinkman and Johnston 2008; Brinkman and Johnston 2012). However, field biomonitoring studies and mesocosm exposures find that aquatic insects are rather sensitive to

metals at relatively low concentrations (Clements et al. 2000, Clements 2004; Buchwalter et al. 2007; Clements et al. 2013). Lower metals thresholds observed in these studies may be the result of improved ecological realism, such as the inclusion of dietary exposures, species interactions, trophic cascades, and differences in invertebrate size assemblage structure (Clements et al. 2013; Rogers et al. 2016). Because early-instar invertebrates are typically too small to collect, or manipulate in the field or laboratory, mesocosm studies which integrate naturally colonized communities have been the primary method for inclusion of early instar toxicity assessments (Kiffney and Clements 1996; Clements et al. 2013). Body size might partly explain the discrepancy between single species and mesocosm results. Evidence of an age class or size class influence on susceptibility to toxicants will help guide policy and standard building in the future.

Aquatic toxicity trials that compare early and late life stages of aquatic invertebrate species often find greater susceptibility in smaller size classes (Powlesland and George 1986; McCahon et al. 1989; Diamond et al. 1992; Stuhlbacher et al. 1993; Kiffney and Clements 1996). Susceptibility of smaller organisms may be due, but not limited to, a large surface area to volume ratio, lower fat to protein ratios, more rapid accumulation of toxicants in organs, less developed antioxidant systems and less developed physical structures. Buchwalter et al. (2008) used body size as a significant cofactor in seminal work documenting phylogenetic differences in metal tolerance. Potential or maximum body size is considered an important biotic trait in predicting aquatic macroinvertebrate colonization and (or) occupancy in anthropogenically disturbed habitats, and it has been used as a biotic indicator of trace metal pollution (Statzner et al. 2001; Archaimbault et al. 2005; Statzner et al. 2005; Doledec and Statzner 2008; Statzner and Beche 2010)

Aquaculture techniques for fish are well established, which improves the ability of researchers to conduct experiments with early life stages. However, culture techniques for aquatic insects are poorly developed and are limited to a handful of species (e.g. *Chironomus riparius*). Traditional single-species experiments exposing aquatic insects to toxicants generally use mature individuals because they are less fragile and more easily collected, identified and enumerated without microscopic assistance. Failure to properly characterize susceptibility of aquatic insects may result in standards that are not protective of the food sources that sustain Colorado fisheries.

Acute and chronic standards use species or genus sensitivity distributions as the basis of deriving safe water quality standards (Stephen et al. 1985) as phylogenetic differences in toxicity are well established. Yet, if species are difficult to obtain at an early age or are difficult to culture in the laboratory, it is unlikely that these taxonomic groups are well characterized. Although many standardized procedures give preference to early life stages or full life cycle trials, such experiments are rare, especially for aquatic insects. If small size classes of aquatic insects are significantly more susceptible than larger age classes, standards, predictive tools and toxicity models used to estimate risk and protect aquatic species might overestimate safe pollution levels.

To test the hypothesis that early life-stage invertebrates are relatively more sensitive to contaminants compared to mature invertebrates, a series of studies were conducted to experimentally test if small, less-developed aquatic insects were more susceptible to aqueous metals compared to large, mature aquatic insects.

First, we examined archived samples from three mesocosm studies which exposed naturally colonized benthic macroinvertebrate communities to metals in different combinations (Cu, Zn, Cd). The sensitivity of each taxon present in each experiment was compared to average wet mass of that taxon (Study 1). We expected taxa with low body mass would generally exhibit greater sensitivity to metals compared to taxa with larger body mass. Additionally, we predicted the distribution of sensitive taxa would be limited to only small body mass, while tolerant taxa would include a larger range of body mass. Next, head capsule widths of the mayfly *Baetis sp.* were compared across treatment levels to determine if smaller organisms were selected against at higher metal concentrations levels (Study 2). With improved microscopy methodologies for measurements of head capsule widths, a fourth mesocosm experiment was conducted to assess the distribution of head capsule widths of four major aquatic insect orders across a metals treatment gradient (Study 3). We expected the distribution of head capsule widths to change across treatment levels, with the relative proportion of smaller organisms decreasing with increasing concentrations of metals, but that the magnitude of these relationships would vary among different aquatic insect orders. Lastly, methods for conducting acute toxicity tests on nearly microscopic size classes of mayflies were developed. The concentration of zinc (Zn) needed to reduce the population by 50% (LC_{50} values) of early (<24 hour post hatch) and mid (approximately 1 month post hatch) instars were compared to the LC_{50} value derived from using late instar (nearly adult) mayflies of the same species (Study 4). We expected to see LC_{50} values of earlier age classes to be well below that of larger age classes.

Methods

Study I

Three mesocosm exposures were conducted exposing naturally colonized communities of aquatic macroinvertebrates to mixtures of Copper (Cu; 25 September 2007), Copper and Zinc (Cu+Zn; 24 October 2007), and Copper, Zinc and Cadmium (Cu+Zn+Cd; 22 August 2010) as described by Clements et al. (2013). Cumulative Criterion Units (CCUs) were used as a unit of metal concentration for the mixtures (Table 1.1.2-1; Clements 2004; Clements et al. 2013). CCUs are the summation of multiples of the hardness adjusted criterion value for each metal. Hardness adjusted chronic criterion values in use at the time of analysis were used over Biotic Ligand Model criterion for reasons described by Clements et al. 2013. For each taxon in each

experiment the inverse of the slope of log-log transformed survival data was used as a measure of susceptibility to the toxicant mixture. Wet mass of every organism in the controls of the three mesocosm experiments was measured. Preserved organisms (20% water, 80% ethanol) were identified and placed on dry filter paper on a Buchner funnel for 30 seconds and then weighed on an O'Hause GS200D (0.00001g resolution). Average mass of each taxon was calculated and log transformed (ln(g+1)). Weighted linear regression for each order was performed to determine if body mass was correlated to susceptibility to heavy metal toxicants. Weighted regression assigned more weight to taxa abundant in the naturally colonized substrate used in the experiments. Members of Tipulidae were removed from consideration as they gain considerable mass when preserved.

After natural log transformations of survival (ln(population+1) and concentration (ln(CCU+1)) from each mesocosm experiment the LM function in package 'car' (Fox and Weisberg 2011) in R Studio (version 3.5.0) was used to create slopes of survival over metal exposure for each taxa in the 2007 and 2009 experiments per Clements et al. (2013). These slopes served as a value of tolerance with highly negative slopes suggesting less tolerance than organisms with slopes near 0. The average wet mass of each taxon from each experiment was then compared to the slope from each experiment using weighted linear regression ('weights='). Population of each taxon in the controls of each experiment was used as the weight for linear regression. This ensured poorly represented taxa did not have a disproportionate influence on the relationship between mass and tolerance. This was conducted for the orders Trichoptera (Caddisflies), Plecoptera (Stoneflies) and Ephemeroptera (Mayflies) and a composite of all organisms. Low diversity of Diptera (true flies) and Coleoptera (beetles) taxa prevented assessment of these orders.

Study II

Head capsule widths are commonly used to measure invertebrate body size because of their high correlation to one another (Benke et al. 1999). Head capsules of the mayfly *Baetis spp*. were measured in all treatment levels of Cu, Cu+Zn and Cu+Zn+Cd experiments described in Study I. A stereo microscope (Meji EMZ-TR) with a reticle SFW20x eyepiece provided 0.1 mm resolution.

Head capsule width distributions across treatments were examined to test the hypothesis that mortality was relatively greater in smaller organisms compared to larger organisms in higher treatments. Because complete mortality of *Baetis spp*. occurred among our high treatments, we limited our analysis to lower CCU treatment levels which had adequate mayfly densities for our analyses. To enable comparisons between large and small head capsule sizes, Baetis were

assigned to four size classes of equal abundances based on control abundances of the three experiments.

Analysis of covariance (ANCOVA) using package 'car' (Fox and Weisberg 2011) in R statistical computing (R Core Team v3.5.1) was used to test the hypothesis that the metals treatment effect was dependent on insect body size (e.g. head capsule width), with the interaction model term representing this relationship. Akaike Information Criterion (AIC) was used to support the interaction model in best explaining the relationship of body size and metals treatment. To determine which instar size slopes were statistically significant from one another, we used the estimated marginal means of linear trends (emtrends) function in package 'emmeans' (Lenth 2018), and the 'multcompLetters' package (Graves et al. 2012) with a TukeyHSD multiple comparison adjustment.

Study III

A 14 day mesocosm exposure of Cu and Zn to benthic communities collected from the Arkansas River, Colorado was conducted in September 2015. Details of the Stream Research Laboratory mesocosm and water quality parameters are described by Clements et al. (2013). CCU calculations were derived from the same hardness-adjusted equations used by Clements et al. (2013), but with the observed hardness measured during the course of this experiment. CCU exposures ranged from 0.64 to 59.27 CCU. Benthic samples were preserved in ethanol, and individuals were enumerated and identified to the lowest taxonomic resolution possible. Four dominant taxa from four major aquatic insect orders were chosen to measure head-capsule widths, specifically, *Baetis spp.* (Ephemeroptera), Orthocladiinae (Diptera), *Hydropsyche sp.* (Trichoptera), and *Isoperla spp.* (Plecoptera). Head capsule widths were measured using a high-definition microscopy camera (ACCU-SCOPE® Excelis Camera AU-600-HD) attached to a stereoscope (Meji EMZ-TR), and displayed on a monitor. A calibration slide micrometer (0.01 mm precision) was used to calibrate measurements with the microscopy camera before every sample series. Three measurements were taken on each individual insect and reported as an average.

To formally separate size classes for each aquatic insect order package 'ggplot2' (Wickham 2016) in R statistical computing (R Core Team v 3.5.1) was used to fit histograms to predefined numbers of bins (6 or 7). The macroinvertebrate abundance data was normalized to proportion mortality compared to observed control values for each size class by dividing the raw abundance data by the respective average control (n = 3 for each size class) abundances. A two-factor analysis of variance model (package 'car') was used to test the hypothesis that proportion

mortality was explained by the interaction of treatment effect and insect body size (e.g. head capsule width).

Study IV

Over numerous years, development of meiofaunal benthic organisms that resembled Beatis sp. were observed in a 4th order mountain stream (Cache la Poudre River, Larimer County, USA). Members of Baetidae (Ephemeroptera) oviposit eggs on the bottom of cobble. On September 29th of 2014, lenticular cobble 6 cm to 30 cm in diameter was collected from riffle areas. Egg masses (Figure 1.1.2-1) were carefully removed from rocks using stainless steel razor blades (Figure 1.1.2-2) and placed in 35mm polystyrene Petri dishes with river water. Petri dishes were transported in coolers to Colorado Parks and Wildlife Aquatic Toxicology Laboratory where they were maintained in an incubator at temperatures observed at the sample site (12-15° C). Water received gentle aeration though Pasteur pipettes. Water was replaced daily. Eggs were assessed for hatching organisms twice daily. Immediately upon hatch, organisms (head capsule 113.5 micron SD=10 *n*=7) were fed a suspension of the diatoms *Navicula sp*. and *Synedra sp* (Carolina Bio Supply. Burlington, NC, USA).

November 16th of 2015, substrate was collected from the same sample location and rinsed into white sorting pans. Transfer pipettes were used to collect organisms that appeared to have the body shape, size and behavior of *Baetis sp.* at approximately one month of age. Organisms (head capsule 260.1 micron SD=25 *n*=7) were transported in coolers and used in experiments beginning the same day. Prior to use in experiments both 24 hour post hatch and approximately one month old organisms were identified under a microscope and a sub-sample was preserved for genetic analysis.

Fine stainless steel mesh (105 x 125 micron pore size) was affixed to the bottom of 24 borosilicate glass tubes (8 mm I.D.) which contained instars during exposure (Figure 1.1.2-3). Small strips of fine mesh (3 x 30mm) were placed inside each tube as substrate for organisms. Continuous-flow serial diluters (Benoit et al. 1982) delivered 40 ml/min dechlorinated municipal drinking water (Fort Collins, CO, USA) to test tubes (25 mm diameter x 15 cm; Figure 1.1.2-4) partly submerged in a chilled water bath (Figure 1.1.2-5). Glass exposure tubes and tubes delivering aerated water from the serial diluter were mechanically raised and lowered 3 cm in each test tube every 30 seconds. This change in hydraulic head (Figure 1.1.2-6) continuously replenished water in exposure tubes with highly oxygenated water from the serial diluter without losing or damaging organisms. Under a stereo dissecting microscope (Meji EMZ-TR with 20x eyepieces) 10-20 instars were transferred to exposure tubes using a 100 µl pipette. Organisms were enumerated at 0 (prior to exposure), 48 and 96 hours of exposure to Zn. After range finding

experiments first instars were exposed to 0, 133, 300, 642, 1433, and 3263 µg/L Zn (26 Oct 2016) and mid-instars were exposed to 0, 4600, 9380, 20450, 46550, 84800 µg/L Zn (16 Nov 2016) using standard ASTM compliant laboratory methods detailed by Brinkman and Johnston (2012).

To enumerate organisms, stoppers were placed in the top of the exposure tubes (Figure 1.1.2-7). The negative pressure in the top of the tube suspended water and organisms during assessment of survival. Exposure tubes were placed horizontally in the focal range of the microscope. Temperature was maintained by resting the exposure tubes on slate tiles chilled to -20 deg C. During experiments using first instars, each experimental unit received 10 ml of algal suspension at 0 and 43 hours of exposure. A sub-sample of surviving organisms from each experiment was preserved for genetic analysis. Phenotypic characteristics used to identify these species are typically not developed until organisms are large and mature (late instar). Genetic analysis by The Centre for Biodiversity Genomics, Biodiversity Institute of Ontario, was employed to ensure test organisms were properly identified. Total genomic DNA was extracted using a validated spin column DNA extraction protocol. The target genetic marker was amplified using the Polymerase Chain Reaction and was analyzed by high-voltage capillary electrophoresis on an automated ABI 3730xL DNA Analyzer. DNA sequences recovered from the unknown samples were compared against the species sequence reference library in the Barcode of Life Data System (BOLD). Samples from both experiments were a 99.69% BOLD match to Baetis tricaudatus. 96 hour LC_{50} values for first instar and mid-instar size classes were estimated using probit analysis (PROC PROBIT) using SAS (v9.3).

Results

Study I

Water chemistry observations from the 2010 mesocosm experiment were similar to experiments in 2005 (Cu+Zn and Cu; Clements et al. 2013) and are representative of oligotrophic headwater streams. Sensitivity decreased with an increase in body size for Ephemeropterans, Plecopterans and Tricopterans (EPT) (Slope: -0.5689, p<2.71e-08; Figure 1.1.2-8). As predicted, the distribution of sensitivity for small organisms varied from no sensitivity to the highest observed sensitivity to metals, while the largest body sizes were limited to taxa with only low sensitivity values. This wedge shaped response distribution contributed to the low r^2 value (r^2=0.42). This trend was largely influenced by Plecopterans (Slope: -0.532, p<0.00159, r^2:0.496), and Ephemeropterans (Slope: -0.6182, p=0.00224, r^2:0.339). Tricopterans were insignificant (Slope: -0.0347, p=0.2453, r^2:0.08883). Although Dipterans and Coleopterans followed this trend

(Figure 1.1.2-9), the number of taxa observed in mesocosm experiments was limited and they were not included in analysis.

Numerous genera and species were present in multiple experiments and represent two or three bubbles in Figure 1.1.2-8 and 1.1.2-9. The measure of sensitivity of a "Taxon" in Study 1 was dependent on the body size and the experiment. Largely the trend of smaller organisms having a greater susceptibility was observed for each taxon. As predicted, large organisms were found to only be tolerant while the distribution of sensitivity was widespread for small taxa.

Study II

Adequate abundances of Baetis spp. allowed for treatment comparisons of 0-5.1 CCU for Cu, 0-7.0 CCU for Cu+Zn, and 0-12.9 CCU for Cu+Zn+Cd. Instar regression slopes were negative for all instar size groups, except for instar size 0.8-1.0 mm in the Cu only exposure (Figure 1.1.2-10). Significant ANCOVA (alpha 0.05) interaction terms were observed for Cu (p=0.0082) and Cu+Zn+Cd (p= 0.0171; Table 1.1.2-2), but were not significant for Cu+Zn (p=0.2395). The most negative slopes among instar size groups were early- to mid-instars in the Cu and Cu+Zn+Cd exposures; however, the opposite trend was observed in the Cu+Zn treatment, with the most negative slope observed in late-instar individuals (> 1 mm). AIC results support the use of the ANCOVA interaction term for all mesocosm models compared to models that excluded the metals (CCU) and size class interaction, indicating that an interaction and (or) an additive effect of both size and CCU best explained trends in mortality across treatments (Table 1.1.2-3)

Study III

CCU metals treatment effects were highly significant (p<0.0001) for Ephemeroptera, Diptera, and Plecoptera, with the highest treatment resulting in greater than 50% mortality. Metals treatment effects for Trichoptera overall were significant (p-value 0.0023), but the smallest instars (<0.30 mm) were particularly sensitive, with average proportion mortality greater than 50% in the lowest treatment. Interaction terms for all taxa were statistically significant (Table 1.1.2-4), except for *Isoperla spp.* (p= 0.0762). In general, the greatest proportion mortality was observed in smaller instar classes showing negative slopes approaching larger instars (indicating lower proportion mortality as organisms get larger). Trends in proportion mortality in Orthocladiinae were consistent among treatments, indicating size-dependent relationships through the treatment gradient. However, size-dependent responses in *Baetis* and *Isoperla* were less pronounced in higher treatments, likely because at high metals concentration toxicity is so high that size does not strongly influence mortality in these taxa.

Study IV

Use of the same laboratory, identical equipment and the same water sources produced very similar water chemistries as published by (Brinkman and Johnston 2012). *Baetis tricaudatus* tolerance to acute Zn exposure increased with the size and age of the organisms (Figure 1.1.2-1.1.2-11). First-instar *B. tricaudatus* responded (LC_{50}) to zinc 451 µg/L. This is <5% of the LC_{50} value reported for late instars (10,020 µg/L) obtained from the field in August of 2005. Mid-instar organisms responded at a moderately high concentration of 4,741µg/L Zn.

Discussion

The naturally colonized benthic communities used in our mesocosm studies incorporated diverse size assemblage structure within and among taxa. This enabled us to evaluate responses to metals among numerous developmental size classes. At metals exposure levels in which partial mortality was observed, smaller organisms were generally disproportionately reduced by trace metals compared to larger organisms. Size-dependent responses of Baetis were confirmed in all four mesocosm experiments, as well as in the laboratory using first, mid and late-instar test organisms. Across all taxa, body mass was inversely correlated with sensitivity. Small organisms had a wide range of sensitivity to aqueous metals, but large organisms were consistently tolerant. Together, these results suggest that aquatic insect body size is a strong predictor of susceptibility to aqueous metals, with smaller, less-mature invertebrates displaying greater sensitivity relative to larger, mature invertebrates.

In Study 3, small size classes generally observed increased mortality relative to larger size classes. At the highest exposure levels, *Baetis* and *Isoperla* experienced nearly complete extirpation, suggesting that all size classes were susceptible. Small, less mature *Hydropsyche* were extirpated at the highest exposures, but mature individuals were tolerant and minimally reduced. Toxicity assessments of *Hydropsyche* suggest that this genera is highly metal tolerant (Cain and Luoma 1998), but these assessments did not incorporate a range of size classes, including early instars. In our study, *Hydropsyche* had the greatest head capsule size range, and importantly, the most pronounced size-dependent treatment effects.

We generally observed disproportional mortality in less mature individuals. However, the opposite trend was observed in *Baetis* in the Cu+Zn exposure in Study 2, with the most negative slopes observed in the largest individuals. It is possible that the reduced abundance of these large individuals was a result of toxicant avoidance through increased rates of metamorphosis to their adult life stage. Joachim et al. (2017) observed increased rates of emergence in numerous Diptera taxa in response to Cu exposure in mesocosms. Key life stage transitions such as mating,

oviposition, diapause, emergence and molting might complicate the general trend that smaller size classes are more susceptible.

In Study 2, analysis of covariance (ANCOVA) was used because replication (*n*=2) was too limited to employ analysis of variance (ANOVA) of the mortality proportional to controls. Slopes showing abundance of each size class responding to metals were potentially influenced by the abundance of each age class in the controls. For example, it appeared all age classes were lost at the same exposure level and greater negative slopes were observed in those size classes with greater control abundance. Study 3 was designed to account for this problem through improved replication and improved technologies to measure head capsule width.

Small size classes represented in Study 1 were both sensitive and tolerant, but taxa represented by large size classes were only tolerant. Phylogenetic differences in acclimating to stressors is perhaps of greatest importance at small age classes. All aquatic macroinvertebrates hatch as small individuals and selection against sensitive taxa is most likely to be observed at this age class. Observational studies find maximal body size is a trait commonly associated with taxa present in contaminated sites (Statzner et al. 2001; Archaimbault et al. 2005; Statzner et al. 2005; Doledec and Statzner 2008; Statzner and Beche 2010). It is unlikely that the trait of maximal body size is somehow genetically correlated to tolerance at all age classes. Observational studies are limited in addressing these relationships because individuals may immigrate from undisturbed site. It is possible that maximal body size might be a trait that predicts which taxa can immigrate to and survive at a site while minimal body size might better explain what species can sustainably reproduce at a site. Most field research examining aquatic insects is limited to organisms which are retained through a 500 or 350 micron mesh sampling equipment. As technologies improve to detect the presence of early instars in the field our ability to characterize the relationship of size-sensitivity to contaminants will too improve.

Acute laboratory trials exposing late instar Baetis tricaudatus to zinc estimated an LC_{50} value of 10,020 µg/L (Brinkman and Johnston 2012) after adjusting for the emergence from each experimental unit. In the same study *Drunella doddsi*, *Ephemerella* sp., *Cinygmula* sp., *Lepidostoma* sp. and Chloroperlidae stoneflies had LC_{50} values estimated between 32,000 and greater than 64,000 µg/L. In all these studies larvae were large enough to be collected from river cobble by hand and survival was easily assessed with the naked eye. These single species LC_{50} values are orders of magnitude higher than thresholds reported in mesocosm experiments and field studies. Under the same laboratory conditions and the same applied statistical methodology we found first instar Baetis tricaudatus respond at <5% the level of zinc. This shows a clear size progression of sensitivity and might explain, in part, why laboratory experiments typically find aquatic insects to be tolerant to metals while mesocosm and field studies show that they are quite sensitive.

The physical and chemical cues which influence the phenology of macroinvertebrates in the field probably influence their spatiotemporal sensitivity to contaminants. For example, environmental cues such as degree days, steam flow, and day length influence hatching, adult aquatic insect emergence, and primary production. The seasonal changes in water chemistry associated with snowmelt and monsoonal patterns of the Rocky Mountains may interact with the sensitive life stages of some taxa and not others based on timing of development and environmental cues. Kiffney and Clements (1996) suggested a similar interplay might explain altitudinal changes of aquatic insect distribution. Use of single species experiments conducted on late instar insects should be limited to short term (seasonal) standards that match the developmental stage of the tolerant life stage.

Major policies and standard testing methods (American Society for Testing and Materials 1993; Stephen et al. 1985; U.S. Environmental Protection Agency - USEPA Office of Water 2002) have long noted the importance of using early life stages in toxicity trials. However, no requirements are made to use sensitive or early life stages nor do methodologies for deriving water quality standards give added weight to experiments that use early life stages. In our study, AIC model selection suggests that models including organism size and toxicant dose are better predictors of mortality than models using treatment alone.

A bias towards the use of large organisms is common in aquatic toxicology assessments given the logistical challenges of collecting organisms, providing care, and assessing condition and survival. While policies were developed with good intentions, some standardized methods may have inadvertently dictated the use of mature organisms in toxicity testing. For example, guidelines for conducting toxicity experiments often mandate that mortality in controls not exceed 5% or 10% (American Society for Testing and Materials 1993; Stephen et al. 1985; U.S. Environmental Protection Agency - USEPA Office of Water 2002). This requirement was intended to limit the risk of a Type I error, in which a toxic effect is determined erroneously. High survival in controls superficially suggests that the abiotic (e.g., appropriate test system water quality) and biotic (e.g., disease, feeding) requirements of organisms in all experimental units are realized to associate causation with the respective toxicant. However, the goal of risk assessments and water quality policy is to protect populations in nature where few early life classes of freshwater taxa see mortality rates compliant with ASTM standards.

Starting in the early twentieth century ecologists have used the concept of survivorship curves (Figure 1.1.2-13) to describe the natural rates of mortality throughout the life of any given species (Pearl and Miner 1935; Deevey 1947). Fish and aquatic insects generally occupy a Type II or Type III survivorship curve. Therefore, some mortality in experimental controls should be expected if using the early life stages. Given the inconvenience and spurious mortality exhibited by aquatic species at young age classes (dashed box of Figure 1.1.2-13) published data from

these age classes are perhaps less common than older age classes (solid box of fig 1.1.2-13). Fish hatcheries have long existed to shelter fish eggs and fry during high rates of mortality (dashed box Figure 1.1.2-13), with the release of larger fish when survival is greater (solid box of figure 1.1.2-13). But even in hatchery settings mortality naturally occurs. Aquaculture techniques largely pioneered by hatcheries have enabled toxicity trials of many fish species to be conducted at early age classes without excessive mortality in the controls. Culture techniques for most aquatic insects, algae, freshwater meiofauna, and some rare fish species are lacking. While invertebrate species such as Cladocerans and *Chironomus riparius* are easily cultured, repeatable, and convenient, freshwater invertebrate diversity is extensive and surrogate test species simply fail to represent the complexity of sensitivity of species found in natural communities (Cairns 1986). The methodologies devised in this paper, along with genetic assessment of species before phenological characteristics develop, enable "non-culturable" species to be evaluated at earlier age classes (dashed box of Figure 1.1.2-13). By testing a diversity of sizes among ecological relevant invertebrate species we improve the accuracy of effect concentration derivations for communities in natural systems which we seek to protect. Experiments that are more ecologically relevant or that use early life stages may be prematurely discounted from species sensitivity distributions because of poor survival in controls. In such situations proportion of survival or mortality relative to controls should be considered in lieu of nominal survival counts.

The goal of risk assessment, water quality standards, and policy is to prevent species loss and reduced function of the ecosystems that support us physically and economically. The field of environmental toxicology should be more concerned with reducing Type II errors or the risk that a pollutant is erroneously deemed safe. However standardized testing procedures concerned with Type I errors often preclude use. For this reason, toxicity trials using small age classes should be mandated or be given larger weight in assigning sensitivity thresholds. To further reduce the risk of creating underprotective standards, species sensitivity distributions should perhaps be expanded to include microscopic and meiofaunal community members. Moreover, data obtained from mesocosm testing and field studies should be expanded because these approaches implicitly include the spatiotemporal realism of organism developmental size diversity and include species interactions lacking from laboratory trials.

Altogether, these laboratory and mesocosm approaches demonstrate that smaller invertebrates are more susceptible to aqueous metals compared to larger, mature invertebrates. Toxicity models which incorporate the developmental size progression of organisms have the potential to improve our accuracy in predicting effects in the field. Moreover, the toxicity testing methodology which researchers use, such as the test system introduced in this study, as well as mesocosm testing, should be "acceptable" testing procedures to obtain these data. Additional field sampling methods should be developed to quantify early-instar densities along contaminant

and stressor gradients, and more laboratory and mesocosm tests need to specifically address these size-dependent contaminant relationships.

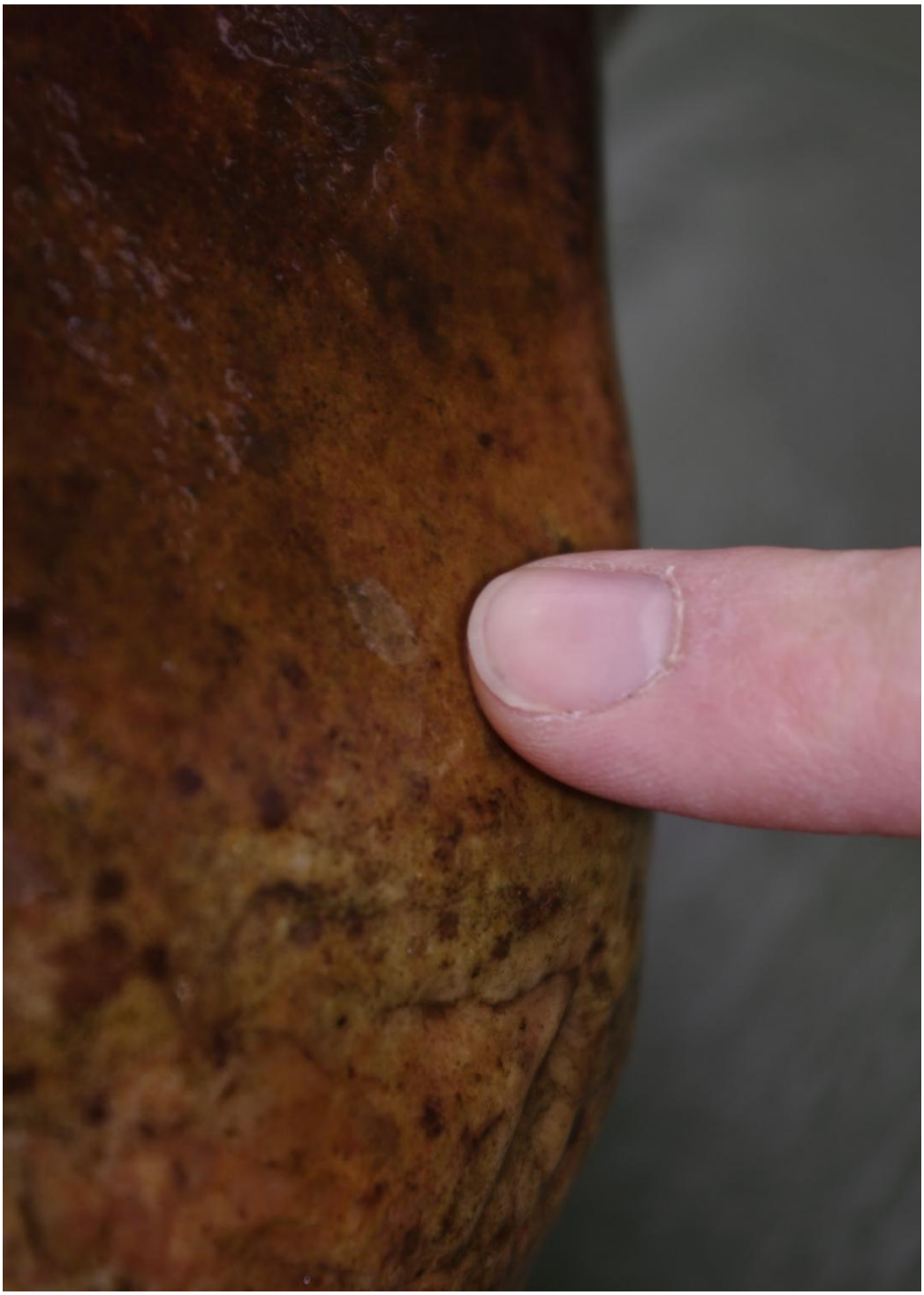

Figure 1.1.2-1. *Baetis tricaudatus* egg mass. Almost translucent the bumpy white patch of eggs covers less than half a square centimeter.

Figure 1.1.2-2. Egg mass being gently removed from cobble using stainless steel razor blade

Figure 1.1.2-3. Borosilicate tubes (8mm ID. 12 mm OD) were used to house organisms during exposure and enumeration.

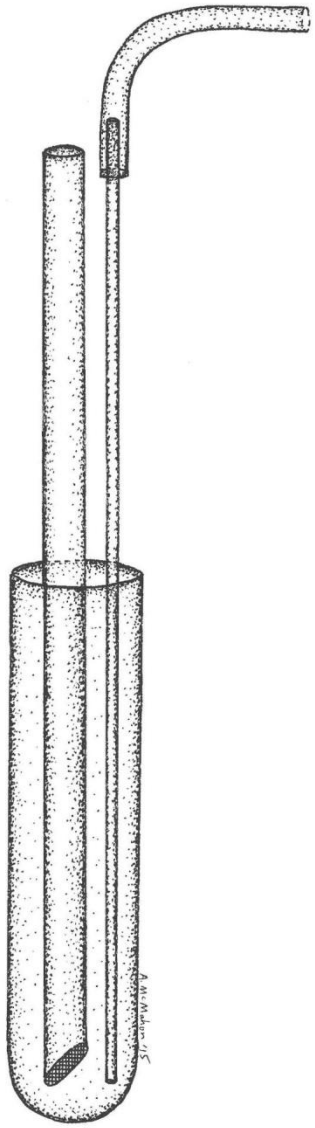

Figure 1.1.2-4. Exposure tube and toxicant delivery tube were mechanically raised and lowered in a test tube held in a chilled water bath. Illustration by Amy E. McMahon.

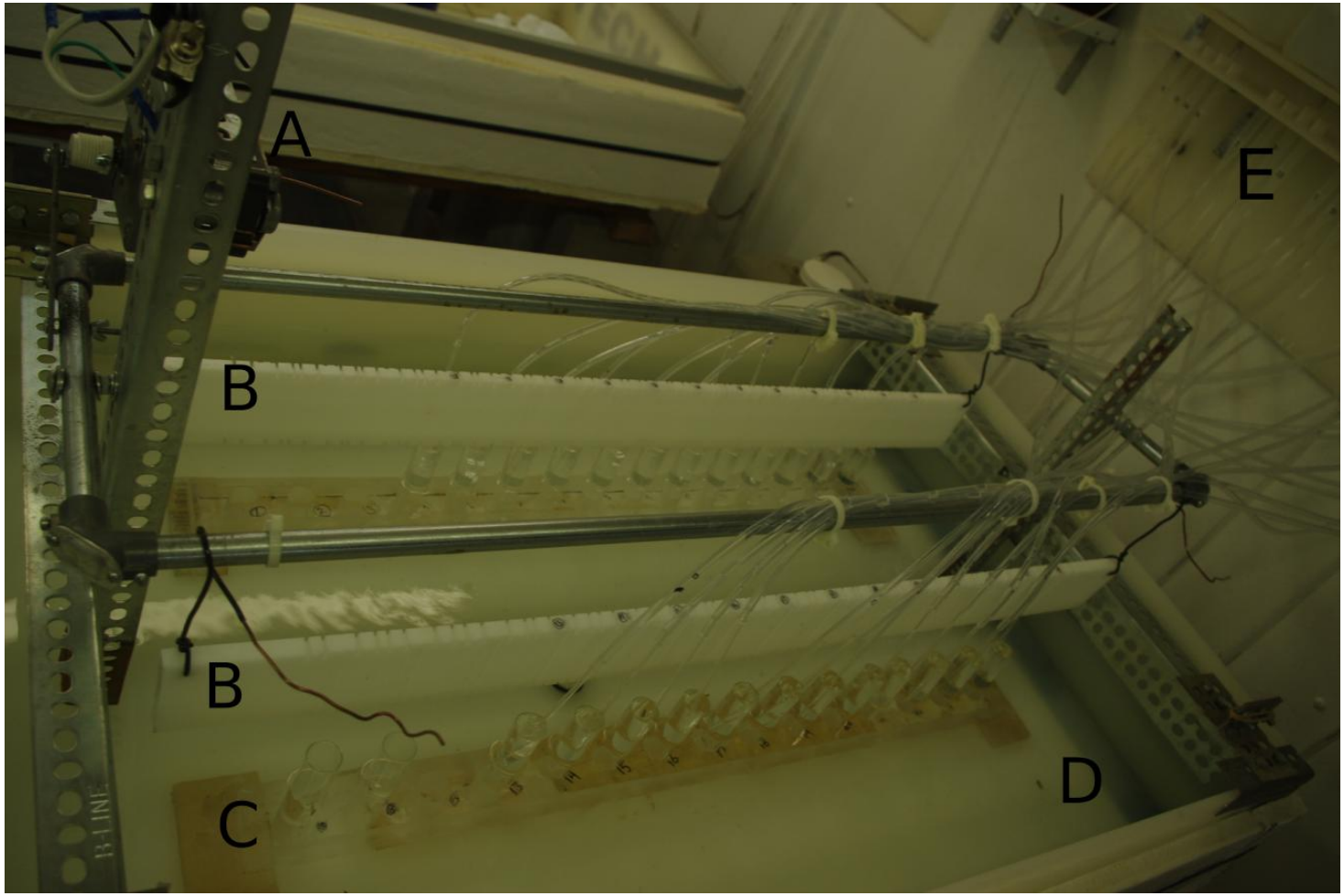

Figure 1.1.2-5. Motorized teeter totter powered by a motor (A) was constructed of perforated angle iron and metal electrical conduit. Wire suspended white high density polyethylene boards (B) on each arm of the teeter totter. Dado cuts accommodated rubber bands (not shown) which held exposure tubes and toxicant delivery tubes perfectly aligned with test tubes. Test tubes were held in heavy acrylic test tube racks (C) in a water bath (D). Both the water bath and flow from the serial diluter (E) were chilled at 12 deg C.

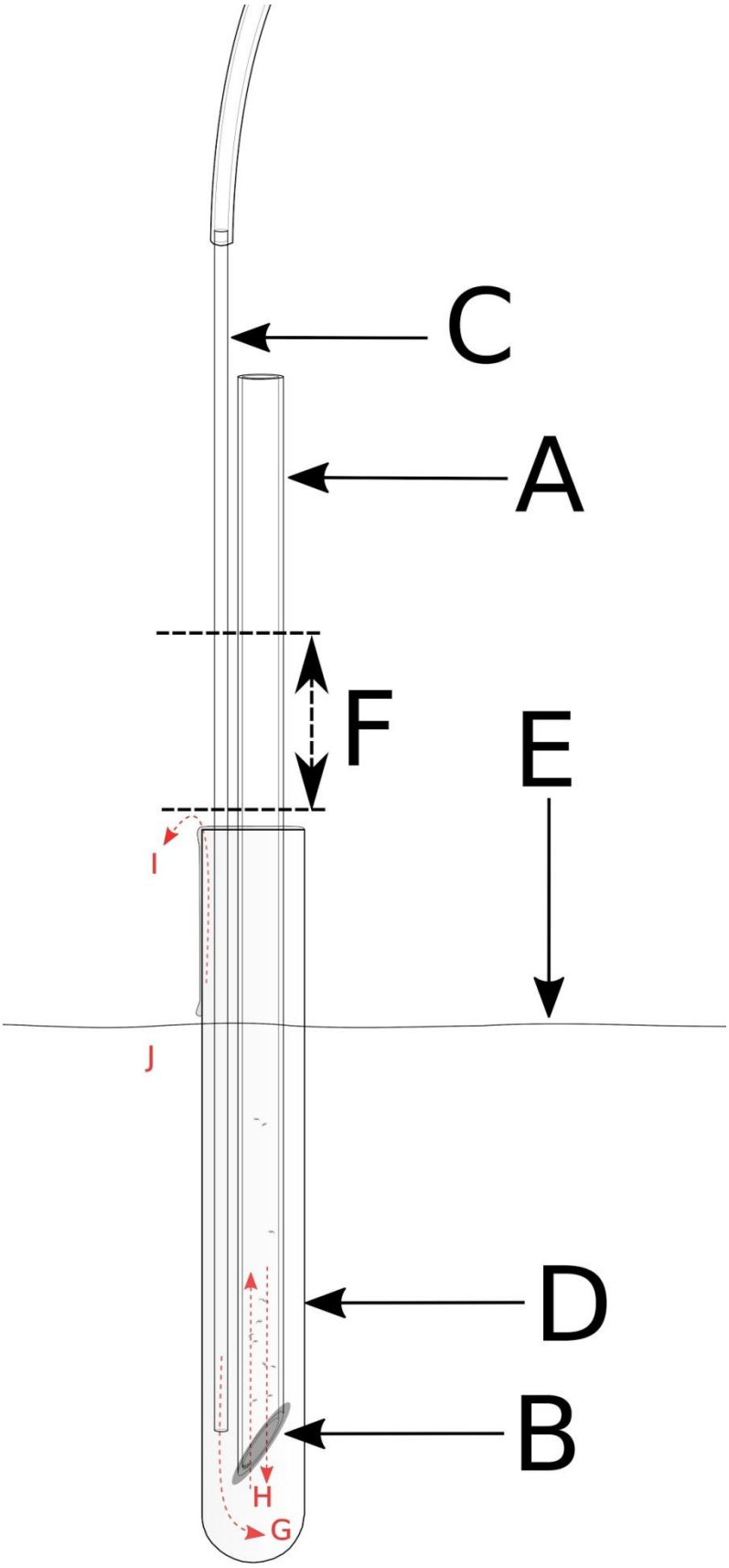

Figure 1.1.2-6. Organisms were contained in exposure tubes (A) constructed of borosilicate glass tubes (8mm ID, 12mm OD, 15 cm long) that were cut to a 30° bevel. Fine stainless steel mesh (B) was affixed to the end of exposure tubes. Vinyl food grade tubing and fine glass tubes (C; 3 mm ID. 5 mm OD) was used to transport toxicants and aerated water from the serial diluter to the test tubes (B; 23 mm ID). Test tubes were held in test tube racks placed in a chilled water bath with a standpipe holding the water level at E. Exposure tubes and toxicant delivery tubes were affixed by rubber band to a motorized teeter totter (Figure S4) dipping and raising the tubes approximately 3cm (F). Highly oxygenated water and toxicants from the serial diluter enter the system at G. The change in hydraulic head forces water in and out (H) of exposure tubes. Water and toxicants overflow (I) into the water bath (J).

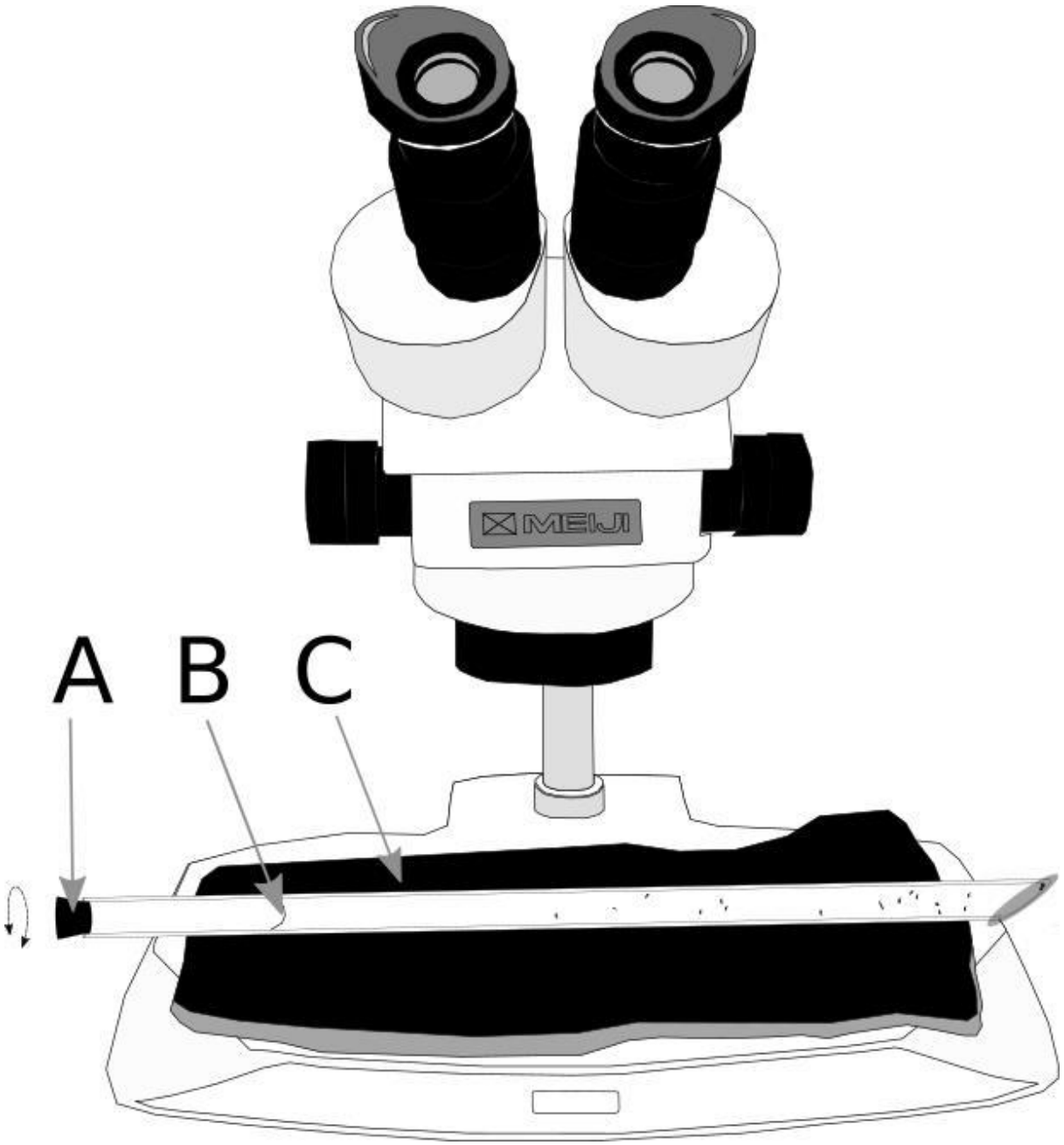

Figure 1.1.2-7. Rubber stoppers (A) were placed in exposure tube during enumeration of surviving organisms. This held the water level in the exposure tube at approximately B. The exposure tube was rotated in the focal field of the microscope for enumeration. Chilled slate tiles (C) were used to maintain temperature in exposure tubes during microscopy.

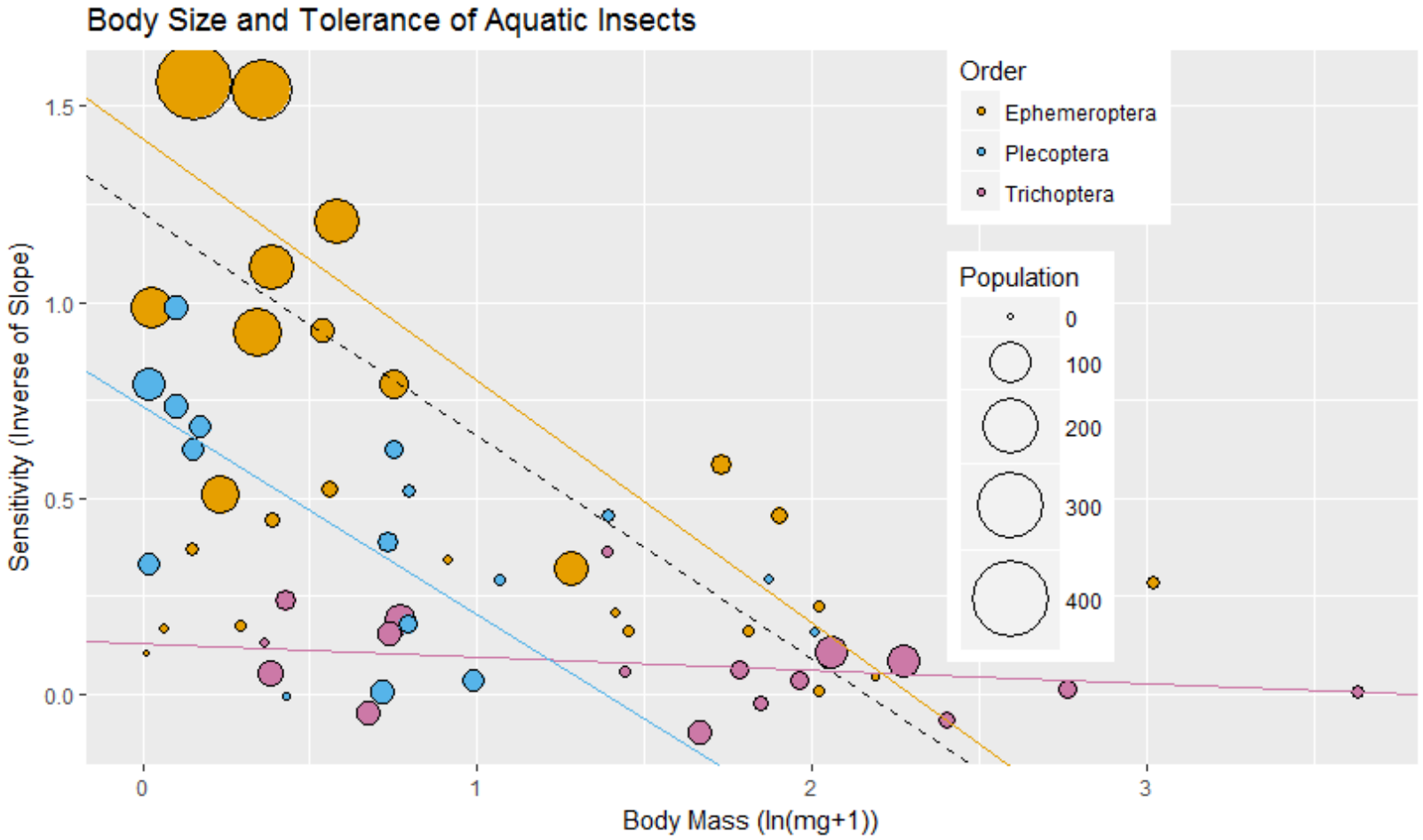

Figure 1.1.2-8. Relationship of body mass and sensitivity of aquatic insects. Across all EPT taxa organisms of smaller mean body mass were more sensitive than organisms of larger body mass (black dashed lines). This trend was significant for Ephemeroptea (Orange) and Plecoptera (Blue) but was insignificant for Trichoptera (Pink).

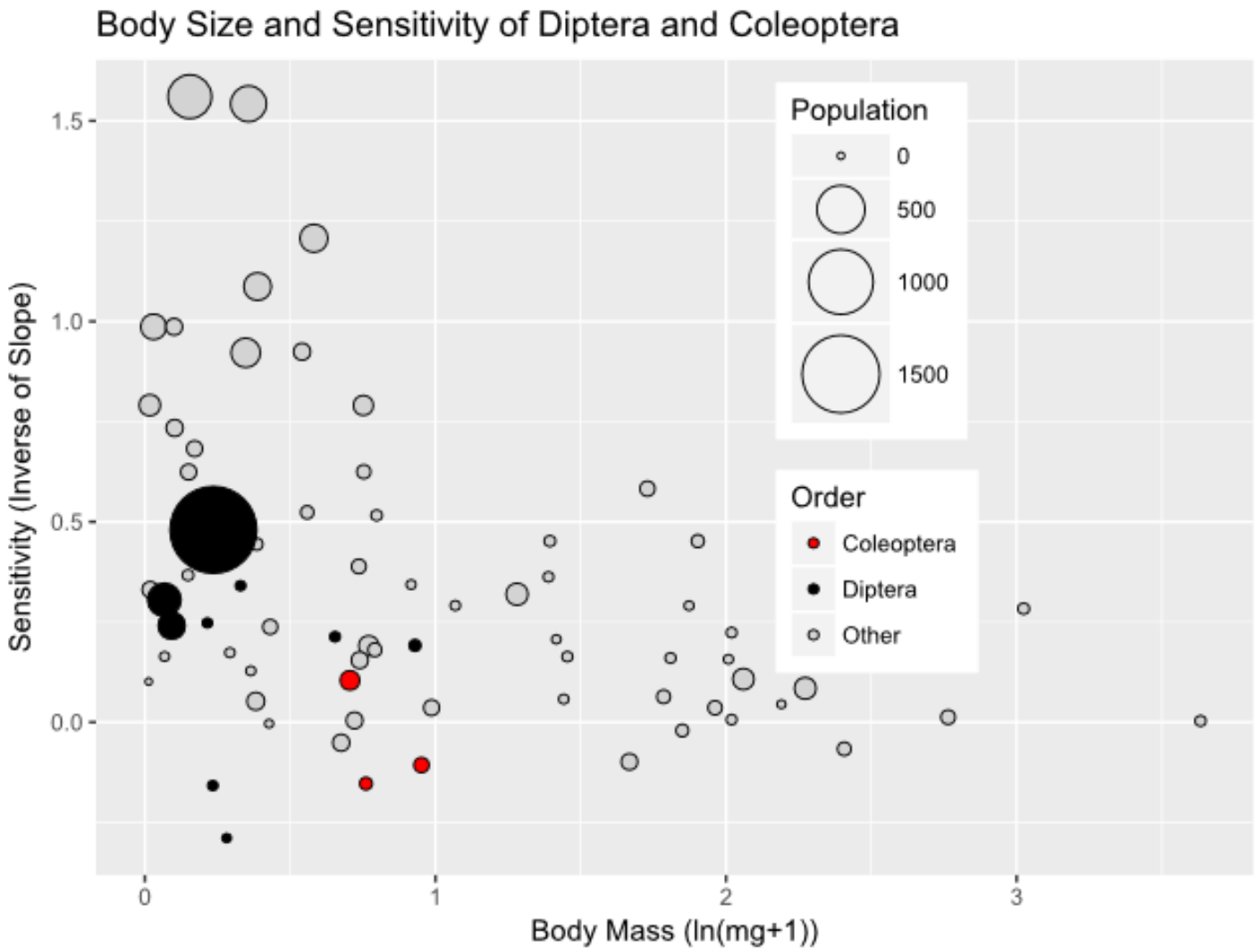

Figure 1.1.2-9. Diptera and Coleoptera followed the general pattern of EPT species. However, the number of taxa was too limited to warrant analysis.

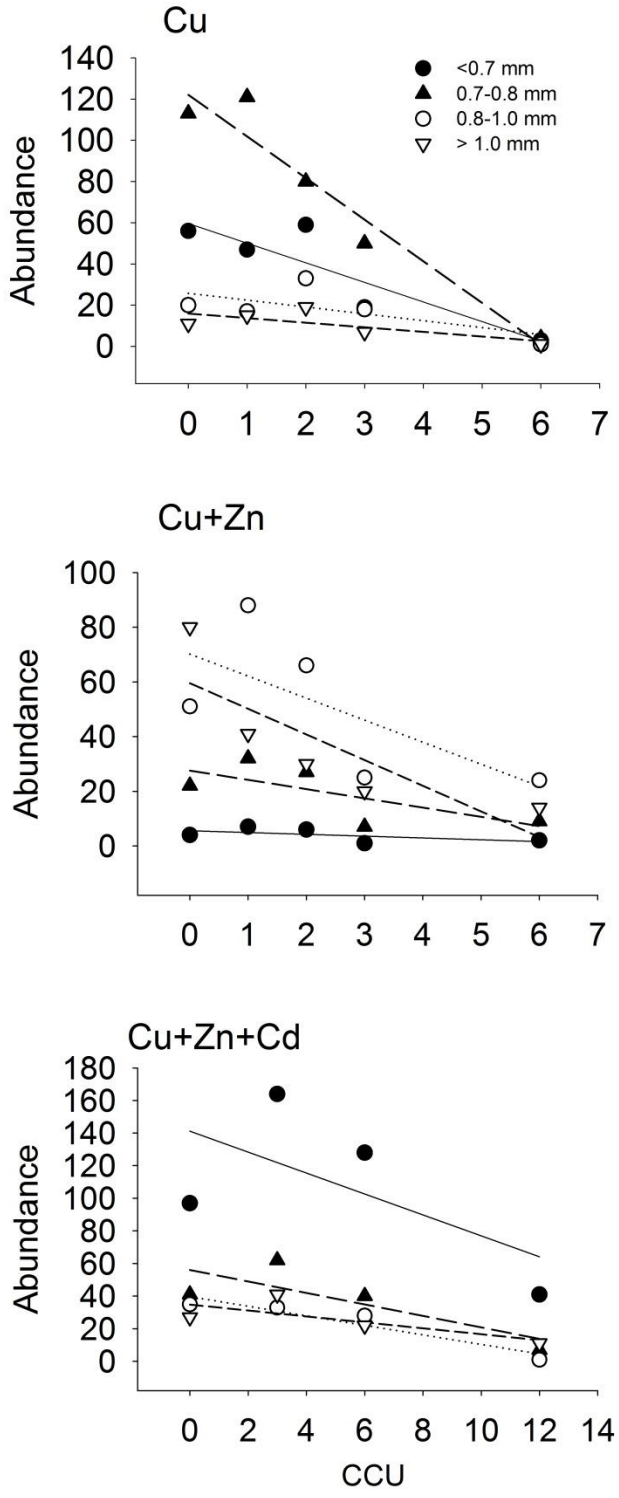

Figure 1.1.2-10. Study 2 response curves for four size classes of *Baetis spp.* across a gradient of aqueous metal(s). ANCOVA approach was used to incorporate CCU as covariate. Here we see slopes for size classes differ. See Table 1.1.2-2 for significance, multiple comparisons and see Table 1.1.2-3 for AIC selection of important predictors.

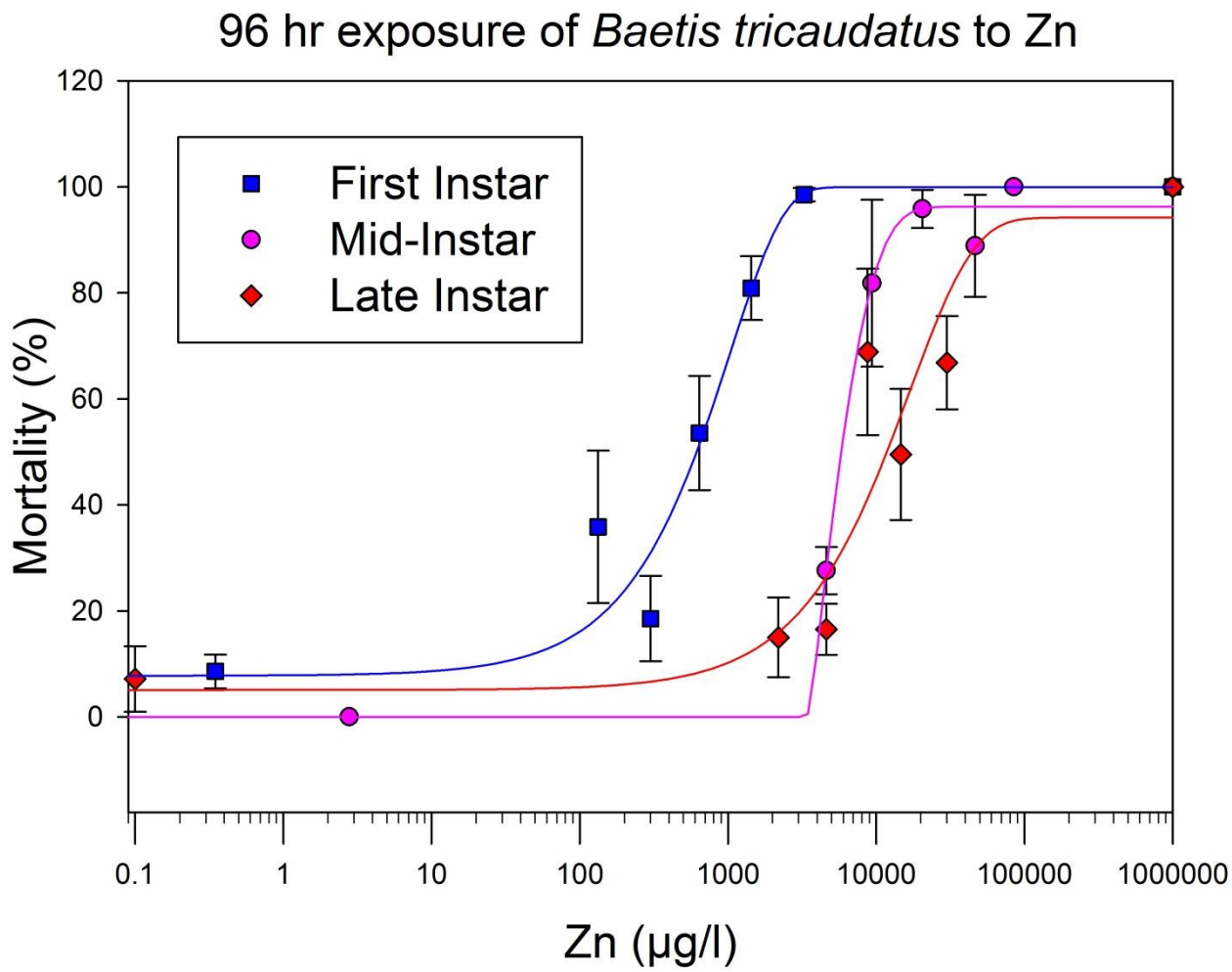

Figure 1.1.2-11. Dose response curves from First Instar (blue), Mid Instar (red) and Late Instar (Brinkman and Johnston 2012; pink). Please note use of a log scale on X axis.

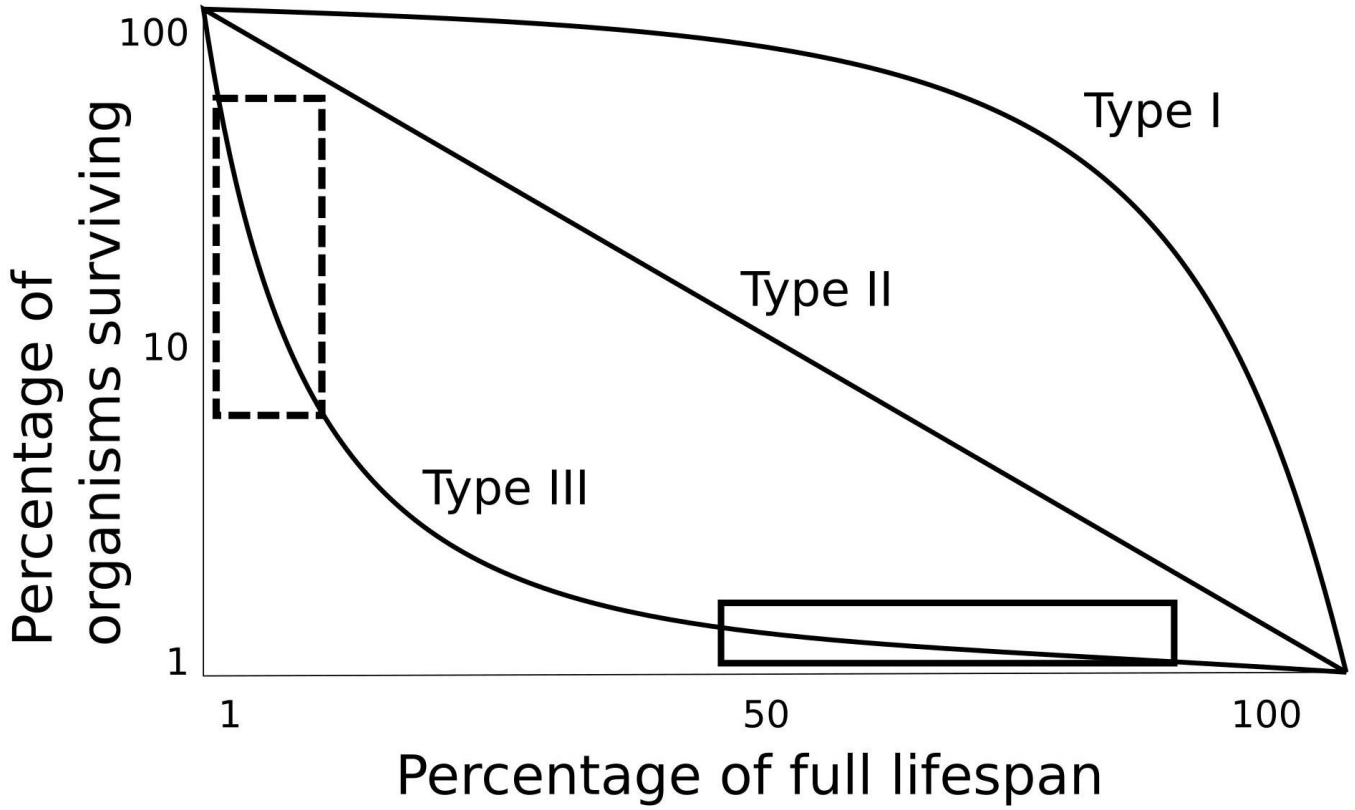

Figure 1.1.2-12. Theoretical survivorship curves are commonly used by ecologists to explain life history traits. Dashed box includes early, more sensitive life stages. Solid box represents larger, more tolerant age classes. Please note the appropriate use of a log scale on the Y-axis.

Table 1.1.2-1. Hardness Adjusted Criterion Values in place at the time of analyzing the 2005 Mesocosm studies in this publication were used throughout to make results consistent with previous publications and (Clements et al. 2013) and make fair comparisons to other experiments in this publication.

	2007 (Cu, Cu + Zn) **2010 (Cu + Zn + Cd)**		**2015 (Cu + Zn)**		
	2007 & 2010 Hardness (mg/L $CaCO_3$)	Criterion Value (µg/L)	2015 Average Hardness (mg/L $CaCO_3$)	Criterion Value (µg/L)	
Cu	35	5	30	4.3	EXP(0.9422*(LN(Hardness))-1.7)*0.96
Zn	35	48.1	30	42.2	EXP(0.8473*(LN(Hardness))+0.884)*0.978
Cd	35	0.7	30	0.6	EXP(1.0166*(LN(Hardness))-3.924)*(1.137-(LN(Hardness)*(0.041)))

Table 1.1.2-2. Study 2 analysis of covariance, EMTRENDS slope estimates and multiple comparisons of slopes.

ANCOVA				**EMTRENDS ESTIMATES & MULTIPLE COMPARISON OF SLOPES**			
Treatment	**Model Term**	**F-Value**	**P-value**	**Instar Size**	**Slope**	**Confidence Limits**	**Mult-Comp of Slopes**
Baetis Cu (0 - 5.1 CCU)	CCU	11.53	0.0023	<0.7 mm	-3.928	(-0.38, -7.48)	A
	Instar Size	29.44	<0.0001	0.7-0.8 mm	-7.898	(-11.45, -4.35)	AB
	CCU*Instar Size	4.94	0.0082	0.8-1.0 mm	0.431	(-3.12, 3.98)	B
				> 1 mm	-0.298	(-3.85, 3.25)	B
Baetis Cu + Zn (0 - 7.0 CCU)	CCU	7.06	0.0137	<0.7 mm	-0.184	(-2.94, 2.57)	A
	Instar Size	7.10	0.0014	0.7-0.8 mm	-1.019	(-3.77, 1.75)	A
	CCU*Instar Size	1.50	0.2395	0.8-1.0 mm	-1.901	(-4.65, 0.85)	A
				> 1 mm	-3.989	(-6.74, -1.24)	A
Baetis Cu + Zn + Cd (0 - 12.9 CCU)	CCU	44.68	<0.0001	<0.7 mm	-3.266	(-4.33, -2.19)	A
	Instar Size	26.49	<0.0001	0.7-0.8 mm	-1.580	(-2.65, -0.51)	AB
	CCU*Instar Size	3.86	0.0171	0.8-1.0 mm	-1.329	(-2.41, -0.28)	AB
				> 1 mm	-0.891	(-1.96, 0.18)	B

Table 1.1.2-3. Study 2 AIC Model Selection for interaction terms for ANCOVA Analysis.

Treatment	Model Term	AIC	Delta-AIC
Baetis Cu			
0 - 5.1 CCU	CCU	280.45	45.81
	Instar Size	250.36	15.72
	CCU+Instar Size	244.03	9.39
	CCU*Instar Size	234.64	0
Baetis Cu + Zn			
0 - 7.0 CCU	CCU	265.45	16.40
	Instar Size	252.96	5.08
	CCU+Instar Size	247.88	0
	CCU*Instar Size	249.05	1.17
Baetis Cu + Zn + Cd			
0 - 12.9 CCU	CCU	368.07	55.18
	Instar Size	346.31	33.42
	CCU+Instar Size	319.18	6.29
	CCU*Instar Size	312.89	0

Table 1.1.2-4. Study 3 two-factor ANOVA based on proportional mortality.

Experiment	Model Term	F-Value	P-value
Baetis spp.	CCU Treatment	100.12	<0.0001
	Instar Size	14.68	<0.0001
	CCU Treatment*Instar Size	4.50	<0.0001
Orthocladiinae	CCU Treatment	53.43	<0.0001
	Instar Size	17.99	<0.0001
	CCU Treatment*Instar Size	2.13	0.0076
Hydropsyche spp.	CCU Treatment	4.56	0.0023
	Instar Size	53.73	<0.0001
	CCU Treatment*Instar Size	2.75	0.0008
Isoperla spp.	CCU Treatment	14.75	<0.0001
	Instar Size	4.54	0.0014
	CCU Treatment*Instar Size	1.62	0.0762

American Society for Testing and Materials. 1993. Standards on aquatic toxicology and hazard evaluation. ASTM Publication Code 03-547093-16. American Society for Testing and Materials, Philadelphia, PA.

Archaimbault, V., P. Usseglio-Polatera, and J. P. Vanden Bossche. 2005. Functional differences among benthic macroinvertebrate communities in reference streams of same order in a given biogeographic area. Hydrobiologia 551:171-182.

Benke, A. C., A. D. Huryn, L. A. Smock, and J. B. Wallace. 1999. Length-mass relationships for freshwater macroinvertebrates in North America with particular reference to the southeastern United States. Journal of the North American Benthological Society 18(3):308-343.

Brinkman, S. F., and W. D. Johnston. 2008. Acute toxicity of aqueous copper, cadmium, and zinc to the mayfly *Rhithrogena hageni*. Archives of Environmental Contamination and Toxicology 54(3):466-472.

Brinkman, S. F., and W. D. Johnston. 2012. Acute toxicity of zinc to several aquatic species native to the Rocky Mountains. Archives of Environmental Contamination and Toxicology 62(2):272-281.

Brinkman, S.F., and N. Vieira. 2008. Federal aid in fish and wildlife restoration. Job Progress Report F-243R-15. Colorado Division of Wildlife, Fort Collins, CO, USA.

Brix, K. V., D. K. Deforest, M. Burger, and W. J. Adams. 2005. Assessing the relative sensitivity of aquatic organisms to divalent metals and their representation in toxicity datasets compared to natural aquatic communities. Human and Ecological Risk Assessment 11:1139-1156.

Buchwalter, D. B., D. J. Cain, W. H. Clements, and S. N. Lucima. 2007. Using biodynammic models to reconcile differences between laboratory toxicity tests and field biomonitoring with aquatic insects. Environmental Science & Technology 41:4821-4828.

Buchwalter, D. B., and coauthors. 2008. Aquatic insect ecophysiological traits reveal phylogenetically based differences in dissolved cadmium susceptibility. Proceedings of the National Academy of Sciences of the United States of America 105:8321-8326.

Cain, D. J., and S. N. Luoma. 1998. Metal exposures to native populations of the caddisfly *Hydropsyche* (Trichoptera : Hydropsychidae) determined from cytosolic and whole body metal concentrations. Hydrobiologia 386:103-117.

Cairns, J. 1986. The myth of the most sensitive species. Bioscience 36:670-672.

Clements, W. H. 2004. Small-scale experiments support causal relationships between metal contamination and macroinvertebrate community responses. Ecological Applications 14:954-967.

Clements, W. H., P. Cadmus, and S. F. Brinkman. 2013. Responses of aquatic insects to Cu and Zn in stream microcosms: understanding differences between single species tests and field responses. Environmental Science & Technology 47:7506-7513.

Clements, W. H., D. M. Carlisle, J. M. Lazorchak, and P. C. Johnson. 2000. Heavy metals structure benthic communities in Colorado mountain streams. Ecological Applications 10:626-638.

Deevey, E. S. 1947. Life tables for natural populations of animals. The Quarterly Review of Biology 22:283-314.

Diamond, J. M., E. L. Winchester, D. G. Mackler, and D. Gruber. 1992. Use of the mayfly *Stenonema modestum* (Heptageniidae) in subacute toxicity assessments. Environmental Toxicology and Chemistry 11:415-425.

Doledec, S., and B. Statzner. 2008. Invertebrate traits for the biomonitoring of large European rivers: an assessment of specific types of human impact. Freshwater Biology 53:617-634.

Fox, J., and S. Weisberg. 2011. An R companion to applied regression. SAGE Publications, Thousand Oaks, California

Graves, S., H. P. Piepho, L. Selzer, and S. Dorai-Jai. 2012. MultcompView: visualizations of paired comparisons. R Package Version 0.1-5.

Joachim, S., and coauthors. 2017. A long-term copper exposure in a freshwater ecosystem using lotic mesocosms: invertebrate community responses. Environmental Toxicology and Chemistry 36:2698-2714.

Kiffney, P. M., and W. H. Clements. 1996. Size-dependent response of macroinvertebrates to metals in experimental streams. Environmental Toxicology and Chemistry 15:1352-1356.

Lenth, R. 2018. Estimated marginal means, aka least-squares means. URL: https://github.com/rvlenth/emmeans

McCahon, C. P., A. J. Whiles, and D. Pascoe. 1989. The toxicity of cadmium to different larval instars of the trichopteran larvae *Agapetus fuscipes* (Curtis) and the importance of life-cycle information to the design of toxicity tests. Hydrobiologia 185:153-162.

Pearl, R., and J. R. Miner. 1935. Experimental studies on the duration of life XIV The comparative mortality of certain lower organisms. Quarterly Review of Biology 10:60-79.

Powlesland, C., and J. George. 1986. Acute and chronic toxicity of nickel to larvae of *Chironomus riparis* (Meigen). Environmental Pollution Series A-Ecological and Biological 42:47-64.

Rogers, H. A., and coauthors. 2016. Bifenthrin causes trophic cascade and altered insect emergence in mesocosms: implications for small streams. Environmental Science & Technology 50:11974-11983.

Statzner, B., P. Bady, S. Doledec, and F. Scholl. 2005. Invertebrate traits for the biomonitoring of large European rivers: an initial assessment of trait patterns in least impacted river reaches. Freshwater Biology 50:2136-2161.

Statzner, B., and L. A. Beche. 2010. Can biological invertebrate traits resolve effects of multiple stressors on running water ecosystems? Freshwater Biology 55:80-119.

Statzner, B., B. Bis, S. Doledec, and P. Usseglio-Polatera. 2001. Perspectives for biomonitoring at large spatial scales: a unified measure for the functional composition on invertebrate communities in European running waters. Basic and Applied Ecology 2:73-85.

Stephen, C. E., and coauthors. 1985. Guidelines for deriving numerical standards for the protection of aquatic organisms and their uses. PB85-227049. U.S. Environmental Protection Agency - USEPA Office of Water, editor, Washington, D.C.

Stuhlbacher, A., M. C. Bradley, C. Naylor, and P. Calow. 1993. Variation in the development of cadmium resistance in *daphnia magna* (Straus) effect of temperature, nutrition, age and genotype. Environmental Pollution 80:153-158.

U.S. Environmental Protection Agency - USEPA Office of Water. 2002. Methods for measuring the acute toxicity of effluents and receiving waters to freshwater and marine organisms (4303T). U.S. Environmental Protection Agency, 1200 Pennsylvania Avenue, NW. Washington, DC 20460.

Wickham, H. 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag, New York.

Sub-Action #1.2.2: Field 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. CPW, EPA and federal and state land management agencies will use these data to and told 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.

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.

● 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.
-Fixed a new colorimetric analyzer and developed methods for nutrients
-Examined laboratory policies and procedures and laid the ground work for improving good laboratory practice and sample storage.

-Began reviewing QAQC policies to improve results, turn-around time of results, reporting information.
-Researched and advised on equipment options for replacing an ICP-OES with an ICP-MS
-Explored cost saving measures.

● Provided Pulse Amplitude Modulated Fluorimetry analysis of laboratory and field experiments that will inform metal toxicity models and create predictive tools that will allow help triage and prioritize mine sites. These studies are primarily the work of Colorado School of Mines and Colorado State University.

● Aided in interagency planning working towards a stream bank mitigation assessment tool that is ecologically sound.

● Collaborated with Colorado Department of Public Health and Environment to investigate effects of Selenium on fish (White Suckers & 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.

●Aided in interagency efforts to prioritize abandoned mine restoration efforts across Colorado.

● 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.